UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA, TOXICOLOGÍA Y MEDICINA LEGAL

"DISEÑO, CARACTERIZACIÓN Y EVALUACIÓN DE LA SEGURIDAD DE ENVASES ACTIVOS DESARROLLADOS CON ACEITES ESENCIALES"

Memoria que presenta la Licenciada MARÍA LLANA RUIZ-CABELLO

para optar al título de Doctor por la Universidad de Sevilla con la Mención Internacional

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CERTIFICA: Que la Tesis Doctoral titulada "**DISEÑO, CARACTERIZACIÓN Y EVALUACIÓN DE LA SEGURIDAD DE ENVASES ACTIVOS DESARROLLADOS CON ACEITES ESENCIALES**", presentada por la Lda. MARÍA LLANA RUIZ-CABELLO para optar al grado de Doctor por la Universidad de Sevilla con la Mención Internacional, ha sido realizada en el Área de Toxicología de este Departamento bajo la dirección de la Dra. Ana María Cameán Fernández, la Dra. Silvia Pichardo Sánchez y la Dra. María Puerto Rodríguez. Así mismo, certifica la estancia internacional predoctoral llevada a cabo en el Interdisciplinary Centre of Marine and Environmental Research de Oporto, Portugal, bajo la dirección del Dr. Alexandre Campos.

Y para que así conste, firmo el presente en Sevilla, a 24 de abril de 2017.

Ana María Cameán Fernández

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INFORMAN:

Que siendo directoras de la Tesis Doctoral de la Licenciada Dª MARÍA LLANA RUIZ-CABELLO, con título "**DISEÑO, CARACTERIZACIÓN Y EVALUACIÓN DE LA SEGURIDAD DE ENVASES ACTIVOS DESARROLLADOS CON ACEITES ESENCIALES**", AVALAN la idoneidad de la presentación de la misma en la modalidad de COMPENDIO POR PUBLICACIONES.

Y para que así conste a los efectos oportunos, se firma en Sevilla, a 24 de abril de 2017.

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CERTIFICAN:

Que la Tesis Doctoral titulada "DISEÑO, CARACTERIZACIÓN Y EVALUACIÓN DE LA SEGURIDAD DE ENVASES ACTIVOS DESARROLLADOS CON ACEITES ESENCIALES" ha sido realizada por la Licenciada Dª MARÍA LLANA RUIZ-CABELLO en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar a la mención de Doctor Internacional por la Universidad de Sevilla

Y para que así conste a los efectos oportunos, se firma en Sevilla, a 24 de abril de 2017.

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Este trabajo ha sido realizado en el Área de Toxicología del Departamento de Nutrición y Bromatología, Toxicología y Medicinal Legal de la Facultad de Farmacia de la Universidad de Sevilla, y se ha financiado con los siguientes Proyectos de Investigación:

- AGL2012-38357-C02-01, Plan Nacional de 2012 (Ministerio de Economía y Competitividad): Evaluación de la Seguridad para Contacto Alimentario de Envases Activos Basados en Polímeros Biodegradables y Extractos Naturales (Investigadora Principal: Ana María Cameán Fernández).
- P11-AGR-7252, Proyecto de Excelencia de la Junta de Andalucía: Desarrollo y Evaluación Toxicológica de Envases Activos Aptos para Contacto Alimentario (Investigadora Principal: Ana María Cameán Fernández).

La Doctoranda Dª María Llana Ruiz-Cabello ha disfrutado de una ayuda Predoctoral de Personal Investigador en Formación (PIF), asociada a un Proyecto de Excelencia de la Junta de Andalucía (P11-AGR- 7252), desde Junio de 2014.

Así mismo, para la realización de la estancia en el extranjero, la Doctoranda Dª María Llana Ruiz-Cabello ha disfrutado de una Ayuda de Movilidad derivada del VI Plan Propio de Investigación de la Universidad de Sevilla (2016).

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ABTS: 2,20 -azinobis-(ácido 3-etil-benzotiazolin-6-sulfonico

AE: Aceites esenciales

AECOSAN: Agencia Española de Consumo, Seguridad Alimentaria y Nutrición

AEO: aceite esencial de orégano

AEs: aceites esenciales

ADME: absorción, distribución, metabolismo y excreción

ADN: ácido desoxirribonucleico

bw: body weight

CA: aberraciones cromosómicas

Caco-2: línea celular humana de adenocarcinoma de colon

CE: Comisión Europea

CITIUS: Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla

DMT: dosis máxima tolerable

DAS: dialil sulfuro

DADS: dialil disulfuro

DATS: dialil trisulfuro

DPS: dipropil sulfuro/ dipropyl sulphide

DPDS: dipropil disulfuro/ dipropyl disulphide

DPPH: 2,2-difenil-1-picrilhidrazilo

DSC: calorimetría diferencial de barrido/ differential scanning calorimetry

EC_{50}: concentración efectiva media

EEUU: Estados Unidos

EFSA: Autoridad Europea de Seguridad Alimentaria/ European Food Safety Authority

Endo-III: endonucleasa III/ endonuclease III

ERO: especies reactivas de oxígeno

FCM: materials en contacto con alimentos/ food contact materials

FDA: Food and Drug Administration

FPG: formamidopirimidina ADN glicosilasa/ formamidopyrimidine DNA glycosylase

g: gramo

GC: cromatografía gaseosa

GRAS: Generally Recognized as Safe

GSH: glutatión/ glutathione

h: horas

HepG2: línea celular humana de hepatoma

IP: ioduro de propidio

IRMS: espectrometría de masas de relación isotópica

kg: kilogramo

M: molar

mg: miligramo

mL: mililitro

mM: milimolar

MN: micronúcleos/ micronucleus

MS: espectrometría de masa

MTD: máximum tolerated dose

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium

MTT: bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol

NIAS: sustancias añadidas involuntariamente/ non-intentionally added substances

NOAEL: No-Observed-Adverse-Effect-Level

OCDE: Organización para la Cooperación y Desarrollo Económico

OECD: The Organisation for Economic Co-operation and Development

OEO: oregano essential oil

OS: compuestos organosulfurados

pc: peso corporal

PE: polietileno/ polyethylene

PLA: ácido poliláctico/ polylactic acid

PP: polipropileno/ polypropilene

PT: proteínas totales

PTSO: propil propano tiosulfonato/ propyl propane thiosulphonate

PUFAs: ácido grasos poliinsaturados/ polyunsaturated fatty acids

Py-GC/MS: pirólisis analítica/ analytical pyrolysis

RN: rojo neutro

ROS: reactive oxigen species

S9: fracción metabólica externa

TGA: termogravimetría/. Thermogravimetric analysis

UE: Unión Europea

µM: micromolar

II. RESUMEN / SUMMARY

RESUMEN

Las nuevas tendencias alimentarias constituyen un reto para la industria alimentaria que debe lanzar al mercado productos atractivos para los consumidores, cada vez más informados y preocupados por el binomio calidad-seguridad. Por otro lado, la globalización alimentaria hace necesario desarrollar nuevos materiales de envasado que permitan a las mercancías recorrer largas distancias y permanecer más tiempo en condiciones óptimas en los canales de distribución.

Una de las estrategias que la industria ha encontrado para cubrir esta necesidad ha sido el diseño de envases activos. Estos materiales que incorporan sustancias con propiedades biológicas, permiten aumentar la vida útil de los alimentos manteniendo sus cualidades nutricionales y su seguridad al crear, en el caso que nos ocupa, una atmósfera protectora en contacto con el alimento.

Los aceites esenciales (AEs), productos aromáticos sintetizados en distintas partes de las plantas, han mostrado a lo largo de los siglos un gran atractivo tanto por su agradable aroma, como por las propiedades biológicas (antioxidantes, antimicrobianas, antiparasitarias, etc.) con las que se han relacionado. En este sentido, y teniendo en cuenta que los consumidores responden de manera positiva a la sustitución de aditivos sintéticos por nuevos aditivos naturales con mejor percepción de seguridad, la industria alimentaria se hace eco y pone el foco en este tipo de sustancias. Para que un AE sea aprobado como aditivo alimentario o como sustancia activa y pueda ser incorporada legalmente al envasado activo, la Unión Europea (UE), a través de Autoridad Europea de Seguridad Alimentaria (EFSA), exige una exhaustiva evaluación de la toxicidad de estas sustancias para confirmar la seguridad asociada a su uso.

Uno de los aspectos fundamentales que se exigen para esta evaluación es el estudio del daño al material genético que las sustancias pueden producir. Por ello, nos pareció fundamental revisar la bibliografía existente relacionada con la genotoxicidad de los AEs utilizados habitualmente en alimentación y de manera más concreta de aquellos aceites seleccionados para desarrollar envases activos. El estudio de la bibliografía publicada mostró ausencia de potencial mutagénico y genotóxico para una amplia variedad de los AEs evaluados, así como para sus componentes mayoritarios. Sin embargo, la mayoría de estos estudios se han llevado a cabo respetando solo en parte las recomendaciones de la EFSA o las guías propuestas por la Organización para la Cooperación y el Desarrollo Económicos (OCDE). Por este motivo, se hace necesario reevaluar estas sustancias siguiendo las directrices propuestas por las autoridades europeas. La revisión bibliográfica citada ha dado lugar a la siguiente publicación:

- ***IN VITRO TOXICOLOGICAL EVALUATION OF ESSENTIAL OILS AND THEIR MAIN COMPOUNDS USED IN ACTIVE FOOD PACKAGING: A REVIEW.*** *(Llana-Ruiz-Cabello et al., 2015; Food and Chemical Toxicology 81, 9-27)*

Con objeto de reevaluar en profundidad, y de acuerdo a la nueva legislación europea, las posibles sustancias utilizadas para el desarrollo de envase activos, se seleccionaron para la presente tesis doctoral los extractos de aliáceas y el aceite esencial de orégano (AEO), tanto por sus propiedades activas como por la ausencia o contradicción de los resultados toxicológicos existentes.

Para diseñar la evaluación de la toxicidad, se tuvo en cuenta que la vía oral va a ser la de exposición preferente para estos AEs puesto que los consumidores estarán expuestos a ellas a través del alimento. Así, para la evaluación *in vitro* se seleccionaron modelos celulares del aparato digestivo. En primer lugar, las células de hepatocarcinoma humano (HepG2) como modelo hepático por ser el hígado el órgano principal involucrado en la biotransformación de xenobióticos. Por otro lado, las células de adenocarcinoma de colon humano (Caco-2) por ser el intestino el órgano encargado de la absorción y donde existe un mayor tiempo de contacto con las sustancias estudiadas.

Para evaluar la toxicidad *in vitro* de los componentes mayoritarios de los AEs, las líneas celulares fueron expuestas a compuestos sulfurados presentes en los extractos de aliáceas (dipropil sulfuro (DPS), dipropil disulfuro (DPDS) y propil propano tiosulfonato (PTSO)) y a compuestos fenólicos mayoritarios del AEO (carvacrol y timol). Dentro de la batería de ensayos llevados a cabo se incluyeron estudios de citotoxicidad basal, análisis de la morfología celular, estrés oxidativo (producción de especies reactivas de oxígeno (ERO) y contenido en glutatión reducido (GSH)), muerte celular por citometría de flujo y actividad antioxidante (incluyendo protección y reversión del daño inducido por oxidantes, como peróxido de hidrógeno). Además, se evaluó el potencial mutagénico de estas sustancias mediante el test de Ames, utilizando 5 cepas de *Salmonella thyphimurium* auxotrófas para histidina y se incluyó el ensayo cometa estándar y modificado con enzimas de restricción endonucleasa III (Endo-III) y formamidopirimidina glicosila (FPG), para evaluar el potencial genotóxico. Por último, para completar la evaluación del PTSO, se realizó un ensayo de toxicidad oral aguda para determinar la dosis máxima tolerable (DMT) en roedores.

De manera general, tanto los compuestos sulfurados DPS y DPDS como el timol, mostraron ausencia de toxicidad a las concentraciones ensayadas. En los casos de PTSO y carvacrol se observaron daños, a concentraciones superiores a las que se utilizarían en envasado

activo, estableciéndose una DMT en roedores de 55 mg/Kg peso corporal (pc) para PTSO. Además, los resultados han revelado que el carvacrol induce actividad mutagénica y presenta capacidad para oxidar las bases púricas del ADN. Los resultados obtenidos de estos ensayos han dado lugar a las siguientes publicaciones:

- ***ESTUDIO IN VITRO DE LA VIABILIDAD DE CÉLULAS CACO-2 EN PRESENCIA DE COMPONENTES DEL ACEITE ESENCIAL DE ALLIUM SPP.*** *(Llana-Ruiz-Cabello et al., 2013, Revista Española de Toxicología 30, 144-148).*

- ***CYTOTOXIC AND MUTAGENIC IN VITRO ASSESSMENT OF TWO ORGANOSULFUR COMPOUNDS DERIVED FROM ONION TO BE USED IN THE FOOD INDUSTRY.*** *(Llana-Ruiz-Cabello et al., 2015. Food Chemistry 166, 423-431).*

- ***ACUTE TOXICOLOGICAL STUDIES OF THE MAIN ORGANOSULFUR COMPOUND DERIVED FROM ALLIUM SP. INTENDED TO BE USED IN ACTIVE FOOD PACKAGING.*** *(Llana-Ruiz-Cabello et al., 2015. Food and Chemical Toxicology 82, 1-11).*

- ***CYTOTOXICITY AND MORPHOLOGICAL EFFECTS INDUCED BY CARVACROL AND THYMOL ON THE HUMAN CELL LINE CACO-2.*** *(Llana-Ruiz-Cabello et al., 2014. Food and Chemical Toxicology 64, 281-290).*

- ***EVALUATION OF THE MUTAGENICITY AND GENOTOXIC POTENTIAL OF CARVACROL AND THYMOL USING THE AMES SALMONELLA TEST AND ALKALINE, ENDO-III AND FPG-MODIFIED COMET ASSAYS WITH THE HUMAN CELL LINE CACO-2.****(Llana-Ruiz-Cabello et al., 2014. Food and Chemical Toxicology 72, 122-128).*

- ***IN VITRO PRO-OXIDANT/ANTIOXIDANT ROLE OF CARVACROL, THYMOL AND THEIR MIXTURE IN THE INTESTINAL CACO-2 CELL LINE.*** *(Llana-Ruiz-Cabello et al., 2015. Toxicology in Vitro 29, 647-656).*

Tras valorar todos los resultados obtenidos *in vitro*, se impuso la necesidad de evaluar *in vivo* la toxicidad de las sustancias. En primer lugar, porque la legislación europea (EFSA 2016) exige llevar a cabo un estudio de toxicidad oral subcrónica a 90 días para todas aquellas sustancias que vayan a ser incluidas en materiales de envasado alimentario y cuya exposición se prevea superior a 1,5 µg/kg pc al día. Se seleccionaron ratas Wistar a las que se administró AEO durante 90 días. Transcurrido el período de exposición, los animales fueron sacrificados, y se extrajeron los órganos y la sangre, para llevar a cabo una evaluación histopatológica, bioquímica y hematológica. Los resultados obtenidos en este estudio no mostraron efectos adversos para

ninguna de las dosis administradas, y de ellos se dedujo un NOAEL de 200 mg/kg pc para AEO (*Origanum vulgare* L. *virens*) para el citado modelo experimental. Estos resultados dieron lugar a la siguiente publicación:

- ***A SUBCHRONIC 90-DAY ORAL TOXICITY STUDY OF ORIGANUM VULGARE ESSENTIAL OIL IN RATS.*** *(Llana-Ruiz-Cabello et al., 2017. Food and Chemical Toxicology 101, 36-47).*

La segunda razón que nos llevó a estudiar la toxicidad *in* vivo fue que la guía para evaluar la genotoxicidad de las sustancias (EFSA, 2011) insta a confirmar *in vivo* la genotoxicidad contradictoria o no concluyente obtenida *in vitro*. Teniendo en cuenta las recomendaciones del citado documento, se seleccionaron el ensayo de micronúcleos (MN) en médula ósea; y el ensayo cometa, en estómago, hígado y sangre periférica, para evaluar el potencial genotóxico de carvacrol y AEO *in vivo*. Ambas sustancias mostraron resultados negativos para los dos ensayos, y también ausencia de alteraciones histopatológicas, en todos los tejidos evaluados. Según las propuestas por la OCDE para llevar a cabo estos ensayos, la ausencia de toxicidad debe ser corroborada con algún método que confirme que los tejidos diana has estado expuestos a las sustancias objeto de estudio (OCDE 474 y OCDE 489). En este sentido, y por primera vez según nuestro conocimiento, se ha llevado a cabo pirolisis analítica como técnica para demostrar presencia de compuestos naturales en muestras biológicas. Los resultados obtenidos de estos trabajos se recogen en las siguientes publicaciones:

- ***GENOTOXICITY EVALUATION OF CARVACROL IN RATS USING A COMBINED MICRONUCLEUS AND COMET ASSAY.*** *(Llana-Ruiz-Cabello et al., 2016. Food and Chemical Toxicology 98, 240-250).*

- ***COMBINED MICRONUCLEUS AND COMET ASSAY TO EVALUATE THE GENOTOXICITY OF OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) IN RATS ORALLY EXPOSED FOR 90 DAYS.*** *(Llana-Ruiz-Cabello et al., 2017. Food Research International (en revisión/under revision))*

Durante la realización de esta tesis, la doctoranda llevó a cabo varias estancias nacionales (ITENE (Valencia), y DOMCA S.A.U. (Granada)) para desarrollar y caracterizar materiales activos diseñados con las sustancias objeto de estudio. Se desarrollaron envases a base de matrices plásticas tradicionales como el polipropileno (PP), y otros utilizando nuevos polímeros biodegradables como el ácido poliláctico (PLA). En estas matrices se incorporaron mediante extrusión un extracto de aliáceas (Proallium®) y AEO a diferentes concentraciones y se procedió a

la caracterización físico-mecánica de los nuevos materiales, evaluando además las propiedades ópticas y térmicas. Por otro lado, se llevó a cabo la evaluación de la funcionalidad de estos nuevos envases como materiales antioxidantes y antimicrobianos y su capacidad para intervenir en la vida útil de lechugas de cuarta gama y jamón cocido. De manera general, todos los materiales diseñados mostraron propiedades físico-mecánicas, ópticas y térmicas compatibles con el envasado alimentario. Sin embargo, la vida útil de los alimentos envasados en ellos no se ha visto incrementada en ninguno de los casos, incluso cuando los materiales han mostrado actividad antioxidante y/o antimicrobiana. Los resultados obtenidos de estos ensayos se incluyen en las siguientes publicaciones:

- ***CHARACTERISATION AND EVALUATION OF PLA FILMS CONTAINING AN EXTRACT OF ALLIUM SPP. TO BE USED IN THE PACKAGING OF READY-TO-EAT SALAD UNDER CONTROLLED ATMOSPHERES.*** *(Llana-Ruiz-Cabello et al., 2015. LWT-Food Science and Technology 64, 1354-1361).*

- ***DEVELOPMENT OF PLA FILMS CONTAINING OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) INTENDED FOR USE IN FOOD PACKAGING.*** *(Llana-Ruiz-Cabello et al., 2016. Food Additives and Contaminants: Part A 33, 1374-1386).*

- ***CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ACTIVE POLYPROPYLENE FILMS CONTAINING ORIGANUM AND ALLIUM EXTRACTS***. (Llana-Ruiz-Cabello et al., 2017. *LWT-Food Science and Technology (en revisión/under revision))*

Teniendo en cuenta la alta volatilidad de los extractos naturales y las altas temperaturas que se alcanzan en los procesos de extrusión de plásticos, es importante determinar que las concentraciones de activo en el material final siguen siendo funcionales. Habitualmente, la termogravimetría (TGA) o la calorimetría diferencial de barrido (DSC) han sido utilizadas con este fin y por ello han sido incluidas en la caracterización de los materiales de la presente tesis. Sin embargo, y tras valorar el potencial de la pirolisis analítica (Py-GC/MS) como técnica para determinar compuestos naturales en matrices bio, se han llevado a cabo diferentes estudios para confirmar el contenido de sustancias activas en las matrices poliméricas utilizando esta técnica. Los resultados obtenidos mediante pirolisis analítica reflejados en las siguientes publicaciones, son muy similares a los proporcionados por las técnicas tradicionales (TGA y DSC), demostrando que la pirólisis es una técnica rápida y útil para identificar y cuantificar aditivos naturales en matrices poliméricas biodegradables.

- ***CHARACTERISATION OF A BIO-BASE PACKAGING CONTAINING A NATURAL ADDITIVE FROM ALLIUM SPP. USING ANALYTICAL PYROLYSIS AND CARBON STABLE ISOTOPES.*** *(Llana-Ruiz-Cabello et al., 2016. Journal of Analytical and Applied Pyrolysis 120, 334-340).*

- ***MOLECULAR CHARACTERISATION OF A BIO-BASED ACTIVE PACKAGING CONTAINING ORIGANUM VULGARE L. ESSENTIAL OIL USING PYROLYSIS GAS CHROMATOGRAPHY-MASS SPECTROMETRY.*** *(Llana-Ruiz-Cabello et al., 2016. Journal of the Science of Food and Agriculture 96, 3207-3212).*

- ***MONITORING NATURAL ADDITIVES IN POLYLACTIC ACID ACTIVE FOOD PACKAGES. PYROLYSIS-GAS CHROMATOGRAPHY-ISOTOPE RATIO MASS SPECTROMETRY ANALYSIS.*** *(Llana-Ruiz-Cabello et al., 2017. Journal of Chromatography A. (en revisión/under revision))*

Tras observar que la vida útil no había aumentado en ninguno de los modelos experimentales, decidimos valorar si el perfil nutricional del alimento sufría cambios durante la etapa de almacenamiento en los envases activos en relación a los cambios que se producen en envasado tradicional. Para ello seleccionamos los films diseñados a partir de PLA con AEO y estudiamos, mediante Py-GC/MS, el perfil nutricional de la lechuga envasada en estos materiales. Los resultados han mostrado que los films de PLA con AEO consiguen mantener los niveles de ácidos grasos poliinsaturados (PUFA) y de fitosteroles durante el tiempo de vida útil de la lechuga. Estos resultados darán lugar a la siguiente publicación:

- ***EFFECT OF AN ACTIVE BIO-PACKAGE IN THE PRESERVATION OF NUTRITIONALLY IMPORTANT PHYTOCONSTITUENTS IN READY-TO-EAT LETTUCE.*** *(Llana-Ruiz-Cabello et al., 2017. Journal of Food Composition and Analysis (en revision/under revision))*

Además, y teniendo en cuenta que ambos activos (Proallium® y AEO) tienen actividades complementarias, habiendo mostrado el Proallium® mayor efecto antimicrobiano y el AEO mayor efecto antioxidante, consideramos interesante diseñar un material de envasado que incorporase ambos. Se ha utilizado una matriz de PLA y se han incorporado los activos mediante extrusión, procediéndose a desarrollar la caracterización físico-mecánica llevada a cabo para el resto de materiales y la evaluación de la funcionalidad en lechugas de cuarta gama para poder comparar los resultados con los obtenidos para los materiales que solo incorporan un activo. Los resultados de estos estudios darán lugar a la siguiente publicación:

- ***CHARACTERISATION OF A BIO-BASED POLYLACTIC ACID PACKAGING CONTAINING PROALLIUM AND OEO*** *(Título provisional)*

Por último, para confirmar la seguridad derivada del uso de esta combinación, decidimos evaluar la toxicidad de la mezcla de los componentes mayoritarios de ambos activos (PTSO y carvacrol) en las líneas celulares HepG2 y Caco-2 mediante ensayos de citotoxicidad y alteraciones morfológicas ultraestructurales. Los resultados de estos estudios darán lugar a la siguiente publicación:

- ***CITOTOXICITY, MORPHOLOGICAL EFFECTS AND SINERGIES OBSERVED IN MIXTURES CONTAINING PTSO AND CARVACROL*** *(Título provisional)*

SUMMARY

New trends in consumer demand, increasingly informed and concerned about the quality and safety of food, are affecting the food industry, which need to launch to the market attractive products to consumers. Moreover, changes in retailing practices due to globalization force the industry to develop new packaging materials that allow food to travel long distances and to maintain its properties in perfect conditions through the distribution channels.

One of the strategies that the industry has found to fulfill this need has been the design of active packaging. Active food contact materials can incorporate substances with biological properties, which are intended to be released into the headspace of packages with the aim of increasing the shelf-life of food maintaining its nutritional profiles and safety.

Essential oils (EOs), secondary metabolites which are produced in different parts of the plants, have been traditionally proved to have many applications due to their flavor and fragrance for flavoring foods, as well as due to their bioactive properties such as antioxidant or antimicrobial characteristics. In this sense, considering that consumers are willing the substitution of synthetic additives for natural ones with a relative safe status, food industry has focused on using such substances. In Europe, the EOs allowed to be used as additive or as active substance in food packaging should be approved by the European Commission after the safety assessment developed by the European Food Safety Authority (EFSA), because the safety of these substances should be guaranteed before being commercialized.

One of the main aspects required in the safety assessment is to evaluate the mutagenic and genotoxic potential of these substances. Therefore, it is important to carry out a comprehensive review of the genotoxicity data published in the scientific literature up to now regarding EOs intended to be used in food, and more specifically, regarding those oils selected to develop active packaging. The study of the published literature showed absence of mutagenic and genotoxic potential for a wide variety of EOs evaluated, as well as for their main compounds. However, most of these studies has only partially followed the recommendations by EFSA and the Organization for Economic Co-operation and Development (OECD) protocols. Consequently, it is necessary to perform complete studies following the guidelines proposed by the European Authorities. This review has led to the following publication:

- ***IN VITRO TOXICOLOGICAL EVALUATION OF ESSENTIAL OILS AND THEIR MAIN COMPOUNDS USED IN ACTIVE FOOD PACKAGING: A REVIEW.*** *(Llana-Ruiz-Cabello et al., 2015; Food and Chemical Toxicology 81, 9-27)*

In order to evaluate in depth the safety of EOs according to the European legislation, oregano essential oil (OEO), *Allium* spp. extracts and their main compounds, were selected as the main topic of study in this Doctoral Thesis due to their active properties and because of the results published are scarce or contradictory.

In order to design the toxicological assessment, the experimental models were chosen to represent oral exposure to these substances taking into account that consumers will be exposed to them through the food. Therefore, for the *in vitro* assessment, human hepatoma cell line (HepG2) was selected because the liver is the most active organ in metabolism of substances and colon adenocarcinoma cell line (Caco-2) was chosen because the intestine is considered as site-of-contact tissue and main absorption site.

For the *in vitro* evaluation of the major components of EOs cell lines were exposed to the main organosulfur compounds in *Allium* spp. extracts (dipropyl sulphide (DPS), dipropyl disulphide (DPDS) and propyl propane thiosulfonate (PTSO)), and were also exposed to phenolic compounds observed in OEO (carvacrol and thymol). The experiments performed were: basal cytotoxicity, cell morphology, oxidative stress (production of reactive oxygen species (ROS) and glutathione content (GSH)), mechanisms of death with flow cytometry and antioxidant activity (including protection and reversion effects against hydrogen peroxide). Moreover, the mutagenic effects of these substances were developed by the Ames test using five different strains of *Salmonella thyphimurium* that carry mutations in genes involved in histidine synthesis. Furthermore, the genotoxicity of these compounds were evaluated through the standard and modified-comet assays using endonuclease III (ENDO-III) and formamidopyrimidine DNA glicosilase (FPG) to measure oxidized pyrimidines and purines, respectively. Finally, in order to complete the PTSO evaluation, an *in vivo* study was conducted in rats orally expose to this substance to establish it maximum tolerated dose (MTD).

In general, no toxicity was observed for DPS, DPDS and thymol at the concentrations assayed. However, toxic effects were observed in both cell lines after exposure to PTSO and carvacrol at concentrations higher than those which are expected to be used in active packaging. Moreover, the MTD of PTSO was set at 55 mg/kg body weight (bw). In addition, results showed that carvacrol were able to produce mutagenic activity and ability to oxidize purine bases. The results of these experiments have led to the following publications:

- ***ESTUDIO IN VITRO DE LA VIABILIDAD DE CÉLULAS CACO-2 EN PRESENCIA DE COMPONENTES DEL ACEITE ESENCIAL DE ALLIUM SPP.*** *(Llana-Ruiz-Cabello et al., 2013, Revista Española de Toxicología 30, 144-148).*

- ***CYTOTOXIC AND MUTAGENIC IN VITRO ASSESSMENT OF TWO ORGANOSULFUR COMPOUNDS DERIVED FROM ONION TO BE USED IN THE FOOD INDUSTRY.*** *(Llana-Ruiz-Cabello et al., 2015. Food Chemistry 166, 423-431).*

- ***ACUTE TOXICOLOGICAL STUDIES OF THE MAIN ORGANOSULFUR COMPOUND DERIVED FROM ALLIUM SP. INTENDED TO BE USED IN ACTIVE FOOD PACKAGING.*** *(Llana-Ruiz-Cabello et al., 2015. Food and Chemical Toxicology 82, 1-11).*

- ***CYTOTOXICITY AND MORPHOLOGICAL EFFECTS INDUCED BY CARVACROL AND THYMOL ON THE HUMAN CELL LINE CACO-2.*** *(Llana-Ruiz-Cabello et al., 2014. Food and Chemical Toxicology 64, 281-290).*

- ***EVALUATION OF THE MUTAGENICITY AND GENOTOXIC POTENTIAL OF CARVACROL AND THYMOL USING THE AMES SALMONELLA TEST AND ALKALINE, ENDO-III AND FPG-MODIFIED COMET ASSAYS WITH THE HUMAN CELL LINE CACO-2.****(Llana-Ruiz-Cabello et al., 2014. Food and Chemical Toxicology 72, 122-128).*

- ***IN VITRO PRO-OXIDANT/ANTIOXIDANT ROLE OF CARVACROL, THYMOL AND THEIR MIXTURE IN THE INTESTINAL CACO-2 CELL LINE.*** *(Llana-Ruiz-Cabello et al., 2015. Toxicology in Vitro 29, 647-656).*

After having studied all the results obtained *in vitro*, the need to evaluate the *in vivo* toxicity of the substances was evidenced. First of all because the European legislation request *in vivo* toxicity evaluation of EOs and their main compounds in the tiered approach to toxicity testing, demanding an extended 90-day oral toxicity study in rodents for substances intended to be used in food contact materials and whose estimating exposure level for humans are greater than 1.5 µg/kg bw per day (EFSA, 2016). To develop the repeated-dose oral assay, wistar rats were exposed to OEO during 90 days. At the end of the exposure period, the animals were sacrificed, and organs and blood were removed for histopathological, biochemical and haematological evaluation. The results obtained in this study did not show adverse effects for any dose administered. Therefore, the oral no-observed-adverse-effect level (NOAEL) was set at 200 mg/kg bw of OEO (*Origanum vulgare* L. *virens*). The results obtained are compiled in the following publication:

- ***A SUBCHRONIC 90-DAY ORAL TOXICITY STUDY OF ORIGANUM VULGARE ESSENTIAL OIL IN RATS.*** *(Llana-Ruiz-Cabello et al., 2017. Food and Chemical Toxicology 101, 36-47).*

Secondly, in the case of inconclusive or contradictory results, as well as positive results from the *in vitro* assays, it may be appropriate to conduct further *in vivo* tests (EFSA, 2011). According to the European recommendations, micronucleus (MN) assay in bone marrow and comet assay in stomach, liver and blood samples were developed to evaluate the *in vivo* genotoxic potential of OEO and its main compound, carvacrol. Results revealed absence of genotoxicity for OEO and carvacrol in both assays. Moreover, no histopathological changes were observed in any of the tissues evaluated. Finally, according to OECD protocols the negative results must be corroborated by a method that confirms that target tissues have been exposed to the substances under study (OECD 474 and OECD 489). As far as we know, analytical pyrolysis (Py-GC/MS) was developed for the first time to verify that the test chemical reached the target tissue, or general circulation. The results are summarized in the following publications:

- ***GENOTOXICITY EVALUATION OF CARVACROL IN RATS USING A COMBINED MICRONUCLEUS AND COMET ASSAY.*** *(Llana-Ruiz-Cabello et al., 2016. Food and Chemical Toxicology 98, 240-250).*

- ***COMBINED MICRONUCLEUS AND COMET ASSAY TO EVALUATE THE GENOTOXICITY OF OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) IN RATS ORALLY EXPOSED FOR 90 DAYS.*** *(Llana-Ruiz-Cabello et al., 2017. Food Research International (en revisión/under revision))*

For the fulfillment of this thesis, the PhD student performed an internship in ITENE (Valencia, Spain) and DOMCA S.A.U (Granada, Spain) in order to develop and characterize different active materials. New active films based on traditional plastic matrices such as polypropylene (PP), and others using new biodegradable polymers such as polylactic acid (PLA) containing Proallium® and OEO were obtained by melt blending and its evaluation was performed through different assays: physical, mechanical, optical and thermal properties. Moreover, antioxidant and antimicrobial properties of these new films were also evaluated *in vitro* and in real food models such as lettuce and cooked ham. In general, the new material showed good physical and mechanical properties compatible food packaging. However, the shelf-life of perishable food has not been improved in any case, even when the films exhibited antioxidant and antimicrobial properties. The results obtained in these experiments have led to the following publications:

- ***CHARACTERISATION AND EVALUATION OF PLA FILMS CONTAINING AN EXTRACT OF ALLIUM SPP. TO BE USED IN THE PACKAGING OF READY-TO-EAT SALAD UNDER CONTROLLED ATMOSPHERES.*** *(Llana-Ruiz-Cabello et al., 2015. LWT-Food Science and Technology 64, 1354-1361).*

- ***DEVELOPMENT OF PLA FILMS CONTAINING OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) INTENDED FOR USE IN FOOD PACKAGING.*** *(Llana-Ruiz-Cabello et al., 2016. Food Additives and Contaminants: Part A 33, 1374-1386).*

- ***CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ACTIVE POLYPROPYLENE FILMS CONTAINING ORIGANUM AND ALLIUM EXTRACTS***. (Llana-Ruiz-Cabello et al., 2017. *LWT-Food Science and Technology (en revisión/under revision))*

Due to the high volatility of EOs and the high temperatures reached in the plastic extrusion processes, it is necessary to ensure that the active substances remain in the desired quantities in the final manufactured polymer. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) are common techniques to evaluate this aspect; and therefore they have been included in the characterization of the materials of the present thesis. However, after assessing the potential of analytical pyrolysis as a technique for determining natural compounds in biomatrices, different studies have been carried out to confirm the content of active substances in the polymer matrices using this technique. Results obtained for Py-GC/MS revealed similar data than obtained by TGA and DSC; hence, the Py-GC/MS seems to be a convenient method for inexpensive and relatively rapid analysis of synthetic or bio-based polymers including EOs. The results obtained in these experiments were summarized in the following publications:

- ***CHARACTERISATION OF A BIO-BASE PACKAGING CONTAINING A NATURAL ADDITIVE FROM ALLIUM SPP. USING ANALYTICAL PYROLYSIS AND CARBON STABLE ISOTOPES.*** *(Llana-Ruiz-Cabello et al., 2016. Journal of Analytical and Applied Pyrolysis 120, 334-340).*

- ***MOLECULAR CHARACTERISATION OF A BIO-BASED ACTIVE PACKAGING CONTAINING ORIGANUM VULGARE L. ESSENTIAL OIL USING PYROLYSIS GAS CHROMATOGRAPHY-MASS SPECTROMETRY.*** *(Llana-Ruiz-Cabello et al., 2016. Journal of the Science of Food and Agriculture 96, 3207-3212).*

- ***MONITORING NATURAL ADDITIVES IN POLYLACTIC ACID ACTIVE FOOD PACKAGES. PYROLYSIS-GAS CHROMATOGRAPHY-ISOTOPE RATIO MASS SPECTROMETRY***

ANALYSIS. *(Llana-Ruiz-Cabello et al., 2017. Journal of Chromatography A. (en revisión/under revision))*

Considering that the new materials were not able to improve the shelf-life of food, we decided to assess if the nutritional profile of the food underwent changes during the storage stage in the active materials in relation to the changes that occur in traditional packaging. The evaluation of the nutritional profile of lettuce packaged with PLA films containing OEO has been developed using Py-GC/MS. Results showed that PLA containing OEO allow maintaining the profile of polyunsaturated fatty acids (PUFAs) and phytosterols during the shelf life lettuce. This data are compiled in the following publication:

- ***EFFECT OF AN ACTIVE BIO-PACKAGE IN THE PRESERVATION OF NUTRITIONALLY IMPORTANT PHYTOCONSTITUENTS IN READY-TO-EAT LETTUCE.*** *(Llana-Ruiz-Cabello et al., 2017. Journal of Food Composition and Analysis (en revision/under revision))*

Moreover, taking into account that both active (Proallium® and OEO) have complementary activities, Proallium® have shown greater antimicrobial effect and OEO greater antioxidant effect, films containing Proallium® and OEO were developed. These new films were designed to include both active substances by melt blending into PLA matrices. Physical and mechanical characterization was carried out and the antimicrobial activity of films in ready to eat salad was also assayed. These results will be included in the following publication:

- ***CHARACTERISATION OF A BIO-BASED POLYLACTIC ACID PACKAGING CONTAINING PROALLIUM AND OEO*** *(provisional title)*

Finally, in order to confirm the safety of the Proallium®-OEO mixture, the *in vitro* cytotoxicity and morphological effects of the mixture of their main compounds, PTSO and carvacrol, respectively in HepG2 and Caco-2 cells were developed. These results will be included in the following publication:

CITOTOXICITY, MORPHOLOGICAL EFFECTS AND SINERGIES OBSERVED IN MIXTURES CONTAINING PTSO AND CARVACROL *(provisional title)*

I. INTRODUCCIÓN / INTRODUCTION

Actualmente, con la apertura al comercio internacional, los alimentos que llegan a nuestras mesas proceden de todo el mundo, entre otros motivos porque los consumidores desean tener acceso a alimentos considerados "de temporada" durante todo el año. Este aumento de las importaciones y exportaciones de alimentos hace que los riesgos de la seguridad alimentaria no conozcan fronteras internacionales (Fukuda, 2015). Lo que en el pasado eran problemas regionales o nacionales, actualmente podrían llegar a afectar a Europa o al mundo entero si el causante del incidente es un alimento que procede de un área geográfica diferente.

En este marco mundial actual, en el que la alimentación se ha convertido en un bien globalizado, la industria del envasado alimentario pone el foco en el desarrollo de envases capaces de transportar alimentos perecederos a través de largas distancias sin que sus características organolépticas, nutricionales y de seguridad se vean comprometidas (Battini et al., 2016).

Para llevar a cabo esta empresa, la industria alimentaria ha debido adaptarse a las nuevas demandas de los consumidores. Por un lado, el incremento de la sensibilidad de la población con respecto al medio ambiente y los movimientos conocidos como "green consumerism" han obligado a la industria a decantarse por opciones con perspectivas más ecológicas y sostenibles (Ramos et al., 2012). Por otro lado, tras las grandes crisis alimentarias de los años 90 (encefalopatía espongiforme bovina, fiebre aftosa, dioxinas), la preocupación de los consumidores por temas relacionados con la seguridad alimentaria ha experimentado un enorme incremento. La población se ha vuelto más crítica y exige información más rigurosa en relación a la calidad y a la inocuidad de los alimentos que se consumen (Grunert, 2005).

Por todo ello, el desarrollo de esta nueva generación de envases, que debe contemplar el uso de matrices ecológicas, tiene también como reto disminuir el uso de aditivos sintéticos en los alimentos y sustituirlos por sustancias de origen natural que sean capaces de mantener o incrementar la vida útil del alimento durante la etapa de distribución del mismo, generando así, alternativas de envasado que respondan a las necesidades del sistema de alimentación global, a la "conciencia eco" y a la exigencia de los consumidores de productos más saludables.

Es en este contexto dónde nace el envasado activo que, aunque es un término relativamente reciente, como concepto ya venía desarrollándose desde hace siglos en regiones de África, Asia o Sudamérica donde las hojas de los árboles han sido y continúan siendo utilizadas para envolver, cocinar y conservar los alimentos, beneficiándose así de las

propiedades antioxidantes, antibacterianas o aromáticas que confiere el contacto de las sustancias naturales de las hojas con los alimentos (Dainelli et al., 2008).

En Europa, los materiales activos se legislan en conjunto con otros envases innovadores conocidos como inteligentes que, a diferencia de los envases activos, son diseñados para monitorizar el estado de los alimentos envasados o de su entorno y que incorporan sustancias inteligentes que van a informar al consumidor de esta situación.

1. ENVASADO ACTIVO

Introducidos en Japón a mediados de 1970, los envases activos llamaron la atención de Europa y EEUU mucho más tarde, a mediados de los años 90 (Restucia et al., 2010).

1.1 Definición

La primera definición de envase activo aparece en la legislación europea en el artículo 2 del Reglamento (CE) Nº 1935/2004 sobre los materiales y objetos destinados a entrar en contacto con alimentos que recoge que los "materiales y objetos activos en contacto con alimentos" son:

"los materiales y objetos destinados a ampliar el tiempo de conservación, o a mantener o mejorar el estado de los alimentos envasados, y que están diseñados para incorporar deliberadamente componentes que transmitan sustancias a los alimentos envasados o al entorno de éstos o que absorban sustancias de los alimentos envasados o del entorno de éstos."

El envase alimentario se diseñó inicialmente para constituir una barrera física que protegiera al alimento de influencias externas que pudieran provocar la degradación de los mismos; como la luz, la humedad o la temperatura. Incluso pretendían proteger de olores, microorganismos, golpes o polvo ambiental (Dainelli et al., 2008). Una de las grandes novedades que plantean estos nuevos materiales activos es, precisamente, que se basan en la interacción con el alimento. En el envasado tradicional, se perseguía que los materiales actuasen únicamente como barrera física que aislara al alimento del ambiente externo "hostil"; por lo que eran más interesantes cuanto más inertes, impermeables y resistentes (Dainielli et al., 2008).

En los envases activos, las sustancias funcionales que interaccionarán con el alimento o el entorno del mismo pueden ser incluidas en contenedores diferentes, y bien diferenciados del envase principal, o bien directamente incorporadas en las matrices de los materiales de envasado (CE, 2004, 2009). Independientemente de la modalidad que se seleccione, estos envases activos pretenden conseguir que los alimentos lleguen a los consumidores en perfectas condiciones organolépticas y nutricionales gracias a un aumento de su vida útil. El concepto de vida útil se define como el período anterior a la fecha de duración mínima o a la «fecha de caducidad», es decir, fecha hasta la cual dicho producto alimenticio mantiene sus propiedades específicas siempre que el producto se guarde en condiciones de conservación adecuadas (CE, 2005).

En los envases que se desarrollan y evalúan en la presente tesis doctoral, este aumento de vida útil se produciría gracias a un mecanismo de difusión que permite que la sustancia activa migre desde el envase hasta la superficie del alimento o bien que se disuelva en la atmósfera que lo rodea, conocida como espacio de cabeza, para allí ejercer su función (Nerín et al, 2006).

1.2 Marco normativo respecto a envasado activo en Europa

La mayor parte de la legislación alimentaria se establece a nivel europeo, en forma de reglamentos o directivas, con el objetivo de unificar los requisitos de todos los Estados miembros y garantizar la libre circulación de los alimentos dentro la Unión Europea (UE).

1.2.1 Reglamento 1935/2004/CE

La tendencia europea de legislar mediante directivas estaba generando una dispersión normativa y agravios comparativos entre los diferentes Estados miembros puesto que las transposiciones no respetaban debidamente lo exigido por la directiva. Así, las instituciones de la UE comenzaron a regular mediante reglamentos para evitar esta situación de desigualdad y favorecer el mercado común.

En el año 2004 se publicó el Reglamento 1935/2004/CE que constituye el marco legislativo para materiales de envasado alimentario, común en toda la UE. Esta norma indica, en el artículo 1, que su finalidad es garantizar el funcionamiento del mercado interior en relación con la comercialización en la Comunidad Europea de los materiales en contacto con

alimentos, garantizando un elevado nivel de protección de la salud humana y de los intereses de los consumidores.

Los requisitos generales para todos los materiales en contacto con alimentos se detallan en el artículo 3, donde se establece que todos los materiales deben ser fabricados siguiendo las buenas prácticas de fabricación para evitar que se transfieran los componentes en cantidades que puedan representar un peligro para la salud humana, provocar modificación inaceptable en la composición del alimento o alterar sus características organolépticas.

Aunque en el Reglamento se hace referencia a todos los materiales en contacto con alimentos, esta norma ofrece, por primera vez de manera explícita, la posibilidad a los envases activos de ser utilizados en Europa, estableciendo los requisitos necesarios para ponerlos en el mercado (Sanches-Silva et al., 2014). Así, en el artículo 4 se concreta para envases y sus agentes activos, que en el caso de producirse modificaciones en la composición o la organoléptica del alimento, las sustancias que los provocan deben haber sido aprobadas como aditivos y deben cumplir la normativa aplicable a los mismos (CE, 2004). Más adelante aclararemos cuándo las sustancias activas podrían considerarse aditivos y cuándo se consideran únicamente sustancias activas.

El Reglamento 1935/2004 abarca la totalidad de modalidades de envasado alimentario y genera un marco normativo general para todos ellos. Por este motivo, se indica expresamente que en legislaciones futuras, la Comisión podrá implementar medidas específicas para cada tipo de material en concreto. En este sentido, en la letra b del apartado 1 del artículo 5, se incluye entre las posibles medidas específicas la creación de "una o varias listas de sustancias autorizadas incorporadas en materiales y objetos activos e inteligentes destinados a entrar en contacto con alimentos y, cuando resulte necesario, condiciones específicas para la utilización de dichas sustancias y de los materiales y objetos en las que están incorporadas" (CE, 2004).

Es aquí, en la evaluación de seguridad de estas nuevas sustancias, donde interviene la Autoridad Europea en Seguridad Alimentaria (EFSA). Uno de los aspectos importantes del Reglamento es que cualquier nuevo envase activo y sus componentes deben ser evaluados por la EFSA antes de que la Comisión adopte una opinión sobre los mismos y regule su uso. Basándose en el resultado de esta evaluación, la Comisión concedería la autorización al solicitante, de manera individual como titular de la autorización, para las sustancias activas evaluadas.

1.2.2 Reglamento 450/2009/CE

El Reglamento Nº 450/2009 de la Comisión, de 29 de mayo de 2009, sobre materiales y objetos activos e inteligentes destinados a entrar en contacto con alimentos, establece requisitos específicos para la comercialización de este tipo de materiales de envasado, completando aquellos dispuestos en el Reglamento 1935/2004.

Este Reglamento especifica que las sustancias responsables de la función activa deben ser evaluadas y, además, según lo descrito en su artículo 4 letra e, para que un material activo se ponga en circulación en la UE solo podrá incorporar sustancias activas incluidas en la lista comunitaria de sustancias autorizadas. Esta lista que se confeccionará en base a las solicitudes presentadas de acuerdo con el artículo 9 del Reglamento 1935/2004, no va a ser establecida hasta que la EFSA complete las evaluaciones de riesgo y publique las opiniones científicas sobre cada una de las sustancias.

Mientras se produce la publicación de la lista positiva de sustancias, el Reglamento propone una serie de excepciones que permiten desarrollar envasado activo. En una de estas excepciones, incluidas en el artículo 5, se indica que pueden utilizarse sin estar incluidas en las listas positivas las “sustancias activas liberadas”, que deberán hacerlo cumpliendo plenamente las disposiciones comunitarias y nacionales pertinentes aplicables a los alimentos.

1.2.3 Matrices poliméricas en envasado activo

Los materiales activos pueden estar compuestos por una o más capas o partes de diferentes tipos de materiales, como plástico, papel y cartón o revestimientos y barnices. Los requisitos aplicables a estos materiales no están armonizados aun a escala comunitaria, a excepción de los materiales plásticos para los que se publicó un reglamento europeo en el año 2011 (Reglamento 10/2011), y por este motivo la Comisión permite que los reglamentos comunitarios en materia de envasado alimentario (Reglamento 1935/2004 y Reglamento 450/2009) se apliquen sin perjuicio de las disposiciones nacionales que regulen esos materiales (CE, 2009).

De la amplia variedad de materiales de envasado alimentario, los plásticos son los más empleados debido a que presentan óptimas propiedades mecánicas, ópticas y de barrera (Ramos et al., 2012). Aquellos derivados del petróleo, como las poliolefinas entre las que

destacan el polietileno (PE) o el polipropileno (PP), presentan grandes ventajas, como son: buen rendimiento mecánico, buenas propiedades de sellado, disponibilidad a gran escala y bajos costes de producción. Sin embargo, al tratarse de materiales que procede de fuentes no renovables con baja o nula biodegradabilidad, su impacto en el medio ambiente se ha convertido en un hándicap que limita su uso y que, junto al aumento de la conciencia ecológica ha demandado la creación de alternativas más respetuosas con el medio (Siracusa et al., 2008).

Los biomateriales representan una alternativa interesante a los polímeros convencionales derivados de petróleo. Destacan los polímeros termoplásticos, donde se incluye el ácido poliláctico o poli (ácido láctico) (PLA), por su equilibrio de propiedades y por poder ser procesados por las mismas técnicas que los polímeros tradicionales (Drumright et al., 2000). El PLA es un polímero biodegradable derivado del ácido láctico que se produce a partir de maíz, remolacha, trigo y otros productos ricos en almidón y que presenta propiedades similares al PE que lo convierten en un candidato perfecto para desarrollar envases alimentarios. En este sentido, el PLA esta aprobado como GRAS (Generally Recognized as Safe) por la Autoridad Americana de Alimentación (Food and Drug Administration (FDA) (Jamshidiam et al., 2010) y el ácido láctico, monómero de este material, está reconocido en Europa como sustancia de partida para la creación de polímeros plásticos destinados a entrar en contacto con alimentos en el Reglamento (UE) Nª 10/2011 de la Comisión de 14 de enero de 2011 sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos.

1.3 Situación comercial

El envase alimentario es esencial para proteger a los alimentos durante las etapas de transporte, almacenamiento o distribución. En las últimas décadas, el envasado activo se ha abierto hueco en el mercado mundial, revolucionando los empaquetados de alimentos y bebidas (Kotler y Keller, 2006). Esta nueva tecnología ha permitido mantener la calidad organoléptica y nutricional de los alimentos, así como prolongar su vida útil, disminuyendo además la generación de residuos en caso de ser diseñados en matrices biodegradables.

Aunque estos materiales introducen grandes ventajas, la demanda de la industria alimentaria no sigue el mismo patrón en las distintas regiones del planeta. Actualmente, es Japón el país que más consume envasado activo, mientras que en Estados Unidos (EEUU) y Europa se está observado un incremento más gradual en su uso.

Algunos autores han intentado dar explicación a esta diferencia. En el caso de EEUU, Restucia et al. (2010) señalaron que la implementación de estos nuevos envases se aceleraría, si se desarrollara una nueva generación de materiales con propiedades mejoradas a un precio más competitivo. Estos autores ponen énfasis en el hecho de que se trata de una tecnología cara que incrementa el precio del producto final y lo hace poco interesante para el consumidor medio. En Europa, el bajo interés por esta nueva tecnología se ha atribuido a dos hechos fundamentales; el primero el alto coste económico de estos nuevos materiales y el segundo la baja aceptación de los consumidores (Dainelli et al., 2008) que consideran que los envases que actualmente están en el mercado son adecuados (Restucia et al., 2010). Es posible que en Europa, la mentalidad más conservadora, la legislación más restrictiva y la ausencia de una lista de sustancias autorizadas esté agravando la situación de desinterés. En este sentido, los consumidores, preocupados cada vez más por la seguridad de los alimentos que se ponen en el mercado, no encontrarían garantía de que las sustancias que se utilizan en envasado activo fueran seguras, y la industria no vería interés comercial en desarrollar este tipo de materiales al no poder comercializarlo dentro de las fronteras de la UE.

Podríamos decir que el diseño y desarrollo de este tipo de materiales, así como la evaluación de seguridad de los compuestos activos, podría contribuir a aumentar el interés de los consumidores europeos por estos envases y agilizar su legalización en Europa.

2. LOS ACEITES ESENCIALES EN ENVASADO ACTIVO

Como se ha mencionado anteriormente, la población actual selecciona alimentos libres de aditivos, y si estos no están disponibles se decantan por aquellos que contienen aditivos naturales frente a los que incorporan aditivos sintéticos (Carocho et al., 2013).

Las tradiciones ancestrales de las zonas tropicales del planeta han facilitado la búsqueda de sustancias activas naturales que respondan a las necesidades de la industria del envasado alimentario que requiere de compuestos capaces de aumentar la vida útil de los alimentos para poder así cubrir las demandas de los consumidores y los largos tiempos de distribución provocados por la globalización alimentaria (Dainelli et al., 2008). Entre las sustancias de origen natural, los aceites esenciales y extractos vegetales se han convertido en diana de estudio gracias a sus propiedades antioxidantes, antimicrobianas y organolépticas.

2.1 Definición aceite esencial

Los aceites esenciales (AEs) son mezclas complejas, fundamentalmente oleosas, que se presentan en estado líquido a temperatura ambiente y proceden del metabolismo secundario de diferentes especies de plantas (Bajpai et al., 2012). Se obtienen a partir de raíces, hojas, sumidades floridas, etc. por diferentes técnicas, entre las que destacan presión, destilación o extracción por disolvente orgánico (Burt, 2004). La composición y calidad de los AEs se ve afectada por multitud de factores como son, la etapa de desarrollo de la planta, variedad, origen geográfico, parte de la planta utilizada y estación de recolección, entre otros. Pequeñas modificaciones en el proceso extractivo también pueden influir en la composición y, con ello, en las propiedades, actividades o en la toxicidad de los AEs (Azmir et al., 2013; Németh-Zamboriné, 2016).

Los AEs presentan fragancias características debido a la presencia, en su composición, de compuestos altamente volátiles que suelen ser de naturaleza aromática o de origen terpénico (Bakkali et al., 2008). En la Tabla 1 se muestran los principales AEs utilizados en la industria alimentaria y sus compuestos mayoritarios.

Nombre Común	**Nombre latino**	**Compuesto mayoritario**
Ajo	*Allium sativum*	Alil disulfuro, dialil disulfuro, dialil trisulfuro
Canela	*Cinnamomum zeylanicum*	Trans-cinamaldehido
Cebolla	*Allium cepa*	Dipropil disulfuro
Cilantro	*Coriandrum sativum*	Linalol
Clavo	*Syzygium aromaticum*	Eugenol
Orégano	*Origanum vulgare*	Carvacrol Timol
Romero	*Rosmarinus officinalis*	α- pineno

Tabla 1. Compuestos mayoritarios de aceites esenciales con aplicación en alimentación (Shaat y Flores, 1998; Burt, 2004; Romeilah et al., 2010; Tsai et al., 2013).

Estos compuestos confieren a los AEs las propiedades antioxidantes, antimicrobianas y organolépticas que los han hecho interesantes en medicina, agricultura, o en las industrias

alimentaria y de perfumería. Suelen constituir en torno al 85 % de la composición total de los aceites y definen, en la mayoría de los casos, las propiedades biológicas que presentarán los mismos. Sin embargo, aunque los componentes mayoritarios definan la actividad, los AEs han de evaluarse *per se* ya que el 15 % que suponen los compuestos minoritarios, que pueden encontrarse incluso en concentraciones traza, tiene un papel importante en la modulación de la actividad, pudiendo producir efectos aditivos, y actuar de manera sinérgica o antagonista con los componentes mayoritarios (Burt, 2004; Escobar et al., 2012).

2.1 1 Aceite esencial de orégano

El aceite esencial de orégano (AEO), obtenido de diferentes especies del género *Origanum,* así como las hierbas secas de estas plantas, han sido utilizadas tradicionalmente por sus propiedades organolépticas como aromatizante en alimentación (Liju et al., 2013). Además, estas plantas y sus extractos han demostrado propiedades antioxidantes y antimicrobianas que diferentes autores han relacionado con la presencia de compuestos fenólicos como el carvacrol, el timol o sus precursores γ-terpineno y ρ-cimeno (Dundar et al., 2008; Liolios et al., 2009).

Teniendo en cuenta lo comentado anteriormente respecto a las variaciones que sufren las composiciones de los aceites en función de la especie, época de recogida o condiciones de secado, se hace necesario concretar la especie y la composición del aceite evaluado. En este sentido, el AEO utilizado en la presente tesis doctoral, extraído de la especie *Origanum vulgare* L. *virens* y recolectado en febrero de 2013, fue proporcionado por la empresa El Jarpil S.L. (Almería, España) y según ficha técnica, sus constituyentes mayoritarios son carvacrol (56%) y timol (5%) (Figura 1).

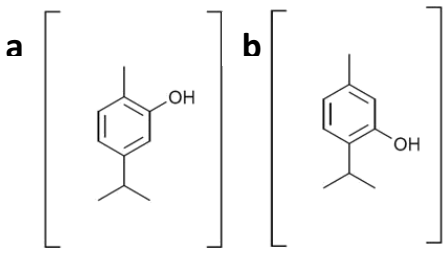

Figura 1. Estructuras químicas de los compuestos mayoritarios de *Origanum vulgare* L. *virens*, carvacrol (a) y timol (b).

2.1.2 Extractos de Allium spp.

Las plantas del género *Allium* son conocidas por su característico aroma azufrado que se asocia a la gran cantidad de compuesto organosulfurados (OS) que forman parte de su composición; y a los que se deben las propiedades biológicas y funcionales de estas plantas (Corzo- Martínez et al., 2007).

El ajo (*Allium sativum*) y la cebolla (*Allium cepa)*, especies representativas de este género, se han utilizado tradicionalmente por sus propiedades farmacológicas y terapéuticas. Además de sus propiedades organolépticas, los AEs de estas especies poseen propiedades antioxidantes y antimicrobianas (Benkeblia, 2004) que han hecho de estas plantas magníficos conservantes naturales usados tradicionalmente en gastronomía (Corzo- Martínez et al., 2007).

En el caso del ajo, las propiedades biológicas se asocian a compuestos OS entre los que destacan el dialil sulfuro (DAS) (Figura 2a), dialil disulfuro (DADS) (Figura 2b) y dialil trisulfuro (DATS) (Figura 2c) (Romeilah et al., 2010; Tsai et al., 2013). El dipropil disulfuro (DPDS) (Figura 2d) es el componente mayoritario de la cebolla y su aceite esencial y al que se le asignan las propiedades funcionales de esta especie (Shaat y Flores, 1998).

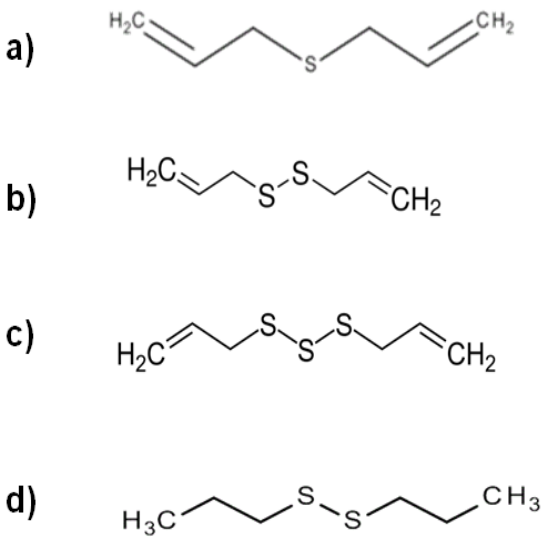

Figura 2. Estructuras químicas de los compuestos mayoritarios de *Allium sativum*, DAS (a), DADS (b), DATS (c) y de *Allium cepa*, DPDS (d).

La evaluación de los aceites esenciales y extractos del género *Allium* se complica por el hecho de que los compuestos OS son muy inestables y la composición de estas mezclas suele ir variando con el tiempo y las condiciones de almacenamiento (Lanzotti et al., 2006). Para evitar esta situación, la empresa DOMCA S.A.U. (Granada, España) ha diseñado un preparado comercial estabilizando un compuesto OS en una matriz orgánica. El propil propano tiosulfonato (PTSO) (Figura 3) es el compuesto azufrado mayoritario (14,5%) de este preparado conocido como Proallium® y ha demostrado mantener las propiedades antimicrobianas características de los extractos de *Allium* spp. (Peinado et al., 2012, 2013)

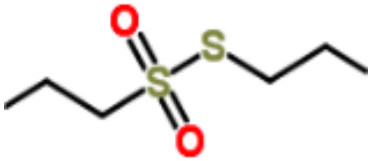

Figura 3. Estructura química de Propil propano tiosulfonato (PTSO), componente mayoritario de Proallium®.

2.2 Situación legislativa de los aceites esenciales en envasado activo en Europa

En el Reglamento 450/2009 se establecen varias excepciones en cuanto a la obligatoriedad de que las sustancias activas utilizadas en materiales de envasado estén incluidas en la lista comunitaria de sustancias autorizadas. Una de estas excepciones hace referencia a las sustancias que se añaden para ser liberadas al alimento o al entorno de éstos, como es el caso de los AEs y de los extractos naturales que tratamos en la presente tesis doctoral. Se indica en el punto 1 del artículo 9 que estas sustancias, las añadidas para ser liberadas, deberán utilizarse cumpliendo las disposiciones comunitarias y nacionales pertinentes aplicables a los alimentos, así como las disposiciones del Reglamento 1935/2004 (CE, 2009).

Si estas sustancias activas van a tratarse entonces como ingredientes tecnológicos, es importante conocer que cualquier sustancia utilizada como tal debe estar aprobada por la autoridad competente en materia de seguridad alimentaria que, en la mayoría de los casos, regula las cantidades y los alimentos, piensos o materiales de envasado alimentario en los que estas sustancias pueden adicionarse. En Europa, al igual que comentábamos anteriormente, el organismo encargado de esta tarea es la Comisión Europea (CE).

Actualmente no existe una legislación armonizada globalmente que delimite el uso de AEs en la industria alimentaria a nivel mundial. Las normativas nacionales y comunitarias varían entre estados y países, y los grados de exigencia en cuanto a los requisitos necesarios para la aprobación de estas mezclas distan mucho de ser similares.

Mientras que en EEUU los AEs están reconocidos como sustancias GRAS por la FDA, homóloga a la EFSA; la legislación europea, mucho más restrictiva, necesita evaluaciones más profundas para incorporar los AEs a las listas comunitarias que incluyen las sustancias autorizadas por la CE.

En EEUU, los AEs pasaron rápidamente a estar aprobados como aditivos por el uso tradicional que de ellos se ha hecho a lo largo de la historia. El historial de seguridad asociado al consumo de estos aceites y de sus plantas de origen ha sido suficiente para que la FDA permita su adición a alimentos, piensos y envases alimentarios. En Europa, estas sustancias presentan también un historial de uso seguro y están reconocidas como sustancias N2 por el Consejo de Europa (EFSA, 2010). Las sustancias incluidas en esta categoría son aquellas que habitualmente se han utilizado en alimentación en pequeñas cantidades como aderezos,

condimentos o especias (Knights, 2010). Sin embargo, la EFSA considera que esta presunción de seguridad no es aplicable a las condiciones de uso de los AEs como aditivos en alimentación (EFSA, 2010) y que no es comparable su uso como hierbas aromáticas para evaluar su seguridad (EFSA, 2016). Además, el Comité Científico añade que, debido al aumento de los canales a través de los cuales estas sustancias llegan al consumidor (alimentos, complementos alimenticios, etc.), es esencial evaluar la calidad y la seguridad del uso de estas preparaciones botánicas (EFSA, 2016). Por tanto, independientemente de que los AEs vayan a utilizarse como sustancias activas, aromas o aditivos, estos extractos deben ser evaluados por la EFSA y autorizados por la Comisión.

Teniendo en cuenta todo lo anterior, los extractos botánicos y entre ellos los AEs, podrían utilizarse en envasado activo de manera legal en el momento en el que fueran incluidos en cualquiera de las listas comunitarias de ingredientes tecnológicos (aditivos, aromas, enzimas, coadyuvantes o sustancias activas). Sin embargo, los AEs no están incluidos en ninguna de las listas de sustancias autorizadas y la UE no dispone actualmente de un procedimiento de autorización común para AEs. En la legislación vigente, únicamente se indica que estas sustancias deben cumplir los requerimientos generales propuestos por Reglamento (CE) Nº 178/2002 del Parlamento Europeo y del Consejo de 28 de enero de 2002 por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria (CE, 2002). Sin embargo, este reglamento no incorpora ninguna guía para la evaluación de la seguridad de este tipo de sustancias y se hace necesario recurrir a las propuestas publicadas para los diferentes ingredientes tecnológicos.

Una situación diferente aplica a los compuestos mayoritarios de estos AEs, aquellos a los que se les asigna la función activa. Actualmente, muchos de ellos forman parte de las listas de sustancias autorizadas como aromas o aditivos. Es el caso de los compuestos mayoritarios del AEO (timol, carvacrol) o de los extractos de *Allium* sp. (sulfuros, disulfuros y trisulfuros) que están aprobados como aromas por la legislación europea (CE, 2008b) y por tanto sí podrían ser utilizados como activos para el desarrollo de materiales de envasado según la excepción comentada anteriormente.

2.3 Proceso de autorización de aceites esenciales para envasado activo alimentario

Como se ha comentado anteriormente, los AEs podrían ser incorporados a materiales de envasado activo sin haber sido incluidos en la lista comunitaria de sustancias activas autorizadas, siempre que hayan sido aprobados como ingrediente tecnológico, puesto que están destinadas a ser liberados y contactar directamente con el alimento. Debido a que se añaden de manera intencionada deben emplearse cumpliendo las normas que regularían su uso directo en alimentos. Si esas disposiciones, las que regulan sus usos en alimentos, prevén un procedimiento de autorización de la sustancia, ésta y su uso deben cumplir los requisitos de tal procedimiento conforme a la legislación sobre alimentos de que se trate (CE, 2009), por ejemplo, la legislación sobre aditivos alimentarios.

En Europa, el procedimiento de autorización de todas las sustancias consideradas ingredientes tecnológicos está unificado en el Reglamento 1331/2008, de 16 de diciembre de 2008, por el que se establece un procedimiento de autorización común para los aditivos, las enzimas y los aromas alimentarios. Por tanto, en la situación en la que se encuentran actualmente los AEs, se podrían solicitar su inclusión en las listas de sustancias autorizadas por dos vías diferentes. La primera como ingrediente tecnológico y la segunda como sustancia activa, y cualquiera de las dos sería válida para legalizar el uso de estos aceites en envasado activo.

Tanto la información exigida en la solicitud como los procedimientos de autorización propuestos para ambas vías son similares. Así, para obtener la autorización, el expediente debe contener un apartado con información administrativa que haga referencia a la identidad del solicitante, nombre del operador, direcciones o fecha de presentación de la solicitud y un dosier técnico en el que se proporcione información sobre la naturaleza química de la sustancia, proceso de fabricación y datos toxicológicos, autorizaciones o evaluaciones de riesgo existentes.

Con respecto al procedimiento, en el Reglamento 1331/2008 se indica que la solicitud de inclusión (o retirada) en la lista comunitaria puede ser solicitada por la propia CE, los Estados miembros o por una persona física o jurídica. Esta solicitud se emite a la Comisión, que la transmite a la EFSA para que ésta, en el plazo de nueve meses, emita una opinión científica con la que la Comisión tomará la decisión final (Figura 4a).

Para la solicitud de autorización de sustancias activas, el Reglamento 450/2009 remite al artículo 9 del Reglamento 1935/2004. Aquí se indica, que la solicitud para sustancias activas se emite a la autoridad competente del Estado miembro, que en España sería la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN), encargada de

transmitirla a la EFSA. Ésta emite un dictamen en el plazo de 6 meses, con una posible prórroga de otros 6 meses, y lo presenta a la Comisión que, finalmente, tomará la decisión (Figura 4b).

Al final de ambos procedimientos, y en caso de que se acepte la solicitud, la sustancia es incluida en la lista positiva y ésta se hace pública.

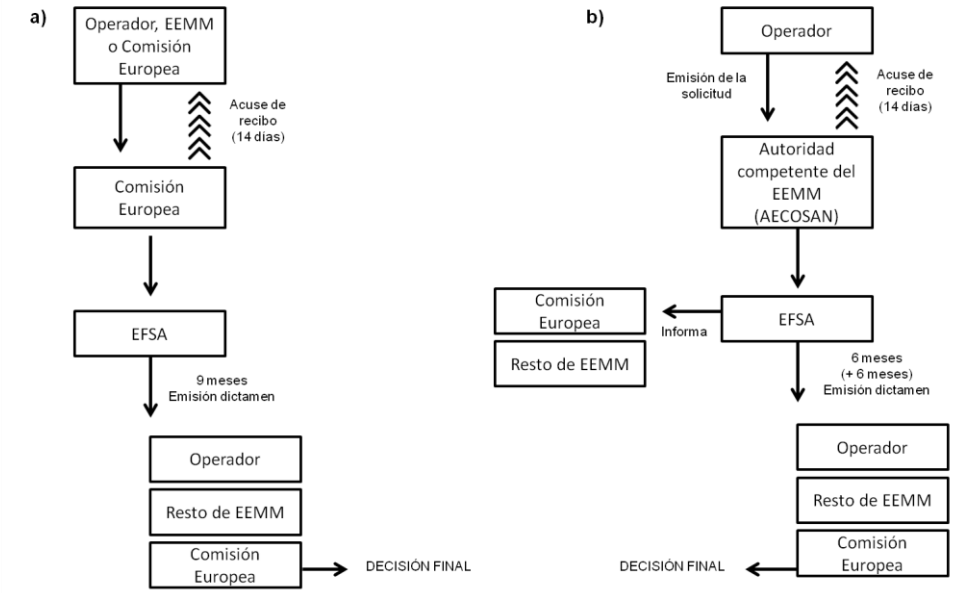

Figura 4. Proceso de autorización de nueva sustancia según Reglamento de aditivos (a) y según Reglamento de sustancias activas (b). (Basado en Reglamento 1331/2008 y Reglamento 450/2009, respectivamente).

2.4 Aceites esenciales en matrices biodegradables

Una de las limitaciones del uso de AEs en alimentación es su intenso aroma. Habitualmente, cuando los AEs son adicionados directamente para ejercer su función en contacto con los alimentos, la concentración requerida para conseguir la función conservante excede los niveles organolépticos aceptables, impactando en el sabor natural de determinados alimentos (Ponce et al., 2008; Goñi et al., 2009).

Además de la adición directa, los AEs pueden ejercer su acción en el alimento a través del envasado activo. La ventaja de aplicar los aceites a través de esta nueva tecnología es que se consigue el mismo objetivo con cantidades menores de aceite, limitando así la transmisión de olores y sabores a los alimentos (Salgado et al., 2013). Sin embargo, al incorporar los aceites

al polímero, estos entran a formar parte de la estructura química de la matriz e interaccionan con el polímero y las sustancias que se añadan como plastificantes (Ruiz-Navajas et al., 2013). Estas interacciones electrostáticas entre las sustancias activas y las cadenas poliméricas, los cambios estructurales inducidos por la adición de aceites o los procesos de ósmosis pueden afectar al ratio de difusión de los agentes activos y limitar así la funcionalidad del material (Avila-Sosa et al., 2012).

Por otro lado, el uso de AEs en envasado activo tiene como objetivos principales reducir la peroxidación lipídica y/o minimizar o eliminar la presencia de microorganismos sin afectar a la organoléptica del alimento. Para ello, los aceites pueden incluirse en sistemas independientes (como puede ser saquitos o bolsitas que contengan las sustancias activas) o ser incorporados en el propio material de envasado. En este último caso, los materiales que incorporan AEs pueden encontrarse como films o como recubrimientos. Los films son películas delgadas preformadas que en un paso posterior se utilizarán para envolver al alimento y los recubrimientos se definen como una matriz continúa que polimeriza en contacto con el propio alimento por aplicación directa de una solución que contiene el agente activo (Krotcha, 2002).

Aunque el campo de los recubrimientos comestibles está abriéndose paso en el mercado, la presente tesis doctoral se centra en el uso de matrices poliméricas a las que se adicionan aceites esenciales para desarrollar films activos debido a que los materiales plásticos continúan siendo los más empleados por la industria alimentaria en la fabricación de envases (Robertson, 2016). En este sentido, y teniendo en cuenta los inconvenientes medioambientales que generan los plásticos tradicionales, las matrices biodegradables entre ellas el PLA descrito anteriormente, representan una interesante alternativa para desarrollar envases alimentarios (Azeredo, 2009).

Muchos son los AEs que están siendo estudiados para desarrollar envases activos, por ejemplo aquellos obtenidos a partir de albahaca (*Ocimum basilicum* L.), camomila (*Chamomila* L.), cardamomo (*Elettaria cardamomum* (L) Maton) o romero (*Rosmarinum officinalis* L.). La presente tesis doctoral se centra en los extractos naturales obtenidos de orégano (*Origanum vulgare* L.) y aliáceas como ajo y cebolla (*Allium* sp.) y en sus compuestos mayoritarios.

2.5 Estudios físico-mecánicos. ¿Cómo se ve afectada la matriz tras la adición de AEs?

La incorporación de aceites a matrices poliméricas para desarrollar films activos puede llevarse a cabo mediante multitud de métodos, entre los que destacan la extrusión o el

fundido (Krochta et al., 2002). En estos procesos se pueden alcanzar temperaturas superiores a las temperaturas de volatilización de los aceites, provocando pérdidas de sustancia activa en el material final y limitando la funcionalidad de estos envases (Coma, 2008). Además, las condiciones propias del proceso de elaboración de estos materiales, incluidas las temperaturas alcanzadas, pueden provocar la aparición de productos de degradación conocidos como "sustancias añadidas involuntariamente" o por su sigla en inglés como NIAS (non-intentionally added substances) (Aznar et al., 2012). Estas sustancias que se generan como consecuencia de reacciones de descomposición de aditivos o interacciones entre los componentes del envase, han sido ignoradas durante años en las evaluaciones de seguridad (Koster et al., 2014). Sin embargo, en el año 2011 el Reglamento 10/2011 reconoció la importancia de tomar en consideración este tipo de impurezas y de incluirlas en la evaluación de riesgo.

Por otro lado, la evaluación de las propiedades térmicas, mecánicas, ópticas y de barrera constituyen aspectos importantes en la caracterización de materiales de envasado alimentario. Las propiedades mecánicas como la elongación a rotura o el modulo elástico se completan con análisis químicos y físicos de la estructura del material como son el grosor, la solubilidad, la actividad de agua, permeabilidad al oxigeno y al dióxido de carbono, color, transparencia, etc.

Se han descrito muchos factores que afectan a estas propiedades cuando se añaden determinadas sustancias a los polímeros. En el caso de los AEs, se ha observado que las propiedades del aceite, las cantidades utilizadas, su efecto plastificante y la interacción con la propia matriz constituyen factores que pueden modificar las propiedades del material de partida (Ramos et al., 2013).

2.5.1 Propiedades térmicas

Las técnicas termoanalíticas han sido y continúan siendo utilizadas ampliamente en la caracterización de materiales (Dobkowski, 2006). Como se ha comentado anteriormente, las condiciones de fabricación de los materiales son decisivas en las propiedades finales del mismo y estas técnicas son imprescindibles en el proceso de control que nos permitirá, entre otras, determinar si la concentración final de activo en el film es funcional y si se han producido NIAS que deban ser tenidas en cuenta a la hora de desarrollar la evaluación toxicológica de los envases. Entre las más utilizadas para estudiar los efectos de la incorporación de aceites a matrices poliméricas están la calorimetría diferencial de barrido (en inglés DSC, Diferential

Scaning Calorimetry) y el análisis termogravimétrico o termogravimetría (en inglés TGA, Thermogravimetric Analysis).

2.5.2 Propiedades mecánicas

Las propiedades mecánicas de los polímeros se estudian habitualmente forzando la elongación del film hasta su rotura y registrando la fuerza aplicada con respecto al tiempo o a la distancia a la que se produce esa rotura. Los parámetros más utilizados para reportar estos datos son el módulo elástico, directamente relacionado con la rigidez del material; la resistencia a la tracción como medida de resistencia del film; y el porcentaje de elongación que indica la elasticidad del material. Todas estas propiedades dependen de multitud de factores entre los que se incluyen las características microestructurales de los polímeros (Krochta & Jonhson, 1997).

La adición de AEs a la matriz polimérica puede producir multitud de efectos dependiendo de las características del aceite y de su capacidad para interaccionar con la misma (Pires et al., 2011). A veces, estos efectos pueden ser interesantes tecnológicamente como en el caso de la adición de aceites a PLA. Esto ocurre porque una de las mayores debilidades del PLA es su baja flexibilidad y como consecuencia su comportamiento frágil (Yahyaoui et al., 2016). La adición de AEs suele producir un efecto plastificante que algunos autores asocian a una disminución de la resistencia a la tracción que aumenta la flexibilidad del PLA y además le confiere características de material activo (Chieng et al., 2014).

2.5.3 Propiedades ópticas

Dos de las propiedades ópticas que afectan a la apariencia de los films y más comprometen la aceptación de los nuevos envases por parte de los consumidores son color y transparencia (Yahyaoui et al., 2016).

Los cambios en el color de los materiales activos que contienen AEs se han relacionado directamente con el tipo y la concentración del aceite incorporado (Du et al., 2009). Los aceites suelen producir un pardeamiento de las matrices poliméricas, variando su color desde amarillos hasta marrones suaves (Yahyaoui et al., 2016).

Por otro lado, diferentes autores han puesto de manifiesto que la adición de AEs produce una reducción en la transparencia de los films como consecuencia de una disminución en la transmisión de la luz a través del material. Este hecho ha sido relacionado por algunos autores con la dispersión de la luz en las gotículas de aceite que quedan incorporadas a la matriz polimérica (Hosseini et al., 2009; Siripatrawan et al., 2010; Pires et al., 2013). La transparencia de un film permite ver con claridad la apariencia del alimento a través de él y esto es algo que los consumidores demandan. Sin embargo, los recubrimientos más opacos podrían proteger de manera más efectiva frente a la luz en alimentos fotosensibles.

2.6 Eficacia antioxidante y antimicrobiana de envases activos con AEO y *Allium* sp y sus componentes mayoritarios

Una vez incorporados a la matriz polimérica, los compuestos activos son liberados de manera controlada al espacio de cabeza del envase con objeto de mejorar o mantener las propiedades organolépticas y la integridad microbiológica de los alimentos (CE, 2009).

La eficacia de los envases activos, incluidos aquellos que contiene AEs, se evalúa mediante técnicas *in vitro* o, preferiblemente, observando su actividad directamente en alimentos envasados con los prototipos (Gomez-Estaca et al., 2010).

Como se muestra en la tabla 2, muchos son los prototipos de envases que han incluido en su composición aceites, objeto de estudio en la presente tesis doctoral.

Nombre Común	Nombre latino	Matriz polimérica
Ajo	*Allium sativum*	Chitosan con AE de ajo al 0.5, 1 y 1.5 %
		Polietileno de baja densidad (LDPE)- 2, 4, 6 y 8 % AE
		LDPE/etilvinilacetato (EVA)- 2, 4, 6 y 8 % AE
		Polímero de proteínas de pescado- 0.1µg AE/cm^2film
Orégano	*Origanum vulgare*	Films de alginato- 05., 1 y 1.5 % AE
		Films comestible de residuos de

		piel de patata- 0.97 y 1.92 % AE (185 y 286 mf AE/g film) PP y polietileno tereftalato (PET)- 4, 6 y 8 % AE Films de gelatina de pescado y chitosan- 2-1. %)
		Copolímero de PET/PE/EVOH/PE- 1.5-2 g/cm^2 Films de proteína de merluza- 0.001:4 AE
	Zataria multiflora	Envases de papel- 2, 4 y 6 % AE Fils de gelatina- 2, 4, 6 y 8 % AE Carboximetilcelulosa (CMC)- 1, 2 y 3 % AE Films de almidón de maíz- 1, 2 y 3 % AE Films de polisacárido de soja- 1, 2 y 3 % AE EVOH- 5 %AE
	Thymus capitatus	Films de proteína de pescado- 0.1 µL AE/cm^2 material

Tabla 2: Envases activos desarrollados y evaluados por sus propiedades antioxidantes y/o antimicrobianas (Maisanaba et a., 2017)

3. EVALUACIÓN TOXICOLÓGICA DE ADITIVOS Y SUSTANCIAS ACTIVAS

La EFSA se creó en el año 2002 como responsable de la evaluación científica de los riesgos de la cadena alimentaria y con capacidad para comunicar estos riesgos de un modo independiente, lo cual contribuiría a aumentar la confianza en los alimentos disponibles en Europa, el mercado interior y el comercio internacional (Libro Blanco sobre seguridad alimentaria, 2002).

Así, la EFSA tiene dos objetivos fundamentales dentro de la UE: el asesoramiento científico integrado con carácter independiente, de calidad y en el momento oportuno sobre

los riesgos de la cadena alimentaria desde la granja a la mesa; y la comunicación abierta de estos riesgos a todas las partes interesadas y al público en general. Como ya se ha comentado, la EFSA es la encargada de llevar a cabo la evaluación de riesgo de aquellas sustancias que van a ser añadidas a los alimentos, piensos o envases alimentarios y para este propósito, la Autoridad necesita recabar toda la información disponible sobre la toxicidad de la sustancia objeto de estudio.

3.1 Requerimientos de la EFSA. Datos toxicológicos para sustancias activas

En el año 2009, el panel de Materiales en Contacto con Alimentos o Food Contact Materials (FCM) de la EFSA publicó las directrices para la evaluación de la seguridad de las sustancias empleadas en materiales y objetos activos tal y como había adelantado en el punto 2 del artículo 8 del Reglamento 450/2009 (EFSA, 2009). El documento exige, para las sustancias activas, los mismos ensayos que para cualquier sustancia que vaya a ser utilizada en envasado alimentario y remite a la guías publicadas por la Comisión (CE, 2001). En cuanto a la metodología, la EFSA recomienda la utilización de protocolos aceptados universalmente como los descritos por la Organización para la Cooperación y el Desarrollo Económicos (OCDE) o los recogidos en las Directivas 87/432/EEC y 67/548/EC (EFSA, 2009).

En estas guías, la premisa que determina los ensayos de toxicidad que la Autoridad va a exigir para cada sustancia se basa en la afirmación más célebre, quizás, de esta área de conocimiento; la pronunciada por Paracelso en el siglo XVI: *sola dosis facit venenum* (Barlow, 2009). Así, la cantidad de datos requeridos estará directamente relacionada con el nivel de exposición a esa sustancia que se prevé; de manera que a mayor nivel de exposición previsto, mayor cantidad de estudios necesarios para la evaluación (Tabla 3) (EFSA, 2016).

Niveles y especificaciones	**Datos toxicológicos requeridos**	**Consideraciones adicionales**

Nivel 1: Exposición inferior a 1,5 µg/kg pc por día o, exposición menor a 30 µg/kg pc por día para sustancias clasificadas como Clase I en la escala Cramer	- Estudios de genotoxicidad: *in vitro*: test de Ames & MN. Si hay resultados positivos, contradictorios o no concluyentes, *in vivo*: MN y/o cometa y/o aberraciones cromosómicas (CA) - Información previa disponible	En este nivel no se requieren otros estudios de toxicidad, excepto: - si existen datos que indiquen que la sustancia puede producir daño potencial al sistema neurológico o endocrino; - sustancia susceptible de sufrir acumulación en el organismo; - nanomateriales, incluso si ya han sido evaluados y aprobados como FCM.
Nivel 2: Exposición comprendida entre 1.5 µg/kg pc por día y 80 µg/kg pc por día	Datos toxicológicos del nivel 1 más: - Estudios de toxicidad oral de 90 días en roedores	- En el caso de sustancias con potencial de acumulación, debería llevarse a cabo un estudio de absorción, distribución, metabolismo y excreción (ADME). - si existen datos que sugieran que la sustancia produce alteraciones endocrinas por exposición prenatal se debe considerar realizar un ensayo de 90 días con un periodo de exposición prenatal o bien extenderlo a un estudio de toxicidad sobre la reproducción de una generación
Nivel 3: Exposición superior a 80 µg/kg pc por día	Datos toxicológicos de los niveles 1 y 2 más: - Estudio ADME - Estudios de toxicidad reproductiva y de toxicidad de desarrollo - Estudio de toxicidad/carcinogenicidad de larga duración	- si existen datos que sugieran que la sustancia produce alteraciones endocrinas por exposición prenatal se debe considerar realizar un ensayo crónico con un tratamiento de exposición prenatal

Tabla 3. Enfoque escalonado propuesto por la EFSA para evaluar la toxicidad de las sustancias a incorporar en FCM (EFSA, 2016).

Como podemos observar en la tabla 3, el mínimo imprescindible que se requiere para cualquier nivel de exposición implica la evaluación de los riesgos genotóxicos de la sustancia. En el año 2011, la EFSA publicó una estrategia a este respecto y en ella define y clarifica los conceptos de genotoxicidad y mutagenicidad (EFSA, 2011).

Describe genotoxicidad como un término amplio que incluye todo proceso que altera la estructura del material genético, la información contenida en el ADN o que produce la segregación del mismo. Incluye formación de aductos, rotura de la hebra de ADN, intercambio de cromátidas hermanas, etc. La mutagenicidad es un tipo de genotoxicidad y se refiere únicamente a la inducción de cambios permanentes en la cantidad o la estructura del material genético y que se transmiten entre generaciones. En este sentido, podríamos diferenciar entre sustancias clastogénicas, que serían aquellos mutágenos que producen alteración en la estructura del material genético produciendo, por ejemplo, rotura de cromosomas; o sustancias aneugénicas, que serían aquellas capaces de alterar el número de cromosomas produciendo aberraciones numéricas. Una vez definidos los conceptos, la guía hace hincapié en la importancia de encontrar estrategias de evaluación que cubran todas las modalidades de daño genotóxico que pueden producir las sustancias.

En el citado documento, la Autoridad propone dos ensayos *in vitro* para el primer paso de la evaluación: mutación reversa en bacterias (OCDE 471) y micronúcleos (MN) *in vitro* en células de mamífero (OCDE 487). Esta combinación permite estudiar todos los marcadores necesarios para evaluar este aspecto utilizando el mínimo número de ensayos posible. Así, el ensayo de mutación reversa evaluaría la producción de mutaciones génicas y el ensayo de micronúcleos la producción de aberraciones cromosómicas tanto estructurales como numéricas.

Aquellas sustancias que produzcan resultados positivos o equívocos en los ensayos *in vitro* deben ser evaluadas *in vivo* y para ello, la guía propone distintos protocolos: micronúcleos *in vivo* (OCDE 474), ensayo cometa *in vivo* (OCDE 489) y ensayo de mutación génica en roedores transgénicos (OCDE 488). El primero de ellos cubriría aberraciones cromosómicas estructurales y numéricas y es adecuado para comprobar si las sustancias que muestran actividad clastogénica o aneugénica *in vitro* se produce también *in vivo*. El ensayo cometa evalúa las lesiones en el ADN identificando las sustancias que podrían actuar como mutágenos o produciendo aberraciones cromosómicas estructurales *in vivo*. Este ensayo es una herramienta muy útil para evaluar la genotoxicidad en órganos en los que es complicado el desarrollo de otro tipo de ensayo. Por su parte, el ensayo en ratones transgénicos es interesante porque puede llevarse a cabo en cualquier tejido para detectar mutaciones puntuales o pequeñas delecciones. Según la guía, se debe seleccionar uno de estos tres ensayos en función de la información obtenida en los estudios *in vitro*. Si el resultado del test es positivo no es necesario llevar a cabo más estudios y se puede afirmar que la sustancia es genotóxica *in vivo*. Sin embargo, en caso de obtener resultados negativos en el primer ensayo

es posible que sea necesario asegurar la ausencia de toxicidad llevando a cabo un segundo test en un tejido u órgano alternativo. En este sentido, y con idea de disminuir el uso de animales, así como el tiempo y el coste de los ensayos, Bowen et al. (2011) propusieron un protocolo combinado para estudiar la genotoxicidad de las sustancias mediante ensayo de MN y cometa *in vivo* introduciendo modificaciones muy leves a los protocolos propuestos por la OCDE.

En el segundo nivel de exposición, además de los ensayos previstos en el nivel 1, se incorpora la necesidad de llevar a cabo un estudio de toxicidad subcrónica oral de 90 días en roedores (OCDE, 1998). Según la guía, este ensayo proporciona información para establecer el perfil toxicológico de la sustancia al definir órganos diana, tejidos afectados, naturaleza y severidad de los daños producidos y relaciones dosis-respuesta. Para llevar a cabo el estudio, la EFSA propone seguir las directrices propuestas por la OCDE en su protocolo 408.

En sus últimas opiniones, la Autoridad ha propuesto combinar los ensayos de toxicidad oral a dosis repetidas con los estudios de genotoxicidad y con aquellos estudios extra que se requieren en el tercer nivel de exposición (estudios de ADME, toxicidad reproductiva, neurotoxicidad, toxicidad endocrina o carcinogenicidad) con el objetivo de respetar la regla de las 3 Rs (reducción, refinamiento, reemplazo) y disminuir el número de animales sometidos a estudios. Con respecto al segundo nivel de exposición y a la integración de la evaluación de la genotoxicidad, la EFSA propone asociar a estos estudios de toxicidad subcrónica o crónica, el ensayo de MN (OECD 474) y/o ensayo cometa (OCDE 489).

3.2 Estudios toxicidad general

El apartado que nos ocupa pretende resumir los ensayos básicos de toxicidad que permiten a la EFSA contextualizar la situación del aditivo, para emitir una opinión acerca de la seguridad derivada de su uso; y proporcionar también una aproximación a la situación en la que se encuentran el aceite esencial de orégano, los extractos de aliáceas y sus componentes mayoritarios.

3.2.1 Ensayos in vitro

En el año 1959 se definió el principio de las 3 R (reducir, refinar y reemplazar). En su libro “Principles of Humane Experimental Technique”, Russel y Burch, dieron el pistoletazo de salida al desarrollo de métodos alternativos que no utilizasen animales para evaluar la

toxicidad de las sustancias. Entre estos métodos, que incluyen modelos matemáticos de predicción o uso de organismos inferiores no protegidos, destacan las técnicas *in vitro* que se llevan a cabo en líneas celulares (Eisenbrand et al., 2002) y que suponen un marco experimental más complejo que los ensayos bioquímicos (Gosslau, 2016).

A la hora de seleccionar las líneas celulares en las que llevar a cabo la evaluación, Gosslau (2016) propone desarrollar un pequeño screening en líneas celulares de interés y posteriormente desarrollar ensayos más específicos en líneas concretas que representen tejidos u órganos diana de ese efecto. En el caso de la presente tesis doctoral se eligieron líneas relacionadas con el aparato digestivo, puesto que la vía oral es la vía de exposición principal a los aditivos alimentarios.

a. Viabilidad y muerte celular

Los ensayos de citotoxicidad permiten determinar la capacidad de una sustancia para producir muerte celular evaluando su habilidad para producir daño en las estructuras básicas de la célula. Además, estos ensayos se utilizan para definir el rango de concentraciones que debería ser utilizado para evaluar, posteriormente, otros parámetros como genotoxicidad, inducción de mutaciones o mecanismos de muerte celular (Eisenbrand et al., 2002).

Entre los métodos para estudiar la citotoxicidad de las sustancias destacan aquellos que evalúan la integridad de la membrana, como el método de azul tripán (Binderup y col., 2002); o aquellos que miden actividad metabólica de la célula, como el ensayo de rojo neutro que evalúa daño en los lisosomas (Borenfreud y Puerner., 1984) o los ensayos de bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) y sal de tetrazolio (3-[4,5,dimetiltiazol-2-il]-5-[3-carboximetoxi-fenil]-2-[4-sulfofenil]-2H-tetrazolio) (MTS) que comprueban la funcionalidad mitocondrial tras la exposición al compuesto (Baltrop y col., 1991).

La determinación de la concentración efectiva media (EC_{50}), a partir de los estudios de citotoxicidad, permite establecer la concentración a la cual el 50 % de la población expuesta experimenta el efecto estudiado, en este caso la pérdida de viabilidad que es un paso previo a la muerte. Esto nos posibilita comparar los resultados obtenidos en distintas líneas celulares. Además, esta concentración suele ser utilizada como dosis de referencia en el diseño de experimentos *in vivo* (Gosslau, 2016).

Son muchos los estudios que se han llevado a cabo para evaluar la toxicidad que provocan los componentes mayoritarios del AEO en diferentes líneas celulares. En este sentido, el carvacrol ha demostrado ser citotóxico en multitud de líneas celulares tales como la línea celular de carcinoma hepático HepG2 (Horváthová et al., 2006; Yin et al., 2012), en la línea de adenocarcinoma de colon Caco-2 (Horváthová et al., 2006), en células de músculo esquelético de ratón CO25 (Akalin y Incesu, 2011), en células de cáncer metastásico de mama (Arunasnaree, 2010) o en células de cáncer de cuello de útero y cérvix HeLa y SiHa (Medhi et al., 2011). En el caso del timol, los resultados son contradictorios debido a que varían en gran medida los rangos de concentraciones estudiadas. Así, mientras diferentes autores han mostrado un descenso de la viabilidad celular en células cancerígenas promielocíticas HL-60 (Deb et al., 2011) y en células de glioblastoma (Hsu et al., 2011), otros no observaron afectación de este parámetro en cultivos primarios de neuronas corticales de ratón (Garcia et al., 2006).

Para los compuestos mayoritarios de aliáceas, todos ellos compuestos sulfurados, encontramos una bibliografía variable en función del origen de estas sustancias. Los derivados alilos como DAS, DADS o DATS que proceden fundamentalmente de *Allium satuvum* (ajo) han sido ampliamente estudiados y han mostrado capacidad de inducir apoptosis en células tumorales intestinales, hepáticas, pulmonares, tiroideas y prostáticas (Sundaram y Milner, 1996; Xiao et al., 2004; De Martino et al., 2006; Shin et al., 2010). En el caso de los derivados de la cebolla (*Allium cepa*) cuyo representante mayoritario es el dipropildisulfuro (DPDS) (Shaath y Flores, 1998) los resultados son menos abundantes y muestran ausencia de actividad citotóxica en líneas celulares tumorales de adenocarcinoma de colón, pulmón, piel y próstata (Sundaram y Milner, 1996; Xiao et al., 2004).

Con respecto al AEO y a los extractos de aliáceas, es importante recordar la variabilidad a la que están sujetas estas mezclas en función de cantidad de parámetros como son el clima de la zona de cultivo, la estación de la recolección, el método extractivos, etc. (Azmir et al., 2013). Todas estas variables producen resultados diversos al evaluar la toxicidad de estas mezclas y hacen necesaria una valoración caso por caso. Además, con respecto a los datos publicados sobre la capacidad citotóxica de los aceites y sus compuestos mayoritarios cabe destacar que en la mayoría de los casos se han realizado teniendo en cuenta un enfoque terapéutico y planteando el uso de estas sustancias como agentes antitumorales (Bakali et al., 2008; Khazaei et al., 2017). Esto hace que la selección de las concentraciones de exposición sean muy diferentes a las que cabría esperar como consecuencia de una exposición alimentaria a estas sustancias.

b. Mecanismo de acción tóxica

b.1 Estrés oxidativo

Se sabe que los mecanismos que producen toxicidad en los humanos son multifactoriales; sin embargo, muchos de los efectos tóxicos aparecen asociados a situaciones de estrés oxidativo y procesos inflamatorios (Reuter et al., 2010).

Las diferentes especies electrofílicas, entre las que se incluyen las especies reactivas de oxígeno (ERO), promueven la situación de estrés oxidativo, producen daño en las membranas lipídicas, en las proteínas celulares y en los ácidos nucléicos. Esta situación termina desencadenando mutaciones y favoreciendo procesos de carcinogénesis que sumados al daño en lípidos y proteínas comprometen la funcionalidad de células, tejidos y órganos (Gosslau, 2016).

La mayoría de la bibliografía muestra las capacidades antioxidantes de los aceites de orégano y sus componentes mayoritarios (Aeschbach et al., 1994; Yanishilieva et al., 1999; Ündeger et al., 2009; Ozkan and Erdogan, 2011) y de los extractos de *Allium* sp. y sus componentes (Takahashi y Shibamoto, 2008; García et al., 2009; Ye et al., 2013). Sin embargo, son muy escasas las publicaciones relacionadas con la capacidad oxidante de los compuestos mayoritarios y casi inexistentes en el caso de los aceites.

Debido a que las sustancias antioxidantes, incluidos los compuestos fenólicos, suelen experimentar comportamientos prooxidantes en función de la concentración o dosis administrada (Ferguson et al., 2001), se hace necesario confirmar las concentraciones a las que estas sustancias no favorecerían situaciones de estrés oxidativo.

b.2 Genotoxicidad

Podemos definir genotoxicidad como la capacidad de una sustancia de producir alteraciones en el material genético (EFSA, 2011). Estas modificaciones pueden producirse de manera directa, cuando el agente interacciona con el ADN, o bien de manera indirecta cuando no se produce esta interacción pero se ven afectadas enzimas de reparación, proteínas de control del ciclo celular o moléculas relacionadas con la apoptosis (Kirsch-Volders et al., 2003). En este sentido, y teniendo en cuenta que la mayoría de los efectos tóxicos que se producen

en el organismo aparecen asociados a situaciones de estrés oxidativo, sería de esperar que, en una situación en la que el organismo es incapaz de eliminar las especies electrofílicas generadas, los radicales libres acumulados oxiden macromoléculas y entre ellas el material genético, produciendo mutaciones (Eisenbrand et al., 2002).

El estudio de la genotoxicidad de los ingredientes tecnológicos, y por ende de los aditivos alimentarios, es fundamental en la evaluación de riesgo de los mismos (EFSA, 2011; Gosslau, 2016) y para armonizar este estudio, la EFSA desarrolló en el año 2011 una guía que indica los pasos a seguir y la batería de ensayos necesarios para cubrir este objetivo tal y como se ha descrito anteriormente.

En esta estrategia, el primer paso evalúa la capacidad de las sustancias de inducir mutaciones puntuales (Eisenbrand et al., 2002). Entendemos por mutación aquellas alteraciones que tienen carácter heredable, ya sea la transformación química de un gen individual (mutación genética o puntual), que altera la función de dicho gen; la reorganización, ganancia o pérdida de una parte de un cromosoma, que puede ser microscópicamente visible (mutación cromosómica); o el cambio en el número de cromosomas de un genoma (mutación genómica). Todas estas situaciones pueden darse tanto en células germinales, heredadas por las generaciones siguientes del organismo, como somáticas, perpetuadas en un linaje celular por división celular (EFSA, 2011).

Como se ha explicado anteriormente, para evaluar este aspecto la guía propone el test de Ames o ensayo de mutación reversa en bacterias. En este ensayo se utilizan cepas de *Salmonella typhimurium* o *Escherichia coli*, auxótrofas para histidina o triptófano, respetivamente. El procedimiento para llevar a cabo el ensayo fue propuesto por la OCDE en 1997 y en él se detalla que el fundamento de esta técnica es detectar la reversión de la auxotrofía de las cepas utilizadas, de manera que se restaure la capacidad normal de estas bacterias de sintetizar estos aminoácidos esenciales. El test de Ames permite detectar así mutaciones puntuales generadas por sustituciones, adiciones o delecciones de uno o varios pares de bases en el ADN, revelando transiciones o transversiones y desplazamientos en el marco de lectura como mecanismos de producción de la mutación (OCDE, 1997).

El test de Ames presenta gran cantidad de ventajas puesto que es un método barato, rápido y sencillo de llevar a cabo. Además, su robustez y reproducibilidad lo han convertido en un método idóneo para evaluar la genotoxicidad de las sustancias (Mahadevan et al., 2011). Ha sido utilizado ampliamente para evaluar la antimutagenicidad de los compuestos sulfurados (Guyonnet et al., 2000, 2001); sin embargo, los resultados sobre potencial

mutagénico son escasos aunque parecen mostrar una ausencia de esta actividad (Shon et al., 2004; Guyonnet et al., 2001). Con respecto al AEO y sus compuestos mayoritarios los resultados obtenidos sobre actividad mutagénica son contradictorios (Zani et al., 1991; Azizan y Blevins, 1995; Stammati et al., 1999), aunque muchos autores si han demostrado la habilidad de estas sustancias para revertir el daño producido por otros agentes (He et al., 1997; Ipek et al., 2004; Zeytinoglu et al., 2003).

El segundo paso de la evaluación abarca el estudio de las aberraciones cromosómicas. Estas alteraciones pueden ser producidas por agentes clastogénicos, que son aquellos que producen alteraciones morfológicas de la estructura de los cromosomas (roturas, delecciones, intercambio o reorganización del material) o bien aquellas producidas por agentes aneugénicos que alteran el número de cromosomas de un genoma. Para estudiar este aspecto, la EFSA propone el ensayo de MN cuyo procedimiento queda detallado en la guía OCDE 487 (OCDE, 2014) y donde se indica que este método permite detectar sustancias que causan daño citogenético provocando la formación de MN que contienen fragmentos cromosómicos o cromosomas completos. Estas estructuras se generan en células que han sufrido división celular durante o después de la exposición al producto químico de prueba y por ello, los MN representan el daño que se ha transmitido a las células hijas.

Con respecto al ensayo de aberraciones cromosómicas utilizado históricamente para evaluar este punto, el ensayo de MN ha demostrado ser más sencillo, más rápido y más eficiente en la detección de sustancias clastogénicas y aneugénicas, convirtiéndose en el método de elección en la evaluación de este tipo de daño *in vitro* (Corvi et al., 2008).

El último de los ensayos que pasamos a describir no está incluido en la batería de test que propone la EFSA para la evaluación de la genotoxicidad *in* vitro; sin embargo, su realización resulta de gran interés ya que permite una primera aproximación a la situación celular tras la exposición a xenobióticos. Se trata del ensayo cometa, que es un método sencillo que permite detectar sustancias que producen daño en el ADN. Bajos unas condiciones controladas, se determina si éstas son capaces de producir roturas en el ADN o sitios álcali-lábiles, apurínicos o apirimidinicos, que surgen por la pérdida de una base dañada y se convierten en lugares de rotura preferente (Collins et al., 2008). Este ensayo puede además asociarse a enzimas de reparación con actividad glicosilasa/endonucleasa como la formamido-pirimidina ADN glicosilasa (FPG) o la endonucleasa-III (Endo III) para detectar daño oxidativo del material genético (Collins et al., 1993). Este ensayo modificado resulta además de gran utilidad porque nos permite poner de manifiesto el efecto genotóxico en sustancias con

potencial efecto prooxidante como es el caso de las sustancias objeto de esta tesis doctoral. Este método presenta una serie de ventajas que lo hacen idóneo para evaluar la genotoxicidad de cualquier sustancia. Además de que es sencillo y rápido, detecta daño en células individuales, por lo que no requiere un número elevado de éstas para ser llevado a cabo y es sensible a niveles bajos de daño en el ADN (Collins et al., 2008; EFSA, 2011; OCDE, 2014).

Uno de los inconvenientes del ensayo cometa es que no diferencia lesiones irreversibles de aquellas que podrían ser resueltas por las maquinarias de reparación celular. Se asemeja a una fotografía que nos permite valorar la situación en la que se encontraba la célula en un momento concreto. Esto hace necesario completar la información obtenida al desarrollar este ensayo con otros métodos que nos clarifiquen si la célula podría recuperarse del daño manifestado o la alteración observada modificaría irreversiblemente el material genético.

Por último, teniendo en cuenta que las sustancias pueden ser tóxicas por sí mismas o bien a través de sus metabolitos, es necesario incorporar a los ensayos llevados a cabo *in vitro* un sistema exógeno de activación metabólica apropiado que permita transformar los xenobióticos. El sistema más utilizado es la fracción S9 obtenida de hígado de ratas tratadas con inductores enzimáticos como el Aroclor 1254 (EFSA, 2011).

Con respecto a los ensayos de genotoxicidad llevados a cabo para las sustancias objeto de la presente tesis doctoral, los resultados publicados hasta el momento son contradictorios y no es posible extraer conclusiones. Además otro de los inconvenientes encontrados es que muchos de estos estudios no se han desarrollado siguiendo los principios de la OCDE por lo que no serían válidos para que la EFSA evaluara las sustancias.

3.2.2 Ensayos in vivo

Los ensayos *in vitro* son muy interesantes para estudiar los mecanismos de toxicidad de las sustancias. Sin embargo, aunque los complejos sistemas de cultivo celular proporcionan una aceptable aproximación a la situación real, no consiguen mimetizar completamente las condiciones que se desarrollan en un organismo completo. Esto hace que, en determinadas situaciones, sea necesario desarrollar estudios *in vivo* en los que la sustancia se administre directamente al animal (Kirkland et al., 2014; Gosslau, 2016).

a. Genotoxicidad *in vivo*

En el caso de los ensayos de genotoxicidad, habitualmente es necesario llevar a cabo una evaluación *in vivo* para confirmar los resultados obtenidos *in vitro* (EFSA, 2009b). Tanto la estrategia propuesta por la EFSA para evaluar la genotoxicidad de las sustancias (EFSA, 2011), como la propuesta para la evaluación de riesgo de las sustancias que van a estar en contacto con alimentos (EFSA, 2016) indican que es necesario llevar a cabo estudios de genotoxicidad con el fin de investigar si los resultados positivos obtenidos *in vitro* se mantienen *in vivo* y para eliminar falsos positivos obtenidos *in vitro*. También es necesario llevar a cabo estudios en animales cuando los resultados obtenidos *in vitro* no son concluyentes.

Los datos publicados hasta el momento sobre la genotoxicidad *in* vivo del AEO, los extractos de aliáceas y sus compuestos mayoritarios son aun más escasos que los publicados *in vitro* y casi no existe bibliografía a la que remitirse. En cuanto a los compuestos mayoritarios, solo se ha encontrado un trabajo que demuestra la capacidad de carvacrol y timol de producir aberraciones cromosómicas en ratas (Azirak y Rencuzogullari, 2008). El resto de publicaciones que relacionan los compuestos de interés de la presente tesis doctoral y los ensayos de genotoxicidad *in vivo* lo hacen planteando la posibilidad de que estos compuestos actúen como antimutagénicos o antigenotóxicos (Shukla y Kalra, 2007) y, aunque es un campo muy interesante, no constituye nuestro objeto de estudio.

b. Estudio de toxicidad subcrónica

Dentro de la batería de ensayos que conforman la evaluación de la toxicidad general se incluyen los estudios *in vivo* de toxicidad subcrónica. En este sentido, el mínimo imprescindible exigido por la Autoridad es el ensayo de toxicidad oral a dosis repetidas de 90 días en roedores. Este ensayo proporciona el valor de NOAEL (non-observed effect level) necesario para llevar a cabo la evaluación de seguridad (EFSA, 2016).

En cuanto a los ensayos de toxicidad subcrónica, no hemos encontrado ningún trabajo que evalúe los aceites y extractos diana de la presente tesis doctoral a excepción de Proallium®, evaluado *in vivo* por Mellado-García et al. (2016) que no observaron signos de toxicidad tras administrar el producto a ratas wistar durante 90 días. Debido a la ausencia de resultados a este respecto, se hace imprescindible abordar este tema.

III. JUSTIFICACIÓN Y OBJETIVOS / SIGNIFICANT & PURPOSES

Tal como se ha comentado anteriormente, los AEs o sus componentes activos van a ser incorporados a las matrices poliméricas con el fin de que se liberen al interior del envase y generen una atmósfera protectora que permita mantener o mejorar las condiciones de almacenamiento de los alimentos. Como consecuencia de esta migración, el consumidor estará expuesto a las sustancias activas al ingerir los alimentos envasados en estos materiales. Esta posible exposición oral hace obligatorio evaluar la toxicidad de estos aditivos antes de permitir su utilización en Europa y tras la revisión bibliográfica llevada a cabo se constata la necesidad de reevaluar los aceites esenciales y sus componentes mayoritarios siguiendo las recomendaciones de la actual normativa europea en materia de seguridad alimentaria.

En una primera aproximación se seleccionaron líneas celulares del aparato digestivo como modelos experimentales *in vitro* para conocer la potencial citotoxicidad y los posibles mecanismos de acción tóxica de estas sustancias. En concreto de extractos de *Allium* y sus componentes mayoritarios (DPS, DPDS y PTSO) y de aceite esencial de orégano, y sus componentes mayoritarios, (carvacrol y timol). Las células HepG2 (hepáticas) y la Caco-2 (intestinales) se seleccionaron por ser el hígado, órgano fundamental en la biotrasformación de xenobióticos; y el intestino, órgano de mayor tiempo de contacto con las sustancias.

Tras la realización de los ensayo *in vitro* se consideró necesario continuar con la evaluación de la toxicidad *in vivo,* para lo cual el AEO fue administrado vía oral a ratas Wistar para llevar a cabo un estudio de toxicidad oral subcrónica durante 90 días, siguiendo las recomendaciones de la EFSA. Por otro lado, y teniendo en cuenta que los resultados obtenidos *in vitro* para la genotoxicidad del AEO y sus componentes mayoritarios no fueron concluyentes, realizamos los pertinentes estudios de genotoxicidad *in vivo* mediante los ensayos de MN y cometa.

En cuanto al diseño de materiales activos, se seleccionaron dos matrices poliméricas, una poliolefina, el PP, y un nuevo bioplástico procedente del maíz conocido como PLA. A ambas matrices se añadieron por extrusión Proallium® y/o AEO como agentes activos y se determinaron las propiedades ópticas, térmicas y físico-mecánicas, así como la funcionalidad como material antioxidante y antimicrobiano de estos nuevos envases activos para compararlos con los materiales tradicionales.

Por último, se utilizó, por primera vez, la pirólisis analítica como técnica útil en la identificación y cuantificación de compuestos naturales tanto en matrices plásticas como en muestras biológicas (tejidos y alimentos).

Para la realización de esta tesis doctoral, la doctoranda llevó a cabo dos estancias nacionales, en la que se abordaron dos objetivos fundamentales: a) desarrollo y diseño de nuevos materiales de envasado activo y aprendizaje de las técnicas de evaluación de propiedades tecnológicas: térmicas, mecánicas y ópticas (ITENE, Valencia, España); b) aprendizaje de métodos de evaluación de la capacidad antioxidante y antimicrobiana de sustancias y materiales activos (DOMCA S.A.U., Granada, España).

Por todo ello, los objetivos específicos establecidos en la presente Tesis Doctoral han sido:

1. Realizar una revisión bibliográfica de los datos de toxicidad *in vitro*, fundamentalmente mutagenicidad y genotoxicidad, de los aceites esenciales (AEs) destinados al diseño de envases activos y de sus componentes mayoritarios.
2. Evaluar el potencial tóxico *in vitro* de los compuestos mayoritarios de extractos de *Allium* spp., dipropil sulfuro (DPS) y dipropil disulfuro (DPDS) en la línea celular de adenocarcinoma de colon (Caco-2), determinando los mecanismos de acción tóxica mediante la realización de ensayos de citotoxicidad, estrés oxidativo y mutagenicidad; y estudiar, mediante microscopía, posibles alteraciones en la morfología celular.
3. Estudiar las posibilidades de uso de propil propano tiosulfonato (PTSO) como agente antimicrobiano contra microorganismos de presencia común en alimentos, así como su potencial citotóxico y citoprotector y su capacidad para producir alteraciones morfológicas *in vitro* en las líneas celulares de órganos diana (HepG2 y Caco-2). Además, estimar la dosis máxima tolerable en roedores tras exposición oral aguda al compuesto.
4. Investigar el potencial tóxico *in vitro* de carvacrol y timol en la línea celular Caco-2, estudiando su mecanismo de acción tóxica mediante ensayos de muerte celular por citometría de flujo, estrés oxidativo, mutagenicidad y genotoxicidad. Además, conocer las posibles alteraciones ultraestructurales producidas en las células tras la exposición a estas sustancias.
5. Estudiar el posible potencial genotóxico *in vivo* de carvacrol en roedores mediante la combinación de los ensayos de micronúcleos (MN) y cometa.
6. Evaluar *in vivo* la posible toxicidad subcrónica del AEO, así como su potencial genotóxico en ratas wistar.
7. Desarrollar y caracterizar films activos con la incorporación de Proallium® o AEO a una matriz de polipropileno (PP) o ácido poliláctico (PLA) mediante la aplicación de técnicas físico-mecánicas, pirolíticas y de evaluación de vida útil (antioxidantes y

antimicrobianas), así como la evaluación el impacto sensorial de estas sustancias en las propiedades organolépticas de los alimentos envasados en los nuevos materiales.

8. Utilizar la pirolisis analítica para caracterizar materiales biopoliméricos con activos de origen natural.
9. Estudiar la utilidad de la pirolisis analítica en la determinación del perfil nutricional de alimentos envasados en materiales activos.

El trabajo experimental se ha llevado a cabo en el Área de Toxicología del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Facultad de Farmacia de la Universidad de Sevilla, haciendo uso así mismo del Servicio de Biología y de Microscopía del Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS). El análisis morfológico a nivel celular ha sido realizado en colaboración con el Dr. F. Javier Moreno del grupo de Citoquímica Ultraestructural del Departamento de Biología Celular de la Facultad de Biología de la Universidad de Sevilla. El diseño y desarrollo de los envases activos se ha llevado a cabo en colaboración con el Área de Materiales del Instituto Tecnológico del Embalaje, Transporte y Logística (ITENE) de Valencia, gracias a la Dra. Susana Aucejo y al Dr. José María Bermúdez. La evaluación de la actividad antimicrobiana y antioxidante de los envases ha sido realizada en DOMCA S.A.U. gracias a la participación de los Ldos. Enrique Guillamón, Cristina Núñez y Paloma Abad y al Dr. Alberto Baños. Por otro lado, los estudios histopatológicos se han realizado en colaboración con el Área de Toxicología y el Departamento de Anatomía y Anatomía Patológica Comparadas de la Universidad de Córdoba, gracias a la colaboración de la Dra. Rosario Moyano y el Dr. Alfonso Blanco. Los Drs. Francisco J. González-Vila y José A. González-Pérez, así como el Ldo. Nicasio T. Jiménez-Morillo, han colaborado activamente en el desarrollo de la técnica de pirólisis analítica llevada a cabo para la caracterización de los materiales desarrollados y la detección de sustancias activas en muestras biológicas.

Siguiendo la normativa de la Universidad de Sevilla, el resumen, la justificación y objetivos, y las conclusiones se redactan tanto en español como en inglés para optar a la "Mención Internacional en el Título de Doctor".

Taking into account all the background, EOs would be included in polymeric matrices to be release in order to preserve the quality of food and to maintain or improve the storage conditions of foods. Because of this migration, consumers would be exposed to these active agents when the packaged food is ingested. The possible oral exposure, a safety assessment should be developed according to the recommendations of EFSA. After a through literature review, it is important to note that EOs and their main compounds toxicity should be re-evaluated according to the current European legislation.

In a first approach to the toxicity assessment, target cell lines from organs involved in the digestive process were selected to develop the *in vitro* evaluation and to study the cytotoxicity potential and the main mechanisms of toxicity of *Alliums pp*. extracts, their main compounds (DPS, DPDS and PTSO), OEO and their main compounds (carvacrol and thymol). Hence, human hepatoma cell line (HepG2) was selected because the liver is the most active organ in metabolism of substances and colon adenocarcinoma cell line (Caco-2) was chosen because the intestine is considered as site-of-contact tissue.

After *in vitro* evaluation, *in vivo* studies were needed. Therefore, the oral subchronic toxicity of OEO *in vivo* was studied by feeding wistar rats with this EO during 90 days, according to the EFSA guidelines. Moreover, considering the contradictory results obtained *in vitro* regarding genotoxicity, MN and comet assay *in vivo* were developed.

Regarding the development of active films, two kinds of matrices were selected, a polyolefin called PP and a new biodegradable plastic from corn called PLA. Proallium® and OEO were included in these polymers and their optical, thermal, physical and mechanical properties as well as the antioxidant and antimicrobial activities of these new films were evaluated and compared with the traditional materials.

Finally, Py-GC/MS was used, for the first time, as a useful technique to identify and quantify natural compounds contained in polymeric matrices and biological samples (tissues and food).

For the fulfilment of this thesis, the PhD student performed two nationals internships, in which two main objectives were addressed: a) design and development of new active packaging materials and learning of new optical, thermical, physical and mechanical characterization techniques (ITENE, Valencia, Spain); b) learning of new methods to evaluate the antioxidant and antimicrobial activities of substances and active films (DOMCA, Granada, Spain).

Therefore, the specific objectives in this PhD thesis were:

1. To perform a comprehensive literature review of the *in vitro* toxicity, focussed on mutagenicity and genotoxicity, of essential oils (EOs) intended to be used in active food packaging and their main compounds.
2. To evaluate the *in vitro* toxic potential of main compounds of *Allium* spp. extracts, dipropyl sulphide (DPS), dipropyl disulphide (DPDS) and propyl propane thiosulfonate (PTSO) in human adenocarcinoma cell line (Caco-2), determining the mechanisms of action by testing the cytotoxicity, oxidative stress and mutagenicity; and to evaluate possible ultrastructural cellular alterations by microscopy.
3. To study the usefulness of propyl propane thiosulphonate (PTSO) as antimicrobial against food spoilage microorganisms; to study PTSO potential cytotoxicity and its effects to protect cell from oxidative damage; as well as its capacity to produced morphological *in vitro* alterations in target cell lines (HepG2 and Caco-2). Beside, to estimate the maximum tolerable dose after oral exposure in rodents.
4. To investigate the *in vitro* toxic potential of carvacrol and thymol in Caco-2 cells; studying the mechanisms of action by cell death assays by flow cytometry, oxidative stress, mutagenicity and genotoxicity. Moreover, to evaluate possible ultrastructural cellular alterations after exposure to these compounds.
5. To study the genotoxic potential *in vivo* of carvacrol in rodents through the combination of micronucleus and comet assays.
6. To evaluate the possible subchronic toxicity of oregano essential oil (OEO), as well as their genotoxic potential in wistar rats
7. To develop and characterize active films of polypropylene (PP) or polylactic acid (PLA) containing Proallium® or OEO by physic-mechanical techniques, analytical pyrolysis (Py-GC/MS) and shelf-life study (antioxidant and antimicrobial properties). Moreover, to evaluate sensory impact of active substances in organoleptical properties of food packaged in new active films.
8. To use analytical pyrolysis to characterize biopolymers containing natural active agents.
9. To study the usefulness of analytical pyrolysis in the determination of the nutritional profile of food packaged in active films.

The experimental work has been performed in the Area of Toxicology, of the Department of Nutrition and Bromatology, Toxicology and Legal Medicine, Faculty of Pharmacy, University of Sevilla, using also the Biology and Microscopy Services of Centro de Investigación, Tecnología e Innovación of the University of Sevilla (CITIUS). Cellular morphological analysis has been

conducted in collaboration with Dr. F. Javier Moreno Onorato, who belongs to the Ultrastructural Cytochemistry group of the Department of Cell Biology, Faculty of Biology, University of Sevilla. The design and development of active materials was carried out in collaboration with the Department of Materials of the Technological Institute of Packaging, Transport and Logistics (ITENE) in Valencia, thanks to Dr. Susana Aucejo and Dr. José María Bermúdez. The research conducted to evaluate the antioxidant and antimicrobial activity of films was carried out in DOMCA S.A.U., thanks to Enrique Guillamón, Cristina Núñez, Paloma Abad and Dr. Alberto Baños. Moreover, histopathological studies have been performed in the Area of Toxicology and in the Department of Anatomy and Comparative Pathology and Anatomy, both from the University of Córdoba, thanks to the collaboration of Dr. Rosario Moyano and Dr. Alfonso Blanco. Analytical pyrolysis developed to characterize new active materials and the identification and quantification of active agents in biological samples were carried out with the collaboration of Drs. Francisco J. González-Vila and José A. González-Perez and Ldo Nicasio T. Jiménez-Morillo.

Following the regulations from the University of Sevilla, the summary, significance and purposes, and conclusions have been written both in Spanish and English to aim for a PhD with International Mention.

IV. RESULTADOS Y DISCUSIÓN / RESULTS & DISCUSSION

CAPÍTULO 1 / CHAPTER 1

María Llana-Ruiz-Cabello, Silvia Pichardo, Sara Maisanaba, María Puerto,, Ana I. Prieto, Daniel Gutiérrez-Praena, Ángeles Jos, Ana M. Cameán

IN VITRO TOXICOLOGICAL EVALUATION OF ESSENTIAL OILS AND THEIR MAIN COMPOUNDS USED IN ACTIVE FOOD PACKAGING: A REVIEW

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Invited Review

In vitro toxicological evaluation of essential oils and their main compounds used in active food packaging: A review

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ABSTRACT

Essential oils (EOs) and their main constituent compounds have been extensively investigated due to their application in the food industry for improving the shelf life of perishable products. Although they are still not available for use in food packaging in the market in Europe, considerable research in this field has been carried out recently. The safety of these EOs should be guaranteed before being commercialized. The aim of this work was to review the scientific publications, with a primary focus on the last 10 years, with respect to different *in vitro* toxicological aspects, mainly focussed on mutagenicity/genotoxicity. In general, fewer genotoxic studies have been reported on EOs in comparison to their main components, and most of them did not show mutagenic activity. However, more studies are needed in this field since the guidelines of the European Food Safety Authority have not always been followed accurately. The mutagenic/genotoxic activities of these substances have been related to metabolic activation. Therefore, in *vivo tests* are required to confirm the absence of genotoxic effects. Considering the great variability of the EOs and their main compounds, a case-by-case evaluation is needed to assure their safe use in food packaging.

1. Introduction

The safety and organoleptic properties of food, such as perishable products, may be altered due to several reactions, which include microbial spoilage or oxidation processes. These reactions decrease the value of the food because of the destruction of substances with beneficial properties (fatty acids, vitamins or proteins); production of off-flavours and odours; and, in the worst case, production of foodborne illnesses related to unsafe food intake (Sanches-Silva et al., 2014). New trends in consumer demands and internationalization of markets have caused changes in the retail and distribution practices, resulting in an increased distribution distance and longer storage time. Hence, the improvement in the shelf life of perishable products has become a large challenge for the food packaging industry worldwide (Vermeiren et al., 1999).

To improve food packaging and extend the optimal quality and freshness of perishable products, the food industry has developed a new packaging system called "active packaging" (Sung et al., 2013). Active food contact materials are "materials that are intended to extend the shelf-life or to maintain or improve the conditions of packaged food" (EFSA, 2009, http://www.efsa.europa.eu/en/efsajournal/doc/1208.pdf). They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food (European Commission, 2004). These components, which would be incorporated into the packaging matrix, include both synthetic and natural substances. Nevertheless, consumers have become more conscious about potential health problems associated with synthetic preservatives and are interested in ingredients from natural sources (Amorati et al., 2013; Bahram et al., 2012). In this sense, the use of essential oils (EOs) in active packaging has become a good alternative for improving the shelf life of perishable products (Sacchetti et al., 2005).

Essential oils are the secondary metabolites of plants, which have been studied due to their flavour and fragrance for flavouring foods, drinks and other goods. Additionally, EOs have been traditionally used to extend the shelf life due to their antimicrobial/antifungal activity (Bajpai et al., 2012; Bakri and Douglas, 2005) or their antioxidant properties (Roby et al., 2013; Teixeira et al., 2012). Currently, their relatively safe status, properties and acceptance by consumers have piqued the interest of industries (Sacchetti et al., 2005). However, the amount of substances added to food will determine the acceptability of products because strong flavours might result in palatability problems. In this sense, the use of EOs as preservatives in food has been limited because high concentrations are required to reach sufficient activity (Hyldgaard et al., 2012). Active

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packaging, where volatile compounds originate in a preservative atmosphere, is a good alternative to the direct incorporation of EOs because this method enables a slow release of the agent onto the food surface, maintaining an adequate concentration of the active compounds to preserve the quality of the food and avoiding the undesirable effects produced by the direct addition of high levels of EOs (Kuorwel et al., 2011; Nerín et al., 2006). Antimicrobial packaging has attracted substantial attention from the food industry, and many EOs and their main compounds can be incorporated in active packaging as antimicrobial agents (Hyldgaard et al., 2012; Seow et al., 2014). Moreover, the antioxidant properties of this active packaging have been reviewed in the literature (Eça et al., 2014; Sanches-Silva et al., 2014).

Many EOs, such as clove, oregano, thyme, nutmeg, basil, mustard or cinnamon, are categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (FDA) (Manso et al., 2014) but there are regulatory limitations on the accepted daily intake of the EOs, and a daily intake survey should be available for the evaluation of the safety. Only a limited range of EO components such as linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene have been accepted as flavouring agents in Europe, all of which are considered to present no health risk to consumers, at the estimated intake level (Hyldgaard et al., 2012). However, in the case of active packaging, the ingredients allowed to be used for this purpose have not been published so far in Europe. Currently, a community list of authorized substances is being established, taking into account the European Food Safety Authority (EFSA) opinion on each substance. The extent of toxicological data needed in order to be included in this list depends on the expected migration into food and it is necessary to perform at least 3 mutagenicity studies *in vitro* (EFSA, 2008a). In non-European countries such as the USA, Japan and Australia, the use of this technology is approved (Dainelli et al., 2008). Due to the market globalization, consumers could be exposed to these substances worldwide. There is therefore a need for more toxicity and exposure data on EOs and their ingredients used for food contact materials in order to confirm their safety (Evandri et al., 2005).

The general biological effects of EOs used in pharmaceutical, sanitary, cosmetic, agricultural and food industries have been reviewed by Bakkali et al. (2008), including some toxicological assessment. Taking into account all these considerations, this review aims to compile evidence on the *in vitro* toxicological aspects, mainly mutagenicity and genotoxicity of the EOs, and their major compounds, that are used in active food packaging. To achieve these objectives, a thorough review of the scientific literature on this topic from the last 10 years was performed; most of the works were published from 2008 (approximately 60%).

2. Main active compounds present in essential oils

Essential oils are low molecular weight, volatile mixtures that are biosynthesized in various organs of plants (Bajpai et al., 2012). In general, the components of EOs are divided into two groups according to their biosynthetic origin, compounds from terpene origin and aromatic compounds (Bakkali et al., 2008). The main terpenes are the monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist. The monoterpenes are the most representative molecules, constituting 90% of the EOs and allowing for a great variety of structures. In this way, terpene compounds comprise alcohols, aldehydes, carbures, ketones, esters, ethers, peroxides and phenols (Bakkali et al., 2008). Aromatic compounds occur less frequently than the terpenes and its constituents comprise aldehydes, alcohol, phenols, methoxy derivates and methylene dioxy compounds (Bajpai et al., 2012; Bakkali et al., 2008).

Essential oils can contain approximately 20–60 components at substantially different concentrations, which are characterized by two or three major components (main compounds) at fairly high concentrations (20–70% or even up to 85%) compared to other components present in trace levels (Bakkali et al., 2008; Burt, 2004). Table 1 shows the main plants containing EOs used for food packaging. Generally, these major components determine the biological properties of the EOs, both the beneficial effects (antibacterial, antifungal, antioxidant, etc.) and the toxicological profile of the substances. Therefore, both the beneficial and prejudicial aspects of EOs should be considered in order to ensure human safety. It is also important to note that the composition of EOs can vary considerably between subspecies of the plant from which they are extracted, origin and harvest time (Smith et al., 2005), and also depending on differences in the cultivation, vegetative stage and growing season of the plants (Vagi et al., 2005). Between different species, the carvacrol content may change considerably from 22.0% in *Origanum compactum* to 64.3% in *Origanum onites* or even exhibit different chemical composition, such as the case for β-caryophyllene and α-thuyene, which are present in *O. compactum* and not detected in *O. onites* (Bostancioglu et al., 2012; Mezzoug et al., 2007). For these reasons also the toxicity of EO extracts can vary based on the above-mentioned differences, which is very important to bear in mind for risk assessors. It is important to highlight that the quantitative and qualitative composition of EO extracts can be modified (in general, larger quantitative changes rather than qualitative ones) depending on the pre-treatment, processing technology and extraction processes.

As already mentioned above, the use of EOs in active packaging in Europe is not yet a reality. Moreover, to our knowledge, no data are available about the use of active packaging containing EOs in non-EU countries. However, the EOs that are more frequently studied to be used for this purpose are oregano, (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), green tea (*Camellia sinensis*), cinnamon (*Cinnamomum zeylanicum*), and clove (*Eugenia caryophyllata*) because of their antimicrobial, antibacterial, antifungal, and antioxidant properties. In this regard, the EOs from “oregano” plants are characterized by the high content of carvacrol (64–22%), a component of particular importance, and also by thymol (19–1.4%) (Bostancioglu et al., 2012; Llana-Ruiz-Cabello et al., 2014a; Mezzoug et al., 2007). Carvacrol (5-isopropyl-2-methylphenol) and its isomer, thymol (2-isopropyl-5-methylphenol), are the major compounds in thyme, marjoram, and savoury EOs (Al-Bandak and Oreopoulou, 2007; De Vincenzi et al., 2004). Carvacrol and thymol are commonly used due to antimicrobial effects on bacteria, fungi, and yeast (Bakkali et al., 2008; Burt, 2004; Lambert et al., 2001; Zhou et al., 2007), as well as insecticidal and antioxidant effects (Akalin and Incesu, 2011). Several studies have reported on the use of carvacrol in packaged food, such as baked chicken, wrapped (Du et al., 2012) or refrigerated fresh fish and meat (Guarda et al., 2011, 2012). Thymol has been incorporated, by direct dissolution, into soy protein isolate-based film to package olive oil (Hu et al., 2012). Both carvacrol and thymol are suitable for their microencapsulated use in a polymer matrix for fresh food preservation (Guarda et al., 2011). Linalool, the main constituent of various EOs (69.2–2.91%), such as basil, cilantro, green tea, lavender and oregano, has potential use in low-density polyethylene-based films with its antimicrobial component that can enhance the quality and safety of cheeses (Suppakul et al., 2008). Linalool has been evaluated by EFSA as a flavouring substance and it has been classified in category A (flavouring substance, which may be used in foodstuffs) (EFSA, 2011a). Moreover, it is stable at relatively high temperatures; therefore, it may have the potential to be incorporated into polymers and used in antimicrobial packaging (AM) (Suppakul et al., 2008). Cinnamon EO from the plant *Cinnamomum zeylanicum* and cinnamaldehyde (with a concentration of 85% in cinnamon EO) have high antioxidant,

Table 1
Plant species containing main compounds with applications in active food packaging, including their composition in their respective EOs (percentages).

Main compounds of selected EOs				
Plant	Specie	Parts of plant	Main compounds (approximate %)[a]	References
Basil	*Ocimum basilicum*	Leaves	Linalool (69.2),α-Cadinol (2.56), γ-Cadinene (2.5), estragol (2.4), δ-Guaiene (2.10)	Stajkovic et al., 2007; Beric et al., 2008
Cilantro	*Coriandrum sativum*	Leaves	Linalool (26), E-2-decanol (20)	Delaquis et al., 2002
Cinnamon	*Cinnamomum zeylanicum*	Bark	(E)- Cinnamaldehyde (68.95)	Unlu et al., 2010
Citronella	*Cymbopogon winterianus*	Leaves	Citronellal (20–30), Citronellol (10–15)	Sinha et al., 2014
Clove	*Eugenia caryophyllata*	Flowers	Eugenol (58.2–48.82), caryophyllene (36.94–13.99), eugenol acetate (22.34–3.89)	Guan et al., 2007
Garlic	*Allium sativum*	Bulbs	2 Propenyl thioacetonitril (43.25), trisulfide methyl 2-propenyl (23.40), disulfide di-2-propenyl (20.87)	Kirkpinar et al., 2011
Green tea	*Camellia sinensis*	Leaves	Linalool (56.73), α-terpineol (9.37), p-cresol, 2,6-di-tert-butyl (11.63), nonanaldehyde (5.00), hexanal (4.49), 3-methylbutanal (3.41)	Tontul et al., 2013
Lemongrass	*Cymbopogon citratus*	Leaves	Citral (50–88)	Sinha et al., 2014
Marjoram	*Origanum majorana*	Inflorescences	Apigenin (35.23), methyl rosmarenate (31.58), rosmarenic acid (15.15)	Roby et al., 2013
Onion	*Allium cepa*	Bulbs	Propionaldehyde (78.41–13.56), dipropyldisulfide (78.7–10.6), 3,4-dimethylthio-phene(13.23–0.35)	Colina-Coca et al., 2013
Oregano	*Origanum compactum*	Leaves	γ-Terpinene (22.90),carvacrol (22.0), thymol (19.36), p-Cymene (13.26), β-caryophyllene (3.47), α-terpinene (3.26), linalool (2.91), myrcene (2.58), α-Thuyene (1.51), α-Pinene (1.15)	Mezzoug et al., 2007
	Origanum onites		Carvacrol (64.30), linalool (13.80), p-cymene (7.10), γ-terpinene (3.50), myrcene (1.6), thymol (1.4), α-terpinene (1.4), α-pinene (1.0)	Bostancioglu et al., 2012
			Linalool (50.53), carvacrol (24.52), thymol (15.66)	Özkan and Erdogan, 2011
Palmarosa	*Cymbopogon martini*	Leaves	Geraniol (65–85), geranyl acetate (5–20)	Sinha et al., 2014
Pennyroyal	*Mentha pulegium*	Leaves and inflorescences	Menthone (35.90), neo-menthol (9.20), 8-Hydroxy- δ-4(5)-p-menthen-3-one (2.10)	Teixeira et al., 2012
Rosemary	*Rosmarinus officinalis*	Leaves and calyx	Verbenone (21.76), camphor (14.6), safrole (12.3), borneol (10.4), 1,8-cineole (7.26), α-pinene (6.65), β-caryophyllene (6.17), geraniol (5.75), β-selinene (3.61), α-selinene (3.57)	Sacchetti et al., 2005
Thyme	*Artemisia campestris*	Leaves	β-pinene (41.0), p-cymene (9.9), α-terpinene (7.9), limonene (6.5), myrcene (4.1) β-phellandrene (3.4), α-Pinene (3.2)	Neffati et al., 2008
	Thymus vulgaris L.		Cinnamic acid (28.54), apigenin (8.88), luteolin-7-o-rutinose (7.65)	Roby et al., 2013

[a] % approximate of selected main compounds detected in EO extracts under specific conditions.

antibacterial, and anti-inflammatory activities, and they are currently widely used in the food industry (Xing et al., 2014). Cinnamon EOs and cinnamon fortified with cinnamaldehyde have been incorporated by coating into polypropylene and polyethylene/ethylene vinyl acetate copolymers for bakery products (López et al., 2007) or into paper packaging for strawberries (Rodríguez et al., 2007) and cherry tomatoes (Rodríguez-Lafuente et al., 2010). Eugenol is an important antioxidant that is isolated from black pepper, dill, marjoram, nutmeg, red pepper, thyme, turmeric and clove oils (58.2–48.82%) (Guan et al., 2007; Sanla-Ead et al., 2012; Suhaj, 2006). It has demonstrated antimicrobial activity and potential application by direct dissolution in antimicrobial packaging film or coating, such as methyl cellulose films (Sanla-Ead et al., 2012).

In many cases, EOs have more beneficial effects than chemically synthesized, pure compounds. This may be due to the synergistic action of the complex mixture of components that can interact with multiple molecular sites. Moreover, some constituents of EOs may affect the absorption rates or bioavailability of other components (Seow et al., 2014). Considering that some authors have demonstrated the induction or changes in the potency induced by EOs compared with their isolated components, this review is focussed on assessing some toxicological aspects of both the EOs and their main components.

3. Toxicological studies

Antimicrobial, antioxidant, and antimutagenic/antigenotoxic effects of EOs may be beneficial effects, but besides these properties, EOs may also have potential toxic effects such as mutagenicity/genotoxicity, which need to be assessed before being used in food contact materials. Moreover, the incorporation of EOs in active food packaging can result in a higher human exposure to these compounds. Therefore, more research is needed to establish effective and safe concentrations of EOs (Seow et al., 2014) as well as to assess their mutagenic/genotoxic potential, identify possible toxic/mutagenic components, and try to construct an almost complete profile of the risks and benefits of their use (Bakkali et al., 2008; Rossi et al., 2013).

3.1. Evaluation of mutagenicity and genotoxicity

Although the detailed mutagenic potential of the extracts of common spices, such as garlic, ginger, pepper and turmeric, have been studied, less work has been performed on the EOs extracted from these spices (Jeena et al., 2014). The growing interest in the use of these EOs with diverse applications, including to treat minor health problems, makes it necessary to assess their genotoxic potential and identify the mutagenic components (Evandri et al., 2005) because the random and inappropriate use of the EOs may increase the risks to human health due to mutational events, carcinogenic effects and genetic damage (Sousa et al., 2010).

The process of assessing the genotoxic potential of a compound is a crucial and mandatory step because of the relevance for human safety in relation to the potential induction of carcinogenesis and hereditary defects (Guzmán et al., 2008). No single genotoxicity test is capable of detecting all relevant genotoxic agents; therefore, in accordance with the Guidelines of the Scientific Committee on Food for safety assessment of substances used in food contact materials (European Commission, 2001), which has been updated (http://www.efsa.europa.eu/en/efsajournal/doc/21r.pdf), the core set of tests consists of the following 3 mutagenicity studies *in vitro*: (1) a test of induction of gene mutations in bacteria; (2) a test for induction of gene mutations in mammalian cells (preferably the mouse lymphoma tk assay), and (3) a test for induction of

chromosomal aberrations in mammalian cells. More recently, the Scientific Committee of EFSA reviewed the current state-of-the-science on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011b; http://www.efsa.europa.eu/en/efsajournal/doc/2379.pdf). According to this opinion, a step-wise approach is recommended for the generation and evaluation of data on genotoxic potential, beginning with a basic battery of 2 *in vitro* tests, comprising (1) a bacterial reverse mutation assay and (2) an *in vitro* micronucleus assay. In the event of negative *in vitro* results, it can be concluded that the substance has no genotoxic potential. In case of inconclusive, contradictory or equivocal results, it may be appropriate to conduct further testing *in vitro*. In case of positive *in vitro* results, an appropriate *in vivo* study to assess whether the genotoxic potential observed *in vitro* is expressed *in vivo* is recommended. Suitable *in vivo* tests are the mammalian erythrocyte micronucleus test, transgenic rodent assay, and Comet assay. The approach to *in vivo* testing should be also step-wise.

A review of the most recent mutagenicity and genotoxicity studies performed on EOs and their main compounds available in the literature is presented in Tables 2 and 3, respectively. Comparisons of the genotoxicity profiles of EOs in different tests allow us to conclude that different compounds present in these EOs are responsible for their genotoxicity in different tests. The identification of the genotoxic compounds in EOs is of great importance for two main reasons: (1) to perform the genetic risk assessment of EOs based on genotoxicity of their individual compounds and (2) to improve the safety of EOs by eliminating or reducing the amount of these genotoxic compounds (Lazutka et al., 2001).

3.1.1. Mutagenicity/genotoxicity studies

In relation to the induction of gene mutations in bacteria, several studies have been performed on EOs, mainly focussing in their major components, the most abundant of which are those from the oregano, allium, rosemary, and basil oils.

To assess the mutagenicity spectrum of EOs and the main compounds, a test for gene mutation in bacteria (OECD, 1997a) with *Salmonella typhimurium* and *Escherichia coli* should be conducted to detect all possible single base pair changes as well as frameshift mutation. In this guideline, 5 strains are required with and without metabolic activation and maximum test concentration for non-toxic substances up to 5000 μg/plate. Many authors have used this *Salmonella*/microsome assay (Table 2) (Di Sotto et al., 2008, 2013; Ipek et al., 2005; Llana-Ruiz-Cabello et al., 2014b; Shon et al., 2004; Stammati et al., 1999; Zegura et al., 2011). However, very few of them have performed the tests following the guideline for this assay accurately. Thus, only two strains are frequently selected in mutagenicity studies (TA98 and TA100). On the other hand, in the *Escherichia coli* reversion assay, the strains selected are *E. coli* K12 strains (SY252, IB103, IB105, IB111, IB127, IC102 and IC185).

In general, the majority of EOs with applications in food packaging does not have mutagenic effects (Table 2). Similarly, the main compounds generally did not induce mutagenicity, whatever the organism, i.e. bacteria, yeast or insect, with or without metabolic activation (Bakkali et al., 2008). However, some exceptions or contradictory results have been reported, such as carvacrol (as seen in Table 3). Fewer genotoxic studies have been performed on EOs (Hamedo and Abdelmigid, 2009; Liju et al., 2013; Sinha et al., 2014; Thirugnanasampandan et al., 2012; Zegura et al., 2011) in comparison to their main components (approximately two-fold higher).

Following the battery of genotoxicity assays recommended by the Guidelines of the Scientific Committee on Food (European Commission, 2001), as previously mentioned, the most frequent genotoxic assays in mammalian cells *in vitro* for EOs and their components are the following: induction of chromosomal aberrations (CA) (OECD, 1997b), the sister chromatid exchange test (SCE) (OECD, 1986), micronucleus assay (MN) (OECD, 2010) and, especially, the standard Comet assay (alkaline single-cell gel electrophoresis tests (Tables 2 and 3).

The genotoxic response may be affected by the experimental model chosen and the range of concentrations assayed. For example, the origin of the cell line exposed to thymol influenced the result obtained. Whereas Aydin et al. (2005a, 2005b) found DNA damage in human lymphocytes, no genotoxic response was observed in the HepG2 cell line from liver hepatocellular carcinoma (Horváthová et al., 2006), nor in Caco-2 cells derived from a human colon carcinoma (Llana-Ruiz-Cabello et al., 2014b). In addition, the genotoxicity of EOs could also depend on the biogeographical conditions of the plant and on the manufacturer from which it was obtained (Lazutka et al., 2001).

The *in vitro* MN assay is being increasingly used in the evaluation of EOs (Di Sotto et al., 2011, 2013; Liju et al., 2013; Maistro et al., 2011; Nicolella et al., 2014) rather than the *in vitro* CA assay (Hamedo and Abdelmigid, 2009; Lazutka et al., 2001; Lorge et al., 2006). This finding may be due to the incorporation of the MN assay in the core set of genotoxicity tests recommended by EFSA together with the bacterial reverse mutation test (EFSA, 2011b). Moreover, the MN assay offers some advantages compared with the CA test, such as the high number of analysable cells, simplicity of the technique, possibility of automation, and ability to more accurately detect aneugens (Nesslany and Marzin, 1999; Vanderkerken et al., 1989).

The alkaline version of the Comet assay (Collins et al., 1997; Singh et al., 1988) is probably the most widely used test for both the EOs (Liju et al., 2013; Sinha et al., 2014; Thirugnanasampandan et al., 2012; Zegura et al., 2011) and their main compounds. The enzyme-modified Comet assay (Endo III and formamidopyrimidine DNA glycosylase (FPG)), has only been used to evaluate carvacrol and thymol in V70 Chinese hamster lung fibroblast cells, showing no DNA damage (Ündeger et al., 2009), whereas in Caco-2 cells, carvacrol (460 μM) induced DNA-oxidative effects, indicating damage to the purine bases (Llana-Ruiz-Cabello et al., 2014b). Additionally, it is important to note that, to the best of our knowledge, the mouse lymphoma tk assay (OECD, 1997c) has not been used to evaluate the genotoxic potential of EOs used in the food industry.

In vivo genotoxicity tests with tissues can be used when *in vitro* positive results have been obtained, which, unlike *in vitro* tests, better reflect the absorption, excretion, distribution, and metabolism of chemicals. As already mentioned, according to the Scientific Opinion of EFSA, *in vivo* testing should be step-wise. Although EFSA (2011b) recommend only one *in vivo* test in case of clear positive or negative results, in some cases, a second *in vivo* test may be necessary (e.g. if the first test is negative but more than one endpoint in the *in vitro* tests are positive). The combination of assessing different endpoints in different tissues in the same animal *in vivo* should also be considered (EFSA, 2011b). In this regard, integrating the *in vivo* Comet and *in vivo* MN assays has been successfully performed for follow-up testing of positive *in vitro* results. They are also potential tools for assessing local genotoxicity (e.g. site of contact), especially for tissues or cell types that cannot be easily measured with other standard testing methods (Kang et al., 2013). Following this recommendation, it would be very useful to apply both assays in further development of EOs with potential use in food packaging.

The genotoxic responses of EOs and their components could be due in part to induction of oxidative stress. In this sense, the literature concerning the prooxidant effects of EOs is very scarce, whereas that on the prooxidant effects of single compounds from EOs is much more extensive. Considering EOs, rosemary EO has been demonstrated to induce lipid and protein oxidation at high doses in white pig frankfurters (Estévez and Cava, 2006). In the case of the main compounds, Bakkali et al. (2008) stated that polyphenolic and phenolic compounds from EOs can mainly act as prooxidants through the intermediate of their volatile constituents. Several authors have

Table 2
Mutagenicity and genotoxicity assays of essential oils (EOs) used in food industry: percentages in main compounds, assays and experimental setup, and the main results reported.

Essential oil	Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Basil (*Ocimum basilicum* L.)	Linalool (69.2%), α-canidol (2.560%), α-canidiene (2.5%), estragole (2.4%)	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA 98, TA 100, TA102)	0.5–2 μL/plate	Not mutagenic response; however, antimutagenic effects against several mutagens	Stajkovic et al., 2007
Basil (*Ocimum basilicum* L.)	Essential oil	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA98, TA100, TA102) and *E. coli* (WP2, SY252, IB101)	0–2 μl/plate	There was no mutagenic response to the EO The EO exhibited antimutagenic potential against *t*-BOOH-treated cells	Beric et al., 2008
Basil (*Ocimum basilicum* L.)	Iso-pinocamphone (35.10%), carvone (39.70%)	Ames test	*S. typhimurium* strains (TA 98, TA 100)	96–480 μg/plate	No mutagenic activity was observed at the conditions tested	De Martino et al., 2009
Cilantro, Coriander (*Coriandrium sativum* L.)	Linalool (75.4%) γ-Terpinene ((4.9%) α-Pinene (4.8%) Geranyl-acetate (3.0%) Geraniol (2.8%)	Ames test	*S. typhimurium* strains (TA 97, TA 102)	0.4–8.0 μg/mL	Mutagenicity was present in high concentrations in both tested strains (1.6–8 μg/mL)	Reyes et al., 2010
Cinnamon (*Cinnamomum zeylanicum*)	Cinnamaldehyde, cinnamic acid, eugenol, limonen, cinnamyl alcohol	Ames test	*S. typhymurium strain* (TA100)	50–2000 μg/mL	Negative results were obtained with and without S9	Shoeibi et al., 2009
Citronella (*Cymbopogon winterianus* Jowitt)	Citronella	Comet assay DNA diffusion assay	Human lymphocytes	100–2000 μg/mL	DNA damage at high concentrations (1000 μg/mL and above)	Sinha et al., 2014
Curcuma longa L.	Turmeric essential oil (TEO)	Ames test Micronucleus test (MN) Chromosome aberrations (CA) Comet assay	*S. typhimurium* strains (TA 98, TA 100, TA102, TA1535) Bone marrow, spleen and intestine of rats (oral)	100–3000 μg/plate 1 mg/kg 2 weeks	TEO did not produce any mutagenicity to *S. typhimurium* with or without metabolic activation TEO did not produce any CA or MN in rat bone marrow cells, and any DNA damage was observed in the organs studied	Liju et al., 2013
Ginger essential oil (*Zingiber officinale* R.) (GEO)	α-Zingiberene (31.08%) and ar-curcumene (15.4%), and α-sesquiphellandrene (14.02%)	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA98, TA100, TA102)	10–3000 μg/plate	No mutagenic effects with and without S9 metabolic fraction GEO showed antimutagenicity activity against direct-acting mutagens	Jeena et al., 2014
Green tea (*Camellia sinensis*)	Extract of green tea	Sister chromatid exchange (SCE) Replication index (antigenotoxicity assays)	Human lymphocytes	$1.075–3.15*10^{-4}$ g/mL	Antigenotoxic effects were obtained against two anabolic steroids	Gupta et al., 2009
Green tea (*Camellia sinensis*)	Extract of green tea	Comet assay	Jurkat cells	10 mg/mL	The extract did not protect against H_2O_2-induced DNA damage	Bhatt et al., 2010
Lemongrass (*Cymbopogon citratus*)	Citral (50–88%), linalool, Myrcene, geraniol, geranyl acetate, camphene	Comet assay DNA diffusion assay	Human lymphocytes	0–800 μg/mL	DNA damage at 100 μg/mL and above	Sinha et al., 2014
Onion (red onions)	Ethyl acetate extracts	Ames test (antimutagenicity assay)	*S. typhimurium* strains (TA98, TA100)	0–3000 μg/plate	All type of onions contained antimutagens and the extracts showed similar levels of anitimutagenic activity, which depends on the mutagen and dose levels	Shon et al., 2004
Oregano (*Origanum onites*)	Carvacrol (74%); Linalool (7.2%); Thymol (4.4%); p-Cymene (3.0%); β-bisabolone (1.4%) and Caryophyllene oxide (1.3%)	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA98, TA100)	0.01–0.50 μl/plate	No mutagenicity was found in the oil to both strains with or without S9 mixture. Moreover, EO inhibited mutagenicity induced by 4-NPD and 2-AF	Ipek et al., 2005
Oregano (*Origanum vulgare* L.)	O-cymene (41.90%), carvacrol (44.10%)	Ames test	*S. typhimurium* strains (TA98, TA100)	93–467 μg	The essential oil tested, at any dose, did not show mutagenic activity with or without metabolic activation	De Martino et al., 2009
Oregano (*Origanum vulgare)*	Extract essential oil	Mitotic index CA	*Vicia faba* seeds	0.1–1%	Reduction in mitotic activity, chromosomal and nuclear irregularities	Hamedo and Abdelmigid, 2009

(*continued on next page*)

Table 2 (*continued*)

Essential oil	Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Oregano (*Lippia graveolens* Kunth)	Total phenol content: 211–270 mg gallic acid equivs/dried extract	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA98, TA100)	0.01 mL extracts or 200 μg gallic acid/mL	None of the extracts was toxic to the tested strains; Antimutagenic activity was exhibited by the extracts at a concentration equivalent to 200 μg of gallic acid	Martinez-Rocha et al., 2008
Oregano (*Origanum vulgare*)	Extracts: Methanol, n-butanol, and from these extracts the following compounds were isolated: luteolin-7-O-glucuronide, luteolin-7-O-xyloside	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA1535, TA1537) and *E. coli* (wP2uvrA)	0.4–2 μM/plate	Not mutagenic activity; however, antimutagenic effects on *E. coli* strain and TA1537, but not in TA1535	Gulluce et al., 2012
Palmarosa (*Cymbopogon martini*)		Comet assay DNA diffusion assay	Human lymphocytes	100–2000 μg/mL	DNA damage at high concentrations for palmarosa (1000 μg/ml and above)	Sinha et al., 2014
Pituranthos chloranthus (Coss. Et Dur.)	Gabes, Medenine and Benguerdane essential oils (GEO, MEO, BEO), thymol and carvacrol being among the monotepenoids predominants	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA98, TA100, TA1535)	30–100 μg/plate essential extracts	None of the tested essential oils induced mutagenic effects within the tested dose range; They reduced significantly BaP and SA-induced mutagenicity	Neffati et al. 2009
Rosemary (*Rosmarinus officinalis*)	10–12% of carnosic acid (88%) and carnosol (12%)	Ames test (antimutagenicity assay)	*S. typhimurium* strain (TA102)	0–0.8 mg/mL	The rosemary extract significantly and dose-dependently reduced the *t*-BOOH-induced mutagenicity	Minnunni et al., 1992
Rosemary (*Rosmarinus officinalis*)	Water soluble AquaROX® and oil soluble VivOX® extracts	Ames test (mutagenicity/ antimutagenicity assays) Comet assay	*S. typhimurium* strains (TA98) HepG2 cells	0.05–0.2 mg/plate 0.05–5 μg/mL	None of the extracts was mutagenic in the absence and presence of S9 Antimutagenic activity against promutagen IQ Both extracts protected HepG2 cells against stress induced by *t*-BOOH, and against indirect acting mutagens (BaP and PhIP)	Zegura et al., 2011
Rosemary (*Rosmarinus officinalis*)	Extract essential oil	Mitotic index CA	*Vicia faba* seeds	0.1–1%	Reduction in mitotic activity, chromosomal and nuclear irregularities	Hamedo and Abdelmigid, 2009
Teucrium ramosissimum	Essential oil	Ames test (mutagenicity/ antimutagenicity assays)	*Salmonella typhimurium* strains (TA98, TA100, TA1535)	0–250 μg/plate	No mutagenicity was detected for the different doses in the three strains systems Antimutagenic effects against SA, AFB1, BaP, 4-NPD were obtained	Sghaier et al., 2010
Thyme (*Artemisia campestris*)	β-Pinene (41.0%), p-Cymene (9.9%), α-Terpinene (7.9%), Limonene (6.5%), Myrcene (4.1%), β-Phellandrene (3.4%), a-Pinene (3.2%)	Ames test (mutagenicity/ antimutagenicity assays)	*Salmonella typhimurium* strains (TA97, TA98)	10–100 μg/mL	Mutagenicity response did not present in the conditions tested; however, antimutagenic activity against BaP was observed	Neffati et al., 2008
Thyme (*Thymus Vulgaris* L.)	*o*-Cymene (56.20%), carvacrol (24.44%), thymol (8.75%)	Ames test	*Salmonella typhimurium* strains (TA98, TA100)	93–463 μg/plate	Mutagenic activity did not obtain with or without metabolic activation, at any dose assayed	De Martino et al., 2009
Toddalia asiatica (L.) Lam	β-Phellandrene (21.35%), cis-β-ocimene (12.87%), α-phellandrene (9.01%), viridiflorol (6.74%), β-bisabolol (5.24%), α-pinene (4.49%)	Comet assay	Human lymphocytes	25–100 μg/mL	The oil protects 50% of DNA against H_2O_2 induced genotoxicity (100 μg/ mL)	Thirugnanasampandan et al., 2012

AFB1: Aflatoxin B_1; 2-AF: 2-aminofluorene; 4-NPD: 4-nitro-*o*-phenylenediamine; IQ: 2-amino-3-methyl-3H-imidazo[4,5-F]quinoline; BaP: benzo(*a*)pyrene; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SA: Sodium azide; *t*-BOOH-: *t*-Butyl-hydroperoxide.
HepG-2: human hepatocellular carcinoma cell line; Jurkat: human lymphoblast acute T cell leukemia cell line.

Table 3
Mutagenicity and genotoxicity assays of main compounds of EOs used in food industry: assays and experimental setup, and the main results reported.

Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Apigenin	Micronucleus (MN)	Human lymphocytes	2–25 μg/mL	Apigenin did not induce increase in MN frequency at lower concentrations (2.5–10 μg/mL) although increased MN at the highest concentration assayed. In irradiated cells MN frequency decreased as the concentration of apigenin increased.	Rithidech et al., 2005
Apigenin	MN	Peripheral human lymphocytes	10–100 μM	Apigenin induced micronuclei in a dose dependent manner, indicating potential genotoxic hazard in humans.	Noel et al., 2006
Apigenin	Chromosomal aberrations (CA) (Genotoxicity/antigenotoxicity assays) Sister chromatid exchanges (SCE) (Genotoxicity/antigenotoxicity assays)	Human lymphocyte culture	1–20 μM	Not genotoxic. The treatment of apigenin results in a dose dependent decrease in the genotoxic damage, induced by MMC and CP.	Siddique et al., 2008
Apigenin	SCE CA	HepG2 cells	1–50 μM	Apigenin exhibited dose-dependent genotoxic potential in HepG2 cells.	Papachristou et al., 2013
β-Caryophyllene	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA 98, TA100) and *E. coli* (WP2uvrA)	Mutagenicity assay: 9.0–2.3 mg/plate Antimutagenicity assay: 0.17–6.40 mg/plate	No mutagenic effects were observed at the conditions assayed. Inhibition of the mutagenic effect against 2NF, 2AA and MMS.	Di Sotto et al., 2008
β-Caryophyllene	MN (clastogenicity/ anticlastogenicity assays)	Human lymphocytes	Clastogenicity assay: 0–200 μg/mL Anticlastogenic assay: 0–100 μg/mL with three protocols: pretreatment, co-treatment, and post-treatment	Up to 100 μg/mL did not induce any genotoxic effect. The compound (0–100 μg/mL) reduced the MN frequency induced by EMS in pre- and co-treatment protocols. In the post-treatment, it was not antigenotoxic. No significant reduction of COL induced MN frequency was found.	Di Sotto et al., 2010
β-caryophyllene oxide	Ames test MN	*S. typhimurium* strains (TA 98, TA100) and *E. coli* (WP2uvrA) Human lymphocytes	0.23–0.90 mg/plate 1–50 μg/mL	No mutagenic effects were observed with or without S9. The compound did not induce genotoxicity at chromosomal level.	Di Sotto et al., 2013
Camphor	Ames test, *E. coli* reversion assay (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA100, TA102), *Escherichia coli* (K12SY252, IB112, WP2) and *Sacharomycces cerevisiae* D7	0–20 μL/plate	Mutagenic potential was not detected. Antimutagenic effects against UV-induced mutations were observed.	Vukovic-Gacic et al., 2006
Camphor	*E. coli reversion* assay (mutagenicity/ antimutagenicity assays) Comet assay (genotoxicity/ antigenotoxicity assays)	*E. coli* K12 strains (SY252, IB103, IB105, IB111, IB127, GY7066, GY8281, GY8252) Vero cells	0–15 μL/plate 5–750 μM 20 h	No mutagenic effect was obtained. Antimutagenic potential against UV- and 4NQO-induced mutagenesis was detected. Genotoxic effects were observed at higher tested concentrations. Antigenotoxic potential (low concentrations) against 4NQO.	Nikolic et al., 2011
Carnosic acid and carnosol	Ames test (antimutagenicity assay)	*S. typhimurium* strain (TA102)	Carnosic acid: 1g/mL Carnosol: 20 mg/mL	Only carnosic acid presented a significantly and dose dependent reduction.	Minnunni et al., 1992
Carvacrol	Ames test	*S. typhimurium strains* (TA98, TA100)	0.1 M 6.25–25μl/plate	No significant increase was observed in both strains at the concentrations assayed.	Stammati et al.,1999
Carvacrol	SCE	Human peripheral blood lymphocytes	0.1–5.0 μl/ml (v/v)	All doses did not increase the formation of SCE, whereas the rate of SCE induced by MMC was inhibited.	Ipek et al., 2003
Carvacrol	Comet assay (genotoxicity/ antigenotoxicity assays)	Human lymphocytes	0.0005–2 mM	At the concentration of 0.1 mM carvacrol itself induced DNA damage. Carvacrol protects from the genotoxic effects induced by IQ and MMC at concentrations below 0.05 mM.	Aydin et al., 2005a

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Table 3 (*continued*)

Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Carvacrol	Comet assay (genotoxicity/ antigenotoxicity assays)	Human lymphocytes	0.0005–2 mM	Carvacrol at concentrations above 0.05 mM induced significantly DNA damage Carvacrol protected from the genotoxic effects of 0.1 mM H_2O_2 at concentrations below 0.1 mM.	Aydin et al., 2005b
Carvacrol	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* (TA98, TA100)	0.01–1 μL/plate	Mutagenicity was induced by carvacrol, almost in absence of S9. Antimutagenicity was observed against 4-NPD and 2-AF.	Ipek et al., 2005
Carvacrol	Comet assay (genotoxicity/ antigenotoxicity assays)	HepG2 cells Caco-2 cells	0–200 μM 0–300 μMl	Not genotoxic. Carvacrol led to a significant protection of the cells studied towards DNA damage induced by H_2O_2.	Horváthová et al., 2006
Carvacrol	Standard comet assay Post-treatment Fgp protein	V79 cells	1–25 μM	No significant increase in DNA strand breakage was detected with carvacrol concentrations up to 25 μM. In post-treatment with Fgp enzyme, no significant increase of Fpg-sensitive sites was observed.	Ündeger et al. 2009
Carvacrol	SOS chromotest (antigenotoxicity assay)	*E. coli* strain (PQ37)	6.3–812.0 mM	Carvacrol produced significant reduction of bleomycin-induced genotoxicity at a dose between 50.7 and 812.0 mM.	Vicuña et al., 2010
Carvacrol	Comet assay (antigenotoxicity assay) FPG-sensitive sites	HepG2 cells	200 μM	Carvacrol protected against oxidative damage induced by H_2O_2 but did not protect against DNA damage induced by *t*-BHP.	Slamenova et al., 2013
Carvacrol	Comet assay	Cultured primary rat neurons N2a cells	0–400 mg/L	DNA damage was not found significantly different from the control values in both cell cultures.	Aydin et al., 2014
Carvacrol	Ames test Comet assay and Endo III and FPG-modified comet assay	*S. typhimurium* strains (TA97A, TA98, TA100, TA102, TA104) Caco-2 cells	29–460 μM	Carvacrol exhibited mutagenic potential, displaying greater activity in presence of S9 fraction. In the standard comet assay no effects were observed. The FGP-modified comet assay showed that carvacrol (460 μM) caused oxidative DNA damage.	Llana-Ruiz-Cabello et al., 2014b
Citral	SOS chromotest (genotoxicity/ antigenotoxicity)	*E. coli* strain (PQ37)	0–2915 mM	Citral did not induce genotoxic damage and showed significant reduction in bleomycin-induced genotoxicity.	López et al., 2011
Citral	Comet assay DNA diffusion assays	Lymphocytes from healthy male volunteers	0–800 μg/ml	DNA strand breaks at 25 μg/ml and above.	Sinha et al., 2014
Hydro-alcoholic solution of terpenes from *Boswellia sacra* (Burseraceae) resin, *Commiphora myrrae* (Burseraceae) resin and *Hyssopus decumbens* (Lamiaceae)	Ames test	*S. typhimurium* strains (TA98, TA100, YG1024)	1:100–1:750 doses hydro-alcoholic solution of plants	No significant differences between samples and negative control were observed.	Lupi et al., 2009
Estragole	SCE Comet assay Plasmid DNA strand-break assay ^{32}P-postlabelling analysis	V79 cells CHO cells: AA8 cells EM9 cells	0–1000 μM (2h) 0–750 μM (1h) 500–1000 μM (2–8 h)	Estragole induced DNA strand breakage in comet assay without S9. Estragole, besides being metabolized to genotoxic metabolites, is a weak direct-acting genotoxin that forms DNA adducts.	Martins et al., 2012
Eucalyptol (1,8 cineole)	Ames test, *E. coli* reversion assay (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA100, TA102), *Escherichia coli* (K12SY252, IB112, WP2) and *S. cerevisiae* D7	0–20 μL/plate	Mutagenic potential was not detected. Antimutagenic effects against UV-induced mutations were observed.	Vukovic-Gacic et al., 2006

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Table 3 (*continued*)

Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Eucalyptol (1,8-cineole)	*E. coli* reversion assay (mutagenicity/ antimutagenicity assays) Comet assay (genotoxicity/ antigenotoxicity assay)	*E. coli strains* (IC185 and IC202) HepG2 cells NC–NC cells	0.05–1.5 mg/plate (or 0.32–9.72 mM) 0–1 μg/mL	There was no mutagenic effect. However, antimutagenic effects in IC202 (33%) but not in IC185 were observed. Pre-treatments: in NC–NC cells, eucalyptol reduced *t*-BOOH induced DNA damage (about 50% reduction at 1.0 μg/ml). In HepG2 cells eucalyptol reduced DNA damage by 40%. Co-treatment experiments: the repair of *t*-BOOH induced DNA damage in HepG2 cells was not affected.	Mitic-Culafic et al., 2009
Eucalyptol	*E. coli* reversion assay (mutagenicity/ antimutagenicity assays) Comet assay (genotoxicity/ antigenotoxicity assays)	*E. coli* K12 strains (SY252, IB103, IB105, IB111, IB127, GY7066, GY8281, GY8252) Vero cells	0–15 μl/plate 5–500 μM 20 h	No mutagenicity response was obtained, but antimutagenic effects against 4NQO and UV were observed. Genotoxic effect was observed at higher tested concentrations. Strong antigenotoxic potential of low concentrations was determined using a pre-treatment with 4NQO.	Nikolic et al., 2011
Eugenol	CA Topoisomerase II (TopoII)	V79 cells	100–3000 μM 5–7500 μM	Eugenol is genotoxic (induced CA) and raises the possibility of having Topo II inhibiting activity.	Maralhas et al., 2006
Eugenol	Comet assay The γ-H2AX assay	CHO cells: AA8 cells and EM9 cells	0–500 μM	Eugenol produced DNA strand breaks and double strand breaks.	Martins et al., 2011
Ferulic acid	Comet assay MN	HTC cells	50–1500 μM	Not genotoxic in comet assay. The MN test showed an increase in the frequency of micronucleated cells.	Maistro et al., 2011
Geraniol	Comet assay DNA diffusion assays	Lymphocytes from healthy male volunteers	0–2000 μg/ml	No genotoxicity was shown.	Sinha et al., 2014
Limonene	Ames test, *E. coli* reversion assay (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA100, TA102), *E. coli* (K12SY252, IB112, WP2) and *Saccharomycces cerevisiae* D7	0–20 μL/plate	Mutagenic potential was not detected. Antimutagenic effects against UV-induced mutations were observed.	Vukovic-Gacic et al., 2006
R-(+)limonene	Ames test Comet assay	*Salmonella typhimurium* strain (TA100) V79 cells	1.36 μM	Genotoxic damage	Saverini et al., 2012
Linalool	Ames test (mutagenicity/ antimutagenicity assays) Comet assay	*S. typhimurium* strains (TA98, TA100, TA102) and *E. coli* (WP2, SY252, IB101) *S. cerevisiae* 3A cells	0.25–2.0 μl/plate 0–0.5 μM	There was no mutagenic response to linalool. Moreover, it exhibited antimutagenic potential against *t*-BOOH-treated cells and significantly decreased the number of H_2O_2-induced comets.	Beric et al., 2008
Linalool	*E. coli* reversion assay (mutagenicity/ antimutagenicity assays) Comet assay (genotoxicity/ antigenotoxicity assay)	*E. coli strains* (IC185, IC202) HepG2 cells NC–NC cells	0.05–1.5 mg/plate (or 0.32–9.72 mM) 0–1 μg/mL	No mutagenicity was observed and -strongly suppressed *t*-BOOH induced mutagenesis. Pretreatment experiments: In NC–NC cells linalool reduced *t*-BOOH induced-DNA damage by about 50% at 0.01 μg/ml; in HepG2 cells reduced DNA damage by 34%. Co-treatment experiments: The repair of *t*-BOOH induced DNA damage, studied in HepG2 cells, was not affected.	Mitic-Culafic et al., 2009
Linalool	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA 98, TA100) and *E. coli* (WP2uvrA)	0.17–0.9 mg/plate	Linalool showed neither mutagenic nor antimutagenic effects against 2NF, SA, 2AA.	Di Sotto et al., 2008
Linalool	MN	Human lymphocytes	0.5–100 μg/ml	Not genotoxic	Di Sotto et al., 2011
Linalyl acetate	Ames test	*S. typhimurium* strains (TA 98, TA100, TA1535, TA1537, TA1530)	100–25000 nl/plate	Not mutagenic response was observed.	Letizia et al., 2003

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Table 3 (*continued*)

Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
Linalyl acetate	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA 98, TA100) and *E. coli* (WP2uvrA)	1.7–9.0 mg/plate	Mutagenic in WP2uvrA, with and without S9 mixture	Di Sotto et al., 2008
Linalyl acetate	MN	Human lymphocytes	0.5–100 μg/ml	Genotoxic in a concentration-dependent manner	Di Sotto et al., 2011
Monoterpenoids constituents from EOs: (±)-Camphor, 1,8-cineole, citral, citronellal, (−)-menthol and terpineol	Ames test	*S. typhimurium* strains (TA97A, TA98, TA100, TA102)	0–3000 μg/plate	With the exception of terpineol, the monoterpenoid compounds tested were not mutagenic.	Gomes-Carneiro et al., 1998
Manool	MN (genotoxicity/antigenotoxicity assay)	V79 cells HepG2 cells	0.5–6.0 μg/mL 0.5–8.0 μg/mL	The highest concentration tested demonstrated genotoxic effect. Manool exhibited a protective effect against chromosome damage induced by MMS in HepG2 cells, but not in V79 cells.	Nicolella et al., 2014.
β-Myrcene	Ames test	*S. typhimurium* strains (TA97a, TA98, TA100, TA1535)	0–5000 μg/plate	No mutagenicity was detected.	Gomes-Carneiro et al., 2005
Myrcene	SCE	V79 cells HTC cells	100–500 μg/ml	Not genotoxic with and without metabolic activation. In V79 inhibited SCEs induced by CP and AFB_1 in a dose-dependent manner, but it had no effects on SCE induction by BaP and DMBA. In HTC cells also reduced CP-induced SCE frequencies.	Roscheisen et al., 1991
Myrcene	*E. coli* reversion assay (mutagenicity/ antimutagenicity assay) Comet assay (genotoxicity/ antigenotoxicity assay)	*E. coli* strains (IC202, IC185) HepG2 cells NC–NC cells	0.36–11.01 mM 0–1 μg/mL	No mutagenicity was observed, but suppression of mutagenesis induced by *t*-BOOH induced mutagenesis (43–80%) was observed. Pretreatment experiments: In NC–NC cells reduced *t*-BOOH induced DNA damage by about 50% at 0.01 μg/ml. In HepG2 cells did not reduce DNA damage. Co-treatment experiment: It showed weak, but significant reduction of t-BOOH induced DNA damage.	Mitic-Culafic et al., 2009
Organosulfur compounds (OSC): diallyl sulfide (DAS), diallyl disulfide (DADS), dipropyl sulfide (DPS), dipropyl disulfide (DPDS)	Ames test (mutagenicity/ antimutagenicity assays)	*S. typhimurium* strains (TA 98, TA 100)	0–1500 μg/plate	DADS showed antimutagenic effects against some carcinogens (DMN, BaP, N-PiP) although it increased the mutagenicity of PhIP. DAS and DPS strongly inhibited DMN mutagenicity, but at the same time enhanced the activation of BaP, CP, N-PiP and PhIP. DPDS enhanced the mutagenicity of all mutagens.	Guyonnet et al., 2000; Guyonnet et al., 2001
Organosulfur compounds: allicin (DADSO), diallyl sulfide (DAS), diallyl disulfide (DADS), S-allyl-cysteine (SAC), allyl mercaptan (AM)	Comet assay (genotoxicity/ antigenotoxicity assays)	HepG2 cells	5–100 μM	Pre-treatment protocol: All the OSC except AM reduced genotoxicity of Aflatoxin B_1, and DADS was the most effective reducing B(*a*)P genotoxicity. Moreover, SAC and AM decreased DNA breaks induced by DMN. Co-treatment: all the OSC compounds decreased the genotoxicity of the direct-acting compounds, H_2O_2 and MMN.	Belloir et al., 2006
Organosulfur compounds: dipropyl sulfide (DPS) and dipropyl disulfide (DPDS)	Ames test	*S. typhimurium* strains (TA97A, TA98, TA100, TA102 and TA104)	0.1–200 μM	No mutagenic activity was detected for both compounds.	Llana-Ruiz-Cabello et al., 2015b
(−)-α-pinene	Ames test	*S. typhimurium* strains (TA97A, TA98, TA100, TA1535)	0–5000 μg/plate	No mutagenicity	Gomes-Carneiro et al., 2005
(±)α-pinene	Ames test Comet assay	*S. typhimurium* strain (TA100) V79 cells	100 μL/plate 0.4 μM	Not mutagenic in the presence and absence of S9 mix. Genotoxic damage	Saverini et al., 2012
(+)β-pinene	Comet assay	V79 cells	3.6 μM	Genotoxic damage	Saverini et al., 2012

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Table 3 (*continued*)

Main compounds	Assays performed	Experimental model	Concentration ranges	Main results	References
α-Terpinene	Ames test	*S. typhimurium* strains (TA97A, TA98, TA100, TA1535)	0–5000 μg/plate	No mutagenicity was detected.	Gomes-Carneiro et al., 2005
Thymol	Ames test	*S. typhimurium strain* (TA98, TA100)	0.1 M 6.25–25μl/plate	No significant increase was observed in both strains at the concentrations assayed.	Stammati et al.,1999
Thymol	Comet assay (genotoxicity/ antigenotoxicity assays)	Human lymphocytes	0.005–2 mM	No increase in DNA strand breakage was observed at concentrations below 0.1 mM, but at 0.2 mM significant increases in DNA damage were observed. However, thymol significantly reduced the DNA strand breakage induced by IQ and MMC at the lower concentrations studied.	Aydin et al., 2005a
Thymol	Comet assay (Genotoxicity/antigenotoxicity assays)	Human lymphocytes	0.005–2 mM	Concentrations above 0.1 mM thymol significantly induced DNA damage. Thymol protected from the genotoxic effects of 0.1 mM H_20_2 at concentrations below 0.2 mM.	Aydin et al., 2005b
Thymol	Comet assay (genotoxicity/ antigenotoxicity assays)	HepG2 cells Caco-2 cells	0–500 μmol 0- 600 μmol	Not genotoxic effects were observed. Thymol reduced the level of DNA lesions induced by H_2O_2.	Horváthová et al., 2006
Thymol	SCE CA, MN	Human peripheral lymphocytes	25–100 μg/ml 24 and 48h	Thymol significantly increased SCE and induced structural CA and frequency of MN at all concentrations.	Buyukleyla and Rencuzogullari, 2009
Thymol	Standard comet assay Fgp protein post-treatment	V79 cells	1–25 μM	No significant increase in DNA strand breakage was detected at thymol concentrations of 1–5 μM. Only 25 μM of thymol caused some clastogenic DNA damage. Post-treatment with Fgp enzyme revealed no significant increase of Fpg-sensitive sites at all concentrations assayed.	Ündeger et al. 2009
Thymol	SOS chromotest (antigenotoxicity assay)	*E. coli.*strain (PQ37)	7.1–912.0 mM	Thymol produced a significant decrease in the bleomycin-induced genotoxicity at a dose between 28.5 and 912.0 mM, showing complete inhibition at doses between 228.0 and 912.0 mM.	Vicuña et al., 2010
Thymol	Ames test Comet assay and Endo III and FPG-modified comet assay	*S. typhimurium* strains (TA97A, TA98, TA100, TA102, TA104) Caco-2 cells	15.6–250 μM 0–250 μM	Thymol did not show any mutagenic activity. In the standard comet and FPG-modified comet assays no effects were observed.	Llana-Ruiz-Cabello et al., 2014b

2AA: 2-aminoanthracene; AFB_1: Aflatoxin B_1; BaP: Benzo(a)pyrene; CP: Cyclophosphamide; DMBA: 9,10-dimethyl-1,2-benz[a]anthracene; DMN:dimethylnitrosamine.
t-BHP or t-BOOH-: *t*-Butyl-hydroperoxide; COL: Colcemid; DMSO: Dimethylsulfoxide; EMS: Ethyl methanesulfonate; Fpg-protein: Formamido pyrimidine glycosylase protein; IQ: 2-amino-3-methyl-3H-imidazo[4,5-F]quinoline; MMC: Mitomycin C; MMS: Methyl methanesulfonate; *N*-PiP: *N*-nitrosopiperidine; 2NF: 2-nitrofluorene; 4NPD: 4-nitro-o-phenylenediamine; 4NQO: 4-nitroquinoline-1-oxide; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SA: Sodium azide.
Vero: kidney ephithelial cell line; Caco-2: human colorectal adenocarcinoma cell line; HepG-2: human hepatocellular carcinoma cell line; AA8: Chinese hamster ovary epithelial cell line; CHO: Chinese hamster ovary cell line; EM9: DNA repair mutant of CHO; HCT-15: human colon tumour cell line; V79: Chinese hamster lung cell line; NC–NC: human, Caucasian, lymphoblastoid B cell line; NA2: neuroblastoma cell line.

described that high doses of the monoterpenoid phenols, carvacrol and thymol, increase the levels of malondialdehyde (MDA), resulting in membrane damage, and 8-hydroxy deoxyguanosine, causing DNA damage in different cell lines (Özkan and Erdogan, 2012; Ündeger et al., 2009). Recently, Llana-Ruiz-Cabello et al. (2015a) demonstrated that carvacrol, thymol, and their combination (10:1) induce oxidative stress in Caco-2 cells. Another monoterpenoid phenol, α-pinene (present in rosemary EO), can also increase the MDA levels and activity of several antioxidant enzymes (Singh et al., 2006), and γ-terpinene (0.1 mM), present as a minor component in many EOs, significantly induces DNA damage in human lymphocytes (Aydin et al., 2005a). Fujisawa et al. (2002) described that eugenol (present in clove EO) could be oxidized in an enzymatic or non-enzymatic manner to phenoxyl radicals, which are involved in oxidative stress production; in fact, it induced a reactive oxygen species (ROS)-mediated apoptosis in HL-60 human promyelocytic leukaemia cells (Yoo et al., 2005). Quercetin, a component for example in green tea extract, greatly accelerates the generation of hydroxyl radicals from H_2O_2 in the presence of Fe^{3+}-EDTA at neutral pH (Laughton et al., 1989). Concerning sulfur compounds from garlic, several authors have described that diallyl sulfide (DAS) and diallyl disulfide (DADS) could induce, in many cellular models, apoptosis through the generation of ROS and, consequently, oxidative stress (Truong et al., 2009; Yi and Su, 2013).

Data summarized in this review note that high concentrations of the single compounds are needed to induce oxidative damage. Hence, these naturally occurring substances can have prooxidant effects. For this reason, prooxidant studies should be performed to establish the safety range of the concentrations of EOs and their compounds when they are intended for use in the food industry for food packaging (Llana-Ruiz-Cabello et al., 2015a).

3.1.2. Antimutagenicity/antigenotoxicity

Many studies have reported the antimutagenic effects of EOs (Table 3) (Beric et al., 2008; Di Sotto et al., 2008; Ipek et al., 2005; Jeena et al., 2014; Knezevic-Vukcevic et al., 2005; Martinez-Rocha et al., 2008; Mitic-Culafic et al., 2009; Neffati et al., 2009; Nikolic et al., 2011; Sghaier et al., 2010; Vukovic-Gacic et al., 2006; Zegura et al., 2011). The antimutagenic effects of EOs depend on the mutagen and oil concentrations (Bakkali et al., 2008; Neffati et al., 2009) as well as on the balance between activation and detoxification (Guyonnet et al., 2000, 2001). The efficiency of EOs as antimutagens has been ascribed to the presence of several major compounds that exhibit antimutagenic activities (De Martino et al., 2009; Ipek et al., 2005; Neffati et al., 2008). The mechanism of this antimutagenicity may be due to the antioxidant activity, as suggested by several authors (Kulisic et al., 2004; Ruberto and Baratta, 2000). But, in general, the anti-mutagenic properties of EOs have been attributed to different factors, including (1) the inhibition of mutagen penetration into the cells, (2) activation of cell antioxidant enzymes, (3) mutagen neutralization by direct scavenging activity or inactivation of radicals produced by mutagens, (4) inhibition of metabolic conversion of pro-mutagens into mutagens by microsomal enzyme pools, (5) activation of enzymatic detoxification of mutagens, (6) interferences with DNA repair systems, and (7) general and unspecified hepatoprotective activity (Edris, 2007; Rossi et al., 2013).

Similarly, the EOs and their main compounds may exhibit a dual genotoxic/antigenotoxic effect. This is the case for carvacrol and thymol (Aydin et al., 2005a, 2005b; Horváthová et al., 2006; Slamenova et al., 2013), eucalyptol, myrcene and linalool (Mitic-Culafic et al., 2009; Nikolic et al., 2011), garlic sulfur compounds (Belloir et al., 2006) and β-caryophyllene (Di Sotto et al., 2010). To investigate the mechanisms of the antigenotoxic action of these compounds, some studies have followed three different experimental protocols, before (pre-treatment), during (co-treatment) and after (post-treatment) with the mutagens (Di Sotto et al., 2011). There are very few studies on the molecular mechanisms of action of potential antigenotoxic compounds present in EOs. Several garlic organo sulfur compounds (OSCs) displayed antigenotoxic activity in HepG2 cells, suggesting an effect on the cell metabolic pathways (Belloir et al., 2006). According to Mitic-Culafic et al. (2009) differences in responses between co- and pre-treatments with potential antigenotoxic compounds (linalool, myrcene, and eucalyptol) are often considered to indicate the induction of cellular defense mechanisms.

3.1.3. Evaluation of specific essential oils and their main compounds with applications in food packaging

Considering that genotoxicity/mutagenicity studies are among the core set of toxicological studies required for the authorization of substances to be used in food contact materials, a thorough review of the scientific literature on the topic of EOs and their main compounds is of interest. A summary of the main results reported in the scientific literature about the mutagenic and genotoxic potential of several EOs and their main compounds (including antimutagenicity and antigenotoxic effects) is presented below case-by-case, in alphabetical order.

*3.1.3.1. Basil essential oil (*Ocimum basilicum *L.) and main compounds.* The major constituent of this EO, linalool (approximately 70%), has not induced a mutagenic response in any of the assays performed (Beric et al., 2008; Di Sotto et al., 2008; Letizia et al., 2003; Mitic-Culafic et al., 2009), with the exception of Di Sotto et al. (2008), who demonstrated a positive mutagenicity profile in the *E. coli* bacterial strain. Similarly, no mutagenicity has been reported for this EO (Beric et al., 2008; De Martino et al., 2009; Stajkovic et al., 2007). However, none of these studies were performed completely according to OECD guidelines (471).

Regarding the genotoxicity of their components, Beric et al. (2008) showed that linalool at higher concentrations (3–5 μM) produced alkali-labile sites using the yeast Comet assay. Further studies indicated that it did not induce DNA damage in HepG2 or NC–NC cells according to the Comet assay (Mitic-Culafic et al., 2009). Di Sotto et al. (2011) found that whereas linalool (0–100 μg/ml), metabolite of linalyl acetate, did not increase the frequency of MN in peripheral human lymphocytes, the parent compound did from 10 μg/ml. These findings suggest that with metabolism, there is a loss of the genotoxicity activity of the parent compound, highlighting the need for *in vivo* studies and that linalool should be preferable to its parent compound as a flavouring agent. With respect to estragole, another constituent of basil oil, Martins et al. (2012) reported an increase in SCE without S9 in V79 cells and a decrease in its presence; positive results were also observed in the Comet assay without S9. However, positive results observed up to 1000 μM could not be attributed to toxicity, because at these dose levels, toxicity is relatively low. Overall, the authors suggested that estragole is a weak, direct-acting genotoxin that forms DNA adducts.

Moreover, linalool and linalyl acetate exerted a good antimutagenicity response against several known carcinogens, *t*-Butyl hydroperoxide (*t*-BOOH), 2-nitrofluorene (2NF), 2-aminoanthracene (2AA), sodium azide (SA), and methyl methane sulfonate (MMS) (Beric et al., 2008; Di Sotto et al., 2008; Mitic-Culafic et al., 2009). Only Di Sotto et al. (2008) demonstrated that there was no antimutagenic response with linalool. Beric et al. (2008) also found that linalool at lower concentrations (0.5 μM) than those inducing genotoxicity (above mentioned) has protective effects against oxidative DNA damage induced by *t*-BOOH or H_2O_2. These results indicated that linalool has substantial protective effects against oxidant-induced genotoxicity (Mitic-Culafic et al., 2009). In addition, the antimutagenic potential of this basil EO in different bacterial

strains against mutagens has been also reported (Beric et al., 2008; Stajkovic et al., 2007).

3.1.3.2. Cinnamomum cassia *essential oil and main compounds.* The major volatile constituent contained in cinnamon powder, cinnamic aldehyde, is the only α,β-unsaturated aldehyde that is FDA-approved for use in foods and given GRAS status by the Flavor and Extract Manufacturers' Association (FEMA). The first studies about the genotoxicity of this compound showed negative results in the Ames test, and *E. coli* reversion tests also produced negative results, while the results in *Bacillus subtilis* and in the *E. coli* DNA repair tests were positive, indicating a direct reaction with DNA (Azizan and Blevins, 1995; Stammati et al., 1999). According to the results obtained in the genetic toxicology studies conducted by the National Toxicology Program (NTP, 2004), trans-cinnamaldehyde was mutagenic in the *S. typhimurium* strain TA100 in the presence of induced mouse liver S9 activation enzymes. All other strains and the activation combinations yielded negative results. Additionally, it induced SCE exchanges in CHO cells with and without induced rat liver S9 activation. There was no significant increase in the frequency of CA in CHO cells with or without rat liver S9. In the case of cinnamic acid, Maistro et al. (2011) reported no genotoxic response in the Comet assay in hepatoma tissue cells, whereas the MN test showed an increase in the frequency of micronucleated cells from 50 μM.

The cinnamic aldehyde showed an antimutagenic effect on spontaneous mutation in *S. typhimurium* TA104 (Shaughnessy et al., 2001). Moreover, cinnamic aldehyde and an ethanolic cinnamon extract displayed significant potency when tested for cell protection against oxidative stress-induced genotoxicity and cytotoxicity (H_2O_2-genoprotection) in human colon cancer cells (Wondrak et al., 2010).

As far as we know the cinnamon EO has been very little studied, and it did not show any mutagenic response in the only Ames test carried out (with the *S. typhimurium* TA100 strain) at any of the assayed concentrations (50–2000 μg/mL), with or without S9 fraction (Shoeibi et al., 2009).

*3.1.3.3. Clove oil (*Eugenia caryophyllata*) and main compounds.* Eugenol is present in a variety of EOs, such as clove oil (85–95%), clove oleoresin (60–90%) and in cinnamon leaf oil (70–90%) (National Toxicology Program, NTP, 1983; Guan et al., 2007). The genotoxicity of eugenol was evaluated by NTP (1982) and it induced CA in CHO cells. Hikiba et al. (2005) observed an induction of CA in Syrian hamster embryo cells, which was enhanced in the presence of exogenous metabolic activation. Maralhas et al. (2006) demonstrated that eugenol induces CA at 2500 μM, including exchanges, in V79 cells, in the absence and presence of rat liver S9 mix, which suggests biotransformation to reactive metabolites. The authors concluded that caution is needed in considering a threshold approach for eugenol and related compounds since they have been shown to be genotoxic in various assays. Later, Martins et al. (2011) demonstrated its direct genotoxicity using the Comet assay (DNA strand breaks) and the c-H2AX assay (induction of double strand breaks), the formation of ROS in CHO cells and the induction of DNA fragmentation detected by TUNEL assay at 750 μM.

β-Caryophyllene and β-caryophyllene oxide occur in EOs from various medicinal and edible plants, such as clove (*E. caryophyllata* and *Syzygium aromaticum* (L.) Merr. et Perry). Due to its potential hazardous chemical structure (epoxide group), the EFSA reported that a safety assessment for β-caryophyllene oxide in this compound is pending (EFSA, 2008b). In this sense, Di Sotto et al. (2008) demonstrated that β-caryophyllene does not have mutagenic activity; in contrast, it showed a strong antimutagenic activity against 2-NF. Moreover, it did not increase the MN frequency in human lymphocytes up to 100 μg/ml, and also showed protective properties against the clastogenic effect of ethyl methanesulfonate (EMS) (Di Sotto et al., 2010). Later, the same authors confirmed the lack of mutagenicity of β-caryophyllene oxide in different bacterial strains, which are sensitive to frameshift, base substitution mutations, and oxidative damage (Di Sotto et al., 2013). Likewise, there was no mutagenicity in presence of the exogenous metabolic activator, showing that no genotoxic derivatives were produced from the test compound by CYP450-mediated biotransformation. Furthermore, the compound did not induce genotoxicity at the chromosomal level, as observed in the MN assay. The consistency between results in bacteria and mammalian cells is important because the lack of genotoxicity was verified in terms of different endpoints, both at the gene level, as a frameshift or a base-substitution mutagen, and at the chromosomal level (clastogenicity and aneugenicity).

*3.1.3.4. Coriander essential oil (*Coriandrum sativum*) and main compounds.* Linalool, the major constituent of coriander oil (55–74%), has been reviewed to be non-mutagenic in several different genotoxicity assays (Burdock and Carabin, 2009). This compound is also found in other EOs and the genotoxicity of linalool has been already described above.

Coriander oil is approved for food use by the FDA, FEMA and Council of Europe, and its safety-in-use has been revised by Burdock and Carabin (2009). Little is known about the mutagenic potential of coriander EO, and the results for the spice and some extracts are mixed. Earlier works have indicated that the extracts were not mutagenic in *S. typhimurium* strains TA98 and TA100 with and without S9 metabolic activation (Higashimoto et al., 1993), whereas coriander fruit extract has been reported to be mutagenic in the Ames assay (*S. typhimurium* strains TA98 and TA100) (Mahmoud et al., 1992). Reyes et al. (2010) demonstrated that a water extract of coriander leaves was mutagenic in the tested range of 1.6–8 μg/mL according to the Ames test (*S. typhimurium* TA97 and TA102), which was associated with metabolic activation by S9 mix. Despite the existing studies, the mutagenicity studies on this EO have only included 2 strains of *S. typhimurium*. Taking into account these data, coriander EO cannot be considered safe.

*3.1.3.5. Garlic (*Allium sativum*) and onion (*Allium cepa*) essential oils and main compounds.* The main compounds responsible for the biological properties of garlic and onion EOS are the organo sulfur compounds (OSC), and differences between both essential oils are not only related to the content in OSC (three times higher in the case of garlic) but also in the profile of these compounds (Lanzotti, 2006). In the case of garlic, allyl sulfides are most abundant, whereas in onion, propyl sulfides are more frequently found (Llana-Ruiz-Cabello et al., 2015b).

The studies concerning the mutagenicity/genotoxicity of garlic and onion are practically non-existent. The mutagenicity of dipropyl sulfide (DPS) and dipropyl disulfide (DPDS) and their mixture was assessed by the Ames test following the OECD test guideline (471), showing lack of mutagenicity in the range of concentrations assayed (0–200 μM) with and without S9 metabolic activation (Llana-Ruiz-Cabello et al., 2015b). Diallyl sulfide (DAS) and diallyl disulfide (DADS) were tested in a Chinese hamster ovary (CHO) cell line, and both compounds were able to induce small, but significant numbers of CA and SCE (Musk et al., 1997). The influence of the S9 activation mix on the metabolism of these compounds appeared to be complex because it enhanced the generation of CA by DADS (but not by DAS), reduced the induction of SCEs by both compounds, and radically altered the parameters of the survival curves. Thus, mutagenic and genotoxic assays are needed in order to elucidate the potential effects of these compounds and extracts from these EOs.

Although few mutagenic/genotoxic effects have been described due to OSC, they have been shown to exert a protective effect against DNA damage. Guyonnet et al. (2000) studied the antimutagenic effects of DAS, DADS, DPS and DPDS using the Ames test (only with

two strains of *S. typhimurium*). Some of these compounds (DAS, DPS and DPDS) significantly induced the activation of several genotoxic compounds (see Table 3), while DADS only increased the mutagenicity of 2-amino-1-methyl-6-phenylimidazo4,5-pyridine (PhIP). DAS, DADS and DPS strongly inhibited the dimethylnitrosamine (DMN) mutagenicity, while DPDS enhanced it. Similarly, Belloir et al. (2006) investigated protective effects of the following five garlic OSCs towards DNA damage induced by carcinogens in the cell line HepG2: allicin (DADSO), DAS, DADS, S-allyl cysteine (SAC) and allyl mercaptan (AM). In the pre-treatment, aflatoxin B1 (AFB_1) genotoxicity was significantly reduced by all the OSCs tested, except AM. For all the OSCs tested, antigenotoxic properties have been observed at the dose of 5 μM, which could be a physiologic concentration. Both antimutagenic/antigenotoxic properties of OSC were related to the modification of specific oxidative enzymes (CYP) involved in their activation, and by the induction of phase II enzymes involved in the detoxification of carcinogens (Belloir et al., 2006; Guyonnet et al., 2001). On the other hand, allicin, which is activated when garlic is cut or crushed, showed antigenotoxic activity in cultured mammalian cells against MMS and estradiol-17β-induced genotoxic damage (Siddique and Afzal, 2004; Siddique et al., 2010). In addition, antimutagenic effects have been also observed in some extracts from white, yellow and red onions (Shon et al., 2004), by using *S. typhimurium* strains, and were related to their phenol and flavonoid components.

*3.1.3.6. Marjoram (*Origanum majorana*) and main compounds.* Apigenin (4′,5,7-trihydroxyflavone) is the major compound of *O. majorana*, (Roby et al., 2013). Contradictory results about its genotoxic potential have been found in the scientific literature. Rithidech et al. (2005) reported that low concentrations of apigenin (2.5–10 μg/ml) did not induce an increase in MN frequency in human lymphocytes, but at higher concentrations (25 μg/ml) enhanced the MN frequency. Some authors demonstrated its clastogenic activity in Chinese hamster V79 cells and peripheral human lymphocytes at a dose of 100 μM (Noel et al., 2006; Snyder and Gillies, 2002), and Papachristou et al. (2013) reported a dose-dependent genotoxic potential of this compound in HepG2 cells using CA and SCE assays (1–50 μM). Nevertheless, Siddique et al. (2008) demonstrated the antigenotoxic potential of apigenin both in the absence as well as in the presence of S9 in human lymphocytes.

3.1.3.7. Origanum *essential oils, (*O. onites *L.,* O. vulgare *L.* Lippia graveolens *Kunth).* It is known that there is a relationship between the high activity (antibacterial, antifungal, antioxidant, etc.) of the oregano-type oils and the presence of phenolic components, such as carvacrol, thymol and their precursors, γ-terpinene and p-cymene (Dundar et al., 2008; Liolios et al., 2009). The main components of *O. onites* essential oil are carvacrol, linalool and thymol, whose contents in the same species (*O. onites* from Western Anatolia, Turkey) may change considerably, from 64.3 to 24.52%, 13.8 to 50.53% and 1.4 to 15.66%, respectively (Bostancioglu et al., 2012; Özkan and Erdogan, 2011). The differences could be due to different dates of collection, or climate conditions, because in both cases the same species were collected in the same area, extracted (water distillation) and analysed by the same technique (gas chromatography/mass spectrometry analysis).

The mutagenic potential of thymol and carvacrol was studied some time ago, and different and contradictory results have been published, mainly for carvacrol, although all studies have reported their antimutagenic effects (Azizan and Blevins, 1995; Ipek et al., 2005; Llana-Ruiz-Cabello et al., 2014b; Stammati et al., 1999). Several studies related to thymol concluded the absence of mutagenic potential at the range of concentrations and conditions tested: 6.67 mM (Azizan and Blevins, 1995) or to 0.1 M (Stammati et al., 1999), although these assays have only used two or three strains in the *S. typhimurium* assay (TA97, TA98 and TA100). Nevertheless, for carvacrol, several authors have reported the absence of mutagenic activity of this monoterpene in the *Salmonella*/microsome reversion test, using TA100 and TA98 strains (at 0.1 M carvacrol) with and without S9 activation (Stammati et al., 1999) and in the sister chromatid exchange (SCE) assay (0.1–5.0 μl/mL) (Ipek et al., 2003). In contrast, according to Ipek et al. (2005), carvacrol is a strong, direct-acting mutagen in the Ames test system and its metabolites showed weaker mutagenicity at lower concentrations, at least for TA98 strains of *S. typhimurium*. Recently, studies from our laboratory found that thymol did not show any mutagenic activity in the Ames test (strains TA97A, TA98, TA100, TA102, TA104, with and without S9) at concentrations intended for use in food packaging (up to 250 μM), whereas carvacrol exhibited *per se* mutagenic potential, which was more active in the presence of the metabolic fraction S9 (29–460 μM) (Llana-Ruiz-Cabello et al., 2014b). In conclusion, additional studies following the test guideline for gene mutation in bacteria (OECD, 1997a), using five Salmonella strains, and with and without metabolic activation, should be considered to confirm the latest results and to fully characterize their safety before they are used in the food industry.

Regarding the genotoxicity evaluation of carvacrol and thymol by Comet assay, several studies are available with differing results according to the range of concentrations or the cell system studied (Aydin et al., 2005a, 2005b; Horváthová et al., 2006; Llana-Ruiz-Cabello et al., 2014b; Ündeger et al., 2009). Aydin et al. (2005a) demonstrated that carvacrol (0.1 mM) and thymol (0.2 mM) induced DNA damage in human lymphocytes. However, Horváthová et al. (2006) concluded that carvacrol (0–200 μM) and thymol (0–500 μM) are not associated with a DNA-damaging effect on human hepatoma HepG2 and colonic Caco-2 cells. Similarly, a lack of genotoxicity activity for thymol and carvacrol at biologically relevant concentrations (1–25 μM, taking into account previous cytotoxicity test and *in vivo* pharmacokinetic assay) has been reported in V70 Chinese hamster lung fibroblast cells using the standard Comet assay, and only at the highest concentration assayed thymol (25 μM) caused some DNA damage (Ündeger et al., 2009); post-treatment with FPG protein revealed no additional DNA damage at any of the thymol and carvacrol concentrations assayed. Moreover, Llana-Ruiz-Cabello et al. (2014b) have evaluated the genotoxic effects in Caco-2 cells, and the standard Comet assay did not reveal effects of either carvacrol (0–460 μM) or thymol (0–250 μM). We have demonstrated that after application of the FPG modified-Comet assay, DNA damage was shown for the first time with carvacrol at the highest concentration assayed of 460 μM, indicating damage in purine bases. In healthy neurons and neuroblastoma cells, carvacrol did not induce any genotoxic damage using Comet assay (0–400 μg/L) (Aydin et al., 2014). Overall, contradictory results were obtained for thymol and carvacrol, with human lymphocytes and hamster lung fibroblasts being more sensitive in comparison to Caco-2 cells. In this cell line, we can conclude that thymol and carvacrol did not induce genotoxic damage at approximately 250 μM and 460 μM, respectively, although further studies using the enzyme-modified version are necessary to confirm the potential oxidative damage induced by carvacrol. This recommendation is extended to other assays, such as CA, SCE and frequency of MN, due to the scarce studies available. In this regard, Buyukleyla and Rencuzogullari (2009) reported that thymol induces damage in human peripheral lymphocytes using CA, SCE, and frequency of MN, concluding that this compound poses a genotoxic risk for humans.

The studies performed with the *O. onites* L. essential oil demonstrated that under the conditions assayed, there were no mutagenic activities (Ipek et al., 2005).

Regarding the antimutagenic profile, Ipek et al. (2005) found notable effects against several strong mutagens, such as 4-nitro-o-phenylenediamine (4-NPD) and 2-aminofluorene (2-AF), and concluded that they could probably be due to the concomitant

presence of different components, thymol or linalool, which could be antimutagenic (De Martino et al., 2009; Ipek et al., 2005). In relation to their antigenotoxic profile, Aydin et al. (2005a, 2005b) found that below DNA damaging concentrations, both compounds protected lymphocytes against DNA damage induced by mitomycin C (MMC), 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) and H_2O_2. Similarly, carvacrol and thymol led to a significant protection of the Caco-2 and HepG2 cells from DNA strand breaks induced by H_2O_2 (Horváthová et al., 2006; Slamenová et al., 2007). Moreover, pretreatment of V79 cells with thymol (0–100 μg/mL) reduced radiation-induced MN as well as the percent of DNA in the tail (Archana et al., 2011).

Other active compounds isolated from *O. vulgare* L. spp. extracts were also evaluated using Ames *S. typhimurium* strains (TA1535, TA1537) and *E. coli* (*wPuvrA*) bacterial assays systems. Neither luteolin 7-*O*-glucuronide nor luteolin 7-*O*-xyloside showed mutagenic activity at the assayed concentrations (0.4–2 μM/plate). Moreover, both isolated compounds provided significant antimutagenic activity against 9-aminoacridine (9-AA) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Gulluce et al., 2012). These authors concluded that in *in vitro* bacterial mutagenicity and antimutagenicity test systems, flavonoids could act in four different ways, similar to other natural or synthetic substances. They can have antimutagenic, comutagenic, promutagenic or directly mutagenic effects on living organisms. Some flavonoids have one or more simultaneous effects, depending on the tested concentrations (Gulluce et al., 2012). The studies related to the mutagenicity of the essential oil of this species (*O. vulgare*) are very scarce, and De Martino et al. (2009) reported that it did not show mutagenic effects on TA98 and TA100 *S. typhimurium* strains at any concentrations assayed (up to 467 μg) with or without metabolic activation.

On the other hand, Martinez-Rocha et al. (2008) evaluated the mutagenic and antimutagenic effects of gallic acid extracts from the essential oil of *Lippia graveolens* Kunth, a Mexican oregano, which has a high level of phenolic compounds for which flavonoids are the major constituents. The results obtained from the antimutagenic assays were the most relevant, showing approximately 40 and 30% antimutagenic effects against two carcinogenic compounds selected, 4-NPD (16–500 μg/mL) and sodium azide (SA) (50–800 μg/mL), respectively.

*3.1.3.8. Palmarosa (*Cymbopogon martini*), Lemongrass (*Cymbopogon citrates*), Citronella (*Cymbopogon winterianus *Jowitt) and main compounds.* Recently, Sinha et al., (2014) investigated the genotoxic potential of these EOs and monoterpenoids (citral and geraniol) on human lymphocytes. Palmarosa and citronella oils could induce significant DNA damage at concentrations of 1000 μg/mL and above, and their major component geraniol did not have any genotoxicity. DNA strand breaks induced by lemongrass oil and its major component citral exhibited significant DNA damaging potential on human lymphocytes at 100 and 25 μg/mL and higher, respectively. The DNA diffusion assay showed significant DNA damaging potential similar to the Comet assay, and demonstrated the formation of DNA fragments at high concentrations, which might be due to apoptosis/necrosis. Based on the results, the authors concluded that these EOs are considered safe for human consumption at low concentrations. The genotoxicity of citral was also assayed using the SOS Chromotest in *E. coli* (López et al., 2011) and it did not induce the SOS response, whereas it induced a significant reduction in bleomycin-induced genotoxicity at 182 mM.

*3.1.3.9. Rosemary essential oil (*Rosmarinus officinalis *L.) and main compounds.* Among the main compounds of rosemary EO, the following monoterpenoid constituents are noteworthy: camphor (14.6%), 1,8-cineole (7.26%), α-pinene (6.65%) and β-caryophyllene (6.12%), which have been widely used also as flavouring food additives, and carnosic acid and carnosol, which have antioxidant components. No mutagenic effects were found in the *Salmonella*/microsome assay and *E. coli* reversion assay of the aforementioned compounds (Di Sotto et al., 2013; Gomes-Carneiro et al., 1998, 2005; Mitic-Culafic et al., 2009; Nikolic et al., 2011), although these tests were not performed according to OECD guidelines. The genotoxicity of camphor was assayed in the Vero cell line by the alkaline Comet assay and no genotoxicity up to 100 μM was found, although higher tested concentrations induced DNA strand breaks (Nikolic et al., 2011).

All these compounds showed antimutagenic/antigenotoxic potential against several carcinogens, such as *t*-BOOH, 2-NF, 2-aminoanthracene (2-AA), SA, and MMS (Di Sotto et al., 2013; Gomes-Carneiro et al., 1998, 2005; Mitic-Culafic et al., 2009; Nikolic et al., 2011), including camphor against 4-nitroquinoline-1-oxide (4-NQO) (Nikolic et al., 2011). The antimutagenic effect, at least in the case of carnosic acid, has been explained because this compound was an inhibitor of lipid peroxidation in microsomal liposomes by acting as a powerful hydroxyl radical scavenger and, thereby, inhibiting iron-dependent $^{\cdot}OH$ generation (Minnunni et al., 1992).

Little is known about the mutagenic potential of the pure oil of *Rosmarinus officinalis*. The only report available concluded the absence of mutagenicity in the Ames test (only one strain was assayed of *S. typhimurium*) of two *R. officinalis* extracts (Zegura et al., 2011), AquaROX® containing rosmarinic acid (17%) and VivOX® 40, which contained carnosic acid (50.27%) and carnosol (5.65%). This EO showed a significant, dose-dependent decrease of the mutagenic effects of different strong mutagens, such as *t*-BOOH, 4-NQO, and IQ (Minnunni et al., 1992; Zegura et al., 2011). Additionally, the extracts showed antigenotoxic effects against *t*-BOOH, benzo (*a*) pyrene (BaP) and PhIP in HepG2 cells by applying the comet assay.

*3.1.3.10. Thyme essential oil (*Thymus vulgaris *L.,* Artemisia campestris*) and main compounds.* Two species of thyme, *Artemisia campestris* and *Thymus vulgaris*, present different chemical composition, β-pinene, p-cymene, α-terpinene, limonene, myrcene, β-phellandrene, α-pinene in the case of *A. campestris* versus cinnamic acid, apigenin, luteolin-7-o-rutinose in *T. vulgaris* (Neffati et al., 2008; Roby et al., 2013). With respect to the mutagenicity of the main components of thyme EO, such as myrcenes or α-terpinene, there was no demonstrated mutagenic activity (Gomes-Carneiro et al., 2005; Mitic-Culafic et al., 2009). β-myrcene did not induce SCE in V79 cells with and without S9 mix, and it modulated the genotoxicity of indirect-acting mutagens by inhibiting certain forms of the CYP-450 enzymes required for activation of premutagens, such as CP and aflatoxin B_1 (AFB_1) (Roscheisen et al., 1991).

Studies regarding the mutagenic profile of the *T. vulgaris* L. or *A. campestris* are very scarce. For *A. campestris*, Neffati et al. (2008) reported that the EO did not show an increase in the number of revertants in the three *S. typhimurium* strains assayed, at the range of concentrations tested (10–100 μg/ml), whereas it induced a reduction of the mutagenicity produced by BaP, which could be related to the presence of some major components. Similarly, De Martino et al. (2009) evaluated the mutagenic potential of the *T. vulgaris* L., and none of the concentrations tested showed mutagenic activity in *S. typhimurium* strains.

Mitic-Culafic et al. (2009) also demonstrated the antimutagenic activity of myrcene against *t*-BOOH and this protective effect in cultured human cells could be predominantly mediated by its radical scavenging activity.

3.1.3.11. Other essential oils and main compounds. The EOs obtained from an aerial part of *Pituranthos chloranthus* (Tunisia) were not mutagenic for *S. typhimurium* strains and significantly reduced the BaP and SA-induced mutagenicity at the range of concentrations assayed (Neffati et al., 2009). The turmeric essential oil (TEO) prepared from the rhizome of *Curcuma longa* L. and *Zingiber officinale*

R., commonly known as Ginger essential oil, did not produce any revertants in the Ames test with or without metabolic activation (Liju et al., 2013), showing antimutagenic activity against different direct-acting mutagens (SA, 4-NPD, MNNG, tobacco extract, and 2-AAF) (Jeena et al., 2014). Similarly, the essential oil of *Teucrium ramosissimum* (Beric et al., 2008; Sghaier et al., 2010) did not induce mutagenicity in three *S. typhimurium* strains and it has been shown to possess antimutagenic effects against direct and indirect acting mutagens. Also, the non-polyphenolic fraction of green tea showed strong suppressive activity against the damage induced by MMC in *S. typhimurium* (TA 1535) (Okai and Higashi-Okai, 1997). In the case of an extract of green tea (*Camellia sinensis*), no antigenotoxic effects were found in Jurkat cells against H_2O_2-induced DNA damage (Bhatt et al., 2010), although these effects were demonstrated in human lymphocytes exposed to anabolic steroids (Gupta et al., 2009).

4. Conclusions

Considering the increasing interest in EOs and their components in active food packaging, the exposure of consumers can be substantially enhanced. Despite extensive research performed in this field, their use in the EU is not allowed since the community list of authorized substances has not been published so far. In order to be included in this list, data about the genotoxicity of these substances are needed. In the present review, the more relevant mutagenic and genotoxic aspects of the EOs and their main components have been described. In general, fewer genotoxic studies have been reported on EOs in comparison to their main components. The step-wise approach recommended by EFSA to evaluate their genotoxic potential has not always been followed and it is necessary to perform complete studies according to OECD protocols. In this regard, the mouse lymphoma *tk* assay, has not been used so far for this purpose. Most of EOs and their main compounds have been reported to be not mutagenic/genotoxic. However, the genotoxic response may be affected by the experimental model chosen and the range of concentrations assayed, and this could be due in part to the induction of oxidative stress. However, it is important to point out that some of them can be metabolically activated, such as cinnamaldehyde, or they can be metabolized to a substance without genotoxic activity, such as in the case of linalool. Therefore, *in vivo* genotoxic tests should be performed to confirm the *in vitro* results. Finally, all of the data compiled in the present review should be considered when selecting appropriate concentrations of the active substances that are planned for use in food packaging.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

Akalin, G., Incesu, Z., 2011. The effects of carvacrol on apoptosis of H-Ras and N-Ras transformed cell lines. Turk. J. Pharm. Sci. 8, 105–116.
Al-Bandak, G., Oreopoulou, V., 2007. Antioxidant properties and composition of *Majorana syriaca* extracts. Eur. J. Lipid Sci. Tech. 109, 247–255.
Amorati, R., Foti, M.C., Valgimigli, L., 2013. Antioxidant activity of essential oils. J. Agric. Food Chem. 61, 10835–10847.
Archana, P.R., Nageshwar, R.B., Satish Rao, B.S., 2011. Modulation of gamma ray-induced genotoxic effect by thymol, a monoterpene phenol derivative of cymene. Integr. Cancer Ther. 10, 374–383.
Aydin, E., Türkez, H., Keles, M.S., 2014. The effect of carvacrol on healthy neurons and N2a cancer cells: some biochemical, anticancerogenicity and genotoxicity studies. Cytotechnology 66, 149–157.
Aydin, S., Basaran, A.A., Basaran, N., 2005a. Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. J. Agric. Food Chem. 53, 1299–1305.
Aydin, S., Basaran, A.A., Basaran, N., 2005b. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutat. Res. 581, 43–53.
Azizan, A., Blevins, R.D., 1995. Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay. Arch. Environ. Contam. Toxicol. 28, 248–258.
Bahram, S., Rezaei, M., Soltani, M., Kamali, A., Ojagh, S.M., Abdollahi, M., 2012. Whey protein concentrate edible film activated with cinnamon essential oil. J. Food Process. Pres. 38, 1251–1258.
Bajpai, V.K., Baek, K.H., Kang, S.C., 2012. Control of *Salmonella* in foods by using essential oils: a review. Food Res. Int. 45, 722–734.
Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – a review. Food Chem. Toxicol. 46, 446–475.
Bakri, I.M., Douglas, C.W.I., 2005. Inhibitory effects of garlic extract on oral bacteria. Arch. Oral Biol. 50, 645–651.
Belloir, C., Singh, V., Daurat, C., Siess, M.H., Le Bon, A.M., 2006. Protective effects of garlic sulphur compounds against DNA damage induced by direct- and indirect-acting genotoxic agents in HepG2 cells. Food Chem. Toxicol. 44, 827–834.
Beric, T., Nikolic, B., Stanojevic, J., Vukovic-Gacic, B., Knezevic-Vukcevic, J., 2008. Protective effect of basil (*Ocimum basilicum* L.) against oxidative DNA damage and mutagenesis. Food Chem. Toxicol. 46, 724–732.
Bhatt, P.R., Pandya, K.B., Sheth, N.R., 2010. *Camellia sinensis* (L): the medical beverage: a review. Int. J. Pharm. Sci. Rev. Res. 3, 6–9.
Bostancioglu, R.B., Kürkcüoglu, M., Can Baser, K.H., Koparal, A.T., 2012. Assessment of anti-aniogenic and anti-tumoral potentials of *Origanum onites* L. essential oil. Food Chem. Toxicol. 50, 2002–2008.
Burdock, G.A., Carabin, I.G., 2009. Safety assessment of coriander (*Coriandrum sativum* L.) essential oil as a food ingredient. Food Chem. Toxicol. 47, 22–34.
Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. 94, 223–253.
Buyukleyla, M., Rencuzogullari, E., 2009. The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes. Ecotox. Environ. Safe 72, 943–947.
Colina-Coca, C., González-Peña, D., Vega, E., de Ancos, B., Sánchez-Moreno, C., 2013. Novel approach for the determination of volatile compounds in processed onion by head space gas chromatography–mass spectrometry (HSGC–MS). Talanta 13, 137–144.
Collins, A.R., Mitchell, D.L., Zunino, A., de Wit, J., Busch, D., 1997. UV-sensitive rodent mutant cell lines of complementation groups 6 and 8 differ phenotypically from their human counterparts. Environ. Mol. Mutagen. 29, 152–160.
Dainelli, D., Gontard, N., Spyropoulosc, D., Van den Beuken, E.Z., Tobback, P., 2008. Active and intelligent food packaging: legal aspects and safety concerns. Trends Food Sci. Tech. 19 (S1), S103–S112.
De Martino, L., De Feo, V., Nazzaro, F., 2009. Chemical composition and *in vitro* antimicrobial and mutagenic activities of seven Lamiaceae essential oils. Molecules 14, 4213–4230.
De Vincenzi, M., Stammati, A., De Vincenzi, A., Silano, M., 2004. Constituents of aromatic plants: carvacrol. Fitoterapia 75, 801–804.
Delaquis, P.J., Stanich, K., Girard, B., Mazza, G., 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Int. J. Food Microbiol. 74, 101–109.
Di Sotto, A., Evandri, M.G., Mazzanti, G., 2008. Antimutagenic and mutagenic activities of some terpenes in the bacterial reverse mutation assay. Mutat. Res. Gen. Tox. En. 653, 130–133.
Di Sotto, A., Mazzanti, G., Carone, F., Hrelia, P., Maffei, F., 2010. Inhibition of β-caryophyllene of ethyl methanesulfonate-induced clastogenicity in cultured human lymphocytes. Mutat. Res. 699, 23–28.
Di Sotto, A., Mazzanti, G., Carbone, F., Hrelia, P., Maffei, F., 2011. Genotoxicity of lavender oil, linalyl acetate, and linalool on human lymphocytes *in vitro*. Environ. Mol. Mutagen. 52, 69–71.
Di Sotto, A., Maffei, F., Hrelia, P., Castelli, F., Sarpietro, M.G., Mazzanti, G., 2013. Genotoxicity assessment of β-caryophyllene oxide. Regul. Toxicol. Pharmacol. 66, 264–268.
Du, W.X., Avena-Bustillos, R.J., Woods, R., Breksa, A.P., McHugh, T.H., Friedman, M., et al., 2012. Sensory evaluation of baked chicken wrapped with antimicrobial apple and tomato edible films formulated with cinnamaldehyde and carvacrol. J. Agric. Food Chem. 60, 7799–7804.
Dundar, E., Gurlek Olgun, E., Isuksoy, S., Kurkçuoglu, M., Baser, K.H.C., Bal, C., 2008. The effects of intra-rectal and intra-peritoneal application of *Origanum onites* L. essential oil on 2,4,6-trinitrobenzenesulfonic acid-induced colitis in the rat. Exp. Toxicol. Pathol. 59, 399–408.
Eça, K.S., Sartori, T., Menegalli, F.C., 2014. Films and edible coatings containing antioxidants – a review. Braz. J. Food Technol. 17, 98–112.

Edris, A.E., 2007. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. Phytother. Res. 21, 308–323.
Estévez, M., Cava, R., 2006. Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: contradictory effects in different types of frankfurters. Meat Sci. 72, 348–355.
European Commission, 2001. Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorization. <http://ec.europa.eu/food/fs/sc/scf/out82_en.pdf> (accesed date 14.04.15.).
European Commission, 2004. Regulation (EC) No. 1935/2004 of the European Parliament and the Council of 17 October 2004, on materials and articles intended to come into contact with food and repealing Directives 80/509/EEC and 89/109/EEC. Official J. Eur. Comm. 338, 4–17.
European Food Safety Authority, 2008a. Guidance document on the submission of a dossier on a substance to be used in Food Contact Materials for evaluation by EFSA by the Panel on food additives, flavourings, processing aids and materials in contact with food (AFC). <http://www.efsa.europa.eu/en/efsajournal/doc/21r.pdf>.
European Food Safety Authority, 2008b. Scientific opinion of the panel on food, additives, flavorings, processing aids and materials in contact with food on a request from the commission on the use of rosemary extracts as a food additive. EFSA J. 721, 1–3.
European Food Safety Authority, 2009. Opinion of the Panel on food contact materials, enzymes, flavourings and processing aids (CEF) Guidelines on submission of a dossier for safety evaluation by the EFSA of active or intelligent substances present in active and intelligent materials and articles intended to come into contact with food. EFSA J. 1208, 2–11. <http://www.efsa.europa.eu/en/efsajournal/doc/1208.pdf>.
European Food Safety Authority, 2011a. Scientific Opinion on Flavouring Group Evaluation 18, Revision 2 (FGE.18Rev2): aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical groups 6 and 8. EFSA J. 9 (5), 1847.
European Food Safety Authority, 2011b. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J. 9 (9), 2379, 69 pp. doi:10.2903/j.efsa.2011.2379; <www.efsa.europa.eu/efsajournal>.
Evandri, M.G., Battinelli, L., Daniele, C., Mastrangelo, S., Bolle, P., Mazzanti, G., 2005. The antimutagenic activity of *Lavandula angustifolia* (lavender) essential oil in the bacterial reverse mutation assay. Food Chem. Toxicol. 43, 1381–1387.
Fujisawa, S., Atsumi, T., Kadoma, Y., Sakagami, H., 2002. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Toxicology 177, 39–54.
Gomes-Carneiro, M.R., Felzenszwalb, I., Paumgartten, F.J.R., 1998. Mutagenicity testing of (±)-camphor, 1,8-cineole, citral, citronellal, (−)-menthol and terpineol with the *Salmonella*/microsome assay. Mutat. Res. 416, 129–136.
Gomes-Carneiro, M.R., Viana, M.E.S., Felzenszwalb, I., Paumgartten, F.J.R., 2005. Evaluation of β-myrcene, α-terpinene and (+) and (−)-α-pinene in the *Salmonella*/microsome assay. Food Chem. Toxicol. 43, 247–252.
Guan, W., Li, S., Yan, R., Tang, S., Quan, C., 2007. Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. Food Chem. 101, 1558–1564.
Guarda, A., Rubilar, J.F., Miltz, J., Galotto, M.J., 2011. The antimicrobial activity of microencapsulated thymol and carvacrol. Int. J. Food Microbiol. 146, 144–150.
Guarda, A., Rubilar, J., Galotto, M.J., 2012. A process for obtaining a film comprised of the incorporation of naturally-sourced antimicrobial agents in a polymeric structure to develop packages for increasing the shelf life of refrigerated meat, preferentially refrigerated fresh salmon. Universidad de Santiago de Chile, Chile, 43 pp.
Gulluce, M., Karadayi, M., Guvenalp, Z., Ozbek, H., Arasoglu, T., Baris, O., 2012. Isolation of some active compounds from *Origanum vulgare* L. spp. *vulgare* and determination of their genotoxic potentials. Food Chem. 130, 248–253.
Gupta, J., Siddique, Y.H., Beg, T., Ara, G., Afzal, M., 2009. Protective role of green tea extract against genotoxic damage induced by anabolic steroids in cultured human lymphocytes. Biol. Med. 1, 87–99.
Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2000. Liver subcellular fractions from rats treated by organosulfur compounds from *Allium* modulate mutagen activation. Mutat. Res. 466, 17–26.
Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2001. Antimutagenic activity of organosulfur compounds from *Allium* is associated with phase II enzyme induction. Mutat. Res. 495, 135–145.
Guzmán, A., García, C., Marín, A.P., Proudlock, R.J., Fernández de Henestrosa, A.R., Ruíz, M.T., et al., 2008. Evaluation of the genotoxic potential of three phenyltetrahydropyrinidyl butylazole-derived sigma-receptor ligand candidates. Mutat. Res. 653, 91–98.
Hamedo, H., Abdelmigid, H.M., 2009. Use of antimicrobial and genotoxicity potentially for evaluation of essential oils as food preservative. Open Biotechnol. J. 3, 50–56.
Higashimoto, M., Purintrapiban, J., Kataoka, K., Kinouchi, T., Vinitketkumnuen, U., Akimoto, S., et al., 1993. Mutagenicity and antimutagenicity of extracts of three spices and a medicinal plant in Thailand. Mutat. Res. 303, 135–142.
Hikiba, H., Watanabe, E., Barrett, J.C., Tsutsui, T., 2005. Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. J. Pharmacol. Sci. 97, 146–152.
Horváthová, E., Sramkova, M., Labaj, J., Slamenova, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured *in vitro*. Neuro Endocrinol. Lett. 27, 44–47.
Hu, C.Y., Chen, M., Wang, Z.W., 2012. Release of thymol, cinnamaldehyde and vanillin from soy protein isolate films into olive oil. Packag. Technol. Sci. 25, 97–106.
Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interaction with food matrix components. Front. Microbiol. 3, 12.
Ipek, E., Tüylü, B.A., Zeytinoglu, H., 2003. Effects of carvacrol on sister chromatid exchanges in human lymphocyte culture. Cytotechnology 43, 145–148.
Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B.A., Kurkcuoglu, M., Can Baser, K.H., 2005. Genotoxicity and antigenotoxicity of *Origanum* oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. Food Chem. 93, 551–556.
Jeena, K., Liju, V.B., Viswanathan, R., Kuttan, R., 2014. Antimutagenic potential and modulation of carcinogen-metabolizing enzymes by ginger essential oil. Phytother. Res. 28, 849–855.
Kang, S.H., KwoN, J.Y., Lee, J.K., Seo, Y.R., 2013. Recent advances in *in vivo* genotoxicity testing: prediction of carcinogenic potential using comet and micronucleus assay in animal models. J. Cancer Prev. 4, 277–288.
Kirkpinar, F., Bora Unlu, H., Ozdemir, G., 2011. Effects of oregano and garlic essential oils on performance, carcase, organ and blood characteristics and intestinal microflora of broilers. Livest. Sci. 137, 219–225.
Knezevic-Vukcevic, J., Vukovic-Gacic, B., Stevic, T., Stanojevic, J., Nikolic, B., Simic, D., 2005. Antimutagenic effect of essential oil of Sage (*Salvia officinalis* L.) and its fractions against UV-induced mutations in bacterial and yeast cells. Arch. Biol. Sci. 57, 163–172.
Kulisic, T., Radonic, A., Katalinic, V., Milos, M., 2004. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85, 633–640.
Kuorwel, K., Cran, M.J., Sonneveld, K., Miltz, J., Bigger, S.W., 2011. Antimicrobial activity of biodegradable polysaccharide and protein-based films containing active agents. J. Food Sci. 76, 90–102.
Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462.
Lanzotti, V., 2006. The analysis of onion and garlic. J. Chromatogr. A 1112, 3–22.
Laughton, M.J., Halliwell, B., Evans, P.J., Hoult, J.R.S., 1989. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. Biochem. Pharmacol. 38, 2859–2865.
Lazutka, J.R., Mierauskiene, J., Slapsyte, G., Dedonyte, V., 2001. Genotoxicity of dill (*Anethum graveolens* L.), peppermint (*Menthaxpiperita* L.) and pine (*Pinus sylvestris* L.) essential oils in human lymphocytes and *Drosophyla melanogaster*. Food Chem. Toxicol. 39, 485–492.
Letizia, C.S., Cocchiara, J., Lalko, J., Api, A.M., 2003. Fragrance material review on linalool. Food Chem. Toxicol. 41, 943–964.
Liju, V.B., Jeena, K., Kuttan, R., 2013. Acute and subchronic toxicity as well as mutagenic evaluation of essential oil from turmeric (*Curcuma longa* L.). Food Chem. Toxicol. 53, 52–61.
Liolios, C.C., Gortzi, O., Lalas, S., Tsaknis, J., Chinou, I., 2009. Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and *in vitro* antimicrobial activity. Food Chem. 112, 77–83.
Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Moreno, F.J., Bermúdez, J.M., Aucejo, S., et al., 2014a. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290.
Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A., et al., 2014b. Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. Food Chem. Toxicol. 72, 123–128.
Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Jos, A., Cameán, A.M., 2015a. In vitro pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. Toxicol. In Vitro 29, 647–656.
Llana-Ruiz-Cabello, M., Maisanaba, S., Gutiérrez-Praena, D., Prieto, A.I., Pichardo, S., Jos, A., et al., 2015b. Cytotoxic and mutagenic *in vitro* assessment of two organosulfur compounds derived from onion to be used in the food industry. Food Chem. 166, 423–431.
López, M.A., Stashenko, E.E., Fuentes, J.L., 2011. Chemical composition and antigenotoxic properties of *Lippia alba* essential oils. Genet. Mol. Biol. 34, 479–488.
López, P., Sánchez, C., Batlle, R., Nerín, C., 2007. Development of flexible antimicrobial films using essential oils as active agents. J. Agric. Food Chem. 55, 8814–8824.
Lorge, E., Thybaud, V., Aardemac, M.J., Oliver, J., Wakata, A., Lorenzon, G., et al., 2006. SFTG international collaborative study on *in vitro* micronucleus test I. General conditions and overall conclusions of the study. Mutat. Res. 607, 13–36.
Lupi, S., Marconi, S., Pajaro, E., Fochesato, A., Gregorio, P., 2009. Mutagenicity evaluation with Ames test of hydro-alcoholic solution of terpenes. J. Prev. Med. Hyg. 50, 170–174.
Mahmoud, I., Alkofahi, A., Abdelaziz, A., 1992. Mutagenic and toxic activities of several spices and some Jordanian medicinal plants. Pharm. Biol. 30, 81–85.
Maistro, E.L., Angeli, J.P.F., Andrade, S.F., Mantovani, M.S., 2011. *In vitro* genotoxicity assessment of caffeic, cinnamic and ferulic acids. Genet. Mol. Res. 10, 1130–1140.
Manso, S., Pezo, D., Gómez-Lus, R., Nerín, C., 2014. Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil. Food Control 45, 101–108.
Maralhas, A., Monteiro, A., Martins, C., Kranendonk, M., Laires, A., Rueff, J., et al., 2006. Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. Mutagenesis 21, 199–204.
Martinez-Rocha, A., Puga, R., Hernández-Sandoval, L., Loarca-Piña, G., Mendoza, S., 2008. Antioxidant and antimutagenic activities of Mexican oregano (*Lippia graveolens* Kunth). Plant Food Hum. Nutr. 63, 1–5.

Martins, C., Doran, C., Laires, A., Rueff, J., Rodrigues, A.S., 2011. Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells. Food Chem. Toxicol. 49, 385–392.
Martins, C., Cação, R., Cole, K.J., Phillips, D.H., Laires, A., Rueff, J., et al., 2012. Estragole: a weak direct-acting food-borne genotoxin and potential carcinogen. Mutat. Res. 747, 86–92.
Mezzoug, N., Elhadri, A., Dallouh, A., Amkiss, S., Skali, N.S., Abrini, J., et al., 2007. Investigation of the mutagenic and antimutagenic effects of *Origanum compactum* essential oil and some of its constituents. Mutat. Res. 629, 100–110.
Minnunni, M., Wolleb, U., Mueller, O., Pfeifer, A., Aeschbacher, H.U., 1992. Natural antioxidants as inhibitors of oxygen species induced mutagenicity. Mutat. Res. 269, 193–200.
Mitic-Culafic, D., Zegura, B., Nikolic, B., Vukovic-Gacic, B., Knezevic-Vukcevic, J., Filipic, M., 2009. Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem. Toxicol. 47, 260–266.
Musk, S.R.R., Clapham, P., Johnson, I.T., 1997. Cytotoxicity and genotoxicity of diallyl sulfide and diallyl disulfide towards Chinese hamster ovary cells. Food Chem. Toxicol. 35, 379–385.
National Toxicology Program, 1982. NTP studies on eugenol. In vitro cytogenetics – chromosome aberrations. Study ID: 416109. <http://ntp-apps.niehs.nih.gov/ntp_tox/>.
National Toxicology Program, 1983. Carcinogenesis studies of eugenol (Cas No. 97-53-0) in F344/N-rats and B6C3F1 mice (feed studies). Technical Report Series No. 223. NHI Publication No. 84–1779. U.S. DHHS, PHS, NIH, NTP, Research Triangle Park, NC.
National Toxicology Program, 2004. NTP technical report on the toxicology and carcinogenesis studies of trans-cinnamaldehyde. Technical Report 514, NIH Publication N° 04–4448.
Neffati, A., Skandrani, I., Ben Sghaier, M., Bouhlel, I., Kilani, S., Ghedira, K., et al., 2008. Chemical composition, mutagenic and antimutagenic activities of essential oils from (tunisian) *Artemisia campestris* and *Artemisia herba-alba*. J. Essent. Oil Res. 20, 471–477.
Neffati, A., Limem, I., Kilani, S., Bouhlel, I., Skandrani, I., Bhouri, W., et al., 2009. A comparative evaluation of mutagenic, antimutagenic radical scavenging and antibacterial activities of essential oils of *Pituranthos chloranthus* (Coss. Et Dur.). Drug Chem. Toxicol. 32, 372–380.
Nerín, C., Tovar, L., Djenane, D., Camo, J., Salafranca, J., Beltrán, J.A., et al., 2006. Stabilization of beef meat by a new active packaging containing natural antioxidants. J. Agric. Food Chem. 54, 7840–7846.
Nesslany, F., Marzin, D., 1999. A micromethod for the *in vitro* micronucleus assay. Mutagenesis 14, 403–410.
Nicolella, H.D., De Oliveira, P.F., Munari, C.C., Dias Costa, G.F., Rodrigues Moreira, M., Veneziani, R.C.S., et al., 2014. Differential effect of manool – a diterpene from *Salvia officinalis*, on genotoxicity induced by methyl methanesulfonate in V79 and HepG2 cells. Food Chem. Toxicol. 72, 8–12.
Nikolic, B., Mitic-Culafic, D., Vukovic-Gacic, B., Knezevic-Vukcevic, J., 2011. Modulation of genotoxicity and DNA repair by plant monoterpenes camphor, eucalyptol and thujone in *Escherichia coli* and mammalian cells. Food Chem. Toxicol. 49, 2035–2045.
Noel, S., Kasinathan, M., Rath, S.K., 2006. Evaluation of apigenin using *in vitro* cytochalasin blocked micronucleus assay. Toxicol. In Vitro 20, 1168–1172.
OECD (Organisation for Economic Cooperation and Development), 1986. Guideline for the testing of chemicals: *in vitro* sister chromatid exchange assay in mammalian cells, Guideline 479. pp 1–20.
OECD (Organisation for Economic Cooperation and Development), 1997a. Guideline for the testing of chemicals: bacterial reverse mutation test, Guideline 471. pp 1–11.
OECD (Organisation for Economic Cooperation and Development), 1997b. Guideline for the testing of chemicals: *in vitro* mammalian chromosome aberration test, Guideline 473. pp 1–14.
OECD (Organisation for Economic Cooperation and Development), 1997c. Guideline for the testing of chemicals: *in vitro* mammalian cell gene mutation test, Guideline 476. pp 1–10.
OECD (Organisation for Economic Cooperation and Development), 2010. Guideline for the testing of chemicals: *in vitro* mammalian cell micronucleus test, Guideline 487. pp 1–24.
Okai, Y., Higashi-Okai, K., 1997. Potent suppressing activity of the non-polyphenolic fraction of green tea (*Camellia sinensis*) against genotoxin-induced umu C gene expression in *Salmonella typhimurium* (TA1535/pSK1002)-association with pheophytins a and b. Cancer Lett. 120, 117–123.
Özkan, A., Erdogan, A., 2011. A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (*Lamiaceae*) and its two major phenolic components. Turkish J. Biol. 35, 735–742.
Özkan, A., Erdogan, A., 2012. A comparative study of the antioxidant/prooxidant effects of carvacrol and thymol at various concentrations on membrane and DNA of parental and drug resistant H1299 cells. Nat. Prod. Commun. 7, 1557–1560.
Papachristou, F., Chatzaki, E., Petrou, A., Kougioumtzi, I., Katsikogiannis, N., Papalambros, A., et al., 2013. Time course changes of anti- and pro-apoptotic proteins in apigenin-induced genotoxicity. Chin. Med. 8, 1–9.
Reyes, M.R., Reyes-Esparza, J., Ángeles, O.T., Rodríguez-Fragoso, L., 2010. Mutagenicity and safety evaluation of water extract of *Coriander sativum* leaves. J. Food Safety 75, T6–T12.
Rithidech, K.N., Tungjai, M., Whorton, E.B., 2005. Protective effect of apigenin on radiation-induced chromosomal damage in human lymphocytes. Mutat. Res. 585, 96–104.
Roby, M.H.H., Sarhana, M.A., Selima, K.A.H., Khalela, K.I., 2013. Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. Ind. Crops Prod. 43, 827–831.
Rodríguez, A., Batlle, R., Nerín, C., 2007. The use of natural essential oils as antimicrobial solutions in paper packaging. Part II. Prog. Org. Coat. 60, 33–38.
Rodríguez-Lafuente, A., Nerin, C., Batlle, R., 2010. Active paraffin-based paper packaging for extending the shelf life of cherry tomatoes. J. Agric. Food Chem. 58, 6780–6786.
Roscheisen, C., Zamith, H., Paumgartten, F.J.R., Speit, G., 1991. Influence of fl-myrcene on sister-chromatid exchanges induced by mutagens in V79 and HTC cells. Mutat. Res. 269, 43–49.
Rossi, D., Guerrini, A., Paganetto, G., Bernacchia, G., Conforti, F., Statti, G., et al., 2013. *Croton lechleri* Müll. Arg. (Euphorbiaceae) stem bark essential oil as possible mutagen-protective food ingredient against heterocyclic amines from cooked food. Food Chem. 139, 439–447.
Ruberto, G., Baratta, M.T., 2000. Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem. 69, 167–171.
Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., et al., 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chem. 91, 621–632.
Sanches-Silva, A., Costa, D., Albuquerque, T.G., Buonocore, T.G., Ramos, F., Castilho, M.C., et al., 2014. Trend in the use of natural antioxidants in active food packaging: a review. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 31, 374–395.
Sanla-Ead, N., Jangchud, A., Chonhenchob, V., Suppakul, P., 2012. Antimicrobial activity of cinnamaldehyde and eugenol and their activity after incorporation into cellulose-based packaging films. Packag. Technol. Sci. 25, 7–17.
Saverini, M., Catanzaro, I., Sciandrello, G., Avellone, G., Indelicato, S., Marcì, G., et al., 2012. Genotoxicity of citrus wastewater in prokaryotic and eukaryotic cells and efficiency of heterogeneous photocatalysis by TiO_2. J. Photochem. Photobiol. B. 108, 8–15.
Seow, Y.X., Yeo, C.R., Chung, H.L., Yuk, H.G., 2014. Plant essential oils as active antimicrobial agents. Crit. Rev. Food Sci. Nutr. 54, 625–644.
Sghaier, N.B., Boubaker, J., Neffati, A., Limem, I., Skandrami, I., Bhouri, W., et al., 2010. Antimutagenic and antioxidant potentials of *Teucrium ramosissimum* essential oil. Chem. Biodivers. 7, 1754–1763.
Shaughnessy, D.T., Setzer, R.W., De Marin, D.M., 2001. The antimutagenic effect of vanillin and cinnamaldehyde on spontaneous mutation in *Salmonella* TA104 is due to a reduction in mutations at GC but not AT sites. Mutat. Res. 480–481, 55–69.
Shoeibi, S., Rahimifard, N., Pirouz, B., Yalfani, R., Pakzad, S.R., Mirab Samiee, S., et al., 2009. Mutagenicity of four natural flavors: clove, cinnamon, thyme and *Zataria multiflora* Boiss. J. Med. Plants 8, 89–96.
Shon, M.Y., Choi, S.D., Kahng, G.G., Nam, S.H., Sung, N.J., 2004. Antimutagenic, antioxidant and free radical scavenging activity of ethylacetate extracts from white, yellow and red onions. Food Chem. Toxicol. 42, 659–666.
Siddique, Y.H., Afzal, M., 2004. Antigenotoxic effect of allicin against SCEs induced by methylmethane sulphonate in cultured mammalian cells. Indian J. Exp. Biol. 42, 437–438.
Siddique, Y.H., Ara, G., Beg, T., Faisal, M., Ahmad, M., Afzal, M., 2008. Antigenotoxic role of *Centella asiatica* L. extract against cyproterone acetate induced genotoxic damage in cultured human lymphocytes. Toxicol. In Vitro 22, 10–17.
Siddique, Y.H., Ara, G., Beg, T., Afzal, M., 2010. Anticlastogenic effect of apigenin in human lymphocytes treated with ethinylestradiol. Fitoterapia 81, 590–594.
Singh, H.P., Batish, D.R., Kaur, S., Arora, K., Kohli, R.K., 2006. α-Pinene inhibits growth and induces oxidative stress in roots. Ann. Bot. London 98, 1261–1269.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Sinha, S., Jothiramajayam, M., Ghosh, M., Mukherjee, A., 2014. Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes. Food Chem. Toxicol. 68, 71–77.
Slamenová, D., Horvathova, E., Sramkova, M., Marsalkova, L., 2007. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*. Neoplasma 54, 108–112.
Slamenova, D., Kozics, K., Hunakova, L., Melusova, M., Navarova, J., Horvathova, E., 2013. Comparison of biological processes induced in HepG2 cells by tert-butyl hydroperoxide (t-BHP) and hydroperoxide (H_2O_2): the influence of carvacrol. Mutat. Res. 757, 15–22.
Smith, R.L., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., et al., 2005. A procedure for the safety evaluation of natural flavour complexes used as ingredients in food: essential oils. Food Chem. Toxicol. 43, 345–363.
Snyder, R.D., Gillies, P.J., 2002. Evaluation of the clastogenic, DNA intercalation, and topoisomerase II-interactive properties of bioXavonoids in Chinese hamster V79 cells. Environ. Mol. Mutagen. 40, 266–276.
Sousa, S.M., Silva, P.S., Viccini, L.F., 2010. Cytogenotoxicity of *Cymbopogon citratus* (DC) Stapf (lemongrass) aqueous extracts in vegetal test systems. An. Acad. Bras. Cienc. 82, 305–311.
Stajkovic, O., Beric-Bjedov, T., Mitic-Culafic, D., Stankovic, S., Vukovic-Gacic, B., Simic, D., et al., 2007. Antimutagenic properties of basil (*Ocimum basilicum* L.) in *Salmonella typhimurium* TA100. Food Technol. Biotechnol. 45, 213–217.
Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823.

Suhaj, M., 2006. Spice antioxidants isolation and their antiradical activity: a review. J. Food Comp. Anal. 19, 531–537.
Sung, S.Y., Sin, L.T., Tee, T.T., Bee, S.T., Rahmat, A.R., Rahman, W.A.W.A., et al., 2013. Antimicrobial agents for food packaging applications. Trends Food Sci. Tech. 33, 110–123.
Suppakul, P., Sonneveld, K., Bigger, S.W., Miltz, J., 2008. Efficacy of polyethylene-based antimicrobial films containing principal constituents of basil. LWT-Food Sci.Technol. 41, 779–788.
Teixeira, B., Marques, A., Ramos, C., Batista, I., Serrano, C., Matos, O., et al., 2012. European pennyroyal (*Mentha pulegium*) from Portugal: chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil. Ind. Crops Prod. 36, 81–87.
Thirugnanasampandan, R., Jayakumar, R., Prabhakaran, M., 2012. Analysis of chemical composition and evaluation of antigenotoxic, cytotoxic and antioxidant activities of essential oil of *Toddalia asiatica* (L.) Lam. Asia Pac. J. Trop. Biomed. S1276–S1279.
Tontul, I., Torun, M., Dincer, C., Sahin-Nadeem, H., Topuz, A., Turna, T., et al., 2013. Comparative study on volatile compounds in Turkish green tea powder: impact of tea clone, shading level and shooting period. Food Res. Int. 53, 744–750.
Truong, D., Hindmarsh, W., O'Brien, P.J., 2009. The molecular mechanisms of diallyl disulfide and diallyl sulfide induced hepatocyte cytotoxicity. Chem. Biol. Interact. 180, 79–88.
Unlu, M., Ergene, E., Unlu, G.V., Zeytinoglu, H.S., Vural, N., 2010. Composition, antimicrobial activity and in vitro cytotoxicity of essential oil from *Cinnamomum zeylanicum* Blume (Lauraceae). Food Chem. Toxicol. 48, 3274–3280.
Ündeger, U., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.
Vagi, E., Rapavi, E., Hadolin, M., Vásárhelyiné Perédi, K., Balázs, A., Blázovics, A., 2005. Phenolic and triterpenoid antioxidants from *Origanum majorana* L. herb and extracts obtained with different solvents. J. Agric. Food Chem. 53, 17–21.
Vanderkerken, K., Vanparys, P., Verschaeve, L., KirschVolders, M., 1989. The mouse bone marrow micronucleus assay can be used to distinguish aneugens from clastogens. Mutagenesis 4, 6–11.
Vermeiren, L., Devlieghere, F., van Beest, M., de Kruijf, N., Debevere, J., 1999. Developments in the active packaging of food. Trends Food Sci. Tech. 10, 77–86.
Vicuña, G.C., Stashenko, E.E., Fuentes, J.L., 2010. Chemical composition of the *Lippia origanoides* essential oils and their antigenotoxicity against bleomycin-induced DNA damage. Fitoterapia 81, 343–349.
Vukovic-Gacic, B., Nikcevic, S., Beric-Bjedov, T., Knezevic-Vukcevic, J., Simic, D., 2006. Antimutagenic effect of essential oil of sage (*Salvia officinalis* L.) and its monoterpenes against UV-induced mutations in *Escherichia coli* and *Saccharomyces cerevisiae*. Food Chem. Toxicol. 44, 1730–1738.
Wondrak, G.T., Villeneuve, N.F., Lamore, S.D., Bause, A.S., Jiang, T., Zhang, D.D., 2010. The cinnamon-derived dietary factor cinnamic aldehyde activates the Nrf2-dependent antioxidant response in human epithelial colon cells. Molecules 15, 3338–3355.
Xing, F., Hua, H., Selvaraj, J.N., Zhao, Y., Zhou, L., Liu, X., et al., 2014. Growth inhibition and morphological alterations of *Fusarium verticillioides* by cinnamon oil and cinnamaldehyde. Food Control 46, 343–350.
Yi, L., Su, Q., 2013. Molecular mechanisms for the anti-cancer effects of diallyl disulfide. Food Chem. Toxicol. 57, 362–370.
Yoo, C.B., Han, K.T., Cho, K.S., Ha, J., Park, H.J., Nam, J.H., et al., 2005. Eugenol isolated from the essential oil of *Eugenia caryophyllata* induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. Cancer Lett. 255, 41–52.
Zegura, B., Dobnik, D., Niderl, M.H., Filipic, M., 2011. Antioxidant and antigenotoxic effects of rosemary (*Rosmarinus officinalis* L.) extracts in *Salmonella typhimurium* TA98 and HepG2 cells. Environ. Toxicol. Pharmacol. 32, 296–305.
Zhou, F., Ji, B., Zhang, H., Jiang, H., Yang, Z., Li, J., et al., 2007. The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella typhimurium*. J. Food Safety 27, 124–133.

CAPÍTULO 2 / CHAPTER 2

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ESTUDIO IN VITRO DE LA VIABILIDAD DE CÉLULAS CACO-2 EN PRESENCIA DE COMPONENTES DEL ACEITE ESENCIAL DE ALLIUM SPP.

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Estudio *in vitro* de la viabilidad de células Caco-2 en presencia de componentes del aceite esencial de *Allium spp*

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Resumen: El aceite esencial de los componentes del género *Allium,* principalmente ajo y cebolla, presenta propiedades antioxidantes y antibacterianas debidas a la presencia de compuestos azufrados en su composición. La industria alimentaria ha comenzado a desarrollar nuevos sistemas de envasado activo a partir de polímeros seleccionados, a los que se incorporan aceites esenciales que, por sus propiedades, contribuyen a aumentar la vida útil de los alimentos perecederos. En este sentido, se hace necesario evaluar la seguridad asociada al uso de estas sustancias en envases alimentarios que van a estar en contacto con el consumidor a través del alimento. El objetivo del presente estudio fue determinar la citotoxicidad producida por dipropil sulfuro y dipropil disulfuro, dos de los componentes del aceite esencial de ajo y cebolla, en la línea celular Caco-2, células humanas procedentes de carcinoma de colon. Los biomarcadores ensayados fueron el contenido total de proteínas, la captación de rojo neutro y la reducción de la sal de tetrazolio (3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4sulfofenil)-2H-tetrazolio). Las células fueron expuestas durante 2, 4 y 8 h a concentraciones comprendidas entre 0 y 200 µM. Los resultados no mostraron diferencias significativas frente al control para ninguno de los tres marcadores, lo que demuestra que bajo las condiciones de los ensayos ambos compuestos azufrados no son citotóxicos para esta línea celular gastrointestinal y podrían ser útiles en la industria alimentaria para desarrollar envases activos.

Palabras clave: aceite esencial de *Allium spp*., citotoxicidad, dipropil sulfuro, dipropil disulfuro, Caco-2

Abstract: *In vitro* study of the viability of Caco-2 cells in presence of two compound of *Allium spp* essential oil. *Allium spp*. essential oil, mainly from garlic and onion, possesses different beneficial properties, for example antioxidant and antimicrobial effects, due to the presence of sulfur compounds. Food industry is developing new active packaging systems that include the essential oil of garlic in their structure, in order to improve the shelf-life of perishable products. Therefore it is necessary to evaluate the safety associated with the use of these substances in food packaging that will be in contact with the consumer through food. The aim of our study was to evaluate *in vitro* the cytotoxicity of dipropyl sulfide and dipropyl disulfide. For this purpose, we used the human Caco-2 cell line, from human small intestinal mucosa carcinoma. The assayed cytotoxicity biomarkers were the total protein content, neutral red uptake and reduction of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboximethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt. Cells were exposed to dipropyl sulfide and dipropyl disulfide in concentrations between 0-200 µM for 2, 4 and 8 h. After periods of exposure, no alterations were observed in any of the biomarkers assayed. These results suggest that both organosulfur compounds are safety options for food industry and could be a choice in the development of active packaging.

Keywords: *Allium spp* essential oil, cytotoxicity, dipropyl sulfide, dipropyl disulfide, Caco-2.

Introducción

El ajo (*Allium sativum*) y la cebolla (*Allium cepa*) se han utilizado tradicionalmente por sus propiedades farmacológicas y terapéuticas [1-3], sobre todo en el tratamiento de enfermedades cardiovasculares [4], inflamación [5,6], carcinogénesis [7,8] e infecciones víricas [9] o bacterianas [10,11].

Los aceites esenciales de ajo y cebolla se obtienen mediante un proceso de destilación por arrastre de vapor. La cantidad y variedad de compuestos azufrados que forman parte de la composición de este aceite puede variar en función de distintos factores como son, el proceso de extracción o la madurez del bulbo [12,13]. Se ha observado que, los aceites de ajo y cebolla, pueden inhibir el crecimiento de células tumorales *in vitro* [14], inducir enzimas que intervienen en la destoxificación de xenobióticos [15,16]; e incluso el aceite de ajo es capaz de activar mecanismos de apoptosis [17]. El interés en estos aceites esenciales dentro del ámbito alimentario radica en la creciente preocupación, por parte de los consumidores, del uso de conservantes, antioxidantes y antibacterianos artificiales en los alimentos. Para responder a esta inquietud, comienzan a desarrollarse en Europa los denominados envases activos, que incorporan sustancias naturales con el fin de preservar los alimentos.

Según el Reglamento (CE) No 1935/2004 del Parlamento Europeo y del Consejo, de 27 de octubre de 2004, sobre los materiales y objetos destinados a entrar en contacto con alimentos [18], los envases activos son "materiales y objetos destinados a ampliar el tiempo de conservación, o a mantener o mejorar el estado de los alimentos envasados, y que están diseñados para incorporar deliberadamente componentes que transmitan sustancias a los alimentos envasados o al entorno de éstos o que absorban sustancias de los alimentos envasados o del entorno de éstos". La liberación de sustancias desde el envase al alimento no debe provocar una modificación inaceptable de la composición del alimento en virtud del reglamento citado anteriormente. Por ello, se permite la fabricación de materiales plásticos que no liberen más de 10 mg de sustancias por dm^2 de la superficie del material plástico [19].

La posible incorporación del aceite esencial de ajo o cebolla, por las propiedades descritas anteriormente, a polímeros plásticos para desarrollar estos nuevos envases, hace necesario estudiar la seguridad derivada de su uso en la industria alimentaria. Amagase [20] atribuyó

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a los compuestos azufrados del ajo sus propiedades antioxidantes. Estos compuestos son derivados de la descomposición de la alicina [21] y presentan una gran variabilidad, destacando el dialil sulfuro (dAS), dialil disulfuro (dAdS) y dialil trisulfuro (dAtS) [22] como componentes mayoritarios; frente a otros, menos abundantes, como el dipropil sulfuro (dPS) y dipropil disulfuro (dPdS) cuyas estructuras constan de uno y dos átomos de azufre respectivamente (Fig 1) [23]. No obstante, ambos compuestos son componentes principales de la cebolla, siendo el dPdS el componente mayoritario de su aceite esencial [24].

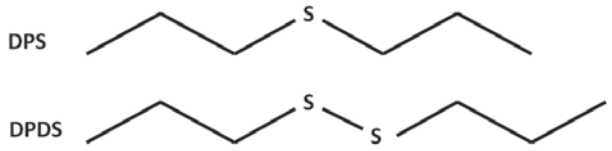

Figura 1. Estructuras químicas del dipropil sulfuro y dipropil disulfuro [23]

Las propiedades de los derivados alilos han sido ampliamente estudiadas y existe una abundante bibliografía que demuestra la capacidad de estos compuestos para inducir apoptosis *in vitro* en una gran variedad de tipos celulares como son las células de carcinoma de colon HCT-15 [25], Caco-2 y HT-29 [26], de cáncer de mama MCF-7 [27], de cáncer de próstata PC-3 y DU145 [28] o las de cáncer de tiroides anaplásico ATC [29]. Además, Tsai y col. [30] muestran la capacidad antioxidante del dAtS frente a las especies reactivas de oxígeno (ERO) generadas en miocitos cardíacos (H9c2) expuestos a altas concentraciones de glucosa. Por otro lado, Yin y col. [31] reportaron el efecto antioxidante no mediado por enzimas del dAS y dAdS como potenciales componentes de envases alimentarios. Asimismo, la actividad antibacteriana de los alilos frente a *Staphylococcus aureus, Pseudomonas aeruginosa y Escherichia coli* también ha sido demostrada [32].

Con respecto a los compuestos minoritarios en el aceite de ajo, como el dPS y el dPdS, los estudios son más escasos, habiéndose encontrado que ambos disminuyen el daño oxidativo producido en el ADN tras la exposición de N-nitrosaminas en células HepG2 [33]. Además, se ha observado que el dPdS presenta efectos antiapoptóticos en células HepG2 expuestas a N-nitrosaminas [34].

Teniendo en cuenta la escasa bibliografía publicada hasta el momento acerca de los efectos que producen los componentes dipropilos de los aceites de ajo y cebolla, dPS y dPdS, éstos fueron el objetivo de nuestro estudio. Con el fin de evaluar la seguridad derivada de su uso en el envasado de alimentos, se seleccionó la línea celular intestinal humana Caco-2. Por todo ello, el objetivo del presente trabajo fue realizar un screening de la citotoxicidad *in vitro* del dPS y dPdS tras 2, 4 y 8 horas de exposición en las células Caco-2.

Material y métodos.

Materiales y productos químicos

El medio de cultivo Eagle Minimum Essential Medium (EMEM), suero bovino fetal y aminoácidos no esenciales (AANE) fueron proporcionados por BioWittaker (Lonza, Verviers, Bélgica) y la L-glutamina, gentamicina y piruvato sódico por Gibco (Paisley, Reino Unido). Los compuestos azufrados (dPS y dPdS) fueron suministrados por Sigma Aldrich (Madrid, España). El resto de reactivos y materiales empleados se obtuvieron de Termo Fisher Scientific (Dinamarca), Sigma Aldrich (Madrid, España), VWR International Eurolab (Madrid, España) y BioRad (Madrid, España).

Modelo celular

La línea celular Caco-2, derivada de un adenocarcinoma de colon humano (ATCC® HTB-37), se mantuvo a 37°C, en atmósfera de CO2 al 5% y un 95% de humedad relativa (CO2 incubator, NuAire®, España) en medio EMEM suplementado con 10% de suero bovino fetal, 1% de aminoácidos no esenciales, 1 mM de piruvato sódico, 2 mM de L-glutamina y 50 µg/mL de gentamicina. Las células se mantuvieron hasta confluencia en frascos de plástico de 75 cm^2 estériles. Los pases celulares de trabajo se encontraban entre el 14 y 19.

Ensayos de citotoxicidad basal

Para los ensayos de citotoxicidad, las células Caco-2 se sembraron en placas de 96 pocillos a la densidad de $7{,}5 \cdot 10^5$ cel/mL, y se incubaron durante 24 h a las condiciones adecuadas. Transcurrido este tiempo, se procedió a la exposición de las células. Las soluciones de dPS y dPdS se prepararon en medio de cultivo EMEM, sin suero bovino fetal, a partir de una disolución stock de dPS y dPdS en 0.1% DMSO. Se prepararon disoluciones seriadas para la exposición de manera que la concentración final de DMSO fuera inferior al 0,1%. Las concentraciones de exposición se calcularon teniendo cuenta el porcentaje de estos compuestos en el aceite esencial de cebolla (0-20%) y la cantidad de aceite que se podría emplear en la elaboración de envases activos. Dichas concentraciones de exposición fueron 0,25; 0,5; 1; 10; 20; 50; 100; 200 µM para ambos compuesto azufrados, además de un grupo control sin la presencia de ninguno de los compuestos objeto de estudio y otro con una concentración 0,1% en DMSO. Una vez expuestas, las células se incubaron durante 2, 4 y 8 h a 37ºC. Dichos tiempos de exposición fueron seleccionados con el fin de realizar una estimación más realista del daño que una ingestión de los compuestos azufrados puede provocar en las células intestinales durante el proceso digestivo.

Los diferentes ensayos de citotoxicidad basal realizados fueron la estimación del contenido proteico total (PT), la reducción de la sal de tetrazolio (3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4sulfofenil)-2H-tetrazolio) (MTS) y la captación del colorante rojo neutro (RN).

El ensayo de PT se realizó según el protocolo de Bradford [35] que utiliza Azul brillante de Coomassie G-250 para determinar el número total de células presentes. La absorbancia se midió a 595nm en el lector de placas Infiniti 200 (Tecan Austria GmbH, Grödig, Austria). El ensayo RN permite determinar la viabilidad celular. Se lleva a cabo siguiendo el procedimiento descrito por Borenfreund y Puerner [36]. La reducción de MTS se realiza siguiendo el procedimiento de Baltrop y col. [37] y es llevada a cabo por deshidrogenasas presentes en la mitocondria.

Cálculos y análisis estadístico

Los experimentos se llevaron a cabo tres veces con sextuplicados para cada concentración. Los datos se representan como porcentaje con respecto al grupo control ± sus respectivas desviaciones estándar (DE).

El análisis estadístico se realizó mediante el análisis de varianza (ANOVA), seguido del test de Dunnet, utilizando el programa GraphPadInStat software (GraphPadSoftware Inc., La Jolla, EE.UU.)

Resultados

Tras incubar las células Caco-2 a concentraciones entre 0 y 200 µM de dPS se midieron tres biomarcadores de la viabilidad celular. Los resultados obtenidos se compararon con un control, no expuesto a dPS, al que se le asignó el valor de 100% de viabilidad. Después de 2, 4 y 8 h de exposición, el número de células vivas, mediante la cuantificación de PT, relacionadas directamente con la proliferación celular, no experimentó cambios significativos en todas las concentraciones ensayadas con respecto al grupo control (Fig 2A). Para todos los tiempos y concentraciones de exposición, el ensayo de RN, basado en la incorporación del colorante supravital rojo neutro a los lisosomas de células viables no dañadas, mostró valores de viabilidad cercanos al 100% (Fig 2B). Así mismo, la reducción de MTS, indicador del grado de afectación de las mitocondrias, se comportó de forma similar al contenido proteico y RN, sin experimentar cambios en la proliferación celular con respecto al control (Fig 2C).

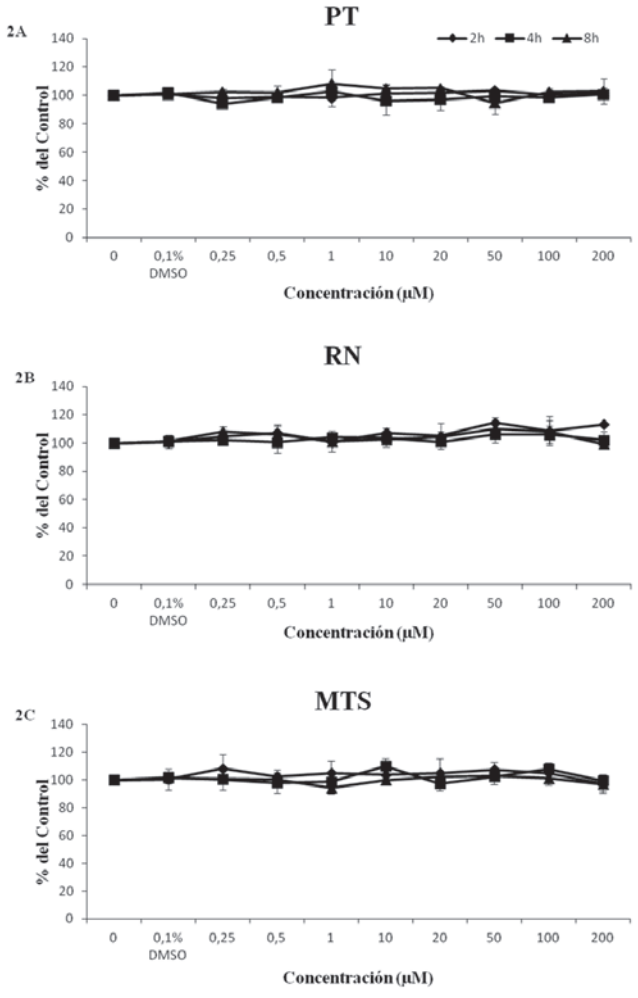

Figura 2. Contenido proteico total (A), Captación de Rojo Neutro (B) y Reducción de la sal de tetrazolio MTS (C) en células Caco-2 expuestas a dPS durante 2, 4 y 8 h. Valores expresados como media ± DE.

De forma similar, las células Caco-2 expuestas a dPdS a las mismas condiciones de exposición que su homólogo sulfurado, no sufrieron ningún efecto tóxico. El contenido proteico no experimentó alteraciones significativas con respecto al grupo control (Fig. 3A). Igualmente, ni el ensayo de RN (Fig. 3B) ni la reducción de MTS (Fig. 3C) mostraron afectación notable a ninguna concentración ni tiempo de exposición ensayados para el dPdS.

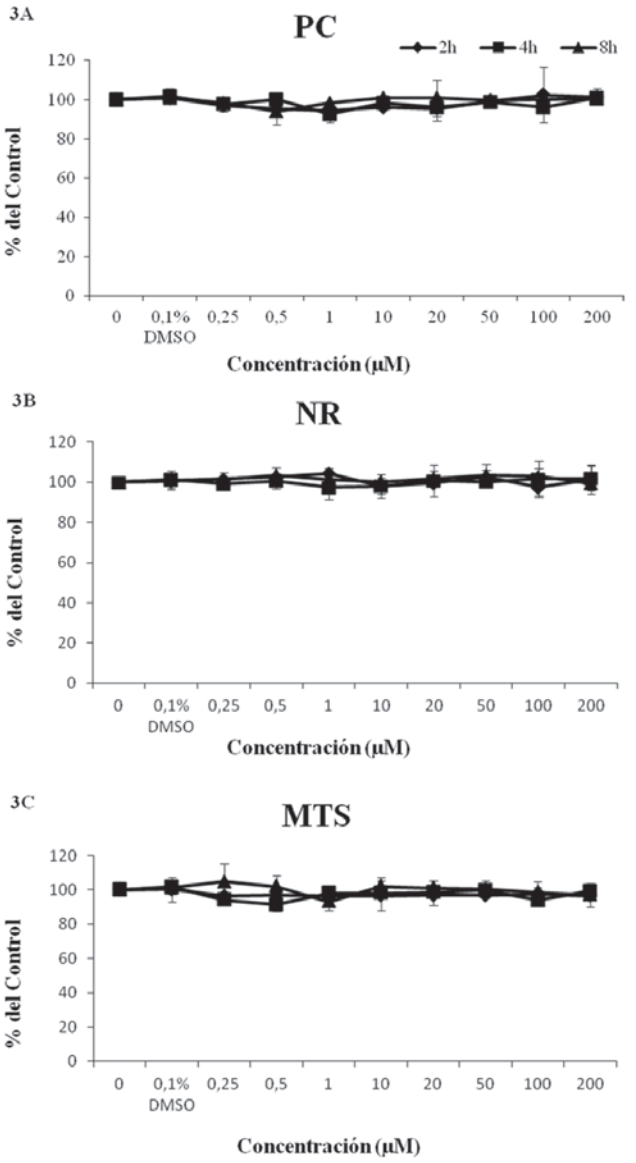

Figura 3. Contenido proteico total (A), Captación de Rojo Neutro (B) y Reducción de la sal de tetrazolio MTS (C) en células Caco-2 expuestas a dPdS durante 2, 4 y 8 h. Valores expresados como media ± DE.

Discusión

Las investigaciones realizadas concernientes a los efectos producidos por los compuestos dPS y dPdS son muy escasas y no existen datos de migración de estos compuestos desde el envase al alimento. Como consecuencia, teniendo en cuenta el peor escenario posible de exposición, la máxima concentración ensayada fue superior a la concentración prevista en el envase para asegurar que, en el rango real de uso, el compuesto sea seguro. En nuestro estudio, los marcadores de citotoxicidad ensayados, no revelaron cambios significativos a ninguna de las concentraciones estudiadas (0-200 µM dPS). Del mismo modo, Xiao y col. [28] no encontraron una disminución de la viabilidad celular al estudiar el biomarcador Sulforhodamina B,

similar al ensayo de PT que determina la cantidad de proteínas, en células de cáncer de próstata PC-3 expuestas a dPS (0-160 μM) durante 24 h. Así mismo, tras la exposición de las células Caco-2 a su homólogo propílico, el dPdS, no se manifestaron efectos citotóxicos. En este sentido, Sundaram y Milner [25], tras exponer a 100 μM de dPdS durante 24h a células de cáncer de colon HTC-15, piel SK MEL-2 y pulmón A549, no observaron disminución de la viabilidad en las diferentes líneas celulares. Así mismo, Mehri y col. [38] demostraron la ausencia de citotoxicidad en células de leucemia mieloide aguda U937 expuestas a un rango de 0-100 μM de dPdS durante 72 h.

Na y col. [27] estudiaron el efecto de un análogo estructural del dPS, el dAS, que difiere del primero por la presencia de dos dobles enlaces, sobre el biomarcador MTT en células de cáncer de mama (MCF-7) expuestas a una concentración 50 μM de dAS durante 48 h, tras las cuales no se observaron alteraciones. Estos resultados coinciden con los observados en nuestro caso para el biomarcador MTS en células Caco-2 tras la exposición a dPS. Por el contrario, Hong y col. [39] observaron efectos citotóxicos para este compuesto por reducción de MTT tras la exposición de dos líneas celulares de cáncer de pulmón (H460 y H1299) a 25 μM de dAS durante 1 h. Además, Shin y col. [29] también demostraron una disminución de la viabilidad celular en células cancerígenas de tiroides (ARO) expuestas a dAS (0-400 μM) durante 24 h. Por tanto, los dobles enlaces presentes en la estructura del dAS podrían incrementar la toxicidad de este compuesto en comparación con el dPS, si bien se ha observado que no produce daños en todas las líneas celulares, por lo que también podrían deberse a la distinta sensibilidad de cada una de ellas frente a estos compuestos.

Por otro lado, Fukao y col. [40] sugirieron que el número de átomos de azufre juega un papel crucial en las actividades biológicas, siendo el dialil trisulfuro (dAtS) el principal responsable de la protección frente a tetracloruro de carbono (CCl_4) en experimentos *in vitro*. En este sentido, se ha demostrado que la actividad de los organosulfurados está directamente relacionada con el número de átomos de azufre que presenta el compuesto [41]. Jakubíková y Sadlák [26] comprobaron que, de los derivados alilos, el compuesto con mayor citotoxicidad es el dAtS, seguido del dAdS y del dAS en las células Caco-2 y HT-29 por lo que cabría esperar que el dPS sea, de los derivados propilos, el menos citotóxico. No obstante, en nuestro estudio ambos compuestos mostraron ausencia de efectos tóxicos.

Nuestro estudio muestra la ausencia de citotoxicidad de ambos compuestos azufrados, dPS y dPdS, en células de adenocarcinoma de colon humano Caco-2. Constituye el primer paso para la idoneidad del empleo de dichos compuestos en envases destinados a la industria alimentaria.

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Bibliografía

1. Agarwal K (1996) Therapeutic actions of garlic constituents. Med Res Rev 16(1): 111-124.
2. Reuter HD (1995) *Allium sativum* and *Allium ursinum*: Part 2. Pharmacology and medicinal application. Phytomedicine 2(1): 73-91.
3. Corzo-Martínez M, Corzo N, Villamiel M (2007) Biological properties of onions and garlic. Trends Food Sci Technol 18: 609-625.
4. Rahman K, Lowe GM (2006) Garlic and cardiovascular disease: a critical review. J Nutr 136: 736S-740S.
5. Wilson E, Demming-Adams B (2007) Antioxidant, anti-inflammatory, and antimicrobial propertis of garlic and onions. NFS 37: 178-183.
6. Takahashi M, Shibamoto T (2008) Anti-inflammatory Activities of Steam Distillate from Freeze-Dried Onion (*Allium cepa* L.) Sprout. J. Agric. Food Chem. 56: 10462-10467.
7. Galeone C, Pelucchi C, Levi F, Negri E, Franceschi S, Talamini R, Giacosa A, La Vecchia C (2006) Onion and garlic use and human cáncer. Am J Clin Nutr 84: 1027-1032.
8. Shon M-Y, Choi S-D, Kahng G-G, Nam S-H, Sung N-K (2004) Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. Food Chem Toxicol 42: 659-666.
9. Romeilah R, Fayed S, Mahmound G (2010) Chemical compositions, antiviral and antioxidant activities of seven essential oils. J Appl Sci Res 6: 50-62.
10. Benklebia N (2010) Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). Lebensm Wiss u Technol 37: 263-268.
11. Ye C-H, Dai D-H, Hu W-L (2013) Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.). Food Control 30: 48-53.
12. Fenwick GR, Hanley AB (1985) The genus *Allium*. Part 2. Crit Rev Food Sci Nutr 22: 273-377.
13. Lanzotti V (2006) The analysis of onion and garlic. J Chromatograph A 1112: 3-22.
14. Seki T, Tsuji K, Hayato Y, Tadaaki M, Ariga T (2000) Garlic and onion oils inhibit proliferation and induce differentiation of HL-60 cells. Cancer Lett 160: 29-35.
15. Chen C, Pung D, Leong V, Hebbar V, Shen G, Nair S (2004) Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor NRF2: Effect of chemical structure and stress signals. Free Radicals Biol Med 37: 1578-1590.
16. Kim J, Kim J-S, Park E (2013) Cytotoxic and anti-inflammatory effects of onion peel extract on lipopolysaccharide stimulated human colon carcinoma cells. Food Chem Toxicol 62: 199-204.
17. De Martino A, Filomeni G, Aquilano K, Ciriolo MR, Rotilio G (2006) Effects of water garlic extracts on cell cycle and viability of HepG2 hepatoma cells. J Nutr Biochem 17: 742-749.
18. Reglamento CE nº 1935/2004 del Parlamento Europeo y del Consejo, de 27 de octubre de 2004, sobre los materiales y objetos destinados a entrar en contacto con alimentos y por el que se derogan las Directivas 80/590/CEE y 89/109/CEE.
19. Directiva 90/128/CEE de la Comisión, de 23 de febrero de 1990, relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimentarios.

20. Amagase H (2006) Clarifying the Real Bioactive Constituents of Garlic. J Nutr 136: 716S-725S.

21. Brodnitz MH, Pascale JV, van Derslice LJ (1971) Flavor components of garlic extract. J Agric Food Chem 19: 273-275.

22. Shukla Y, Kalra N (2007) Cancer chemoprevention with garlic and its constituents. Cancer Lett 247: 167-181.

23. Siess M-H, Le Bon A-M, Canivenc-Lavier M-C, Suschetet M (1997) Modification of hepatic drug-metabolizing enzymes in rats treated with alkyl sulfides. Cancer Lett 120: 195-201.

24. Shaath NA, Flores FB (1998) Egyptian onion oil. Develop Food Sci 40: 443–453.

25. Sundaram S, Milner J (1996) Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis 17: 669-673.

26. Jakubíková J, Sedlák J (2006) Garlic-derived organosulfides induce cytotoxicity, apoptosis, cell sycle arrest and oxidative stress in human colon carcinoma cell lines. Neoplasma 53: 191-199.

27. Na H-K, Kim E-H, Choi M-A, Park J-M, Kim D-H, Suth Y-J (2012) Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. Biochem Pharmacol 84: 1241-1250.

28. Xiao D, Choi S, Johnson D, Vogel V, Johnson C, trump D, Lee Y, Singh S (2004) Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulates kinase-mediated phosphorylation of Bcl-2. Oncogene 23: 5594-5606.

29. Shin HA, Cha YY, Park MS, Kim JM, Lim YC (2010) Diallyl sulfide induces growth inhibition and apoptosis of anaplastic thyroid cancer cells by mitochondrial signaling pathway. Oral Oncol 46: 15-18.

30. Tsai C-Y, Wang C-C, Lai T-Y, Tsu T-Y, Wang C-H, Liang H-Y, Kuo W-W (2013) Antioxidant effects of diallyl trisulfide on high glucose-induced apoptosis are mediated by the PI3K/Akt-dependent activation of Nrf2 in cardiomyocytes. Int J Cardiol 168: 1286-1297.

31. Yin M-C, Hwang S-W, Chan K-C (2002) Nonenzymatic antioxidant activity of four organosulfur compounds derived from garlic. J Agric Food Chem 50: 6143-6147.

32. Casella S, Leonardi M, Melai B, Fratini F, Pistelli L (2013) The role of diallyl sulfides and dipropyl sulfides in the *in vitro* antimicrobial activity of the essential oil of garlic, *Allium sativum* L., and leek, *Allium porrum* L. Phytother Res 27: 380-383.

33. Arranz N, Haza AI, García A, Möller L, Rafter J, Morales P (2007) Protective effects of organosulfur compounds towards N-nitrosamine-induces DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Food Chem Toxicol 45: 1662-1669.

34. García A, Morales P, Arranz N, Delgado ME, Rafter J, Haza AI (2009) Antiapoptotic effects of dietary antioxidants toward N-nitrosopiperidine and N-nitrosodibutylamine-induced apoptosis in HL-60 and HepG2 cells. J Appl Toxicol 29: 403-413.

35. Bradford, M (1976) A rapid sensitive method for quantification of icrogramquantitiea of protein utilizing the principke of protein – dye binding. Anal Biochem 72: 248-254.

36. Borenfreud, E, Puerner, JA (1984) A simple quantitative procedure using monolayer culture for cytotoxicity assays. J Tissue Cult Meth 9: 7-9.

37. Baltrop, JA, Owen TC, Cory AH (1991) 5-((3-carboxyphenyl)-3-(4,5-dimethilthiazolyl)-3-(4- sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 2- (4,5- dimethylthiazolyl)- 2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell viability indicators. Bioorg Med Chem Lett 1: 611.

38. Mehri F, Auger J, Rendu F, Bauvois B (2008) *Allium compounds*, dipropyl and dimethyl thiosulfinates as antiproliferative and differentiating agents of human acute myeloid leukemia cell lines. Biologics 2(4): 885-895.

39. Hong Y-S, Ham Y-A, Choi J-H, Kim J (2000) Effects of allyl sulfur compounds and garlic extract on the expressions of Bcl-2, Bax, and p53 in non small cell lung cancer cell lines. Exp Mol Med 32 (3): 127-134.

40. Fukao T, Hosono T, Misawa S, Seki T, Ariga T (2004) The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride. Food Chem Toxicol 42: 743-749.

41. Liu K-L, Chen H-W, Wang R-Y, Lei Y-P, Sheen L-Y, Lii C-K (2006) DATS reduces LPS-Induced iNOS expression, NO production, oxidative stress, and NF-KB activation in RAW 264.7 macrophages. J Agric Food Chem 54: 3472-3478.

CAPÍTULO 3 / CHAPTER 3

María Llana-Ruiz-Cabello, Sara Maisanaba, Daniel Gutiérrez-Praena, Ana I. Prieto, Silvia Pichardo, Ángeles Jos, F. Javier Moreno, Ana M. Cameán

CYTOTOXIC AND MUTAGENIC IN VITRO ASSESSMENT OF TWO ORGANOSULFUR COMPOUNDS DERIVED FROM ONION TO BE USED IN THE FOOD INDUSTRY.

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Cytotoxic and mutagenic *in vitro* assessment of two organosulfur compounds derived from onion to be used in the food industry

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ABSTRACT

Edible members of the *Allium* family are widely used since they exhibit antioxidant and antibacterial related to the organosulphur compounds. One the most promising use of *Allium* species, hence, onion essential oil, could be in the packaging food industry. The present work aims to assess the safety of two organosulphur compounds present in onion essential oil; dipropyl disulphide, dipropyl sulphide and their mixture. For this purpose, cytotoxicity, reactive oxygen species and glutathione contents, and ultrastructural cellular damages were studied in the human intestinal cells, Caco-2, exposed to these organosulphur compounds. Moreover, their potential mutagenicity was also assessed. The results revealed no significant adverse effects. Additionally, reactive oxygen species scavenger activity was observed for both compounds. Therefore, they could be a good natural alternative to other synthetic antioxidant and antibacterial substances used in the food industry.

1. Introduction

Onion (*Allium cepa*) and garlic (*Allium sativum*) are two food ingredients widely used in gastronomy (Corzo-Martínez, Corzo, & Villamiel, 2007). The *Allium* family components possess antibacterial and antioxidant activities, as well as other biological properties (Benkeblia, 2004). For this reason, they are also used in the treatment and prevention of many diseases (Augusti & Mathew, 1974; Lanzotti, 2006; Corzo-Martínez et al., 2007). These activities are related to the thiosulphinates (Benkeblia & Lanzotti, 2007); however, they are very unstable compounds and give rise to further rearrangements leading to a wide variety of derived sulphur compounds (Lanzotti, 2006). In the case of garlic, there are numerous lipid-soluble sulphur-containing byproducts such as diallyl disulphide (DADS), diallyl sulphide (DAS), diallyl trisulphide (DATS), allylmethyl trisulphide, and diallyl tetrasulphide, which activities have been extensively studied (Romeilah, Fayed, & Mahmoud, 2010; Tsai et al., 2013). Similarly, biological action of onion is ascribed to organosulphur compounds, highlighting dipropyl disulphide (DPDS). The content of these compounds in onion essential oil is directly related to the time of storage; the longer the storage the greater the level of this compound (Shaath & Flores, 1998). However, the active principles in garlic and onion have not been fully characterised (Amagase, Petesch, Matsuura, Kasuga, & Itakura, 2001). In addition, their complex chemistry makes it plausible that variations in processing can yield quite different content in sulphur compounds (Amagase, 2006). Therefore, it is necessary to go more in depth on the biological activities of each compound which have not been fully studied yet (Corzo-Martínez et al., 2007).

Despite the beneficial effects attributed to garlic, it is commonly known that excessive consumption can cause problems such as gastrointestinal tract injury, allergic reactions, anemia, weight loss, and toxicity to the heart, liver, and kidney as well as breaks in chromosomes (Amagase et al., 2001; Banerjee, Mukherjee, & Maulik, 2003; Wilson & Demmig-Adams, 2007). Based on such evidence, garlic is classified as a type I allergen (Japee, Bonnekoh, Hausen, & Gollnick, 1999). Differences between garlic and onion essential oils are not only related to the content in organosulphur compounds (three times higher in the case of garlic) but also in the profile of these compounds (Benkeblia, 2004; Lanzotti, 2006). In this sense, allyl sulphides (DADS, DAS and DATS), which are mainly present in garlic, are able to induce apoptosis in human intestinal, hepatic, lung, thyroid and prostate cancer cells (Sundaram & Milner, 1996; Xiao et al., 2004; De Martino, Filomeni, Aquilano, Ciriolo, & Rotilio, 2006; Shin, Cha, Park, Kim, & Lim, 2010). Hence, onion oil, as well as its components, could constitute a safer choice

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in the food industry. However, very little effort has been made to evaluate the safety of the organosulphur compounds present in onion in comparison to those of garlic. Different studies have demonstrated that DPDS and dipropyl sulphide (DPS) have no cytotoxic effects on colonic, lung, skin and prostate tumor cell lines (Sundaram & Milner, 1996; Xiao et al., 2004); although further studies are needed to ensure their safety.

The use of onion in the food industry is of great concern. One the most promising use of *Allium* species is as antioxidant and antibacterial, since they are a natural alternative to chemical substances. In this sense, essential oil from onion could be use in "active packaging" (Appendini & Hotchkiss, 2002). This technology consists of the used of active agents that are incorporated into the packaging walls from which they are released to the food surface at a controlled rate (Cerisuelo, Alonso, Aucejo, Gavara, & Hernandez-Muñoz, 2012). The incorporation of essential oil components, such as organosulphur compounds, in food-packaging films allows the controlled release of these active substances into the food, reducing the undesirable flavors caused in the case of the direct addition of these compounds into food (Ramos, Jiménez, Peltzer, & Garrigós, 2012). However, as far as we know, any component of *Allium* essential oils has been authorised for this purpose so far. For this reason, toxicological testing is needed to ensure the safety of each product, since this is a major factor in the quality control of *Allium* species preparations (Amagase, 2006).

Considering all this background, the present work aims to perform for the first time a thorough safety assessment of two organosulphur compounds present in the essential oil of onion, DPDS and DPS, in the human intestinal cell line Caco-2 to be used in the food packaging industry. The cytotoxicity, morphological alterations as well as alteration in the oxidative status and glutathione content were investigated in Caco-2 cells after short-term exposure to these compounds. Moreover, the potential mutagenic activity was assessed by the Ames test.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, foetal bovine serum (FBS) and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma–Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Model systems

Caco-2 cell line derived from human colon carcinoma (ATCC® HTB-37) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) in a medium consisting of Eagle's medium supplemented with 10% FBS, 1% non-essential amino acids, 50 μg/ml gentamicin, 1.25 μg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by the Trypan Blue exclusion test. Caco-2 cells were plated at density of 7.5×10^5 cells/ml to perform the experiments.

2.3. Test solutions

The ranges of DPDS and DPS concentrations for the tests were selected considering the content of these active compounds to be incorporated in the packaging materials and the possible migration to the food. In this sense, the maximum concentration was calculated considering that the active compounds completely migrate from the active package into the food (worst scenario of exposure). Considering that the presence of organosulphur compounds could vary from up to 20% in the onion essentials oil (Shaath & Flores, 1998; Takahashi & Shibamoto, 2008; Romeilah et al., 2010), and that a 2–10% of the onion essential oil can be incorporated in 5.5–6.5 g of packaging film needed to pack 1 kg of food, the potential concentration released to the food will be around 200 μM for these compounds. Therefore, serial test solutions of DPDS and DPS as well as the mixture 1:1 of these substances (0–200 μM) were prepared from both stock solutions of organosulphur compounds (0.2 M) in DMSO (0.1%).

2.4. Cytotoxicity assays

Culture medium with and without 0.1% DMSO was used as control group. After replacing the previous medium, the exposure solutions were added to the systems and incubated at 37 °C for 24 h and 48 h according to INVITOX cytotoxicity protocols. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR), and MTS tetrazolium salt reduction (MTS).

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage in independence of the toxic mechanism involved (Pichardo et al., 2007). PC was analysed *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 (BioRad, Madrid, Spain) in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the percentage of cells present in the culture in comparison to the control group.

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. NR uptake was performed according to Borenfreund and Puerner (1984). Briefly, NR in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. The NR can be extracted from lysosomes for quantitative measurement of viable cells and cytotoxicity of xenobiotics (Zhang, Lipsky, Trump, & Hsu, 1990).

MTS reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good biomarker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop, Owen, Cory, & Cory (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt) tetrazolium compound (Promega Biotech Ibérica, Madrid, Spain) added to the medium is bioreduced by cells into a coloured formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 h of incubation at 37 °C in the dark.

2.5. Oxidative stress study

After replacing the previous medium, exposure solutions were added to cells and incubated at 37 °C for 24 h and 48 h. Culture medium with and without 0.1% DMSO was used as control group. The oxidative stress endpoints measured were reactive oxidative species (ROS) content and glutathione (GSH) levels.

The production of ROS was assessed in 96-well microplates using the dichlorofluorescein (DCF) assay. The probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Invitrogen) readily diffuses through the cell membrane and is hydrolysed by intracellular esterases to non-fluorescent compound (DCFH), which is rapidly oxidised in the presence of ROS to the highly fluorescent DCF. Specifically, cells were incubated with 200 μl 20 μM DCFH-DA in culture medium at 37 °C for 30 min, and then washed with phosphate buffered saline (PBS) and resuspended in 200 μl of PBS. The formation of the fluorescent oxidised derivative of DCF-DA was monitored at emission wavelength of 535 nm and

excitation wavelength of 485 nm. ROS production was expressed as fluorescent arbitrary units (Puerto et al., 2010).

GSH content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos, Camean, Pflugmacher, & Segner, 2009). This molecule forms a thioether adduct with GSH in a reaction catalysed by the enzyme GST. After the cell exposure to the organosulphur compounds, medium was discarded and cells were incubated at 37 °C for 20 min in the presence of 40 μM mBCl. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the excitation/emission wavelengths of 380/460. Results were expressed as arbitrary units.

2.6. Morphology

Cells were exposed to the highest concentration assayed (200 μM) of DPSP and DPS as well as the mixture 1:1 of these substances. Afterwards, cultured cells were fixed directly in the cell culture dish in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4 °C. They were all postfixed in 1% osmium tetroxide for 60 min at 4 °C. Subsequently, cells were scraped and pelleted by centrifugation. Samples were washed in 0.1 M cacodylate buffer, pH 7.3. Then, samples were dehydrated in ethanol at progressively higher concentrations and embedded in Epon (epoxy embedding medium). Toluidine blue-stained semi-thin sections (0.5 μm thick) used as controls were viewed in a Leitz (Aristoplan) light microscope. The presence of lipids was confirmed by Sudan III cytochemical technique.

Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope (FEI, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV. The cell growth and development of morphology damage was checked using a Leica DMIL inverted microscope by phase contrast.

2.7. Ames test

The incorporation version of the Ames test was performed according to the recommendations of Maron and Ames (1983) and following the principles of OCDE guideline 471 (1997). Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used for the assay. Cultures of each tester strain were prepared from their main strain plates, and use in late exponential growth phase. Each test substance was assessed in three independent experiments, each conducted in absence and presence of S9 metabolic activation system from rats livers (Aroclor 1254, Sigma, Spain), using triplicate plates for each test substance concentration. DPDS, DPS and their mixture were tested in six different concentrations (0.1, 1, 10, 50, 100 and 200 μM). Briefly, 0.1 ml of test substance, 0.1 ml of bacterial culture and 0.5 ml of S9 mix, were added to 2 ml molten agar at 42° and poured onto Vogel Bonner-E minimal glucose agar plates. Plates were inverted and incubated 65–70 h at 37 °C in the dark. Revertant colonies were counted and background lawn was inspected for signs of toxicity or compound precipitation. 2-Nitrofluorene (2-NF) (0.1 μg/plate) and sodium azide (NaN_3) (1 μg/

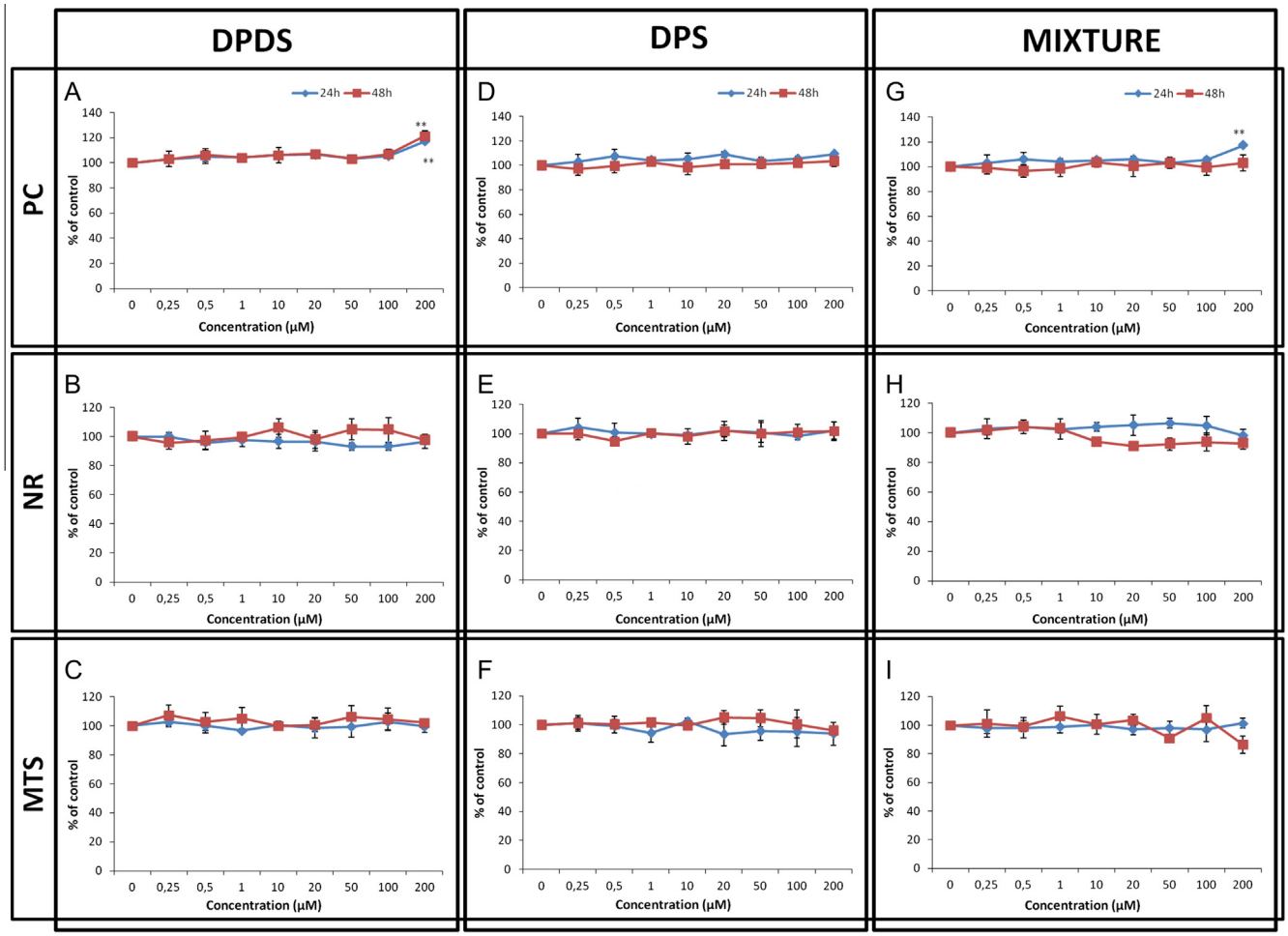

Fig. 1. Protein content (TP), neutral red uptake (NR) and reduction of tetrazolium salt (MTS) of Caco-2 cells exposed for 24 h and 48 h to 0–200 μM of DPDS (A–C); DPS (D–F) and a mixture 1:1 of DPDS/DPS (G–I). All values are expressed as mean ± SD ** very significantly different from control ($P < 0.01$).

plate) were selected as positive controls for assays performed without metabolic activation system. 2-Aminofluorene (2-AF) was the positive control used in assays employing S9 mix. MilliQ water (100 µl/plate) and DMSO (10 µl/plate) were selected as negative controls for this assay.

2.8. Calculations and statistical analysis

All experiments were performed three times and in duplicate per concentration. Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $P < 0.05$. All values passed the normality tests (Kolmogorov & Smirnov's test, and Bartlett's test).

3. Results

3.1. Cytotoxicity assays

No differences were observed in both control groups (culture medium with and without 0.1% DMSO).

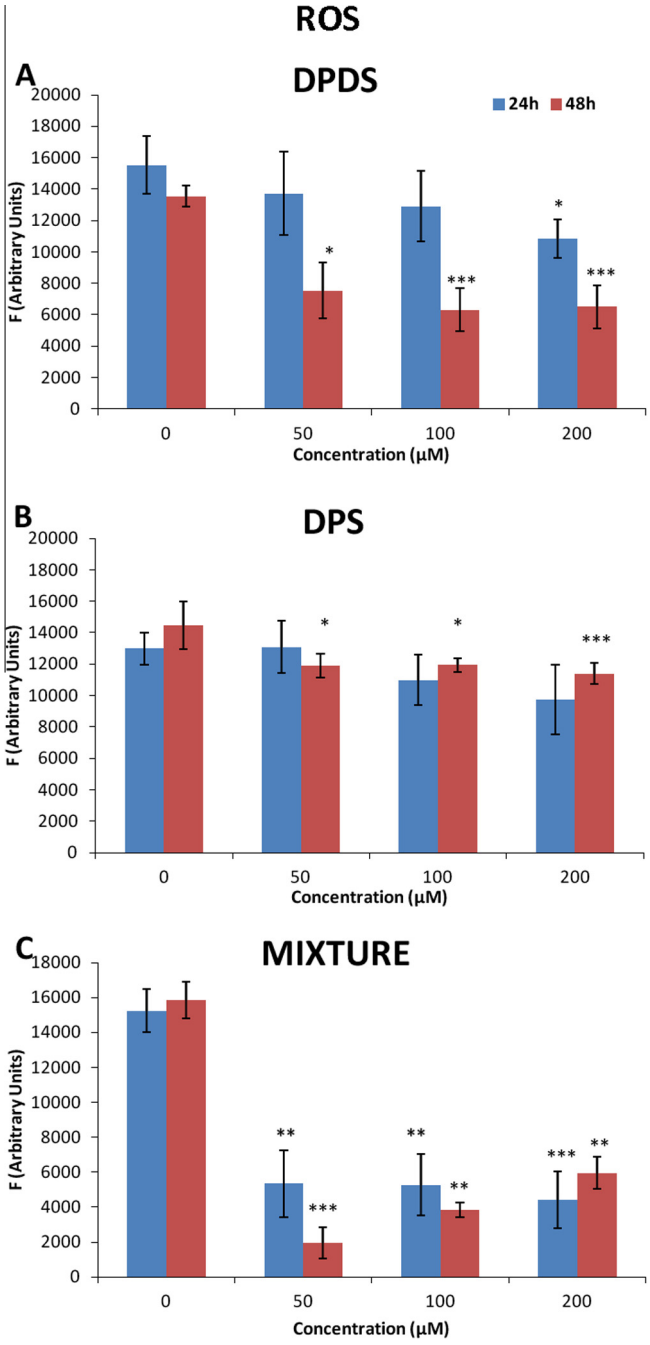

Fig. 2. ROS content of 200 µM DPDS (A); 200 µM DPS (B); and a mixture 1:1 200 µM DPDS/DPS (C) in Caco-2 cells after 24 h and 48 h of exposure. All values are expressed as mean ± SD * significantly different from control ($P < 0.05$); ** very significantly different from control ($P < 0.01$) and *** extremely significantly different from control ($P < 0.01$).

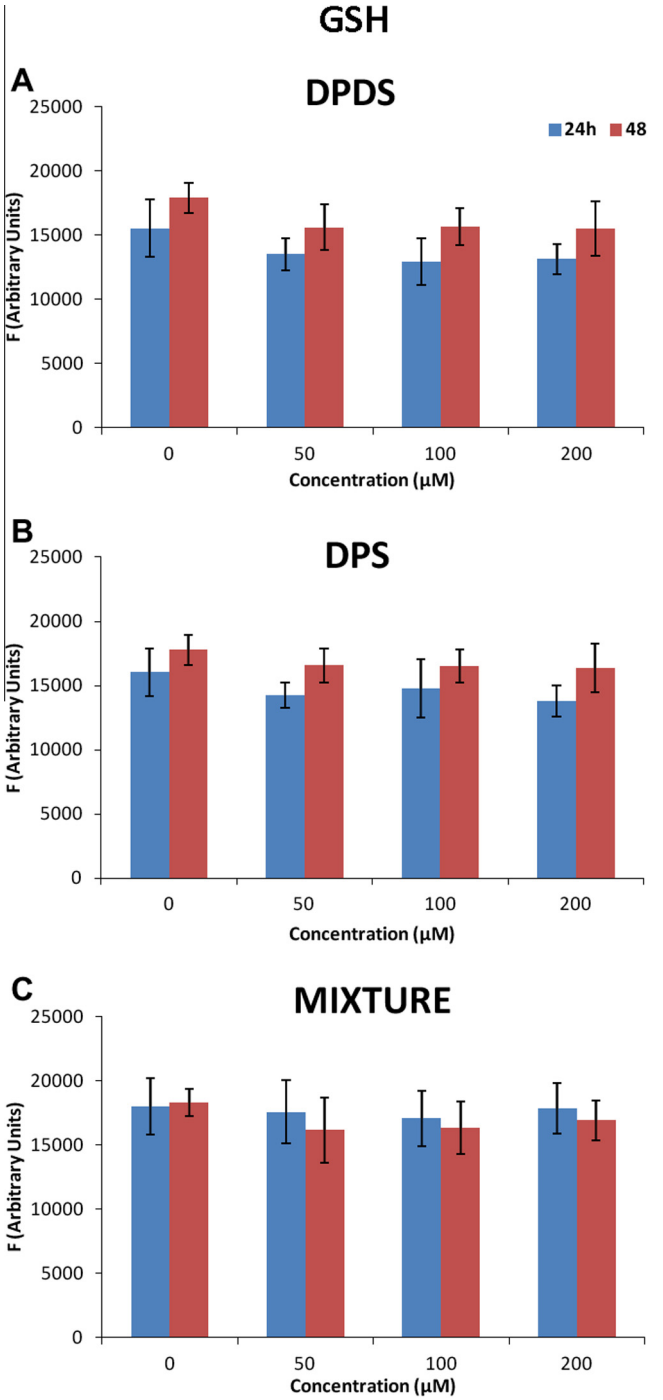

Fig. 3. GSH content of 200 µM DPDS (A); 200 µM DPS (B); and a mixture 1:1 200 µM DPDS/DPS (C) in Caco-2 cells after 24 h and 48 h of exposure. All values are expressed as mean ± SD

When Caco-2 cells were exposed to DPDS no significant change was recorded with respect to the control group after 24 h and 48 h at NR uptake and MTS metabolization assays, although PC assay showed a significant increase at the highest concentration used (200 μM) at both times of exposure (Fig. 1A–C). Similarly, Caco-2 cells exposed to DPS remained unaltered with respect to the control cells after 24 h and 48 h for all the endpoints assayed (Fig. 1D–F). In addition, no significant change was observed in cells exposed to the mixture 1:1 of DPDS/DPS in any of the biomarkers employed except for the PC assay, showing a significant increase at 200 μM after 24 h of exposure (Fig. 1G–I).

3.2. Oxidative stress study

3.2.1. ROS

Caco-2 cells exposed to DPDS underwent significant reductions in ROS content only at 200 μM after 24 h of exposure. However, ROS content decreased significantly in all the concentrations assayed after 48 h (Fig. 2A). Similarly, no change in ROS content was recorded when cells were treated with DPS after 24 h, but significant reduction were observed after a longer exposure of time, 48 h (Fig. 2B). Higher reductions were shown when Caco-2 cells were exposed to the mixture DPDS/DPS, being significant in all the treatments and exposure times (Fig. 2C).

3.2.2. GSH

No significant changes were observed in the GSH content of Caco-2 cells in any of the treatment with the organosulphur compounds after 24 h and 48 h of exposure (Fig. 3).

3.3. Morphology study

3.3.1. Electron microscope observation of control cells

Unexposed Caco-2 cells observed under electron microscope showed large euchromatic nuclei with irregular border. Moreover, cells showed compact nucleoli where large fibrillar centres, dense fibrillar component and abundant granular component are clearly observed (Fig. 4A). In the perinuclear region of the cytoplasm, mitochondria with dense matrix, lipid drops and dictyosomes with associated vesicular system showing light content are shown. The cytoskeleton is shown as intermediate filaments displayed in the cellular periphery (Fig. 4B).

3.3.2. Electron microscope observation of cells exposed to DPDS, DPS and their mixture

When Caco-2 cells were exposed to the organosulphur compounds, similar morphological features were observed after 24 h or 48 h of exposure. Therefore, the results described in the present work are related to 48 h of exposure.

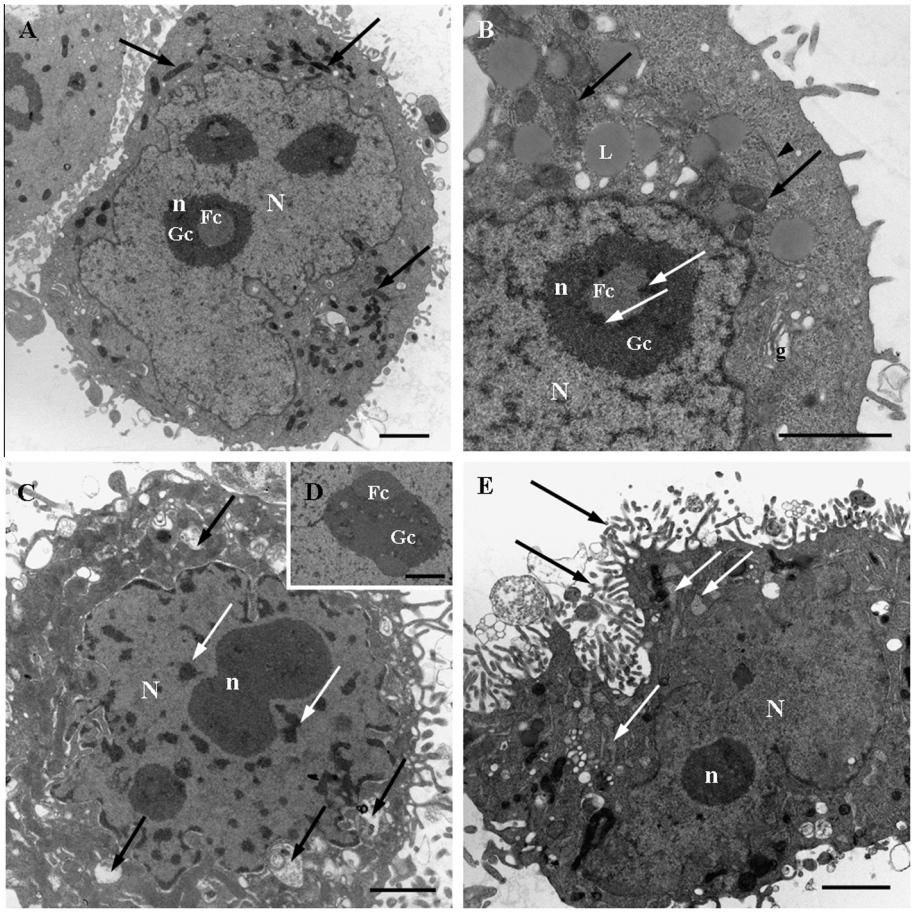

Fig. 4. Morphology of Caco-2 cells after 48 h of treatment observed by electron microscopy. Bars = 2 μm. Unexposed control cultures (A,B), and Caco-2 cells exposed to 200 μM DPDS (C,D) and 200 μM DPS (E). (A) Unexposed cells with high density in the nucleus (N) and nucleolus (n) with fibrillar centres (Fc) and granular component (Gc); mitochondria (arrow) are also visible. (B) Control cells showing nucleus (N) and nucleolus (n) with fibrillar centres (Fc), dense fibrillar component (white arrow) and granular component (Gc); mitochondria (arrow); lipid drops (L); Golgi apparatus (g); intermediate filaments (arrow heads). (C) Autophagic vacuoles (black arrow) and chromatin condensation (white arrows) are observed. Nucleus (N) and nucleus (n) are also observed. (D) Detail of granular component (Gc) and fibrillar component (Fc). (E) Apical cytoplasm membrane with microvilli (black arrow) and dilatation in the cisternae from the ER (white arrow). Nucleus (N) and nucleus (n) are also observed.

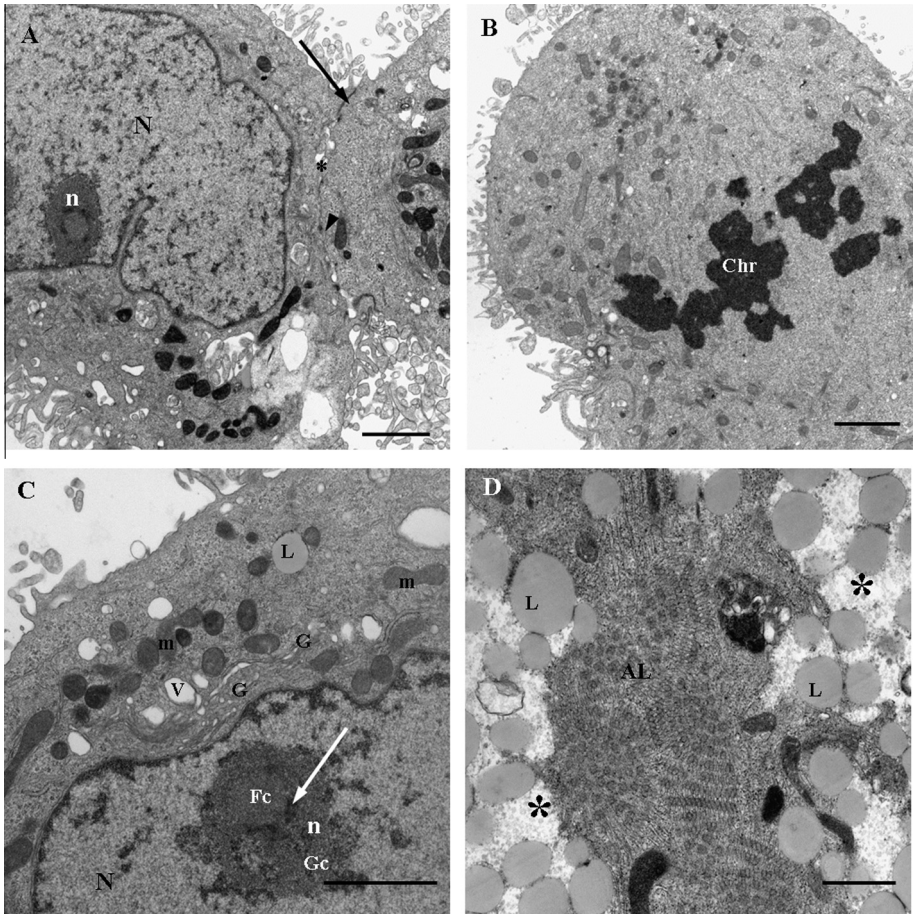

Fig. 5. Morphology of Caco-2 cells after 48 h of treatment observed by electron microscopy. Bars (A–C) = 2 μm. Bar (D) = 1 μm. Caco-2 cells exposed to 200 μM DPS (A,B) and 200 μM of a mixture 1:1 DPDS:DPS (C,D). (A) Cells exposed to DPS showed intercellular links (black arrow) and desmosomes (arrow head); as well as large intercellular spaces (asterisk). Nucleus (N) and nucleus (n) are also observed. (B) Cells under mitosis process, showing chromatin (Chr). (C) Euchromatic nucleus (N) with compact nucleoli (n) is observed. Dense fibrillar component can be distinguished (white arrow); well-developed Golgi apparatus (G) with Golgi vesicles (v), as well as dense mitochondria (m) are shown. (D) Annulate lamellae (AL) are observed in the cytoplasm associated with glycogen accumulations (asterisk) and lipid drops (L).

Cells exposed to 200 μM DPDS maintained certain proliferative activity, although they underwent characteristic morphological features of activation and/or apoptotic process induction. Moreover, cells showing vacuoles in the cytoplasm which could have an autophagic origin are frequently observed (Fig. 4C). The nucleus exhibited an irregular border with light chromatin condensation and nucleoli in segregation process (Fig. 4C and D).

When cells were exposed to 200 μM DPS, some ultrastructural features related to the differentiation process were observed. Apical cytoplasm membrane showed microvilli, evidencing the polarity of the cell. Moreover, in the cytoplasm dilatation in the cisternae from the endoplasmic reticulum (ER) was observed (Fig. 4E). Cellular interactions could be possible due to the presence of narrow links, adherent links and desmosomes. However, large intercellular spaces are observed between neighbouring cells (Fig. 5A). Therefore, culture cells exposed to DPS were induced to cellular differentiation, although certain proliferative activity remained unaltered as showed by the presence of cells undergoing mitosis (Fig. 5B).

The morphological study of cells exposed to the mixture of DPDS/DPS reveal similar results to those obtained in the control group. Nevertheless, the organelles involved in biosynthetic pathways are more developed. Cells showed euchromatic nuclei with irregular border. Compact nucleoli are also observed showing fibrillar centres, dense fibrillar component and granular component, which are morphological features of an intense transcriptional activity. In the cytoplasm, Golgi apparatus are present in dictyosomes, which showed many developed cisternae surrounded by light-content vesicles. Moreover, in the perinuclear regions, mitochondria with dense matrix are observed (Fig. 5C). Moreover, annulate lamellae are also observed in the cytoplasm associated with cisternae from ER, glycogen accumulations and lipid drops. Annulate lamellae are shown as stacks of flattened membrane cisternae perforated by numerous spaced pores similar to those observed in the nuclear membrane, which may indicate a high metabolic activity, as well as growing and cell proliferation (Fig. 5D).

3.4. Ames study

After exposure to DPDS, DPS and their mixture 1:1, there was no increase in the number of revertants and no indication of mutagenic activity in any of the treated strains up to the highest concentration (200 μM) of each compound and the mixture, either in the presence or absence of S9 (Table 1). Toxicity, measured as a decrease in background lawn and in revertant frequency, was not observed at any concentration tested. Positive controls produced statistically significant increases ($P < 0.01$) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix. The results showed that both organosulphur

Table 1
Results of Ames test conducted with DPDS, DPS and the mixture 1:1 for three independent experiments. MilliQ water was used as negative control and DMSO as solvent for positive controls. Data are given as mean ± SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/TA98/TA102/TA104 without S9 mix: 2-NF (0.1 μg/plate) and TA100 without S9 mix: NaN_3 (1 μg/plate). Positive controls for all strains with S9: 2-AF.

	Concentration (μM)	TA97A		TA98		TA100		TA102		TA104	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
DPDS	Negative controls	217 ± 7	244 ± 37	20 ± 4	23 ± 1	103 ± 3	144 ± 27	218 ± 19	208 ± 31	378 ± 39	368 ± 34
	0.1	301 ± 20	331 ± 8	15 ± 4	24 ± 4	95 ± 8	109 ± 15	260 ± 43	158 ± 39	321 ± 52	405 ± 37
	1	271 ± 60	352 ± 27	23 ± 2	23 ± 9	127 ± 4	115 ± 6	293 ± 47	316 ± 5	365 ± 22	305 ± 6
	10	326 ± 40	338 ± 14	18 ± 2	25 ± 3	85 ± 3	99 ± 6	253 ± 52	265 ± 17	340 ± 52	522 ± 56
	50	295 ± 6	273 ± 3	16 ± 3	28 ± 3	116 ± 4	106 ± 7	231 ± 13	189 ± 8	227 ± 8	216 ± 7
	100	330 ± 14	322 ± 4	17 ± 4	23 ± 5	95 ± 13	116 ± 7	349 ± 6	341 ± 3	424 ± 18	429 ± 28
	200	297 ± 13	256 ± 7	19 ± 4	24 ± 3	100 ± 19	81 ± 1	293 ± 28	253 ± 25	339 ± 34	278 ± 52
	Positive controls	715 ± 17**	714 ± 25**	1153 ± 45**	1083 ± 78**	1237 ± 56**	1079 ± 78**	771 ± 29**	885 ± 118**	859 ± 37**	980 ± 109**
DPS	Negative controls	342 ± 25	355 ± 40	18 ± 6	23 ± 1	144 ± 9	143 ± 12	233 ± 35	211 ± 10	258 ± 21	223 ± 24
	0.1	312 ± 34	326 ± 9	22 ± 5	23 ± 7	140 ± 2	160 ± 41	149 ± 10	153 ± 6	231 ± 21	191 ± 26
	1	327 ± 14	339 ± 10	25 ± 3	24 ± 3	145 ± 17	115 ± 14	176 ± 2	134 ± 7	213 ± 19	171 ± 32
	10	384 ± 30	349 ± 18	29 ± 2	29 ± 2	110 ± 12	129 ± 11	226 ± 15	183 ± 11	244 ± 20	178 ± 20
	50	396 ± 67	319 ± 28	25 ± 4	25 ± 5	138 ± 4	123 ± 6	203 ± 9	172 ± 5	205 ± 22	163 ± 32
	100	424 ± 28	500 ± 50	23 ± 9	23 ± 9	130 ± 9	122 ± 16	183 ± 17	185 ± 14	232 ± 19	178 ± 31
	200	360 ± 35	353 ± 6	18 ± 3	26 ± 5	148 ± 15	172 ± 8	177 ± 18	194 ± 8	235 ± 22	222 ± 13
	Positive controls	750 ± 18**	718 ± 20**	1064 ± 41**	1185 ± 110**	1034 ± 60**	1193 ± 190**	514 ± 14**	518 ± 6**	904 ± 105**	1138 ± 226**
DPDS:DPS (1:1)	Negative controls	292 ± 28	332 ± 66	23 ± 1	25 ± 3	111 ± 25	100 ± 1	331 ± 25	297 ± 29	334 ± 26	329 ± 29
	0.1	393 ± 33	441 ± 22	21 ± 10	22 ± 9	124 ± 10	155 ± 23	360 ± 12	341 ± 12	386 ± 49	479 ± 26
	1	271 ± 37	308 ± 14	23 ± 3	25 ± 3	102 ± 11	98 ± 2	273 ± 62	350 ± 44	425 ± 33	548 ± 84
	10	279 ± 25	237 ± 11	35 ± 8	30 ± 2	106 ± 7	106 ± 5	293 ± 73	335 ± 17	424 ± 14	465 ± 19
	50	340 ± 31	343 ± 12	18 ± 1	19 ± 1	130 ± 19	142 ± 11	370 ± 20	404 ± 26	435 ± 43	528 ± 26
	100	319 ± 18	320 ± 15	21 ± 5	27 ± 3	128 ± 10	107 ± 7	302 ± 27	262 ± 29	341 ± 54	437 ± 49
	200	362 ± 35	298 ± 19	27 ± 3	32 ± 6	143 ± 19	127 ± 3	254 ± 62	250 ± 19	284 ± 38	377 ± 32
	Positive controls	756 ± 10**	672 ± 67**	1317 ± 96**	1624 ± 200**	1065 ± 31**	1159 ± 217**	768 ± 29**	694 ± 44**	925 ± 14**	1113 ± 206**

** $P < 0.01$ significantly different from control.

compounds as well as the mixture were not mutagenic in this bacterial assay.

4. Discussion

Although onions have been reported to possess antioxidant and antibacterial properties in less extend than garlic (Benkeblia, 2004; Wilson & Demmig-Adams, 2007), studies focused on these properties are of current interest due to their potential application in food systems as an alternative to traditional food preservatives (Ye, Dai, & Hu, 2013). In fact, the main organosulphur compound of onion oil, DPDS, has been proved to be a more efficient ROS scavenger in HL-60 and HepG2 cells in comparison to its allylic analogous, DADS, present in the garlic essential oil (García et al., 2009). However, to the best of our knowledge, no previous research group has examined in depth the toxic effects of the organosulphur compounds of onion, DPDS and DPS. In this regard, the present work aims to assess the toxicity induced by these compounds to ensure their safety to be potentially used in the food industry.

The results obtained in the cytotoxicity assays revealed that no significant damage was produced by the organosulphur compounds, except for a significant increase in the PC assay at the highest concentration used, 200 μM, of DPDS and the mixture DPDS/DPS. However, this enhancement was not accompanied by any alteration in the other endpoints assayed. Similarly, 100 μM DPDS did not cause growth depression after 24 h of exposure in any of the human tumor cell lines, HCT-15 (colon), A549 (lung) and SK MEL-2 (skin); (Sundaram & Milner, 1996). However, its allylic counterpart, DADS, reduced significantly the cell viability in all the cell lines at the same exposure conditions. Moreover, the viability of the human prostate cell line PC-3 was not affected in exposure up to 160 μM of DPDS and DPS (Xiao et al., 2004), whereas DADS and DATS did. In addition, no effect in the growth of the human acute myeloid leukemia cell line U937 exposed during 3 days to 100 μM DPDS and dimethyl disulphide were reported; although their related thiosulphinates, in the same range of concentration and exposure time, exhibited antiproliferative activity (Merhi, Auger, Rendu, & Bauvois, 2008). Collectively, these results indicated that the presence of allyl and sulphoxide groups could affect the activity of the organosulphur compounds against cell proliferation (Xiao et al., 2004; Merhi et al., 2008). Therefore, the cytotoxic effects observed on HT-29 human colon carcinoma cells exposed to 250 μg/ml of onion peel extract (Kim, Kim, & Park, 2013), as well as the inhibition of cell growth in HL-60 cells exposed to 20 μg/ml of garlic and onion oil (Seki, Tsuji, Hayato, Moritomo, & Ariga, 2000) may be related to other compounds different of DPDS and DPS.

Onion oil exhibits a moderate antioxidant activity in comparison to other synthetic antioxidants such as BHT (Ye et al., 2013). This property has been attributed to its flavonoid compounds, mainly quercetin (Corzo-Martínez et al., 2007). However, further studies demonstrated that the volatile sample from dried onion sprouts showed the most potent antioxidant activity in comparison to water and methanol samples (Takahashi & Shibamoto, 2008). The main constituents of the latter volatile sample were the organosulphur compounds, as instance, DPDS. In addition, ROS production induced by N-nitrosamines was reduced by DPDS; this compound being a more efficient scavenger of ROS at the lowest concentration (1 μM) in both HL-60 and HepG2 cells, in comparison to its allylic analogous (DADS) (García et al., 2009). This finding correlates well with the results obtained in the present work, since we have demonstrated the scavenging power against ROS of two pure organosulphur compounds DPDS, DPS and their mixture. However, GSH content remained unaltered; therefore, the scavenging activity seems to be not related to GSH activity. In this sense, Ye et al. (2013) were also not able to elucidate the mechanism of the antioxidant activity of the essential oil from onion by analysing the capacity of the oil to scavenge free radicals (ABTS and DPPH assays), metal cheating and reducing power. Moreover, it is interesting to point out that the reduction in ROS content observed in the present work when Caco-2 cells were

exposed to the mixture DPDS/DPS was higher than those obtained after the treatment with each organosulphur compound separately. Similarly, compounds in garlic work synergistically to provide health benefits (Amagase, 2006).

The most remarkable morphological changes observed in Caco-2 cells exposed to DPDS were an increase in the number of autophagic vacuoles, light chromatin condensation and nucleoli in segregation process, which could be signs of cell degradation. However, no significant damage was observed in comparison to the control group. In addition, a slight increase in apoptosis death was also observed. Similarly, another human colon tumour cells, HTS-15, treated with 100 and 500 μM DPDS did not result in any significant apoptosis (Sundaram & Milner, 1996). However, electron microscopy revealed in the latter work that morphological changes characteristic of apoptosis occurred in cells treated with 100 μM DADS. Similarly, allyl sulphur compounds induced apoptosis in PC-3 cells, with DATS showing the highest effects in comparison to DAS or DADS (Xiao et al., 2004). Therefore, the presence of the allyl group and the length of the oligosulphide chain influence the induction of apoptosis. In addition, our results showed that DPS is able to enhance cell differentiation. Also, Merhi et al. (2008) found out the ability of two organosulphur compounds (diallyl thiosulphinate and dipropyl thiosulphinate) to inhibit the proliferation of the monoblastic cell line U937, assessed by phenotypic and morphological features. Furthermore, onion and garlic oils induced differentiation in another leukemia cell line, HL-60 cells (Seki et al., 2000). Additionally, in the present work, when Caco-2 cells were exposed to the mixture 1:1 DPDS/DPS, cell metabolism was increased, highlighting the beneficial effects that these compounds exhibit.

Finally, in order to complete the assessment of the safety of DPDS and DPS to be used in food packaging, the Ames test was performed, which is required in the Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorisation (European Commission, 2001). In the present study, any of the organosulphur compounds have shown mutagenic potential with and without metabolic activation. This finding correlates well with a previous work that reported the antimutagenic activity of onion (Shon, Choi, Kahng, Nam, & Sung, 2004). Additionally, DPDS and DPS can inhibit both early and late stages of carcinogenesis (Guyonnet, Siess, Le Bon, & Suschetet, 1999). However, the antimutagenic effect of the latter compounds has resulted to be lower than the allylic analogous (DADS and DAS) (Guyonnet, Belloir, Suschetet, Siess, & Le Bon, 2001).

5. Conclusions

The results obtained in the present work revealed no significant cytotoxicity in Caco-2 cells exposed up to 200 μM of DPDS and DPS. Moreover, no outstanding morphological change or mutagenic effects were recorded. Additionally, the ROS scavenger activity was observed for both organosulphur compounds, with a synergic activity when the colonic cells were exposed to their mixture. In conclusion, the present work provides first evidences of the safety of the organosulphur compounds DPDS and DPS, considering that no remarkable toxic effects have been observed. Therefore, these organosulphur compounds could be a good natural alternative to other synthetic antioxidant and antibacterial substances used in the food industry.

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References

Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S., & Itakura, Y. (2001). Intake of garlic and its bioactive components. *The Journal of Nutrition, 131*, 955S–962S.

Amagase, H. (2006). Clarifying the real bioactive constituents of garlic. *The Journal of Nutrition, 136*(3), 716S–725S.

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies, 3*, 113–126.

Augusti, K. T., & Mathew, P. T. (1974). Lipid lowering effect of allicin (diallyl disulfide oxide) on long-term feeding in normal rats. *Experientia, 30*, 468–470.

Baltrop, J. A., Owen, T. C., Cory, A. H., & Cory, J. G. (1991). 5-((3-Carboxyphenyl)-3-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)) tetrazolium, inner salt (MTS) and related analogs of 2-(4,5-dimethylthiazolyl)-2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell-viability indicators. *Bioorganic and Medicinal Chemistry Letters, 1*, 611.

Banerjee, S. K., Mukherjee, P. K., & Maulik, S. K. (2003). Garlic as an antioxidant: the good, the bad and the ugly. *Phytotherapy Research, 17*, 97–106.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *Lebensmittel-Wissenschaft und Technologie-Food Science and Technology, 37*(2), 263–268.

Benkeblia, N., & Lanzotti, V. (2007). Allium thiosulfinates: chemistry, biological properties and their potential utilization in food preservation. *Food, 1*, 193–201.

Borenfreund, E., & Puerner, J. A. (1984). A simple quantitative procedure using monolayer culture for cytotoxicity assays. *Journal of Tissue Culture Methods, 9*, 7–9.

Bradford, M. (1976). A rapid sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cerisuelo, J. P., Alonso, J., Aucejo, S., Gavara, R., & Hernandez-Muñoz, P. (2012). Modifications induced by the addition of a nanoclay in the functional and active properties of an EVOH film containing carvacrol for food packaging. *Journal of Membrane Science, 423–424*, 247–256.

Corzo-Martínez, M., Corzo, N., & Villamiel, M. (2007). Biological properties of onions and garlic. *Trends in Food Science and Technology, 18*, 609–625.

De Martino, A., Filomeni, G., Aquilano, K., Ciriolo, M. R., & Rotilio, G. (2006). Effects of water garlic extracts on cell cycle and viability of HepG2 hepatoma cells. *Journal of Nutritional Biochemistry, 17*, 742–749.

Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorisation (2001). Scientific Committee on Food, European Commission, Health & Consumer Protection Directorate-General.

García, A., Morales, P., Arranz, N., Delgado, M. E., Rafter, J., & Haza, A. I. (2009). Antiapoptotic effects of dietary antioxidants towards N-nitrosopiperidine and N-nitrosodibutylamine- induced apoptosis in HL-60 and HepG2 cells. *Journal of Applied Toxicology, 29*, 403–413.

Guyonnet, D., Siess, M. H., Le Bon, A. M., & Suschetet, M. (1999). Modulation of phase II enzymes by organosulfur compounds from Allium vegetables in rat tissues. *Toxicology and Applied Pharmacology, 154*, 50–58.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M. H., & Le Bon, A. M. (2001). Antimutagenic activity of organosulfur compounds from Allium is associated with phase II enzyme induction. *Mutation Research, 495*, 135–145.

Jappe, U., Bonnekoh, B., Hausen, B. M., & Gollnick, H. (1999). Garlic-related dermatoses: case report and review of the literature. *American Journal of Contact Dermatitis, 10*, 37–39.

Jos, A., Camean, A. M., Pflugmacher, S., & Segner, H. (2009). The antioxidant glutathione in the fish cell lines EPC and BCF-2: response to model pro-oxidants as measured by three different fluorescent dyes. *Toxicology in Vitro, 23*, 546–553.

Kim, J., Kim, J. S., & Park, E. (2013). Cytotoxic and anti-inflammatory effects of onion peel extract on lipopolysaccharide stimulated human colon carcinoma cells. *Food and Chemical Toxicology, 62*, 199–204.

Lanzotti, V. (2006). The analysis of onion and garlic. *Journal of Chromatography A, 1112*, 3–22.

Maron, D. M., & Ames, B. N. (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutation Research, 113*, 173–215.

Merhi, F., Auger, J., Rendu, F., & Bauvois, B. (2008). Allium compounds, dipropyl and dimethyl thiosulfinates as antiproliferative and differentiating agents of human acute myeloid leukemia cell lines. *Biologics, 2*(4), 885–895.

OCDE (1997). Guidline for Testing of Chemicals 471: Bacterial Reverse Mutation Test. pp. 1–11.

Pichardo, S., Jos, A., Zurita, J. L., Salguero, M., Cameán, A. M., & Repetto, G. (2007). Acute and subacute toxic effects produced by microcystin-YR on the fish cell lines RTG-2 and PLHC-1. *Toxicology in Vitro, 21*, 1460–1467.

Puerto, M., Pichardo, S., Jos, A., Prieto, A. I., Sevilla, E., Frias, J. E., et al. (2010). Differential oxidative stress responses to pure Microcystin-LR and Microcystin-containing and non-containing cyanobacterial crude extracts on Caco-2 cells. *Toxicon, 55*, 514–522.

Ramos, M., Jiménez, A., Peltzer, M., & Garrigós, M. C. (2012). Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *Journal of Food Engineering, 109*, 513–519.

Romeilah, R. M., Fayed, S. A., & Mahmoud, G. I. (2010). Chemical compositions, antiviral and antioxidant activities of seven essential oils. *Journal of Applied Sciences Research, 6*(1), 50–62.

Shaath, N. A., & Flores, F. B. (1998). Egyptian onion oil. *Developments in Food Science, 40*, 443–453.

Seki, T., Tsuji, K., Hayato, Y., Moritomo, T., & Ariga, T. (2000). Garlic and onion oils inhibit proliferation and induce differentiation of HL-60 cells. *Cancer Letters, 160*, 29–35.

Shin, H. A., Cha, Y. Y., Park, M. S., Kim, J. M., & Lim, Y. C. (2010). Diallyl sulfide induces growth inhibition and apoptosis of anaplastic thyroid cancer cells by mitochondrial signaling pathway. *Oral Oncology, 46*, e15–e18.

Shon, M. Y., Choi, S. D., Kahng, G. G., Nam, S. H., & Sung, N. K. (2004). Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food and Chemical Toxicology, 42*, 659–666.

Sundaram, S. G., & Milner, J. A. (1996). Diallyl disulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice. *The Journal of Nutrition, 126*, 1355–1361.

Takahashi, M., & Shibamoto, T. (2008). Chemical compositions and antioxidant/anti-inflammatory activities of steam distillate from freeze-dried onion (*Allium cepa L.*) sprout. *Journal of Agricultural and Food Chemistry, 56*, 10462–10467.

Tsai, C. Y., Wang, C. C., Lai, T. Y., Tsu, T. Y., Wang, C. H., Liang, H. Y., et al. (2013). Antioxidant effects of diallyl trisulfide on high glucose-induced apoptosis are mediated by the PI3K/Akt-dependent activation of Nrf2 in cardiomyocytes. *International Journal of Cardiology, 168*, 1286–1297.

Wilson, E. A., & Demmig-Adams, B. (2007). Antioxidant, anti-inflammatory, and antimicrobial properties of garlic and onions. *Nutrition and Food Science, 37*(3), 178–183.

Xiao, D., Choi, S., Johnson, D. E., Vogel, V. G., Johnson, C. S., Trump, D. L., et al. (2004). Diallyl trisulfide induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. *Oncogene, 23*, 5594–5606.

Ye, C. H., Dai, D. H., & Hu, W. L. (2013). Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa L.*). *Food Control, 30*, 48–53.

Zhang, S. Z., Lipsky, M. M., Trump, B. F., & Hsu, I. C. (1990). Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell Biology and Toxicology, 6*, 219–234.

CAPÍTULO 4 / CHAPTER 4

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ACUTE TOXICOLOGICAL STUDIES OF THE MAIN ORGANOSULFUR COMPOUND DERIVED FROM ALLIUM SPP. INTENDED TO BE USED IN ACTIVE FOOD PACKAGING.

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Acute toxicological studies of the main organosulfur compound derived from *Allium* sp. intended to be used in active food packaging

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ABSTRACT

Some plant extracts have been proposed as potential alternative to the use of synthetic preservatives in the food industry. Among those, extracts from *Allium* species exhibit interesting antimicrobial and antioxidant properties for the food packaging industry. The present work aims to assess the usefulness and potential safety of the major organosulfur compound present in a commercial *Allium* sp. extract (PROALLIUM AP®), namely propyl thiosulfinate oxide (PTSO). For this purpose, its antimicrobial activity was studied in a wide range of microorganisms. Moreover, cytotoxicity and ultrastructural cellular damages caused by PTSO were studied in two human cell lines, Caco-2 and HepG2, being the colonic cells more sensitive to this compound. Finally, the protective role of PTSO against an induced oxidative situation was evaluated in the human intestinal Caco-2 cells. The results revealed damage at high concentration, although no significant adverse effects were recorded for the concentration to be used in food packaging. Moreover, the *in vivo* study also revealed the potential safety use at the established concentrations. In addition, the antimicrobial properties and the antioxidant role of PTSO were confirmed. Therefore, this compound could be considered as a good natural alternative to synthetic preservatives used in the food packaging industry.

1. Introduction

Garlic (*Allium sativum*) and onion (*Allium cepa*) have been traditionally used for its antibacterial, antifungal and antioxidant properties (Corzo-Martínez et al., 2007; Wilson and Demmig-Adams, 2007). The widespread use of these plants as flavouring agents in food is well known (Romeilah et al., 2010); however, new applications in the food industry are arising due to their beneficial properties. Evidence from investigations suggests that the biological and medical functions of garlic and onion are related to their high organosulfur compound content (Corzo-Martínez et al., 2007). Nevertheless, depending on the different conditions, the content of these compounds can substantially vary (Benkeblia and Lanzotti, 2007). The increasing interest into the utilization of natural biologically active compounds and the development of specific packaging, mainly active packaging, is evident. Moreover, organosulfur compounds aroused much interest for the improvement of the shelf-life and safety of perishable foods and their potency as food preservatives and substitutes for chemicals (Benkeblia and Lanzotti, 2007). In this concern, one of the main inconveniences is the great variability in the composition of the extracts from *Allium* plants, due in part to the low stability of their components. In order to avoid this problem, some stable well characterized active compounds have been recently obtained. In this regard, propyl thiosulfinate oxide (PTSO) is an organosulfur compound, obtained by decomposition of initial compounds present in *Allium* plants as propiin by the DMC Research Center (Granada, Spain). Then, it is incorporated and stabilized in an inert commercial alimentary support (propylene glycol and polysorbate) to produce PROALLIUM AP®.

The antioxidant and antibacterial properties of the PTSO (Peinado et al., 2012, 2013) as well as PROALLIUM AP® (Ramos-Morales et al., 2013; Ruiz et al., 2010) have been previously studied. In fact, the antibacterial properties of PTSO have been studied in animal pathogens; being the use of this complement very useful in animal nutrition applications (Kim et al., 2012; Peinado et al., 2013). However, little is known about their safety for human consumption. In this sense, despite the beneficial effects attributed to *Allium*

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plants, it is commonly known that excessive consumption can cause problems such as gastrointestinal tract injury, allergic reactions, anaemia, weight loss, and toxicity to the heart, liver, and kidney as well as breaks in chromosomes (Wilson and Demmig-Adams, 2007). Based on such evidence, garlic is classified as a type I allergen (Jappe et al., 1999), the allergens being identified as diallyl disulfide, allylpropyl disulfide, allylmercaptan, and allicin (Tsai et al., 2012). In contrast, in the case of onion, which is the main natural source of PTSO, the adverse effects reported are not very severe, although gastrointestinal upsets, flatulence, changes in the intestinal flora, and dermatological problems in susceptible individual have been described (Corzo-Martínez et al., 2007). Therefore, the evaluation of the safety of the compounds present in *Allium* sp. extracts is needed, since safety is a major factor in the quality control of *Allium* species preparations (Amagase, 2006). Previous studies performed in our laboratory have evidenced that two of the minor components of onion essential oil (dipropyl disulfide and dipropyl sulfide) could be safe for their use in the food industry (Llana-Ruiz-Cabello et al., 2015). However, they exhibited limited beneficial properties in comparison to other organosulfur compounds (Sundaram and Milner, 1996).

In the present work, the suitability of PTSO, intended to be used in "active packaging", is studied. This technology consists of the use of active agents that are incorporated into the packaging walls from which they are released to the food surface at a controlled rate (Llana-Ruiz-Cabello et al., 2014). Thus, the undesirable flavours caused by the direct addition of these compounds into food could be reduced (Ramos et al., 2012). For this purpose, once the antibacterial activity of PTSO was assessed, the cytotoxic effects of this compound were studied in the human Caco-2 and HepG2 cell lines. Moreover, the morphological alterations induced by this compound in the same cell lines were observed by light and electron microscopy in order to determine ultrastructural cellular damages. Besides, the potential protection and reversion abilities against an induced oxidative situation were also investigated in Caco-2 cells, in order to know the mechanism effects (antioxidant) of this compound in regard to its potential use in food packaging. Finally, a preliminary *in vivo* study was conducted in rats orally exposed to PTSO in order to establish the maximum tolerated dose (MTD) of this compound.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, foetal bovine serum (FBS) and cell culture reagents were purchased from BioWhittaker (Spain), ATCC and Gibco (New Zealand). Chemicals were purchased from Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was provided by BioRad (Spain).

2.2. Cell culture

The Caco-2 cell line derived from human colon carcinoma (ATCC® HTB-37) was maintained as explained in Gutiérrez-Praena et al. (2012a). The HepG2 cell line was derived from human hepatocellular carcinoma (ATCC® HB-8065). Cells were maintained according to Maisanaba et al. (2013).

2.3. Synthesis of propyl thiosulfinate oxide (PTSO)

Proallium AP®, a product containing 14.5% of PTSO, was provided by DOMCA S.A. (Granada, Spain). The biosynthesis of PTSO is made from propiin (*S*-propyl-L-cysteine sulfoxide), an amino acid derived from L-cysteine found in *Allium* species. The first step of the biosynthesis is the formation of the sulfenic acid (in this case propyl-1-sulfenic acid; $CH_3CH_2CH_2SOH$) plus pyruvate and ammonia. However, these compounds (sulfenic acids) are highly reactive, so they immediately produce thiosulfinates (in this case, propyl propane thiosulfinate, PTS) by a condensation reaction. In the last step, oxidation of PTS induces its dismutation in PTSO and propyl disulfide. Propyl disulfide can be oxidized and transformed to PTSO and that way the oxidation of PTS to PTSO is completed.

2.4. Bacterial strains and growth conditions

A total of 26 strains, including foodborne spoilage and pathogenic bacteria, were employed in this study. Thus, 13 Gram-negative bacteria (different strains of *Salmonella enterica*, *Campylobacter jejuni*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Cronobacter sakazakii*, *Vibrio cholerae* and *Vibrio parahaemolyticus*), 9 Gram-positive bacteria (strains of *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Clostridium perfringens*), and 4 moulds (*Penicillium digitatum*, *Penicillium expansum*, *Fusarium oxysporum* and *Aspergillus flavus*) were selected due to their relevance in the food industry. Most of microorganisms were purchased from the Spanish Culture Collection (CECT) (Valencia, Spain). The rest were isolated from clinic cases and identified according to PCR-amplified 16S rRNA genes (Murray and Baron, 2003).

2.5. Antimicrobial activity test

In order to evaluate the *in vitro* antimicrobial activity against different strains, the Minimum Bactericidal Concentration (MBC) was determined by a microdilution assay. At first, bacteria and moulds were grown on commercial plate count agar provided by Difco (Difco Laboratories, Detroit, Michigan). When the growth was optimal, they were resuspended in normal saline solution and diluted to 0.5 McFarland density (10^8 colony-forming-units). Moreover, a series of increasing concentrations of PTSO were prepared in the broth medium. Each tube was uniformly inoculated with the microbial suspensions and incubated at 37 °C for 24 h. Afterwards, the tubes were cultured on specific solid agar for each microorganism. The plates were incubated at 37 °C for 24–48 h for pathogenic bacteria or at 25 °C during 72 h for moulds. The MBC was defined at the lowest concentration of the antibacterial agent that killed the 99.9% of the existing microorganisms and was expressed as micrograms of PTSO per millilitre (μg/mL).

2.6. Cytotoxicity assays

The range of PTSO concentrations for the cytotoxicity assays was selected considering the content of this active compound to be incorporated in the packaging materials and the possible migration to the food. In this sense, the maximum concentration was calculated taking into account that the compound completely migrates from the active package into the food (worst scenario of exposure). Considering that, the presence of PTSO in PROALLIUM AP® is about 14.5% (information provided by the suppliers, DOMCA S.A.) and that a 2–6.5% of this commercial preparation can be incorporated in 5.5–6.5 g of packaging film needed to pack 1 kg of food, the potential maximum concentration released to the food would be around 300 μM PTSO. Therefore, in order to establish the safety concentration range of this compound, the highest concentration assayed in the cytotoxicity study was above the maximum concentration of migration (500 μM PTSO). The concentrations employed for the oxidative and morphological studies were calculated according to the EC_{50} obtained in the cytotoxicity studies.

Culture medium with and without 0.1% DMSO was used as control group. After replacing the previous medium, the exposure solutions (25–450 μM) were added to the systems and incubated at 37 °C for 24 h and 48 h according to INVITOX cytotoxicity protocols. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR), and MTS tetrazolium salt reduction (MTS). All assays were performed according to Gutiérrez-Praena et al. (2012a).

2.7. In vitro *morphological study*

Light and electron microscopic observations were performed according to Gutiérrez-Praena et al. (2012a). Cultured cells were exposed to three different concentrations of PTSO, the EC50 value and their fractions (EC50/2, EC50/4). Caco-2 cells were exposed to 92.5, 185 and 370 μM; and HepG2 were exposed to 100, 200 and 400 μM.

2.8. Antioxidant study

The antioxidant ability of PTSO was evaluated taking into account its capacity to protect Caco-2 cells against a further exposition to H_2O_2 100 μM or its ability to revert the damage induced by this substance after a previous exposition. These abilities were evaluated measuring both reactive oxidative species (ROS) content and glutathione (GSH) levels, using the EC_{10} (261.1 μM), $EC_{10}/2$ (130.6 μM) and the $EC_{10}/4$ (65.3 μM) values of PTSO obtained from the cytotoxicity assays.

After discharging the previous medium, exposure solutions were added to the cells and incubated at 37 °C for 24 h and 48 h. Culture medium was used as control group. The oxidative stress endpoints measured were ROS content and GSH levels. The production of ROS was assessed in 96-well microplates using the dichlorofluorescein (DCF) assay, as described in Gutiérrez-Praena et al. (2012b). Glutathione content in cells was evaluated by reaction with the fluorescent probe monochlorobimane assay (Gutiérrez-Praena et al., 2012b). The results of both assays were expressed as fluorescence arbitrary units.

2.9. In vivo study

In order to establish safety margins of PTSO, the OECD 425 guideline for testing of chemicals was carried out (OECD, 2008). Considering that this substance was supposed to exhibit low toxicity, so the limit test was first conducted and followed by the main test. Female rats (8–10 weeks old) weighed around 300 g were single dosed using a stomach tube (Vygon, Ecouen, France) with decreasing doses of PTSO in order to establish the LD50. Housing and feeding conditions are described in OECD (2008). Doses of PTSO were prepared in water in a final volume of 1.5 mL. Once the experiment was finished, tissues were extracted and weighed. Tissue samples for histological examination (light microscopy) were taken from the liver and intestines of control and exposed rats. Samples were first fixed in 10% buffered formalin for 24 h at 4 °C, and then immediately dehydrated in graded series of ethanol, immersed in xylol and embedded in paraffin wax using an automatic processor. Sections of 3–5 μm were mounted. After they had been deparaffinized, the sections were rehydrated, stained with haematoxylin and eosin, and mounted with Cristal/Mount (Paraplast, Oxford Labware, St. Louis, MO). Liver tissue sections were also stained with periodic acid Schiff (PAS) (Sigma-Aldrich Química, SA, Madrid, Spain) for glycogen content assessment.

2.10. Calculations and statistical analysis

All experiments were performed three times and in duplicate per concentration. The statistical analysis was carried out according to Gutiérrez-Praena et al. (2012a).

3. Results

3.1. Antimicrobial study

PTSO was able to inhibit the growth of all organisms tested (Table 1). In general, the Gram-negative strains exhibit higher slightly MBC values than Gram-positive strains tested. In addition, moulds were more sensitive than bacteria. *V. parahaemolyticus* CECT 511 had the lowest MBC value overall, while *C. jejuni* CECT 8119 and *E. faecalis* CECT 5254 had the highest MBC of all organisms tested.

3.2. Results of the cytotoxicity assays

The PC showed a significant decrease from 350 μM PTSO in Caco-2 cells after 24 h of exposure, this reduction being remarkable at 300 μM PTSO when cells were exposed for 48 h (Fig. 1A). The EC_{50} for this endpoint were 369.8 ± 3.4 μM for 24 h and 350.9 ± 1.5 μM for 48 h. In addition, NR uptake experienced a reduction at 400 μM in both exposure times (Fig. 1B) with EC_{50} of 367.9 ± 2.8 μM and 366.5 ± 1.9 μM for 24 h and 48 h, respectively. Similarly, Caco-2 cells exposed to PTSO underwent a significant decrease in MTS with respect to the control cells after 24 h and 48 h (Fig. 1C). Similar EC_{50} values were also obtained in this endpoint at both exposure times, 374.2 ± 2.3 μM and 388.3 ± 1.6 μM. Similar results were observed in HepG2 cells exposed to PTSO. After 24 h, the hepatic cells underwent a significant decrease in PC from 250 μM PTSO and from 300 μM PTSO after 48 h of exposure. In the treatment to 500 μM PTSO all cells died and detached from the flask since no protein was measured (Fig. 1D). The EC_{50} for this endpoint were 380.8 ± 2.7 μM for 24 h and 368.1 ± 3.1 μM for 48 h. In the NR uptake and MTS assay, concentration-dependent reductions were also observed, being significant from 350 μM PTSO at both exposure times (Fig. 1E and F). Similar EC_{50} were obtained, 400.7 ± 1.4 μM for 24 h and 395.7 ± 2.6 μM for 48 h, in the case of NR uptake, and 415.14 μM for 24 h and 401.6 μM for 48 h, in the case of MTS assay.

3.3. Results of the morphological study

3.3.1. Light and electron microscopic observation in Caco-2 cells

Unexposed Caco-2 cells observed under light microscope showed large nuclei with irregular border, some of them undergoing a mitotic process as regards the telophase observed, evidencing proliferative activity (Fig. 2A). When cells were exposed to 92.5 μM PTSO for 24 h, large intercellular spaces are shown. In addition, nuclei evidenced segregated nucleoli (Fig. 2B). After 48 h to the same concentration, similar results were obtained, although the damage induced by PTSO was more intense, appearing autophagosomes (Fig. 2C). A concentration and time-dependent impairment of cell integrity was observed, with the highest concentration assayed (370 μM PTSO) showing the greater damages. After 24 h of

Table 1
Minimal bactericidal concentration (MBC) expressed as μg/mL of PTSO for each strain.

Bacterial strain	MBC (μg/mL)	Comment
Gram-negative		
Salmonella enterica subsp. *enterica* (group E1) ser. London CECT 4376	**0.5**	
Salmonella enterica subsp. *enterica* ser. Typhimurium CECT 4156	**5**	
Salmonella enterica subsp. *enterica* (9,12:g,m:–) serovar. *enteritidis* CECT 7160	**2**	
Salmonella enterica subsp. *enterica* (9,12:g,m:–) serovar. *enteritidis* CECT 7159	**2.5**	
Salmonella enterica subsp. Arizonae – *Salmonella choleraesuis* subsp. Arizonae CECT 4395	**2.5**	
Salmonella enterica subsp. *enterica* ser. Derby. CTC1022	**1.25**	
Salmonella enterica subsp. *enterica* Typhimurium/DT014 DSM – 26529	**1.25**	
Campylobacter jejuni CECT 8119	**10**	
Escherichia coli O157:H7 CECT 4972	**0.5**	
Pseudomonas aeruginosa CECT 118	**5**	
Cronobacter sakazakii CECT 858	**2.5**	
Vibrio cholerae CECT 514	**0.5**	Isolated from faeces
Vibrio parahaemolyticus CECT 511	**0.25**	
Gram-positive		
Listeria monocytogenes CECT 4032, serotype 4b,	**1.25**	Clinical isolated
Listeria monocytogenes CECT 911 serovariety 1/2c	**1.25**	
Listeria monocytogenes serotype 1/2a strain	**2.5**	Clinical isolated
Enterococcus faecalis CECT 795	**5**	
Enterococcus faecalis CECT 5254	**10**	Clinical isolated, vancomycin-resistant
Staphylococcus aureus CECT 976	**1.25**	producer of enterotoxin A
Staphylococcus aureus CECT 4438	**2**	Methicillin-resistant
Staphylococcus aureus CECT 4439	**2**	Methicillin-resistant
Clostridium perfringens CECT 486	**2.5**	
Fungui		
Penicillium digitatum CECT 2954	**0.5**	
Penicillium expansum CECT 2279	**0.5**	
Fusarium oxysporum CECT 2154	**1.25**	
Aspergillus flavus CECT 2687	**5**	

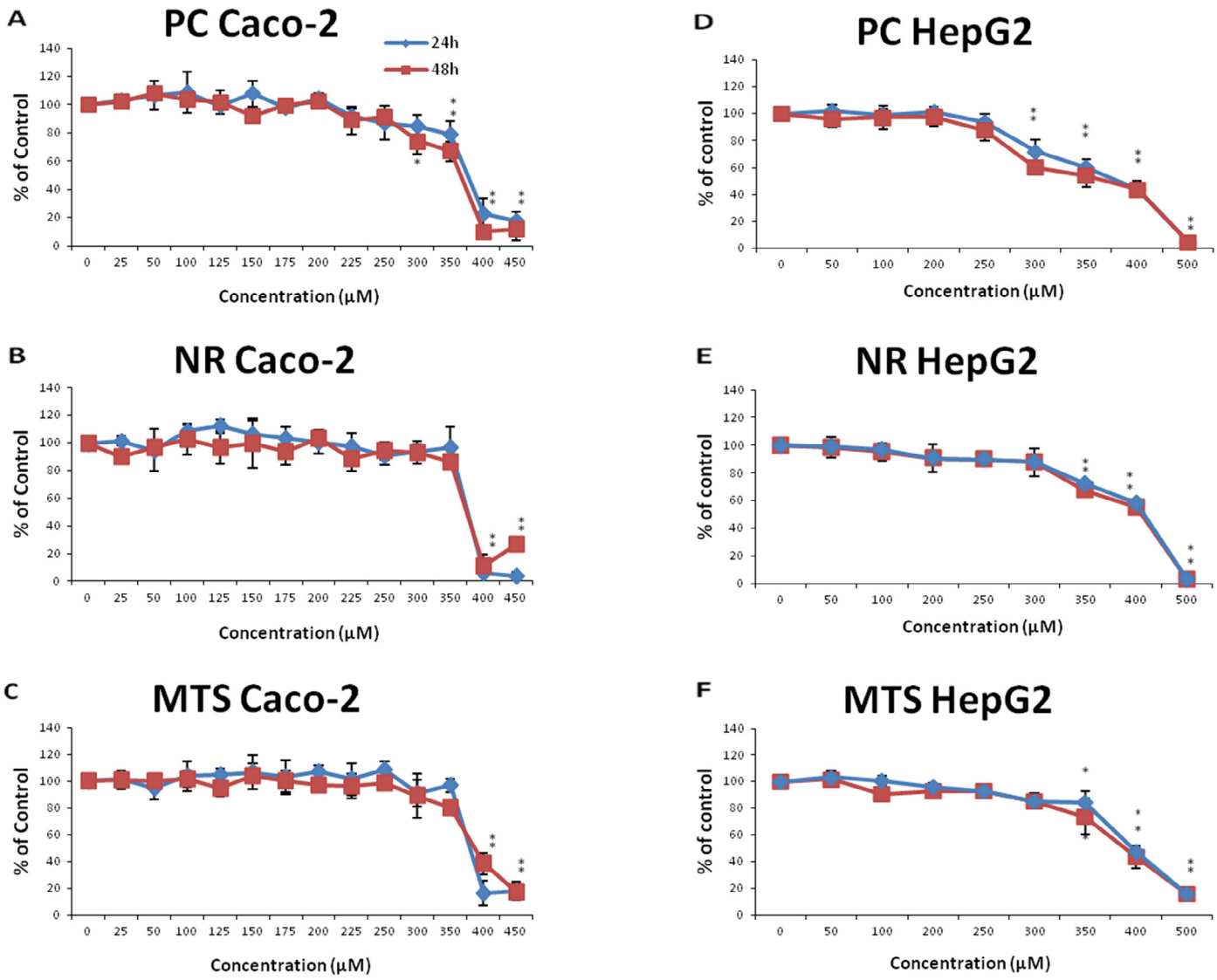

Fig. 1. Protein content (PC), neutral red uptake (NR) and reduction of tetrazolium salt (MTS) of Caco-2 (A–C) and HepG2 (D–F) cells exposed for 24 and 48 h to 0–450 µM and 0–500 µM of PTSO, respectively. All values are expressed as mean ± S.D. *Very significantly different from control ($p < 0.01$).

exposure to 370 µM PTSO, the cells experienced a severe loss of viability, showing damaged nuclei and necrotic cytoplasm (Fig. 2D).

Unexposed Caco-2 cells observed under electron microscope showed large euchromatic nuclei with irregular bordering and compact nucleoli, which are formed by several fibrillar centres rounded by dense fibrillar and granular component. Caco-2 cells showed intercellular interactions by means of the differentiation in the plasmatic membrane, which allow cellular linking and communication. In the cytoplasm mitochondria with dense matrix and transversal crests are observed as well as the presence of glycogen reservoirs. In the intercellular spaces and in the apical surface, the plasmatic membrane exhibits signs of differentiation process such as microvilli (Fig. 3A). Cells exposed to 92.5 µM for 48 h evidenced characteristic morphological features of degenerative process such as autophagic vacuoles and mitochondria with scarce and thin transversal crests (Fig. 3B). Cellular interactions could be possible due to the presence of desmosomes. However, large intercellular spaces are observed between neighbouring cells evidencing cell shrinkage (Fig. 3C). The damage previously described was enhanced in cells exposed to 185 µM for 24 h. Damaged nuclei and nucleoli are observed. In the cytoplasm, dilatation in the cisternae from the endoplasmic reticulum (ER) is visible, which is a morphological feature of alteration in the membranes traffic, both from the secretory and constitutive pathways (Fig. 3D). In addition, mitochondria were altered, with loss of transversal crests (Fig. 4A). When cells were exposed to the highest concentration assayed (370 µM PTSO) damage was more marked. Nucleus showed damaged nucleolus and lipid drop inclusions (Fig. 4B). In the necrotic cytoplasm damages in the cell membrane were distinguished. Moreover, necrotic organelles were also observed (Fig. 4C). Some of the cells were hastily damaged since mitosis was stopped, as regards the chromatin condensation (Fig. 4D). Moreover, cells are profusely damaged; organelles are completely destroyed, with visible organelle debris. After 48 h of exposure to this concentration (370 µM PTSO), cells were completely injured, losing attachment to the flask.

3.3.2. *Light and electron microscopic observation in HepG2 cells*

Control HepG2 cells observed under light microscopy show large euchromatic nuclei and well-developed nucleoli. Secretory vesicles are observed in the cytoplasm (Fig. 5A). No ultrastructural features indicative of morphological changes are observed in HepG2 cells exposed to 100 µM PTSO for 24 and 48 h. Similarly to the finding

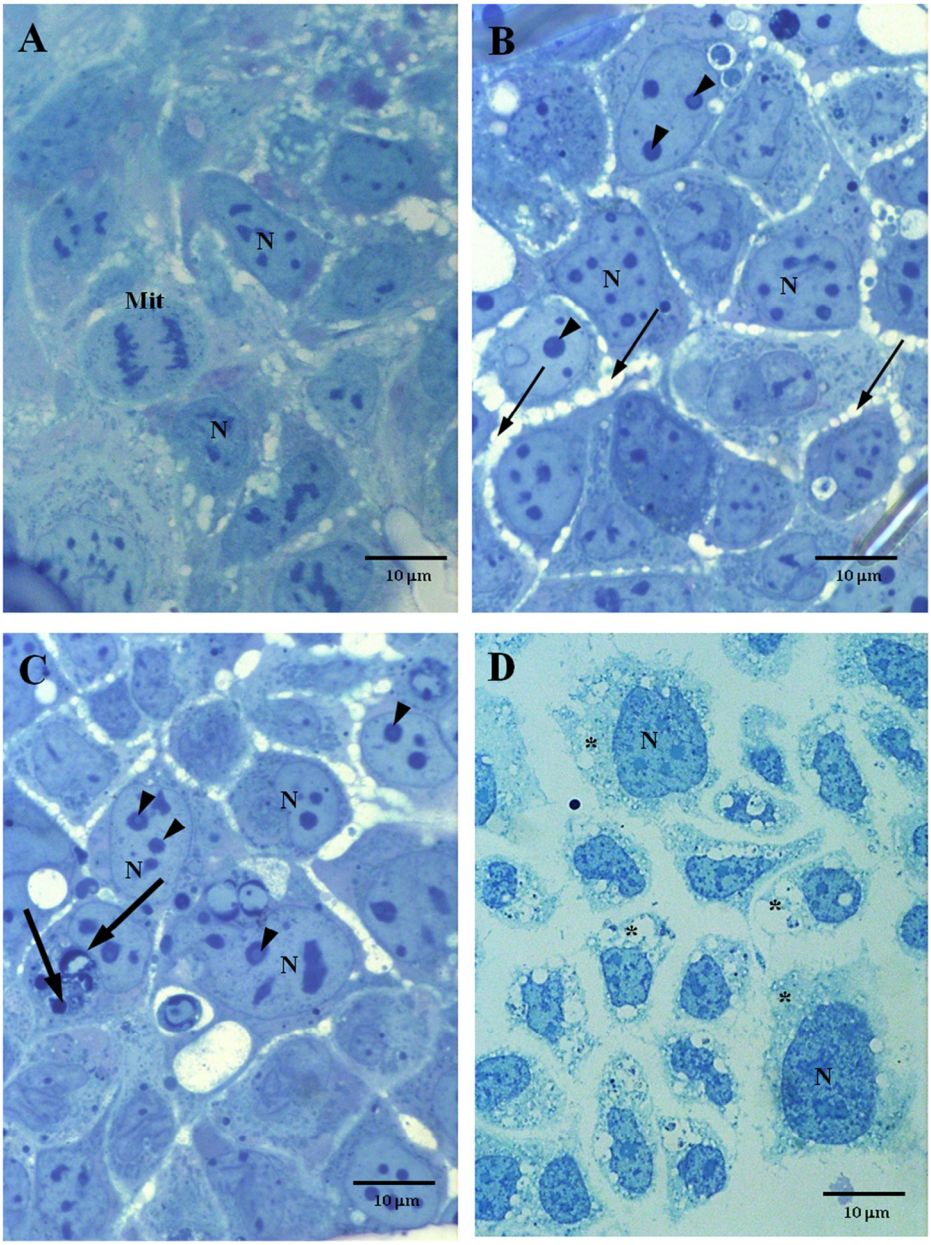

Fig. 2. Morphology of Caco-2 cells after 24 h (A, B, D) and 48 h (C) of treatment observed by light microscopy. Bars = 10 µm. Unexposed control culture (A), and Caco-2 cells exposed to 92.5 µM PTSO (B, C) and 370 µM PTSO (d). (A) Control cells showing large nucleus (N), some of them in telophase (Mit). (b) Nuclei (N) with segregated nucleoli (arrow heads) and intercellular spaces (black arrows). (C) Nuclei (N) with segregated nucleoli (arrow heads) and autophagosomes (black arrows). (D) Damage nuclei (N) and necrotic cytoplasm (asterisk).

recorded in the control group, in these cells secretory vesicles are also observed (Fig. 5B). The most remarkable cellular damage is observed at the highest concentration assayed (400 µM). At this concentration, the cellular growth is stopped, undergoing cell death. This finding is mainly due to necrotic process although the induction of apoptotic mechanisms is also observed. Under light microscopy, HepG2 cells exposed to 400 µM PTSO for 24 h showed morphological characteristics of early apoptosis, such as heterochromatic clusters in peripheral regions of the nucleus, directly in contact with the nuclear membrane (Fig. 5C). This morphological feature was also observed under electron microscopy (Fig. 5D). After 48 h of exposure to 400 µM, cellular debris is frequently observed in culture media subsequent to necrosis (Fig. 5E) In addition, cells showing micronuclei are also found together nuclei exhibiting irregular bordering (Fig. 5E).

3.4. Results of the antioxidant activities in Caco-2 cells

In the protection assay, no significant effect was recorded in ROS content after 24 h. However, in Caco-2 cells pre-treated during 48 h to PTSO prior exposure to H_2O_2, ROS level remained similar to the control group and lower than those observed after exposure to H_2O_2 (Fig. 6A). Similarly, no significant differences were observed in GSH levels between the H_2O_2 group and those exposed to PTSO for 24 h. Nevertheless, after 48 h of exposure to PTSO, GSH levels were significantly higher than in the H_2O_2 group, being similar to the control group (Fig. 6B).

In the reversion assay, PTSO reduced ROS levels significantly with respect to the H_2O_2 group at all concentrations assayed after both pre-treatment times (Fig. 6C). With respect to GSH levels, no significant differences were observed in groups exposed to PTSO in

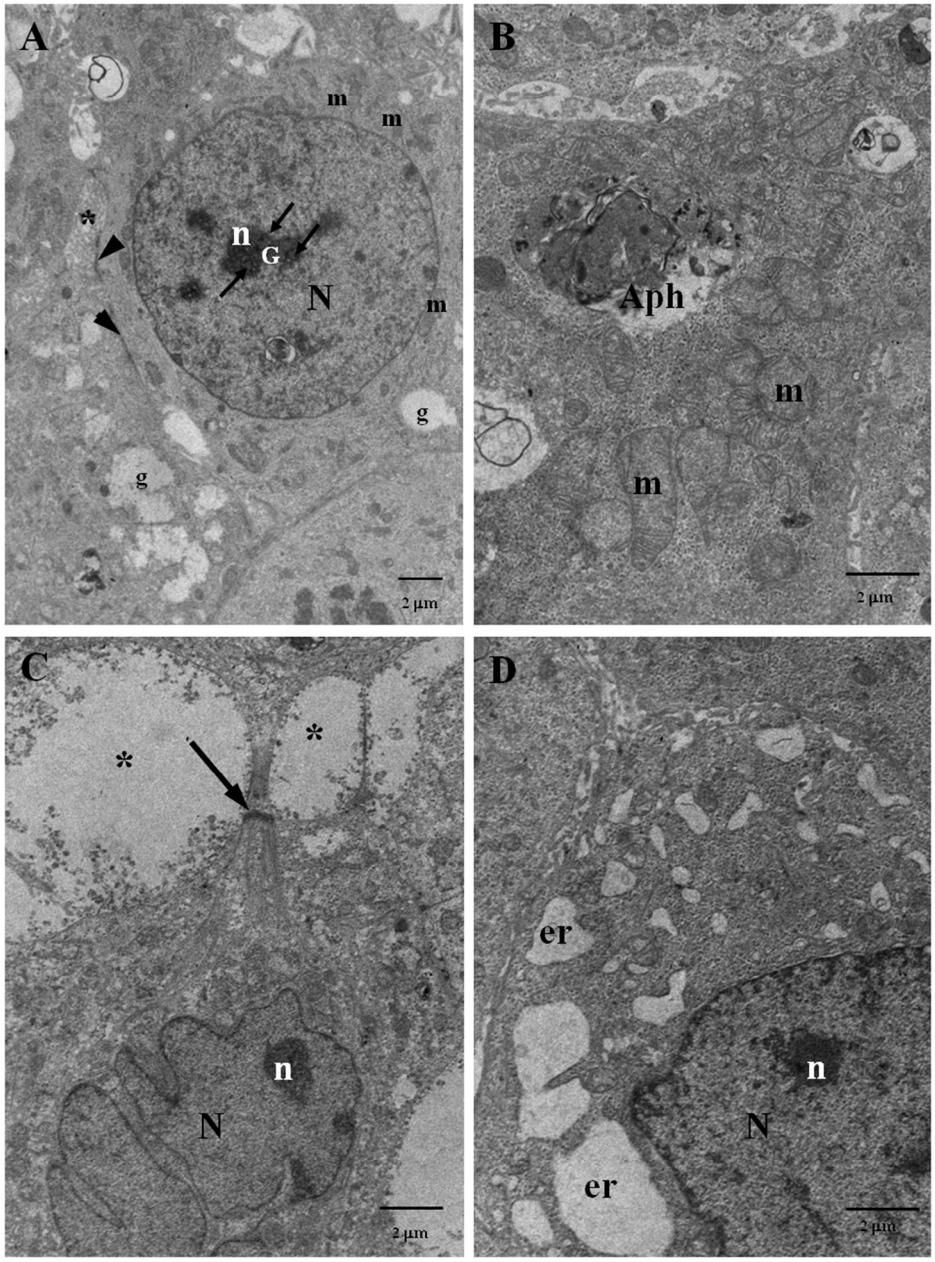

Fig. 3. Morphology of Caco-2 cells after 24 h (D) and 48 h (A, B, C) of treatment observed by electron microscopy. Bars = 2 µm. Unexposed control culture (A), and Caco-2 cells exposed to 92.5 µM PTSO (B, C) and 185 µM PTSO (D). (A) Control cells showing large nuclei (N), compact nucleoli (n) with dense fibrillar component (arrow) and granular component (G). Intercellular interactions (black arrow head), glycogen reservoirs (g) and mitochondria with dense matrix and transversal crests (m) are also observed. Signs of differentiation process are visible such as microvilli (asterisk). (B) Cells showed autophagosomes (Aph) and mitochondria with scarce crests (m). (C) Large nuclei with irregular bordering (N) and altered nuclei (n) are shown. Desmosomes (black arrow) and intercellular spaces (asterisk) are also observed. (D) Damage nuclei (N) and nucleoli (n) are observed. In the cytoplasm, dilatation in the cisternae from the ER is visible (er).

comparison to the H_2O_2 group at any of the concentrations assayed after both times of treatment (Fig. 6D).

3.5. *Results of the* in vivo *study*

The limit test was first conducted. For this test, one female rat was dosed 2000 mg/kg body weight (bw) of PTSO. The animal died within 24 h, therefore, the main test was further carried out. Following the recommendations of the OECD guidelines for testing chemicals (OECD, 2008), dosing was initiated at 175 mg /kg bw. This dose induced the death of the animal, so the dosing was now established at 55 mg/kg bw. Three consecutive animals survived the dose of 55 mg/kg bw. Consequently, the MTD of PTSO was set at 55 mg/kg bw. Inflammation was observed macroscopically in the intestine and liver of rats gavaged with the highest doses (2000 and 175 mg/kg bw). Similarly, light microscopy revealed damage at those doses. In the liver of rats exposed to 2000 mg/kg bw degenerative process is observed with hepatic parenchyma revealing loss of structure and numerous hepatocytes in necrotic process (Fig. 7A). Intestinal pathological changes were also observed at this dose, with the intestinal mucosa showing necrotic enteritis and severe oedema in the muscles layers (Fig. 7B). Tissues of rats exposed to 175 mg/kg bw also underwent histological damage, although it was less intense. Hepatic parenchyma was slightly altered, showing the presence of some binucleated hepatocytes (Fig. 7C). Desquamation process is observed in the intestinal mucosal epithelium (Fig. 7D).

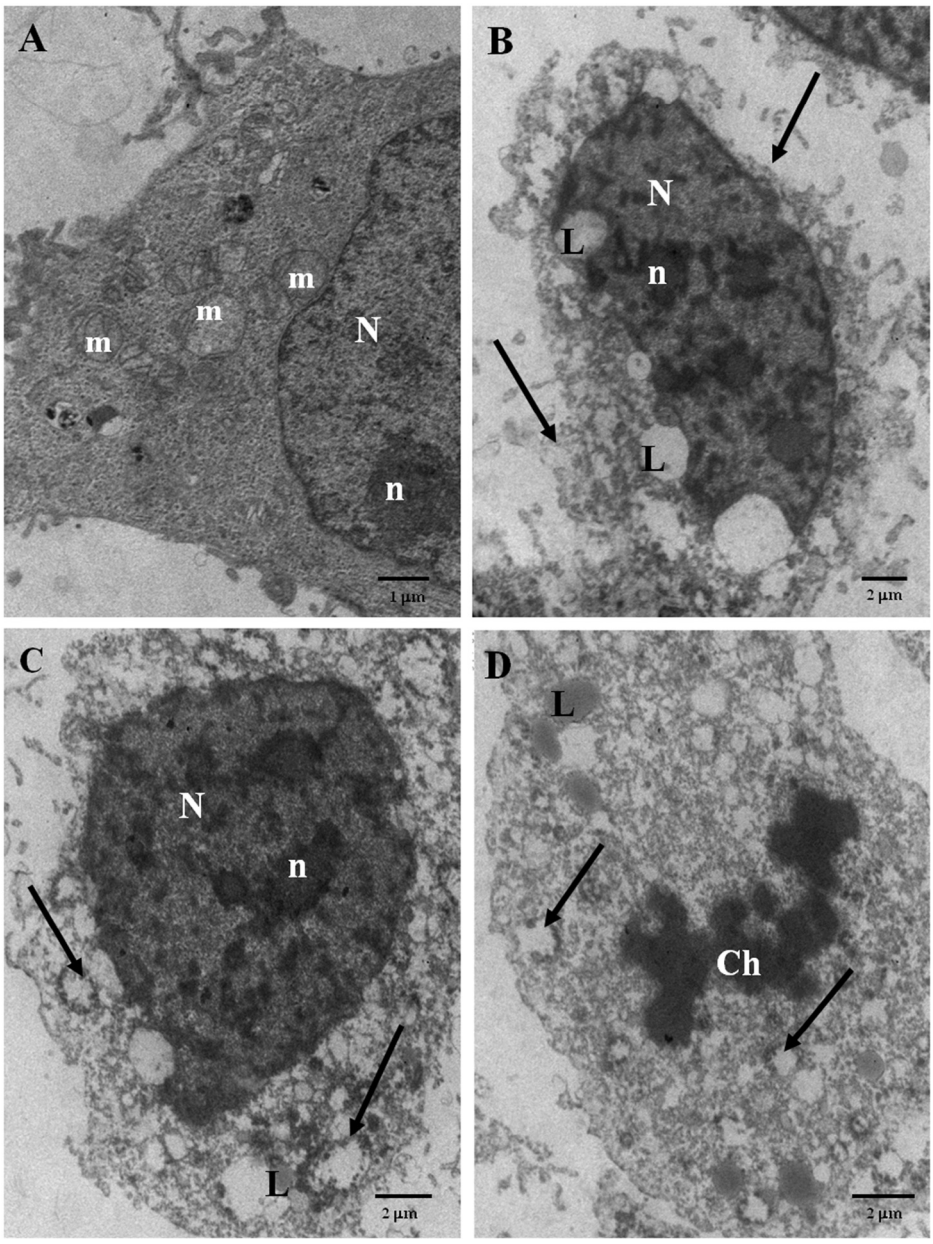

Fig. 4. Morphology of Caco-2 cells after 24 h of treatment observed by electron microscopy. Bars = 2 µm. Caco-2 cells exposed to 185 µM PTSO (A) and 370 µM PTSO (B, C, D). (A) Damaged mitochondria, with complete loss of crests (m). Nucleus (N) and nucleolus (n) are also observed. (B) Nucleus (N) with damaged nucleolus (n) and lipid drops (L). Necrotic cytoplasm showing damaged cell membrane (black arrows). (C) Nucleus (N) with damaged nucleolus (n) and lipid drops (L). Necrotic organelles are also observed (black arrows). (D) Chromatin condensation (Ch) and organelles debris are visible (black arrows).

However, when rats were orally exposed to 55 mg/kg bw, no remarkable damage was observed. Both tissues were apparently normal. Hepatic parenchyma showed normal structure with unaltered hepatocytes set in the cord-like architecture with normal nuclei (Fig. 7E). Intestinal mucosal epithelium was also observed apparently normal (Fig. 7F).

4. Discussion

In the present work, the suitability of PTSO for its use in active food packaging has been assessed. Previous studies have evidenced the antimicrobial activity of this organosulfur compound for its use in feedstuffs (Peinado et al., 2012, 2013). However, the latter work evidenced the safety of PTSO for its use with veterinary purposes, but no research has been performed so far to assess its usefulness and safety for human consumption. In this study, we have now confirmed the antimicrobial activity of PTSO against pathogens frequently found in food. Most of the pathogens assayed were selected due to their ability to cause foodborne diseases. Therefore, the antimicrobial activity of PTSO in these strains evidences a suitable use in food packaging. In our study, the most sensitive microorganism was *V. parahaemolyticus* (MCB = 0.25 µg/mL), which is related to human intoxication for shellfish consumption (Su and Liu, 2007). Also strains of *S. enterica*, *E. coli*, *V. cholerae* and two species of *Penicillium* were highly inhibited by exposure to PTSO, with a MCB of 0.5 µg/mL. Similarly, the antimicrobial activity of essential oil extracts from *Allium* plants (garlic and onions) against two bacteria, *S. aureus* and *S. enteritidis*, and three fungi, *Aspergillus niger*, *Penicillium cyclopium* and *F. oxysporum*, was corroborated, with garlic showing the highest growing inhibition (Benkeblia, 2004). Other

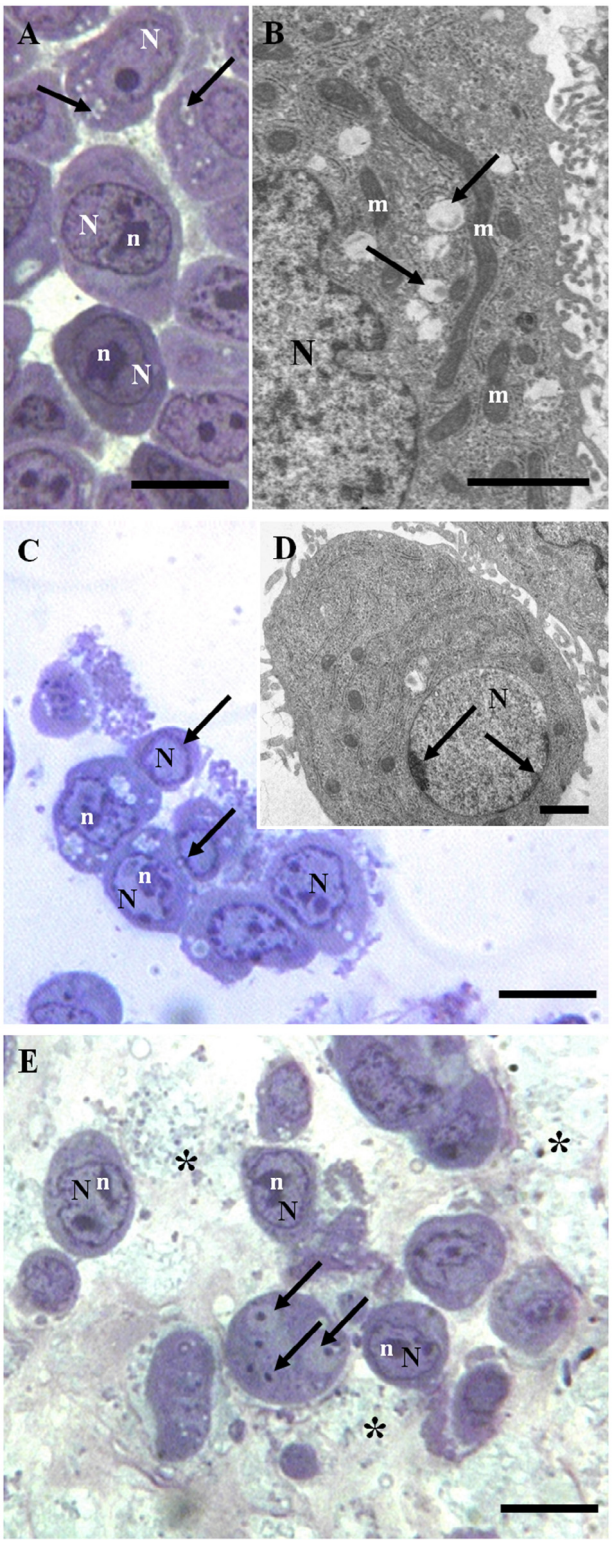

Fig. 5. Morphology of HepG2 cells after 24 and 48 h of treatment observed by light (A, C, E) and electron microscopy (B, D). Bars (A, C, E) = 10 μm; bar (B) = 2 μm; bar (D) = 1 μm. Unexposed control culture (A), and HepG2 cells exposed to 100 μM PTSO (B) and 400 μM PTSO (C, D, E). (A) Unexposed HepG2 cells show large euchromatic nuclei (N) and well-developed nucleoli (n). Secretory vesicles are observed in the cytoplasm (arrow). (B) Normal cells are observed, secretory vesicles (arrow) and normal nuclei (N) and mitochondria (m) are shown. (C) Morphological characteristics of early apoptosis are shown, such as heterochromatic clusters in the peripheral regions of the nucleus (N), directly in contact with the nuclear membrane (arrow). (D) Detail of early apoptosis showing heterochromatic clusters (arrow) in the nucleus (N). (E) Cellular debris is observed (asterisk) with cells showing micronuclei (arrow) and nuclei exhibiting irregular bordering (N).

microbes inhibited by garlic included *Bacillus subtilis*, *E. coli*, and the yeast *Saccharomyces cerevisiae* (Lai and Roy, 2004). Moreover, antibacterial and antidermatophytic activities of onion oil have been corroborated, highlighting the remarkable antibacterial properties against Gram-positive bacteria (Zohril et al., 1995). Similar results were also obtained *in vivo* supplementing broiler diet with onion, which induced favourable influences on performance and ileum microflora composition (Goodarzi et al., 2014). The antimicrobial activity of several preparations of garlic has also been evaluated by aerobic plate count in chicken sausages for 21 days, evidencing a weakest activity for the garlic oil in comparison to fresh and powder garlic. This finding was explained due to the losses of sulfur compounds and also due to the nature of garlic oil itself, which is volatile and hydrophobic (Sallam et al., 2004). Therefore, the antimicrobial activity of *Allium* sp. components should not be taken for granted, and this activity should be confirmed in each component, in our case, PTSO.

Considering that PTSO has exhibited interesting antimicrobial activity to be used in the food industry, it is important to study the potential cytotoxicity in human cell lines (Caco-2 and HepG2) to assess its safety. The cytotoxicity study revealed that PTSO did not reduce cell viability in a wide range of concentrations; in most of the case cells were not damaged when exposed up to 350 μM. From this concentration, a marked drop was observed in all endpoints. However, ultrastructural changes were observed from 92.5 μM in Caco-2 cells, which is higher than the real exposure concentration. Similar results were observed in other active substances from essential oils, evidencing that the morphological study is a more sensitive assay to detect toxicity in comparison to the cytotoxic endpoints (Llana-Ruiz-Cabello et al., 2014, 2015). In contrast, in the present work, the morphological study of HepG2 cells exposed to PTSO did not reveal significant changes between the unexposed cells and those treated with 100 μM PTSO. Therefore, according to the cytotoxicity and morphological results, Caco-2 cells resulted to be more sensitive to PTSO than HepG2 cells. In general, the cytotoxicity study reveals loss of cell viability; although cells can be damaged without resulting in death; hence, in the morphological study we can detect earlier damages. The most remarkable morphological changes observed in Caco-2 cells exposed to PTSO were lipid drop inclusions, dilatation in the cisternae from ER and altered mitochondria which are signs of cell degradation. Lipid drop inclusions are related to the damages observed in the cell membrane (Gubern et al., 2009). The relationship of *Allium* plants and lipid metabolism is well known since they are used to reduce serum lipid (Corzo-Martínez et al., 2007). In addition, cell shrinkage, as well as other cellular features characteristic of cell death, was observed in our study for both cell types. Also, Zhang et al. (2014) reported apoptosis in MCF-7 breast cancer cells induced by organosulfur compounds derived from *Allium* vegetables. Moreover, allyl sulfides from garlic arrested proliferation of human A549 lung tumor cells (Sakamoto et al., 1997). Similarly, Sigounas et al. (1997) evidenced apoptosis in erythroleukaemia cell lines exposed to S-Allylmercaptocysteine. Another compound present in *Allium* plants, ajoene, also induces apoptosis in human promyleoleukaemic cells (Dirsch et al., 1998).

The application of organosulfur compounds in food systems via exogenous addition may be feasible and benefit for antioxidant protection (Yin et al., 2002). Numerous studies have demonstrated organosulfur compounds from *Allium* sp. to be potent antioxidants by displaying radical-scavenging activity and modulating cellular antioxidant enzyme activity (Tsai et al., 2012). In this sense, considering the highest sensitivity of Caco-2 cell in comparison to HepG2, the protection of PTSO from the oxidative damage induced by H_2O_2 was tested in Caco-2 cells, being this protection more evident after 48 h of exposure. In addition, PTSO seemed to be able to revert the oxidative situation caused by H_2O_2 since the levels of ROS were

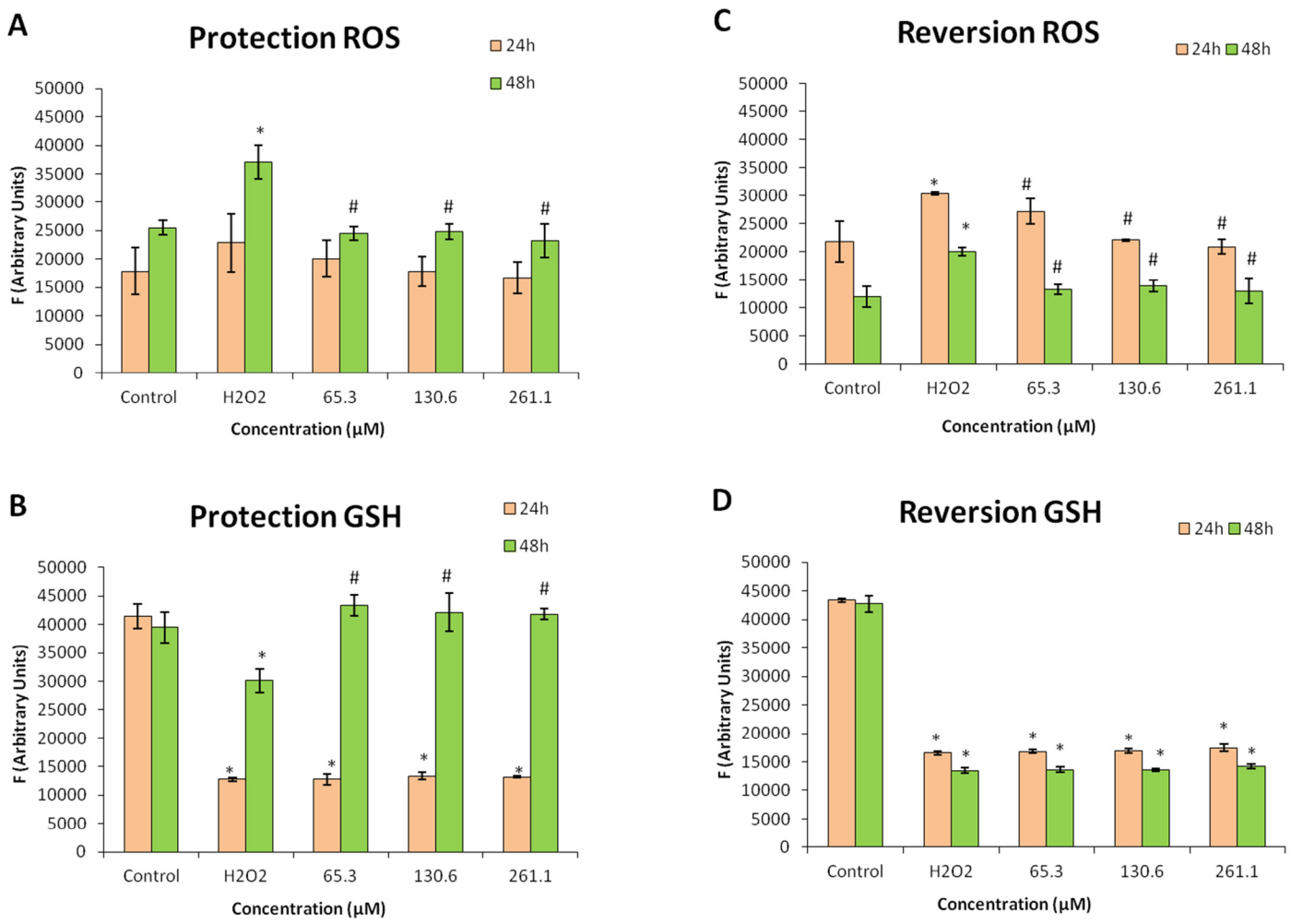

Fig. 6. ROS (A) and GSH (B) content in Caco-2 cells first pre-treated for 24 or 48 h with PTSO (0–261.1 μM), and a later exposure for 2 h to H_2O_2 100 μM. ROS (C) and GSH (D) content in Caco-2 cells first exposed to H_2O_2 100 μM for 2 h and a later 24 or 48 h treatment with PTSO (0–261.1 μM). All values are expressed as mean ± S.D. Differences were considered significant with respect to the control group from $p < 0.05$ (*), and significant with respect to the H_2O_2 control group from $p < 0.05$ (#).

restored. However, this effect was not evident with regard to the GSH level study. Hassan et al. (2010) reported that the oral administration of garlic oil to rats daily for 3 months significantly inhibited the oxidative stress induced in brain by $NaNO_2$ ingestion. Similarly, garlic extract injections reverted lindane-induced oxidative damages in the testes and brain of rats (Hfaiedh et al., 2011). Moreover, garlic powder feeding was able to prevent the increase in markers of oxidative and nitrosative stress in the kidney of rats previously exposed to potassium dichromate (Pedraza-Chaverri et al., 2008). Aged garlic extract has been also reported to exert a protective effect against an induced oxidative situation both *in vivo* (Chowdhury et al., 2008; Maldonado et al., 2003) and *in vitro* (Chowdhury et al., 2008). Similarly, garlic extract has been proven to be as effective as N-acetylcysteine in lessening ROS formation and GSH depletion induced by acetaminophen in rat primary hepatocytes (Anoush et al., 2009). In this regard, Ola-Mudathir et al. (2008) reported that aqueous extracts of onion and garlic can protect against Cd-induced oxidative damage. Furthermore, the protective effect of onion extract as well as flavonoids (catechin and quercetin) was also observed on rats subjected to oxidative stress by mercuric chloride treatment (Jaiswal et al., 2013). In addition, Yin et al. (2002) reported nonenzymatic antioxidant protection of four organosulfur compounds, evidencing that they are potent agents for enhancing lipid stability.

In rats orally exposed to 2000 mg/kg bw, PTSO induced inflammation in the intestine and liver. This finding, together with the desquamation in the intestinal mucosal epithelium of rats dosed with 175 mg/kg bw, can be related to the irritant property of this substance reported in the safety data sheet of the provider (DOMCA S.A., Granada, Spain). In contrast, no remarkable morphological changes were observed in rats gavaged with 55 mg/kg bw. Therefore, the MTD was set at this dose. This result is of high importance considering that the safety of PTSO has not been addressed so far. The use of *Allium* sp. extract, such as PROALLIUM AP® containing PTSO or even directly PTSO, could result in a higher intake of the organosulfur compounds in comparison to the ingestion of garlic or onion. In this concern, in the present work the range of concentrations assayed was selected considering the quantity of PTSO that could migrate to food from the package containing PROALLIUM AP®. However, this estimation was carried out per kilogram of food. Considering that the most likely application of this packaging would be lettuce, a more real consumption would be 132.53 g per person per day (http://aesan.msssi.gob.es/), therefore, in the worst scenario only 37.5 μM PTSO could reach the consumer, which is 10-fold lower than the concentration inducing toxic effects. In addition, considering the *in vivo* study, the MTD value would correspond to 3300 mg of PTSO that could be ingested by a person weighting 60 kg without suffering adverse effects. In the worst scenario of

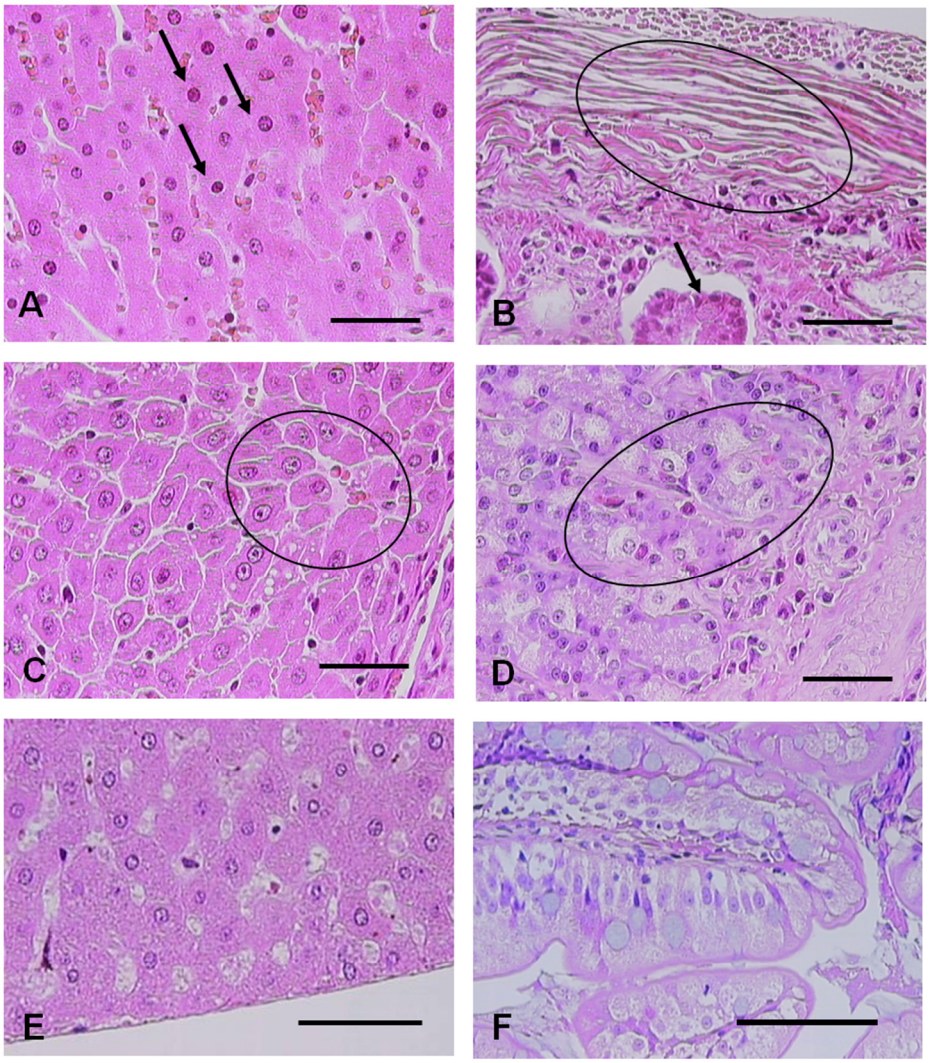

Fig. 7. Histopathological changes of rats treated with 2000 mg/kg bw of PTSO (A, B), 175 mg/kg bw of PTSO (C, D) and 55 mg/kg bw of PTSO (E, F) observed by light microscopy. Bars = 100 μm. (A) Hepatic parenchyma evidenced loss of structure and numerous hepatocytes in necrotic process (arrow). (B) Intestinal mucosa showing necrotic enteritis with enterocytes undergoing necrosis (arrow) and severe oedema in the muscles layers (circle). (C) Hepatic parenchyma was slightly altered, showing the presence of some binucleated hepatocytes (circle). (D) Desquamation in the intestinal mucosal epithelium (circle). (E) Hepatic parenchyma showed normal structure with unaltered hepatocytes set in the cord-like architecture with normal nuclei. (F) Intestinal mucosal epithelium apparently normal.

exposure, a human could ingest 6.87 mg PTSO, around 500-fold lower than those considered to be safe in our study. In addition, the lowest dose inducing damage in rats was 175 mg/kg bw PTSO, this would correspond to 10,500 mg of PTSO that could be harmful, which is more than 1500-fold the quantity that a consumer could ingest in the worst scenario of exposure. Therefore, no potential risk associated to the consumption of this substance derived from its potential used in food packaging could be predicted.

5. Conclusions

The results obtained in the present work revealed the usefulness and safety of PTSO in its potential use in the food packaging industry. PTSO has been confirmed to exhibit antimicrobial activity and a protective role against an induced oxidative situation. In addition, despite damage was observed when Caco-2 and HepG2 cells were exposed to high concentrations of PTSO, no cytotoxic effect was recorded in the concentrations intended to be used in the food packaging. Moreover, the *in vivo* study also revealed the potential safety use at the established concentrations. Considering all the results obtained in this work, PTSO is a promising active substance to be used in active food packaging.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

Amagase, H., 2006. Clarifying the real bioactive constituents of garlic. J. Nutr. 136 (3), 716S–725S.

Anoush, M., Eghbal, M.A., Fathiazad, F., Hamzeiy, H., Kouzehkonani, N.S., 2009. The protective effects of garlic extract against acetaminophen-induced oxidative stress and glutathione depletion. Pak. J. Biol. Sci. 12, 765–771.

Benkeblia, N., 2004. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). LWT – Food Sci. Technol. 37 (2), 263–268.

Benkeblia, N., Lanzotti, V., 2007. *Allium* thiosulfinates: chemistry, biological properties and their potential utilization in food preservation. Food 1, 193–201.

Chowdhury, R., Dutta, A., Chaudhuri, S.R., Sharma, N., Giri, A.K., Chaudhuri, K., 2008. *In vitro* and *in vivo* reduction of sodium arsenite induced toxicity by aqueous garlic extract. Food Chem. Toxicol. 46, 740–751.

Corzo-Martínez, M., Corzo, N., Villamiel, M., 2007. Biological properties of onions and garlic. Trends Food Sci. Technol. 18, 609–625.

Dirsch, V.M., Gerbes, A.L., Vollmar, A.M., 1998. Ajoene, a compound of garlic, induces apoptosis in human promyleoleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kB. Mol. Pharmacol. 53, 402–407.

Goodarzi, M., Nanekarani, S., Landy, N., 2014. Effect of dietary supplementation with onion (*Allium cepa* L.) on performance, carcass traits and intestinal microflora composition in broiler chickens. Asian Pac. J. Trop. Dis. 4 (1), S297–S301.

Gubern, A., Casas, J., Barceló-Torns, M., Barneda, D., de la Rosa, X., Masgrau, R., et al., 2009. Group IVA phospholipase A is necessary for the biogenesis of lipid droplets. J. Biol. Chem. 283, 27369–27382.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Moreno, F.J., Cameán, A.M., 2012a. Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2. Water Res. 46 (5), 1566–1575.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Moreno, F.J., Cameán, A.M., 2012b. Alterations observed in the endothelial HUVEC cell line exposed to pure Cylindrospermopsin. Chemosphere 89, 1151–1160.

Hassan, H.A., Hafez, H.S., Zeghebar, F.E., 2010. Garlic oil as a modulating agent for oxidative stress and neurotoxicity induced by sodium nitrite in male albino rats. Food Chem. Toxicol. 48, 1980–1985.

Hfaiedh, N., Murat, J.C., Elfeki, A., 2011. Protective effects of garlic (*Allium sativum*) extract upon lindane-induced oxidative stress and related damages in testes and brain of male rats. Pestic. Biochem. Phys. 100, 187–192. <http://aesan.msssi.gob.es/> (accessed 30.10.14).

Jaiswal, N., Kumar, D., Rizvi, S.I., 2013. Red onion extract (*Allium cepa* L.) supplementation improves redox balance in oxidatively stressed rats. Food Sci. Hum. Wellness 2, 99–104.

Jappe, U., Bonnekoh, B., Hausen, B.M., Gollnick, H., 1999. Garlic-related dermatoses: case report and review of the literature. Am. J. Contact Dermat. 10, 37–39.

Kim, D.K., Lillehoj, H.S., Lee, S.H., Lillehoj, E.P., Bravo, D., 2012. Improved resistance to *Eimeria acervulina* infection in chickens due to dietary supplementation with garlic metabolites. Br. J. Nutr. 109, 76–88.

Lai, P.K., Roy, J., 2004. Antimicrobial and chemopreventive properties of herbs and spices. Curr. Med. Chem. 11, 1451–1460.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Javier Moreno, F., Bermúdez, J.M., Aucejo, S., et al., 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290.

Llana-Ruiz-Cabello, M., Maisanaba, S., Gutiérrez-Praena, D., Prieto, A.I., Pichardo, S., Jos, A., et al., 2015. Cytotoxic and mutagenic in vitro assessment of two organosulfur compounds derived from onion to be used in the food industry. Food Chem. 166, 423–431.

Maisanaba, S., Puerto, M., Pichardo, S., Jordá, M., Moreno, F.J., Aucejo, S., et al., 2013. *In vitro* toxicological assessment of clays for their use in food packaging. Food Chem. Toxicol. 57, 266–275.

Maldonado, P.D., Barrera, D., Medina-Campos, O.N., Hernández-Pando, R., Ibarra-Rubio, M.E., Pedraza-Chaverri, J., 2003. Aged garlic extract attenuates gentamicin induced renal damage and oxidative stress in rats. Life Sci. 73, 2543–2556.

Murray, P.R., Baron, E.J. (Eds.), 2003. Manual for Clinical Microbiology. ASM Press, Washington.

OECD (Organisation for Economic Cooperation and Development), 2008. Guideline for the testing of chemicals: Acute oral toxicity – Up-and-Down-Procedure (UDP), Guideline 425. pp 1–27.

Ola-Mudathir, K.F., Suru, S.M., Fafunso, M.A., Obioha, U.E., Faremi, T.Y., 2008. Protective roles of onion and garlic extracts on cadmium-induced changes in sperm characteristics and testicular oxidative damage in rats. Food Chem. Toxicol. 46, 3604–3611.

Pedraza-Chaverri, J., Yam-Canul, P., Chirino, Y.I., Sánchez-González, D.J., Martínez-Martínez, C.M., Cruz, C., et al., 2008. Protective effects of garlic powder against potassium dichromate-induced oxidative stress and nephrotoxicity. Food Chem. Toxicol. 46, 619–627.

Peinado, M.J., Ruiz, R., Echávarri, A., Rubio, L.A., 2012. Garlic derivative PTS-O is effective against broiler pathogens *in vivo*. Poult. Sci. 91, 2148–2157.

Peinado, M.J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., Rubio, L.A., 2013. Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. Anim. Feed Sci. Technol. 181, 87–92.

Ramos, M., Jiménez, A., Peltzer, M., Garrigós, M.C., 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. J. Food Eng. 109, 513–519.

Ramos-Morales, E., Martínez-Fernández, G., Abecia, L., Martin-García, A.I., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2013. Garlic derived compounds modify ruminal fatty acid biohydrogenation and induce shifts in the *Butyrivibrio* community in continuous-culture fermenters. Anim. Feed Sci. Technol. 184, 38–48.

Romeilah, R.M., Fayed, S.A., Mahmoud, G.I., 2010. Chemical compositions, antiviral and antioxidant activities of seven essential oils. J. Appl. Sci. Res. 6 (1), 50–62.

Ruiz, R., García, M.P., Lara, A., Rubio, L.A., 2010. Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota *in vitro*. Vet. Microbiol. 144, 110–117.

Sakamoto, K., Lawson, L.D., Milner, J.A., 1997. Allyl sulfides from garlic suppress the *in vitro* proliferation of human A549 lung tumor cells. Nutr. Cancer 29 (2), 152–156.

Sallam, K.I., Ishioroshi, M., Samejim, K., 2004. Antioxidant and antimicrobial effects of garlic in chicken sausage. LWT – Food Sci. Technol. 37, 849–855.

Sigounas, G., Hooker, J.L., Li, W., Anagnostou, A., Steiner, M., 1997. S-Allylmercaptocysteine, a stable thioallyl compound, induces apoptosis in erythroleukemia cell lines. Nutr. Cancer 28 (2), 153–159.

Su, Y.C., Liu, C., 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol. 24 (6), 549–558.

Sundaram, S.G., Milner, J.A., 1996. Diallyl disulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice. J. Nutr. 126, 1355–1361.

Tsai, C.W., Chen, H.W., Sheen, L.Y., Lii, C.K., 2012. Garlic: health benefits and actions. Biomedicine 2, 17–29.

Wilson, E.A., Demmig-Adams, B., 2007. Antioxidant, anti-inflammatory, and antimicrobial properties of garlic and onions. Nutr. Food Sci. 37 (3), 178–183.

Yin, M.C., Hwang, S.W., Chan, K.C., 2002. Nonenzymatic antioxidant activity of four organosulfur compounds derived from garlic. J. Agric. Food Chem. 50, 6143–6147.

Zhang, W., Xiao, H., Parkin, K.L., 2014. Apoptosis in MCF-7 breast cancer cells induced by S-alkenylmercaptocysteine (CySSR) species derived from *Allium* tissues in combination with sodium selenite. Food Chem. Toxicol. 68, 1–10.

Zohril, A.N., Abdel-Gawadl, K., Saber, S., 1995. Antibacterial, antidermatophytic and antitoxigenic activities of onion (*Allium cepa L.*) oil. Microbiol. Res. 150, 167–172.

CAPÍTULO 5 / CHAPTER 5

María Llana-Ruiz-Cabello, Daniel Gutiérrez-Praena, Silvia Pichardo, F. Javier Moreno, José M. Bermúdez, Susana Aucejo, Ana M. Cameán

CYTOTOXICITY AND MORPHOLOGICAL EFFECTS INDUCED BY CARVACROL AND THYMOL ON THE HUMAN CELL LINE CACO-2.

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Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2

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ABSTRACT

Essential oils used as additives in the food industry due to its flavour, antimicrobial and antioxidant properties. Therefore, human can be exposed orally to these compounds through the ingestion of foods. In this sense, the present work aims to assess toxicological effects of oregano essential oil on the digestive tract. In concrete, the cytotoxic effects of two components of the oregano essential oils, carvacrol and thymol, and their mixture, on the intestinal cells line Caco-2 after 24 and 48 h of exposure are studied. The basal cytotoxicity endpoints assayed (total protein content, neutral red uptake and the tetrazolium salt reduction) and the annexin/propidium iodide staining indicated that carvacrol and the mixture carvacrol/thymol induced toxic effects. Moreover, a morphological study was performed in order to determine the ultrastructural cellular damages caused by these substances. The main morphological alterations were vacuolated cytoplasm, altered organelles and finally cell death. In addition, although no cytotoxic effects were recorded for thymol at any concentration and time of exposure, ultrastructural changes evidenced cellular damage such as lipid degeneration, mitochondrial damage, nucleolar segregation and apoptosis.

1. Introduction

Essential oils are aromatic oily liquids obtained from plant materials. They have been traditionally used in food products and perfume preparations because of their flavours. Many species of *Origanum* have a high amount of phenolic contents in their essential oils (Özkan and Erdoğan, 2011). In this sense, carvacrol and thymol have been reported as major phenolic constituents of many of the essential oils of oregano species (Ündeger et al., 2009). Carvacrol (5-isopropyl-2-methylphenol) is the predominant monoterpenic phenol, and it presents antibacterial, antifungal, insecticidal, and antioxidant effects (Akalin and Incesu, 2011). Thymol (2-isopropyl-5-methylphenol) has been widely used as a general antiseptic in the medical practice, agriculture, cosmetics, and food industry (Szentandrassy et al., 2004). In this sense, due to these properties, carvacrol and thymol are being used as alternatives to synthetic chemical products to protect the ecological equilibrium (Bakkali et al., 2008). In addition, the relatively recent of interest of the consumers for in "green and organic" products has lead to a renewal of scientific interest in these substances (Burt, 2004).

On the other hand, a recent packaging technology named "active packaging" is being increasingly developed in the last years (Appendini and Hotchkiss, 2002; Suppakul et al., 2003). This technology consists in the incorporation of active agents into the packaging walls from which they are released to the food surface at a controlled rate (Cerisuelo et al., 2012). In this sense, the incorporation of carvacrol and thymol in food-packaging films allows the controlled release of these active substances into the food reducing the undesirable flavours caused in the case of the direct addition into food (Ramos et al., 2012).

As flavouring, carvacrol and thymol are normally used in foods at low concentrations. However, the use of these compounds in other applications such as in active packaging may require higher doses that will increase the concern regarding exposure to these compounds (Stammati et al., 1999). In addition, carvacrol and thymol can be used alone or in combination, for example in the treatment of oral infectious diseases (Ündeger et al., 2009). Therefore, the evaluation of this combination is also required in order to assess its safety. The few *in vivo* toxicity data available in the literature mainly concern acute and subacute effects on different animal species, and suggest that such compounds may not pose a risk to human health (Jenner et al., 1964; Hagan et al., 1964; Domaracky et al., 2007). However, the toxic mechanism of these compounds has not been completely elucidated so far. It seems

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that mitochondrial membranes are first damaged by permeabilization, resulting in a pro-oxidant status and induction of apoptosis thereafter (Bakkali et al., 2008; Deb et al., 2011; Yin et al., 2012).

Various *in vitro* models have been used to assess the toxicity of these essential oils and its constituents. In this sense, time and dose-dependent cytotoxic effects and morphological alterations on a mouse skeletal muscle cell line, CO25, exposed to carvacrol have been reported (Akalin and Incesu, 2011). Horváthová et al. (2006) also observed cytotoxic effects on the human cell lines HepG2 and Caco-2, which were not associated with a DNA-damaging effect. Bostancioglu et al. (2012) reported that three concentrations of *Origanum onites* essential oil (125, 250 and 500 μg/mL) could markedly inhibit cell viability and induced apoptosis of rat adipose tissue endothelial cells (RATECs) and rat embryonic fibroblasts (5RP7). Furthermore, cytotoxic effects and apoptosis induction were observed in human cervical cancer cell lines (HeLa and SiHa) exposed to carvacrol at the concentration of 50 mg/L after 48 h of exposure (Mehdi et al., 2011). In this sense, several authors have suggested that carvacrol induces apoptosis by direct activation of the mitochondrial pathway on HepG2 cells (Yin et al., 2012) and on human metastatic breast cancer cells (Arunasree, 2010). This mechanism of toxicity has been also found for thymol. Deb et al. (2011) reported apoptosis induced by thymol in the promyelotic cancer cell line HL-60 at concentrations of 75 and 100 μM after 24 h of exposure. Similarly, Hsu et al. (2011) found that human glioblastoma cells exposed during 24 h to 200, 400 and 600 μM of thymol died by an apoptotic pathway. However, García et al. (2006) found that thymol did not affect cellular viability in primary cultures of mouse cortical neurons at concentrations ranging from 0 to 1 mM.

A limited number of studies on tissue damages and alterations in cellular models exposed to carvacrol and thymol have been reported. In this sense, no studies regarding the ultrastructural alterations have been performed so far. The morphological alterations induced by carvacrol that have been observed include: inhibition in the cellular growing (Bostancioglu et al., 2012); rounding (Akalin and Incesu, 2011; Koparal and Zeytinoglu, 2003); cytoplasmic blebbing and detachment from the disk (Koparal and Zeytinoglu, 2003); and cell death mainly by apoptosis (Stammati et al., 1999; Yin et al., 2012; Bostancioglu et al., 2012).

Therefore, considering the increasing use of carvacrol and thymol in the food industry, like for instance in active food packaging, the use of the permanent intestinal cell line Caco-2 (a commonly used enterocytic model established from a human colon carcinoma) to assess the safety of these compounds is of great interest. Therefore, the present work aims to assess, for the first time, the cytotoxic effects caused by carvacrol, thymol and their mixture in the Caco-2 cell line. Moreover, a thorough morphological study was performed in order to determine ultrastructural cellular damages that could help to explain the mechanism of action of these substances.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma–Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Model systems

Caco-2 cell line derived from human colon carcinoma (ATCC® HTB-37) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 50 μg/ml gentamicin, 1.25 μg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by exclusion of Trypan Blue. Caco-2 cells were plated at density of 7.5×10^5 cells/ml to perform the experiments.

2.3. Test solutions

The range of carvacrol and thymol concentrations for the tests was selected considering the content of these active compounds to be incorporated in the packaging materials and the possible migration to the food. In this sense, the maximum concentration was calculated considering that the active compounds completely migrate from the active package into the food (worst scenario of exposure).

Following that a 10% of the oregano essential oil (El Jarpil S.L., Almería, Spain) will be incorporated in 5.5–6.5 g of packaging film needed to pack 1 kg of food, and following that this oregano essential oil contains a 56% of carvacrol and 5% thymol (proportion around 10:1), the potential concentration released to the food will be around 2500 μM and 250 μM for carvacrol and thymol, respectively.

Serial test solutions of carvacrol (0–2500 μM), thymol (0–250 μM), and their combination in the proportion 10:1 (0–2500:0–250 μM), were prepared from both stock solutions of carvacrol (2.5 M) and thymol (0.5 M) in DMSO, being the final concentration in DMSO bellow 0.1%.

2.4. Cytotoxicity assays

Culture medium with 0.1% DMSO was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37 °C for 24 and 48 h according to INVITOX cytotoxicity protocols. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR), and tetrazolium salt reduction (MTS).

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage in independence of the toxic mechanism involved (Pichardo et al., 2007). PC was analysed *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 (BioRad, Madrid, Spain) in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the percentage of cells present in the culture in comparison to the control group.

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, neutral red (NR) (Sigma–Aldrich, Madrid, Spain) in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. The NR can be extracted from lysosomes for quantitative measurement of cells viability and cytotoxicity of xenobiotics (Zhang et al., 1990).

MTS reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good biomarker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) tetrazolium compound (Promega Biotech Ibérica, Madrid, Spain) added to the medium is bioreduced by cells into a coloured formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 h of incubation in the dark.

The concentrations used in further assays were calculated based on the cytotoxicity study. The EC_{50} values obtained in the most sensitive endpoint for carvacrol and its mixture with thymol at 24 h of exposure, MTS reduction in both cases, were chosen as the highest exposure concentration for the detection of apoptosis and the morphological study, along with the fractions $EC_{50}/2$ and $EC_{50}/4$. In the case of thymol, since no cytotoxic effect was recorded, the highest concentration used and its fractions were maintained for further studies.

2.5. Detection of apoptosis by flow cytometry

The detection of apoptosis was performed following the method described by Cárdeno et al. (2013) with modifications. Briefly, Caco-2 cells (7.5×10^5 cells/mL) were seeded in 6-well plates. After 24 h of incubation, cells were treated in presence of different concentrations of carvacrol (115, 230 and 460 μM), thymol (62.5, 125, and 250 μM), and their mixture in the selected proportion (75:7.5, 150:15, and 300:30 μM). Moreover, a negative control without the presence of any compound, a positive control with the presence of curcumin 40 μM (Sigma–Aldrich, Madrid, Spain), and a 1% DMSO control were prepared. After 24 h of exposure, media were collected and cells were detached by trypsinization (0.05% Trypsin–EDTA; Gibco, Paisley, United Kingdom) and collected in 0.5 mL of MEM without serum. Afterwards, cells were centrifuged at 4500 rpm during 3 min at 4 °C, resuspended and washed with ice-cold PBS, centrifuged, and resuspended in ice-cold 1× binding buffer (BB) to 5×10^5 cells/mL. Cells were incubated with 25 μL/mL Annexin V-FICT and 20 μg/mL propidium iodide (PI) solution (Annexin V-FICT Apoptosis Detection Kit, eBioscience, Vienna, Austria). Four different groups of cells were obtained based on their stainability: those unstainable with annexin V or PI [annexin(−)/PI(−)]: viable cells (quadrant E3); those stainable with annexin V but unstainable with PI [annexin(+)/PI(−)]: early apoptotic cells (quadrant E4); those

stainable with both annexin V and PI [annexin(+)/PI(+)]: late apoptotic cells (quadrant E2); and those unstainable with annexin V but stainable with PI [annexin(−)/PI(+)]: primary necrotic cells (quadrant E1). The untreated population was used to define the basal level of apoptotic and dead cells.

2.6. Morphology

Cells were exposed to different concentrations of carvacrol (115, 230 and 460 μM), thymol (75, 125, 250 μM) and the mixture of carvacrol/thymol 10:1 (75:7.5; 150:15; 300:30 μM) during 2, 4, 6, 24 and 48 h of exposure. Afterwards, cultured cells were fixed directly in the cell culture dish in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4 °C. They were all postfixed in 1% osmium tetroxide for 60 min at 4 °C. Subsequently, cells were scraped and pelleted by centrifugation. Samples were washed in 0.1 M cacodylate buffer, pH 7.3. Then, samples were dehydrated in ethanol at progressively higher concentrations and embedded in Epon (epoxy embedding medium). Toluidine blue-stained semi-thin sections (0.5 μm thick) used as controls were viewed in a Leitz (Aristoplan) light microscope. The presence of lipids was confirmed by Sudan III cytochemical technique.

Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope (FEI, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV. The cell growth and development of morphology damage was checked using a Leica DMIL inverted microscope by phase contrast.

2.7. Calculations and statistical analysis

All experiments were performed three times and in duplicate per concentration. Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. The distribution of data was checked using Kolmogorov and Smirnov's test, and all the values passed the normality test. Considering that variances were not homogeneous (tested by Bartlett's test), a non parametric statistical frame has been performed. Briefly, a Kruskal–Wallis test was carried out. Differences were considered significant from $p < 0.05$. If those tests were statistically significant, multiple comparisons were performed using Bonferroni/Dunn's Multiple Comparison Tests (the level of significance was set at $\alpha = 0.00625$ for a comparison to be significant).

EC_{50} values (mean effective concentration, concentration that modified each biomarker by 50%, positive or negative, in comparison with appropriate untreated controls) were determined by probit analysis.

In order to compare the EC_{50} values from 24 and 48 h the analysis of variance (ANOVA) was used, followed by Dunnett's multiple comparison tests. The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test and Bartlett's test, respectively. Differences were considered significant from $p < 0.05$.

3. Results

3.1. Results of the cytotoxicity assays

Caco-2 cells exposed to carvacrol underwent a time-dependent decrease in all the endpoints studied. The PC assay showed significant changes respect to the control from 750 μM for 24 h ($p = 0.0043$) and from 500 μM for 48 h ($p = 0.0022$) (Fig. 1A). Similarly, NR uptake and MTS metabolism performed by Caco-2 cells exposed to carvacrol also indicated significant reductions in the cellular viability from 500 μM for 24 and 48 h ($p = 0.0043$ in NR 24 h and $p = 0.0022$ in NR 48 h and MTS in both exposure times) (Fig. 1B and C). The EC_{50} values obtained for all the endpoints also evidenced a greater effect in the cells exposed a longer time (Table 1). After 24 h of exposure EC_{50} were significantly different from those observed at 48 h ($p < 0.0001$ in all the endpoints).

On the contrary, cells exposed to thymol remained unaltered with respect to the control cells after 24 and 48 h for all the endpoints assayed (Fig. 2).

Cells exposed to the mixture carvacrol/thymol, in a proportion 10:1, experienced a higher time-concentration decrease in comparison to those exposed only to carvacrol. The PC and MTS assays revealed significant differences from 500:50 μM for 24 h ($p = 0.0043$ for PC and $p = 0.0022$ for MTS) and from 100:10 μM for 48 h ($p = 0.0022$ for both endpoints) (Fig. 3A and C). The NR uptake assay appeared to be more sensitive, showing significant changes respect to the control from 100:10 μM for both exposure times ($p = 0.0022$ for 24 h an $p = 0.0043$ for 48 h) (Fig. 3B). The EC_{50} values are included in Table 1. All the EC_{50} values determined at 24 h were significantly different from those observed at 48 h ($p < 0.0001$ in all the endpoints).

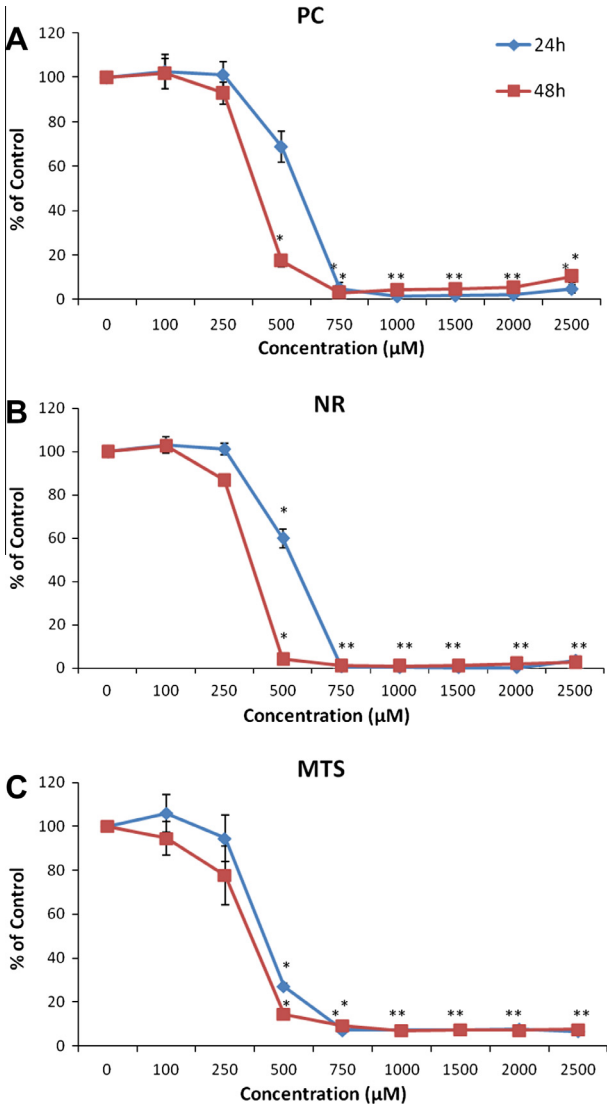

Fig. 1. Protein content, TP (A); neutral red uptake, NR (B); and reduction of tetrazolium salt, MTS (C) on Caco-2 cells after 24 h and 48 h of exposure to 0–2500 μM carvacrol. All values are expressed as mean ± s.d. *Significantly different from control ($p \leqslant 0.05$).

Table 1
EC_{50} values obtained in Caco-2 cells exposed to carvacrol and the mixture carvacrol/thymol for 24 and for 48 h. Data are expressed as μM ± S.D.

	PC (μM)	NR (μM)	MTS (μM)
Carvacrol			
24 h	543 ± 2.6	518.8 ± 5.9	460 ± 3.6
48 h	377.4 ± 6.8	340.1 ± 3.4	343 ± 7.4
Mixture			
24 h	360 ± 0.8:36 ± 1.3	385.6 ± 3.7:38.5 ± 2.1	300 ± 1.5:30 ± 0.3
48 h	209 ± 3.9:20.9 ± 0.5	186.8 ± 6.4:18.6 ± 1.5	105.5 ± 2.6:10.5 ± 0.9

3.2. Detection of apoptosis

An annexin-V/PI double staining assay was performed to detect apoptotic and necrotic cells after exposure to different

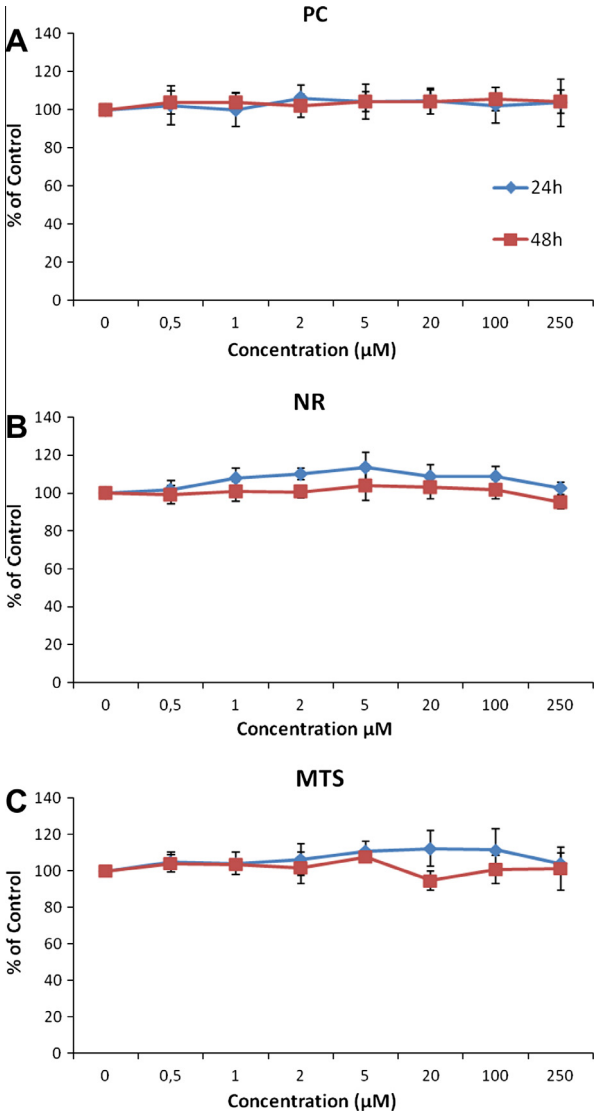

Fig. 2. Protein content, TP (A); neutral red uptake, NR (B); and reduction of tetrazolium salt, MTS (C) on Caco-2 cells after 24 h and 48 h of exposure to 0–250 μM thymol. All values are expressed as mean ± s.d. *Significantly different from control ($p \leqslant 0.05$).

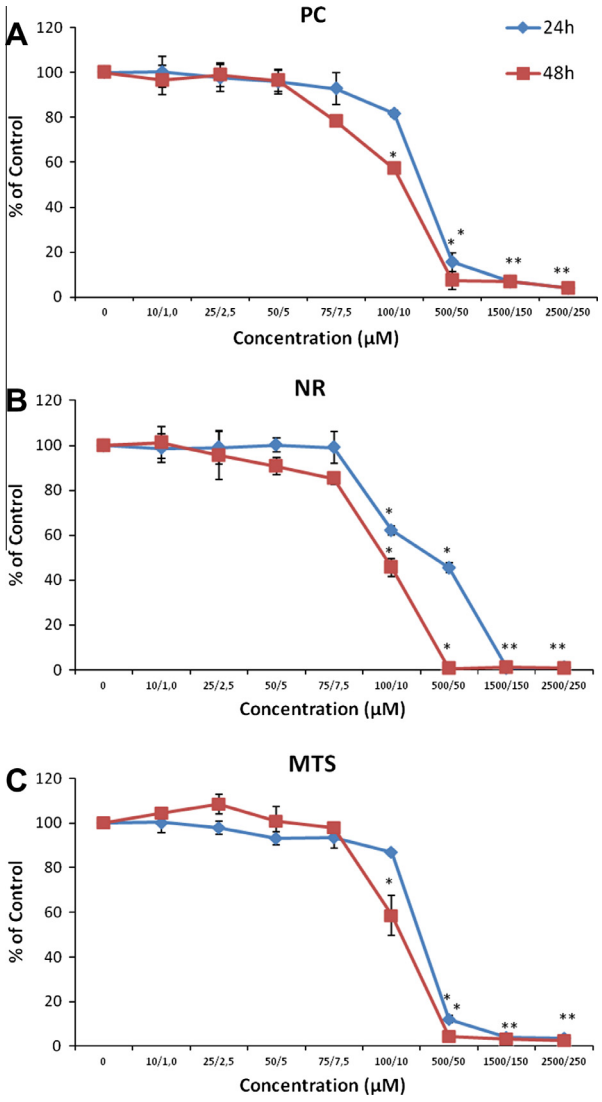

Fig. 3. Protein content, TP (A); neutral red uptake, NR (B); and reduction of tetrazolium salt, MTS (C) on Caco-2 cells after 24 h and 48 h of exposure to 0–2500:0–250 μM carvacrol/thymol. All values are expressed as mean ± s.d. *Significantly different from control ($p \leqslant 0.05$).

concentrations of carvacrol, thymol, and their mixture during 24 h. In the case of exposing the cells to carvacrol, a significant increase respect to the control group of early apoptosis appeared only at the concentration of 115 μM. At 230 and 460 μM, significant changes appeared in late apoptosis and necrosis stages, although at the highest concentration assayed most of the cells may die via necrosis (Fig. 4A). In the case of thymol, it did not produce any apoptotic or necrotic situations at any of the concentrations assayed (62.5, 125, and 250 μM) in comparison to the control group (Fig. 4B). In the case of exposing the cells to the mixture of carvacrol and thymol, significant changes were observed in late apoptosis and necrosis at the combination of 150:15 μM carvacrol/thymol, and in necrosis at 300:30 μM carvacrol/thymol (Fig. 4C).

3.3. Results of the morphology study

3.3.1. Electron microscope observation of control cells

Unexposed Caco-2 cells observed under microscope showed very heterogeneous phenotype depending on the passages of the culture studied. Moreover, in the culture cell, a great variability in morphology, size and morphological features of organelles could be distinguished. In order to exclude this finding, the morphological study carried out in the present work was performed only on the same passage and on cells showing the same characteristics, discharging aneuploids cells as well as cells undergoing ultrastructural feature changes.

Control cells showed high density of nuclear volume with loose chromatin structures. In the cytoplasm, free ribosomes were abundant as well as cisternae from the rough endoplasmic reticulum (RER) and mitochondria with scarce and thin transversal crests. Moreover, lipid drops and endosomic vesicles were also visible in the cytoplasm. Golgi apparatus was located near the nucleus, formed by scarce cisternae (Fig. 5A).

3.3.2. Electron microscope observation of cells exposed to carvacrol

Cells exposed to 115 μM carvacrol were able to grow in the culture, increasing the density in the plate frequently showing mitotic figures. No significant utrastructural changes were observed in Caco-2 cells after 24 h of exposure to 115 μM carvacrol compared to the control group. However, after 48 h the presence

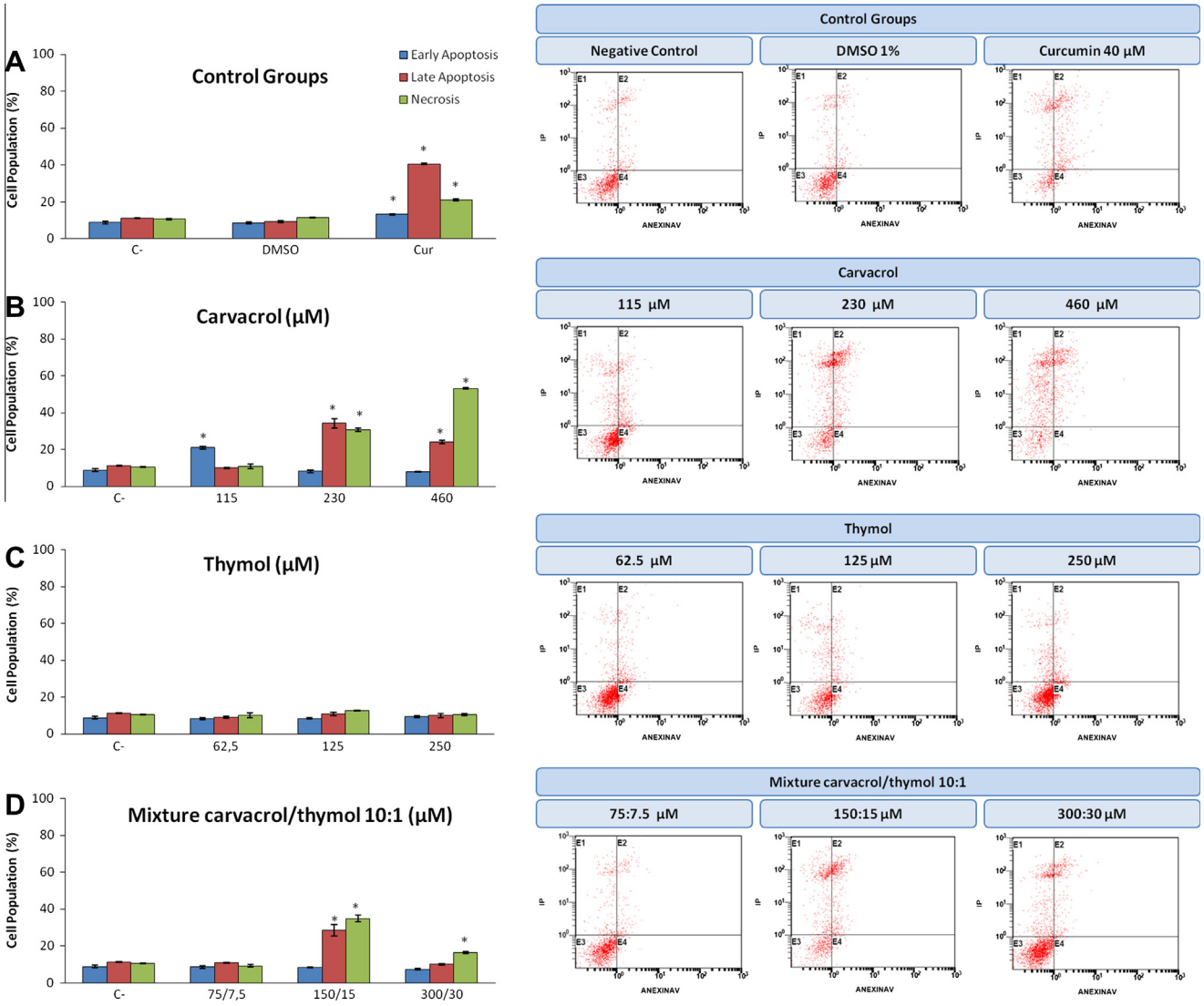

Fig. 4. Early and late apoptotic cells (quadrants E4 and E2, respectively) and primary necrotic cells (quadrant E1) detected by flow cytometry with Annexin V-FICT conjugated with PI staining after 24 h of exposure to 460, 230, and 115 µM of carvacrol (A), 250, 125, and 62.5 µM of thymol (B), and their mixture in the selected proportion 300:30, 150:15, and 75:7.5 µM (C). Moreover, a negative control without the presence of any compound, a positive control with the presence of curcumin 40 µM, and a DMSO 1% control were used.

of lysosomes in the cytoplasm increased as well as the alteration of the mitochondrial crests (Fig. 5B and 5C). After 24 h of exposure to 230 µM carvacrol, an increase in the chromatin condensation and invagination in the nuclear covering were observed. In the cytoplasm, an increase in lipid drops, disorganized mitochondrial crest and presence of autophagic vesicles were observed (Fig. 5D). In the plasmatic membrane, fusion of lipid drops could be observed resulting in exocytic processes (Fig. 5E). Moreover, intense vacuolization in the cytoplasm was observed, ending up the fusion of these vacuoles in a segmentation of cytoplasm which was released to the culture media (Fig. 5F). These morphological features were related to the apoptotic cellular process.

When cells were exposed to the highest concentration of carvacrol (460 µM), damage was observed in the first 2 h of exposure. Mitochondria were enlarged with disorganized crests, forming membranous figures in the interior (Fig. 6A). In addition, dilatation in the cisternae from the RER was observed (Fig. 6B). The cellular damage observed after 4 h of exposure to the highest concentration of carvacrol was similar to those previously described for the medium concentration exposed for 24 h, highlighting dilation of cisternae from the Golgi apparatus (Fig. 6C). Irregular cellular edges formed by globular cytoplasmic projections were also distinguished (Fig. 6D). After 6 h of exposure to 460 µM lipid drops were more abundant, which were fused into lipid bodies with irregular borders. Additionally, the appearance of membranous figures which will turn into heterophagosomes was observed (Fig. 6E). A remarkable decrease in the cellular growth including cell death was observed when they were in contact with 460 µM of carvacrol for 24 and 48 h. The scarce living cells showed vacuolated cytoplasm with plenty lipid drops (Fig. 6F). In the culture cells, most of cells lost their adhesion detaching from the flask and dying via necrosis. (Fig. 6G).

3.3.3. Electron microscope observation of cells exposed to thymol

Cells exposed to 75 µM of thymol, showed a higher differentiation degree than unexposed cells after 24 and 48 h. Also, irregular thread-like projections covered by plasmatic membrane were observed in the apical cytoplasm, which were similar to microvilli. Moreover, glycogen aggregates linked to lipid drops were observed (Fig. 7A). In the cytoplasm, mitochondria with light matrix and these irregular crests were shown. Lipid drops were frequently associated to cisternae from the RER. Some ultrastructural features

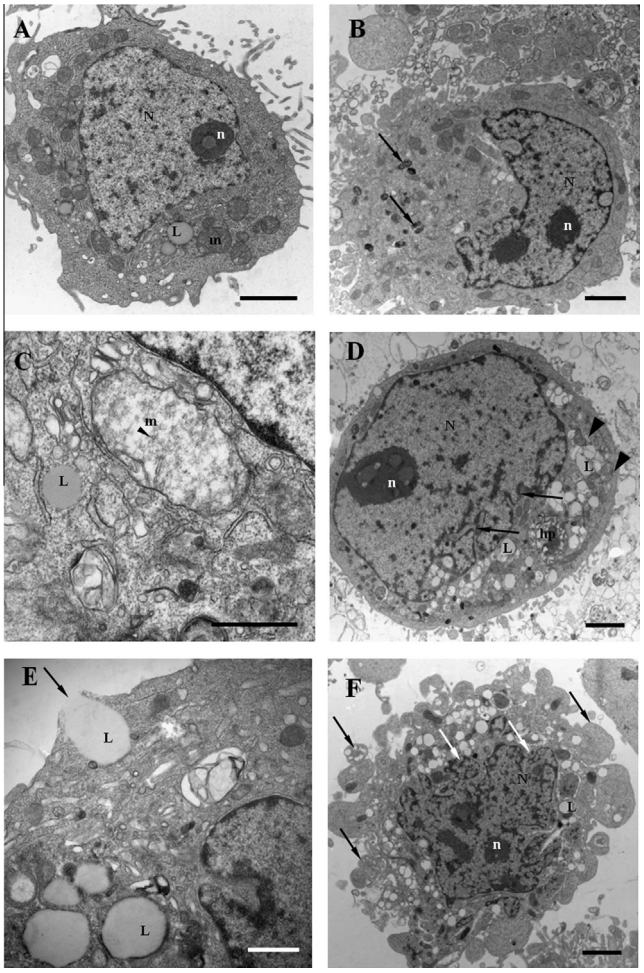

Fig. 5. Morphology of Caco-2 cells after 24 h (A, D, E) and 48 h (B, C, F) of treatment observed by electron microscopy. Bars (A, B, D) = 2 μm. Bars (B, E, F) = 1 μm. Unexposed control cultures (A) and Caco-2 cells exposed to 115 μM (B, C) and 230 μM carvacrol (D, E, F). (A) Unexposed cells with high density in the nucleus (*N*) and lipid drops (*L*); nucleolus (*n*) and mitochondria (*m*) are also visible. (B) Increase presence of lysosomes in the cytoplasm (black arrow). (C) Alteration of the mitochondrial crests (black arrow head) and lipid drops (L). (D) Increase in the chromatin condensation (*N*) and in lipid drops are observed (L); as well as invagination in the nuclear covering (black arrow), disorganized mitochondrial crest (black arrow head) and presence of autophagic vesicles (hp). (E) Fusion of lipid drops (*L*) resulting in exocytic processes (black arrow). (F) Chromatin condensation (*N*) showing the nucleus an irregular bordering (white arrow). Intense vacuolization in the cytoplasm (black arrow).

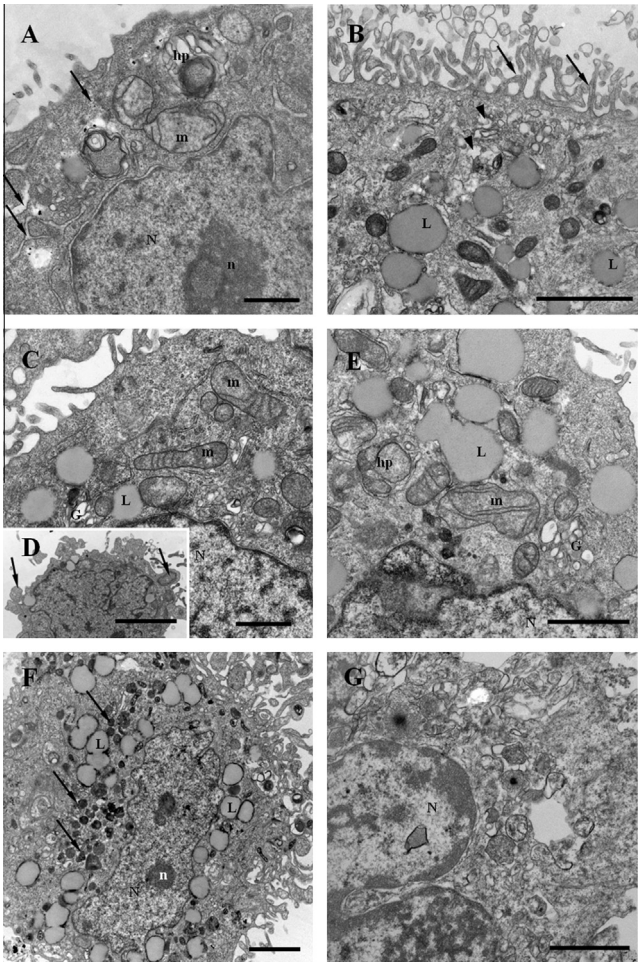

Fig. 6. Morphology of Caco-2 cells exposed to 460 μM carvacrol for 2 h (A and B), 4 h (C and D), 6 h (E), 24 h (F) and 48 h (G) observed by electron microscopy. Bars (A, C, D) = 1 μm. Bars (B, E, F, G) = 2 μm. (A) Enlarged mitochondria are with disorganized crests (m) and dilation of cisternae from the RER (black arrow). (B) Increase in lipid drops (*L*), dilatation in the cisternae from the RER (black arrow head), and presence of microvilli (black arrow). (C) Mitochondrial hypertrophy with disorganized crests (*m*), presence of lipid drops (*L*) and dilation of cisternae from the Golgi apparatus (G). (D) Irregular cellular edges formed by globular cytoplasmic projections (black arrow). (E) Fusion of lipid drops (*L*), dilated cisternae from the Golgi apparatus (G), and presence of heterophagosomes (hp). (F) Vacuolated cytoplasm with plenty lipid drops (*L*) and heterophagosomes (black arrow). (G) Necrotic cell.

that were observed in relation to the differentiation process were the presence of intermediate filaments, and membrane differentiations as desmosomes allowing interaction between cells (Fig. 7B). The increase in lipid drops in the cytoplasm and dilatation of cisternae from the Golgi apparatus were the most remarkable morphological changes observed in cells exposed to 125 μM thymol after 24 and 48 h (Fig. 7C). Moreover, vesicles from the Golgi apparatus were fused forming autophagosomes (Fig. 7D).

The damage previously described was enhanced after 24 h of exposure to 250 μM of thymol (Fig. 8A). Moreover, cells showed compact nucleoli where fibrillar centres and dense fibrillar component were clearly observed (Fig. 8B). Segregation of nucleolar components (Fig. 8C) as well as lipid drops with tendency to confluence releasing the content to the extracellular media by microexocytic mechanism (Fig. 8D) were observed when cells were exposed to 250 μM of thymol for 48 h. In addition, the changes previously described on mitochondria were also found after 24 h. Finally, cells showed condensation of chromatin, lobules in the nucleus, increase of lipid drops and vacuolization of the cytoplasm with dilatation of the cisternae from the RER, evidencing the apoptotic process (Fig. 8E and F).

3.3.4. Electron microscope observation of cells exposed to the mixture carvacrol/thymol

Cells exposed to the lowest concentration of the mixture carvacrol/thymol (75:7.5 μM) for 24 h showed a large nucleus with irregular borders and invaginations (Fig. 9A). Fibrillar centres and dense fibrillar component could be distinguished in the large compact nucleoli. A large development in the cisterns from the RER is shown, that were dividing the cytoplasm was observed after 48 h of exposure. Moreover, mitochondria with dense matrix were also observed (Fig. 9B). Cells were largely vacuolated when exposed to the concentration of 150:15 μM carvacrol/thymol for 24 and 48 h (Fig. 9C). Also, cell membrane formed lobes involving segregation and release of cytoplasm portions (Fig. 9C). On the other hand, the highest concentration of the mixture carvacrol/thymol assayed (300:30 μM) induced greater damage than the lower concentrations (Fig. 9D).

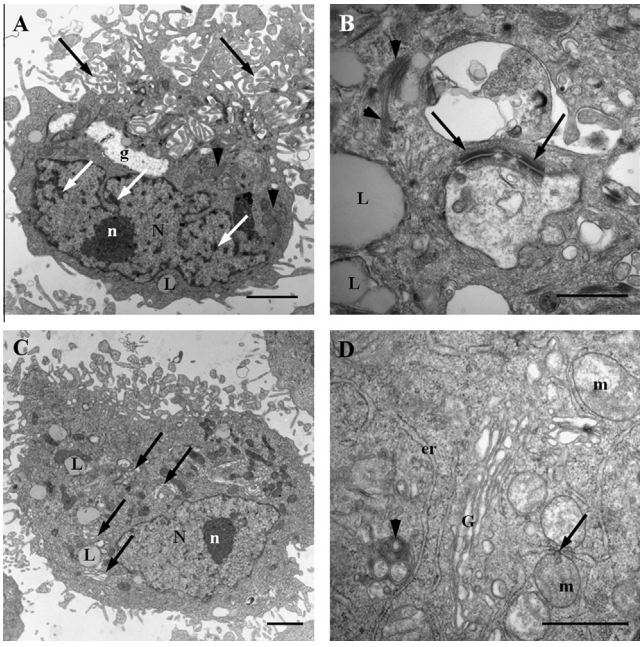

Fig. 7. Morphology of Caco-2 cells after 24 h (A and C) and 48 h (B and D) of treatment observed by electron microscopy. Bars (A and C) = 2 μm. Bars (B and D) = 1 μm. Caco-2 cells exposed to 62.5 μM thymol (A and B) and 125 μM thymol. (A) Apical cytoplasm with microvilli (black arrow), irregular bordering in the nucleus (*N*), heterochromatin (white arrow), glycogen aggregates linked to lipid drops (*g*), and damaged mitochondria (black arrow head). (B) Lipid drops associated to the RER (*L*), presence of intermediate filaments (black arrow head), and desmosomes (black arrow). (C) Increase in lipid drops (*L*) and dilatation of cisternae from the Golgi apparatus (black arrow). (D) Mitochondria with destroyed crest (*m*) fused with vesicles from the Golgi apparatus (black arrow) and autophagosomes (black arrow head).

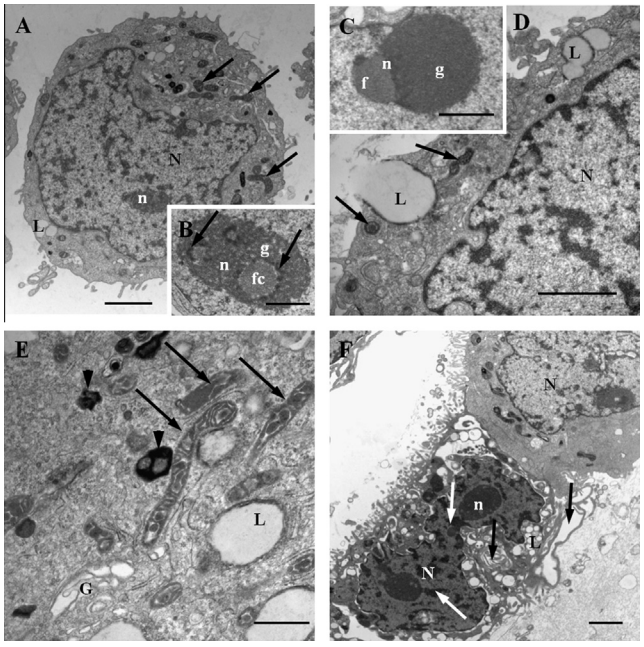

Fig. 8. Morphology of Caco-2 cells exposed to 250 μM thymol for 24 h (A and B) and 48 h (C, D, E and F) observed by electron microscopy. Bars (A, D, F) = 2 μm. Bars (B, C, E) = 1 μm. (A) Increase of lipid drops (*L*) and dilated mitochondria (black arrow). (B) Compact nucleoli (*n*) with fibrillar centres (fc), dense fibrillar component (arrow) and granular component (*g*). (C) Segregation of nucleolar components (*n*), fibrillar component (*f*) and granular component (*g*). (D) Lipid drops with tendency to confluence which are excreated (*L*) and dilated mitochondria (black arrow). (E) Damaged mitochondria (black arrow), lipid drops (*L*), dilatation of cisternae from the Golgi apparatus (G), and presence of autophagosomes (black arrow head). (F) Condensation of chromatin (white arrow) and presence lobules in the nucleus (*N*), increase of lipid drops (*L*) and vacuolization of the cytoplasm with dilatation of the cisternae from the RER (black arrow).

Cells showed an intense vacuolation due to the dilatation of the cisterns from the RER. The percentage of living cells decreased to 4–7% after 24 h of exposure.

4. Discussion

The incorporation of natural essential oils with known antimicrobial and antioxidant properties into polymeric packaging materials is an innovative technology aiming to increase the shelf-life of food products. In this sense, the oregano essential oil shows very promising properties for this type of application due to their main components; carvacrol and thymol. These compounds, which have been traditionally used as flavouring in food, are categorised as *Generally Recognised as Safe* (GRAS) by US Food and Drug Administration (López et al., 2007). However, due to the new application in the food industry, the intake of carvacrol and thymol may increase, and therefore an accurate toxicological assessment is required. In this concern, the present work aims to study the toxic effects of carvacrol, thymol and their mixture in the intestinal cell line Caco-2, since gastrointestinal tract would be the first target of these compounds when consumed. Previous studies evidenced that essential oil of oregano and its components, carvacrol and thymol, slightly increased the incidence of apoptotic cell death at concentrations used for antimicrobial purposes on Caco-2 cells (Dusan et al., 2006). However, doses of essential oils able to completely inhibit bacterial growth showed also relatively high cytotoxicity to Caco-2 cells (Dusan et al., 2006). Moreover, pro-apoptotic effects of ethanolic extracts from *Origanum vulgare* on Caco-2 cells have been reported (Savini et al., 2009). Nevertheless, no study of the mixture of carvacrol and thymol has been performed so far. In addition, the intracellular target of carvacrol and thymol remains unknown.

The results of the present study revealed a concentration- and time-dependent decrease in all the endpoints assayed for carvacrol and the mixture carvacrol/thymol, although no effect was recorded for thymol in the cytotoxicity study. García et al. (2006) also reported that thymol (0–1 mM) did not affect cellular viability in primary cultures of mouse cortical neurons measured by the MTT and LDH leakage assays. However, in the hepatic cell line HepG2, the essential oil of oregano resulted to be less cytotoxic than carvacrol and thymol by themselves (Özkan and Erdoğan, 2011). Similarly, a dose-dependent decrease of NR uptake and PC was observed in HepG2 cells exposed to carvacrol and thymol (Stammati et al., 1999). Horváthová et al. (2006) found that HepG2 cells were more sensitive than Caco-2 cells after 24 h of exposure to carvacrol and thymol. These discrepancies can be partially due to the different metabolic ability of each cell line. It has been shown that Caco-2 cells are able to glucuronidate various aglycones, including thymol. Intestinal glucuronidation represents the first metabolic line of defense against ingested toxic xenobiotics, and it could also affect the cytotoxicity of thymol in our experiments (Dusan et al., 2006). Moreover, different effects were also recorded in different cells exposed to carvacrol. In this sense, cytotoxic effects were observed in HepG2 cells exposed to 0.05–0.4 mM of carvacrol, while no changes in the MTT assay were recorded in another hepatic cell line, LO2 (Yin et al., 2012). Moreover, no cytotoxic effects were found on primary fibroblasts and colonocytes when exposed to 300 μg/mL of oregano extract for 12, 24, 48 and 72 h, whereas Caco-2 cells decreased significantly their viability measured by MTT reduction after 24 h of exposure (Savini et al., 2009). Similarly, a concentration- and time-dependent decrease on cell viability in 5RP7 and RATEC cells exposed to 125, 250 and 500 μg/mL of oregano essential oil after 24, 48, 72 and 96 h was observed, resulting

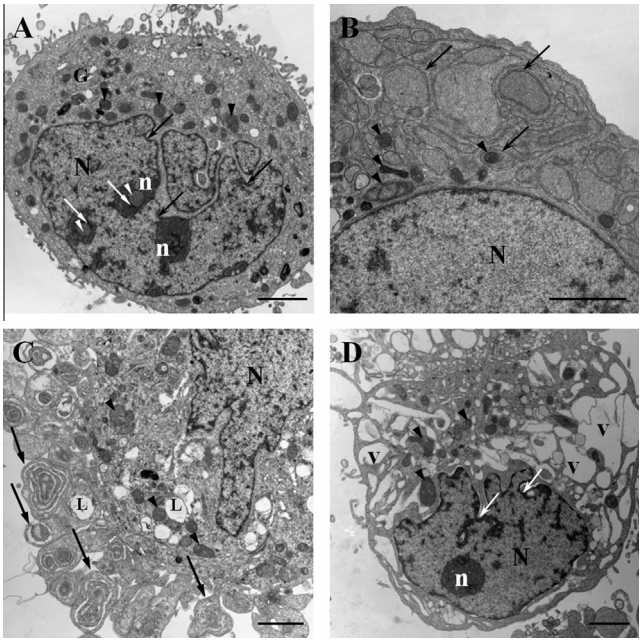

Fig. 9. Morphology of Caco-2 cells after 24 h (A and D) and 48 h (B and C) of treatment observed by electron microscopy. Bars = 2 μm. Caco-2 cells exposed to a mixture of carvacrol/thymol of 75:7.5 μM (A and B), 150:15 μM (C) and 300:30 μM. (A) Large nucleus with irregular borders and invaginations (black arrow), large compact nucleoli (*n*) with fibrillar centres (white arrow head) and dense fibrillar component (white arrow), altered mitochondria undergoing autophagy (black arrow head). (B) Cisterns from the RER well developed (black arrow) and mitochondria with dense matrix (black arrow head). (C) Cell membrane lobes (black arrow), lipid drops (*L*) and altered mitochondria in autophagy process (black arrow head). (D) Intense vacuolation (*V*), invagination in the nuclear membrane (white arrow) and altered mitochondria (black arrow head).

more toxic in 5RP7 cells, which is a cancer cell line (Bostancioglu et al., 2012). This latter cell line also underwent concentration- and time-dependent cytotoxic effects when exposed to carvacrol, as well as CO25 cells, although 5RP7 cells were more sensitive (Akalin and Incesu, 2011). Additionally, other cell lines such as SiHa, HeLa (Mehdi et al., 2011) and MDA-MB (Arunasree, 2010) have suffered cytotoxic effects when exposed to carvacrol. Considering these differences in the sensibility the use of a cell line representative of the exposure pathway for these compounds is essential for a suitable toxicity assessment.

It is interesting to point out that cell viability was more affected when Caco-2 cells were exposed to the mixture carvacrol/thymol in comparison to individual exposure to carvacrol. The synergism study could not be performed since no cytotoxic effects were recorded in Caco-2 cells exposed to thymol. Synergism has been observed between carvacrol and its precursor p-cymene in the antibacterial activity (Ultee et al., 2000). However, no synergic effect has been reported so far for carvacrol and thymol, although Bakkali et al. (2008) stated that it is possible that the activity of the main components of essential oils could be modulated by other minor molecules. In this sense, carvacrol and thymol were found to give an additive effect when tested against different bacterial species (Lambert et al., 2001). Moreover, the mixture carvacrol/thymol provokes a higher late apoptosis and necrosis at 150/15 than at 300/30. This finding could be explained because the damage induced in the highest concentration may cause debris of dead cells which is discharged in the annexin-V/PI staining assay. As consequence, lower apoptosis and necrosis was recorded in the concentrations of 300/30 in comparison to 150/15.

Cell death induced by oregano essential oil and its components, carvacrol and thymol, has been extensively studied in several culture cells. Oregano extract have been reported to induce both apoptosis and necrosis in Caco-2 cells (Dusan et al., 2006; Savini et al., 2009), RATECs cells (Bostancioglu et al., 2012), 5RP7 cells (Akalin and Incesu, 2011; Bostancioglu et al., 2012), CO25 cells (Akalin and Incesu, 2011) and human leukemic cells (Abdel-Massiha et al., 2010). Also, the major component of oregano essential oil, carvacrol, can cause cell death by apoptosis and necrosis in Caco-2 cells (Dusan et al., 2006), HepG2 cells (Stammati et al., 1999; Yin et al., 2012), HeLa and SiHa cells (Mehdi et al., 2011), human oral cancer cells (Liang and Lu, 2012) and on human metastatic breast cancer cell line (Arunasree, 2010). However, different results have been reported for thymol. No remarkable effect was observed for thymol in HepG2 cells (Stammati et al., 1999) and primary cultures of mouse cortical neurons (García et al., 2006). Nevertheless, several authors have reported that thymol is able to induce cell death via apoptosis in HL-60 cells but not in human PBMC cells at concentrations up to 100 μM (Deb et al., 2011; Hsu et al., 2011). This induction is lower in comparison to cell death caused by oregano essential oil and carvacrol according to Dusan et al. (2006). In our study, apoptosis as well as necrosis were observed after 24 h of exposure to carvacrol and its mixture with thymol, but no significant cell damage was observed in thymol exposure in the annexin-V/PI staining assay. However, in the morphological study, cell death induction was also observed after to thymol exposure at the highest concentration assayed, as suggested by chromatin condensation, nuclear fragmentation and detaching.

The toxic mechanism of oregano essential oil is linked to its capacity of disrupting cytoplasmic membranes, especially mitochondrial membranes (Bakkali et al., 2008). Several authors have described minor morphological changes in non-intestinal cell lines exposed to carvacrol (Stammati et al., 1999; Koparal and Zeytinoglu, 2003; Arunasree, 2010; Akalin and Incesu, 2011); although they have not pointed any ultrastructural damage. However, our ultrastructural study made possible to identify other intracellular targets besides membranes and mitochondria such as nuclei, Golgi apparatus, and RER. In the present work, we described that Caco-2 cells treated with carvacrol increased the presence of lipid drops. The enhancement of lipid deposits could be a protective mechanism of the cells to survive by means of the recycling of the fatty acids from the membranes (Gubern et al., 2009). Therefore, our results agree the suggestion of cytoplasmic membranes being disrupted by oregano essential oil. In addition, apoptosis induced by carvacrol was observed in Caco-2 cells exposed to 230 μM for 24 h. However, only 4 h were enough for the highest concentration of carvacrol assayed (460 μM) to produce morphological features related to pre-apoptotic process such as globular cytoplasmic projections. Yin et al. (2012) suggested that the carvacrol-induced apoptosis in HepG2 cells may be caused by direct activation of the mitochondrial pathway. In this sense, another study established a relationship between the induction of apoptosis by carvacrol in MDA-MB231 cells and the decrease in the mitochondrial membrane potential of the cells, resulting in a cytochrome c release from mitochondria, caspase activation, and cleavage of poly(ADP-ribose)polymerase (Arunasree, 2010). This finding is in accordance to our results, which showed damage in mitochondria in the morphological study and that the MTS reduction assay was the most sensitive endpoint. MTS is reduced by mitochondrial dehydrogenases, so this endpoint can provide information about the damage induced in this organelle (Gutierrez-Praena et al., 2011). In addition, in the present study, cells also died by a necrotic pathway. This finding was observed in the flux cytometry study as well as in the morphological study. Caco-2 cells exposed to the highest concentration of carvacrol (460 μM) underwent detachment from the disk, cell rounding, and death by necrosis. Therefore, the apoptotic process started at the treatment with the medium concentration of carvacrol may turn into necrosis mechanism when cells are suspended in the culture media.

When Caco-2 cells were exposed to 250 μM of thymol for 24 h cells showed ultrastructural features of transcriptionally active cells (compact nucleoli where fibrillar centres and dense fibrillar component). However, after 48 h of exposure to this concentration, nucleolar segregation was found, evidencing stop in the transcription of ribosomic DNA and protein inhibition. Drug treatments leading to transcriptionally arrested cells induce a phenotype of segregation in which the fibrillar and granular components of nucleoli disengage and form three juxtaposed structures (Hernandez-Verdun et al., 2010). Hence, nucleolar segregation can be observed under some physiological conditions of transcriptional arrest (Shav-Tal et al., 2005). Therefore, in the present work when nucleolar segregation was observed, stop in the transcription of ribosomic DNA and protein inhibition have occurred. This finding is in accordance with the previous results obtained in human breast cells (Arunasree et al., 2010) and in mouse myoblast cells exposed to carvacrol (Zeytinoglu et al., 2003).

Finally, the present work has shown that the ultrastructural study is more sensitive than the cytotoxicity assays, and therefore, it can be used as an early indicator of the damage induced in cells. However, a morphological study is very time-consuming and requires an experienced observer in order to discern the damages caused, so it cannot be used as a rapid screening method. Mitochondria, nuclei, Golgi apparatus and RER have been identified as intracellular targets. Further studies are needed in order to deep in the mechanism of toxicity involved in these damaged observed.

5. Conclusions

In the present work, a significant decrease in the viability of the human intestinal Caco-2 cells was observed after exposure to carvacrol and its mixture with thymol in the cytotoxicity assays, being MTS metabolism the most sensitive endpoint. Moreover, the ultrastructural alterations in the colonic cell line Caco-2 exposed to two components of the oregano essential oil were described for the first time, highlighting mitochondrial damage, lipid degeneration, chromatin condensation as well as cell death via apoptosis and necrosis. Thus, considering our results, carvacrol and thymol can induce damage in the human intestinal cells *in vitro*. This finding is of great concern since these compounds can be used as food additives and as components in the development of active packaging materials. Therefore, further studies are needed in order to assess the toxicity on human and ensure its safety.

6. Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Abdel-Massiha, R.M., Faresa, R., Bazzia, S., El-Chamib, N., Baydoun, E., 2010. The apoptotic and anti-proliferative activity of *Origanum majorana* extracts on human leukemic cell line. Leuk. Res. 34, 1052–1056.

Akalin, G., İncesu, Z., 2011. The effects of carvacrol on apoptosis of H-ras and N-ras transformed cell lines. Turk. J. Pharm. Sci. 8 (2), 105–116.

Appendini, P., Hotchkiss, J.H., 2002. Review of antimicrobial food packaging. Innov. Food Sci. Emerg. 3, 113–126.

Arunasree, K.M., 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB231. Phytomedicine 17, 581–588.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – a review. Food Chem. Toxicol. 46, 446–475.

Baltrop, J.A., Owen, T.C., Cory, A.H., Cory, J.G., 1991. 5-((3-Carboxyphenyl)-3-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)) tetrazolium, inner salt (MTS) and related analogs of 2-(4,5-dimethylthiazolyl)-2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell-viability indicators. Bioorg. Med. Chem. Lett. 1, 611.

Borenfreund, E., Puerner, J.A., 1984. A simple quantitative procedure using monolayer culture for cytotoxicity assays. J. Tiss. Cult. Meth. 9, 7–9.

Bostancioglu, R.B., Kürkçüoglu, M., Baser, K.H.C., Koparal, A.T., 2012. Assessment of anti-angiogenic and anti-tumoral potentials of *Origanum onites* L. essential oil. Food Chem. Toxicol. 50, 2002–2008.

Bradford, M., 1976. A rapid sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burt, A., 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. Int. J. Food Microbiol. 94, 223–253.

Cárdeno, A., Sánchez-Hidalgo, M., Rosillo, M.A., Alarcón de la Lastra, A., 2013. Oleuropein, a Secoiridoid derived from Olive Tree, inhibits the proliferation of human colorectal cancer cell through downregulation of HIF-1α. Nutr. Cancer 65 (1), 147–156.

Cerisuelo, J.P., Alonso, J., Aucejo, S., Gavara, R., Hernandez-Muñoz, P., 2012. Modifications induced by the addition of a nanoclay in the functional and active properties of an EVOH film containing carvacrol for food packaging. J. Membr. Sci. 423–424, 247–256.

Deb, D.D., Parimala, G., Devi, S.S., Chakraborty, T., 2011. Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. Chem.-Biol. Interact. 193, 97–106.

Domaracky, M., Rehak, P., Juhas, S., Koppel, J., 2007. Effects of selected plant essential oils on the growth and development of mouse preimplantation embryos in vivo. Physiol. Res. 56, 97–104.

Dusan, F., Marián, S., Katarína, D., Dobroslava, B., 2006. Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. Toxicol. in Vitro 20, 1435–1445.

García, D.A., Bujons, J., Vale, C., Suñol, C., 2006. Allosteric positive interaction of thymol with the GABA receptor in primary cultures of mouse cortical neurons. Neuropharmacology 50, 25–35.

Gubern, A., Casas, J., Barceló-Torns, M., Barneda, D., de la Rosa, X., Masgrau, R., Picatoste, F., Balsinde, K., Balboa, M.A., Claro, E., 2009. Group IVA phospholipase A is necessary for the biogenesis of lipid droplets. J. Biol. Chem. 283, 27369–27382.

Gutierrez-Praena, D., Pichardo, S., Jos, A., Camean, A.M., 2011. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure Cylindrospermopsin. Ecotoxicol. Environ. Saf. 74, 1567–1572.

Hagan, H.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A., Brouwer, W.I., 1964. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. Food Cosmet. Toxicol. 5, 141–157.

Hernandez-Verdun, D., Roussel, P., Thiry, M., Sirri, V., Lafontaine, D.L.J., 2010. The nucleolus: structure/function relationship in RNA metabolism. Wiley Interdiscip. Rev. RNA 1 (3), 415–431.

Horváthová, E., Šramková, M., Lábaj, J., Slameňová, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured *in vitro*. Neuroendocrinol. Lett. 27 (2), 44–47.

Hsu, S.S., Lin, K.L., Chou, C.T., Chiang, A.J., Liang, W.Z., Chang, H.T., Tsai, J.Y., Liao, W.C., Huang, F.D., Huang, J.K., Chen, I.S., Liu, S.I., Kuo, C.C., Jan, C.R., 2011. Effect of thymol on Ca^{2+} homeostasis and viability in human glioblastoma cells. Eur. J. Pharmacol. 670, 85–91.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L., Fitzhugh, O.G., 1964. Food flavourings and compounds of related structure. I. Acute oral toxicity. Food Cosmet. Toxicol. 2, 327–343.

Koparal, A.T., Zeytinoglu, M., 2003. Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549. Cytotechnology 43, 149–154.

Lambert, R.J.W., Skandamis, P.N., Coote, P., Nychas, G.J.E., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462.

Liang, W.Z., Lu, C.H., 2012. Carvacrol-induced [Ca^{2+}] rise and apoptosis in human glioblastoma cells. Life Sci. 90, 703–711.

López, P., Sánchez, C., Batlle, R., Nerín, C., 2007. Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. J. Agric. Food Chem. 55, 4348–4356.

Mehdi, S.J., Ahmad, A., Irshad, M., Manzoor, N., Rizvi, M.M.A., 2011. Cytotoxic effect of Carvacrol on human cervical cancer cells. Biol. Med. 3 (2), 307–312.

Özkan, A., Erdoğan, A., 2011. A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. Turk. J. Biol. 35, 735–742.

Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Cameán, A.M., Repetto, G., 2007. Acute and subacute toxic effects produced by microcystin-YR on the fish cell lines RTG-2 and PLHC-1. Toxicol. in Vitro 21, 1460–1467.

Ramos, M., Jiménez, A., Peltzer, M., Garrigós, M.C., 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. J. Food Eng. 109, 513–519.

Savini, I., Arnone, R., Catani, M.V., Avigliano, L., 2009. *Origanum vulgare* induces apoptosis in human colon cancer Caco2 cells. Nutr. Cancer 61 (3), 381–389.

Shav-Tal, Y., Blechman, J., Darzacq, X., Montagna, C., Dye, B.T., Patton, J.G., Singer, R.H., Zipori, D., 2005. Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition. Mol. Biol. Cell 16 (5), 2395–2413.

Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823.

Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S.W., 2003. Active packaging technologies with an emphasis on antimicrobial packaging and its applications. J. Food Sci. 68, 408–420.

Szentandrassy, N., Szigeti, G., Szegedi, C., Sarkozi, S., Magyar, J., Banyasz, T., Csernoch, L., Kovacs, L., Nanasi, P.P., Jona, I., 2004. Effect of thymol on calcium handling in mammalian ventricular myocardium. Life Sci. 74, 909–921.

Ultee, A., Slump, R.A., Steging, G., Smid, E.J., 2000. Antimicrobial activity of carvacrol toward Bacillus cereus on rice. J. Food Protect. 63 (5), 620–624.

Ündeger, Ü., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.

Yin, Q., Yan, F., Zu, X.Y., Wu, Y., Wu, X., Liao, M., Deng, S., Yin, L., Zhuang, Y., 2012. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology 64 (1), 43–51.

Zeytinoglu, H., Incesu, Z., Baser, K.H.C., 2003. Inhibition of DNA synthesis by Carvacrol in mouse myoblast cells bearing a human N-RAS oncogene. Phytomedicine 10, 292–299.

Zhang, S.Z., Lipsky, M.M., Trump, B.F., Hsu, I.C., 1990. Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. Cell Biol. Toxicol. 6, 219–234.

CAPÍTULO 6 / CHAPTER 6

María Llana-Ruiz-Cabello, Sara Maisanaba, María Puerto, Ana I. Prieto, Silvia Pichardo, Ángeles Jos, Ana M. Cameán

EVALUATION OF THE MUTAGENICITY AND GENOTOXIC POTENTIAL OF CARVACROL AND THYMOL USING THE AMES SALMONELLA TEST AND ALKALINE, ENDO III- AND FPG-MODIFIED COMET ASSAYS WITH THE HUMAN CEL LINE CACO-2.

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Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2

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ABSTRACT

Currently, direct antimicrobial and antioxidant additives derived from essential oils are used in food packaging and are perceived by consumers as low-health-risk compounds. In this study, we investigated the potential mutagenicity and genotoxicity of carvacrol and thymol, major compounds in several essential oils, using the Ames *Salmonella* test and the alkaline, Endo III- and formamidopyrimidine glycosylase (FPG)-modified comet assays, respectively. Thymol did not show any mutagenic activity at any concentration assayed (0–250 μM), whereas carvacrol exhibited mutagenic potential, displaying greater activity in presence of the metabolic fraction (29–460 μM). The genotoxic effects were evaluated in the human colon carcinoma cell line Caco-2, and the standard comet assay revealed that neither carvacrol (0–460 μM) nor thymol (0–250 μM) had any affects at 24 and 48 h. The FPG-modified comet assay showed that the highest concentration of carvacrol (460 μM) caused DNA damage, indicating damage to the purine bases. These results should be used to identify the appropriate concentrations of carvacrol and thymol as additives in food packaging. Moreover, further studies are necessary to explore the safety and/or the toxicity mechanisms of these compounds.

1. Introduction

Essential oils are products derived from aromatic plants which have a wide range of uses in medicine, crop protection and flavouring and fragrances in the food and perfume industries (Ipek et al., 2005; Bakkali et al., 2008). In the food industry, antimicrobial packaging has garnered increasing attention because of consumer demands for the use of natural additives, which has led to an increase in the number of studies of natural extracts, such as essential oils (Persico et al., 2009; Ramos et al., 2012). The active compounds of these oils have been added to packaging materials via the incorporation of their precursor essential oils (Salafranca et al., 2009). Currently, direct antimicrobial additives derived from essential oils extracted from plants or spices are used in food, which is perceived consumers to be low health risk compounds (Ramos et al., 2012). Studies have shown that these phenolic compounds found in plant essential oils display antioxidant activity because they scavenge free radicals (Mastelic et al., 2008; Özcan and Erdogan, 2011), providing a mechanistic basis for their use in food packaging materials.

Carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol) are major compounds in thyme, oregano, marjoram, and savoury essential oils (De Vincenzi et al., 2004; Al-Bandak and Oreopoulou, 2007). The structure of these compounds is similar; however, they have a hydroxyl group at a different location of the phenolic ring. They have been shown to exhibit antimicrobial effects on bacteria, fungi, and yeast (Lambert et al., 2001; Zhou et al., 2007; Burt, 2004; Bakkali et al., 2008), as well as insecticidal and antioxidant effects (Akalin and Incesu, 2011). These compounds are components of plant essential oils and although they have strong antimicrobial activity, their use as food preservatives is limited by their strong flavour when added in large amounts, which negatively affects the organoleptic properties of food (Zhou et al., 2007). Therefore, the use of the active substances may avoid this problem. In addition, the use of carvacrol and thymol may be advantageous because of the possible synergistic effect produced by the addition of both additives into the polymer matrix (Ditry et al., 1994). In fact, Ramos et al. (2012) demonstrated the potential use of carvacrol and thymol at 8 wt% as active additives in polypropylene (PP) films for food packaging applications with

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the double effect of their controlled antimicrobial release into foodstuffs and their possibility to substitute the common synthetic antioxidants. These authors indicated that additional studies are necessary to evaluate other issues, such as organoleptic behaviour and the release kinetics from the polymer matrix.

Carvacrol and thymol are registered flavourings and foodstuffs for the Council of Europe (Council of Europe, 2000), the Food and Drug Administration (FDA) and the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA, 2000) and are typically used in foods at low concentrations. However, the use of these compounds for other applications, such as active packaging may require higher concentrations, thereby increasing concern regarding exposure to these compounds (Stammati et al., 1999). The available data are primarily about the acute and short-term *in vivo* effects in different animal species and suggest that these compounds may not pose a risk to human health (Hagan et al., 1964; Zani et al., 1991; Stammati et al., 1999). Various *in vitro* models have been used to assess the cytotoxicity of carvacrol (Akalin and Incesu, 2011) and thymol (Deb et al., 2011), although the toxic mechanism of these compounds has not been completely elucidated. Recently, we demonstrated a concentration- and time-dependent decrease in the viability of human intestinal Caco-2 cells after exposure to carvacrol. Although no cytotoxic effects were observed for thymol at any concentration and time of exposure, ultrastructural changes were indicative of cellular damage (Llana-Ruiz-Cabello et al., 2014).

Few studies have been performed investigating on the mutagenicity and/or antimutagenicity of these substances and their genotoxic potential, and the results are contradictory. Several studies have demonstrated strong antimutagenic and antitumourigenic activities of carvacrol using several assays (He et al., 1997; Ipek et al., 2004; Zeytinoglu et al., 2003). Similarly, the essential oil from *Origanum onites* L. and carvacrol (its major constituent) showed significant antimutagenic activity *in vitro* using the Ames *Salmonella*/microsomal test (Ipek et al., 2005), although other authors reported contradictory results using the Ames mutagenicity assay (Zani et al., 1991; Azizan and Blevins, 1995; Stammati et al., 1999). Carvacrol and thymol may protect against peroxide- and mutagen-induced DNA damage in human lymphocytes (Aydin et al., 2005a,b), and they reduced the number of DNA lesions induced by H_2O_2 in human hepatoma HepG2 and colonic Caco-2 cells (Horvathova et al., 2006). By contrast, the genotoxic potential of carvacrol and thymol at non-toxic doses may be weak based on the DNA-repair test and SOS-chromotest (Stammati et al., 1999), although the possible action of carvacrol at the DNA level cannot be excluded because it causes nuclear fragmentation (De Vincenzi et al., 2004). A lack of clastogenic activity for thymol and carvacrol at biologically relevant concentrations has been reported in V70 Chinese hamster lung fibroblast cells using the comet assay (Ündeger et al., 2009). The comet assay (alkaline single-cell gel electrophoresis) is a simple, fast and reliable method for measuring DNA strand breaks. A simple modification, incorporating DNA digestion with lesion-specific enzymes, endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG), allows the measurement of oxidised pyrimidines and oxidised purines, respectively (Collins et al., 1997; Collins, 2004). With these modifications, the comet assay has become a popular method for measuring various types of DNA damage, including oxidative damage inflicted by reactive oxygen species (ROS) (Collins, 2014). For carvacrol and thymol, the enzyme-modified comet assay (FPG-modified assay) has only been used in V70 Chinese hamster lung fibroblast cells, showing no DNA damage (Ündeger et al., 2009).

The objective of this study was to evaluate the following: (1) the potential mutagenic activity of current usage concentrations of carvacrol and thymol via the bacterial reverse-mutation assay (Ames test); and (2) their genotoxic activity using the comet assay on the intestinal cell line Caco-2, including the detection of oxidative DNA damage using the digestion of DNA with lesion-specific enzymes, Endo III and FPG.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, foetal bovine serum and cell culture reagents were obtained from BioWhittaker (Madrid, Spain). Carvacrol (98%) and thymol (99.5%) were purchased from Sigma–Aldrich (Madrid, Spain). All assay chemicals were also purchased from Sigma–Aldrich (Madrid, Spain) and VWR International Eurolab (Madrid, Spain).

2.2. Model systems

The Caco-2 cell line derived from a human colon carcinoma (ATCC® HTB-37) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in Eagle's medium supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 50 μg/mL gentamicin, 1.25 μg/mL fungizone, 2 mM L-glutamine and 1 mM pyruvate. The cells were grown nearly to confluency in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer (Fuchs-Rosenthal, Germany), and the viability was determined by exclusion of trypan blue. The Caco-2 cells were plated at density of 7.5×10^5 cells/mL for all experiments.

2.3. Test solutions

Serial test solutions of carvacrol (0–460 μM) and thymol (0–250 μM) were prepared from stock solutions of carvacrol (2.5 M) and thymol (0.5 M) in DMSO, with a final concentration of DMSO less than 0.1%. Preliminary experiments were performed to determine the cytotoxicity of both chemicals to Caco-2 cells (Llana-Ruiz-Cabello et al., 2014). Based on these results, the highest concentration of carvacrol chosen for testing (460 μM) was the mean effective concentration (EC_{50}) obtained from the tetrazolium salt reduction (MTS) assay after 24 h exposure (Llana-Ruiz-Cabello et al., 2014). For thymol, the exposed cells remained unaltered compared to the control cells after 24 and 48 h for all end-points assayed at 250 μM; therefore, this concentration was selected as the highest concentration tested in this study.

2.4. Ames test

The incorporation version of the Ames test was performed according to the recommendations of Maron and Ames (1983), following the principles of OECD guideline 471 (1997). Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used for the assay. Cultures of each test strain were prepared from their main strain plates and then used in the late exponential growth phase. Each primary experiment included one negative control (distilled water), one positive control for each strain, one solvent control (DMSO) and five concentrations of each compound. Test substances were assessed in three independent experiments, each conducted in the absence and presence of the S9 metabolic activation system from rat livers (Aroclor 1254, Sigma, Spain), using triplicate plates for all test substance concentrations.

Briefly, 0.1 mL test substance, 0.1 mL bacterial culture and 0.5 mL of S9 mix were added to 2 mL molten agar at 42 °C and poured onto Vogel Bonner-E minimal glucose agar plates. The plates were inverted and incubated for 65–70 h at 37 °C in the dark. Revertant colonies were counted, and the background lawn was inspected for signs of either toxicity or compound precipitation. 2-Nitrofluorene (2-NF) (0.1 μg/plate) and sodium azide (NaN_3) (1 μg/plate) were selected as positive controls without the S9 fraction. In presence of S9, 2-aminofluorene (2-AF) (20 μg/plate) was used.

2.5. Comet assay

2.5.1. Standard comet assay

The standard comet assay was performed to detect DNA strand breaks. Caco-2 cells were seeded into 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37 °C in 5% CO_2 to attach. Approximately 3.5×10^5 cells in each well were treated with escalating concentrations of carvacrol (0, 115, 230 or 460 μM) and thymol (0, 62.5, 125 or 250 μM) for 24 and 48 h. To monitor the assay, a negative control (cells treated with medium without foetal calf serum) and a positive control (cells treated with a solution of 100 μM H_2O_2) were included. After treatment, the cells were washed and detached in phosphate buffered saline (PBS). The comet assay was performed as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, the cells were resuspended in PBS at 2.5×10^6 cells/mL. This suspension was mixed with 1% low melting point agarose and placed on a microscope slide. After the gels

solidified, the slides were dipped into lysis solution at 4 °C. All nucleotides were denatured in high-pH buffer. Electrophoresis was performed at approximately 25 V (300 mA), and the DNA was gently reneutralised in PBS and washed with deionized H_2O (>18 MΩ cm^{-1} resistivity (Milli-Q water purification system, Millipore, Spain). After neutralisation, the microscope slides were fixed in 70% ethanol and absolute ethanol. Finally, the DNA was stained with SYBR Gold nuclei acid gel stain (Invitrogen, Life Technologies, USA) and was visualised with an Olympus BX61 fluorescence microscope (20× objective) coupled via a CCD camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei (>100) per experimental point were analysed with image analysis software (Comet Assay IV, Perceptive Instruments, UK).

2.5.2. Enzyme-modified Comet assay

To investigate oxidative DNA damage, the cells were embedded in agarose on microscope slides and stored in lysis buffer (described above) for the standard assay. After removing the slides from the lysis buffer, they were washed 3× for 5 min each with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0 adjusted with 6 M KOH). Two different enzymes were used for these experiments, Endo III, the substrates of which are oxidised pyrimidines, and FPG, which recognises the common oxidised purine 8-oxoGua and ring-opened purines or formamidopyrimidines (FAPY) (Collins et al., 2008; Azqueta et al., 2009). Cells were treated with H_2O_2 (100 μM) as a positive control for Endo III or with Ro19-8022 (2 μM) and white light (1.5 min) on an ice bath to generate predominantly 8-oxo-7,8-dihydroguanine as a positive control for FPG (Lorenzo et al., 2009). After the last wash, the excess liquid was removed turning the coplin jar over, and 30 μL of the following solutions were applied to the slides prepared from each treatment group: 30 μL enzyme buffer alone, 30 μL enzyme buffer (buffer F) containing FPG (10 U/mL) or 30 μL enzyme buffer containing Endo III (14 U/mL). The slides were stored in a humidified metal-box and incubated at 37 °C for 30 min for Endo III and FPG activity. After the enzyme treatment, the slides were placed in a horizontal electrophoresis chamber for the DNA unwinding and electrophoresis as described above for the standard assay. The slides were neutralised, dried, and stained.

2.6. Calculations and statistical analysis

The mutagenicity experiments (Ames test) were performed three times in at least triplicate per concentration. The data are presented as the arithmetic mean percentage ± standard deviation (SD) compared to the control group, which is the unexposed bacterial strain. Statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using GraphPad InStat software (GraphPadSoftware Inc., La Jolla, USA). The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlet́s test, respectively. The differences were considered significant at $p \leqslant 0.05$.

For the comet assays, the data are presented as the means ± SD of three independent experiments. The total scores of the different groups were compared using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U-test when the first test showed differences. The differences were considered significant at $p \leqslant 0.05$.

3. Results

3.1. Ames test

After exposure to carvacrol, no increase in the number of revertants was observed for three of the strains assayed, TA100, TA102, and TA104, at any concentration tested, either in the presence or absence of S9 (Table 1). However, for TA97A, mutagenic activity was observed as an increase in the number of revertants at 29 μM and 115 μM in the absence of S9. Similarly, carvacrol showed mutagenic potential in TA98, both in the presence (29–460 μM) and absence of S9 (115–230 μM). Thereby showing carvacrol has mutagenic potential at the specified concentrations.

For thymol, there was no increase in the number of revertants and no indication of mutagenic activity in any of the treated strains up to the highest concentration (250 μM), either in the presence or absence of S9 (Table 2). Consequently, this shows, thymol is not mutagenic in this bacterial assay at the concentrations tested.

The toxicity of both substances, measured as a decrease in the background lawn and in revertant frequency, was not observed at any concentration tested. The positive controls produced statistically significant increases ($p < 0.01$) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix.

3.2. Comet assay

The comet assay was used to investigate the potential DNA damaging effects of carvacrol and thymol. It was performed as the standard version and with post-treatment with the Endo III and FPG proteins to include oxidative DNA damage. Carvacrol (0–460 μM) and thymol (0–250 μM) did not induce DNA strand-breaks in Caco-2 cells at any concentrations tested after 24 or 48 h of exposure (Fig. 1). Caco-2 cells exposed to 100 μM H_2O_2 (positive control), a significant ($p < 0.001$) increase of DNA damage was observed after 24 h and 48 h of exposure.

Based on the results of the enzyme digestion of the nuclei, no increase in DNA damage was observed when Endo III was used in the cells exposed to carvacrol and thymol after 24 and 48 h (Fig. 2). In addition, there were no differences observed in the frequency of Endo-sensitive sites between samples from the Endo-treated and buffer-treated cells (vehicle control) (data not shown). For cells treated with H_2O_2 (positive control), there was a significant ($p < 0.001$) increase in DNA breakage when the cells were incubated with Endo III (Fig. 2).

The effects of carvacrol and thymol on Caco-2 cells were analysed with FPG post-exposure and are shown in Fig. 3. The results indicate a significant ($p < 0.001$) increase after 48 h exposure to carvacrol at the highest concentration (460 μM) compared to the control group and other treated groups. The per cent DNA in the tail at 460 μM carvacrol was approximately 2-fold higher compared to the other concentrations of carvacrol. Moreover, after 48 h of exposure to 460 μM carvacrol, the damage was greater compared to 24 h ($p < 0.05$). For the Caco-2 cells exposed to thymol and post-treatment with FPG, no significant variations in DNA strand breaks were detected after 24 or 48 h of exposure (Fig. 3).

By contrast, no differences were observed in the Caco-2 cells exposed to 0–230 μM carvacrol dissolved in buffer F compared to cells exposed to carvacrol with FPG post-treatment after 24 or 48 h (Fig. 4a and b, respectively). However, when the Caco-2 cells were exposed to the highest concentration of carvacrol for 24 or 48 h, a difference in DNA damage was measured between buffer F and the FPG-modified comet assays ($p < 0.05$). These results indicate that carvacrol induces the formation of 8-oxoGua, ring-opened purines, or FAPY, which are damaged bases removed by FPG.

4. Discussion

There are major concerns being raised about the food safety of packaging materials relative to the possible migration of the packaging constituents into the food (Maisanaba et al., 2014). The addition of natural essential oils or the direct incorporation of additives with antimicrobial, antifungal or antioxidant properties into the matrix requires an evaluation of their safety at potential usage concentrations. A test for the induction of gene mutations in bacteria is among the core set of toxicological tests required in the Guidelines of the Scientific Committee on Food for the safety assessment of a substance for use in food contact materials prior to its authorisation (European Commission, 2001). Several laboratory studies have used the *Salmonella*/microsome and *Escherichia coli* WP2 reversion tests to investigate the mutagenic and possible antimutagenic effects of diverse essential oils and their major constituents (Evandri et al., 2005; Beric et al., 2008; Aicha et al., 2008), including oregano and thyme oils (De Martino et al., 2009). For carvacrol and thymol, phenolic compounds with antioxidant properties, few reports describing their mutagenic effects exist, and the results are contradictory (Azizan and Blevins, 1995; De Vincenzi et al.,

Table 1
Results of the Ames test conducted with carvacrol (0–460 μM) for three independent experiments. Milli-Q water was used as negative control and DMSO as solvent for positive controls. Data shown as mean ± standard deviation (SD) revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/TA98/TA102/TA104 without S9:2-NF (0.1 μg/plate), and TA100 without S9: NaN_3 (1 μg/plate). 2-AF (20 μg/plate) was used in presence of S9. $^{*}p < 0.05$ significant, and $^{**}p < 0.01$ very significant differences from controls, respectively.

Concentration (μM)	TA97A		TA98		TA100		TA102		TA104	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Carvacrol										
Negative controls	193 ± 28	278 ± 11	15 ± 2	47 ± 6	135 ± 24	106 ± 18	237 ± 35	239 ± 48	273 ± 7	239 ± 25
29	405 ± 35**	374 ± 21	19 ± 2	120 ± 6**	117 ± 11	158 ± 2	300 ± 36	361 ± 20	351 ± 36	418 ± 78
56	369 ± 58	381 ± 12	28 ± 5	123 ± 25**	172 ± 19	134 ± 26	351 ± 31	306 ± 49	320 ± 64	261 ± 21
115	395 ± 6**	395 ± 15	36 ± 10*	220 ± 36**	112 ± 25	88 ± 3	201 ± 5	183 ± 30	196 ± 22	365 ± 57
230	328 ± 22	327 ± 31	37 ± 8*	211 ± 32**	105 ± 4	120 ± 20	282 ± 40	223 ± 6	240 ± 14	456 ± 51
460	330 ± 9	444 ± 26	25 ± 4	116 ± 15*	132 ± 11	144 ± 15	222 ± 12	225 ± 34	197 ± 20	453 ± 64
Positive controls	750 ± 18**	724 ± 21**	1055 ± 205**	1239 ± 196**	1035 ± 48**	1474 ± 106**	620 ± 173**	500 ± 32**	790 ± 62**	906 ± 69**
Solvent controls	194 ± 16	202 ± 14	18 ± 5	34 ± 5	92 ± 16	100 ± 10	260 ± 20	268 ± 17	269 ± 28	271 ± 15

* $p < 0.05$ significant.
** $p < 0.01$ very significant differences from controls.

Table 2
Results of the Ames test conducted with thymol (0–250 μM) for three independent experiments. Milli-Q water was used as negative control and DMSO as solvent for positive controls. Data shown as mean ± standard deviation (SD) revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/TA98/TA102/TA104 without S9:2-NF (0.1 μg/plate), and TA100 without S9: NaN_3 (1 μg/plate). 2-AF (20 μg/plate) was used in presence of S9.

Concentration (μM)	TA97A		TA98		TA100		TA102		TA104	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Thymol										
Negative controls	288 ± 11	261 ± 9	17 ± 3	25 ± 2	118 ± 6	87 ± 3	238 ± 33	252 ± 25	273 ± 7	310 ± 22
15.6	299 ± 41	287 ± 7	17 ± 4	33 ± 4	105 ± 20	112 ± 13	297 ± 40	362 ± 6	330 ± 6	429 ± 43
31.3	314 ± 14	340 ± 35	18 ± 1	31 ± 3	139 ± 17	123 ± 16	364 ± 24	382 ± 28	418 ± 28	498 ± 55
62.5	267 ± 14	306 ± 12	24 ± 8	28 ± 4	80 ± 3	83 ± 13	256 ± 45	311 ± 32	281 ± 18	394 ± 23
125	177 ± 36	206 ± 27	21 ± 4	24 ± 3	70 ± 12	75 ± 7	252 ± 15	192 ± 11	320 ± 13	502 ± 43
250	261 ± 48	273 ± 24	21 ± 3	33 ± 4	105 ± 8	111 ± 11	307 ± 10	346 ± 29	464 ± 17	423 ± 19
Positive controls	617 ± 13**	553 ± 23**	1081 ± 250**	1124 ± 248**	1076 ± 112**	1026 ± 110**	620 ± 173**	542 ± 47**	791 ± 62**	861 ± 122**
Solvent controls	196 ± 11	200 ± 13	20 ± 6	26 ± 10	98 ± 16	105 ± 8	268 ± 22	273 ± 17	296 ± 22	309 ± 15

** $p < 0.01$ very significant differences from controls.

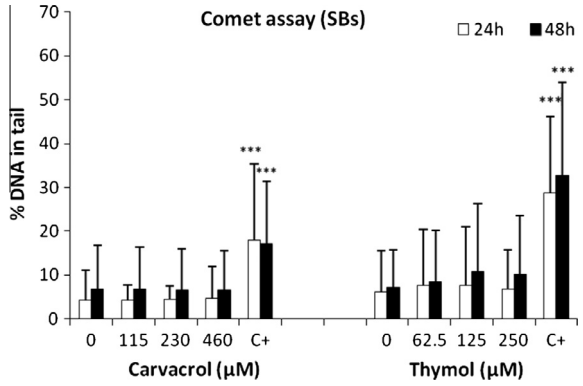

Fig. 1. The level of DNA damage was measured in Caco-2 cells after 24 and 48 h of exposure to 115, 230 or 460 μM carvacrol (left) and 62.5, 125 or 250 μM thymol (right) as the formation of strand breaks by the standard comet assay. The levels of DNA strand-breaks (SBs) are expressed as % DNA in tail. All values are expressed as mean ± s.d. ***Significantly different from control ($p < 0.001$).

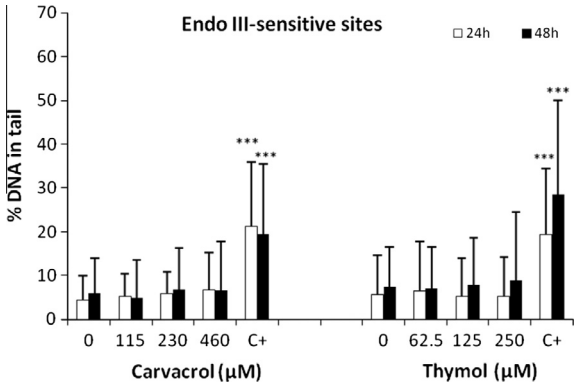

Fig. 2. The level of DNA damage was measured in Caco-2 cells after 24 and 48 h of exposure to 115, 230 or 460 μM carvacrol (left) and 62.5, 125 or 250 μM thymol (right) as End III-sensitive sites by the modified comet assay. The levels of oxidised pyrimidines are expressed as % DNA in tail. All values are expressed as mean ± s.d. ***Significantly different from control ($p < 0.001$).

2004; Ipek et al., 2005). Furthermore, several of these studies were not conducted according to the principles of OECD guideline 471 (1997).

In the present study the Ames test was used to evaluate the mutagenicity of carvacrol and thymol in a range of concentrations added to food packaging (Llana-Ruiz-Cabello et al., 2014). For thymol, revertant colonies were not present in any of the five strains assayed compared to the negative control plates in the presence or absence of S9. The absence of a toxic response to this compound is consistent with the results previously obtained with the Ames test strain TA97, irrespective of metabolic activation (Azizan and Blevins, 1995). By contrast, at non-toxic doses, thymol and carvacrol increased the number of revertants for the TA100 and TA98 *S. typhimurium* strains by 1.5–1.7 times, regardless of metabolic activation. However, these data were considered ambiguous because they produced elevated revertant numbers in the Ames test strain TA100, but not significantly (Stammati et al., 1999). The authors concluded that the mutagenic potential of thymol and carvacrol is very weak, although the possibility of their action at DNA level cannot be excluded, at least for carvacrol, which caused nuclear fragmentation.

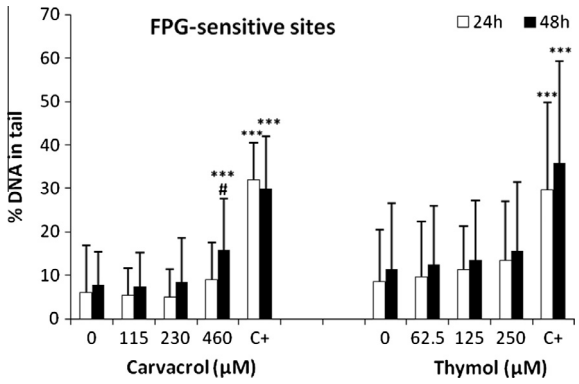

Fig. 3. The level of DNA damage was measured in Caco-2 cells after 24 and 48 h of exposure to 115, 230 or 460 μM carvacrol (left) and 62.5, 125 or 250 μM thymol (right) as FPG-sensitive sites by the modified comet assay. The levels of oxidised purines are expressed as % DNA in tail. All values are expressed as mean ± s.d. ***Significantly different from control ($p < 0.001$); #groups exposed for 48 h in comparison to 24 h ($p < 0.05$).

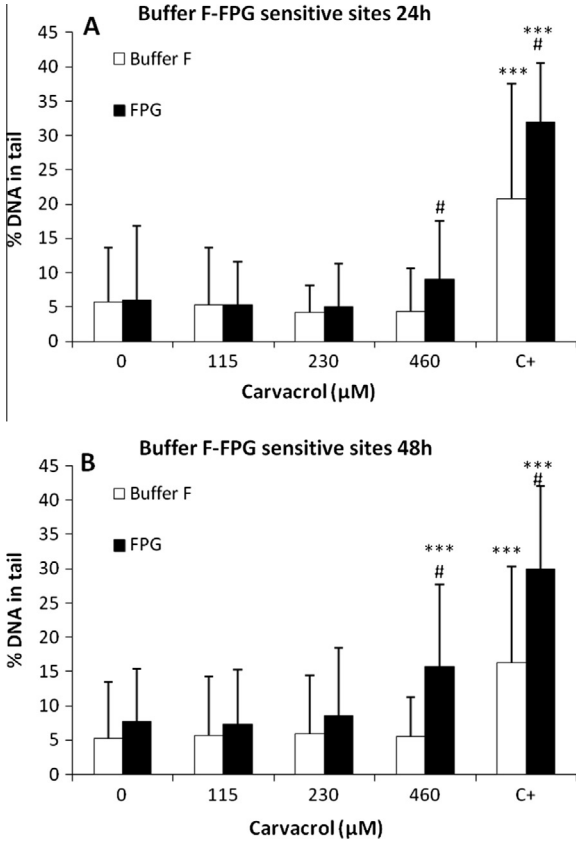

Fig. 4. The level of carvacrol-induced DNA strand-breaks without (white) and with (black) FPG digestion. Caco-2 cells were exposed to 115, 230 or 460 μM carvacrol for 24 h (A) and 48 h (B). The levels of DNA strand-breaks and oxidised purines are expressed as % DNA in tail. All values are expressed as mean ± s.d. ***Significantly different from control ($p < 0.001$). #Significant differences between FPG enzyme-digested and non digested group ($p < 0.05$).

Interestingly, in the present study, carvacrol is a direct acting mutagen in the Ames assay for the TA98 and TA97 strains of *S. typhimurium* (115–230 μM). Furthermore, its metabolites show mutagenicity against TA98 (29–460 μM). These results confirmed those of Ipek et al. (2005), who assessed the genotoxicity and antigenotoxicity of carvacrol and *O. onites* oil using the two *S. typhimurium* strains TA98 and TA100, with or without S9 metabolic activation. No mutagenicity was observed for the oil for the both strains with or without the S9 mixture, whereas significant mutagenic activity was induced by carvacrol in the absence of metabolic activity. By contrast, our results demonstrate that the metabolites of carvacrol are mutagenic at lower concentrations for the TA98 strain. Moreover, prior published research showed that both the oil and carvacrol significantly inhibited mutagenicity induced by two direct acting mutagens in both strains with or without S9, respectively, confirming its antimutagenic and antitumourigenic activities observed using different assays (He et al., 1997; Ipek et al., 2004). The mechanisms of the antimutagenic activity of carvacrol appear to be a result of its antioxidant activity (Kulisic et al., 2004). In fact, it has even been suggested that carvacrol may be used for cancer therapy (Zeytinoglu et al., 2003) based on this mechanism. In addition, De Martino et al. (2009) reported that oregano and thyme oils did not show mutagenic activity at any dose against the TA98 and TA100 *S. typhimurium* strains with or without metabolic activation. The non-mutagenic activity of the oregano essential oil may be due to the concomitant presence of different components, which could be desmutagenic, such as thymol, linalool, and p-cymene (Ipek et al., 2005; De Martino et al., 2009).

In addition to the mutagenicity study, the assessment of genotoxic potential of these compounds is a critical step because of the relevance for human safety in relation to the potential induction of carcinogenesis and hereditary defects. Different experimental models using the comet assay show contradictory results in the case of carvacrol and thymol (Aydin et al., 2005b, 2014; Slamenová et al., 2007; Ündeger et al., 2009). In this work we investigated the potential genotoxicity of these compounds on Caco-2 cells. In the standard comet assay, thymol and carvacrol did not induce DNA stand breaks at any concentration. The absence of a detectable response may be because the DNA damage and repair synthesis either did not occur *in vitro* or occurred in a manner that did not generate detectable strand breaks. These results were in contrast to the positive responses reported by Aydin et al. (2005b) in human lymphocytes because carvacrol induced DNA damage at concentrations greater than 0.05 mM, and thymol induced DNA damage at higher concentrations (0.2 mM). These authors found that below the DNA damaging concentrations, the major compounds and constituents of thyme and its extracts protected lymphocytes against DNA-damage induced by mitomycin C (MMC) and 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) (Aydin et al., 2005a). Similarly, Horvathova et al. (2006) concluded that carvacrol and thymol were not associated with the DNA-damaging effect on human hepatoma HepG2 and colonic Caco-2 cells treated for 24 h, and reduced the level of DNA lesions induced by the potent oxidant H_2O_2. In another study, the incubation of HepG2 and Caco-2 cells in the presence of various concentrations of carvacrol led to significant protection of the cells from DNA strand breaks induced by H_2O_2 (Slamenová et al., 2007). Furthermore, an *in vivo* study in rats demonstrated that carvacrol (20 mg/kg b.w.) did cause DNA damage in rat lymphocytes and hepatocytes (Aristatile et al., 2011), and when coadministered with D-galactosamine (D-GalN), exerted protective effects, likely because of free radical scavenging. Recently, it has been shown that carvacrol is neither genotoxic nor mutagenic to healthy neurons and N2a neuroblastoma cells using the comet assay because the observed mean values of the total scores of cells showing DNA damage were not significantly different from the control values for both cells (Aydin et al., 2014).

The sensitivity and specificity of the comet assay to particular types of DNA lesions can be enhanced by incubating the cells with lesion-specific endonucleases that recognise specific damaged bases and create breaks, increasing the amount of DNA in the comet tail (Collins, 2004). DNA breaks are produced by ROS and other damaging agents, and for more specific detection of oxidation damage, it is necessary to use enzymes that convert

oxidised bases to breaks, such as FPG (Gielazyn et al., 2003). This modification has been widely applied in human biomonitoring and also has applications in ecogenotoxicology and genotoxicity testing to confirm the involvement of ROS (Collins, 2014). In the present study, we incubated cells with the DNA endonucleases Endo III and FPG. The enzyme-modified comet assay showed that carvacrol was more genotoxic than thymol because the cells exposed to the highest concentration of carvacrol (460 μM) for 48 h showed a difference in DNA damage in the buffer and FPG-modified comet assays. However, in addition to its high sensitivity for detecting 8-OH-guanine and other oxidatively damaged purines, the FPG enzyme also detects alkylation damage, such as a basic (AP) sites and ring-opened N-7 guanine adducts (Speit et al., 2004). Therefore, further evidence for oxidative stress induction is required to conclude that the damage observed is caused by oxidative DNA damage to purine bases. In this sense, we have recently demonstrated that Caco-2 cells exhibit a significant increase in ROS when exposed to the highest concentration of carvacrol (460 μM) for 24 h, whereas after 48 h, this increase occurred for 230 μM carvacrol (Llana et al., 2013). The lack of DNA damage induction for the standard comet assay and the significant increase in oxidative DNA damage by the enzyme-modified comet assay has been previously reported for different compounds (Martínez-Alfaro et al., 2006; Gajski et al., 2008; Manjanatha et al., 2014).

In contrast to our results, in V79 Chinese hamster lung fibroblast cells treated with 1, 5, 25 μM thymol and carvacrol, only the 25 μM concentration of thymol caused some clastogenic DNA damage in the standard comet assay (Ündeger et al., 2009). Post-treatment with the FPG protein also revealed increased DNA-damage for the positive control H_2O_2, but no additional DNA damage was detected for the thymol and carvacrol concentrations tested (Ündeger et al., 2009).

Overall, in the present study, carvacrol exhibited the potential for mutagenic activity (115–230 μM, equivalent to approximately to 2.0–3.5 wt% of carvacrol in the essential oil), with metabolites being more active (29–460 μM). We also demonstrated for the first time, the potential genotoxicity of carvacrol using the FPG-modified comet assay (460 μM, equivalent to 7 wt% of carvacrol in the essential oil) in Caco-2 cells, whereas thymol showed no mutagenic or genotoxic effects at any concentration tested. Because the number of studies on the mutagenic and genotoxic effects of these compounds remains limited, further studies are necessary to confirm these findings and elucidate the role of oxidative stress in the induced DNA damage. Ramos et al. (2012) assayed antimicrobial active films on polypropylene (PP) prepared by incorporating thymol and carvacrol, and demonstrated that the addition of both compounds at 8 wt% showed improved product quality and safety in food packaging applications. It is desirable to design new products based on formulations containing mixtures higher in thymol content and lower in carvacrol content. In fact, thymol has received considerable attention as antimicrobial agent with robust antifungal activity. Thymol is also an excellent food antioxidant (Youdim and Deans, 2000; Sánchez-García et al., 2008) and it exhibited a higher antioxidant activity in lipids in comparison to carvacrol (Yanishlieva et al., 1999). Sivropoulou et al. (1996), indicated that thymol is more active than carvacrol against gram-negative bacteria, which is consistent with results from Ditry et al. (1994). Consequently, gradually increasing the proportion of thymol in mixtures or essential oil extracts with packaging purposes, does not necessarily cause decreased effectiveness and may be safer.

5. Conclusion

In the present study, analysis of the mutagenic and genotoxic activities of carvacrol and thymol revealed that carvacrol (29–460 μM) exhibits potential mutagenic activity (Ames *Salmonella*/microsomal assay). We also demonstrated for the first time that carvacrol (460 μM) induced DNA-oxidative effects (FPG-modified comet assay) in Caco-2 cells. Thymol (0–250 μM) did not show any effects in either assay. However, to confirm the safety of these compounds, additional studies should be considered to fully characterise their safety before they are used in food packaging.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

References

Aicha, N., Ines, S., Mohamed, B.S., Ines, B., Soumaya, K., Kametl, G., Mohamed, N., Imed, C., Mohamed, H., Leila, C.-G., 2008. Chemical composition, mutagenic and antimutagenic activities of essential oils from (Tunisian) *Artemisia campesris* and *Artemisia herba-alfa*. J. Essent. Oil Res. 20, 471–477.

Akalin, G., Incesu, Z., 2011. The effects of carvacrol on apoptosis of H-ras and N-ras transformed cell lines. Turk. J. Pharm. Sci. 8 (2), 105–116.

Al-Bandak, G., Oreopoulou, V., 2007. Antioxidant properties and composition of *Majorana syriaca* extracts. Eur. J. Lipid Sci. Technol. 109, 247–255.

Aristatile, B., Al-Numair, K.S., Al-Assaf, A.H., Pugalendi, K.V., 2011. Pharmacological effect of carvacrol on D-galactosamine-induced mitocondrial enzymes and dNA damage by single-cell gel elctrophoresis. J. Nat. Med. 65, 568–577.

Aydin, S., Basaran, A.A., Basaran, N., 2005a. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutat. Res. 581, 43–53.

Aydin, S., Basaran, A.A., Basaran, N., 2005b. Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. J. Agric. Food Chem. 53, 1299–1305.

Aydin, E., Türkez, H., Keles, M.S., 2014. The effect of carvacrol on healthy neurons and N2a cancer cells: some biochemical, anticancerogenicity and genotoxicity studies. Cytotechnology 66, 149–157.

Azizan, A., Blevins, R.D., 1995. Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay. Arch. Environ. Contam. Toxicol. 28, 248–258.

Azqueta, A., Shaposhnikov, S., Collins, A.R., 2009. DNA oxidation: investigating its key role in environmental mutagenesis with the comet assay. Mutat. Res. 674, 101–1086.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – a review. Food Chem. Toxicol. 46, 446–475.

Beric, T., Nikolic, B., Stanojevic, J., Vukovic-Gacie, B., Knezevic-Vukcevic, J., 2008. Protective effect of basil (*Ocimum basilicum* L.), against oxidative DNA damage and mutagenesis. Food Chem. Toxicol. 46, 724–732.

Burt, A., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. 94, 223–253.

Collins, A.R., 2004. The comet assay for DNA damage and repair. Principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Collins, A.R., 2014. Measuring oxidative damage to DNA and its repair with the comet assay. Biochim. Biophys. Acta 1840, 794–800.

Collins, A.R., Mitchell, D.L., Zunino, A., de Wit, J., Busch, D., 1997. UV-sensitive rodent mutant cell lines of complementation groups 6 and 8 differ phenotypically from their human counterparts. Environ. Mol. Mutagen. 29, 152–160.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143-15.

Corcuera, L.A., Arbillaga, L., Vettorazzi, A., Azqueta, A., López de Cerain, A., 2011. Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. Food Chem. Toxicol. 49, 2883–2889.

Council of Europe, 2000. Partial Agreement in the Social and Public Health Field. Chemically-defined Flavoring Substances. Group 5.2 Aromatic Aldehydes, vol. 102. Council of Europe Publishing, Strasbourg, France, p. 119.

De Martino, L., De Feo, V., Nazzaro, F., 2009. Chemical composition and *in vitro* antimicrobial and mutagenic activities of seven Lamaceae essential oils. Molecules 14, 4213–4230.

De Vincenzi, M., Stammati, A., De Vincenzi, A., Silano, M., 2004. Constituents of aromatic plants: carvacrol. Fitoterapia 75, 801–804.

Deb, D.D., Parimala, G., Devi, S.S., Chakraborty, T., 2011. Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. Chem.-Biol. Interact. 193, 97–106.

Ditry, N., Dureuil, L., Pinkas, M., 1994. Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. Pharm. Acta Helv. 69, 25–28.
European Commission, 2001. Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorisation. Scientific Committee on Food. SCF/CS/PLEN/GEN/100 Final 19 December 2001.
Evandri, M.G., Battinelli, L., Daiele, C., Mastrangelo, S., Bolle, P., Mazzanti, G., 2005. The antimutagenic activity of *Lavandula angustifolia* (lavender) essential oil in the bacterial reverse mutation assay. Food Chem. Toxicol. 43, 1381–1387.
Gajski, G., Garaj-Vrhovac, V., Orescanin, V., 2008. Cytogenetic status and oxidative DNA-damage induced by atorvastatin in human peripheral blood lymphocytes: Standard and Fpg-modified comet assay. Toxicol. Appl. Pharmacol. 231, 85–93.
Gielazyn, M.L., Ringwood, A.H., Piegorsch, W.W., Stancyk, S.E., 2003. Detection of oxidative DNA damage in isolated marine bivalve hemocytes using the comet assay and formamidopyrimidine glycosylase (Fpg). Mutat. Res. 542, 15–22.
Hagan, H.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A., Brower, W.I., 1964. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. Food Cosmet. Toxicol. 5, 141–157.
He, L., Mo, H., Hadisusilu, S., Quresni, A.A., Elson, C.E., 1997. Isoprenoids suppress the growth of murine B16 melanomas in vitro and in vivo. Biochem. Mol. J. Nutr. 127, 668–674.
Horvathova, E., Šramkova, M., Labaj, J., Slamenova, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured in vitro. Neuroendocrinol. Lett. 27, 44–47.
Ipek, E., Tüylü, B.A., Zeytinoglu, H., 2004. Effects of carvacrol on sister chromatid exchanges in human lymphocyte culture. Cytotechnology 43, 145–148.
Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B.A., Kurkcuoglu, M., Can Baser, K.H., 2005. Genototoxicity and antigenotoxicity of Origanum oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. Food Chem. 93, 551–556.
Joint Expert Committee on Food Additives (JECFA), 2000. Cinnamy alcohol and related flavouring agents. WHO Food Additives Series: 46. Prepared by the Fifty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives, June 6–15, Geneva, Switzerland.
Kulisic, T., Radonic, A., Katalinic, V., Milos, M., 2004. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85, 633–640.
Lambert, R.J.W., Skandamis, P.N., Coote, P., Nychas, G.J.E., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462.
Llana, M., Gutiérrez-Praena, D., Pichardo, S., Jos, A., Bermúdez, J.M., Aucejo, S., Cameán, A.M., 2013. Oxidative stress induced by carvacrol and its mixture with thymol in the Caco-2 cell line. Rev. Esp. Toxicol. 30 (1), 52.
Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Moreno, F.J., Bermúdez, J.M., Aucejo, S., Cameán, A.M., 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290.
Lorenzo, Y., Azqueta, A., Luna, L., Bonilla, F., Domínguez, G., Collins, A.R., 2009. The carotenoid beta-cryptoxanthin stimulates the repair of DNA oxidation damage in addition to acting as an antioxidant in human cells. Carcinogenesis 30, 308–314.
Maisanaba, S., Pichardo, S., Jordá-Benayto, M., Aucejo, S., Cameán, A.M., Jos, A., 2014. Cytotoxicity and mutagenicity studies on migration extracts from nanocomposites with potential use in food packaging. Food Chem. Toxicol. 66, 366–372.
Manjanatha, M.G., Bishop, M.E., Pearce, M.G., Kulkarni, R., Lyn-Cook, L., Ding, W., 2014. Genotoxicity of Doxorubicin in F344 rats by combining the comet assay, flow-cytometric peripheral blood micronucleus test, and pathway-focused gene expression profiling. Environ. Mol. Mutagen. 55, 24–34.
Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.
Martínez-Alfaro, M., Palma-Tirado, L., Sandoval-Zapata, F., Cárabez-Trejo, A., 2006. Correlation between formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites determined by a comet assay, increased MDA, and decreased glutathione during long exposure to thinner inhalation. Toxicol. Lett. 163, 198–205.
Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovic, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brcic-Kostic, K., Vicic-Topic, D., Müller, N., 2008. Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J. Agric. Food Chem. 56, 3989–3996.
OECD, 1997. Guideline for Testing of Chemicals 471: Bacterial Reverse Mutation Test, pp. 1–11.
Özcan, A., Erdogan, A., 2011. A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. Turk. J. Biol. 35, 735–742.
Persico, P., Ambrogi, V., Carfagna, C., Cerruti, P., Ferrocino, I., Mauriello, G., 2009. Nanocomposite polymer films containing carvacrol for antimicrobial active packaging. Polym. Eng. Sci. 49, 1447–1455.
Ramos, M., Jimenez, A., Peltzer, M., Garrigos, M.C., 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. J. Food Eng. 109, 513–519.
Salafranca, J., Pezo, D., Nerin, C., 2009. Assessment of specific migration to aqueous simulants of a new active food packaging containing essential oils by means of an automatic multiple dynamic hollow fibre liquid phase microextraction system. J. Chromatogr. A 1216 (18), 3731–3739.
Sánchez-García, M.D., Ocio, M.J., Gimenez, E., Lagaron, J.M., 2008. Novel polycaprolactone nanocomposites containing thymol of interest in antimicrobial film and coating applications. J. Plast. Film Sheet. 24, 239–251.
Sivropoulou, A., Papanikolau, E., Nikolau, C., Kokkini, S., Lanaras, T., Arsenakis, M., 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. J. Agric. Food Chem. 44, 1202–1205.
Slamenová, D., Horváthová, E., Sramková, M., Marsálková, L., 2007. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured in vitro. Neoplasma 54, 108–112.
Speit, G., Schütz, P., Bonzheim, I., Trenz, K., Hoffmann, H., 2004. Sensitivity of the FPG protein towards alkylation damage in the comet assay. Toxicol. Lett. 146, 151–158.
Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823.
Ündeger, U., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.
Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., Raneva, V.G., 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem. 64, 59–66.
Youdim, K.A., Deans, S.G., 2000. Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. Br. J. Nut. 83, 87–93.
Zani, F., Masimo, G., Benvenuti, S., Biachi, A., Albasini, A., Melegari, M., Vampa, G., Belloti, A., Mazza, P., 1991. Studies on the genotoxic properties of essential oils with *Bacillus subtilis* rec-assay and Salmonella/microsome reversion assay. Planta Med. 57, 237–241.
Zeytinoglu, H., Incesu, Z., Baser, K.H.C., 2003. Inhibition of DNA synthesis by carvacrol in mouse myoblast cells bearing a human N-ras oncogene. Phytomedicine 10, 292–299.
Zhou, F., Ji, B., Zhan, H., Jiang, H., Yang, Z., Li, J.L.J., Yan, W., 2007. The antibacterial effect of cinnamadlhehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella typhimurium*. J. Food Saf. 27, 124–133.

CAPÍTULO 7 / CHAPTER 7

María Llana-Ruiz-Cabello, Daniel Gutiérrez-Praena, María Puerto, Silvia Pichardo, Ángeles Jos,

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IN VITRO PRO-OXIDANT/ANTIOXIDANT ROLE OF CARVACROL, THYMOL AND THEIR MIXTURE IN THE INTESTINAL CACO-2 CELL LINE.

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In vitro pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line

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ABSTRACT

The food industry needs to provide consumers with fresh and healthy products. In this context, food packaging plays an important role. Thus, certain essential oils are being incorporated into plastic polymers to confer better preservative properties. The oregano essential oil contains carvacrol and thymol, two important polyphenols. Considering their increasing use in active food packaging, the evaluation of their suitability and safety is of great interest. In the present work, a concentration-dependent increase in the antioxidant effects of carvacrol, thymol, and their mixture (10:1) was determined using DPPH and ABTS assays. In addition, the safety of these compounds was tested *in vitro*. Reactive oxygen species and glutathione levels were measured after exposing cells for 24 and 48 h to different concentrations of carvacrol, thymol and their mixture. The abilities of these compounds to protect against or revert the effects of H_2O_2 on cells were also studied. The results showed that oxidative stress plays a role in the damage induced by carvacrol and the mixture at high concentrations. However, at lower concentrations, both compounds and their mixture were shown, for the first time, to protect cells against the damage induced by the H_2O_2.

1. Introduction

Origanum species (Lamiaceae) are a wide group of plants that are rich in several compounds, such as monoterpene hydrocarbons, oxygenated monoterpenes, phenolic monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and phenolics (Ortega-Nieblas et al., 2011). Amongst these compounds, two phenolic monoterpenes are their major components, carvacrol and thymol (Ozkan and Erdogan, 2011; Teixeira et al., 2013). Carvacrol (2-methyl-5-(1-methylethyl)-phenol) (Fig. 1A) presents a broad variety of properties and uses, such as antifungal, insecticidal, antibacterial, antiparasitic, and antioxidant activities (Ramos et al., 2012; Suntres et al., 2013). Thymol (5-methyl-2-(1-methylethyl)-phenol) is isomeric with carvacrol (Fig. 1B) and presents similar applications (Ramos et al., 2012). Both compounds are extensively used in the food industry as flavouring without any restriction and according to good manufacturing practices (Regulation CE 1334/2008) because they are both considered safe for consumption by the European Commission (1999).

The interest recently aroused by carvacrol and thymol is their possible application in food packaging, due to their stated antimicrobial and antioxidant properties (Didry et al., 1994; Yanishlieva et al., 1999). Moreover, they could serve as an interesting alternative to synthetic compounds such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), which have presented several undesirable side effects (Branen, 1974). Therefore, the relatively recent increase in the interest in "green" consumerism has led to a renewal of the scientific interest in these phenolic substances (Burt, 2004). In this context, the usefulness of carvacrol and thymol in food packaging is being studied by several authors (Ramos et al., 2013; Llana-Ruiz-Cabello et al., 2014) because it would be possible to allow a controlled release of active substances into the food over time. This approach could also reduce the strong flavours caused by the direct addition of this type of additive into food (Ramos et al., 2012). It is also important to note that, at present, there is no regulation concerning the quantity of oregano essential oil or any of its main compounds to be used in food packaging.

As flavourings, carvacrol and thymol are usually used in low concentrations in food intended for human consumption. However, their use in food packaging may require higher doses, increasing the level of exposure of humans to these compounds.

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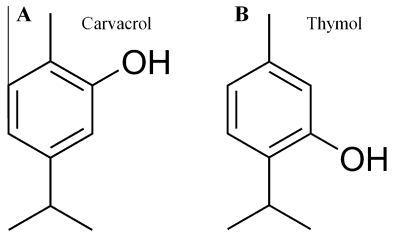

Fig. 1. (A) Carvacrol chemical structure. (B) Thymol chemical structure.

For this reason, new studies concerning the safety of carvacrol and thymol are needed (Stammati et al., 1999). Carvacrol and thymol can also be used in combination because they have demonstrated a higher effectiveness against bacteria in concomitant use (Pei et al., 2009). Therefore, the evaluation of this mixture is also required.

Various *in vitro* models have been used to assess the toxicity of carvacrol and thymol. Several authors have reported cytotoxic effects in different cell lines, such as HepG2, Caco-2, CO25, H1299, HeLa, and SiHa, exposed to these compounds (Akalin and Incesu, 2011; Horváthová et al., 2006; Mehdi et al., 2011; Ozkan and Erdogan, 2012). There are some reports concerning thymol that indicate that this compound does not alter cell viability (García et al., 2006; Llana-Ruiz-Cabello et al., 2014). However, other authors have described apoptosis induction by carvacrol and thymol (Arunasree, 2010; Deb et al., 2011; Hsu et al., 2011; Llana-Ruiz-Cabello et al., 2014; Yin et al., 2012). Little is known with respect to the mixture of carvacrol and thymol as pure compounds. In this sense, our research group has previously demonstrated that the mixture of carvacrol and thymol in a proportion of 10:1 produced cytotoxic effects at the highest concentrations assayed, as well as induction of apoptosis from 150:15 μM in Caco-2 cells (Llana-Ruiz-Cabello et al., 2014).

Taking into account that carvacrol, thymol and their mixture are able to induce toxic effects and their potential use in the food packaging industry, it would be desirable to study the mechanism underlying this toxicity to establish safe limits. In this context, oxidative stress has been noted as one possible toxic mechanism (Ozkan and Erdogan, 2012). Several studies have shown that carvacrol and thymol induce oxidative stress in several cell lines (Ozkan and Erdogan, 2012; Ündeger et al., 2009). In addition, it has been described that it is not uncommon for phenolic compounds to show both antioxidant and pro-oxidant activities at different doses (Ferguson, 2001; Ozkan and Erdogan, 2012).

Due to the increasing use of carvacrol and thymol in the food industry, for instance in active food packaging, the evaluation of the safety of these compounds is of great interest. Therefore, the present work aims to evaluate the scavenging properties of carvacrol, thymol and their mixture (10:1) *per se*, as well as the oxidative stress status and the protective and reversion abilities of these compounds against an oxidant agent in the intestinal Caco-2 cell line. This information will help to elucidate the best concentrations of these compounds to be used in food packaging to obtain remarkable beneficial results.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, foetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma–Aldrich (Spain) and VWR International Eurolab (Spain).

2.2. Model system

The Caco-2 cell line, derived from human colon carcinoma (ATCC® HTB-37), was maintained at 37 °C, in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's medium supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids, 50 μg/mL gentamicin, 1.25 μg/mL fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were grown near confluence in 75-cm^2 sterile plastic flasks and harvested twice a week with 0.25% trypsin. Viability was determined by the Trypan Blue exclusion test. Caco-2 cells were plated at density of 7.5×10^5 cells/mL to perform the experiments.

2.3. Test solutions

The range of carvacrol and thymol concentrations for the tests was selected considering the content of these active compounds to be incorporated in the packaging materials and their possible migration to food. The maximum concentration was calculated considering that the active compounds completely migrate from the active package into food (worst scenario of exposure).

Considering that 10% oregano essential oil (El Jarpil S.L., Almería, Spain) would be incorporated in 5.5–6.5 g of packaging film needed to pack 1 kg of food and that the oregano essential oil used contains 56% carvacrol and 5% thymol (proportion approximately 10:1), the potential concentration released to the food would be approximately 2500 μM and 250 μM for carvacrol and thymol, respectively. For the oxidative stress assays, as well as for the protection and the reversion assays, the concentrations of carvacrol, thymol and the mixture used in the assays were calculated based on a cytotoxicity study previously performed (Llana-Ruiz-Cabello et al., 2014). The concentrations for these assays were selected depending on the effect intended to study. Thus, for the oxidative stress assays, the mean effective concentrations (EC50) as well as its fraction were used (EC50/2 and EC50/4). Thymol did not show any cytotoxic effect, therefore, 250 μM was assessed as the highest concentration for thymol in the oxidative stress assay. Serial test solutions of carvacrol (0–460 μM), thymol (0–250 μM), and their combination in the proportion 10:1 (0–300:0–30 μM), were prepared from both stock solutions of carvacrol (2.5 M) and thymol (0.5 M) in DMSO. The final concentration of DMSO was below 0.1%.

For the protection and the reversion assays, concentrations of the compounds corresponding to cell viability greater than 90% (EC_{10}) were selected in the case of carvacrol and its mixture 10:1 with thymol. Moreover, the $EC_{10}/2$ and the $EC_{10}/4$ concentrations were also used. In the case of thymol, since no toxicity has been recorded, the highest concentration that could migrate from the package to the food (250 μM) and its fractions have been used. In the case of thymol, as no toxicity has been recorded, the highest concentration (250 μM) and its fractions have been used. These concentrations were selected in agreement with Horváthová et al. (2006), Slamenova et al. (2007), Ozkan and Erdogan (2012), and Slamenova et al. (2013), which used subcytotoxic concentrations of carvacrol and/or thymol to evaluate the protective role of these compounds against an induced oxidative damage (H_2O_2), avoiding any possible damage induced by higher concentrations.

2.4. Antioxidant activity assays

2.4.1. DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assay was performed according to Wu et al. (2005) with modifications in 96-well plates. To each well, 25 μL of different concentrations of carvacrol (0–2500 μM), thymol (0–2500 μM)

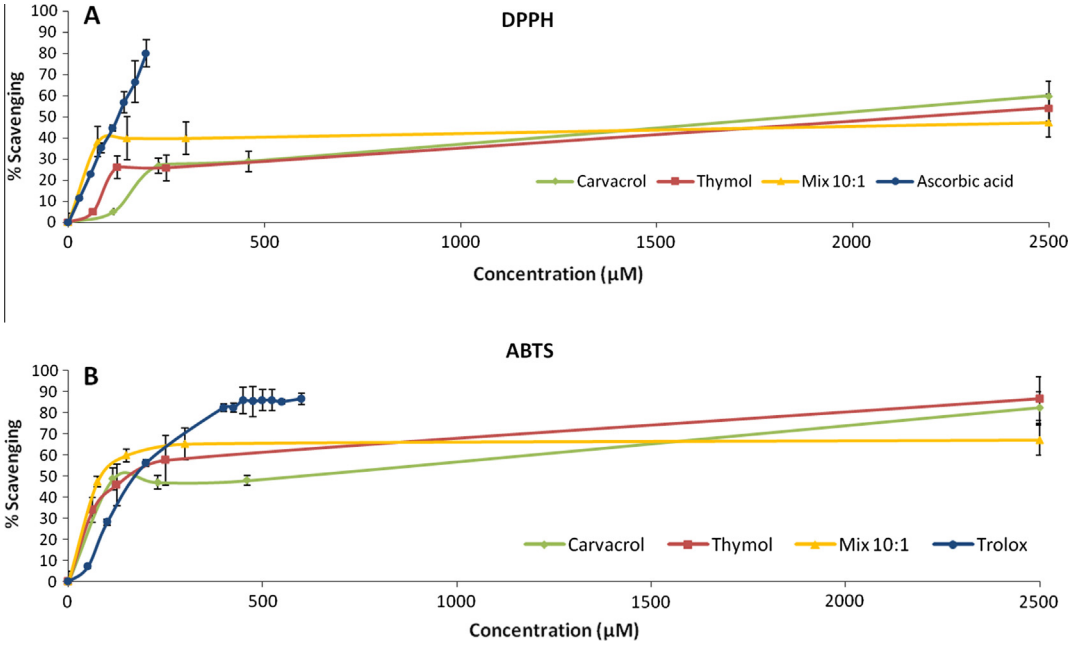

Fig. 2. Antioxidant activity of different concentrations (0–2500 μM) of carvacrol, thymol and their mixture (10:1) expressed as inhibition %. (A) DPPH assay. (B) ABTS assay. All values are expressed as mean ± S.D.

or their mixture (10:1) (0:0–2500:250 μM) were added. Then, a 100 μM DPPH methanolic solution was added to a final volume of 200 μL. The plates were shaken for 20 s and stored into the dark at room temperature for 30 min. After this time, the absorbance was measured at 517 nm (Infinite M200, Tecan, Austria). Ascorbic acid was used as a positive antioxidant control. The reduction of DPPH radicals (I%) was calculated as follows:

$$I\% = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

where A_{DPPH} is the absorbance of the uninhibited radical cation and A_S is the absorbance measured at 0, 30 and 60 min after the addition of carvacrol, thymol and their mixture (10:1). The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against sample concentration (μM). All experiments were performed in triplicate.

2.4.2. ABTS assay

The ABTS (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) radical cation decolourisation assay was performed following the method described by Re et al. (1999) with modifications. Trolox, a vitamin E analogue, was used as an antioxidant control. The ABTS radical cation ($ABTS^+$) stock solution was prepared by mixing a 7 mM ABTS water solution with a 140 mM potassium persulphate solution, obtaining a final concentration of 2.45 mM potassium persulphate. The stock solution was stored in the dark at room temperature for 12–16 h before its use. During this period, the $ABTS^+$ was produced. After the incubation period, the $ABTS^+$ stock solution was diluted with ethanol (1:88) to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30 °C. Different concentrations of carvacrol (0–2500 μM), thymol (0–2500 μM) or their mixture (10:1) (0:0–2500:250 μM) together with the diluted $ABTS^+$, were added to the plates. The absorbance was measured at 734 nm (Infinite M200, Tecan, Austria). All experiments were performed in triplicate. The reduction (I%) was calculated according to the following equation:

$$I\% = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the non-scavenged radical cations and A_S is the absorbance measured after addition of antioxidant samples. The results were also expressed as TEAC units (Trolox Equivalent Antioxidant Capacity), which is the concentration of Trolox required to reach the same antioxidant activity of 1 mM solution of carvacrol, thymol or their mixture.

2.5. Oxidative stress assays

After discarding the previous medium, exposure solutions were added to the cells and incubated at 37 °C for 24 h and 48 h. Unexposed cells were used as control group. The oxidative stress endpoints measured were reactive oxidative species (ROS) content and glutathione (GSH) levels. The production of ROS was assessed in 96-well microplates using the dichlorofluorescein (DCF) assay, as described in Gutiérrez-Praena et al. (2012). Glutathione content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (Gutiérrez-Praena et al., 2012). The results of both assays were expressed as relative light units (RLU).

2.6. Antioxidant ability assays

For the estimation of the protection and the reversion abilities of carvacrol, thymol and their mixture in Caco-2 cells, H_2O_2 (100 μM) was administered for 2 h because Wijeratne et al. (2005) found that this concentration of H_2O_2 for this exposure time induced changes in the cell membranes and antioxidant system of Caco-2 cells . Both abilities were evaluated by measuring the ROS levels, following the protocol of the ROS-Glo™ H_2O_2 Assay kit (Promega, Wisconsin, USA), and the GSH content, following the protocol of the GSH-Glo™ Glutathione Assay kit (Promega, Wisconsin, USA). A control of 0.1% DMSO was included in all plates.

For the protection assay, Caco-2 cells were first treated with EC_{10}, $EC_{10}/2$ and $EC_{10}/4$ values of carvacrol (214, 107 and 53.5 μM), thymol (250, 125 and 62.5 μM) and their mixture (85:8.5, 42.5:4.25 and 21.25:2.125 μM) for 24 h or 48 h. After the treatment time, the medium was discarded, and the cells were exposed to H_2O_2 (100 μM) for 2 h.

For the reversion assay, Caco-2 cells were first exposed to H_2O_2 (100 μM) for 2 h. Afterwards, the medium was discarded, and the cells were treated with the EC_{10}, $EC_{10}/2$ and $EC_{10}/4$ values of carvacrol, thymol and their mixture for 24 h and 48 h.

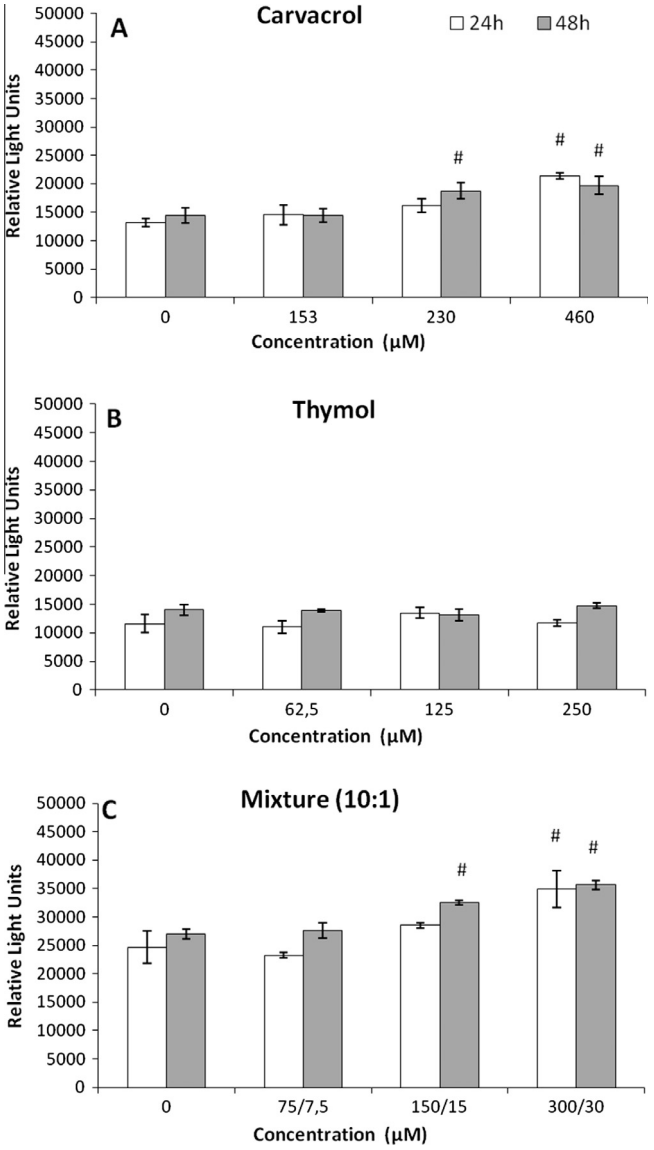

Fig. 3. ROS content in Caco-2 cells after 24 and 48 h of exposure to (A) carvacrol (0–460 μM), (B) thymol (0–250 μM) and (C) their mixture (10:1) (0–300/30 μM). All values are expressed as mean ± S.D. Differences were considered significant compared to the control group from $p < 0.01$ (#).

2.7. Calculations and statistical analysis

All experiments were performed three times per assay and six times *per* concentration. The data for all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant in respect to the control group at $p < 0.01$ (#), significant compared to the H_2O_2 control group at $p < 0.05$ (∗) and at $p < 0.01$ (§).

3. Results

3.1. Antioxidant activity assays

Both the DPPH and the ABTS assays were carried out to evaluate the free radical scavenging activities of different concentrations of carvacrol, thymol and their mixture (10:1).

In the DPPH assay (Fig. 2A), the addition of sub-cytotoxic concentrations of both compounds separately and their mixture to the DPPH solution resulted in a higher optical density compared with the positive antioxidant control, ascorbic acid, which reached an inhibition percentage (I%) of 80%. Thus, the I% reached by carvacrol, thymol and their mixture when their EC_{10} (214, 62.5 and 85:8.5 μM, respectively) was used were 27 ± 3.7%, 26 ± 8% and 39.2 ± 3.6%, respectively. Similar results were obtained when the EC_{50} of carvacrol (460 μM), thymol (250 μM) and their mixture (300:30 μM) was used (29 ± 1.1%, 26 ± 8% and 40 ± 4.2%, respectively). Moreover, carvacrol and thymol, but not their mixture, scavenged the DPPH radical in a dose-dependent manner. The IC_{50} values (concentration required to produce half of the scavenging effect) of each compound were 1832 ± 0.11 μM (carvacrol) and 2155 ± 1.24 μM (thymol). The scavenging activity of the mixture (10:1) was not linear, although its IC_{50} value was similar to those obtained for each compound separately (2342 ± 1.25 μM).

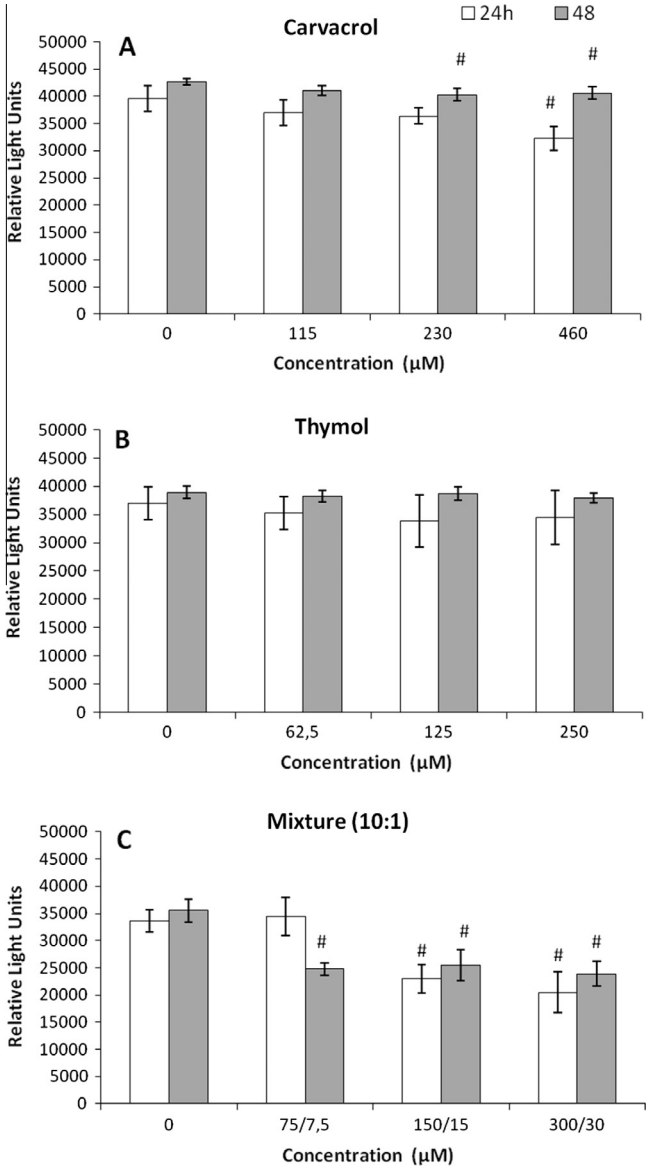

Fig. 4. GSH content in Caco-2 cells after 24 and 48 h of exposure to (A) carvacrol (0–460 μM), (B) thymol (0–250 μM) and (C) their mixture (10:1) (0–300/30 μM). All values are expressed as mean ± S.D. Differences were considered significant compared to the control group from $p < 0.01$ (#).

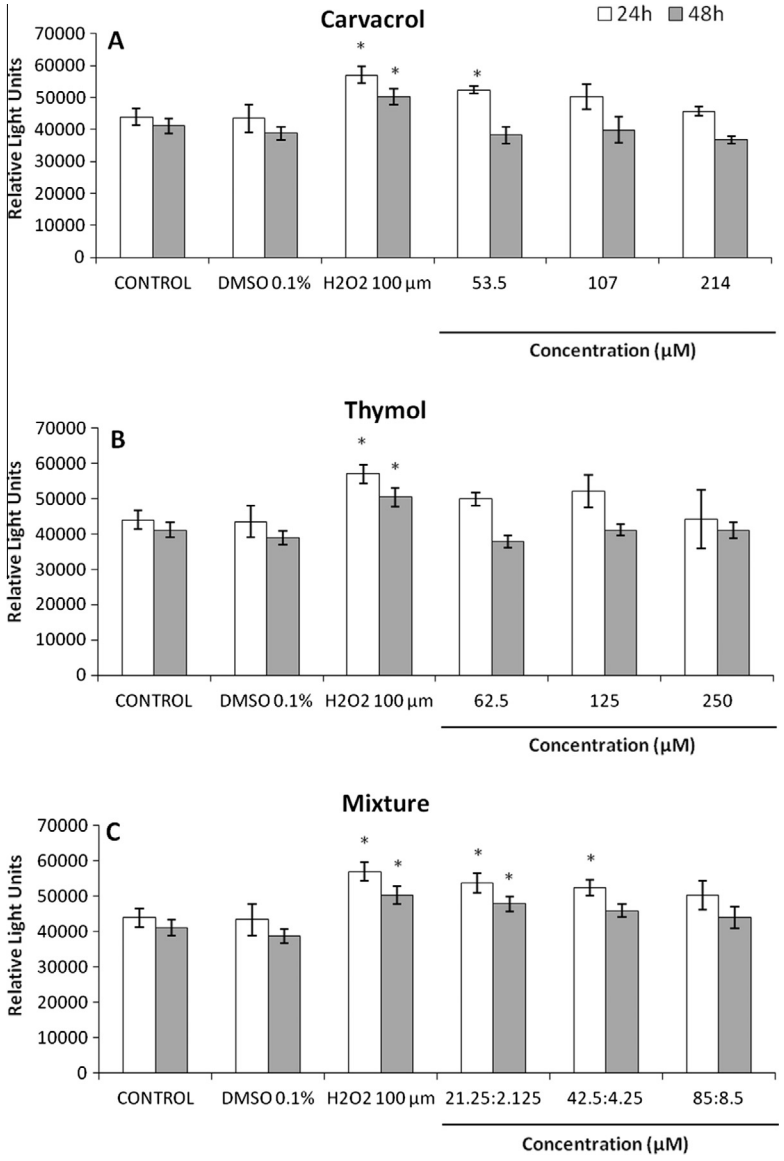

Fig. 5. ROS content in Caco-2 cells first pre-treated for 24 or 48 h with (A) carvacrol (0–214 µM), (B) thymol (0–250 µM) and (C) their mixture (10:1) (0–85/8.5 µM), and a later exposure for 2 h to H_2O_2 100 µM. All values are expressed as mean ± S.D. Differences were considered significant in respect to the control group from $p < 0.01$ (#), significant in respect to the H_2O_2 control group from $p < 0.05$ (∗) and from $p < 0.01$ (§).

The ABTS assay (Fig. 2B) showed that I% values at subcytotoxic concentrations obtained for carvacrol and the mixture were similar for EC_{10} and EC_{50}. Thus, carvacrol reached a 48 ± 4.3% scavenging percentage at EC_{10} and a 48 ± 1.2% scavenging percentage at EC_{50}, while the mixture presented an I% of 62 ± 3.7% and 65 ± 5.2% for the EC_{10} and the EC_{50}, respectively. However, thymol showed a higher I% at the highest concentration (46 ± 2.5% for the EC_{10} value and 57 ± 2.2% for the EC_{50} value). This finding was corroborated when these results were expressed as TEAC values, with carvacrol (264 µM), thymol (260 µM) and their mixture (261 µM) showing similar antioxidant abilities.

3.2. Oxidative stress assays

Caco-2 cells experienced a significant increase in ROS levels when they were exposed to the highest concentration of carvacrol (460 µM) after 24 h, while this increase occurred with 230 µM carvacrol after 48 h (Fig. 3A). Cells exposed to thymol did not show any variation at any of the concentrations assayed (Fig. 3B). The mixture of carvacrol and thymol (10:1) followed the same pattern as carvacrol, that is, it showed a significant increment of ROS levels at the highest concentration assayed (300:30 µM) after 24 h and at 150:15 µM after 48 h of exposure (Fig. 3C).

Cells exposed to carvacrol suffered a significant GSH depletion compared to the control after 24 h when the highest concentration (460 µM) was used. This reduction occurred at 230 µM after 48 h (Fig. 4A). Cells exposed to thymol did not show any change in GSH at any of the concentrations and exposure times assayed (Fig. 4B). GSH levels of Caco-2 cells exposed to the mixture (10:1) appeared significantly reduced in respect to the control group from 150:15 µM after 24 h of exposure and from 75:7.5 µM after 48 h (Fig. 4C).

3.3. Antioxidant assays

The antioxidant ability of carvacrol, thymol and their mixture was evaluated taking into account their capacity to protect Caco-2 cells against a further exposure to H_2O_2 or their capacity to revert

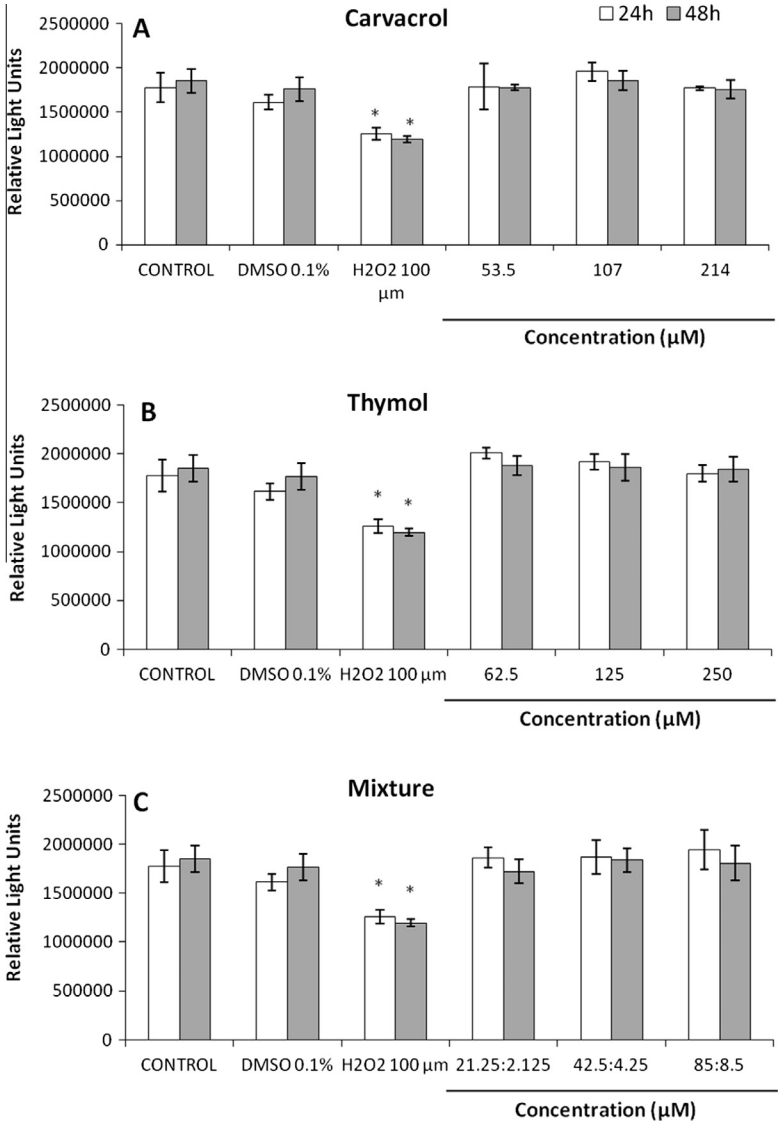

Fig. 6. GSH content in Caco-2 cells first pre-treated for 24 or 48 h with (A) carvacrol (0–214 µM), (B) thymol (0–250 µM) and (C) their mixture (10:1) (0–85/8.5 µM), and a later exposure for 2 h to H_2O_2 100 µM. All values are expressed as mean ± S.D. Differences were considered significant in respect to the control group from $p < 0.01$ (#), significant in respect to the H_2O_2 control group from $p < 0.05$ (∗) and from $p < 0.01$ (§).

the damage induced by this substance after a previous exposure. These abilities were evaluated by measuring both ROS and GSH levels.

In the protection assay, after pre-treatment with carvacrol, thymol or their mixture (10:1) for 24 h or 48 h, Caco-2 cells were exposed to H_2O_2 100 µM for 2 h. The results showed that carvacrol protected cells at the highest concentrations assayed (107 and 214 µM) after 24 h or 48 h. Thymol protected cells at all concentrations assayed, and the mixture only protected cells at the highest concentration assayed (85:8.5 µM) after 24 h and starting at 42.5:4.25 µM after 48 h (Fig. 5). GSH levels were not affected by the administration of H_2O_2 (100 µM) (Fig. 6).

In the reversion assay, after exposure to H_2O_2 (100 µM) for 2 h, Caco-2 cells were treated with carvacrol, thymol or their mixture (10:1) for 24 h or 48 h. Carvacrol did not significantly reduced ROS levels compared to the H_2O_2 (100 µM) control group at any of the concentrations and exposure time assayed, whereas thymol and the mixture reduced ROS levels down to basal levels (Fig. 7). With respect to GSH levels, carvacrol, thymol and their mixture managed to significantly increase GSH levels compared to the H_2O_2 (100 µM) control group, showing values similar to the control group (Fig. 8).

4. Discussion

The food industry is trying to take advantage of the antioxidant and antimicrobial properties of some essential oils to develop new active packaging systems for improving the food quality and the shelf-life of perishable products. In this context, the essential oil of oregano, the main components of which are carvacrol and thymol, is a good option for this purpose. Moreover, these compounds are categorised as Generally Recognised As Safe (GRAS) by the US Food and Drug Administration (López et al., 2007); therefore, their use should not entail toxicological problems. However, due to the possible use in food packaging of higher quantities of carvacrol and thymol than those approved, a toxicological assessment is required.

According to Schleiser et al. (2002), the antioxidant activity of phytochemicals cannot be evaluated by a single method due to

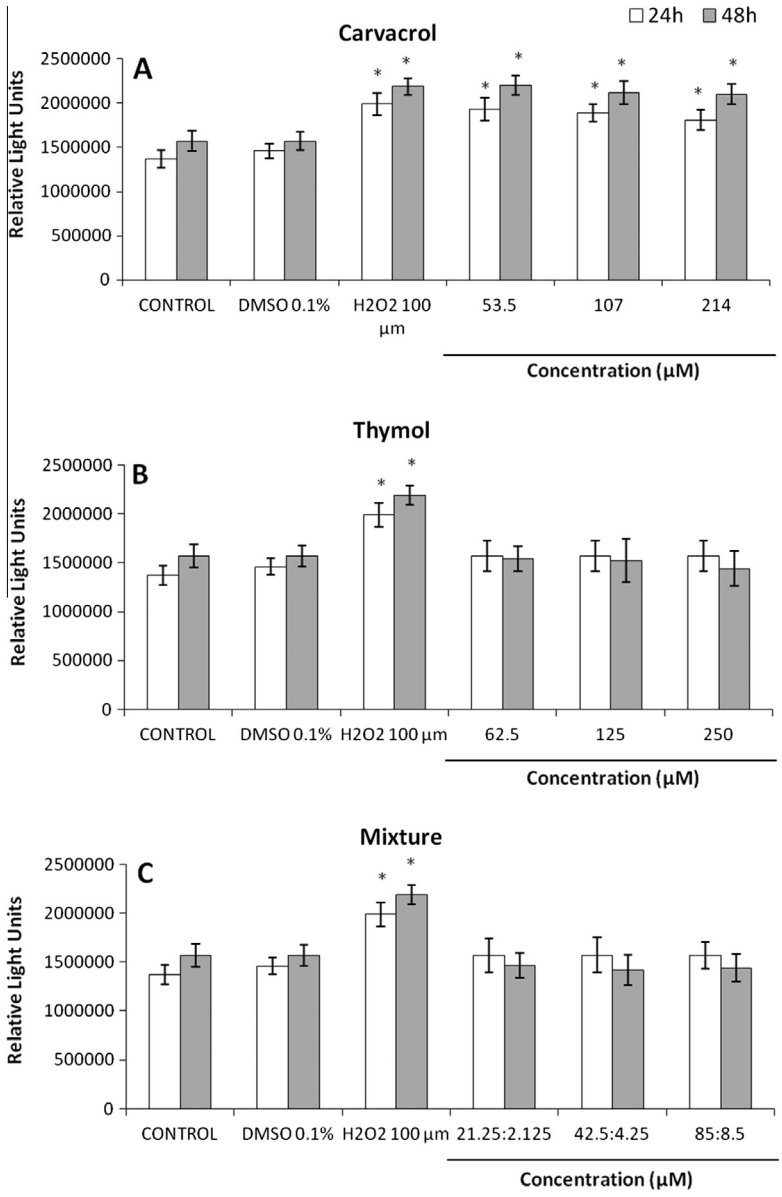

Fig. 7. ROS content in Caco-2 cells first exposed to H_2O_2 100 µM for 2 h and a later 24 or 48 h-treatment with (A) carvacrol (0–214 µM), (B) thymol (0–250 µM) and C. Their mixture (10:1) (0–85/8.5 µM). All values are expressed as mean ± S.D. Differences were considered significant in respect to the control group from $p < 0.01$ (#), significant in respect to the H_2O_2 control group from $p < 0.05$ (∗) and from $p < 0.01$ (§).

their complex reactive nature; therefore, at least two methods are recommended. For this reason, the DPPH and the ABTS assays were used to evaluate carvacrol, thymol and their mixture (10:1) in the present work, as they have been evaluated for other compounds used in active packaging (López de Dicastillo et al., 2011; Park et al., 2012; Perdones et al., 2014). The radical scavenging ability presented by these compounds showed a lower reduction of DPPH radicals compared to ascorbic acid. This is in agreement with Kulisic et al. (2004), who found the same behaviour for the oregano essential oil as well as carvacrol and thymol. These authors stated that the constituents are involved in one or more secondary reactions, resulting in a low reduction of DPPH radicals. Thus, the ROS reducing power presented could be due to a combination of the enhancement of the GSH levels and the direct action against the radicals. In contrast, Shahat et al. (2002) found that carvacrol possesses a strong free radical scavenging ability during oxidative stress. This was also corroborated by Aristatile et al. (2010), who found that carvacrol exhibited marked DPPH and ABTS radical scavenging activities. Slamenova et al. (2013) also determined the DPPH-scavenging activity of carvacrol, establishing that this activity was concentration-dependent, which has also been observed in the present study in both antioxidant assays. Moreover, Ündeger et al. (2009) also found that carvacrol and thymol had significant antioxidant activities, measured by the ABTS assay, similar to Trolox. In the present study, 2500 µM of carvacrol and thymol exhibited the same TEAC values as Trolox, whereas the mixture presented constant values with the increment of the concentration after a certain point (300:30 µM). Ündeger et al. (2009) also found that the antioxidant capacity of carvacrol was significantly higher than that of thymol, whereas in our experiment, these differences were not evident. These conflicting results could be due to the differences between the concentrations of carvacrol and thymol assayed, which are related to the proportion of both compounds in the mixture (10:1). This fact was more evident when a concentration of 2.5 mM of carvacrol and thymol was assayed because a similar antioxidant activity of both compounds was

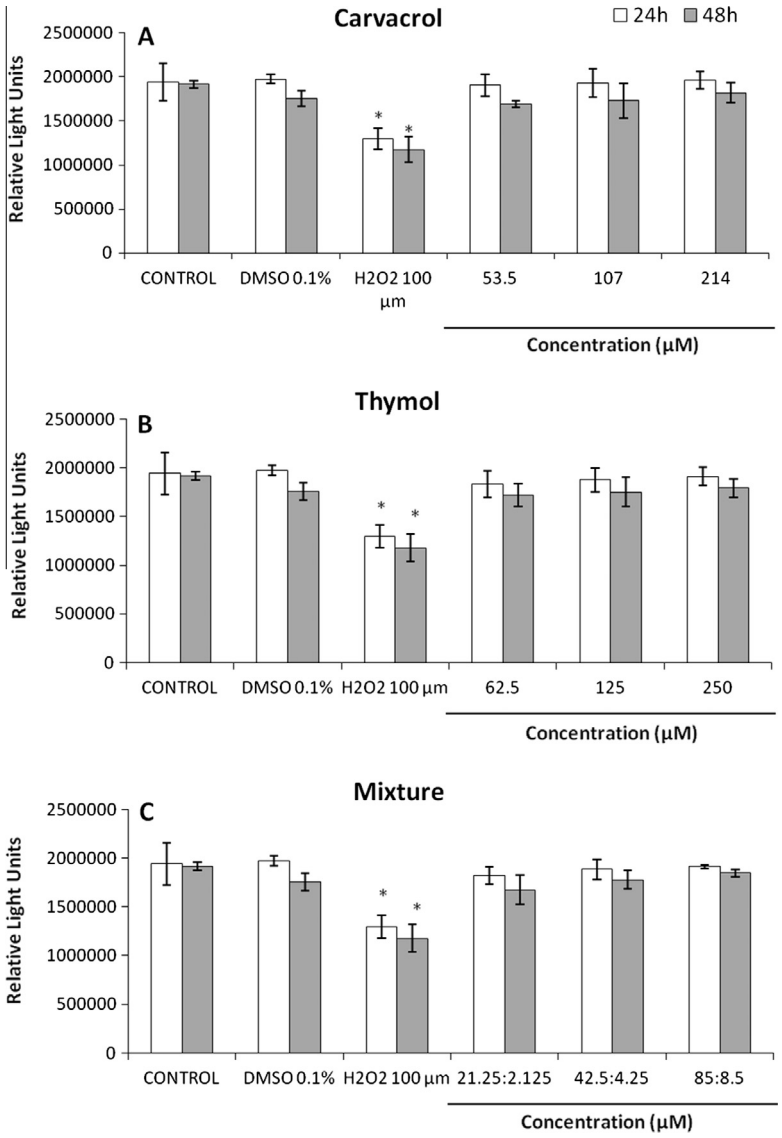

Fig. 8. GSH content in Caco-2 cells first exposed to H_2O_2 100 µM for 2 h and a later 24 or 48 h-treatment with (A) carvacrol (0–214 µM), (B) thymol (0–250 µM) and (C) their mixture (10:1) (0–85/8.5 µM). All values are expressed as mean ± S.D. Differences were considered significant in respect to the control group from $p < 0.01$ (#), significant in respect to the H_2O_2 control group from $p < 0.05$ (∗) and from $p < 0.01$ (§).

observed. Moreover, as Schleiser et al. (2002) stated, the sensitivity of the methods used may vary as a function of the compound evaluated.

As carvacrol and thymol could be used in active packaging due to their antioxidant properties, their toxic effects should be studied. Previous studies have shown that these compounds and their mixture can induce cytotoxic effects in Caco-2 cells, as well as ultrastructural alterations and cell death via apoptosis and necrosis (Llana-Ruiz-Cabello et al., 2014). Most of the literature addresses the *in vitro* antioxidant properties of carvacrol and thymol (Aeschbach et al., 1994; Yanishlieva et al., 1999; Ündeger et al., 2009; Ozkan and Erdogan, 2011), but little is known about the oxidant ability of these two compounds. The present work reveals a concentration-dependent increase of ROS levels, as well as a concentration-dependent decrease of GSH levels, for carvacrol and its mixture with thymol (10:1). In both cases, the mixture had a stronger effect, indicating oxidative stress. However, thymol did not affect the oxidation status of cells at any of the concentrations assayed and exposure times. Ündeger et al. (2009) also found that carvacrol induced a significant ROS increase in V79 Chinese hamster lung fibroblast cells exposed to 100 µM of carvacrol for 24 h. These same authors found that thymol reduced ROS levels at concentrations between 1 and 100 µM, although this reduction was not significant compared to the control group. Moreover, Ozkan and Erdogan (2012) reported that carvacrol, at higher concentrations than 380 µM, increased MDA and 8-OHdG levels in H1299 lung cancer cells after 24 h and 48 h, causing membrane damage and DNA damage, respectively. These same authors described that thymol also induced similar effects, although the concentration used in this study was higher (497 µM) than that used in ours (250 µM).

According to Ferguson (2001), it is common for phenolic compounds to present pro-oxidant or antioxidant activities depending on the dose. As it has been previously described, in the present work, carvacrol was able to induce oxidative stress, but it also showed important protective and reversion roles in oxidative stress. Similar results were obtained for thymol and the mixture of both components. Thus, carvacrol, thymol and their mixture were able to protect cells against an oxidant agent (H_2O_2), and they also reverted the damage previously induced by such agent. Consistently, Aeschbach et al. (1994) described that carvacrol and thymol inhibited phospholipid liposome induced-peroxidation in

a concentration-dependent manner (from 0.1 mM to 0.664 mM). Moreover, during autoxidation of lipids at room temperature, Yanishlieva et al. (1999) found that thymol is a more effective and more active antioxidant than carvacrol, although they also stated that their inhibiting action depends on the character of the lipidic medium. Pretreatment with carvacrol (0–300 μM) and thymol (0–600 μM) for 24 h has also been shown to protect Caco-2 and HepG2 cells against the DNA-damaging effects of H_2O_2 (250 μM) (Horváthová et al., 2006). In agreement with these findings, Aherne et al. (2007) described that a pre-treatment for 24 h with an extract of oregano essential oil (60 μg/mL), which is rich in carvacrol and thymol, protected Caco-2 cells against H_2O_2-induced DNA damage. The essential oil from *Origanum onites* (containing 24.52% carvacrol and 15.66% thymol) has been demonstrated to be more effective than carvacrol and thymol separately against the lipidic peroxidation induced by H_2O_2 in HepG2 cells (Ozkan and Erdogan, 2011). These same authors also stated that carvacrol and thymol at lower concentrations ($<IC_{50}$) protected H1299 cells against H_2O_2-induced membrane and DNA damage (Ozkan and Erdogan, 2012). Recent studies have also corroborated the protective role of carvacrol on isolated pancreas islet cells of rats (Dagli Gul et al., 2013). These authors found that carvacrol application protected cells from lipid peroxidation and protein oxidation induced by different concentrations of H_2O_2 (0–300 μM), suggesting a 20 mg/kg/day dose as the more effective. Although many studies have already stated the protective effects of carvacrol and thymol, our study shows, for the first time, that their mixture also provides this activity.

The reversion ability of carvacrol, thymol and their mixture (10:1) after oxidative stress previously induced by H_2O_2 (100 μM) was also demonstrated for the first time in the present work. This ability could be related to their own scavenging activity, as the results of the DPPH and ABTS assays corroborated. Generally, the protective abilities of both compounds and their mixture were stronger than their reversion abilities.

5. Conclusions

This work presents a wide assessment of the pro-oxidant and antioxidant profiles of carvacrol, thymol and their mixture (10:1), which, to our knowledge, had not been studied previously in the existing literature. At high concentrations, carvacrol and its mixture with thymol (10:1) induced oxidative stress. However, at low concentrations, all exposures assayed presented a protective role against an induced oxidative stress. These findings are of great interest because these compounds can be components of active packaging in the food industry, enhancing potential human exposure to them. Therefore, the proportion of these polyphenols in active packaging should be examined to ensure their safety.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

References

Aeschbach, R., Löliger, J., Scott, B.C., Murcia, A., Butler, J., Halliwell, B., Aruoma, O.I., 1994. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem. Toxicol. 32, 31–36. http://dx.doi.org/10.1016/0278-6915(84)90033-4.

Aherne, S.A., Kerry, J.P., O'Brien, N.M., 2007. Effects of plant extracts on antioxidant status and oxidant-induced stress in Caco-2 cells. Br. J. Nutr. 97, 321–328. http://dx.doi.org/10.1017/S0007114507250469.

Akalin, G., Incesu, Z., 2011. The effects of carvacrol on apoptosis of H-ras and N-ras transformed cell lines. Turk. J. Pharm. Sci. 8, 105–116.

Aristatile, B., Al-Numair, K.S., Al-Assaf, A.H., Veeramani, C., Pugalendi, K.V., 2010. Protective effect of carvacrol on oxidative stress and cellular DNA damage induced by UVB irradiation in human peripheral lymphocytes. J. Biochem. Mol. Toxicol. http://dx.doi.org/10.1002/jbt.20355.

Arunasree, K.M., 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. Phytomedicine 17, 581–588. http://dx.doi.org/10.1016/j.phymed.2009.12.008.

Branen, A.L., 1974. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J. Am. Oil Chem. Soc. 52, 59–62.

Burt, A., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. 94, 223–253. http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Commission Decision 1999/217/EC of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No. 2232/96 of the European Parliament and of the Council of 28 October 1996 (1999/217/EC). Official Journal of the European Communities.

Dagli Gul, A.S., Fadillioglu, E., Karabulut, I., Yesilyurt, A., Delibasi, T., 2013. The effects of oral carvacrol treatment against H_2O_2 induced injury on isolated pancreas islet cells of rats. Islets 5, 149–155. http://dx.doi.org/10.4161/isl.25519.

Deb, D.D., Parimala, G., Devi, S.S., Chakraborty, T., 2011. Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. Chem. Biol. Interact. 193, 97–106. http://dx.doi.org/10.1016/j.cbi.2011.05.009.

Didry, N., Dubreuil, L., Pinkas, M., 1994. Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. Pharm. Acta Helv. 69, 25–28. http://dx.doi.org/10.1016/0031-6865(94)90027-2.

Ferguson, L.R., 2001. Role of plant polyphenols in genomic stability. Mutat. Res. 475, 89–111. http://dx.doi.org/10.1016/S0027-5107(01)00073-2.

García, D.A., Bujons, J., Vale, C., Sunol, C., 2006. Allosteric positive interaction of thymol with the GABA receptor in primary cultures of mouse cortical neurons. Neuropharmacology 50, 25–35. http://dx.doi.org/10.1016/j.neuropharm.2005.07.009.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Moreno, F.J., Cameán, A.M., 2012. Alterations observed in the endothelial HUVEC cell line exposed to pure Cylindrospermopsin. Chemosphere 89, 1151–1160. http://dx.doi.org/10.1016/j.chemosphere.2012.06.023.

Horváthová, E., Šramkova, M., Labaj, J., Slamenova, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured *in vitro*. Neuroendocrinol. Lett. 27, 44–47.

Hsu, S.S., Lin, K.L., Chou, C.T., Chiang, A.J., Liang, W.Z., Chang, H.T., Tsai, J.Y., Liao, W.C., Huang, F.D., Huang, J.K., Chen, I.S., Liu, S.I., Kuo, C.C., Jan, C.R., 2011. Effect of thymol on Ca^{2+} homeostasis and viability in human glioblastoma cells. Eur. J. Pharmacol. 670, 85–91. http://dx.doi.org/10.1016/j.ejphar.2011.08.017.

Kulisic, T., Radonic, A., Katalinic, V., Milos, M., 2004. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85, 633–640. http://dx.doi.org/10.1016/j.foodchem.2003.07.024.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Moreno, F.J., Bermúdez, J.M., Aucejo, S., Cameán, A.M., 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290. http://dx.doi.org/10.1016/j.fct.2013.12.005.

López de Dicastillo, C., Nerín, C., Alfaro, P., Catalá, R., Gavara, R., Hernández-Muñoz, P., 2011. Development of new antioxidant active packaging films based on ethylene vinyl alcohol copolymer (EVOH) and green tea extract. J. Agric. Food Chem. 59, 7832–7840. http://dx.doi.org/10.1021/jf201246g.

López, P., Sánchez, C., Batlle, R., Nerín, C., 2007. Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. J. Agric. Food Chem. 55, 4348–4356. http://dx.doi.org/10.1021/jf063295u.

Mehdi, S.J., Ahmad, A., Irshad, M., Manzoor, N., Rizvi, M.M.A., 2011. Cytotoxic effect of carvacrol on human cervical cancer cells. Biol. Med. 3, 307–312.

Ortega-Nieblas, M.M., Robles-Burgueño, M.R., Acedo-Félix, E., González-León, A., Morales-Trejo, A., Vázquez-Moreno, M.L., 2011. Chemical composition and antimicrobial activity of oregano (Lippia palmeri S. Wats) essential oil. Rev. Fitotec. Mex. 34, 11–17.

Ozkan, A., Erdogan, A., 2011. A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. Turk. J. Biol. 35, 735–742. http://dx.doi.org/10.3906/biy-1011-170.

Ozkan, A., Erdogan, A., 2012. A comparative study of the antioxidant/prooxidant effects of carvacrol and thymol at various concentrations on membrane and DNA of parental and drug resistant H1299 cells. Nat. Prod. Commun. 7, 1557–1560.

Park, H.Y., Kim, S.J., Kim, K.M., You, Y.S., Kim, S.Y., Han, J., 2012. Development of antioxidant packaging material by applying Corn-Zein to LLDPE film in combination with phenolic compounds. J. Food Sci. 77, 273–279. http://dx.doi.org/10.1111/j.1750-3841.2012.02906.x.

Pei, R.S., Zhou, F., Ji, B.P., Xu, J., 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against E. coli with an improved method. J. Food Sci. 74, 379–383. http://dx.doi.org/10.1111/j.1750-3841.2009.01287.x.

Perdones, A., Vargas, M., Atares, L., Chiralt, A., 2014. Physical, antioxidant and antimicrobial properties of chitosan–cinnamon leaf oil films as affected by oleic acid. Food Hydrocoll. 36, 256–264. http://dx.doi.org/10.1016/j.foodhyd.2013.10.003.

Ramos, M., Jiménez, A., Peltzer, M., Garrigós, M.C., 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. J. Food Eng. 109, 513–519. http://dx.doi.org/10.1016/j.jfoodeng.2011.10.031.

Ramos, M., Beltrán, A., Valdés, A., Peltzer, M.A., Jiménez, A., Garrigós, M.C., Zaikov, G.E., 2013. Carvacrol and thymol for fresh food packaging. J. Bioequiv. Availab. 5, 154–160.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237. http://dx.doi.org/10.1016/S0891-5849(98)00315-3.

Regulation (EC) No. 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No. 1601/91, Regulations (EC) No. 2232/96 and (EC) No. 110/2008 and Directive 2000/13/EC.

Schleiser, K., Harwat, M., Bohm, V., Bitsch, R., 2002. Assessment of antioxidant activity by using different *in vitro* methods. Free Radic. Res. 36, 177–187.

Shahat, A.A., Cos, P., De Bruyne, T., Apers, S., Hammouda, F.M., Ismail, S.I., Azzam, S., Claeys, M., Goovaerts, E., Pieters, L., Vanden-Berghe, D., Vlietinck, A.J., 2002. Antiviral and antioxidant activity of flavonoids and proanthocyanidins from Crataegus sinaica. Planta Med. 68, 539–541. http://dx.doi.org/10.1055/s-2002-32547.

Slamenova, D., Horvathova, E., Sramkova, M., Marsalkova, L., 2007. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*. Neoplasma 54, 108–112.

Slamenova, D., Kozics, K., Hunakova, L., Melusova, M., Navarova, J., Horvathova, E., 2013. Comparison of biological processes induced in HepG2 cells by tert-butyl hydroperoxide (t-BHP) and hydroperoxide (H_2O_2): the influence of carvacrol. Mutat. Res.: Genet. Toxicol. Environ. Mutagen. 757, 15–22. http://dx.doi.org/10.1016/j.mrgentox.2013.03.014.

Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823. http://dx.doi.org/10.1016/S0278-6915(99)00075-7.

Suntres, Z.E., Coccimiglio, J., Alipour, M., 2013. The bioactivity and toxicological actions of carvacrol. Crit. Rev. Food Sci. Nut. http://dx.doi.org/10.1080/10408398.2011.653458.

Teixeira, B., Marques, A., Ramos, C., Serrano, C., Matos, O., Neng, N.R., Nogueira, J.M.F., Saraiva, J.A., Nunes, M.L., 2013. Chemical composition and bioactivity of different oregano (*Origanum vulgare*) extracts and essential oil. J. Sci. Food Agric. 93, 2707–2714. http://dx.doi.org/10.1002/jsfa.6089.

Ündeger, U., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043. http://dx.doi.org/10.1016/j.fct.2009.05.020.

Wijeratne, S.S.K., Cuppet, S.L., Schlegel, S., 2005. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. J. Agric. Food Chem. 53, 8768–8774. http://dx.doi.org/10.1021/jf0512003.

Wu, J.H., Tung, Y.T., Wang, S.Y., Shyur, L.F., Kuo, Y.H., Chang, S.T., 2005. Phenolic antioxidants from the heartwood of Acacia confuse. J. Agric. Food Chem. 53, 5917–5921. http://dx.doi.org/10.1021/jf050550m.

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., Raneva, V.G., 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem. 64, 59–66. http://dx.doi.org/10.1016/S0308-8146(98)00086-7.

Yin, Q., Yan, F., Zu, X.Y., Wu, Y., Wu, X., Liao, M., Deng, S., Yin, L., Zhuang, Y., 2012. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology 64, 43–51. http://dx.doi.org/10.1007/s10616-011-9389-y.

CAPÍTULO 8 / CHAPTER 8

María Llana-Ruiz-Cabello, Sara Maisanaba, Maria Puerto, Silvia Pichardo, Ángeles Jos, Rosario Moyano, Ana M. Cameán

A SUBCHRONIC 90-DAY ORAL TOXICITY STUDY OF ORIGANUM VULGARE ESSENTIAL OIL IN RATS.

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A subchronic 90-day oral toxicity study of *Origanum vulgare* essential oil in rats

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ABSTRACT

Oregano essential oil (*Origanum vulgare L. virens*) (OEO) is being used in the food industry due to its useful properties to develop new active packaging systems. In this concern, the safety assessment of this natural extract is of great interest before being commercialized. The European Food Safety Authority requests different *in vivo* assays to ensure the safety of food contact materials. One of these studies is a 90 days repeated-dose oral assay in rodents. In the present work, 40 male and 40 female Wistar rats were orally exposed to 50, 100 and 200 mg/kg body weight (b.w.) OEO during 90 days following the OECD guideline 408. Data revealed no mortality and no treatment-related adverse effects of the OEO in food/water consumption, body weight, haematology, biochemistry, necropsy, organ weight and histopathology. These findings suggest that the oral no-observed-adverse-effect level (NOAEL) of this OEO is 200 mg/kg b.w. in Wistar rats, the highest dose tested. In conclusion, the use of this OEO in food packaging appears to be safe based on the lack of toxicity during the subchronic study at doses 330-fold higher than those expected to be in contact consumers in the worst scenario of exposure.

1. Introduction

Oregano (*Origanum vulgare L.*) is an aromatic plant with a wide distribution throughout the Mediterranean area and Asia (Wei et al., 2016). The composition of oregano essential oil (OEO) commonly includes carvacrol, thymol, α-terpinene and ρ-cymene among other compounds (Burt, 2004). The traditional applications of OEO are related to the properties against microorganism and oxidation. Recently, OEO has further application since it is recognized as a natural preservative agent with a strong potential for food preservation (Muriel-Galet et al., 2015). In this sense, the antimicrobial effect of OEO on food has been extensively studied. de Medeiros Barbosa et al. (2016) reported antimicrobial activity of OEO combined with rosemary EO at subinhibitory concentrations in fresh leafy vegetables. Also in meat, OEO has demonstrated to display an antimicrobial effect alone (Soultos et al., 2009; Jayasena and Jo, 2013; Pesavento et al., 2015) and in combination with other EO from clove and cinnamon (Radha Krishnan et al., 2014). In addition, OEO has been also useful in cheese not only as antibacterial (Govaris et al., 2011) but also as antioxidant (Asensio et al., 2015). However, the direct addition of OEO may alter the organoleptic characteristics of food and influence negatively in its acceptance. In this sense, Van Haute et al. (2016) reported that the sensorial properties of the meat/fish marinade with OEO and other EOs (thyme and cinnamon) are inevitably affected when the necessary EO concentrations to extend the microbial shelf life are applied. Similarly, a concentration of 4% OEO in active packaging gave rise to unacceptable oregano smell of fresh beef steaks (Camo et al., 2011). Due to the intense aroma of OEO, it can be used in food matrices to provide a balance between sensory acceptability and properties exerted by the spice (Cattelan et al., 2015). In order to avoid the direct incorporation of OEO into food, the active food packaging is a promising trend that allows using OEO that are gradually release from the package to the food (Llana-Ruiz-Cabello et al., 2016a).

The effectiveness of OEO included in food packaging has been confirmed as a preservative in food. Previous experiments carried out in our laboratory have checked the efficacy of polylactic acid

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films containing 5 and 10% OEO as antioxidant and against microorganism (mainly yeast and molds) in ready-to-eat salads (Llana-Ruiz-Cabello et al., 2016a). A concentration of at least 1% oregano extract in the active packaging system was needed to significantly increase beef display life from 14 to 23 days (Camo et al., 2011). In addition, antioxidant and antimicrobial properties of ethylene vinyl alcohol copolymer films containing OEO and green tea extract components were confirmed (Muriel-Galet et al., 2015). In fact, OEO containing gelatine films exhibited higher *in vitro* antimicrobial and antioxidant properties than films incorporating lavender EO (Martucci et al., 2015).

Along with the usefulness of OEO, included in active food packaging as preservative, its safety should be also confirmed. In this regard, OEO is categorised as 'generally recognized as safe' (GRAS) by the Food and Drug Administration (Manso et al., 2014) and it is classified as a food additive by the European Union (Muriel-Galet et al., 2015). As flavouring, OEO is normally used in foods at low concentrations. However, the use of these compounds in other applications such as in active packaging may require higher doses that will increase the concern regarding exposure to these compounds (Stammati et al., 1999). In addition, according to the Commission Regulation (EC) No 450/2009, only substances that are included in the Community list of authorised substances may be used in components of active packaging. However, no substance has been included in the list so far. Therefore, a toxicological assessment is needed. The Guidelines of the Scientific Committee on Food for safety assessment of substances used in food contact materials (EFSA, 2016) recommend genotoxicity and subchronic studies in the core set of tests. Genotoxicity studies of OEO and its components are very scarce (EFSA, 2008). However, in the case of the OEO used in the present study the genotoxicity has been evaluated. Results obtained in our laboratory have indicated absence of genotoxic effects of this OEO in rats exposed up to 200 mg/kg body weight (b.w.) (Llana-Ruiz-Cabello et al., 2016c). Carvacrol and thymol, two of the main components of OEO have been also studied. Most of the studies have reported that thymol was neither mutagenic nor genotoxic using *in vitro* assays (Azizan and Blevins, 1995; Stammati et al., 1999; Horvathova et al., 2006; Buyukleyla and Rencuzogullari, 2009; Llana-Ruiz-Cabello et al., 2014a; Maisanaba et al., 2015). However, contradictory results have been obtained for carvacrol using *in vitro* test. It exhibited mutagenic potential and oxidative damage in DNA in the comet assay (Llana-Ruiz-Cabello et al., 2014a). Also, carvacrol showed a weak genotoxic potential on L5178Y/Tk ± cells (Maisanaba et al., 2015). Similarly, in mammalian cells, a positive response was obtained in human lymphocytes through the standard comet assay (Aydin et al., 2005a,b). However, other authors have also observed negative results (Stammati et al., 1999; Ündeger et al., 2009; Aydin et al., 2014). In order to complete the toxicological assessment of OEO and its components *in vivo* studies are needed, but those dealing the toxicity of OEO are very scarce. In the case subchronic studies, no previous experiments have been conducted by the moment as far as we know.

Considering all this background, the aim of the present work was to study for the first time the subchronic toxicity of OEO, containing carvacrol/thymol (10:1), intended to be used in active packaging for food applications, in Wistar rats orally exposed to different concentrations of this extract for 90 days. According to the OECD 408 guideline (OECD, 1998), body weight and food and water consumption were recorded. Moreover, clinical observation, haematological and biochemistry parameters, gross and microscopic pathology were performed.

2. Materials and methods

2.1. Supplies and chemicals

Commercial powder neutral gelatine from pork protein (Jesus Navarro S.A., Alicante, Spain) was used as the vehicle for the test item in the dosed groups and control group in the 90-days study. The rest of the chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Oregano essential oil analysis

Oregano essential oil was acquired from El Jarpil® (lot number OR2015) (Almería, Spain). It was analysed according to NF ISO 11024 using a Hewlett Packard 5890 chromatograph interfaced to a Hewlett Packard 5970 Mass selective detector (Hewlett Packard, Palo Alto, USA). The gas chromatograph was equipped with a polar column HP INNOWAX, of 60 m × 0.25 mm x 0.5 μm film thickness. The oven temperature was held at 60 °C for 6 min, raised to 250 °C at 2 °C min^{-1}, and maintained at 250 °C for 10 min. Helium was used as carrier gas at 22 psi and the injection volume was 1 μL. Compound assignment was achieve by single ion monitoring for various homologous series and via comparison with published and stored data (NKS Library). In Table 1 the components found in a percentage above 1% have been listed, being the main components carvacrol (55.82%), ρ-cimene (16.39%), thymol (5.14%), γ-terpine (4.71%) and β-cariophyllene (2.40%).

2.3. Diets

In order to select the doses for the 90-day study, the acute oral toxicity study "Up and Down Procedure", OECD 425 (OECD, 2008) was carried out in Wistar rats. No animal died after dosage up to 2000 mg/kg b.w. of this OEO administered by gavage, so the median lethal dose (LD50) was set above this dose. In addition, a preliminary palatability study evidenced that the maximum dose that was accepted by animals when added to neutral gelatine was 200 mg/kg. Therefore, the selected doses were calculated by dividing this data by a factor of 2, being the doses 50, 100 y 200 mg/kg b.w./day (d). Dietary dose individual formulations were prepared according to Mellado-García et al. (2016).

2.4. Animal and experimental design

The 90-day toxicity study was performed at the Central Service of Experimental Animals from the University of Córdoba (SAE, Córdoba, Spain), in accordance with the OECD Guideline 408

Table 1
Main components of *Origanum vulgare L.* essential oil.

RT (min)	Compound	%
10,6	α-PINENE	1,10
10,8	α-THUYENE	1,69
17,9	β-MYRCENE	1,52
19,2	α-TERPINENE	1,62
23,5	γ-TERPINENE	4,71
25,3	p-CYMENE	16,39
36,8	1-OCTEN-3-OL	1,50
47,4	TERPINENE-4-OL	1,33
47,5	β-CARYOPHYLLENE	2,40
79,8	THYMOL	5,14
81,5	CARVACROL	55,82

RT: retention time. Only the main components were reported. (>1%).

(OECD, 1998), and was codified as 2015-11-SAE. All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes (Directive, 2010/63/EU, Decision, 2012/707/UE and RD 53/2013). All procedures have been approved by the Ethical Animal Experimentation Committee of the University of Córdoba, and by the Junta de Andalucía (project no. 05/10/2015-339).

For the 90-day study, 40 male and 40 female Wistar rats, strain Crl. WI (Han) (type outbred rats), were supplied by Charles River Laboratories S.L. (Kings, NY, USA). Rats were fed during the acclimatization period (1 week) with standard laboratory feed (Harlan Laboratories, Barcelona, Spain) and water *ad libitum*. During acclimatization, the animals were examined by a veterinary surgeon. At the start of dosing, at approximately 8 weeks of age, average body weight of the males was 297.5 ± 2.0 g and of the females was 188.7 ± 3.6 g.

Animals were individually housed in cages. Rats were randomly assigned to groups (10 rats/sex/group) so that mean body weights were similar for each group. The body weight range did not exceed ±20% of the mean weight/sex at beginning of treatment. The maintenance conditions are described in Mellado-García et al. (2016).

2.5. Body weight, food and water consumption

Rats were observed daily, and body weights, food intake, and water consumption were recorded weekly. The mean body weights per group and sex were calculated weekly from the individual animals. The feed conversion efficiency (FCE) was determined by the ratio of food intake (g)/weight gained (g), according to Escobar et al. (2015).

2.6. Haematology and blood chemistry

Blood samples were collected by intracardiac injection (lightly anaesthetized with isoflurane). Animals were fasted overnight prior blood collection (week 13). The haematological parameters studied are described in Mellado-García et al. (2016). Briefly, the haematological parameters evaluated on the automatic haematology analyzer Cell-Dyn 3700 (Abbot, GMI, MI, USA) were: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MHCH), blood platelet count (PLT), red cell volume distribution (RDW), white blood cell count (WBC), and differential leucocyte count (Neutrophils (NE), Lymphocytes (LY), Monocytes (MO), Eosinophils (EO) and Basophils (BA)).

The standard serum biochemistry parameters were analysed with an automatic chemistry analyzer Cobas 6000 (Roche Diagnostics, IN, USA), to evaluate the following serum biochemistry parameters: glucose (GLUC), blood urea nitrogen (BUN), creatinine (CREAT), bile acids, total cholesterol (CHOL), triglycerides (TRIGL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), albumin (ALB), total protein (TOT PROT), sodium (Na^+) and potassium (K^+) ions.

2.7. Necropsy, organ weights and histopathology

Rats were fasted overnight (approximately 18 h) and deeply anaesthetized with isofluorane, then euthanized with carbon dioxide inhalation followed by exsanguinated by intracardiac injection. All animals were macroscopically examined as well as the organs after extraction. The following tissues and organs were collected and weighed wet *in situ* after dissection: brain, liver, intestine, stomach, lungs, heart, spleen, kidneys, thymus, adrenal glands, testes and epididims (males), and uterus with cervix and ovaries (females).

According to the guideline OECD 408 (OECD, 1998), full histopathology was carried on the organs and tissues of all animals in the control and the highest dose (200 mg/kg b.w. OEO) groups. Light microscopy examination of liver, kidney, intestine, stomach, lung, heart, and testicle/ovary was performed. Samples were first fixed in 10% buffered formalin for 24 h at 4 °C, and then immediately dehydrated in graded series of ethanol, immersed in xylol and embedded in paraffin wax using an automatic processor. Sections of 3–5 mm were mounted. Afterwards, tissue sections were deparaffinised, rehydrated, stained with haematoxylin and eosin, and mounted with Cristal/Mount (Paraplat, Oxford Labware, St. Louis, MO.).

2.8. Statistical analysis

Continuous variables, including body weight, body weight gain, food and water consumption, haematology, clinical chemistry, and organ weight, were summarized using standard measures of central tendency and dispersion, mean and standard deviation (SD), and were reported by sex and dosage. One-way analysis of variance (ANOVA) was performed to test differences in continuous variables. Normality assumption was tested using Kolmogorov-Sminorv's test. If non-normality, comparison were carried out with Kruskal-Wallis test. If those tests were statistically significant, multiple comparisons were performed using Tukey-Kramer/Dunn's Multiple Comparisons Tests. Differences were considered significant from $P < 0.05$. All statistical analysis were carried out using Graph-Pad Instant software (GraphPadSoftware Inc., La Jolla, USA).

3. Results

3.1. Survival and clinical observations

No mortality occurred during the study. Clinical and ophthalmological examination showed no abnormalities in all groups during the test period. Overall, there were no remarkable treatment-related changes in behaviour or in locomotor activity during the study period. However, one female rat from the control group showed an increase in the scratch pattern, so it was euthanized for humane reasons on day 89. Samples of skin from this animal were analysed and no alteration was observed.

3.2. Body weight, body weight gain, food consumption, feed conversion efficiency, and water consumption

No significant differences were observed in final body weight, body weight gain or total food consumption between rats in any treatment group in comparison to their control groups (Table 2). In addition, the FCE remained unaltered in rats treated with the three doses of OEO.

The mean body weight per week of male and female rats exposed to OEO (50, 100 and 200 mg/kg b.w./d) was not significantly changed through the experiment (Fig. 1). Similarly, no significant changes were observed in % body weight gain in males (Fig. 2a) and females (Fig. 2b). There was no change in food consumption of animals exposed to OEO for 90 days (Supplementary data). Moreover, no consistent differences were recorded in water consumption at any dosage group when compared to the control group (Supplementary data).

3.3. Haematology and blood chemistry

Haematology parameters measured in rats orally exposed to

Table 2
Effect of 90 days oral exposure to OEO on body weight and food consumption in Wistar rats. Values represent the mean ± SD of 10 rats/sex/group. Differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values).

Parameters	Male				Female			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
	(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)	(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)
	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
Inicial body weight (g)	296.9 ± 9.7	296.6 ± 5.9	297.1 ± 11.2	299.5 ± 6.1	185.1 ± 9.0	187.0 ± 5.1	192.1 ± 8.0	190.8 ± 7.3
	F(36.3) = 0.24 p = 0.88; N.S.				**F(36.3) = 1.90 p = 0.42; N.S.**			
Final body weight (g)	512.6 ± 48.6	495.4 ± 29.4	502.2 ± 39.7	514.1 ± 25.6	255.5 ± 19.3	265.4 ± 15.2	261.6 ± 12.0	258.9 ± 12.5
	F(36.3) = 0.58 p = 0.63; N.S.				**F(36.3) = 0.77 p = 0.94; N.S.**			
Body weight gain	215 ± 44.2	198.8 ± 26.3	205.1 ± 35.7	214.6 ± 23.6	70.4 ± 11.4	78.4 ± 12.6	69.5 ± 15.3	68.1 ± 10.2
	F(36.3) = 0.58 p = 0.63; N.S				**F(36.3) = 1.38 p = 0.27; N.S.**			
Total feed intake (g)	2195.6 ± 159.4	2101.3 ± 168.8	2096.3 ± 138.3	2128.7 ± 93.8	1362.2 ± 120.3	1468.4 ± 128.1	1424.7 ± 92.7	1406.5 ± 61.7
	F(36.3) = 1.02 p = 0.40; N.S.				**F(36.3) = 1.79 p = 0.17; N.S.**			
Feed conversion ratio	10.5 ± 1.8	10.7 ± 0.9	10.4 ± 1.4	10.0 ± 1.0	19.7 ± 3.0	19.0 ± 2.1	21.2 ± 3.7	21.1 ± 3.6
	F(36.3) = 0.44 p = 0.72; N.S.				**F(36.3) = 1.18 p = 0.33; N.S.**			

Values are mean ± SD for 10 rats/sex/group.
F: Statistics ANOVA test; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

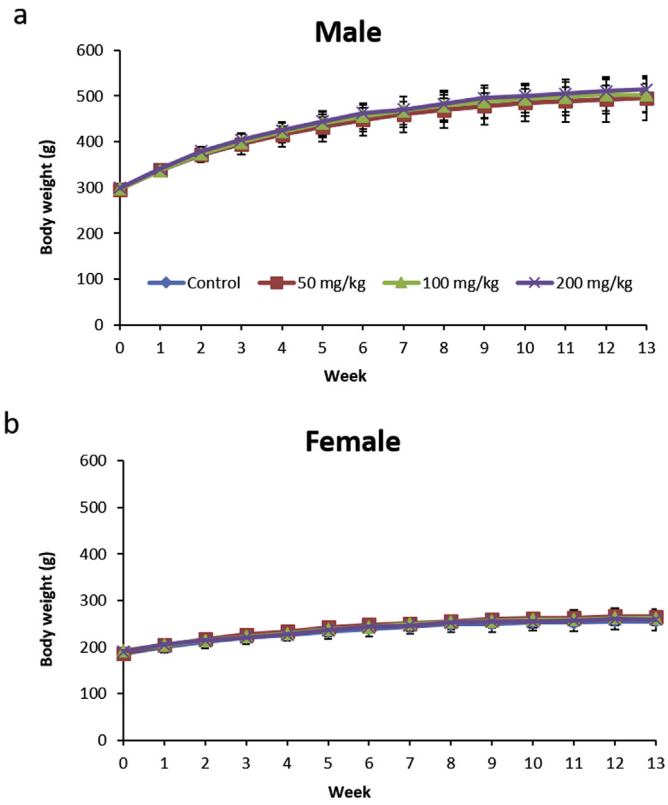

Fig. 1. Mean body weights (g) of a) male and b) female rats orally exposed to 50, 100 and 200 mg/kg b.w./d OEO and control rats for 90 days.

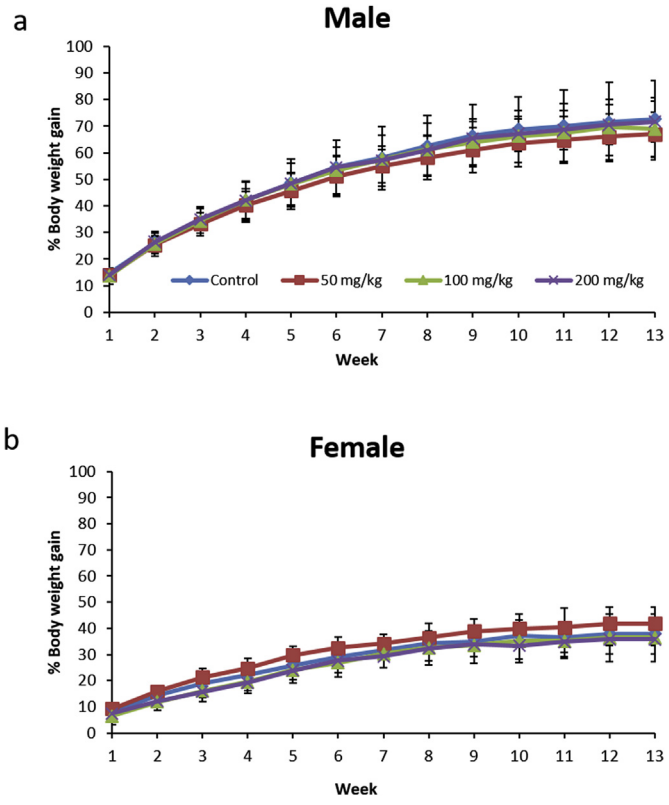

Fig. 2. Mean body weight gain (%) of a) male and b) female rats orally exposed to 50, 100 and 200 mg/kg b.w./d OEO and control rats for 90-day.

OEO are shown in Table 3. All parameters remained unaltered except the RDW that underwent a significant decrease in females fed with the highest dose (200 mg/kg b.w./d OEO). In addition, no significant differences in total and differential leucocyte counts were recorded for rats fed with OEO in comparison to the untreated male and female rats (Table 4).

Clinical biochemistry parameters of rats subchronically exposed to 50, 100 and 200 mg/kg b.w./d OEO are shown in Table 5. These results revealed only significant differences in GLUC levels in male rats exposed to 50 mg/kg/d OEO that decreased in comparison to the control group and in female fed with 200 mg/kg b.w./d OEO that increased when compared to the group exposed to 50 mg/kg/d OEO.

3.4. Necropsy, organ weights and histopathology

Macroscopic examination of organs and tissues from rats subchronically exposed to this OEO revealed no remarkable damage. Moreover, no changes in the organ weight (Table 6) were observed except in kidney in female rats, which weight slightly increased in the group exposed to 200 mg/kg b.w./d OEO in comparison to rats fed with 100 mg/kg b.w./d OEO. Similarly, no significant changes were recorded in the relative organ weight/body weight ratio

Table 3
Haematology parameters of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-days. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). *p < 0.05 in comparison to control group values.

Haematology data summary									
		Male				Female			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)	(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
RBC	MEAN	9.1	9.3	9.4	9.4	7.8	7.8	8.0	7.8
10^6/μl	ST. DEV.	0.6	0.6	0.5	0.6	0.4	0.8	0.4	0.6
		F(36.3) = 0.4373 p = 0.73; N.S.				**F(36.3) = 0.20 p = 0.90; N.S.**			
HGB	MEAN	13.9	13.9	14.4	14.5	12.8	13.4	14.3	13.0
g/dL	ST. DEV.	0.9	1.0	1.2	0.6	1.8	1.4	0.8	1.5
		KW = 3.05 p = 0.39; N.S.				**KW = 4.74 p = 0.19; N.S.**			
HCT	MEAN	69.9	70.6	71.9	70.6	65.3	65.4	66.8	64.4
%	ST. DEV.	3.0	4.3	3.9	3.0	3.5	5.7	3.2	4.4
		F(36.3) = 0.54 p = 0.66; N.S.				**KW = 1.81 p = 0.61; N.S.**			
MCV	MEAN	77.1	75.8	77.0	75.5	83.4	83.6	84.0	83.0
fL	ST. DEV.	2.1	1.9	2.3	2.0	2.3	2.3	1.9	2.1
		F(36.3) = 1.42 p = 0.25; N.S.				**F(36.3) = 0.29 p = 0.83; N.S.**			
MCH	MEAN	15.4	14.9	15.4	15.5	16.4	17.2	18.0	16.7
pg	ST. DEV.	1.7	1.5	1.6	1.3	2.1	1.4	0.5	1.2
		KW = 1.50 = 0.68; N.S.				**F(36.3) = 2.50 p = 0.08; N.S.**			
MCHC	MEAN	20.0	19.6	20.0	20.6	19.6	20.6	19.4	20.1
g/dL	ST. DEV.	1.8	2.0	2.1	1.5	2.2	1.6	6.3	1.5
		KW = 2.50 = 0.48; N.S.				**KW = 2.55 = 0.47; N.S.**			
PLT	MEAN	883.3	901.5	1217.1	1142.0	597.8	755.4	834.4	829.1
10^3/μl	ST. DEV.	256.3	358.0	403.0	418.9	264.2	207.8	135.5	156.8
		KW = 4.75 = 0.19; N.S.				**F(36.3) = 2.76 p = 0.06; N.S.**			
RDW	MEAN	16.4	17.1	16.6	16.8	15.5	15.0	15.4	14.3*
%	ST. DEV.	1.4	1.3	0.8	1.3	1.2	0.7	0.8	0.8
		KW = 1.86 = 0.60; N.S.				**KW = 8.78 = 0.03; *p < 0.05**			

RBC: Erythrocyte count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet (thrombocyte) count; RDW: red blood cell distribution width; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

Table 4
Differential white blood cells count data of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-days. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values).

Differential white blood cells count data summary									
		Male				Female			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)	(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
WBC	MEAN	8.7	10.0	9.4	8.1	6.6	7.6	8.1	11.3
10^3/μL	ST. DEV.	3.3	6.3	1.6	1.1	2.7	3.5	2.2	8.3
		KW = 2.43 p = 0.49; N.S.				**KW = 2.32 p = 0.51; N.S.**			
NE	MEAN	25.4	35.9	33.7	41.9	21.6	23.1	24.8	32.7
%	ST. DEV.	9.2	12.4	12.5	17.8	18.6	13.9	16.3	16.4
		F(36.3) = 2.58 p = 0.07; N.S.				**KW = 2.17 p = 0.54; N.S.**			
LY	MEAN	65.4	53.8	56.2	48.3	73.5	67.3	68.4	57.1
%	ST. DEV.	11.2	13.4	13.6	16.6	19.6	18.3	16.6	20.7
		KW = 7.01 p = 0.07; N.S.				**KW = 2.94 p = 0.40; N.S.**			
MO	MEAN	4.6	5.3	5.1	3.6	4.3	4.7	4.3	4.9
%	ST. DEV.	2.2	3.3	1.2	2.2	2.4	2.5	1.9	2.6
		F(36.3) = 0.85 p = 0.47; N.S.				**F(36.3) = 0.12 p = 0.95; N.S.**			
EO	MEAN	4.3	4.4	4.5	5.6	1.3	4.2	2.0	3.8
%	ST. DEV.	4.0	1.7	2.5	3.1	0.6	5.1	1.0	2.2
		F(36.3) = 0.40 p = 0.75; N.S.				**F(36.3) = 2.07 p = 0.12; N.S.**			
BA	MEAN	0.4	0.7	0.5	0.6	0.5	0.6	0.5	0.5
%	ST. DEV.	0.2	0.2	0.3	0.2	0.2	0.3	0.3	0.3
		F(36.3) = 1.80 p = 0.16; N.S.				**F(36.3) = 0.84 p = 0.48; N.S.**			

WBC: total leukocyte count; NE: neuthrophil; LY: lymphocyte; MO: monocyte; EO: eosinophil; BA: basophil; F: Statistics ANOVA test; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

(Table 7). Overall, no changes were observed in the organ weight/brain weight ratio, although in ovaries it significantly increased only in female rats exposed to 50 mg/kg b.w./d OEO in comparison to the control group (Table 8).

The histological findings described in liver, kidney, intestine, stomach (Fig. 3) and lung, heart and testicle ovary (Fig. 4) of treated

Table 5
Clinical biochemistry of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-days. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). The significance levels observed are $^{*}p < 0.05$ in comparison to control group values, and #p < 0.05 when 50 mg/kg/d and 200 mg/kg/d were compared.

Clinical biochemistry data summary									
		Male				Female			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)	(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
GLUC	MEAN	143.7	122.8*	126.8	129.3	128.5	123.2	129.0	154.3#
tmg/dL	ST. DEV.	18.1	14.6	11.2	13.3	18.2	17.0	11.2	36.2
		F(36.3) = 3.87 *p < 0.05				**F(36.3) = 3.79#p < 0.05**			
UREA	MEAN	38.1	38.0	36.5	35.6	41.0	37.7	37.3	36.5
mg/dl	ST. DEV.	4.2	5.3	6.7	3.8	6.5	5.1	4.3	4.8
		F(36.3) = 0.54 p = 0.66; N.S.				**F(36.3) = 1.41 p = 0.25; N.S.**			
CREAT	MEAN	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3
mg/dL	ST. DEV.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
		F(36.3) = 2.30 p = 0.09; N.S.				**F(36.3) = 0.63 p = 0.60; N.S.**			
Bile acids	MEAN	36.2	31.5	34.2	25.9	54.7	57.0	54.1	44.4
μMol	ST. DEV.	13.3	13.3	12.9	11.2	20.2	30.6	16.3	32.4
		F(36.3) = 1.24 p = 0.31; N.S.				**KW = 1.79 p = 0.62; N.S.**			
CHOL	MEAN	64.9	66.3	67.1	60.9	63.6	59.5	68.8	61.2
mg/dL	ST. DEV.	9.1	9.3	9.8	14.2	12.0	9.8	17.5	8.6
		KW = 2.48 p = 0.48; N.S.				**F(36.3) = 1.06 p = 0.38; N.S.**			
TRIGL	MEAN	128.8	100.8	111.8	122.2	63.2	58.1	69.0	66.6
mg/dL	ST. DEV.	58.6	31.3	32.6	59.2	22.4	12.2	15.9	18.3
		F(36.3) = 0.67 p = 0.58; N.S.				**F(36.3) = 0.72 p = 0.54; N.S.**			
AST	MEAN	136.2	125.9	109.8	117.9	139.3	118.3	136.4	138.89
U/L	ST. DEV.	33.4	20.9	19.2	31.8	51.9	23.4	35.5	35.0
		F(36.3) = 1.74 p = 0.18; N.S.				**F(36.3) = 0.64 p = 0.60; N.S.**			
ALT	MEAN	31.4	35.6	28.5	34.5	31.9	26.8	36.9	37.3
U/L	ST. DEV.	5.4	8.7	6.3	12.7	18.3	10.4	14.9	14.1
		F(36.3) = 1.33 p = 0.28; N.S.				**KW = 4.36 p = 0.23; N.S.**			
ALKP	MEAN	62.1	60.8	54.8	51.8	24.0	25.6	24.9	19.7
U/L	ST. DEV.	11.7	13.7	8.6	6.7	5.6	6.5	6.1	5.5
		KW = 6.10 p = 0.11; N.S.				**F(36.3) = 2.00 p = 0.13; N.S.**			
ALB	MEAN	3.8	3.6	3.6	3.6	3.8	3.7	4.0	3.8
g/dl	ST. DEV.	0.1	0.2	0.2	0.2	0.4	0.4	0.3	0.3
		KW = 0.44 p = 0.93; N.S.				**KW = 2.78 p = 0.43; N.S.**			
TOT PROT	MEAN	6.1	6.1	6.1	6.1	5.8	0.7	6.0	5.7
g/dl	ST. DEV.	0.4	0.2	0.2	0.4	0.4	0.4	0.4	0.4
		KW = 0.06 p = 0.30; N.S.				**F(36.3) = 1.09 p = 0.36; N.S.**			
Na^+	MEAN	127.1	127.9	128.6	129.4	126.4	127.8	127.0	126.6
mmol/L	ST. DEV.	3.5	2.5	3.7	3.6	6.1	1.9	2.1	2.6
		F(36.3) = 0.84 p = 0.48; N.S.				**F(36.3) = 0.30 p = 0.83; N.S.**			
K^+	MEAN	8.6	8.6	7.9	7.7	6.6	6.5	6.1	5.8
mmol/L	ST. DEV.	1.3	1.1	0.6	0.6	1.1	1.1	0.9	0.7
		F(36.3) = 2.27 p = 0.10; N.S.				**F(36.3) = 1.42 p = 0.25; N.S.**			

GLUC: glucose; BUN: blood urea nitrogen; CREAT: creatinine; Bili-T: Bilirubin, total; CHOL: cholesterol, total; TRIGL: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALKP: alkaline phosphatase; ALB: albumin; TOT PROT: protein, total; Na^+:sodium; K^+: potassium.
F: Statistics ANOVA test; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

rats were similar to those observed in the control groups. No remarkable changes were observed in any organ of male and female rats exposed to 50, 100 and 200 mg/kg b.w./d OEO.

4. Discussion

Food industry is undergoing a renewal to increase the shelf-life of food products by developing new food packaging. New preservative compounds are needed to satisfy consumers' demands. The incorporation of OEO in active packaging is under study (Llana-Ruiz-Cabello et al., 2016a), since it could be a good alternative to synthetic compounds, which have presented undesirable side effects (Branen, 1974; Nerin et al., 2006). However, the safety of OEO needs to be confirmed for this new use in the food industry. In order to accomplish with the requirements suggested by the Scientific Committee on Food for safety assessment of substances used in food contact materials (EFSA, 2016), subchronic studies are needed. A 90-day study in rats with the test material administered via the diet would provide a no-observed-adverse-effect level (NOAEL) (EFSA, 2008). As far as we know, any *in vivo* experiments have studied the subchronic toxicity of OEO so far. However, *in vivo* experimental models have been widely used to confirm the antimicrobial and antioxidant effects of OEO. Thus, OEO dietary supplementation in broilers exhibited a significantly positive effect as growth promoter (Peng et al., 2016). In addition, OEO improved antioxidant activity in a similar way than vitamin E in pigs (Zhang et al., 2015), and even higher effects than vitamin E by improving the pigs' antioxidant status after subchronic exposure (Zou et al., 2015). Moreover, OEO exhibited a protective effect against diquat-induced oxidative injury in intestine of rats (Wei et al., 2016). Ranucci et al. (2015) reported that a combination of OEO and sweet chestnut wood extract may be useful to increase the pig antioxidant status, to prevent lipid oxidation and, thus, to increase meat shelf-life.

Despite no previous *in vivo* studies have been conducted to assess the subchronic effect of OEO, there are several experiments

Table 6
Absolute organ weight of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). & $p < 0.05$ when 100 mg/kg/d and 200 mg/kg/d were compared.

Organ weight data summary										
Male						Female				
		Group 1 (0 mg/kg/day) N = 10	Group 2 (50 mg/kg/day) N = 10	Group 3 (100 mg/kg/day) N = 10	Group 4 (200 mg/kg/day) N = 10			Group 1 (0 mg/kg/day) N = 10	Group 2 (50 mg/kg/day) N = 10	Group 3 (100 mg/kg/day) N = 10	Group 4 (200 mg/kg/day) N = 10
BODY W. (g)	MEAN	495.2	477.1	485.3	495.7	BODY W. (g)	MEAN	246.1	255.0	250.7	247.0
	ST. DEV.	48.6	27.8	36.9	25.8		ST. DEV.	16.3	14.4	41.6	12.5
F(36.3) = 0.61 p = 0.61; N.S.						**F(36.3) = 0.82 p = 0.49; N.S.**					
BRAIN (g)	MEAN	2.0	2.0	2.0	2.0	BRAIN (g)	MEAN	1.7	1.7	1.7	1.7
	ST. DEV.	0.2	0.2	0.1	0.1		ST. DEV.	0.2	0.2	0.1	0.1
F(36.3) = 0.05 p = 0.98; N.S.						**F(36.3) = 0.77 p = 0.52; N.S.**					
LIVER (g)	MEAN	15.7	14.0	14.4	14.8	LIVER (g)	MEAN	6.3	6.8	6.9	6.8
	ST. DEV.	2.5	1.4	1.7	1.7		ST. DEV.	0.5	0.8	0.7	0.8
F(36.3) = 1.49 p = 0.23; N.S.						**F(36.3) = 1.21 p = 0.32; N.S.**					
HEART (g)	MEAN	1.5	1.4	1.5	1.3	HEART (g)	MEAN	0.8	0.9	0.9	0.9
	ST. DEV.	0.2	0.2	0.3	0.1		ST. DEV.	0.1	0.1	0.1	0.1
F(36.3) = 1.47 p = 0.24; N.S.						**F(36.3) = 1.85 p = 0.16; N.S.**					
SPLEEN (g)	MEAN	0.7	0.8	0.7	0.8	SPLEEN (g)	MEAN	0.5	0.5	0.5	0.5
	ST. DEV.	0.1	0.1	0.1	0.2		ST. DEV.	0.0	0.1	0.1	0.1
F(36.3) = 0.94 p = 0.43; N.S.						**KW = 0.35 p = 0.95; N.S.**					
KIDNEYS (g)	MEAN	3.3	3.2	3.1	3.2	KIDNEYS (g)	MEAN	1.9	2.0	1.9	2.1[&]
	ST. DEV.	0.4	0.4	0.3	0.2		ST. DEV.	0.2	0.2	0.1	0.2
F(36.3) = 0.57 p = 0.64; N.S.						**F(36.3) = 4.388 [&]p < 0.05**					
THYMUS (g)	MEAN	0.8	0.7	0.8	0.7	THYMUS (g)	MEAN	0.5	0.5	0.6	0.6
	ST. DEV.	0.2	0.2	0.2	0.2		ST. DEV.	0.2	0.2	0.2	0.2
F(36.3) = 1.10 p = 0.35; N.S.						**F(36.3) = 0.67 p = 0.58; N.S.**					
TESTES (g)	MEAN	4.0	3.9	3.9	4.0	UTE./CERV. (g)	MEAN	0.9	1.0	0.8	0.7
	ST. DEV.	0.6	0.4	0.4	0.4		ST. DEV.	0.3	0.3	0.2	0.1
F(36.3) = 0.42 p = 0.73; N.S.						**F(36.3) = 2.18 p = 0.11; N.S**					
EPIDIDIMS (g)	MEAN	2.6	2.3	2.1	2.6	OVARIES (g)	MEAN	0.5	0.7	0.6	
	ST. DEV.	0.7	0.7	0.6	0.5		ST. DEV.	0.0	0.1	0.1	
F(36.3) = 1.45 p = 0.24; N.S.						**KW = 6.23 p = 0.10; N.S**					
ADRENALS (g)	MEAN	0.2	0.2	0.1	0.2	ADRENALS (g)	MEAN	0.2	0.2	0.2	0.2
	ST. DEV.	0.1	0.2	0.1	0.1		ST. DEV.	0.1	0.0	0.1	0.1
KW = 2.10 p = 0.56; N.S.						**KW = 1.10 p = 0.36; N.S**					

F: Statistics ANOVA test; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

carried out on other EO such as ginger, turmeric, peperina and onion (Liju et al., 2013; Jeena et al., 2014; Escobar et al., 2015; Mellado-García et al., 2016), although they are very scarce. In our work the NOAEL was set at 200 mg/kg b.w./d. Similar NOAEL, 500 mg/kg b.w./d, have been reported for turmeric and ginger EOs, 460 mg/kg b.w./d for peperina EO, and 400 mg/kg b.w./d for the onion OE. Higher NOAELs were found in the case of extracts, for the dry spearmint extract a NOAEL of 1948 mg extract/kg b.w./d was reported on Sprague-Dawley rats (Lasrado et al., 2015), and in a polyphenolic extract of clove buds the NOAEL was 1000 mg/kg b.w./d (Vijayasteltara et al., 2016). In all these studies, no remarkable effects were recorded, only in the case of peperina EO there was an increase in weight gain and FCE in rats fed with approximately 66 mg/kg b.w./d, although it did not follow a dose-response pattern (Escobar et al., 2015). Likewise, in the present study, the subchronic oral exposure to OEO did not produce any significant adverse effects in the parameters recorded. The subchronic administration of three doses of OEO (50, 100 and 200 mg/kg/d) had negligible effect on haematological parameters. The percentage of RDW was the single parameter undergoing slight decrease, only in one group and in one gender, which can be considered as a marginal change with no biological relevance. Similarly, this parameter was reported to significantly decrease in male and female exposed to mung bean, but the authors stated that there was no biological significance for this observation (Yao et al., 2015). Also, the significant reduction in the RDW of male rats consuming the 7.5% genetically modified (GM) pork diet compared to the group of 7.5% non-GM pork diets were not considered to be adverse (Zou et al., 2015).

Furthermore, the clinical biochemistry results in the present work only evidenced significant differences in GLUC in male rats exposed to 50 mg/kg/d OEO that decreased in comparison to the control group and in female fed with 200 mg/kg/d OEO that increased when compared to the group exposed to 50 mg/kg/d OEO. Taking into account that these changes did not follow any

Table 7
Relative organ weight/body weight of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values).

Organ weight/body weight ratio data summary											
Male						Female					
		Group 1	Group 2	Group 3	Group 4			Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)			(0 mg/kg/day)	(50 mg/kg/day)	(100 mg/kg/day)	(200 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10			N = 10	N = 10	N = 10	N = 10
BRAIN	MEAN	0.41	0.41	0.41	0.40	BRAIN	MEAN	0.59	0.59	0.62	0.60
(%)	ST. DEV.	0.06	0.04	0.04	0.04	(%)	ST. DEV.	0.13	0.07	0.07	0.06
KW = 0.73 p = 0.87; N.S.						**F(36.3) = 0.29 p = 0.84; N.S.**					
LIVER	MEAN	3.16	2.93	2.96	2.99	LIVER	MEAN	2.26	2.43	2.51	2.45
(%)	ST. DEV.	0.39	0.25	0.23	0.25	(%)	ST. DEV.	0.15	0.27	0.24	0.32
F(36.3) = 1.31 p = 0.29; N.S.						**F(36.3) = 1.81 p = 0.16; N.S.**					
HEART	MEAN	0.30	0.30	0.30	0.27	HEART	MEAN	0.30	0.34	0.34	0.32
(%)	ST. DEV.	0.04	0.03	0.04	0.02	(%)	ST. DEV.	0.03	0.05	0.04	0.05
F(36.3) = 1.97 p = 0.14; N.S.						**F(36.3) = 1.62 p = 0.20; N.S.**					
SPLEEN	MEAN	0.15	0.16	0.15	0.16	SPLEEN	MEAN	0.18	0.19	0.19	0.17
(%)	ST. DEV.	0.02	0.02	0.02	0.03	(%)	ST. DEV.	0.02	0.04	0.04	0.04
F(36.3) = 1.00 p = 0.40; N.S.						**F(36.3) = 0.46 p = 0.71; N.S.**					
KIDNEYS	MEAN	0.67	0.67	0.65	0.64	KIDNEYS	MEAN	0.68	0.71	0.69	0.77
(%)	ST. DEV.	0.07	0.08	0.05	0.04	(%)	ST. DEV.	0.05	0.11	0.07	0.12
F(36.3) = 0.79 p = 0.51; N.S.						**F(36.3) = 1.70 p = 0.18; N.S.**					
THYMUS	MEAN	0.17	0.14	0.16	0.15	THYMUS	MEAN	0.18	0.19	0.23	0.20
(%)	ST. DEV.	0.05	0.04	0.04	0.05	(%)	ST. DEV.	0.07	0.05	0.06	0.06
F(36.3) = 0.86 p = 0.47; N.S.						**F(36.3) = 0.99 p = 0.41; N.S.**					
TESTES	MEAN	0.82	0.82	0.80	0.82	UTE./CERV.	MEAN	0.33	0.36	0.29	0.27
(%)	ST. DEV.	0.13	0.08	0.08	0.07	(%)	ST. DEV.	0.11	0.11	0.09	0.06
F(36.3) = 0.11 p = 0.95; N.S.						**F(36.3) = 1.63 p = 0.20; N.S.**					
EPIDIDIMS	MEAN	0.53	0.48	0.44	0.52	OVARIES	MEAN	0.19	0.23	0.21	0.19
(%)	ST. DEV.	0.13	0.15	0.15	0.10	(%)	ST. DEV.	0.02	0.04	0.04	0.04
KW = 2.84 p = 0.42; N.S.						**F(36.3) = 2.90 p = 0.05; N.S.**					
ADRENALS	MEAN	0.04	0.05	0.03	0.04	ADRENALS	MEAN	0.07	0.07	0.08	0.08
(%)	ST. DEV.	0.02	0.04	0.02	0.01	(%)	ST. DEV.	0.02	0.02	0.02	0.03
KW = 2.30 p = 0.51; N.S.						**F(36.3) = 1.41 p = 0.26; N.S.**					

N.S.: Not Significant.

pattern since they were neither sex-, dose- nor time-dependent they could be considered as not biologically relevant (Pucaj et al., 2011). In fact, the latter authors reported increases in GLUC level in female rats exposed during 90 days to 2.5 mg/kg/d menaquinone-7 and in males fed with the same dose for 44 days; they considered these increases as normal changes. Moreover, GLUC level decreased in male rats fed with 100 mg/kg/d Proallium AP®, this change being not biologically significant (Mellado-García et al., 2016). However, GLUC changes have been also reported to have biological implications. Hence, male rats fed with conjugated linoleic acid underwent significant reductions in GLUC after 8 and 13 weeks of treated and in female after 4 weeks (O'Hagan and Menzel, 2003). These reductions were related to the influence on insulin level.

Besides, no morphological changes were observed in the present work in any of the tissues studied. As far as we know, this is the first ultrastructural study carried out *in vivo* in this type of EO. However, several authors have described some morphological changes and death cells in different cell lines exposed to OEO from different species. Hence, OEO induced apoptosis in the fibroblasts cell line 5RP7 from the concentration of 125 μg/mL, although this EO was from a different oregano specie *Origanum onites* (Bostancioglu et al., 2012). Similarly, cell death was observed in the intestinal cell line Caco-2 cells exposed up to 500 μg/mL OEO from *Origanum vulgare*, both apoptosis and necrosis, (Dusan et al., 2006; Savini et al., 2009). Considering the concentrations of OEO assayed in the present work *in vivo* are higher than those used in previous *in vitro* assays, the higher toxicity observed in the *in vitro* studies may be related to other factors. In this regard, the different experimental model, rats in the case of the *in vivo* study and a human cell line in the case of the *in vitro* studies, can influence the results since differences in metabolism and/or in the bioavailability of the OEO may vary substantially (Llana-Ruiz-Cabello et al., 2016a). Moreover, further morphological features were reported when Caco-2 cells were exposed to the main components of OEO, thymol and carvacrol. Thus, thymol induced lipid degeneration, mitochondrial damage, nucleolar segregation as well as apoptosis; and more severe damage was observed for carvacrol such as vacuolated cytoplasm, altered organelles and finally cell death *in vitro* (Llana-Ruiz-Cabello et al., 2014b). In addition, cell death has been reported in the hepatic cell line HepG2, with an increase in the number of necrotic cells after exposure to thymol, while carvacrol

Table 8
Relative organ weight/brain weight of Wistar male and female rats fed with 50, 100 and 200 mg/kg b.w./d OEO for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). *p < 0.05 in comparison to control group values.

Organ weight/brain weight ratio data summary										
Male						Female				
		Group 1 (0 mg/kg/day) N = 10	Group 2 (50 mg/kg/day) N = 10	Group 3 (100 mg/kg/day) N = 10	Group 4 (200 mg/kg/day) N = 10			Group 1 (0 mg/kg/day) N = 10	Group 2 (50 mg/kg/day) N = 10	Group 3 (100 mg/kg/day) N = 10	Group 4 (200 mg/kg/day) N = 10
LIVER (%)	MEAN	789.8	715.2	731.6	755.4	LIVER (%)	MEAN	360.2	389.8	405.0	411.7
	ST. DEV.	137.3	101.7	103.8	121.6		ST. DEV.	33.6	39.6	41.6	54.8
F(36.3) = 0.77 p = 0.52; N.S.						**F(36.3) = 2.60 p = 0.07; N.S.**					
HEART (%)	MEAN	74.1	73.6	74.2	68.3	HEART (%)	MEAN	48.2	58.0	52.5	52.9
	ST. DEV.	11.3	10.0	15.6	8.1		ST. DEV.	8.8	11.6	6.8	5.1
F(36.3) = 0.62 p = 0.61; N.S.						**F(36.3) = 2.13 p = 0.12; N.S.**					
SPLEEN (%)	MEAN	36.8	38.6	36.4	40.7	SPLEEN (%)	MEAN	29.3	32.3	30.2	29.4
	ST. DEV.	8.1	6.2	6.2	8.6		ST. DEV.	2.9	6.6	5.4	6.1
F(36.3) = 0.71 p = 0.55; N.S.						**F(36.3) = 0.62 p = 0.61; N.S.**					
KIDNEYS (%)	MEAN	167.6	164.1	159.5	161.6	KIDNEYS (%)	MEAN	109.3	123.4	110.6	128.5
	ST. DEV.	26.8	28.9	20.1	17.5		ST. DEV.	13.7	35.9	7.1	18.2
F(36.3) = 0.21 p = 0.89; N.S.						**F(36.3) = 1.85 p = 0.16; N.S.**					
THYMUS (%)	MEAN	41.9	33.5	39.2	37.0	THYMUS (%)	MEAN	31.4	32.6	36.6	33.9
	ST. DEV.	11.5	9.0	8.8	12.6		ST. DEV.	11.3	9.9	10.1	9.5
F(36.3) = 1.13 p = 0.35; N.S.						**F(36.3) = 0.48 p = 0.70; N.S.**					
TESTES (%)	MEAN	194.1	199.5	195.9	207.2	UTE./CERV. (%)	MEAN	56.3	59.8	46.3	45.6
	ST. DEV.	28.8	24.5	20.2	29.4		ST. DEV.	17.5	14.3	12.6	10.1
F(36.3) = 0.48 p = 0.70; N.S.						**F(36.3) = 2.63 p = 0.06; N.S.**					
EPIDIDIMS (%)	MEAN	132.0	117.4	108.5	130.6	OVARIES (%)	MEAN	30.6	39.9*	33.7	32.7
	ST. DEV.	31.1	40.5	34.1	25.0		ST. DEV.	2.4	8.9	6.7	6.1
F(36.3) = 1.14 p = 0.34; N.S.						**F(36.3) = 3.62 *p < 0.05**					
ADRENALS (%)	MEAN	10.9	8.2	7.4	8.7	ADRENALS (%)	MEAN	11.5	12.5	13.6	13.2
	ST. DEV.	5.7	2.9	4.0	3.3		ST. DEV.	3.6	3.3	4.0	4.9
F(36.3) = 1.35 p = 0.27; N.S.						**F(36.3) = 0.54 p = 0.66; N.S.**					

N.S.: No Significant.

caused death cell mainly via apoptosis (Stammati et al., 1999; Yin et al., 2012). Similarly, lung cells A549, breast cancer cells MDA-MB231 and fibroblasts cells 5RP7 treated with carvacrol showed some apoptotic characteristics as well as morphological changes (Koparal and Zeytinoglu, 2003; Arunasree, 2010; Akalin and Incesu, 2011). When comparing the toxicity of OEO and its components, carvacrol and thymol have been reported to induce higher damage than the OEO in HepG2 cells (Özkan and Erdogan, 2011). However, other authors have reported that no single compound seems to be responsible for the cytotoxic effects in Caco-2 cells of the OEO but the whole oil extract (Savini et al., 2009). Overall, many aspects influence the toxicity of OEO such as the concentration assayed, the different composition of each EO and the experimental model used among others.

Provided the NOAEL of the OEO studied in the present work is 200 mg/kg b.w., it is interesting to compare this finding with the real scenario of exposure to consumers. Active packaging containing OEO would be very useful to pack ready-to-eat salads (Llana-Ruiz-Cabello et al., 2016a,b). In this packaging, 5.5 g of active film is required to pack 1 kg of lettuce. Active film containing 5 and 10% of OEO are both useful as antimicrobial and antioxidant agents (Llana-Ruiz-Cabello et al., 2016a). However, the most likely percentage of OEO to be used in the packaging would be 5% from an economical and safety point of view, since 10% did not increase the effectiveness of the active packaging. Therefore, the maximum concentration that could be released from packaging to lettuce would be 0.27 g OEO per kg. Considering that a medium consumption of lettuce would be 132.53 g per day (http://www.aecosan.msssi.gob.es), the maximum quantity of OEO that could be ingested would be 36.4 mg of OEO per person per day. Considering the worst exposure scenario derived from the results analysed in the present work, an adult consumer weighting 60 kg would ingest 12,000 mg of OEO per day in the highest dose (200 mg/kg b.w./d); this would be around 330-fold higher than this maximum potential exposure. Therefore, no subchronic toxic effects are expected in the consumption of the OEO used in this study with applications in active food packaging.

5. Conclusions

The present study reveals lack of subchronic toxic effects of OEO given on diet to rats at doses of 50, 100 and 200 mg/kg b.w./d after 90 days. Male and female rats fed with this OEO did not show any change on body weight, food and water consumption, as well as on biochemical and haematological parameters. In addition, normal histopathological features were observed in all tissues. Therefore, the NOAEL obtained in the present work (200 mg/kg b.w./d) is considerable higher than the maximum potential exposure in its

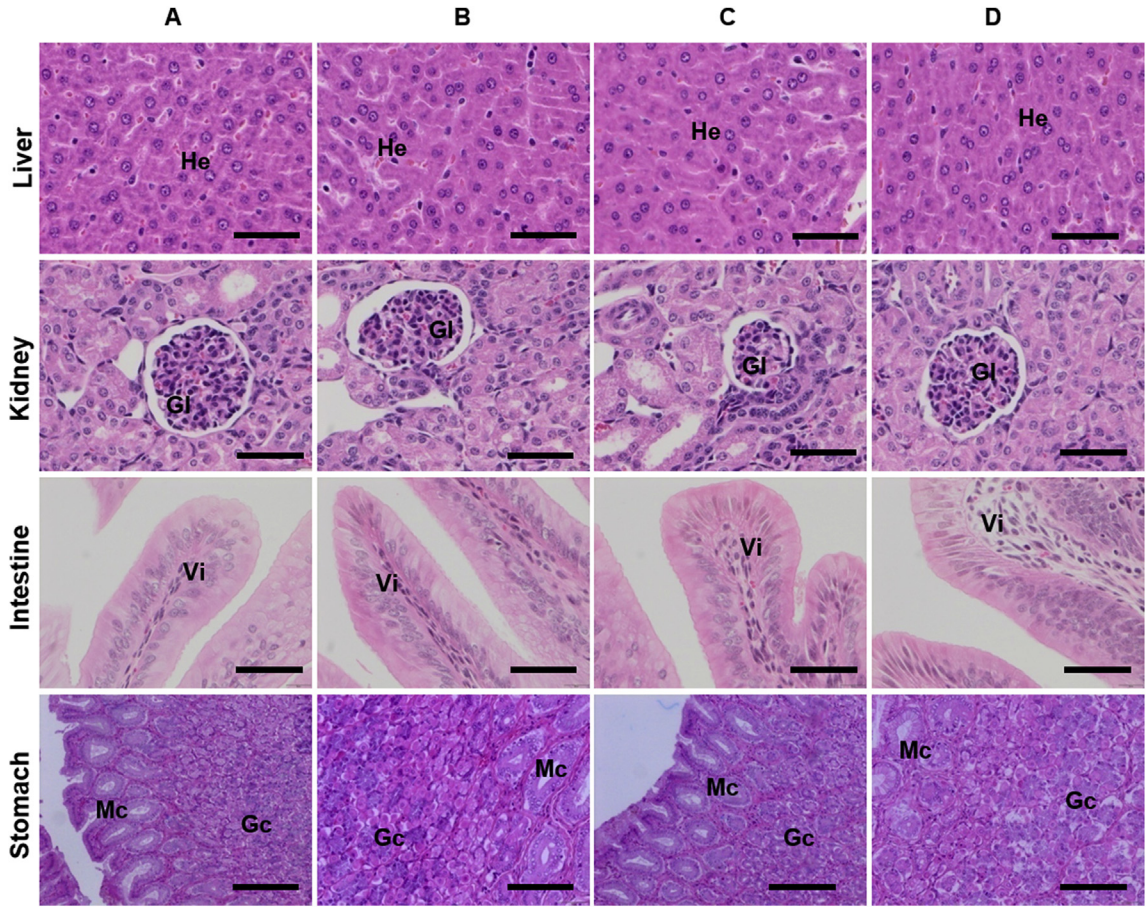

Fig. 3. Histopathological analysis of liver, kidney, intestines and stomach of male (A) and female (C) control Wistar rats, and male (B) and female (D) rats subchronically exposed to 200 mg/kg b.w./d OEO for 90-day (bars = 50 μm). Normal hepatic cords and normal hepatocytes (He) with polyhedral morphology and rounded central nuclei are observed in liver both in control male and female rats (A,C) and rats exposed to the highest dose of OEO. In kidney, renal parenchyma with normal glomeruli (GI) and renal tubules are shown for all the groups. Intestine of rats exposed to OEO are observed as intestinal villi (Vi) with normal enterocytes (A,C). Similarly, well developed villi also appear in control groups (B,D). Gastric mucosa with mucosal (Mc) and glandular (Gc) cells apparently normal are shown in all groups.

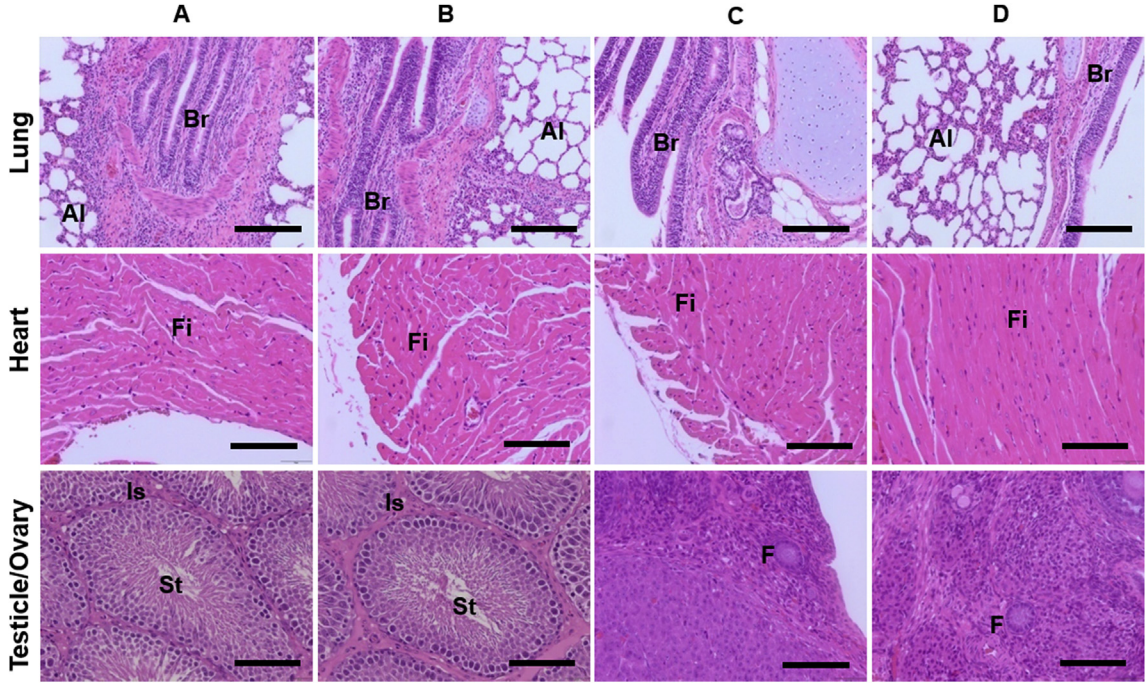

Fig. 4. Histopathological analysis of lung, heart and testicule/ovary of male (A) and female (C) control Wistar rats, and male (B) and female (D) rats subchronically exposed to 200 mg/kg b.w./d OEO for 90-day (bars = 50 μm). In lung, normal bronchial epithelia with bronchia (Br) and alveoli (Al) are observed. No remarkable differences were appreciate in cardiac fibers (Fi) of treated (B,D) and untreated groups (A,C). Detail of the testicles of male rats showing normal seminiferous tubules (St) and interstitial space (Is) (A) are observed in both groups (A,B); Ovaries from rats exposed to OEO remained unaltered in comparison to the control rats, both showing normal follicles (F).

use in food packaging.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.01.001.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.01.001.

References

AECOSAN (Agencia Española de Consumo, Seguridad Alimentaria y Nutrición) http://www.aecosan.msssi.gob.es (Accessed 23 November 2016).

Akalin, G., Incesu, Z., 2011. The effects of carvacrol on apoptosis of H-ras and N-ras transformed cell lines. Turk. J. Pharm. Sci. 8 (2), 105–116.

Arunasree, K.M., 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB231. Phytomedicine 17, 581–588.

Asensio, C.M., Grosso, N.R., Juliani, H.R., 2015. Quality preservation of organic cottage cheese using oregano essential oils. LWT - Food Sci. Technol. 60, 664–671.

Aydin, S., Basaran, A.A., Basaran, N., 2005a. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutat. Res. 581, 43–53.

Aydin, S., Basaran, A.A., Basaran, N., 2005b. Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. J. Agric. Food Chem. 53, 1299–1305.

Aydin, E., Türkez, H., Keles, M.S., 2014. The effect of carvacrol on healthy neurons and N2a cancer cells: some biochemical: anticancerogenicity and genotoxicity studies. Cytotechnology 66, 149–157.

Azizan, A., Blevins, R.D., 1995. Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay. Arch. Environ. Contam. Toxicol. 28, 248–258.

Bostancioglu, R.B., Kürkçüoglu, M., Baser, K.H.C., Koparal, A.T., 2012. Assessment of anti-angiogenic and anti-tumoral potentials of *Origanum onites L.* essential oil. Food Chem. Toxicol. 50, 2002–2008.

Branen, A.L., 1974. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J. Am. Oil Chem. Soc. 52, 59–62.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in food- a review. Int. J. Food Microbiol. 94, 223–253.

Buyukleyla, M., Rencuzogullari, E., 2009. The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes. Ecotox. Environ. Safe 72, 943–947.

Camo, J., Lorés, A., Djenane, D., Beltrán, J.A., Roncalés, P., 2011. Display life of beef packaged with an antioxidant active film as a function of the concentration of oregano extract. Meat Sci. 88, 174–178.

Cattelan, M.G., Castilhos, M.B.M., Silva, D.M.M.N., Conti-Silva, A.N., Hoffmann, F.L., 2015. Oregano essential oil: effect on sensory acceptability. Nutr. Food Sci. 45 (4), 574–582.

Commission Regulation (EC) No 450/2009 of 29 May 2009 on active and intelligent materials and articles intended to come into contact with food. Official Journal of the European Union, L 135/3-L 135/11.

Decision 2012/707/UE. Commission Implementing Decision of 14 November 2012 establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Unofficial%20Consolidated%20version.pdf (Accessed 23 November 2016).

de Medeiros Barbosa, I.M., Medeiros, J.A.C., de Oliveira, K.A.R., Gomes-Neto, N.J., Tavares, J.F., Magnani, M., de Souza, E.L., 2016. Efficacy of the combined application of oregano and rosemary essential oils for the control of *Escherichia coli, Listeria monocytogenes* and *Salmonella enteritidis* in leafy vegetables. Food control. 59, 468–477.

Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. <http://eurlex.europa.eu/legalcontent/EN/TXT/?uri%bc;celex:32010L0063 (Accessed 23 November 2016).

Dusan, F., Marián, S., Katarína, D., Dobroslava, B., 2006. Essential oils-their antimicrobial activity against Escherichia coli and effect on intestinal cell viability. Toxicol. Vitro 20, 1435–1445.

Escobar, F.M., Sabini, M.C., Cariddi, L.N., Sabini, L.I., Ma~nas, F., Cristofolini, A., Bagnis, G., Gallucci, M.N., Cavaglieri, L.R., 2015. Safety assessment of essential oil from *Minthostachys verticillata* (Griseb.) Epling (peperina): 90-days oral subchronic toxicity study in rats. Regul. Toxicol. Pharm. 71, 1–7.

European Food Safety Authority, 2008. Guidance Document on the Submission of a Dossier on a Substance to Be Used in Food Contact Materials for Evaluation by EFSA by the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) (Accessed 23 November 2016). http://www.efsa.europa.eu/en/efsajournal/doc/21r.pdf.

European Food Safety Authority CEF Panel, (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)), 2016. Recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials, pp. 1–28. doi: http://dx.doi.org/10.2903/j.efsa.2016.4357. https://www.efsa.europa.eu/en/efsajournal/pub/4357 (Accessed 23 November 2016).

Govaris, A., Botsoglou, E., Sergelidis, D., Chatzopoulou, P.S., 2011. Antibacterial activity of oregano and thyme essential oils against *Listeria monocytogenes* and *Escherichia coli* O157:H7 in feta cheese packaged under modified atmosphere. LWT - Food Sci. Technol. 44, 1240–1244.

Horvathova, E., Sramkova, M., Labaj, J., Slamenova, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured in vitro. Neuro. Endocrinol. Lett. 27, 44–47.

Jayasena, D.D., Jo, C., 2013. Essential oils as potential antimicrobial agents in meat and meat products: a review. Trends Food Sci. Technol. 34, 96–108.

Jeena, K., Liju, V.B., Kuttan, R., 2014. A preliminary 13-week oral toxicity study of ginger oil in male and female wistar rats. Int. J. Toxicol. 30, 662–670.

Koparal, A.T., Zeytinoglu, M., 2003. Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549. Cytotechnology 43, 149–154.

Lasrado, J.A., Trinker, D., Ceddia, M.A., Herrlinger, K.A., 2015. The safety of a dry spearmint extract *in vitro* and *in vivo*. Regul. Toxicol. Pharm. 71, 213–224.

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A., Cameán, A.M., 2014a. Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames Salmonella test and alkaline, Endo III and FPG-modified comet assays with the human cell line Caco-2. Food Chem. Toxicol. 72, 122–128.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Moreno, F.J., Bermúdez, J.M., Aucejo, S., Cameán, A.M., 2014b. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290.

Llana-Ruiz-Cabello, M., Pichardo, S., Bermúdez, J.M., Baños, A., Núñez, C., Guillamón, E., Aucejo, S., Cameán, A.M., 2016a. Development of PLA films containing oregano essential oil (*Origanum vulgare L. virens*) intended for use in food packaging. Food Addit. Contam. A 33, 1374–1386.

Llana-Ruíz-Cabello, M., Pichardo, S., Jiménez-Morillo, N.T., Abad, P., Guillamón, E., González-Vila, F.J., Cameán, A.M., González-Pérez, J.A., 2016b. Characterisation of a bio-based packaging containing a natural additive from *Allium spp.* using analytical pyrolysis and carbon stable isotopes. J. Anal. Appl. Pyrol 120, 334–340.

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Moyano, R., Jos, A., González-Pérez, J.A., Cameán, A.M., 2016c. Genotoxicity evaluation of carvacrol in rats using a combined micronucleus and comet assay. Food Chem. Toxicol. 98, 240–250.

Liju, V.B., Jeena, K., Kuttan, R., 2013. Acute and subchronic toxicity as well as mutagenic evaluation of essential oil from turmeric (*Curcuma longa L*). Food Chem. Toxicol. 53, 52–61.

Maisanaba, S., Prieto, A.I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R., Cameán, A.M., 2015. In vitro genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays. Mutat. Res. 784–785, 37–44.

Manso, S., Becerril, R., Nerín, C., Gómez-Lus, R., 2015. Influence of pH and temperature on vapor phase action of an antifungal food packaging against five mold strains. Food Control 47, 20–26.

Martucci, J.F., Gende, L.B., Neira, L.M., Ruseckaite, R.A., 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. Ind. Crop. Prod. 71, 205–213.

Mellado-García, P., Puerto, M., Pichardo, S., Llana-Ruiz-Cabello, M., Moyano, R., Blanco, A., Jos, A., Cameán, A.M., 2016. Toxicological evaluation of an Allium-based commercial product in a 90-day feeding study in Sprague Dawley rats. Food Chem. Toxicol. 90, 18–29.

Muriel-Galet, V., Cran, M.J., Bigger, S.W., Hernández-Muñoz, P., Gavara, R., 2015. Antioxidant and antimicrobial properties of ethylene vinyl alcohol copolymer films based on the release of oregano essential oil and Green tea extract components. J. Food Eng. 149, 9–16.

Nerín, C., Tovar, L., Djenane, D., Camo, J., Salafranca, J., Beltrán, J.A., 2006. Stabilization of beef meat by a new active packaging containing natural antioxidants. J. Agr. Food Chem. 54, 7840–7846.

OECD (Organisation for Economic Cooperation and Development), 1998. Guideline for Testing of Chemicals. Repeated Dose 90-day Oral Toxicity Study in Rodents, Guideline, vol. 408, pp. 1–10.

OECD (Organisation for Economic Cooperation and Development), 2008. Guideline for Testing of Chemicals. Acute Oral Toxicity – Up-and-down-procedure (UDP), Guideline, vol. 425, pp. 1–27.

O'Hagan, S., Menzel, A., 2003. A subchronic 90-day oral rat toxicity study and *in vitro* genotoxicity studies with a conjugated linoleic acid product. Food Chem.

Toxicol. 41, 1749–1760.
Özkan, A., Erdogan, A.A., 2011. Comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. Turk. J. Biol. 35, 735–742.
Peng, Q.Y., Li, J.D., Li, Z., Duan, Z.Y., Wu, Y.P., 2016. Effects of dietary supplementation with oregano essential oil on growth performance, carcass traits and jejunal morphology in broiler chickens. Anim. Feed Sci. Technol. 214, 148–153.
Pesavento, G., Calonico, C., Bilia, A.R., Barnabei, M., Calesini, F., Addona, R., Mencarelli, L., Carmagnini, L., Di Martino, M.C., Lo Nostro, A., 2015. Antibacterial activity of Oregano, Rosmarinus and Thymus essential oils against *Staphylococcus aureus* and *Listeria monocytogenes* in beef meatballs. Food control. 54, 188–199.
Pucaj, K., Rasmussen, H., Moller, M., Preston, T., 2011. Safety and toxicological evaluation of a synthetic vitamin K2, menaquinone-7. Toxicol. Mech. Methods 21, 520–532.
Radha Krishnan, K., Babuskina, S., Azhagu Saravana Babu, P., Sasikala, M., Sabina, K., Archana, G., Sivarajan, M., Sukumar, M., 2014. Antimicrobial and antioxidant effects of spice extracts on the shelf life extension of raw chicken meat. Int. J. Food Microbiol. 171, 32–40.
Ranucci, D., Beghelli, D., Trabalza-Marinucci, M., Branciari, R., Forte, C., Olivieri, O., Badillo Pazmay, G.V., Cavallucci, C., Acuti, G., 2015. Dietary effects of a mix derived from oregano (*Origanum vulgare L.*) essential oil and sweet chestnut (*Castanea sativa Mill.*) wood extract on pig performance, oxidative status and pork quality traits. Meat Sci. 100, 319–326.
RD 53/2013, 2013. Real Decreto, se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, 11370e11421 BOE 34. http://boe.es/boe/dias/2013/02/08/pdfs/BOE-A-2013-1337.pdf (Accessed 23 November 2016).
Savini, I., Arnone, R., Catani, M.V., Avigliano, L., 2009. *Origanum vulgare* induces apoptosis in human colon cancer Caco2 cells. Nutr. Cancer 61, 381–389.
Soultos, N., Tzikas, Z., Christaki, E., Papageorgiou, K., Steris, V., 2009. The effect of dietary oregano essential oil on microbial growth of rabbit carcasses during refrigerated storage. Meat Sci. 81, 474–478.
Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823.
Ündeger, U., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.
Van Haute, S., Raes, K., Van der Meeren, P., Sampers, I., 2016. The effect of cinnamon, oregano and thyme essential oils in marinade on the microbial shelf life of fish and meat products. Food control. 68, 30–39.
Vijayasteltara, L., Nair, G.G., Maliakel, B., Kuttana, R., Krishnakumar, I.M., 2016. Safety assessment of a standardized polyphenolic extract of clovebuds: subchronic toxicity and mutagenicity studies. Toxicol. Rep. 3, 439–449.
Wei, H.K., Chen, G., Wang, R.J., Peng, J., 2016. Oregano essential oil decreased susceptibility to oxidative stress-induced dysfunction of intestinal epithelial barrier in rats. J. Funct. Foods 18, 1191–1199.
Yin, Q.H., Yan, F.X., Zu, X.Y., Wu, Y.U., Wu, X.P., Liao, M.C., Deng, S.W., Yin, L.I., Zhuang, Y.Z., 2012. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology 64, 43–51.
Yao, Y., Cheng, X., Ren, G., 2015. A 90-day study of three bruchid-resistant mung bean cultivars in Sprague-Dawley rats. Food Chem. Toxicol. 76, 80–85.
Zhang, T., Zhou, Y.F., Zou, Y., Hu, X.M., Zheng, L.F., Wei, H.K., Giannenas, I., Jin, L.Z., Peng, J., Jiang, S.W., 2015. Effects of dietary oregano essential oil supplementation on the stress response, antioxidative capacity, and HSPs mRNA expression of transported pigs. Livest. Sci. 180, 143–149.
Zou, S., Tang, M., He, X., Cao, Y., Zhao, J., Xu, W., Liang, Z., Huang, K., 2015. A 90-day subchronic study of rats fed lean pork from genetically modified pigs with muscle-specific expression of recombinant follistatin. Regul. Toxicol. Pharm 73, 620–628.

CAPÍTULO 9 / CHAPTER 9

María Llana-Ruiz-Cabello, Sara Maisanaba, Maria Puerto, Ana I. Prieto, Silvia Pichardo, Rosario Moyano, Jose A. González-Pérez, Ana M. Cameán

GENOTOXICITY EVALUATION OF CARVACROL IN RATS USING A COMBINED MICRONUCLEUS AND COMET ASSAY.

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Genotoxicity evaluation of carvacrol in rats using a combined micronucleus and comet assay

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ABSTRACT

Genotoxic data of substances which could be incorporated into food packaging are required by the European Food Safety Authority. Due to its antioxidant and antibacterial properties carvacrol is one of these compounds. This work aims to study for the first time the *in vivo* genotoxic effects produced in rats orally exposed to 81, 256 or 810 mg cavacrol/kg body weight (bw) at 0, 24 and 45 h. A combination of the micronucleus assay (OECD 474) in bone marrow and the standard (OECD 489) and enzyme-modified comet assay was used to determine the genotoxicity on cells isolated from stomach and liver of exposed animals. In addition, a histopathological study was performed on the assayed tissues, and also in the lungs due to the volatility of carvacrol. Direct analytical pyrolysis was used to search for carvacrol in viscera and to ensure that the compound reaches stomach and liver cells. Results from MN-comet assay revealed that carvacrol (81–810 mg/kg bw) did not induce *in vivo* genotoxicity or oxidative DNA damage in any of the tissues investigated. Moreover, no histopathological changes were observed. Altogether, these results suggest lack of genotoxicity of carvacrol and therefore its good profile for its potential application as food preservative.

1. Introduction

Food products are often sold in areas of the world far away from their production sites, hence, several reactions, such as microbial spoilage or oxidation processes, may alter the safety and organoleptic properties of perishable products (Llana-Ruiz-Cabello et al., 2015a). The use of plant extracts and essential oils (EOs) to extend the shelf life of foods are increasing in the food industry, such as in the case of active packaging (Echegoyen and Nerín, 2015; Llana-Ruiz-Cabello et al., 2015b).

Carvacrol, the main compound of Oregano EO is registered as a flavoring in Europe; however, its use for other applications, such as in active food packaging, may require higher concentrations that will increase the concern regarding exposure to these compounds (Llana-Ruiz-Cabello et al., 2014a). In this sense, the European Food Safety Authority (EFSA) panel on Food Contact Materials, Enzymes, Flavorings and Processing Aids (CEF) has recently published a Scientific Opinion on the developments of risk assessment in which the amount of toxicity data needed should be related to the expected human exposure level (EFSA, 2016). This draft remarks that genotoxicity must be evaluated, even if low exposure is expected for substances migrating from food contact materials (FCMs) and recommends performing two tests in the first step-approach: a bacterial reverse mutation (Ames test) and an *in vitro* micronucleus (MN) test (EFSA, 2011, 2016).

The potential mutagenic and carcinogenic activities of carvacrol *in vitro* have been studied previously, and contradictory results have been published (Llana-Ruiz-Cabello et al., 2015a). For the Ames test, carvacrol at different concentrations exhibited a mutagenic response in *Salmonella thyphimurium* strains in absence and presence of S9 (Ipek et al., 2005; Llana-Ruiz-Cabello et al., 2014b), whereas negative results were found by Stammati et al. (1999). On the other hand, for MN test, contradictory results have been also reported: a significant increase of MN was observed in L5178Y/*TK*$^{\pm}$ cells in the absence of S9 (Maisanaba et al., 2015), while negative

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responses have been reported in different cell models, such as HepG2 cells or human lymphocytes (Melusová et al., 2014; Türkez and Aydin, 2013). Moreover, no genotoxic response was found in the mouse lymphoma assay (MLA) after 4 h and 24 h of exposure to carvacrol (Maisanaba et al., 2015). The comet assay has also been recommended by EFSA to evaluate the genotoxicity of substances intended to be used in food packaging (EFSA, 2011, 2016). In this regard, negative results have been reported when different cell lines were exposed to carvacrol (Horváthová et al., 2006; Ündeger et al., 2009; Llana-Ruiz-Cabello et al., 2014b). However, results from the enzyme-modified comet assay, using endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG), developed to detect oxidative pirimidine or purine damage, respectively, showed that carvacrol exerted oxidation in purine DNA bases of Caco-2 cells (Llana-Ruiz-Cabello et al., 2014b). All these contradictory results increase the concerns about carvacrol genotoxicity and make it necessary to elucidate its genotoxic potential.

Kirkland et al. (2011) concluded that there is no convincing evidence that any genotoxic rodent carcinogen or *in vivo* genotoxin would remain undetected in an *in vitro* test battery consisting of Ames and MN assays; however, in the case of inconclusive or contradictory results, as well as positive results from these *in vitro* tests, it may be appropriate to conduct further *in vivo* tests (EFSA, 2011). The CEF panel recommends three different assays to evaluate the genotoxicity of substances: the *in vivo* MN test, the *in vivo* comet assay or the transgenic rodent gene mutation assay (EFSA, 2016). In order to comply with the 3Rs principles (Replace, Reduce and Refine), Bowen et al. (2011) proposed a combined multi-end point *in vivo* assay and evaluated the combination of bone marrow MN test with the comet assay. Previous studies have employed MN as well as comet assay simultaneously to detect the genotoxic effects of various chemicals (Kitamoto et al., 2015), nanoparticles (Downs et al., 2012), drugs (Mughal et al., 2010) or toxins (Corcuera et al., 2015). This combination can improve the sensitivity as well as the reliability of toxicity studies (Mughal et al., 2010).

Considering all this background, the present work aims to perform for the first time an *in vivo* genotoxic evaluation for carvacrol using the combined MN-comet assay in rats. The target tissues selected were bone marrow for MN (OECD 474), and stomach and liver cells from orally exposed rats for the comet assay (OECD 489). Moreover, the comet assay has been combined with bacterial repair enzymes Endo III and FPG, which allow detection of DNA oxidative damage. A histopathological examination was performed on stomach, liver and lungs, in order to complete the genotoxic evaluation and provide useful information to perform the accurate risk assessment required by EFSA for additives in FCMs. Finally, to ensure that carvacrol effectively reached the target tissues, a detailed analytical pyrolysis study was conducted.

2. Materials and methods

2.1. Supplies and chemicals

Carvacrol (98%) was purchased from Sigma–Aldrich (Madrid, Spain). All chemicals, including Endo III and FPG, were purchased from Sigma-Aldrich (Madrid, Spain), VWR Eurolab (Madrid, Spain), C-viral S.L. (Seville, Spain) and BioWhittaker (Madrid, Spain). Endo III (EC3.1.21.5) was purchased from C-Viral S.L. (Sevilla, Spain), and FPG (EC3.2.2.23) from Sigma–Aldrich (Madrid, Spain).

2.2. Animal housing and feeding conditions

The Ethics Committee on Animal Experimentation of the University of Sevilla approved the *in vivo* experiments. Moreover, in compliance with the Directive 2010/63/EU for the protection of animals used for scientific purposes all animals received humane care.

Young adult male and female Wistar rats, strain RjHan:WI (type outbred rats), between 8 and 10 week-old were purchased from the University of Sevilla Center for Animal Production and Experimentation (Espartinas, Spain). Animals were weighed after arrival and housed in polycarbonate cages with stainless steel covers. Then, the animals were acclimatized to the environmental conditions for one week before the experiments, 12 h dark/light cycle, temperature 23 ± 1 °C, relative humidity 55 ± 10%, standard diet (Harlan, 2014; Harlan Laboratories, Barcelona, Spain) and water *ad libitum*.

2.3. Experimental design and treatment

The MN and comet assay OECD protocols (OECD 474 and 489, respectively) recommend a preliminary range-finding study to identify the maximum tolerated dose (MTD), which was defined by Derelanko (2000) as the highest dose to produce toxic effects without causing death and to decrease body weight (bw) by no more than 10% relative to controls. For this purpose, the Acute oral toxicity OECD 425 guideline for testing of chemicals (OECD, 2008) was followed, taking into account the historical report of the LD_{50} in rats described by Jenner et al. (1964). Moreover, considering the OECD 474 (2014a) and OECD 489 (2014b) guidelines, the doses used in the combined MN-comet assay were the MTD and two lower doses (Bowen et al., 2011) appropriately spaced by less than $\sqrt{10}$ (OECD 489, 2014b).

In this study, 23 male and 23 female rats were randomly divided into five groups, two control and three treatment groups: the negative control group (C−) (5 males and 5 females rats) was treated with corn oil (vehicle); the positive control group (C+) (3 males and 3 females rats) was exposed to 200 mg/kg bw ethylmethanesulfonate (EMS) and three exposed groups (5 males and 5 females rats per group) received 81, 256 or 810 mg carvacrol/kg bw, according to the MTD results obtained for carvacrol. The number of animals included in each group was selected according to the OECD 474 (2014a) and OECD 489 (2014b) guidelines: a minimum of 5 analyzable animals of each sex for group (OECD 474) and a minimum of 3 animals of each sex treated with a positive control (OECD 489). All doses were prepared in corn oil at a final volume of 1 mL. According to Bowen et al. (2011), animals for the combined MN and comet endpoints were dosed at 0, 24 and 45 h by gavage using an enteral feeding tube (Vygon, Ecouen, France). Then, animals were sacrificed 3 h after the final dose administration.

During the treatment period, clinical signs, body weight, and food and water consumption were recorded daily.

2.4. Sample collection

Samples of bone marrow, stomach, liver and lungs were collected according to Mellado-García et al. (2016).

2.5. MN assay

The principles of the OECD guideline 474 (OECD, 2014a) and the recommendations of Corcuera et al. (2015) were followed to perform the mammalian erythrocyte MN test. Briefly, the bone marrow cells were smeared on a glass slide, fixed in absolute methanol air dried and stained with Giemsa.

The polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + PCE) ratio and the PCE among NCE ratio were calculated by counting 500 erythrocytes per animal. The incidence of micronucleated immature erythrocytes

(MNPCE) expressed as % MN was analyzed by counting a total of 5000 cells per animal. Five male and 5 female animals were analyzed for the negative control and the dosed groups. Moreover, 3 males and 3 females were analyzed in the case of the positive group.

2.6. Isolation of single-cell suspensions for the comet assay

Single cell suspensions were isolated based on the method of Corcuera et al. (2015) and Mellado-García et al. (2016).

2.7. Standard and enzyme-modified comet assay

The information about DNA damage given by the comet assay reflects the number of single or double stand breaks formed in the cellular DNA before or during the process of electrophoresis. Combining the comet assay with bacterial repair enzymes recognizing specific DNA damage is a necessary step to allow detection of lesions that are not frank breaks (Azqueta et al., 2009). The use of enzymes, Endo III, which responds to oxidized pyrimidines, and FPG, which recognizes altered purine bases including 8-oxo-guanine, induces additional breaks at the sites of oxidized bases and increase the DNA in the tail of the comets (Glei et al., 2016). Therefore, it increases both the sensitivity of the assay in terms of the ability to detect a wider range of damage, and also its specificity (Azqueta et al., 2009).

The standard and modified comet assays were performed as previously described by Mellado-García et al. (2016) and according to the recommendations of the OECD guideline 489 (OECD, 2014b). Briefly, 30 μL cell suspension were mixed with 140 μL pre-warmed low-melting-point agarose in PBS, and immediately twelve aliquots of each cell suspension were placed on agarose precoated glass slides. After lysis, slides were washed 3 × 5 min with enzyme buffer (40 mM HEPES, 0.1M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0 adjusted with 6 M KOH). Afterwards, two gels in each slide were exposed to 30 μL of lysis solution, enzyme buffer alone (buffer F), buffer F containing FPG (10 U/mL) or buffer F containing Endo III (14 U/mL) for up to 30 min in a metal box at 37 °C. Then, nuclei were denatured and electrophoresis was performed. Finally, DNA was neutralized in PBS, washed with water and then fixed with 70% and absolute ethanol before the staining.

The percentages of DNA in tail represents DNA strand breaks and oxidized damage in DNA bases. Five male and 5 female animals were analyzed for the negative control and the dosed groups. Moreover, 3 males and 3 females were analyzed in the case of the positive group.

2.8. Determination of carvacrol in tissues

Analytical pyrolysis was used to identify carvacrol in tissue samples according to Mellado-García et al. (2016). Stomach and liver samples selected to develop pyrolysis were preserved at −80 °C until lyophilisation (Testal Cryodos, Madrid).

Briefly, direct pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) analysis was performed using a double-shot pyrolyzer (Frontier Laboratories, model 2020i) attached to a GC/MS system (Agilent 6890N). Lyophilized tissue samples were thoroughly homogenized and placed (0.5 mg) in small crucible capsules and introduced into a preheated micro-furnace at 500 °C for 1 min. The evolved gases were transferred into the GC/MS for analysis, and the determination was carried out according to Mellado-García et al. (2016). The detector used (Agilent 5973 mass-selective detector) acquired mass spectra at 70 eV ionizing energy. Compounds assignment was achieved via single-ion monitoring for various homologous series, via low-resolution mass spectrometry, and by comparison with published and stored (NIST and Wiley libraries) data.

For estimating the relative abundance of carvacrol, the chromatograms were normalized in high to internal standard peaks: the methoxyphenol vinylguaiacol was used for normalized stomach chromatograms and the nitrogen bearing compound (2,4,6-Trimethyl-1,3-phenylenediamine) for the liver tissue ones.

2.9. Histopathological analysis

The histopathological examination of stomach, liver and lungs by optic and electronic microscopy was performed as described by Mellado-García et al. (2016).

2.10. Statistical analysis

For the MN assay, data are presented as the mean ± SD for each group of animals and statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Moreover, for the standard and the enzyme-modified comet assay, means ± SD of the medians were calculated and total scores of the different groups were compared using the non-parametric Kruskal–Wallis test followed by the Man–Whitney *U* test when the first test showed differences. Analysis was performed using Graph-Pad InStat software (GraphPadSoftware Inc., La Jolla, USA).

3. Results

In the acute toxicity test, the MTD of carvacrol was set at 810 mg/kg bw. At this dose, abnormal behavior and slight body-weight loss were observed, but not death or evidence of pain. Hence, following the OECD guidelines (2014a,b) the doses for testing *in vivo* genotoxicity of carvacrol in the combined MN-comet assay were established at 81, 256 and 810 mg carvacrol/kg bw.

3.1. Micronucleus assay

Table 1 shows that 3 h after the final administration (45 h) of carvacrol, significant differences in the PCE/total erythrocytes ratio were observed from 256 mg/kg bw with respect to the negative control group in males ($p < 0.01$) and females ($p < 0.05$). Regarding PCE/NCE ratio in male rats, a significant ($p < 0.01$) decrease was detected from 256 mg/kg bw. Female rats exposed to carvacrol also showed a significant ($p < 0.05$) decrease of PCE/NCE, but only at the highest dose assayed (810 mg/kg bw). Moreover, carvacrol did not increase the % MN in immature erythrocytes at any dose tested on both sexes.

Treatment with EMS as positive control induced significant decreases ($p < 0.01$) in the PCE/total erythrocytes and PCE/NCE ratios, and significant increases ($p < 0.01$) in the % MN with respect to the negative control.

3.2. Standard and enzyme-modified comet assay

In the standard comet assay, results showed that carvacrol did not induce DNA strand breaks in the stomach cells at any doses assayed, except for the rats exposed to EMS (positive control) (Fig. 1A). Moreover, based on the results of the enzyme digestion of the nuclei, no increase in the frequency of Endo III or FPG-sensitive sites was observed in the stomach cells of rats exposed to carvacrol (Fig. 1B and C, respectively).

With respect to the liver of exposed rats, carvacrol did not induce DNA strand breaks in the standard comet assay at any dose tested (Fig. 1A). Similarly, Fig. 1B and C shows that carvacrol did not

Table 1
Micronucleus assay results. Bone marrow cytotoxicity expressed as polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + PCE), ratio PCE among NCE and the micronuclei induction expressed as % MN. All values are expressed as mean ± SD. The significant levels observed are $^{*}p < 0.05$ or $^{**}p < 0.01$.

Groups	N	Doses	Sex	PCE/Total	PCE/NCE	% MN
Negative Control	5	–	Male	0.51 ± 0.02	1.04 ± 0.07	0.75 ± 0.10
	5		Female	0.53 ± 0.03	1.14 ± 0.12	0.71 ± 0.10
Positive Control (EMS)	3	200 mg/kg	Male	0.31 ± 0.03**	0.45 ± 0.07**	8.17 ± 1.33**
	3		Female	0.31 ± 0.03**	0.46 ± 0.06**	7.55 ± 0.20**
Carvacrol	5	81 mg/kg	Male	0.51 ± 0.09	1.10 ± 0.38	0.74 ± 0.12
	5		Female	0.5 ± 0.15	1.15 ± 0.65	0.73 ± 0.14
	5	256 mg/kg	Male	0.37 ± 0.06**	0.60 ± 0.16*	0.75 ± 0.14
	5		Female	0.40 ± 0.08*	0.69 ± 0.22	0.61 ± 0.23
	5	810 mg/kg	Male	0.40 ± 0.08*	0.68 ± 0.22*	0.86 ± 0.17
	5		Female	0.34 ± 0.03**	0.52 ± 0.07*	0.99 ± 0.17

induce oxidation of pyrimidines and purines bases in exposed rats, as revealed in the modified comet assay by Endo III or FPG enzymes, respectively.

For each test, we found a significantly different response ($p < 0.001$) of the positive control groups treated with 200 mg/kg bw of EMS with respect to the control groups (Fig. 1).

3.3. Presence of carvacrol in stomach and liver of rats

The elucidation of the pyrolysis behavior of carvacrol necessary to identify the compound –or its possible degradation metabolites– in target tissues is shown in Fig. 2. Under the described pyrolytic and chromatographic conditions, a prominent peak was identified with elution time between 7.40 and 7.80 min (Fig. 2A). The average electron ionization mass spectra obtained for this region of the chromatogram allowed us to undoubtedly assign the peak to carvacrol (Fig. 2B). Other minority compounds were also identified, including cumenol, thymol methyl eter, butylcresol and 6-allyl-o-cresol. The analytical pyrolysis of carvacrol does not seem to produce major effects on its chemical structure and therefore was considered an adequate technique to detect the possible presence of carvacrol directly in the tissues.

The analytical pyrolysis of target tissues of control rats was negative to any sign of carvacrol even when searching for the specific mass fragments (m/z 135 and 150). On the other hand, carvacrol was clearly detected in the tissues (stomach and liver) of rats treated with all doses (81, 256 and 810 mg/kg bw) (Fig. 3A). Furthermore, when normalizing the chromatograms to a common peak (vinylguaiacol), a clear dose-response was obtained between the relative intensity of the main carvacrol specific mass fragments and the amount supplied to the animals in the diet (Fig. 3B). A conspicuous difference was found in the dose-response curves between stomach and liver; whereas a direct lineal correlation could be drawn from the former, the response for the latter was best fit to a quadratic equation model (Fig. 3B).

3.4. Histopathological study

No rats died during the experiment and histopathological results from animals orally exposed to carvacrol did not display significant variations. Regarding the stomach, a normal gastric mucosa was observed both under light microscopy (LM) and electron microscopy (EM), for unexposed (Fig. 4A and B) and carvacrol exposed rats (Fig. 4E and F). Moreover, rats intoxicated with EMS (positive control) exhibited necrosis in the mucosa surface under LM (Fig. 4C). Similarly, cells under EM showed abundant phagosomes such as necrotic cell debris with a decrease in the number of mitochondria (Fig. 4D).

The liver histopathology was evaluated and no significant changes were observed in treated groups in comparison with control groups (Fig. 5). In general, under LM as well as under EM normal hepatic cords and sinusoids were observed in the control (Fig. 5A and B) and exposed groups (Fig. 5E and F). Hepatocytes with polyhedral morphology, central nucleus and clear nucleolus and cytoplasm were observed in control (Fig. 5A and B) and treated groups (81, 256 and 810 mg/kg bw) (Fig. 5E and F). However, rats from the positive group (200 mg/kg bw EMS) showed cell swelling with remarkable changes in the nucleus size. Abundant atypical mitosis was also observed in these animals (Fig. 5C and D).

The pulmonary parenchyma in the negative control group did not display significant variations. Optical microscopy evidenced apparently normal bronchi and alveoli (Fig. 6A). Electron microscopy showed morphology and distribution of organelles apparently normal, without alteration of pneumocytes (Fig. 6B). However, in the positive control group treated with EMS slight thickening of the alveolar walls and hyperemia of the capillaries are observed by LM (Fig. 6C). Electron microscopy showed interstitial edema and loss of the lamellar granules from pneumocytes II (Fig. 6D). Overall, the group treated with 810 mg/kg bw carvacrol showed very similar features to those described in the negative control group (Fig. 6E and F).

4. Discussion

Regarding *in vitro* studies of carvacrol, contradictory results on the genotoxicity of this phenolic compound from *Origanum vulgare* EO have been found (Ipek et al., 2005; Aydin et al., 2005a,b; Ündeger et al., 2009; Melusová et al., 2014; Llana-Ruiz-Cabello et al., 2015a). Following the recommendations of the EFSA Scientific Committee (EFSA, 2011) an appropriated *in vivo* study is necessary to ensure whether the genotoxic response *in vitro* is expressed *in vivo*. However, as far as we know, *in vivo* genotoxicity of carvacrol has not been properly studied yet. There is only one report which evaluated the capacity of carvacrol to produce chromosomal aberration (CA) in rats (Azirak and Rencuzogullari, 2008). Therefore, this is the first work which evaluated its *in vivo* genotoxic response combining the MN test in bone marrow cells and the comet assay on cells isolated from stomach and liver of rats. These tissues have been selected according to the OECD 489 guideline (OECD, 2014b) which indicates that the stomach would be a good target because it is a site of direct contact for orally-administered substances, and also indicates that the liver is a relevant organ in xenobiotics metabolism and is exposed to both parent substances and metabolites. In the current study, we chose to perform this *in vivo* MN-comet combined assay, because of its proven benefits in the determination of the genotoxic potential of xenobiotics, such as: 1) improvement of sensitivity and specificity of detection; the MN assay detects the structural and numerical chromosomal damage, while comet assay can determine the short-lived DNA damage, 2) decrease in the number of false negative results, and 3)

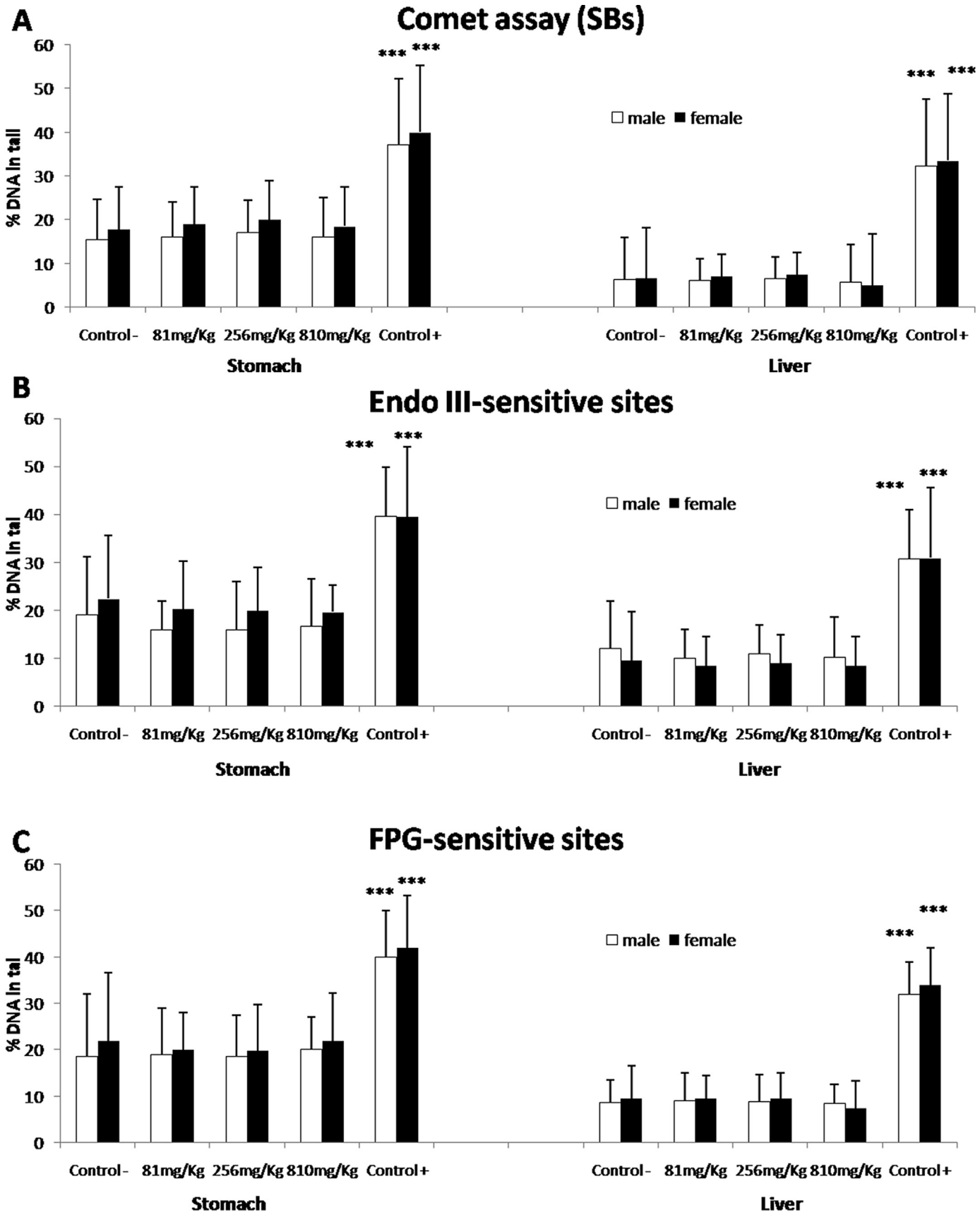

Fig. 1. The level of DNA damage measured on cells isolated from stomach and liver of male and female rats exposed to carvacrol as the formation of strand breaks (SBs) by the standard comet assay (A), and oxidative DNA damage as Endo III-sensitive sites (B) and FPG-sensitive sites (C) by the modified comet assay. The levels of DNA strand breaks and oxidized pyrimidines/purines are expressed as % DNA in tail. All values are expressed as mean ± SD. ***Significantly different from control ($P < 0.001$).

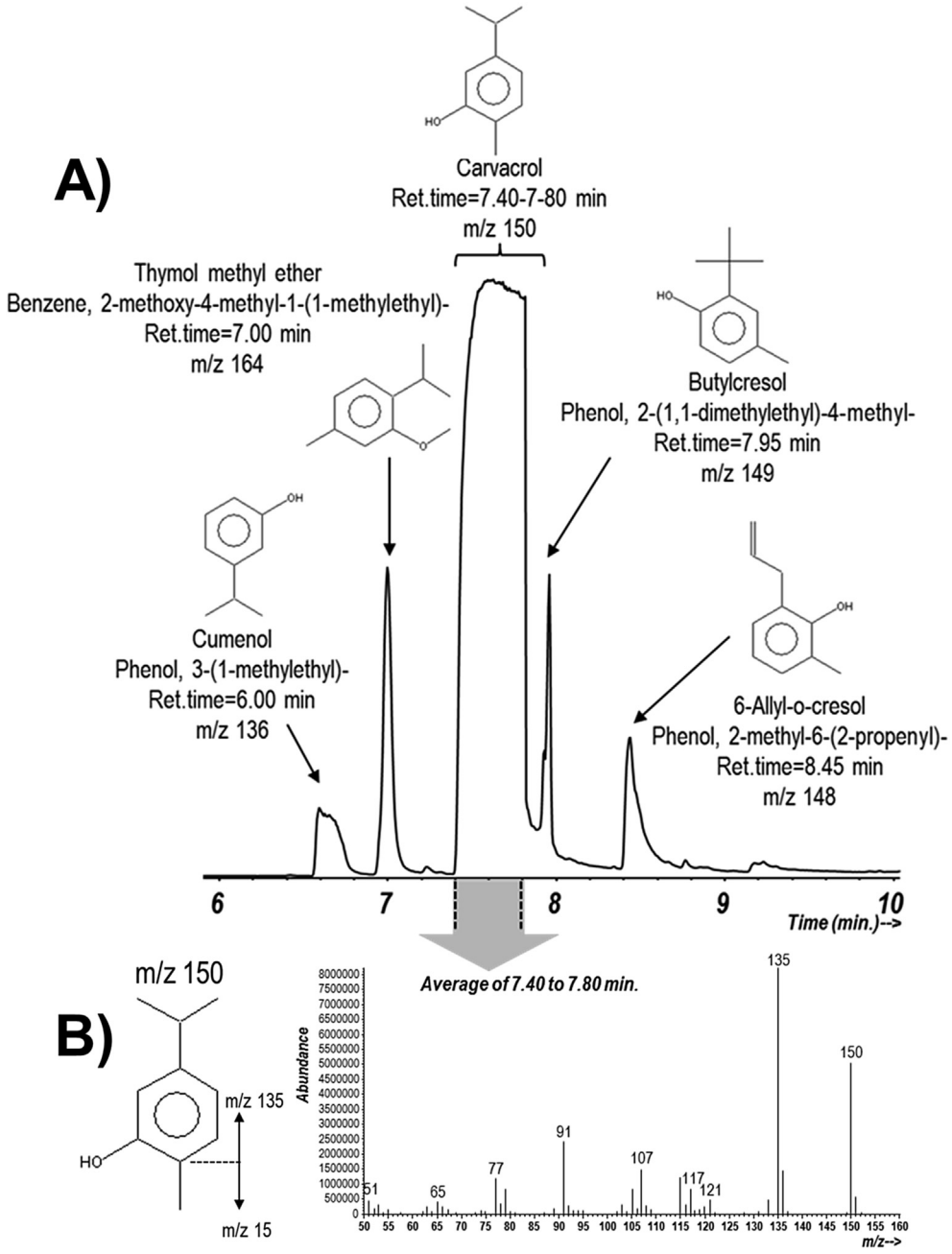

Fig. 2. A) Py-GC/MS total ion current chromatogram (TIC) of carvacrol oil with an indication of the chemical structure of the main compounds released. B) Main mass fragments from carvacrol molecule and average Py-EI-MS spectra corresponding to the carvacrol oil elution domain (min 7.40 to min 7.80 of the chromatogram).

reduction in animal usage in the risk assessment process (Mughal et al., 2010). He et al. (2000) emphasized that measuring the DNA damage using both assays in the same sample can facilitate the estimation of the amount of DNA breakage translated into chromosome and/or genome mutations. Moreover, the use of DNA repair enzymes (Endo III and FPG) increases the sensitivity of the comet assay, since it allows the detection of potential oxidative DNA before the DNA breaks occur (Mellado-García et al., 2016).

In this work, although bone marrow samples collected 24 h after the second dose did not show an increase in the number of micronucleated cells in any of the treated groups, significant decreases in the PCE/NCE ratio were observed in rats exposed to carvacrol (from 256 mg/kg bw). This fact was described previously by Corcuera et al. (2015) in rats exposed to Aflatoxin B1 (0.25 mg/kg b. w.), Ochratoxin A (0.5 mg/kg b. w.) or both mycotoxins as a sign of bone marrow toxicity, and evidenced the exposure of this tissue (e.g. evidence of exposure of the bone marrow to a test substance may include a depression of the immature to mature erythrocyte ratio) (OECD, 2014a). The negative results obtained in the present work in a wide range of concentrations (0–810 mg/kg bw) are in contrast with the positive results reported for carvacrol (0–70 mg/kg bw) in the CA test (Azirak and Rencuzogullari, 2008). In this regard, although the *in vivo* MN assay has been the most widely used *in vivo* test, there are circumstances in which an *in vivo* mammalian bone marrow CA test could be an alternative follow-up test (EFSA, 2011). The discrepancies observed between our results and those mentioned above display differences in the sensitivity of both CA and MN methods. In this sense, Lorge et al. (2006) reported

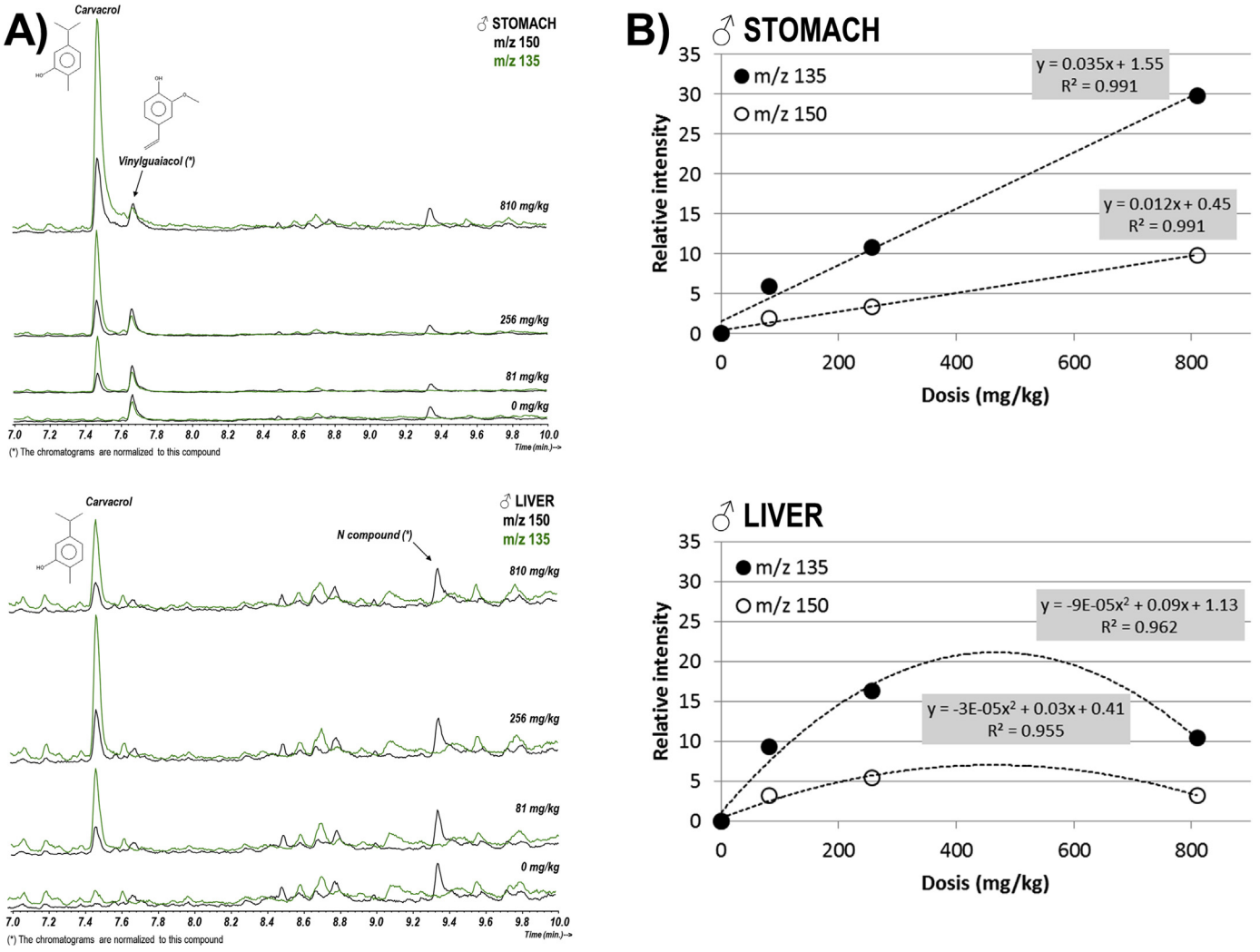

Fig. 3. A) Py-GC/MS selected ion monitoring chromatogram (SIM) for the main carvacrol mass fragments (m/z 35 and m/z 150) in stomach and liver of male rats without and with different dosage of carvacrol in the diet. B) Dose-response and correlation curves of main carvacrol mass fragments (m/z 35 and m/z 150) found after the direct pyrolysis of stomach and liver of male rats without and with different dosage of carvacrol in the diet.

that the high number of analyzable cells, the simplicity of the technique, the possibility of automatization, as well as the capacity to detect aneugens more accurately, led to prefer the *in vivo* MN test (as in this work) to the *in vivo* CA test. Moreover, the negative response obtained in this *in vivo* MN test confirms the previous *in vitro* data in the HepG2 cell line (0–600 μM) (Melusová et al., 2014), as well as in human lymphocytes (0–200 mg/L) (Türkez and Aydin, 2013), and evidences the lack of biological relevance of the positive result obtained in the mouse lymphoma cell line L5178Y/*TK*$^{\pm}$ (Maisanaba et al., 2015). The *in vivo* MN test is preferably recommended in comparison to the *in vitro* MN because it allows evaluating the drug metabolizing, pharmacokinetics and DNA repair processes (Araldi et al., 2015).

Similarly, carvacrol exposure did not produce DNA breaks in cells isolated from either the stomach or the liver of rats in the comet assay, which confirms the negative results reported in *vitro* in different cell lines such as Caco-2 (Horváthová et al., 2006; Llana-Ruiz-Cabello et al., 2014b), HepG2 (Horváthová et al., 2006; Melusová et al., 2014), V79 Chinese hamster lung fibroblast cells (Ündeger et al., 2009), and cultures of primary rat neurons and N2a neuroblastoma cells (Aydin et al., 2014). By contrast, positive results obtained in human lymphocytes (Aydin et al., 2005a; b) could be due to the different sensitivity of cell lines (Llana-Ruiz-Cabello et al., 2015a).

In our study, the application of the enzyme-modified comet assay is useful because of polyphenolic plant extracts belonging to the Lamiaceae family, such as oregano, exhibit antioxidant and pro-oxidant behavior depending on the concentration used (Samec et al., 2015). Previous work reported that Caco-2 cells exposed to carvacrol experienced a significant increase in reactive oxygen species (ROS) (460 μM) after 24 h and concluded that this substance induce oxidative stress at high concentrations; although lower concentrations exhibited DNA-protective effects against H_20_2 in Caco-2 and HepG2 cells lines (Horváthová et al., 2006; Melusová et al., 2014). The increase of ROS may cause irreparable DNA damage leading to mutagenesis and, due to this fact, the oxidative damage inflicted by ROS must be measured in order to evaluate the oxidized bases. In this work, the absence of significant changes observed in Endo III or FPG-sensitive sites in cells isolated from stomach or liver of exposed rats are in agreement with reports obtained *in vitro* in HepG2 cells Melusová et al. (2014), although carvacrol (460 μM) exerted oxidation in purine DNA bases of Caco-2 cells (Llana-Ruiz-Cabello et al., 2014b). The lack of oxidative damage observed in DNA bases *in vivo* could be related to an

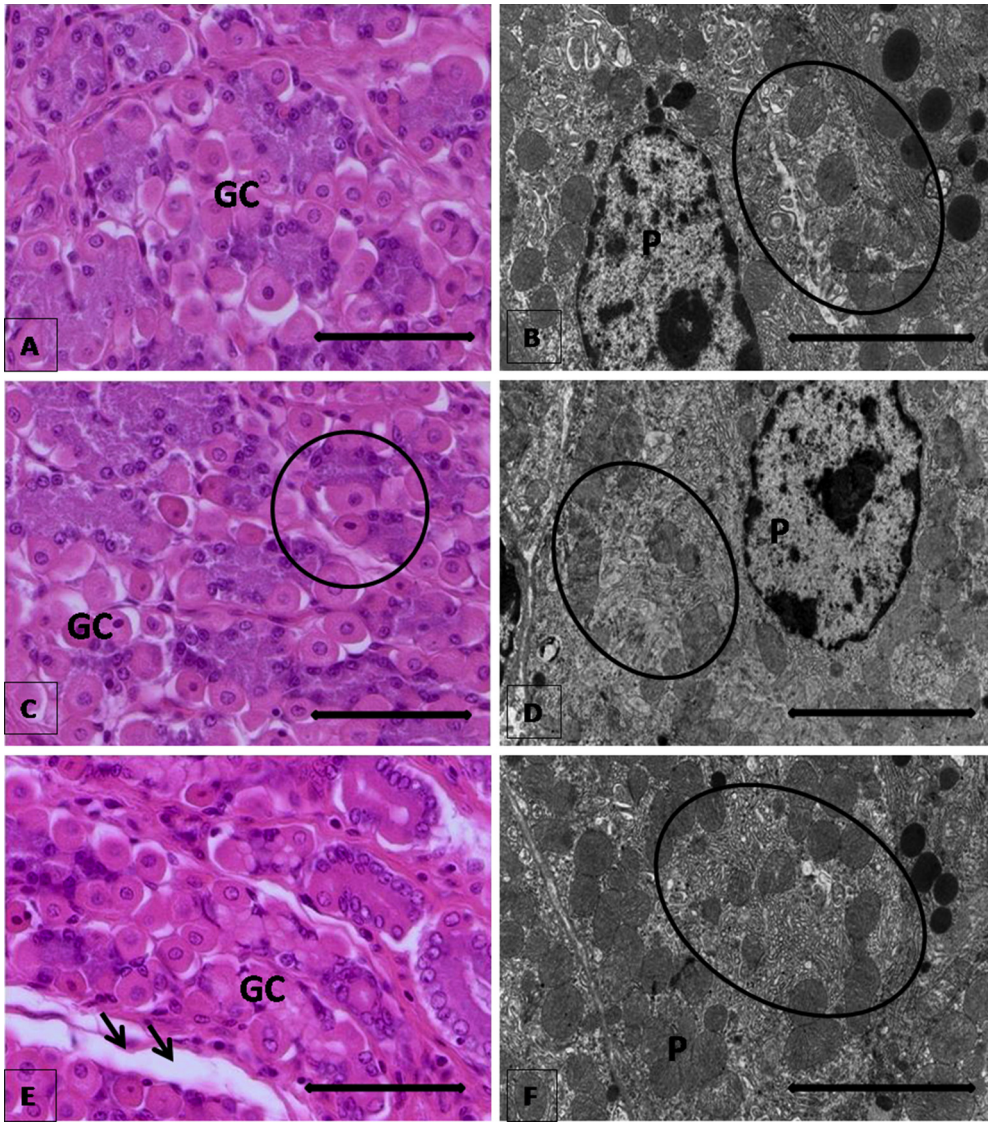

Fig. 4. Histopathological changes in stomach of rats treated with carvacrol. (A,C,E) HE stained stomach section. Bars, 100 μm. (B, D, F) Ultrastructural observations. Bars, 10 μm. (A, B) Negative control rats. (A) Details of gastric mucosa with normal gastric cells (GC). (B) Details of gastric mucosa with normal parietal cells (P) and abundant mitochondria (circle). (C, D) Positive control rats exposed to EMS. (C) Details of the superficial mucosa with very acid-secreting cell (parietal cells) undergoing necrosis (circle). (D) Parietal cells (P) with a decrease in mitochondria rate (circle). (E, F) Rat treated with 810 mg/kg bw carvacrol. (E) Detail of normal gastric cells (GC) and scarce edema in gastric mucosa (arrow). (F) Normal parietal cells (P, circle).

activation of nucleotide excision repair (NER) or base excision repair (BER), two oxidative DNA repair pathways (Azqueta et al., 2009).

In order to complete the genotoxic study of carvacrol following the recommendations of the OECD 489 guideline, we evaluated the histopathological damage and observed no significant changes between control and exposed rats (81–810 mg carvacrol/kg bw) in the stomach, liver and lungs. By contrast, an *in vitro* study found that exposure of human intestinal Caco-2 cells to carvacrol resulted in vacuolated cytoplasm, altered organelles, and other evidences of morphological cellular damage from 230 μM (Llana-Ruiz-Cabello et al., 2014a). There are some discrepancies in the results obtained *in vitro* with those obtained *in vivo*. These discrepancies should be in part due to the different species used as experimental model: rats in the case of the in *vivo* study and a human cell line in the case of the *in vitro* study. In addition, further reasons such as cell culture media, cell line metabolic systems or concentrations tested proposed by Kirkland et al. (2007) could also explain these differences. In this work, the concentrations of carvacrol assayed *in vivo* are higher than those used in previous *in vitro* assays (Llana-Ruiz-Cabello et al., 2014b; Maisanaba et al., 2015). Hence, these differences could be related to other facts such as the differences in metabolism and/or in the bioavailability of the test substances to the target organ (EFSA, 2011). Currently, it is clear that the xenobiotic metabolizing and DNA repair deficiencies are factors which contribute to false positive results *in vitro* (Kirkland et al., 2007; EFSA, 2011).

Although negative results for carvacrol genotoxicity were obtained in this study, we have confirmed the exposure to the compound since the presence of monoterpene has been determined in the target tissues, stomach and liver, by direct analytical pyrolysis. In addition, a decrease of the PCE/NCE ratio in blood samples was demonstrated, which again confirms the exposure of animals. Studies related to the determination of carvacrol in tissues are very scarce. Schroder and Vollmer (1932) detected carvacrol in different tissues such as the stomach, intestine, liver or lung of rats orally

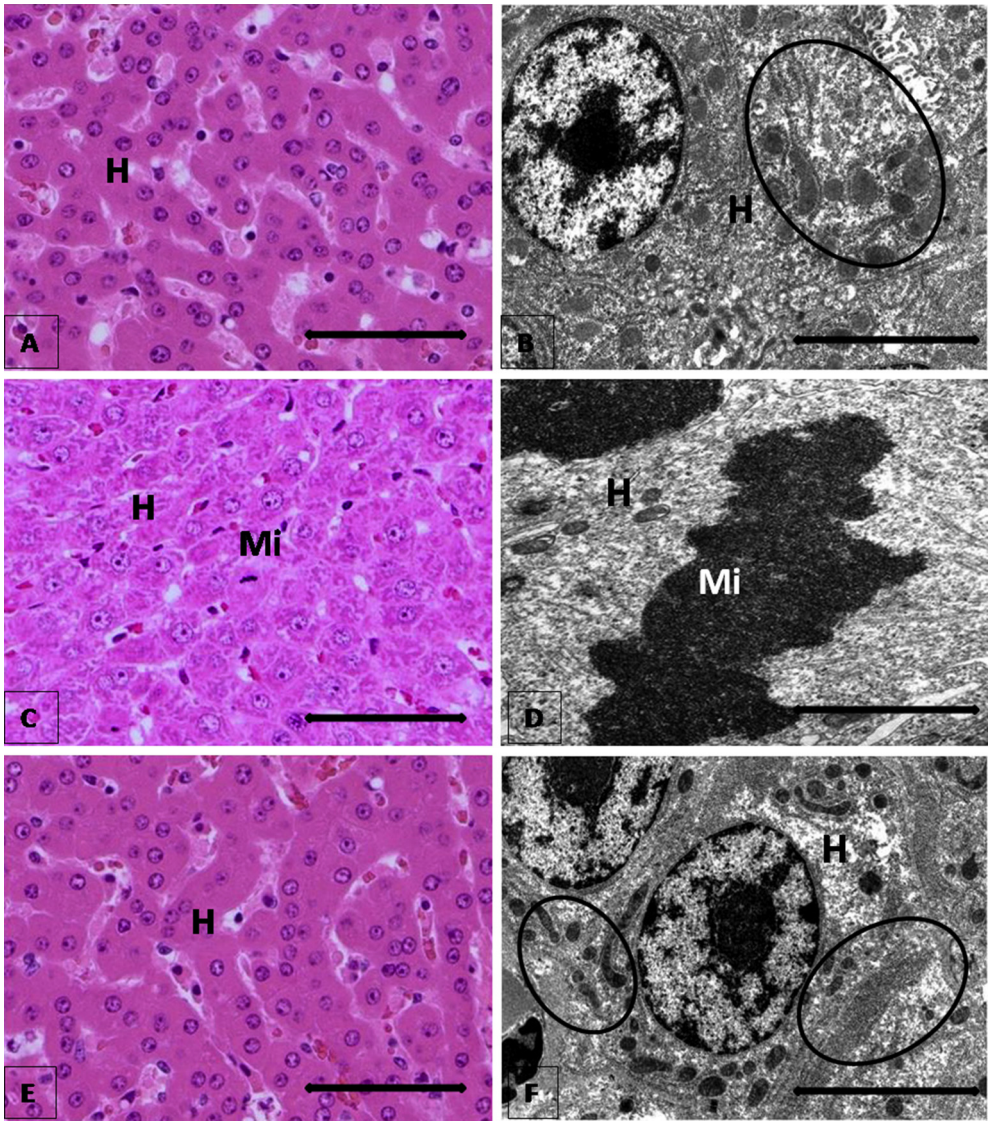

Fig. 5. Histopathological changes in liver of rats treated with carvacrol. (A,C,E) HE stained liver section. Bars, 100 μm. (B, D, F) Ultrastructural observations. Bars, 10 μm. (A, B) Negative control rats. (A) Normal hepatic cordons, (H) polyhedral morphology with clear cytoplasm and central nucleus. (B) Details of apparently normal hepatocyte, (H) with cytoplasmic organelles, reticulum and mitochondria (circle). (C, D) Positive control rats exposed to EMS. (C) Details of liver parenchyma (H) with presence of atypical mitosis (Mi). (D) Detail of hepatocyte (H) in mitosis (Mi). (E,F) Rats treated with 810 mg/kg bw carvacrol. (E) Details of liver parenchyma with normal hepatocytes (H). (F) Apparently normal hepatocyte (H) with intermitotic nuclei and organoids (circle).

exposed to 500 mg/kg of this compound. Moreover, in our work the pyrolysis technique also showed clear and distinct dose-response relationships depending on the tissue evaluated. A non-linear relation was observed in the liver, mostly attributed to the ingestion of concentrations over 256 mg/kg bw; this does not relate to the ratios predicted by the linear model found in the stomach. In this regard, the differences observed in those curves may be related to carvacrol metabolism in rats and the possible occurrence of saturation mechanisms limiting an excess of carvacrol metabolization/presence in liver. Some authors have reported information regarding carvacrol metabolism (Schroder and Vollmer, 1932; Austgulen et al., 1987; Dong et al., 2012a,b). In rats, after oral administration of carvacrol the urinary excretion of metabolites was rapid, and in addition to the parent compound, seven metabolites were identified after extensive oxidation of the methyl and isopropyl groups occurred, resulting in the formation of derivatives of benzyl alcohol and 2-phenylpropanol and their corresponding carboxylic acids; in contrast, ring hydroxylation of phenol was a minor reaction (Austgulen et al., 1987). In humans, carvacrol is oxidized by recombinant human CYP1A2, CYP2B6, and CYP2A6. This later enzyme is the major metabolizing enzyme involved in the metabolism of carvacrol, which is mainly expressed in human liver (Dong et al., 2012a). In rats, only a small amount of unchanged compound is excreted in urine 24 h later, whereas most derivatives were excreted as glucuronides and sulfate conjugates (Austgulen et al., 1987; Friedman, 2014). An *in vitro* study with human microsomes demonstrated that recombinant UGT1A9 was mainly responsible for glucuronidation in liver and rUGT1A7 in intestinal microsomes, forming monoglucuronated metabolites (Dong et al., 2012b). More studies focused on toxicokinetics of this compound are needed to draw conclusions and to define the metabolic derivatives of carvacrol in order to ensure their safety.

5. Conclusions

The combined *in vivo* MN-comet assay protocol demonstrates

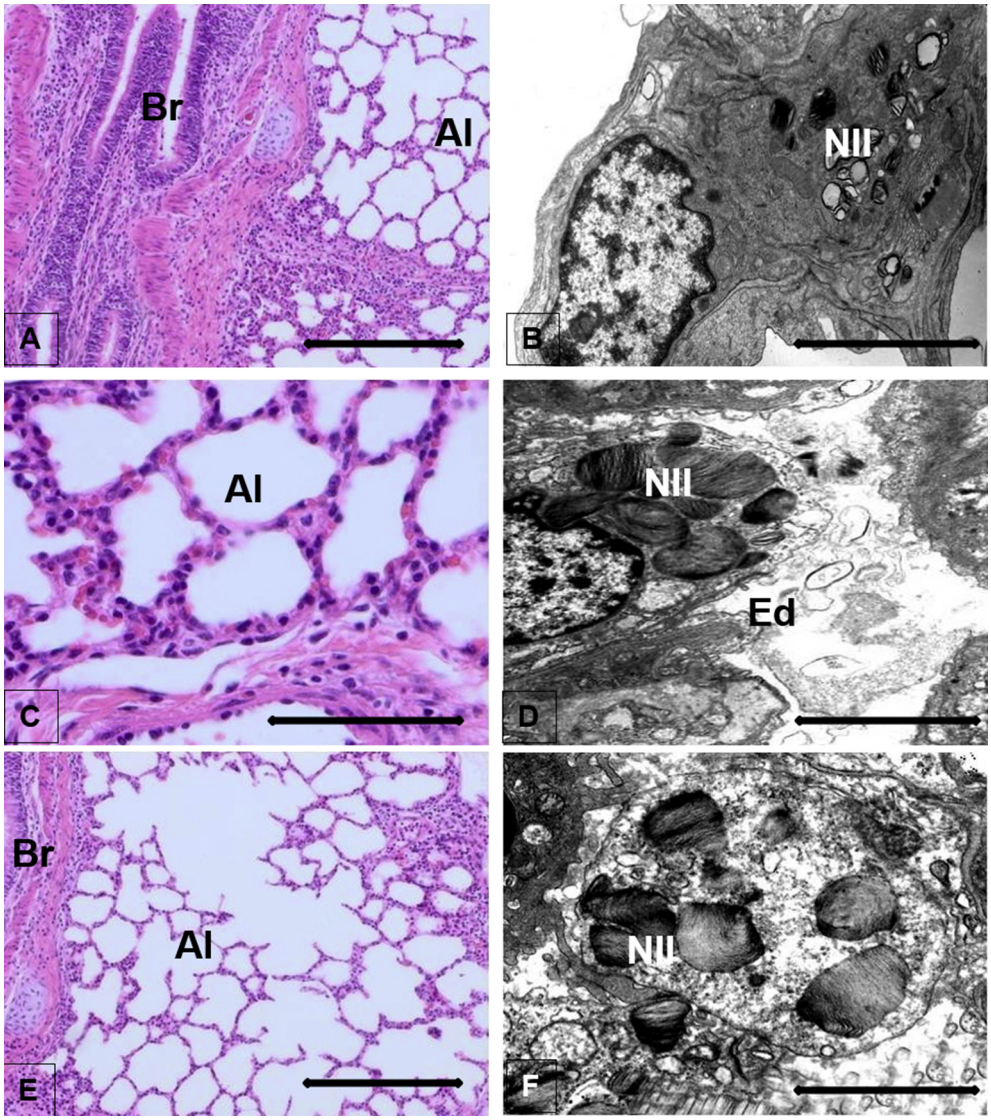

Fig. 6. Histopathological changes in lung of rats treated with carvacrol. (A,C,E) HE stained lung section. Bars, 100 μm. (B, D, F) Ultrastructural observations. Bars, 10 μm. (A, B) Negative control rats. (A) Bronchi (Br) and apparently normal alveolar areas (Al). (B) Presence of normal lamellar granules II from pneumocytes (NII). (C, D) Positive control rats exposed to EMS. (C) Detail of lung parenchyma with slight wall thickening in the alveolar with capillary hyperemia (Al). (D) Presence of interstitial edema (Ed) and partial degranulation of pneumocytes II (NII). (E,F) Rats treated with 810 mg/kg bw carvacrol. (E) Details of lung parenchyma. Apparently normal bronchi (Br) and alveoli (Al). (F) Normal lamellar granules from pneumocytes II (NII).

that carvacrol did not induce genotoxic effects in bone marrow, stomach and liver cells of orally exposed (81–810 mg/kg bw) Wistar rats. Moreover, no oxidative DNA damage was observed when restriction enzymes (Endo III and FPG) were used in the cells isolated from stomach and liver of these animals. In addition, no morphological changes were recorded in any of the tissues evaluated at any of the concentrations assayed. Direct pyrolysis (Py-GC/MS) has allowed the detection of carvacrol in the stomach and liver of treated rats, evidencing the exposure of these tissues to this monoterpene. Therefore, taking into account the lack of reports regarding the *in vivo* genotoxic potential of this substance, more studies are necessary to complete the safety evaluation of this compound before its approval as an active agent in food contact materials.

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Transparency document

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References

Araldi, R.P., Corrêa de Melo, T., Mendes, T.B., Sá Júnior, P.L., Nozima, B.H.N., Ito, E.T., Franco de Carvalho, R., Barreiros de Souza, E., Stocco, R.C., 2015. Using the comet and micronucleus assays for genotocicity studies: a review. Biomed. Pharmacother. 72, 74–82.

Austgulen, L.T., Solheim, E., Scheline, R.R., 1987. Metabolism in rats of p-cymene

derivatives: carvacrol and thymol. J. Pharmacol. Toxicol. 61, 98–102.
Aydin, S., Basaran, A.A., Basaran, N., 2005a. Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. J. Agr. Food Chem. 53, 1299–1305.
Aydin, S., Basaran, A.A., Basaran, N., 2005b. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mut. Res-Gen. Tox. En. 581, 43–53.
Aydin, S., Türkez, H., Keles, M.S., 2014. The effect of carvacrol on healthy neurons and N2a cancer cells: some biochemical, anticancerogenicity and genotoxicity studies. Cytotech 66, 149–157.
Azirak, S., Rencuzogullari, E., 2008. The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells. Environ. Toxicol. 23, 728–735.
Azqueta, A., Shaposhnikov, S., Collins, A.R., 2009. DNA oxidation: investigating its key role in environmental mutagenesis with comet assay. Mut. Res-Gen. Tox. En. 674, 101–108.
Bowen, D.E., Whitwell, J.H., Lillford, L., Henderson, D., Kidd, D., McGarry, S., Pearce, G., Beevers, C., Kirkland, D.J., 2011. Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the comet assay and the flow-cytometric peripheral blood micronucleus test. Mut. Res-Gen. Tox. En. 722, 7–19.
Corcuera, L.A., Vettorazzi, A., Arbillag, L., Pérez, N., Gil, A.G., Azqueta, A., González-Peñas, E., García-Jalón, J.A., López de Cerain, A., 2015. Genotoxicity of Aflatoxin B1 and Ochratoxin A after simultaneous application of the *in vivo* micronucleus and comet assay. Food Chem. Toxicol. 76, 116–124.
Derelanko, M.J., 2000. Toxicologist's Pocket Handbook. CRC Press, Boca Raton, Fla.
Dong, R.H., Fang, Z.Z., Zhu, L.L., Ge, G.B., Cao, Y.F., Li, X.B., Hu, C.M., Yang, L., Liu, Z.Y., 2012a. Identification of CYP isoforms involved in the metabolism of thymol and carvacrol in human liver microsomes (HLMs). Pharmazie 67, 1002–1006.
Dong, R.H., Fang, Z.Z., Zhu, L.L., Ge, G.B., Yang, L., Liu, Z.Y., 2012b. Identification of UDP-glucuronosyltransferase isoforms involved in hepatic and intestinal glucuronidation of phytochemical carvacrol. Xenobiotica 42, 1009–1016.
Downs, T.R., Crosby, M.E., Hu, T., Kumar, S., Sullivan, A., Sarlo, K., Reeder, B., Lynch, M., Wagner, M., Mills, T., Pfuhler, S., 2012. Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. Mut. Res-Gen. Tox. En. 745, 38–50.
Echegoyen, Y., Nerín, C., 2015. Performance of an active paper based on cinnamon essential oil in mushrooms quality. Food Chem. 170, 30–36.
European Food Safety Authority (EFSA), 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J. 9 (2379), 69.
European Food Safety Authority (EFSA), 2016. Recent Developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials. EFSA J. 14 (4375), 28.
Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 2010, 276, 33–79.
Friedman, M., 2014. Chemistry and multibeneficial bioactivities of carvacrol (4-isopropyl-2-methylphenol), a component of essential oils produced by aromatic plants and spices. J. Agric. Food Chem. 62, 7652–7670.
Glei, M., Schneider, T., Schlörmann, W., 2016. Comet assay: an essential tool in toxicological research. Arch. Toxicol. 90, 2315–2336.
He, J.L., Chen, W.L., Jin, L.F., Jin, H.Y., 2000. Comparative evaluation of the *in vitro* micronucleus test and the comet assay for the detection of genotoxic effects of X-ray radiation. Mut. Res-Gen. Tox. En. 469, 223–231.
Horváthová, E., Šramková, M., Lábaj, J., Slamenová, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured in vitro. Neuroendocrinol. Lett. 27, 44–47.
Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B.A., Kurkcuoglu, M., Can Baser, K.H., 2005. Genotoxicity and antigenotoxicity of *Origanum* oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. Food Chem. 93, 551–556.
Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L., Fitzhugh, O.G., 1964. Food flavourings and compounds of related structure I. Acute oral toxicity. Food Cosmet. Toxicol. 2, 327–343.
Kirkland, D., Pfuhler, S., Tweats, D., Aardema, M., Crovi, R., Darroudi, F., Elhajouji, A., et al., 2007. How to reduce false positive results when *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal test: report of an ECVAM Workshop. Mut. Res-Gen. Tox. En. 628, 31–55.
Kirkland, D., Reeve, L., Gatehouse, D., Vanparys, P., 2011. A core *in vitro* genotoxicity battery comprising the Ames test plus the *in vitro* micronucleus test is sufficient to detect rodent carcinogens and *in vivo* genotoxins. Mut. Res-Gen. Tox. En. 721, 27–73.
Kitamoto, S., Matsuyama, R., Uematsu, Y., Ogata, K., Ota, M., Yamada, T., Miyata, K., Kimura, J., Funabashi, H., Saito, K., 2015. Genotoxicity evaluation of benzene, di(2-ethylhexyl) phtalate, and trisodium ethylenediamine tetraacetic acid monohydrate using a combined rat comet/micronucleus assays. Mut. Res-Gen. Tox. En. 786–788, 137–149.
Llana-Ruiz-Cabello, M., Gutierrez-Praena, D., Pichardo, S., Moreno, F.J., Bérmudez, J.M., Aucejo, S., Cameán, A.M., 2014a. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem. Toxicol. 64, 281–290.
Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A., Cameán, A.M., 2014b. Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo-III and FPG- modified comet assays with the human cell line Caco-2. Food Chem. Toxicol. 72, 122–128.
Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A.I., Gutierrez-Praena, D., Jos, A., Cameán, A.M., 2015a. *In vitro* toxicological evaluation of essential oils and their main compounds used in active food packaging: a review. Food Chem. Toxicol. 81, 9–27.
Llana-Ruiz-Cabello, M., Pichardo, S., Baños, A., Nuñez, C., Bermúdez, J.M., Guillamón, E., Aucejo, S., Cameán, A.M., 2015b. Characterisation and evaluation of PLA films containing an extract of *Allium* spp. to be used in the packaging of ready-to-eat salads under controlled atmospheres. LWT- Food Sci. Technol. 64, 1354–1361.
Lorge, E., Thybaud, V., Aardema, M.J., Oliver, J., Wakata, A., Lorenzon, G., Marzin, D., 2006. SFTG international collaborative study on in vitro micronucleus test I. General conditions and overall conclusions of the study. Mut. Res-Gen. Tox. En. 607, 13–36.
Maisanaba, S., Prieto, A.I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R., Cameán, A.M., 2015. *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assay. Mut. Res-Gen. Tox. En. 784–785, 37–44.
Mellado-García, P., Puerto, M., Prieto, A.I., Pichardo, S., Martín-Cameán, A., Moyano, R., Blanco, A., Cameán, A.M., 2016. Genotoxicity of a thiosulfonate compound derived from *Allium* sp. intended to be used in active food packaging: *In vivo* comet assay and micronucleus test. Mut. Res-Gen. Tox. En. 800, 1–11.
Melusová, M., Jantová, S., Horváthová, E., 2014. Carvacrol and Rosemary oil at higher concentrations induce apoptosis in human hepatoma HepG2 cells. Interdiscipli. Toxicol. 7, 189–194.
Mughal, A., Vikram, A., Ramarao, P., Jena, G.B., 2010. Micronucleus and comet assay in the peripheral blood of juvenile rat: establishment of assay feasibility, time of sampling and the induction of DNA damage. Mut. Res-Gen. Tox. En. 700, 86–94.
OECD (Organisation for Economic Cooperation and Development), 2008. Guideline for the Testing of Chemicals: Acute Oral Toxicity – Up-and-down-procedure (UDP), Guideline, vol. 425, pp. 1–27.
OECD (Organisation for Economic Cooperation and Development), 2014a. Guideline for the Testing of Chemicals: Mammalian Erythrocyte Micronucleus Test, Guideline, vol. 474, pp. 1–21.
OECD (Organisation for Economic Cooperation and Development), 2014b. Guideline for the Testing of Chemicals: in Vivo Mammalian Alkaline Comet Assay, Guideline, vol. 489, pp. 1–25.
Samec, D., Durgo, K., Grúz, J., Kremer, D., Kosalec, I., Piljac-Zegarac, J., Salopek-Sondi, B., 2015. Genetic and phytochemical variability of six *Teucrium arduini* L. populations and their antioxidant/prooxidant behavior examined by biochemical, macromoleculate- and cell-based approaches. Food Chem. 186, 298–305.
Schroder, J., Vollmer, H., 1932. The excretion of thymol, carvacrol, eugenol and guaiacol and the distribution of these substances in the organism. Arch. Experimen. Pathol. Pharmakol 168, 331–353.
Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., Von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol. 37, 813–823.
Türkez, H., Aydin, E., 2013. Investigation of cytotoxic, genotoxic and oxidative properties of carvacrol in human blood cells. Toxicol. Ind. Health 1–9.
Ündeger, U., Basaran, A., Degen, G.H., Basaran, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.

CAPÍTULO 10 / CHAPTER 10

María Llana-Ruiz-Cabello, María Puerto, Sara Maisanaba, Remedios Guzmán-Guillén, Silvia Pichardo, Ana M Cameán

COMBINED MICRONUCLEUS AND COMET ASSAY TO EVALUATE THE GENOTOXICITY OF OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) IN RATS ORALLY EXPOSED FOR 90 DAYS.

Food Chemistry (en revisión/under revision), ***2017***

Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

Title: Combined micronucleus and comet assay to evaluate the genotoxicity of oregano essential oil (Origanum vulgare l. Virens) in rats orally exposed for 90 days.

Article Type: Research Article (max 7,500 words)

Keywords: genotoxicity, micronucleus, comet assay, oregano essential, in vivo

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Abstract: Oregano essential oil (OEO) have interesting properties to be used in active food packaging. However, it has been not authorized for this purpose so far. In order to fulfill the requirements of the European Food Safety Authority, the present study addressed for the first time the genotoxicity potential evaluated by a combined micronucleus test (MN) and comet (standard and enzyme-modified) assays in Wistar rats treated with 50, 100 and 200 mg/kg body weight OEO for 90 days. The results obtained for OEO from Origanum vulgare L. virens, containing carvacrol as major compound, indicated absence of genotoxicity in MN and standard comet assay under the conditions tested. Moreover, no oxidative damage was observed in the enzyme-modified comet assay in any of the tissues studied of rats exposed for 90 days to OEO. Therefore, this OEO appears to be safe and could be considered as a potential active in food packaging industry.

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Dear Editor,

We would be very grateful if you consider the manuscript entitled **"Combined micronucleus and comet assay to evaluate the genotoxicity of oregano essential oil (*Origanum vulgare* l. *Virens*) in rats orally exposed for 90 days."** for its publication in "Food Chemistry".

To the extent of our knowledge this is the first work dealing the *in vivo* genotoxicity study in Wistar rats subchronically exposed to oregano essential oil.

The authors declare that there are no conflicts of interest.

I am looking forward to receiving a positive answer from you.

Sincerely,

Silvia Pichardo

***Highlights (for review)**

- Absence of genotoxicity in MN and standard comet assay in rats exposed to OEO
- No oxidative damage was observed in rats exposed for 90 days to OEO
- OEO is safe to be used as a potential active in food packaging industry

Combined micronucleus and comet assay to evaluate the genotoxicity of oregano essential oil (*Origanum vulgare* l. *Virens*) in rats orally exposed for 90 days.

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Abstract

Oregano essential oil (OEO) have interesting properties to be used in active food packaging. However, it has been not authorized for this purpose so far. In order to fulfill the requirements of the European Food Safety Authority, the present study addressed for the first time the genotoxicity potential evaluated by a combined micronucleus test (MN) and comet (standard and enzyme-modified) assays in Wistar rats treated with 50, 100 and 200 mg/kg body weight OEO for 90 days. The results obtained for OEO from *Origanum vulgare* L. *virens*, containing carvacrol as major compound, indicated absence of genotoxicity in MN and standard comet assay under the conditions tested. Moreover, no oxidative damage was observed in the enzyme-modified comet assay in any of the tissues studied of rats exposed for 90 days to OEO. Therefore, this OEO appears to be safe and could be considered as a potential active in food packaging industry.

Keywords: genotoxicity, micronucleus, comet assay, oregano essential, *in vivo*

Abbreviations: Body weight (b.w.); chromosomal aberration (CA); endonuclease III (Endo III); essential oils (EOs); ethylmethanesulfonate (EMS); European Food Safety Authority (EFSA); Food and Drug Administration (FDA); formamidopyrimidine DNA glycosylase (FPG); gas chromatography-mass spectrometry (GC-MS); generally recognize as safe (GRAS); micronucleated inmature erythrocytes (MNPCE); micronucleus test (MN); normochromatic erythrocytes (NCE); Organisation for Economic Co-operation and Development (OECD); oregano essential oil (OEO); phosphate buffered saline (PBS); polychromatic erythrocytes (PCE); repeated-dose toxicity studies (RTD)

1. Introduction

Herbs and spices containing essential oils (EOs) are widely used as flavoring substances in food, soft drinks and beverages (Liju, Jeena & Kuttan, 2013). Moreover, plant extracts obtained from these herbs possess antibacterial, antifungal and antiviral properties, which could be proposed to be used as food additives as an attractive alternative to the synthetic ones in order to improve food shelf life (Hylgaard, Mygind & Meyer, 2012). Therefore, the safety of these EOs should be assessed prior their uses in the food industry both as additive in food and as active substance in food packaging. In this regard, toxicity studies have been published in the scientific literature about botanical preparation and/or its respective components, including results about the genotoxic potential. The disparity in these results has been investigated due to the lack of awareness if some of the compounds presented in the EOs exhibit or not the genotoxic carcinogen profile (Mellado- Garcia et al., 2016a). In Europe, the authorization for the use of EOs as food additives has been not confirmed so far because it is not possible to make a real comparison with their use as fresh or dried aromatic herb in food (EFSA, 2016). Consequently, EOs are not included in the list of approved food additives. Moreover, the positive list for active substances approved to be used in food packaging has not been released so far. However, EOs possesses GRAS status (Generally Recognized as Safe) by the Food and Drug Administration (FDA) from the United States (Llana-Ruiz-Cabello et al., 2016a). Although the GRAS list does not include the dosage of EOs which can be used in food or food packaging. Therefore, exhaustive hazard identification, characterization and a robust demonstration to establish safety levels to be employed in food applications should be performed in order to determine correct levels for human exposure and a concluded risk assessment (EFSA, 2009).

Among the main EOs used due to their extraordinary antimicrobial and antioxidants properties the oregano essential oil (OEO) should be highlighted. This OEO is usually obtained from aerial

68 part of *Origanum* spp. by steam distillation, hydrodistillation or simultaneous distillation-
69 extraction methods (Bayramoglu, Sahin & Summu, 2008). OEO is included as well in the GRAS
70 list by the FDA as mentioned above, although in Europe more studies are required to be
71 approved for this use. Regarding the OEO safety, different results have been reported about its
72 *in vitro* toxicity potential and their main components as it is described in the review published
73 by Llana-Ruiz-Cabello et al. (2015). In general, the OEO tested did not show mutagenic activity
74 with or without metabolic activation in the Ames test at the conditions tested in any case (Ipek
75 et al., 2005; De Martino, De Feo & Nazzaro, 2009; Gulluce et al., 2012); however, a reduction in
76 the mitotic index, chromosomal and nuclear irregularities have been described by Hamedo &
77 Abdelmigid (2009). Moreover, similar contradictory results have been published about its main
78 components, as is the case of carvacrol and thymol, which discrepancies about their
79 mutagenic/genotoxic potential have been related to the conditions established during the
80 experimental setup (assays selected, concentrations assayed, times of exposures,
81 experimental models, etc) (Llana-Ruíz-Cabello et al., 2015). Hence, absence of mutagenic
82 activity of carvacrol in the *Salmonella*/microsome reversion test (Stammatti et al., 1999) and in
83 the sister chromatid exchange assay (Ipek, Tüylü & Zeytinoglu, 2003) has been reported.
84 However, according to Ipek et al. (2005) and Llana-Ruíz-Cabello et al. (2014) carvacrol is a
85 strong, direct-acting mutagen in the Ames test without and with external metabolic activation.
86 Similarly, *in vitro* evaluation of the genotoxic activity of carvacrol has shown both positive
87 (Aydin, Basaran & Basaran, 2005a, b; Llana-Ruíz-Cabello et al., 2014) and negative
88 (Horváthová, Sramkova, Labaj & Slamenova, 2006) results. Moreover, *in vivo*, a lack of
89 genotoxicity of carvacrol has been reported in rats using a combined micronucleus (MN) and
90 comet assays (Llana-Ruiz-Cabello et al., 2016b). All these data could be useful to establish
91 safety limits. Consequently, *in vivo* data as well as an appropriate OEO profile characterization
92 are necessary (EFSA, 2009).

Taking into account that botanical preparations should be characterized due to its variability, in the present work, the chemical composition of this OEO obtained from *Origanum vulgare* L.*virens* grown in Almería (Spain) have been studied. Moreover, combined MN and comet assays were carried out to evaluate *in vivo* the potential genotoxic effects of OEO in bone marrow, and in stomach, liver, and blood cells of Wistar rats exposed to the test OEO during 90 days. In addition, the genotoxicity study was completed by the modified comet assay with restriction enzymes (endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG)) in order to detect DNA oxidative damage.

2. Materials and Methods

2.1. Supplies and chemicals

Chemicals, including Endo III and FPG, were purchased from Sigma-Aldrich (Madrid, Spain), VWR Eurolab (Madrid, Spain), C-viral S.L. (Seville, Spain) and BioWhittaker (Madrid, Spain).

2.2. Isolation of essential oil and gas chromatography analysis

The isolation of the OEO was performed in El Jarpil S.L. (Almería, Spain).Leaves and aerial parts of oregano (*Origanum vulgare* L. *virens*) were collected from Bédar (Almeria, Spain) in June 2012 and were storage during 24-48 h at 23ºC before distillation. Then, 600-800 kg of oregano was placed in an iron tank. A continuous water steam distillation extraction was performed for approximately 3.5 h and the oil was collected and stored at 4°C. The analysis was carried out according to Llana-Ruíz-Cabello et al. (2017), using a Hewlett Packard 5890 chromatograph interfaced to an Hewlett Packard 5970 mass selective detector (Hewlett Packard, Palo Alto, USA). In the Table 1 the components found in a percentage above 0.01% have been listed.

118

119 *2.3. Animal housing and feeding conditions*

120 The Ethical Animal Experimentation Committee of the University of Córdoba and the Junta de
121 Andalucía have been approved the procedures developed in this work (project no.
122 05/10/2015-339). Moreover, all animals received human care in compliance with the
123 guidelines for the protection of animals used for scientific purposes (Directive, 2010/63/EU,
124 Decision, 2012/707/UE and RD 53/2013).

125 Eighty Wistar rats (40 males and 40 females) strain Crl.WI (Han) were randomly distributed
126 into eight groups (10 rats/sex/group), with similar mean body weight (±20%) for each group,
127 and individually housed in cages. Rats were fed with standard laboratory feed (Harlan 2014,
128 Harlan Laboratories, Barcelona, Spain) and water *ad libitum*.

129

130 *2.4. Experimental design and treatment*

131 In order to reduce the animal use in genotoxicity testing, the European Food Safety Authority
132 (EFSA) recommends to integrate both MN and comet assay into repeated-dose toxicity study
133 (EFSA, 2011). Hence, in this work, the MN and comet assay Organisation for Economic Co-
134 operation and Development (OECD) protocols (OECD 474 and 489, respectively) were
135 developed associated to repeated-dose 90-day oral toxicity study (OECD 408).

136 After acclimatization, animals were intoxicated during 90 days with 50, 100 and 200 mg/kg/day
137 of OEO incorporated to neutral gelatin according to Mellado-García et al. (2016a).Acute oral
138 exposure was selected for the positive control group (three male and three female rats) which
139 was exposed to 200 mg/kg/day ethylmethanesulfonate (EMS) using a stomach tube (Vygon,
140 Ecouen, France). In order to obtain the positive controls for both assays three dosage regimen
141 were used as it was described by Bowen et al. (2011) and Mellado-García et al. (2016b).

142

143 *2.5. Sample collection*

144 Samples of bone marrow, stomach and liver were collected according to Mellado-
145 García et al. (2016b). Moreover, blood samples were collected by intracardiac injection (lightly
146 anesthetized with isoflurane) and maintained in Vacutainer® sodium heparin tubes (Becton
147 Dickinson, Rutherford, NJ).

148

149 *2.6. Micronucleus assay*

150 The recommendations of Corcuera et al. (2015) and the principles of OECD guideline 474 were
151 followed to perform the mammalian erythrocyte MN test in bone marrow of Wistar rats
152 exposed to OEO.

153 The polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes
154 (NCE) + PCE) ratio and the PCE among NCE ratio were calculated by counting 500 erythrocytes
155 per animal. The incidence of micronucleated immature erythrocytes (MNPCE) expressed as %
156 MN was analyzed by counting a total of 5000 cells per animal.

157

158 *2.7. Isolation of single-cell suspensions for the comet assay*

159 Single cell suspensions from stomach and liver were isolated based on the method of Corcuera
160 et al. (2015) and Mellado-García et al. (2016b). Besides, heparinized blood samples were mixed
161 v/v (1/1) with phosphate buffered saline (PBS) solution and lymphocytes were isolated with
162 histopaque® (Sigma-Aldrich, Madrid, Spain) and centrifugation (1500 rpm, 30 min). Finally,
163 cells were washed twice with PBS and re-suspended in PBS at a concentration of 2×10^5
164 cells/ml.

165

166 *2.8. Standard and enzyme-modified comet assay*

For blood samples, 30 µl of cells suspension was mixed with 140 µl of 0.5% low-melting point agarose and twelve drops of 5 µl were placed on a microscope slide. For stomach and liver the cell suspensions were mixed with 1% low-melting point agarose and the mixtures were paled on a microscope slide in the same way as blood samples. Then, the standard and modified comet assay were performed as previously described by Mellado-García et al. (2016b) and according to the recommendations of OECD guideline 489 (OECD, 2014b).The percentages of DNA in tail represents DNA strand breaks and oxidized damage in DNA bases.

2.9. Statistical analysis

Results from MN assay are presented as the mean ± SD for each group of animals and statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Moreover, for the standard and the enzyme-modified comet assay, means ± SD were calculated and total scores of the different groups were compared using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U-test when the first test showed differences. Analysis was performed using Graph-Pad InStat software (GraphPadSoftware Inc., La Jolla, USA).

3. Results

3.1 Chemical composition of OEO

Gas chromatography-mass spectrometry (GC-MS) analysis of *Origanum vulgare* L. *virens* essential oil resulted in identification of 108 compounds, representing 99.9% of the essential oil. Based on GC-MS analysis data for *O. vulgare* L. *virens* essential oil: carvacrol (55.82%), p-cimene (16.39%), thymol (5.14%), γ-terpine (4.71%) and β-cariophyllene (2.40%) were found to be the main components, comprising 84.46% of the sample. Other components were present in amounts <2% in the oil (table 1).

192

193 *3.2. Micronucleus assay*

194 Observation from repeated-dose 90-day oral toxicity showed no abnormal clinical signs or
195 mortality in any treated group as described in Llana-Ruíz-Cabello et al. (2017).

196 Results obtained in the MN assay of rats exposed to OEO are shown in table 2. In comparison
197 with their respective control groups, the PCE/total erythrocytes ratio showed significant
198 differences from 100 mg/kg b.w. in male rats ($p<0.05$) and from 200 mg/kg b.w. in female rats
199 ($p<0.01$). Regarding PCE/NCE ratio in males, a significant decrease was observed from 100
200 mg/kg b.w. ($p<0.05$), while in females this significant decreased were detected at the highest
201 dose assayed ($p<0.01$). Finally, the % of MN in immature erythrocytes did not experiment
202 changes in comparison with the control groups at any dose tested on both sexes. In contrast,
203 treatment with EMS (positive control) induced a significant increase in the frequency of MN.

204

205 *3.3. Standard and enzyme-modified comet assay*

206 Figure 1 showed the results obtained in the comet assay after exposure of Wistar rats to this
207 OEO. No DNA strand breaks were observed in stomach (Fig. 1A), liver (Fig. 1B) and blood
208 samples (Fig.1C) in the standard comet assay. Similarly, no increases in the frequency of Endo
209 III or FPG-sensitive sites were observed in any tissue in the enzyme-modified assays.Moreover,
210 a significant different response ($p<0.001$) of control positive groups treated with 200 mg/kg of
211 EMS with respect to the control group was detected in all assays.

212

213 **4. Discussion**

214 *Origanum vulgare* L. EO, as well as other EOs, is intended to be used as additive in food and
215 food packaging due to its antimicrobial and antioxidant properties (Ipek et al., 2005). In fact,
216 the effectiveness of this OEO included in PLA has been check in lettuce packaged with this

217 active film (Llana-Ruiz-Cabello et al., 2016a). However, the lack of data regarding genotoxicity,
218 reproductive and developmental toxicity is delaying the approval of these substances by the
219 European Commission. In this regard, EFSA has reported that genotoxic potential of the
220 botanical preparation and its respective components should be clarified before its
221 authorization in Europe (EFSA, 2016).
222
223 Chemical variations in composition of EOs are widely described and the sources of this
224 variability are hard to determine. Németh-Zámboriné (2016) addressed that the facts that
225 influence the composition of these complex mixtures could be classified in abiotic factors, such
226 as soil, light intensity, temperature or water supplies; and biotic factors, which are related with
227 the genetic differences observed in species from the same botanical genus. Moreover, the
228 extraction method used to obtain the EO plays a determinant role on the final qualitative and
229 quantitative composition of these substances (Azmir et al., 2013). In consequence, the EOs
230 obtained from different sources or by different extraction techniques could present different
231 properties, activities or toxicological behavior. In this regard, the guidance on safety
232 assessment of botanicals remarks that the chemical composition of EOs should be provided,
233 with emphasis on the concentrations of constituents of relevance for the safety assessment
234 (EFSA, 2009). The compositions of different OEOs have been previously determined by several
235 authors. Although the quantity of their major compounds seems to be variable, most of them
236 agreed with the fact that carvacrol, thymol and their precursors, c-terpinene and ρ-cymene,
237 are the major compounds present in oils obtained from *Origanum vulgare* species (Mezzoug et
238 al., 2007; Bostancioglu, Kürkcüoglu, Can Baser & Koparal, 2012). The results obtained in this
239 work agreed with these findings showing that carvacrol (55.82%), followed by its precursor ρ-
240 cimene (16.39%) are the main components in the EO obtained from *Origanum vulgare* L.
241 *virens*. In this study, a proportion carvacrol/thymol 10:1 was found, although according to

242 other authors it could vary between approximately 1:1 (Mezzoug et al., 2007), and 50:1
243 (Bostancioglu et al., 2012).
244
245 In relation to the potential use in the food industry of OEO, the toxicological profile
246 should be determined. As far as we know, there are no *in vivo* studies focused on the
247 genotoxicity of the complete OEO, but individual components have been assessed. In this
248 regard, EFSA (2011) encourage the integration of genotoxicity test, such as comet assay and
249 MN test, into repeated-dose toxicity studies (RTD) because the combined measurement of
250 genotoxicity endpoints offers the possibility for an improved interpretation of genotoxic
251 findings since such data will be evaluated in conjunction with routine toxicological information
252 obtained in the RTD study. In the present work, an absence of significant differences with
253 respect the control group in the % of MN and in the DNA strand breaks in the standard comet
254 assay were observed in rats exposed for 90 days to OEO. Moreover, no oxidative damage was
255 observed in the enzyme-modified comet assay in any of the tissues investigated of the animals
256 subchronically exposed to OEO. This finding is particularly interesting because polyphenolic
257 plant extracts belonging to the Lamiaceae family, such as oregano, exhibit both antioxidant
258 and prooxidant behavior depending on the concentration used (Samec et al., 2015). Therefore,
259 a positive result in this assay could have been expected. In the case of the MN assay, an
260 evidence of exposure of the bone marrow should be demonstrated when negative results are
261 obtained (OECD 474, 2014). In this sense, one of these evidences include a reduction of the
262 PCE/NCE ratio in bone marrow, which has been observed in the present study, confirming the
263 exposure of animals.
264 The *in vivo* results obtained in the present work are very useful to ensure whether the
265 genotoxic potential *in vitro* is expressed *in vivo*. *In vitro* studies related to the
266 mutagenicity/genotoxicity of different extracts obtained from *Oreganum vulgare* are scarce

and the existing studies did not give rise to safety concerns with respect to their genotoxicity (Hamedo & Abdelmigid, 2009). Ipek et al. (2005) and De Martino et al. (2009) reported that OEO did not show mutagenic effects on TA98 and TA100 *Salmonella thyphimurium* strains. Moreover, similar results were observed for *S. thyphimurium* TA1535 and TA1537 and *Escherichia coli* wP2uvrA by Gulluce at al. (2012). In contrast, Hamedo & Abdelmigid (2009) reported reduction in the mitotic index and chromosomal and nuclear irregularities in *Vicia faba* seeds exposed to 0,1% OEO after develop the chromosomal aberration (CA) test.

In general, studies carried out focused on the *in vivo* genotoxicity of EOs also proved to be saved for oral consumption are very scarce (Hwang & Kim, 2012; Escobar et al., 2012, 2015). *Zanthoxylum piperitum*-derived EO produces no bone marrow MN abnormalities, mutagenicity, or chromosomal aberration (Hwang & Kim, 2012). Likewise, *Minthostachys verticillata* EO did not increased the frequency of MN in bone marrow cells of mice (Escobar et al., 2012). The latter authors also confirm the lack of genotoxicity in blood samples studied by both MN and comet assay in rats (Escobar et al., 2015). In these studies, the authors indicated possible synergistic/antagonistic phenomena between the major components present in the EOs, as responsible of the disagreement observed in the toxic effects of the whole oil did not exert cyto-genotoxic effect on the bone marrow of animals, in comparison to some individual constituents which showed a positive mutagenic and genotoxic activities (Escobar et al., 2012, 2015).

Furthermore, EFSA remarks in the guidance on safety assessment of botanicals and botanical preparations intended for use as ingredient in food supplements (EFSA, 2009), the possibility of focusing the toxicological evaluation of botanicals in the compounds of concern when these are well defined. In relation to the components of OEOs most of the studies regarding their genotoxicity have been developed using carvacrol, one of the active and major

292 compounds, which seems to be responsible for antioxidant and antimicrobial properties. The
293 *in vitro* genotoxicity of carvacrol has been largely evaluated and contradictory results have
294 been reported (Stammatti et al., 1999; Ipek et al., 2003; Aydin et al., 2005a,b; Horváthová et
295 al., 2006, Llana-Ruíz-Cabello et al., 2014). However, i*n vivo* genotoxicity of carvacrol has been
296 scarcely studied. As far as we know, there are only two reports dealing with this topic. One of
297 them evaluated the ability of carvacrol to produce CA in rats (Azirak & Rencuzogullari, 2008).
298 Moreover, the second work evaluated the *in vivo* genotoxic potential of carvacrol combining
299 the MN test in bone marrow cells and the comet assay on the stomach and liver cells of rats
300 (Llana-Ruiz-Cabello et al., 2016b). Results from these *in vivo* assays were also contradictory as
301 well as *in vitro* data. Although Azirak & Recunzogulari (2008) found that carvacrol (0-70 mg/kg
302 b.w.) was able to produce chromosomal abnormalities in the chromosomal aberration CA test
303 in rats, the results obtained in MN test and comet assays reported a lack of genotoxicity of
304 carvacrol (0-810 mg/kg b.w.) in bone marrow, stomach and liver cells (Llana-Ruiz-Cabello et al.,
305 2016b). This discrepancy could be related to the different endpoints measured in each assay.
306 All these studies have been carried out after short-term exposure.

307 In the present study, the lack of genotoxicity showed *in vivo* by this OEO is in
308 agreement with the previous results obtained with carvacrol above mentioned (Llana-Ruiz-
309 Cabello, et al., 2016b). According to the scientific literature, the results may differ in exposure
310 to the complete OEO, because the interaction of major and minor components of the EOs can
311 produce additive, antagonist or synergism responses when the effect of EO is greater than the
312 sum of individual compounds (Burt, 2004) and therefore EOs have to be considered as
313 substances acting as a whole (Escobar et al., 2012). In this work, and taking into account the
314 agreement of *in vivo* genotoxicity results obtained, no synergic/antagonistic phenomena in the
315 constituents of this OEO could be considered.

316 Previous toxicological results obtained in our laboratory in the *in vivo* studies have
317 described an absence of clinical, histopathological and biochemical alterations of rats exposed
318 for 90 days to the same OEO used in the present study (Llana-Ruíz-Cabello et al., 2017). The
319 no-observed-adverse-effect level (NOAEL) of this OEO was set at 200 mg/kg b.w. in Wistar rats,
320 which is 330-fold higher than those expected to be in contact consumers in the worst scenario
321 of exposure. In summary, in the present work, neither MN nor comet assays have evidenced
322 for the first time potential *in vivo* genotoxicity of this OEO at doses up to 200 mg/Kg b.w., in
323 Wistar rats for 90 days, reinforcing the absence of genotoxicity previously demonstrated *in*
324 *vivo* by its main component carvacrol. Considering the toxicological assessment performed in
325 Wistar rats subchronically exposed to this OEO following the authority's guidelines, it seems to
326 be safe to be used as food additive.
327
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333

6. References

Aydin, S., Basaran, A. A., & Basaran, N. (2005a). Modulating effects of thyme and itsmajoringredients on oxidative DNA damage in human lymphocytes. *Journal of Agricultural and Food Chemistry*, 53, 1299–1305.

Aydin, S., Basaran, A. A., & Basaran, N. (2005b). The effects of thyme volatiles on theinduction of DNA damage by the heterocyclic amine IQ and mitomycin C. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 581, 43–53.

Azirak, S., & Rencuzogullari, E. (2008). The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells. *Environmental Toxicology*, 23, 728-735.

Azmir, J., Zaidul, I. S.M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N., & Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*, 117, 426-436.

Bayramoglu, B., Sahin, S., & Summu, G. (2008). Solvent-free microwave extraction of essential oil from oregano. *Journal of Food Engineering*, 88, 535-540.

Bowen, D. E., Whitwell, J. H., Lillford, L., Henderson, D., Kidd, D., Mc Garry, S., Pearce, G., Beevers, C., & Kirkland, D. J. (2011). Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the comet assay and the flow-cytometric peripheral blood micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 722, 7-19.

Bostancioglu, R. B., Kürkcüoglu, M., Can Baser, K. H., & Koparal, A. T. (2012). Assessment of anti-angiogenic and anti-tumoral potentials of *Origanum onites* L. essential oil. *Food and Chemical Toxicology*, 50, 2002–2008.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *International Journal of Food Microbiology*, 94, 223-253.

359 Corcuera, L. A., Vettorazzi, A., Arbillag, L., Pérez, N., Gil, A. G., Azqueta, A., González-Peñas, E.,
360 García-Jalón, J. A., & López de Cerain, A. (2015). Genotoxicity of Aflatoxin B1 and Ochratoxin A
361 after simultaneous application of the *in vivo* micronucleus and comet assay. *Food and*
362 *Chemical Toxicology*, 76, 116–124.
363 De Martino, L., De Feo, V., & Nazzaro, F. (2009). Chemical composition and *in vitro*
364 antimicrobial and mutagenic activities of seven *Lamiaceae* essential oils. *Molecules* 14, 4213–
365 4230.
366 Decision 2012/707/UE. Commission Implementing Decision of 14 November 2012 establishing
367 a common format for the submission of the information pursuant to Directive 2010/63/EU of
368 the European Parliament and of the Council on the protection of animals used for scientific
369 purposes <http://
370 http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Unofficial%20Consolidated%20v
371 ersion.pdf [last access 02/03/2017].
372 Directive 2010/63/EU of the European Parliament and of the Council on the protection of
373 animals used for scientific purposes. http://eur-lex.europa.eu/legal-
374 content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN [last access 02/03/17].
375 Escobar, F. M., Cariddi, L. N., Sabini, M. C., Reinoso, E., Sutil, S. B., Torres, C. V., Zanon, S. M. &
376 Sabini, L. I. (2012). Lack of cytotoxic and genotoxic effects of *Minthostachys verticillata*
377 essential oil: Studies *in vitro* and *in vivo. Food and Chemical Toxicology*, 50, 3062–3067.
378 Escobar, F. M., Sabini, M. C., Cariddi, L. N., Sabini, L. I., Mañas, F., Cristofolini, A., Bagnis, G.,
379 Gallucci, M. N. & Cavaglieri, L. R. (2015). Safety assessment of essential oil from *Minthostachys*
380 *verticillata* (Griseb.)Epling (peperina): 90-Days oral subchronic toxicity study in rats. *Regulatory*
381 *Toxicology and Pharmacology*, 71, 1–7.

382 European Food Safety Authority (EFSA) (2009). Guidance on safety assessment of botanicals
383 and botanical preparations intended for use as ingredient in food supplements. EFSA Journal,
384 7(9): 1249 pp.
385 European Food Safety Authority (EFSA) (2011). Scientific Opinion on genotoxicity testing
386 strategies applicable to food and feed safety assessment. EFSA J. 9, 2379, [69 pp.].
387 European Food Safety Authority (EFSA) (2016). Technical report on the outcome of the
388 consultation with Member States and EFSA on the basic substance application for *Origanum*
389 *vulgare* L. essential oil for use in plant protection as a fungicide, bactericide and insecticide.
390 EFSA supporting publication 2016:EN-1054. 54 pp.
391 Gulluce, M., Karadayi, M., Guvenalp, Z., Ozbek, H., Arasoglu, T., & Baris, O. (2012). Isolation of
392 some active compounds from *Origanum vulgare* L. spp. vulgare and determination of their
393 genotoxic potentials. Food Chemistry, 130, 248–253.
394 Hamedo, H., & Abdelmigid, H. M. (2009). Use of antimicrobial and genotoxicity potentially for
395 evaluation of essential oils as food preservative. *The Open Biotechnology Journal*, 3, 50–56.
396 Horváthová, E., Sramkova, M., Labaj, J., & Slamenova, D. (2006). Study of cytotoxic, genotoxic
397 and DNA-Protective effects of selected plant essential oils on human cells cultured *in vitro*.
398 *Neuroendocrinology Letters*, 27, 44-47.
399 Hwang, E. S. & Kim, G. H. (2012). Safety evaluation of *Zanthoxylum piperitum*-derived essential
400 oil by assessing micronucleus abnormalities, mutagenicity, and chromosomal aberration. *Food*
401 *Research International*, 47, 267–271.
402 Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential Oils in Food Preservation: Mode of
403 Action, Synergies, and Interactions with Food Matrix Components. *Frontiers in Microbiology*, 3,
404 12.
405 Ipek, E., Tüylü, B. A., & Zeytinoglu, H. (2003). Effects of carvacrol on sister chromatid exchanges
406 in human lymphocyte culture. *Cytotechnology* 43, 145–148.

Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B. A., Kurkcuoglu, M., Can Baser, K. H. (2005). Genotoxicity and antigentoxicity of Origanum oil and carvacrol evaluated by Ames Salmonella/microsomal test. *Food Chemistry*, 93, 551-556.

Llana-Ruíz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A. I., Pichardo, S., Jos, A., & Cameán, A. M. (2014). Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames Salmonella test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. *Food and Chemical Toxicology*, 72, 123-128.

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A. I., Gutiérrez-Praena, D., Jos A., & Cameán, A.M. (2015). *In vitro* toxicological evaluation of essential oils and their main compounds used in active food packaging: a review. *Food and Chemical Toxicology*. 81, 9–27.

Llana-Ruiz-Cabello, M., Pichardo, S., Bermúdez, J. M., Baños, A., Núñez, C., Guillamón, E., Aucejo, S., & Cameán, A. M. (2016a). Development of PLA containing oregano essential oil (*Origanum vulgare* L *virens*) intended for use in food packaging. *Food Additives and Contaminants: Part A*, 33, 1374-1386.

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A. I., Pichardo, S., Moyano, R., González-Pérez, J. A., & Cameán, A. M. (2016b). Genotoxicity evaluation of carvacrol in rats using a combined micronucleus and comet assay. *Food and Chemical Toxicology*, 98, 240-250.

Llana-Ruíz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A. I., Pichardo, S., Jos, A., Moyano, R., & Cameán, A. M. (2017). A subchronic 90-day oral toxicity study of *Origanum vulgare* essential oil in rats. *Food and Chemical Toxicology, 101*, 36-47.

Liju, V. B., Jeena, K., & Kuttan, R. (2013). Acute and subchronic toxicity as well as mutagenic evaluation of essential oil from turmeric (*Curcuma longa* L). *Food and Chemical Toxicology*, 53, 52-61.

Mellado-García, P., Puerto, M., Pichardo, S., Llana-Ruiz-Cabello, M., Moyano, R., Blanco, A., Jos, A., & Cameán, A. M. (2016a). Toxicological evaluation of an *Allium*-based comercial product in 90-day feeding study in Sprague-Dawley rats. *Food and Chemical Toxicology* , 90, 12-29.

Mellado-García, P., Puerto, M., Prieto, A. I., Pichardo, S., Martín-Cameán, A., Moyano, R., Blanco, A., & Cameán, A. M. (2016b). Genotoxicity of a thiosulfinate compound derived from *Allium* sp. intended to be used in active food packaging: *In vivo* comet assay and micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 800, 1-11.

Mezzoug, N., Elhadri, A., Dallouh, A., Amkiss, S., Skali, N.S., Abrini, J., Zhiri, A., Baudoux, D., Diallo, B., El Jaziri, M., & Idaomar, M. (2007). Investigation of the mutagenic and antimutagenic effects of *Origanum compactum* essential oil and some of its constituents. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 629, 100–110.

Németh-Zámboriné, E. (2016). Natural variability of essential oil components. In Can Baser, K. H., & Buchbauer, G. (Eds.), *Essential oils: Science, technology, and applications* (pp.87-126). CRC Press, Boca Raton.

OECD (Organisation for Economic Cooperation and Development). (1998). Guideline for Testing of Chemicals. Repeated Dose 90-day Oral Toxicity Study in Rodents. Guideline 408, 1-10.

OECD (Organisation for Economic Cooperation and Development). (2014a). Guideline for the testing of chemicals: mammalian erythrocyte micronucleus test. Guideline 474, 1-21.

OECD (Organisation for Economic Cooperation and Development). (2014b). Guideline for the testing of chemicals: *in vivo* mammalian alkaline comet assay. Guideline 489, 1–25.

RD 53/2013, 2013. Real Decreto, se establecen las normas basicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. BOE 34, 11370-11421. http://boe.es/boe/dias/2013/02/08/pdfs/BOE-A-2013-1337.pdf [last access 02.03.17].

456 Samec, D., Durgo, K., Grúz, J., Kremer, D., Kosalec, I., Piljac-Zegarac, J. & Salopek-Sondi, B.
457 (2015). Genetic and phytochemical variability of six Teucrium arduini L. populations and their
458 antioxidant/prooxidant behavior examined by biochemical, macromoleculate- and cell-based
459 approaches. *Food Chemistry*, 186, 298-305.
460 Stammatti, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H. L., & von Wright, A. (1999).
461 Toxicity of selected plant volatiles in microbial and mammalian short-term assays. *Food and*
462 *Chemical Toxicology*, 37, 813-823.
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464

Figure caption

Figure 1.DNA damage in stomach (A), liver (B)and blood (C)of male and female rats: formation of strand breaks detected by the standard comet assay and oxidative DNA damage as Endo III-sensitive sites and FPG-sensitive sites by the modified comet assay (from the left to right). The levels of DNA strand-breaks (SBs) and oxidised pyrimidines/purines are expressed as % DNA in tail. All values are expressed as mean ± SD. ***Significantly different from control (p< 0.0001).

Table caption

Table 1. Main components of *Origanum vulgare L*. essential oil in a percentage above 0.01%.

Table 2. Micronucleus assay results. Bone marrow cytotoxicity expressed as micronucleated polychromatic erythrocytes (PCE) among total erythrocytes; PCE among total erythrocytes (normochromatic erythrocytes (NCE) + PCE) ratio; and the micronuclei induction expressed as % MN. The values are expressed as mean±SD.The significance levels observed are *p < 0.05, **p<0.01 and ***p < 0.001 in comparison tocontrol group values.

Table 1

RT (min)	Compound	%

7,0	2-METHYL BUTANAL	0,02
7,1	ISOVALERALDEHYDE	0,06
7,3	ETHANOL	0,05
10,0	2-METHYLBUTYRATE DE METHYLE	0,10
10,4	ISOVALERATE DE METHYLE	0,02
10,6	α-PINENE	1,10
10,8	α-THUYENE	1,69
12,7	CAMPHENE	0,27
14,9	β-PINENE	0,23
16,1	THUYADIENE	0,04
17,2	Δ3-CARENE	0,13
17,4	ESTER ALIPHATIQUE	0,04
17,9	β-MYRCENE	1,52
18,2	α-PHELLANDRENE	0,17
18,5	ψ-LIMONENE	0,02
19,2	α-TERPINENE	1,62
20,3	LIMONENE	0,38
21,0	1,8-CINEOLE + β-PHELLANDRENE	0,32
22,5	Cis-β-OCIMENE	0,03
23,5	γ-TERPINENE	4,71
23,6	Trans-β-OCIMENE	0,04
23,9	3-OCTANONE	0,74
25,3	p-CYMENE	16,39
26,0	TERPINOLENE	0,17
32,5	3-HEXEN-1-OL	0,03
32,8	3-OCTANOL	0,29
36,6	α,p-DIMETHYLSTYRENE + cis-OXYDE DE LINALOL	0,19
36,8	1-OCTEN-3-OL	1,50
38,0	Trans-THUYANOL	0,10
42,4	CAMPHRE	0,05
43,2	LINALOL	0,08
43,4	β1-CUBEBENE	0,02
43,6	Cis-THUYANOL	0,08
44,7	Trans-p-MENTH-2-EN-1-OL	0,04
45,9	ε-CADINENE	0,04
46,2	ACETATE DE BORNYLE	0,04
47,4	TERPINENE-4-OL	1,33
47,5	β-CARYOPHYLLENE	2,40
48,2	Cis-DIHYDROCARVONE	0,12
48,8	Cis-p-MENTH-2-EN-1-OL	0,03
49,5	Trans-DIHYDROCARVONE	0,06
52,1	α-HUMULENE	0,20
52,3	Cis-PIPERITOL	0,02
52,8	CARVOTANACETONE	0,02
53,0	γ-MUUROLENE	0,02
53,2	α-TERPINEOL	0,13
53,6	BORNEOL	0,39
54,5	GERMACRENE D	0,02
54,9	ACETATE DE NERYLE	0,03
55,0	β-BISABOLENE	0,44
56,1	CARVONE	0,05
56,2	Trans-PIPERITOL	0,02
57,2	δ-CADINENE	0,04

57,7	β-SESQUIPHELLANDRENE	0,02
61,4	Trans-ANETHOLE	0,24
62,1	p-CYMENE-8-OL	0,05
63,8	ACETATE DE CARVACRYLE	0,05
67,2	METHYL CARVACROL	0,03
70,7	OXYDE DE CARYOPHYLLENE	0,28
75,9	CUMINOL	0,03
77,4	SPATHULENOL	0,02
78,8	ISOTHYMOL	0,03
79,4	EUGENOL	0,07
79,8	THYMOL	5,14
80,7	ISOCARVACROL	0,03
81,5	CARVACROL	55,82
84,9	COMPOSÉ PHÉNOLIQUE	0,11
85,6	COMPOSÉ PHÉNOLIQUE	0,03
	TOTAL	99,99

Table 2

Groups	N	Doses	Sex	PCE/Total	PCE/NCE	% MN
Negative Control	5	-	Male	0.49±0.02	0.98±0.07	0.34±0.10
	5		Female	0.50±0.02	1.00±0.10	0.23±0.06
Positive Control (EMS)	3	200 mg/Kg	Male	0.31±0.03**	0.45 ±0.07**	8.17±1.33**
	3		Female	0.31±0.03**	0.46±0.06**	7.55±0.20**
Oregano essential oil	5	50 mg/Kg	Male	0.50±0.01	0.99±0.04	0.33±0.13
	5		Female	0.49±0.03	0.98±0.13	0.21±0.11
	5	100 mg/kg	Male	0.45±0.03*	0.83±0.09*	0.27±0.20
	5		Female	0.47±0.04	0.88±0.15	0.28±0.18
	5	200 mg/kg	Male	0.41±0.02***	0.75±0.12**	0.35±0.21
	5		Female	0.40±0.02**	0.68±0.06**	0.24±0.07

Figure 1

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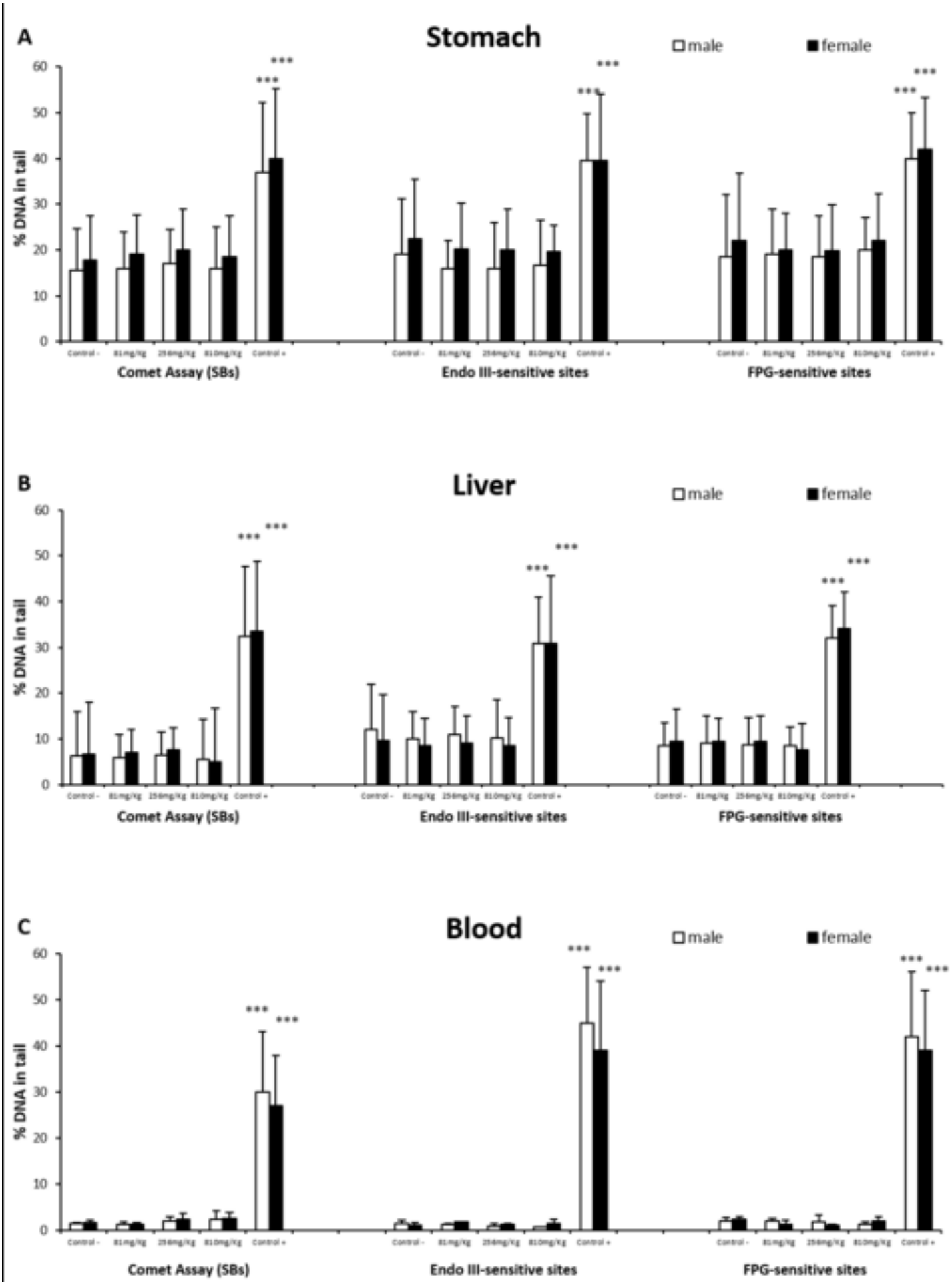

CAPÍTULO 11 / CHAPTER 11

María Llana Ruiz-Cabello, Silvia Pichardo, Alberto Baños, Cristina Nuñez, José M. Bermúdez, Enrique Guillamón, Susana Aucejo, Ana M. Cameán

CHARACTERISATION AND EVALUATION OF PLA FILMS CONTAINING AN EXTRACT OF ALLIUM SPP. TO BE USED IN THE PACKAGING OF READY-TO-EAT SALADS UNDER CONTROLLED ATMOSPHERES.

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Characterisation and evaluation of PLA films containing an extract of *Allium* spp. to be used in the packaging of ready-to-eat salads under controlled atmospheres

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ABSTRACT

New food packaging systems are been developed by food industry. The present work proposes the use of polylactic acid (PLA) containing different percentages of an *Allium* spp. extract (2%, 5%, and 6.5% Proallium®) to be used in the packaging of ready-to-eat salads. Mechanical and optical properties of PLA films studied did not show remarkable changes when the active substance was incorporated. Additionally, no significant antioxidant activity was observed, although remarkable antimicrobial activity was recorded, mainly in films containing 5% and 6.5% of the *Allium* spp. extract. Moreover, the antimicrobial effect *in vivo* was observed in lettuce. All developed films decreased enterobacterial growth in a concentration-dependent manner. Regarding aerobic bacteria, film with the highest concentration of active agent (6.5%) was found to be effective up to 5 days of storage, and even 7 days for moulds. Therefore, the PLA-Proallium® film could be a promising antimicrobial active material, especially for ready-to-eat salads.

1. Introduction

Currently, changes in lifestyle have led to the development of new foods such as ready-to-eat meals. Moreover, due to concerns about the environmental and health, consumers are avoiding the use of petroleum-based conventional packaging, which takes hundreds of years to decompose, and food products containing synthetic additives or preservatives (Debiagi, Kobayashi, Nakazato, Panagio, & Mali, 2014). Consumers demand minimally processed and preservative-free food products (Ramos, Jiménez, Peltzer, & Garrigós, 2012). To preserve food quality and the freshness of these products, it is necessary to identify optimal materials, natural preservatives and innovative packaging technologies (Llana-Ruiz-Cabello, Pichardo, et al., 2015; Souza, Goto, Mainardi, Coelho, & Tadini, 2013).

Biodegradable active packaging containing natural extracts derived from plants with antioxidant or antimicrobial properties is one alternative strategy that can be considered by the food packing industry to reduce the use of environmentally harmful synthetic polymers (Song, Lee, Al Mijan, & Song, 2014). An interesting aliphatic polyester polymer is polylactic acid (PLA), which is made primarily from renewable agricultural resources (corn) following the fermentation of starch and condensation of lactic acid (Del Novile et al., 2009). Moreover, considering that PLA is classified as GRAS (Generally Recognized As Safe, GRAS) by the American Food and Drug Administration (FDA) and is authorised by the European Commission (Commission Regulation No 10/2011), this polymer might be used in contact with food (Sébastien, Stéphane, Copinet, & Coma, 2006). For these reasons, PLA is an excellent candidate for producing a commercial compostable packaging material.

These materials can be used to control the deterioration of perishable food products, to maintain food quality and to extend the shelf life of foods. In this regard, vegetables are perishable products, and their safety and organoleptic properties may be altered during storage. These alterations may be due to different reactions such as microbial spoilage or oxidation processes that decrease food value or produce unsafe food (Sanchez-Silva et al., 2014). PLA incorporated with different substances such us nisin (Jin & Zhang, 2008), silver nanoparticles (Fortunati et al., 2012),

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alpha-tocopherol, BHT or polyethylene glycol (Byun, Kim, & Whiteside, 2010) have been developed in order to improve antimicrobial or antioxidant properties of food packaging with the aim of increasing the length of time that perishable products remain useful. However, the incorporation of natural extracts is less widespread. Film casting method is frequently used to incorporate natural extracts into PLA; however, this method it is normally not feasible for commercial film production (Erdohan, Cam, Turhan, 2013). In the present study, a commercial product based on *Allium* extract (Proallium–SO–DMC®) was incorporated by extrusion into PLA to develop an active food packaging with the aim of improving the shelf life of ready-to-eat salads. Proallium® is a condiment based on vegetal extracts that is composed of organosulfur compounds, which are characteristic of the plant genus *Allium* spp. Propyl thiosulphinate oxide (PTSO) is one of these organosulfur compounds obtained by the decomposition of initial components present in *Allium* spp. and is incorporated in inert commercial food-grade support (cyclodextrin) to produce Proallium® (Llana-Ruiz-Cabello, Gutiérrez-Praena, Puerto, Pichardo, Moreno, et al., 2015).

The antioxidant and antimicrobial effects of the essential oil from *Allium cepa*. have been studied, suggesting that it may be a new potential source as natural antimicrobial and antioxidant agents applied in food systems (Ye, Dai, & Hu, 2013). In this regard, the antibacterial properties of PTSO (Llana-Ruiz-Cabello, Gutiérrez-Praena, Puerto, Pichardo, Moreno, et al., 2015; Peinado, Ruiz, Echávarri, & Rubio, 2012) as well as Proallium® (Ruiz, García, Lara, & Rubio, 2010) have been previously studied.

The present work aimed to develop a new film based on PLA containing Proallium® to be used for packaging ready-to-eat salads. The antioxidant and antimicrobial properties as well as the ability of these materials to improve the shelf life of ready-to-eat salads were studied. In addition, the effects of this *Allium* extract on the mechanical and optical properties of PLA films were also evaluated.

2. Materials and methods

2.1. Supplies and chemicals

The polymer used in this work was polylactic acid (PLA) extrusion-grade (2003D) purchased in pellets from NatureWorks LLC (Minnetonka, USA). Polybutylene succinate (PBS) GS Pla™ FD92WD was purchased from Mitshubishi Chemical Corporation (Tokyo, Japan).

The commercial product based on *Allium* extract, Proallium–SO–DMC®, with 14.5% of PTSO (Llana-Ruiz-Cabello, Gutiérrez-Praena, Puerto, Pichardo, Moreno, et al., 2015) as an active agent, and PTSO (95% purity) were provided by Domca S.L. (Alhendin, Granada, Spain).

Chemicals for the different assays were purchased from Sigma–Aldrich (Spain) and VWR International Eurolab (Spain). Iceberg salad for the *in vivo* assays was purchase in a local market.

2.2. Film preparation

The different active PLA:GSPLA films were obtained by melt blending in a twin-screw extruder (DSE 20-40D, Brabender, Germany). Temperature was set at 200–205 °C and the screw speed was 70 min^{-1}. Proallium® was introduced in the mixer once the polymer was already in the melt state. Different concentrations (2%, 5% and 6.5% w/w) of Proallium® were fed into the barrel through the lateral liquid port at L/D 10 to reduce possible volatility and degradation losses. In the control films, Proallium® was not added. The average width and thickness of the films were approximately 10 cm and 80 μm, respectively. All developed films were stored at −20 °C and darkness before their characterisation.

2.3. Packaging and storage

Several packages were produced containing 200 g of iceberg salad packed in contact with different PLA films (PLA and PLA containing 2, 5% and 6.5% Proallium®). 4 pieces of PLA film 16 × 6 cm each were placed into commercial bags of polypropylene (23 × 19 cm) yielding a ratio of 1.9 cm^2 of film per g of salad, this is 380 cm^2 of film. These commercial bags were made of oriented polypropylene film which has the following characteristics (30 mm thickness, 1300 cm^3 m^{-2} day^{-1} of oxygen transmission rate and 6 g m^{-2} day^{-1} of water vapour transmission rate and antimist treatment). Then, the bags were heat sealed with an initial modified atmosphere composed by 10% O_2 − 10% CO_2 − 80% N_2. Sample bags of iceberg salad were stored at 4 °C for 7 days, simulating commercial conditions of production, transport and commercialisation.

2.4. Quantification of the overall contents of Proallium® incorporated into PLA films

Thermogravimetric analysis (TGA) measures the loss of mass of a given sample as a function of the temperature. In this sense, it can be applied to evaluate the overall content of the compounds from Proallium® incorporated into the PLA films.

TGA of the packaging materials was performed in a thermal analyser Q5000IR (TA Instruments). The procedure was as follows: approximately 6 mg samples were weighed, placed in a pan and heated from room temperature to 900 °C at a heating rate of 20 °C min^{-1} under an inert nitrogen atmosphere (flow rate 50 mL min^{-1}), and then kept in isothermal conditions for 1 min. Three different samples were analysed for each material.

2.5. Quantification of the propyl thiosulphinate oxide (PTSO) incorporated into PLA films

PTSO, the main organosulfur compound of Proallium®, was extracted from the PLA films by immersing the films in isopropanol in the following manner: 0.5 g of each PLA film was accurately weighed and extracted with 10 mL of isopropanol for 24 h with constant shaking at 100 min^{-1}. Then, the solvent extract was recovered and filtered through a Teflon filter of 0.22 microns.

Analyses were performed by a Thermo Finnigan gas chromatograph (GC) equipped with a DB-5MS column (Agilent Technologies, USA) (30 m, 0.25 mm, film thickness 0.25 μm) and a mass spectrometry detector (MS). Helium was used as the carrier gas with a flow rate of 1 ml min^{-1}. The oven temperature was programmed to start at 40 °C (held for 4 min), increase to 250 °C at 15 °C min^{-1} and then to hold at 250 °C for 2 min. The injector and auxiliary temperatures were 250 °C. Injections were performed in split mode with a 1:10 ratio. MS detection was performed in the mass range of 50–450 atomic mass unit (amu) at 4 scans s^{-1}. The solvent delay was 6 min.

Each PLA film was extracted and analysed in triplicate. The results are expressed in terms of the g PTSO per 100 g of PLA film.

Stock solutions of PTSO were prepared by weighting 25 mg of PTSO (95% purity) and dissolving it in 25 mL of methanol. Calibration solutions in the concentration range from 20 to 100 mg L^{-1} were obtained from the stock solutions by the adequate dilution with methanol. Calibration curves showed adequate linearity (coefficient of determination, $R^2 > 0.995$).

Positive identification of PTSO in the samples was obtained when the retention time and mass spectra of the peak obtained matched with those obtained for the PTSO calibration solution at

20 mg L^{-1}. PTSO retention time was 13.4 min while PTSO mass spectra showed the following mass/charge fragments and relative abundancies (in percentage); 76 (100%), 118 (15%), 89 (7.5%) and 182 (5%).

2.6. Physical properties

2.6.1. Light transmission and film transparency

The barrier properties of films against ultraviolet (UV) and visible light were measured at selected wavelengths between 200 and 800 nm, using a Jasco V-630 UV–Visible recording spectrophotometer (Madrid, Spain) according to the method described by Jongjareonrak, Benjakul, Visessanguan, and Tanaka (2008). The transparency of the films was calculated according to Han and Floros (1997), as follows:

Transparency values = $(-\log T_{600})/x$; where T_{600} is the fractional transmittance at 600 nm; and x: is the film thickness in mm.

2.6.2. Thickness

Film thickness was measured in at least six different locations on each film using a digital micrometre Mitutoyo 547-400 Absolute (Kawasaki, Japan). The results are expressed as the median ± standard deviation.

2.7. Mechanical properties

The tensile properties of the films were determined at room temperature using an M350-20CT Universal Testing Machine (Testometric, Rochdale, England) according to UNE-EN-ISO 527-1, with slight modifications.

The mechanical properties of the films were studied by characterising the tensile strength (TS), elongation at break (EAB) and elastic modulus (E). TS is expressed as the maximum force at break per initial cross-sectional area of the film and the elongation as a percentage of the original length; this is the maximum tensile stress that the film can sustain. EAB is the maximum change in length of a test specimen before breaking, while E is a measure of the stiffness of the film.

2.8. Thermal analysis

The glass transition temperature (Tg), crystallisation temperature (Tc), and melting temperature (Tm) of the different PLA films were measured using a Differential Scanning Calorimeter DSC Q2000 (TA Instruments). Specimens weighing 6 mg were heated at the rate of 10 °C min^{-1} from 20 °C to 250 °C. Then, the samples were cooled at 10 °C min^{-1} from 250 °C to −50 °C. Finally, the samples were heated again at 10 °C min^{-1} up to 250 °C.

2.9. Antioxidant activity of the films

Film portions were weighed (0.5 g) and covered with 10 mL of methanol and then sealed and shaken for 48 h at 25 °C. The extract solution was then filtered, and the supernatant was used for testing the films' antioxidant capacities. The radical scavenging activity was measured using two different radicals, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic) acid (ABTS). The DPPH and ABTS assays are based on the ability of the tested substance to scavenge radical cations. Both assays were performed according to Llana-Ruiz-Cabello, Gutiérrez-Praena, Puerto, Pichardo, Jos, et al. (2015).

2.10. Antimicrobial activity

To evaluate the *in vitro* antimicrobial properties of active films containing Proallium®, a total of 13 strains including foodborne spoilage and pathogenic bacteria were assayed in this study. Four Gram-negative, 9 Gram-positive bacteria and 5 moulds and yeasts were selected due to their relevance in the food industry. Most of the microorganisms were purchased from the Spanish Culture Collection (CECT) (Valencia, Spain). Moreover, the antimicrobial activity of the film was evaluated in salad packaged with active films testing total aerobic bacteria, enterobacteria, yeasts and moulds.

2.10.1. Antimicrobial activity of the films

The antimicrobial activity of the films was evaluated using the disc diffusion method and dilution method. For both assays, bacteria, moulds and yeasts were grown on commercial plate count agar provided by Gibco (Paisley, United Kingdom). When the growth was optimal, they were suspended in sterile saline solution and standardized to 0.5 McFarland density (10^8 colony-forming-units/mL (CFU mL^{-1}) to achieve confluent growth. Thereafter, for the disc diffusion procedure, the plates were spread with 0.1 mL of microbial suspension (10^8 CFU mL^{-1}) using a Digralsky spreader. Then, films with and without Proallium® (the control), were aseptically cut into a disc shape of 10 mm diameter and placed on previously seeded plates. The plates were incubated at 37 °C for 24–48 h for bacteria and at 25 °C for 48–72 h for moulds and yeasts. The diameter of the growth inhibition zone around the discs was measured using a metric ruler.

To evaluate the antimicrobial activity of the active films against different strains in liquid media, different tubes containing 6 mL of sterile saline solution with 10 strips (1 cm × 5 cm) of active or control films were uniformly inoculated with 1 mL of microbial suspensions and incubated at room temperature for 3 days. After 0, 1 and 3 days, the tubes were cultured on a microorganism-specific solid agar. Plate Count Agar was used for the bacterial counting and Sabouraud Cloranfenicol Agar was employed for moulds and yeasts. The plates were incubated at 37 °C for 24–48 h for bacteria or at 25 °C for 72 h for moulds and yeast under aerobic or anaerobic conditions depending on the microorganism, and then microbial colonies were counted to evaluate the growth of the strains. The microbiological data were transformed into the logarithm of the number of colony forming units (CFU mL^{-1}). All determinations were performed in triplicate.

2.10.2. Antimicrobial activity in salad

To evaluate the antimicrobial activity of the films in packaged salads 1, 3, 5 and 7 days after packaging, the ISO 4833-2:2014 guidelines with modifications were performed. Three samples per treatment (PLA and PLA containing 2, 5, 6.5% Proallium®) were developed, and the original microbial counts of each iceberg salad packaged in contact with different PLA films were measured. Samples of precut ready-to-eat packaged salad (25 g) were diluted in 225 mL of peptone water (Merck-Spain, Madrid, Spain) and homogenised in a masticator blended (IUL, Barcelona, Spain) for 2 min. Different dilutions were made in sterile saline solution, and 0.1 mL of each dilution was seeded in plates with a Digralski spreader.

Different media (BD™ Difco™, Madrid, Spain) and culture conditions were used for each microorganism group: a) Plate Count Agar for mesophilic aerobic bacteria, incubated at 30 °C for 24 h; b) McConkey Agar for enterobacteria, incubated aerobically at 37 °C for 24 h; c) Sabouraud Cloranfenicol Agar for yeast, incubated for 24–48 h at 30 °C; and d) Rose Bengal Agar for moulds, incubated at 25 °C for 3–5 days. The counts were carried out in a colony counter (Suntex 570 Colony Counter). The results are expressed as the log CFU g^{-1} ± SD (n = 3).

2.11. Statistical analysis

Experiments were performed in triplicate. Data were presented as means ± standard deviation and the differences between groups were considered significant at $p < 0.05$. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between factors and levels. Comparison of the means was done by Dunnet test. The statistical analysis of the data was performed using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

3. Results and discussion

3.1. Content of Proallium® and PTSO incorporated in the films

The TGA curves obtained for Proallium®, PLA and PLA containing different concentrations of Proallium® are depicted in Fig. 1.

The TGA curve for Proallium® showed a first weight loss of around 80% at temperatures up to 150 °C and a second one (the remaining 20%) at temperature above 350 °C. The TGA curve for PLA film showed just one weight loss in the range 350 °C–450 °C. Considering these values, the weight losses obtained in the TGA curves for the different PLA films with Proallium® can be clearly identified. In this sense, the first weight loss at temperatures of approximately 150 °C was related to Proallium® while the second step at 350 °C mainly corresponded to the thermal degradation of the PLA polymer. Therefore, the TGA thermograms confirmed the presence of Proallium® in the polymeric matrix after the extrusion process. The concentrations of Proallium® were calculated from the weight losses observed in Fig. 1 at 150 °C divided by 80% (weigh loss of Proallium® at 150 °C) which gave as results approximately 1.3%, 4.4% and 5.1% w/w for formulations with a nominal amount of 2%, 5% and 6.5% w/w, respectively. Therefore, there was a direct relationship between the nominal content of Proallium® (i.e., the amount introduced into the extruder) and the actual content quantified in the films by TGA. Several authors reported that the losses of essential oils observed were due to volatility during the development of the active packaging systems (Altiok, Altiok, & Tihminlioglu, 2010; Ramos et al., 2012). Similarly, in the present work, the results obtained in the TGA analyses revealed lower quantities of Proallium® than those added in the production process, probably due to the volatility of its components as well.

In addition, GC/MS analysis performed to analyse PTSO clearly revealed the presence of this active agent in all of the different films developed. The content of PTSO was correlated with the amount of Proallium® incorporated. These contents were approximately 0.3%, 1.8% and 2.0% w/w for formulations with a nominal amount of Proallium® of 2%, 5% and 6.5% w/w, respectively.

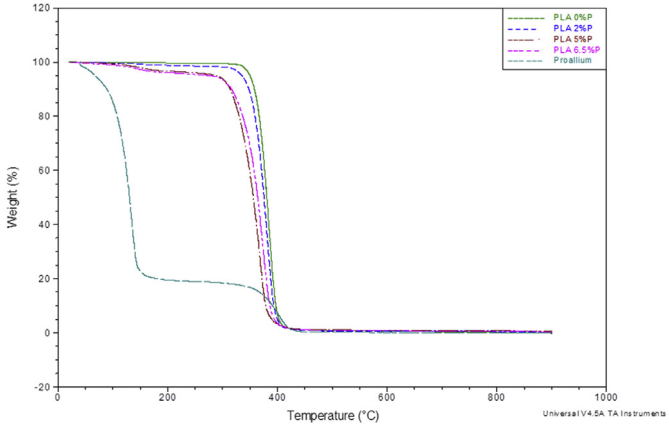

Fig. 1. TGA curves obtained for PLA and formulations with Proallium®.

3.2. Physical properties

The transmission of UV and visible light at selected wavelengths in the range of 200–800 nm by PLA films incorporated with and without Proallium® is presented in Table 1. Proallium® was able to impede light transmission through the film, as demonstrated by the increase in the transparency values ($p < 0.05$) with the increasing concentration of the active agent up to 5%, although a decrease was observed at 6.5% of Proallium®. Transparent films are typically desirable with higher applicability and acceptability in food packaging systems (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Pillin, Montrelay, & Grohens, 2006). Despite our results showing significant decreases in the transparency of the film containing Proallium®, no visual differences with the control film were discernible. Consequently, no effect on their acceptability for the consumer is expected.

3.3. Mechanical properties

Table 2 shows the mechanical properties of the different films. Based on the E and EAB values, PLA films with no additives incorporated can be mechanically described as stiff and brittle, respectively. In addition, the decreased observed in TS when Proallium® was incorporated was significant ($p < 0.05$) in the 5 and 6.5% of Proallium® groups. The reduction in the elongation and elastic modulus observed were related to the concentration of Proallium®. For instance, a decrease of 26.66–40% was found for the films with the highest contents of Proallium®. This means that PLA films with Proallium® were less stiff and more fragile, although PLA films are quite fragile themselves. Similar effects on the mechanical properties have been observed in PLA materials incorporating different antioxidants (ascorbyl palmitate, α-tocopherol and butylated hydroxytoluene) (Byun et al. 2010; Jamshidian et al., 2012). In addition, essential oils are usually liquid at room temperature, and their presence in the film structure in the form of oil droplets can easily be deformed, enhancing film flexibility (Ahmad et al., 2012).

The thickness of active films decreased significantly in comparison with the control ($p < 0.01$). This significant diminution was more noticeable at the highest concentration of Proallium®. This observation was related to a higher fluidity of the PLA and Proallium® mixtures, which had a tendency of melting to generate a non-homogeneous thickness profile along the width of the films (transverse machine direction). However, Erdohan et al. (2013) reported no significant differences in the thickness of PLA when incorporating olive leaf extract.

3.4. Thermal analysis

From the DSC thermograms, three thermal parameters were determined (Tg, Tc and Tm), which are reported in Table 3.

PLA films with no additives exhibited a Tg of approximately 50 °C and a Tc of approximately 93 °C. Two melting temperatures were observed at values of approximately 140 °C and 150 °C, which correspond to the GSPLA additive and PLA, respectively. The addition of Proallium® to the PLA films decreased the Tg, Tc and Tm. In this sense, the addition of Proallium® at nominal contents of 2% provoked a lower modification of the thermal properties (around 10 °C, 5 °C, 5 °C and 1.5 °C less than PLA with no additives for Tg, Tc, Tm1 and Tm2, respectively) than the addition of contents above 5% which provoked a significant decrease ($p < 0.05$) in the Tg, Tc and Tm (around 30 °C, 25 °C, 23 °C and 10 °C less than PLA with no additives for Tg, Tc, Tm1 and Tm2, respectively). In addition,

Table 1
Light transmittance (%) and transparency values of PLA films incorporated with Proallium® at various concentrations.

Film sample	Wavelength (nm)								Transparency values[a]
	200	280	350	400	500	600	700	800	
Control	0.41	63.90	77.62	81.85	85.22	86.84	87.74	88.31	0.59 ± 0.15
2% P®	0.00	64.07	78.66	82.92	85.87	87.43	88.51	89.28	0.77 ± 0.11
5% P®	0.00	37.61	63.46	69.84	77.43	81.30	83.83	85.60	1.11 ± 0.17*
6.5% P®	0.00	50.89	68.62	73.40	80.05	83.28	85.26	86.65	1.25 ± 0.19*

*Significant differences in comparison with control ($p < 0.01$). P®: Proallium–SO–DMC®.
[a] Values are given as the mean ± SD from triplicate determinations.

Table 2
Mechanical properties of the films tested.

Sample	TS (Mpa)	EAB (%)	*E* (Gpa)	Thickness (mm)
PLA	65 ± 7	3.1 ± 0.2	3.0 ± 0.3	0.105 ± 0.002
PLA 2% Proallium®	60 ± 4	2.6 ± 0.2*	2.65 ± 0.14*	0.080 ± 0.008*
PLA 5% Proallium®	33 ± 6*	2.1 ± 0.3*	1.8 ± 0.4*	0.085 ± 0.007*
PLA 6.5% Proallium®	38 ± 8*	1.8 ± 0.7*	2.2 ± 0.2*	0.072 ± 0.010*

Values are given as the mean ± SD. TS: Tensile strength. EAB: Elongation at break. E: Young's module. Reported values are the means of at least 6 replicates ± standard deviation.
*Significant difference in comparison with control ($p < 0.01$).

Table 3
Thermal properties of the films tested (2nd heating cycle).

Sample	Tg (°C)	Tc (°C)	Tm1 (°C)	Tm2 (°C)
PLA	49.2 ± 0.7	93.7 ± 0.5	141.7 ± 0.5	152.7 ± 0.3
PLA 2% Proallium®	39.2 ± 0.5*	88.3 ± 0.3*	136.9 ± 0.7*	152.3 ± 0.4
PLA 5% Proallium®	21.2 ± 0.6*	68.7 ± 0.1*	118.8 ± 1.6*	142.2 ± 0.1*
PLA 6.5% Proallium®	22.9 ± 0.8*	76.0 ± 0.3*	117.4 ± 1.0*	138.5 ± 0.5*

Tg: Glass transition temperature. Tc: Crystallisation temperature. Tm: Melting temperature.
*Significant difference in comparison with control ($p < 0.01$).

Jamshidian et al. (2012) reported a decrease in the Tm and Tc with the addition of 2% α-tocopherol to PLA, which correspond to the observed effects after the addition of plastiziers.

3.5. Antioxidant and antimicrobial activities

3.5.1. Antioxidant activities

The antioxidant activity of the developed films was measured by both DPPH and ABTS radical scavenging methods. The methanol extract of films containing Proallium®, as well as the control films, did not show any scavenging activity in both assays, being the inhibition percentages lower than 5% in both assays. However, while antioxidant activity is expected in an *Allium* extract because it contains a high amount of organosulfur compounds and has been shown to exert antioxidant effects in fish protein films (Teixeira et al., 2014), our results indicate the absence of this effect. In this sense, Benkeblia and Lanzotti (2007) have stated that it is equivocal to attribute antioxidant properties to the thiosulfinates in crude *Allium* preparations, simply because they are present. Villa-Rodriguez, Palafox-Carlos, Yahia, Ayala-Zavala, and Gonzalez-Aguilar (2015) reported that the degradation of antioxidant agents present in fruit and vegetables can affect negatively the organoleptical properties of food. Thus, antioxidant active packaging show positive and promising results to protect the stability of these substances and ensure fruit and vegetables antioxidant potential. In this regard, Gómez-Estaca, López-De-Castillo, Hernández-Muñoz, Catalá, and Gavara (2014) concluded that the development of antioxidant active packaging needs to be optimized before these materials will be implemented by the food industry.

3.5.2. Antimicrobial assays

3.5.2.1. Antimicrobial activity in films. In the diffusion assay no inhibition zones were observed for both the control and active films for *Fusarium* spp., *Alternaria* spp., *Penicilium digitatum* and *Zygosaccharomices bailii*.

Although no antimicrobial activity could be observed in the diffusion assay for any active film, the dilution method clearly evidenced that films containing 5% and 6.5% of Proallium® were able to significantly reduce the growth of all organisms used from the first day of contact. As observed in Table 4, the dilution method used to quantify the bacterial reduction caused by films showed decreases in microbial growth for all of the strains assayed when films containing 5% and 6.5% of Proallium® were used. These decreases were extremely significant for all microorganisms in contact with these concentrations of the active film from the first day. However, the growth of *Staphylococcus aureus* only decreased significantly in the case of 6.5% Proallium® on the first day.

Teixeira et al. (2014) were only able to detect inhibition of *Listeria monocytogenes* in fish protein films containing garlic essential oil by the agar diffusion method, whereas the dilution method revealed inhibition of most of the microorganisms assayed. These findings could be due to the larger film sample used in the latter method in comparison with the diffusion assay. In this regard, Pires et al. (2013) also observed higher inhibition rates in the dilution method than in the agar diffusion method in fish protein films containing citronella, coriander, tarragon or thyme oils. They reported that the limited diffusion of the essential oil active agents through agar medium could explain these results. Therefore, in our opinion, complementary studies to the diffusion assay should be carried out to confirm antimicrobial activity.

The usefulness of *Allium sativum* essential oil as an antimicrobial agent for food packaging plastic film was previously studied in low-density-polyethylene/ethylene-vinyl-acetate (LDPE/EVA) copolymer film (Sung et al., 2014). The authors presented negative results in the agar disk diffusion assay for films with 2–6% garlic essential oil, although the concentration of 8% significantly reduced the concentration of bacteria (*L. monocytogenes*, *B. thermosphacta* and *Escherichia coli*). Similarly, other studies carried out using this method reported different results in films with a plastic matrix containing other essential oils. In this regard, Debiagi et al. (2014) reported no change in the inhibition zones against *S. aureus* and *E. coli* during 15 days of contact with cassava bagasse and polyvinyl alcohol film incorporated with clove and oregano essential oils. In contrast, PLA-containing olive leaf extract significantly increased the inhibition zone in *S. aureus* in a concentration-dependent way (Erdohan et al., 2013). Ramos et al. (2012) observed antibacterial activity of polypropylene with 8% carvacrol and thymol in *S. aureus*, but negative results were shown in *E. coli*.

Results from different works showed that active packaging containing natural extracts are effective against microbial spoilage. However, Patrignani, Siroli, Serrazanetti, Gardini, and Lanciotti (2015) reported that the application of essential oils in real systems is still limited probably due to their strong impact and

Table 4
Antimicrobial activity of the films containing Proallium®.

Microorganisms/Film	Day 1 (log)	Day 3 (log)
Staphylococcus aureus		
Control	5.67 ± 0.18	5.65 ± 0.12
Proallium® 2%	5.61 ± 0.42	5.16 ± 0.26*
Proallium® 5%	5.41 ± 0.31	3.00 ± 0.20**
Proallium® 6.5%	4.90 ± 0.21*	2.62 ± 0.20**
Yersinia enterocolitica		
Control	4.92 ± 0.19	4.91 ± 0.14
Proallium® 2%	4.88 ± 0.26	4.84 ± 0.26
Proallium® 5%	3.00 ± 0.15**	1.45 ± 0.23**
Proallium® 6.5%	2.70 ± 0.14**	1.00 ± 0.14**
Listeria monocytogenes		
Control	5.81 ± 0.21	5.81 ± 0.31
Proallium® 2%	5.79 ± 0.3	5.79 ± 0.16
Proallium® 5%	2.51 ± 0.14**	1.00 ± 0.31**
Proallium® 6.5%	1.70 ± 0.20**	1.00 ± 0.19**
Enterococcus faecalis		
Control	6.58 ± 0.13	6.57 ± 0.16
Proallium® 2%	6.56 ± 0.3	6.49 ± 0.27
Proallium® 5%	3.98 ± 0.14**	3.00 ± 0.23**
Proallium® 6.5%	3.18 ± 0.20**	2.36 ± 0.14**
Salmonella enterica		
Control	4.79 ± 0.16	4.78 ± 0.31
Proallium® 2%	4.76 ± 0.20	4.76 ± 0.37
Proallium® 5%	1.70 ± 0.16**	1.30 ± 0.26**
Proallium® 6.5%	1.70 ± 0.12**	1.00 ± 0.32**
Staphylococcus carnosus		
Control	5.96 ± 0.31	5.95 ± 0.16
Proallium® 2%	5.96 ± 0.23	5.94 ± 0.20
Proallium® 5%	3.48 ± 0.15**	2.13 ± 0.16**
Proallium® 6.5%	3.00 ± 0.30**	1.00 ± 0.12**
Escherichia coli O157:H7		
Control	4.57 ± 0.23	4.56 ± 0.32
Proallium® 2%	4.56 ± 0.13	4.55 ± 0.16
Proallium® 5%	3.08 ± 0.28**	1.45 ± 0.13**
Proallium® 6.5%	2.98 ± 0.21**	1.00 ± 0.26**
Listeria ser. 1/2a		
Control	5.54 ± 0.13	5.54 ± 0.48
Proallium® 2%	5.53 ± 0.37	5.46 ± 0.31
Proallium® 5%	2.70 ± 0.26**	1.48 ± 0.26**
Proallium® 6.5%	2.70 ± 0.12**	1.00 ± 0.15**
Campylobacter jejuni		
Control	4.18 ± 0.23	4.15 ± 0.16
Proallium® 2%	4.11 ± 0.25	4.00 ± 0.31
Proallium® 5%	3.15 ± 0.15**	2.08 ± 0.13**
Proallium® 6.5%	3.02 ± 0.12**	1.45 ± 0.26**
Clostridium perfringens		
Control	4.68 ± 0.31	4.68 ± 0.42
Proallium® 2%	4.68 ± 0.23	4.67 ± 0.23
Proallium® 5%	2.70 ± 0.19**	1.30 ± 0.36**
Proallium® 6.5%	1.70 ± 0.25**	1.00 ± 0.27**
Zygosaccharomyces bailii		
Control	4.91 ± 0.31	4.90 ± 0.30
Proallium® 2%	4.86 ± 0.19	4.83 ± 0.23
Proallium® 5%	2.70 ± 0.42**	1.64 ± 0.37**
Proallium® 6.5%	2.70 ± 0.31**	1.00 ± 0.26**
Candida humicola		
Control	5.64 ± 0.19	5.64 ± 0.32
Proallium® 2%	5.62 ± 0.23	5.61 ± 0.16
Proallium® 5%	2.08 ± 0.37**	1.70 ± 0.23**
Proallium® 6.5%	1.70 ± 0.27**	1.70 ± 0.25**
Fusarium oxysporum		
Control	4.49 ± 0.15	4.49 ± 0.26
Proallium® 2%	4.46 ± 0.31	4.47 ± 0.23
Proallium® 5%	3.08 ± 0.26**	1.78 ± 0.37**
Proallium® 6.5%	2.00 ± 0.15**	1.00 ± 0.19**
Penicillium expansum		
Control	4.51 ± 0.15	4.51 ± 0.42
Proallium® 2%	4.47 ± 0.11	4.45 ± 0.19
Proallium® 5%	2.70 ± 0.36**	1.00 ± 0.31**
Proallium® 6.5%	2.48 ± 0.27**	1.00 ± 0.19**

Values are given as the mean ± SD from 3 different experiments.
*Significantly different from control ($P < 0.05$).
**Highly significantly different from control ($P < 0.01$). The reduction of microbial counts was calculated with respect to the control.

changes they cause in food products.

3.5.2.2. Antimicrobial activity in salads. In our study, the antimicrobial activity of the active film was also assessed in the packaged salad. Changes in the microbial population of the salad during storage time are presented in Fig. 2. In general, the microbial counts had significant differences ($p < 0.05$) during storage time and by packaging treatment. The antimicrobial properties of the films

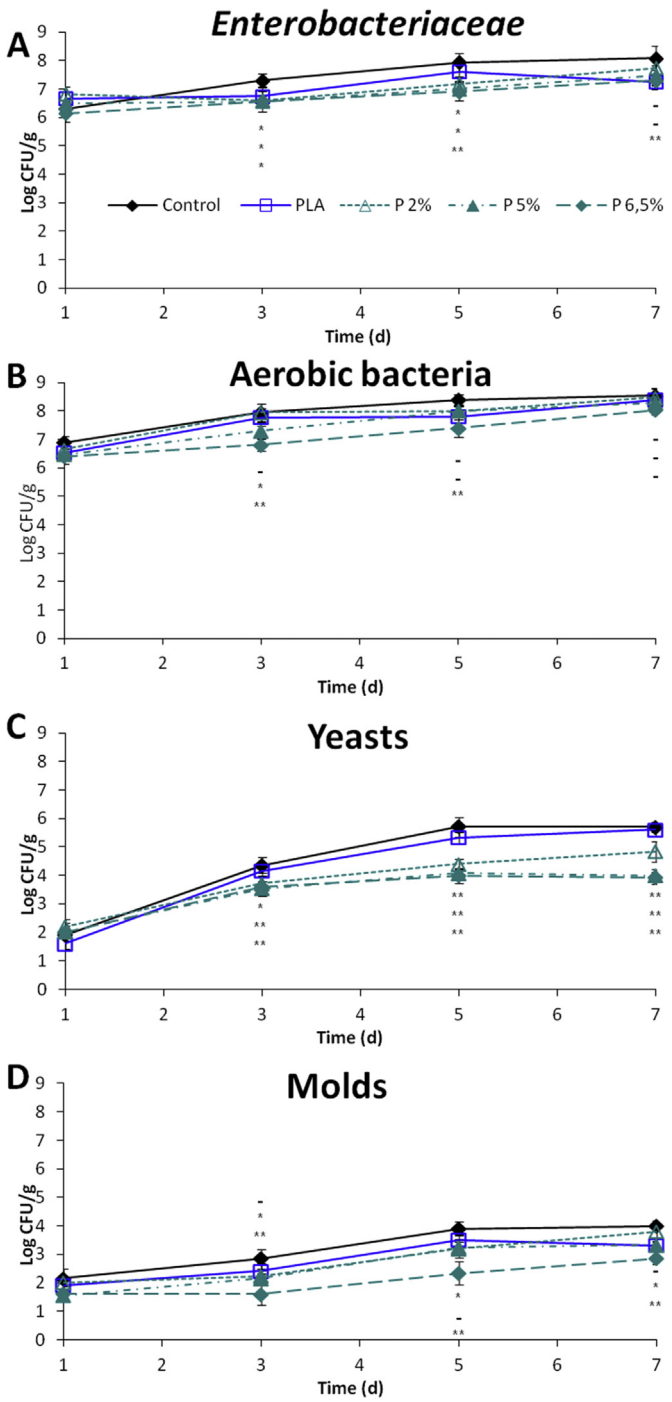

Fig. 2. Antimicrobial activity of films containing Proallium® in packaged salad by storage time for enterobacteriaceae (A), aerobic bacteria (B), yeasts (C) and moulds (D). Values are given as the mean ± SD from 3 different experiments. *significantly different from control ($P < 0.05$); **highly significantly different from control ($P < 0.01$).

incorporated with Proallium® were significantly higher than the controls. The storage Enterobacteriaceae counts increased gradually in the packaged salad at 4 °C and reached maximum levels of 8.08 log units (Fig. 2A). All developed films decreased enterobacterial growth in comparison with the control, and this reduction was related to the concentration of Proallium® in PLA. The cell densities at the end of storage were 7.74, 7.48 and 7.30 log units for PLA containing 2%, 5% and 6.5% of Proallium®, respectively. While the bacterial counts are fairly high, it is important to note that high levels of enterobacteria are common in raw vegetables, and it has been reported that they should not be used to indicate the microbiological quality of fresh vegetables (Little, Roberts, Youngs, & Louvois, 1999). Regarding aerobic bacteria, although PLA 5% ($p < 0.05$) and 6.5% ($p < 0.01$) Proallium® were able to decrease the bacterial counts after 3 days of storage, only the film with the highest concentration of active agent (6.5%) was found to be effective up to 5 days of storage (Fig. 2B). The results showed that yeasts were able to grow in the control salad at 4 °C and reached maximum levels of 5.70 log units (Fig. 2C). All samples packaged with active film had antifungal activity throughout the storage time, and this activity reached a maximum by the fifth day ($p < 0.01$). With respect to moulds, salad packaged with active films (5% and 6.5%) had lower counts on the third day compared with the control group and at 6.5% throughout the 7 day period (Fig. 2D).

Our results show significant reductions in all microbial counts in salad in contact with Proallium®. Sung et al. (2014) also observed that LDPE/EVA films containing garlic essential oil reduced the growth rate of *L. monocytogenes* on cooked beef at 4 °C. Similarly, foal meat packed in PET/PE/EVOH/PE with 2% oregano essential oil and 1% green tea extract exhibited lower counts of microorganisms (lactic acid bacteria, Enterobacteriaceae, yeast and moulds) compared to the control group from day 10 of storage onwards (Lorenzo, Battle, & Gómez, 2014). In the case of minimally processed salad, the enterobacteria counts of salad packaged in the active EVOH films (oregano essential oil and citral) had a significant decrease in comparison with the control sample, whereas no measurable effect was observed regarding storage time (Muriel-Galet et al., 2013).

4. Conclusions

The effectiveness of PLA containing 2%, 5%, and 6.5% of a natural extract of *Allium* spp. was assessed in the packaging of ready-to-eat salad. The incorporation of Proallium® on PLA films slightly improved their mechanical properties. Although no antioxidant effect was observed, the antimicrobial effectiveness was confirmed not only in the film but also in the packaged salad. In this regard, yeast and moulds were more sensitive than enterobacterial and aerobic bacteria to the antimicrobial activity of active films. The highest concentration of active agent (6.5%) was found to be effective throughout the storage time (7 days) against all microorganisms assayed except for aerobic bacteria. Therefore, the developed films, especially those containing 5% and 6.5% Proallium®, exhibit strong potential for development as active food packaging films.

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References

Ahmad, M., Benjakul, S., Prodpran, T., & Agustini, T. W. (2012). Physico-mechanical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils. *Food Hydrocolloids, 28*, 189–199.

Altiok, D., Altiok, E., & Tihminlioglu, F. (2010). Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. *Journal of materials Science: Materials in Medicine, 21*, 2227–2236.

Benkeblia, N., & Lanzotti, V. (2007). Allium thiosulfinates: chemistry, biological properties and their potential utilization in food preservation. *Food, 1*(2), 193–201.

Byun, Y., Kim, Y. T., & Whiteside, S. (2010). Characterization of an antioxidant polylactic acid (PLA) film prepared with α-tocopherol, BHT and polyethylene glycol using film cast extruder. *Journal of Food Engineering, 100*, 239–244.

Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.

Debiagi, F., Kobayashi, R. K. T., Nakazato, G., Panagio, L. A., & Mali, S. (2014). Biodegradable active packaging based on cassava bagasse, polyvinyl alcohol and essential oils. *Industrial Crops and Products, 52*, 664–670.

Del Novile, M. A., Conte, A., Buonocore, G. G., Incoronato, A. L., Massaro, A., & Panza, O. (2009). Active packaging by extrusión processing of recyclable and biodegradable polymers. *Journal of Food Engineering, 93*, 1–6.

Erdohan, Z. O., Cam, B., & Turhan, K. N. (2013). Characterization of antimicrobial polylactic acid based films. *Journal of Food Engineering, 119*, 308–315.

Fortunati, E., Armentano, I., Zhou, Q., Iannoni, A., Saino, E., Visai, I., et al. (2012). Multifuncional bionanocomposite films of poly(lactic acid), cellulose nanocrystals and silver nanoparticles. *Carbohydrate Polymers, 87*, 1596–1605.

Gómez-Estaca, J., López-De-Castillo, C., Hernández-Muñoz, P., Catalá, R., & Gavara, R. (2014). Advances in active antioxidant food packaging. *Trends in Food Science and Technology, 35*, 42–51.

Han, J. H., & Floros, J. D. (1997). Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastics Film Sheeting, 13*, 287–298.

ISO 4833–2. (2014). *Microbiology of the food chain- HORIZONTAL method for the enumeration of microorganisms- Part 2: Colony counts at 30 degrees C by the surface plating technique (ISO 4833-2:2013)*.

Jamshidian, M., Tehrany, E. A., Imran, M., Akhtar, M. J., Cleymand, F., & Desobry, S. (2012). Structural, mechanical and barrier properties of active PLA-antioxidant films. *Journal of Food Engineering, 110*, 380–389.

Jin, T., & Zhang, H. (2008). Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. *Journal of Food Science, 73*, 127–134.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., & Tanaka, M. (2008). Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and α-tocopherol. *Food Hydrocolloids, 22*, 449–458.

Little, C., Roberts, D., Youngs, E., & Louvois, J. D. (1999). Microbiological quality of retail imported unprepared whole lettuces: a PHLS Food Working Group study. *Journal of Food Protection, 62*(4), 325–328.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Jos, A., & Cameán, A. M. (2015). *In vitro* pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. *Toxicology in Vitro, 29*, 647–656.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Moreno, F. J., Baños, A., et al. (2015). .Acute toxicological studies of the main organosulfur compound derived from Allium sp. intended to be used in active food packaging. *Food and Chemical Toxicology, 82*, 1–11.

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A. I., Gutiérrez-Praena, D., et al. (2015). *In vitro* evaluation of essential oils and their main compounds used in active food packaging: a review. *Food and Chemical Toxicology, 81*, 9–27.

Lorenzo, J. M., Battle, R., & Gómez, M. (2014). Extension of the shelf-life of foal meat with two antioxidant active packaging systems. *Food Science and Technology, 59*, 181–188.

Muriel-Galet, V., Cerisuelo, J. P., López-Carballo, G., Aucejo, S., Gavara, R., & Hernández-Muñoz, P. (2013). Evaluation of EVOH-coated PP films with oregano essential oil and citral to improve the shelf-life of packaged salad. *Food Control, 30*, 137–143.

Ojagh, S. M., Rezaei, M., Razavi, S. H., & Hosseini, S. M. H. (2010). Development and avaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. *Food Chemistry, 122*, 161–166.

Patrignani, F., Siroli, L., Serrazanetti, D. I., Gardini, F., & Lanciotti, R. (2015). Innovative strategies based on the use of essential oils and their components to improve safety, shelf-life and quality of minimally processed fruits and vegetables. *Trends in Food Science and Technology*. http://dx.doi.org/10.1016/j.tifs.2015.03.009. XX, 1-12 (in press).

Peinado, M. J., Ruiz, R., Echávarri, A., & Rubio, L. A. (2012). Garlic derivative PTS-O is effective against broiler pathogens in vivo. *Poultry Science, 91*, 2148–2157.

Pillin, I., Montrelay, N., & Grohens, Y. (2006). Thermo-mechanical characterization of plasticized PLA: is the miscibility the only significant factor? *Polymer, 47*, 4676–4682.

Pires, C., Ramos, C., Teixeira, B., Batista, I., Nunes, M. L., & Marques, A. (2013). Hake proteins edible films incorporated with essential oils: physical, mechanical, antioxidant and antibacterial properties. *Food Hydrocolloids, 30*, 224–231.

Ramos, M., Jiménez, A., Peltzer, M., & Garrigós, M. C. (2012). Characterization and

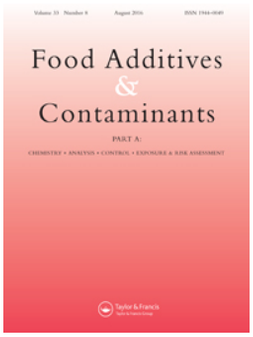
antimicrobial activity studies of polypropilene films with carvacrol and thymol for active packaging. *Journal of Food Engineering, 109*, 513–519.
Ruiz, R., García, M. P., Lara, A., & Rubio, L. A. (2010). Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. *Veterinary Microbiology, 144*, 110–117.
Sanches-Silva, A., Costa, D., Albuquerque, T. G., Buonocore, G. G., Ramos, F., Castilho, M. C., et al. (2014). Trends in the use of natural antioxidants in active food packaging: a review. *Food Additives and Contaminants: Part A, 31*(3), 374–395.
Sébastien, F., Stéphane, G., Copinet, A., & Coma, V. (2006). Novel boidegradable films made from chitosan and poly(lactic acid) with antifungal properties against mycotoxinogen strains. *Carbohydrate Polymers, 65*, 185–193.
Song, N.-B., Lee, J.-H., Al Mijan, M., & Song, K. B. (2014). Development of a chicken feather protein film containing clove oil and its application in smoked salmon packaging. *Food Science and Technology, 57*, 453–460.
Souza, A. C., Goto, G. E. O., Mainardi, J. A., Coelho, A. C. V., & Tadini, C. C. (2013). Cassava starch composite films incorporated with cinnamon essential oil: antimicrobial activity, microstructure, mechanical and barrier properties. *Food Science and Technology, 54*, 346–352.
Sung, S. Y., Sin, L. T., Tee, T. T., Bee, S. T., Rahmat, A. R., & Rahmat, W. A. W. A. (2014). Control of bacteria growth on ready-to-eat beef loaves by antimicrobial plastic packaging incorporated with garlic oil. *Food Control, 39*, 214–221.
Teixeira, B., Marques, A., Pires, C., Ramos, C., Batista, I., Saraiva, J. A., et al. (2014). Characterization of fish protein films incorporated with essential oils of clove, garlic and origanum: physical, antioxidant and antibacterial properties. *Food Science and Technology, 59*, 533–539.
Villa-Rodriguez, J. A., Palafox-Carlos, H., Yahia, E. M., Ayala-Zavala, J. F., & Gonzalez-Aguilar, G. A. (2015). Maintaining antioxidant potential of fresh fruit and vegetables after harvest. *Critical Reviews in Food Science and Nutrition, 55*, 806–822.
Ye, C.-L., Dai, D.-H., & Hu, W.-L. (2013). Antimicrobial and antioxidant activities of the essential oil from onion (Allium cepa L.). *Food Control, 30*, 48–53.

CAPÍTULO 12 / CHAPTER 12

María Llana-Ruiz-Cabello, Silvia Pichardo, José M. Bermúdez, Alberto Baños, Cristina Nuñez, Enrique Guillamón, Susana Aucejo, Ana M. Cameán

DEVELOPMENT OF PLA FILMS CONTAINING OREGANO ESSENTIAL OIL (ORIGANUM VULGARE L. VIRENS) INTENDED TO BE USE IN FOOD PACKAGING.

Food Additives and Contaminants: Part A 33, 1374-1386, ***2016***



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Development of PLA films containing oregano essential oil (*Origanum vulgare* L. *virens*) intended for use in food packaging

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ABSTRACT

Consumers' concerns about the environment and health have led to the development of new food packaging materials avoiding petroleum-based matrices and synthetic additives. The present study has developed polylactic acid (PLA) films containing different concentrations of essential oil from *Origanum vulgare* L. v*irens* (OEO). The effectiveness of this new active packaging was checked for use in ready-to-eat salads. A plasticising effect was observed when OEO was incorporated in PLA films. The rest of the mechanical and physical properties of developed films did not show much change when OEO was included in the film. An antioxidant effect was recorded only for films containing the highest percentages of the active agent (5% and 10%). In addition, films exhibited *in vitro* antibacterial activity against *Staphylococcus aureus*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Enterococcus faecalis* and *Staphylococcus carnosus*. Moreover, in ready-to-eat salads, antimicrobial activity was only observed against yeast and moulds, where 5% and 10% of OEO was the most effective.

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Polylactic acid; oregano essential oil; antioxidant; antimicrobial; active packaging

Introduction

The development of active packaging using polymers from eco-friendly sources has significantly increased in recent years due to new trends in consumer demands for 'green and organic' products (Llana-Ruiz-Cabello et al. 2014).

The special characteristics of polylactic acid (PLA) such as biodegradability and being from a bioresource (derived from corn) put PLA in a unique position for food applications (Jin & Zhang 2008; Del Nobile et al. 2009). In addition, PLA possesses GRAS status (generally recognised as safe) by the USFDA; and it is authorised by the European Commission (Commission Regulation No. 10/2011).

Active packaging containing natural additives with antimicrobial or antioxidant properties has been widely studied in order to respond to consumer concerns about the environment and health (Gutiérrez et al. 2011; Llana-Ruiz-Cabello et al. 2015a). In this regard, several plant extracts possess antibacterial, antifungal and antiviral properties that have been studied as alternative to synthetic drugs (Solórzano-Santos & Miranda-Novales 2012). Essential oils (EOs) are oily liquid secondary metabolites rich in sources of small terpenoids and phenolic compounds that appear to be responsible for much of their activities (Rodríguez-Lafuente et al. 2007). They have been also traditionally used as additives to extend the shelf-life of perishable products due to their antimicrobial or antioxidant properties (Llana-Ruiz-Cabello et al. 2015b). In this regard, Burt (2004) concluded that EOs exhibit antibacterial activity against food-borne pathogens *in vitro* and in foods, being more sensitive Gram-positive organisms. Regarding the antioxidant activity, Nerín et al. (2008) have shown the effectiveness of rosemary EO and its components included in active food packaging by increasing the shelf-life of packaged food. In this sense, Sanches-Silva et al. (2014) reviewed the antioxidant activity of natural substances included in active packaging and concluded that it is necessary to study the safety and the effectiveness of these materials in order to evaluate which food products are more suitable to be packed by each material. In this sense, *Origanum* spp., which are a wide group of plants rich in several compounds, such as monoterpenes, sesquiterpenes and phenolic compounds (Ortega-Nieblas et al. 2011), show promising benefits for active packaging technology due to the properties of

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their main compounds, carvacrol and thymol. Ramos et al. (2012) reported that direct addition of active agents from EOs into food caused undesirable flavours. Taking into account that several works have been published concerning vapour-phase inhibition (López et al. 2007), the drawback of direct addition of active substances could be avoided by the incorporation of EOs in food-packaging films that allow the controlled release of these active substances from the packaging material to the surface of the product during the whole shelf-life of the packaged foodstuff (Gutiérrez et al. 2009). Oregano essential oil (OEO) is categorised as GRAS by the USFDA (Manso et al. 2014); however, it is also important to note that at present in Europe there is no regulation concerning the quantity of OEO or any of its main compounds to be used in food packaging. The incorporation of EOs, such as OEO, into polymeric packaging materials would be an innovative technology aiming to increase the shelf-life of food products (Llana-Ruiz-Cabello et al. 2014). Several authors have been reported the usefulness of OEO contained in different biomatrices, such as queen seed mucilage (Jouki et al. 2014) or gelatine-chitosan (Wu et al. 2014), to improve the shelf-life of perishable products; however, as far as we know, there are no studies dealing with PLA containing OEO. In this regard, a previous work reported that films developed with PLA containing Proallium®, a commercial product based on *Allium* spp. extract, exhibited antimicrobial effectiveness for salad, although antioxidant activity was not observed (Llana-Ruiz-Cabello et al. 2015a). OEO has been widely studied for its antioxidant properties due to the high content of phenolic compounds (Jouki et al. 2014) as well as for its antimicrobial properties (Sivropoulou et al. 1996; Kocic-Tanackov et al. 2014). Once the active agent have been included into the polymeric matrix it is necessary to study its behaviour and ensure its activity (Becerril et al. 2007). Therefore, in the present work the effectiveness of OEO incorporated into a PLA matrix in the preservation of ready-to-eat salad was evaluated. The effect of the addition of OEO into the polymeric matrix was assessed through the mechanical, thermal and optical properties of developed films. Moreover, the ability of these films to exert antioxidant or antimicrobial properties in ready-to-eat salad was also studied.

Materials and methods

Supplies and chemicals

Polylactic acid (PLA) extrusion-grade (2003D) was purchased in pellets from NatureWorks LLC (Minnetonka, MN, USA). GS PLA™ FD92WD from Mitshubishi Chemical Corporation (Tokyo, Japan) was used as a plasticiser. Oregano essential oil was acquired from El Jarpil® (Almería, Spain). Sigma-Aldrich (Spain) and VWR International Eurolab (Spain) provided chemical for the assays. Iceberg salad for the *in vivo* assays was purchased in a local market.

Film preparation

A twin-screw extruder (DSE 20-40D, Brabender, Germany) was used to prepare different PLA films containing nominal OEO contents of 2%, 5% and 10% (w/w) by melt-blending. In order to avoid possible volatility and degradation losses, OEO was introduced into the barrel trough the lateral liquid port at L/D 10. Extrusion temperatures were set at 200–205°C working at a screw speed of 70 min^{-1}. In the control films, OEO was not added. The average width and thickness of the films were around 10 cm and 80 μm, respectively.

Quantification of the overall content of OEO incorporated into PLA films

In order to evaluate the amount of OEO contained in PLA films a thermogravimetric analysis (TGA) was performed according to Llana-Ruiz-Cabello et al. (2015a). Briefly, samples were heated to 900°C and the loss of mass was measured in a thermal analyser Q5000IR (TA Instruments, New Castle, DE, USA). This analysis measures the loss of mass of a given sample as a function of the temperature.

Quantification of carvacrol and thymol incorporated into PLA films

Carvacrol and thymol, monoterpene phenols present in the OEO, were extracted from the PLA films by immersing the films in acetone. Then, 0.5 g of each PLA film was accurately weighed and extracted with 10 ml of acetone for 48 h with constant shaking at 100 rpm. Then, the solvent extract was recovered and filtered through a Teflon filter of 0.22 μm.

Thermo Finnigan gas chromatograph (GC) equipped with a DB-5MS column (Agilent Technologies, USA) (30 m, 0.25 mm, film thickness 0.25 μm) using helium as a carrier gas (flow rate of 1 ml min^{-1}) was used for the analyses of carvacrol and thymol. The detector employed was a MS detector. MS detection was performed in the mass range of 50 to 450 amu at 4 scans s^{-1}. The temperature was set at 40°C for 4 min, and increased afterwards to 250°C at 15°C min^{-1}, being held for 2 min at

this temperature. Injections were performed in split mode with a 1:10 ratio. The solvent delay was 6 min.

The results are the mean of a triplicate analyses in each PLA film extracted and are expressed in terms of g carvacrol or thymol per 100 g of PLA film.

Stock solutions of carvacrol and thymol were prepared by weighting 25 mg of each chemical (> 98% purity) and dissolving them in 25 ml of methanol. Calibration solutions in the concentration range from 20 to 100 mg l^{-1} were obtained from the stock solutions by appropriate dilution with methanol. Calibration curves showed adequate linearity (coefficient of determination, $R^2 > 0.995$). LOD and LOQ were determined as 3 and 10 s criteria, respectively. SD was estimated based on 10 injections of stock standard solutions of 5 mg l^{-1} of carvacrol and thymol. LODs were 0.49 and 0.75 mg l^{-1} for thymol and carvacrol, respectively. LOQs were 1.65 and 2.50 mg l^{-1} for thymol and carvacrol, respectively.

Positive identification of carvacrol and thymol in the samples was obtained when the retention time and mass spectra of the peak obtained matched with those obtained for the carvacrol and thymol calibration solution at 20 mg l^{-1}. Retention times were 10.8 and 10.9 min for carvacrol and thymol, respectively. Mass spectra showed the following mass/charge fragments and relative abundancies (%) for both substances: 135 (100%), 150 (35%) and 115 (10%).

Physical properties

Light transmission and film transparency

The light transmission properties of the films were measured using a Jasco V-630 UV-Visible recording spectrophotometer (Madrid, Spain) according to the method described by Jongjareonrak et al. (2008). The transparency of the films was calculated according to Han and Floros (1997) as follows:

$$\text{Transparencyvalues} = (-\log T_{600})/x$$

where T_{600} is the fractional transmittance at 600 nm; and x is film thickness (mm).

Thickness

The thickness of films was measured using a digital micrometre Mitutoyo 547-400 Absolute (Kawasaki, Japan). Six different locations across each film were used for average thickness determination, and SD was also expressed.

Mechanical properties

Prior to testing the mechanical properties films were conditioned for 48 h at 25°C and 50 ± 5% RH. Tensile strength (TS), elongation at break (EAB) and elastic modulus (E) were determined according to Llana-Ruiz-Cabello et al. (2015a) and UNE-EN-ISO 527-1 using a M350-20CT Universal Testing Machine (Testometric, Rochdale, UK). TS was calculated by dividing the maximum force by the initial specimen cross-sectional area. EAB is the maximum change in length of a test specimen before breaking and is expressed as a percentage of the original length. E is a measure of the stiffness of the film.

Thermal analysis

Thermal analysis was carried out with a differential scanning calorimeter DSC Q2000 (TA Instruments). Glass transition temperature (Tg), crystallisation temperature (Tc) and melting temperature (Tm) of the different PLA films were measured according to Fan et al. (2004) and Llana-Ruiz-Cabello et al. (2015a). Samples of 6 mg were weighed accurately into aluminium pans, sealed and then heated from 20 to 250°C at a rate of 10°C min^{-1} in the first scan. Then samples were cooled to −50°C at 10°C min^{-1} and then the second heating scan was performed in the same way as the first.

Antioxidant activity of the films

The antioxidant activity of films containing OEO was evaluated using two different radicals, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic) acid (ABTS). Both methods are based on the ability of the tested substance to scavenge radical cations. The DPPH assay is an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts (Huang et al. 2005). This assay was performed according to Wu et al. (2005) with modifications. Moreover, the radical measured in the ABTS assay can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Prior et al. 2005). ABTS assay was performed following the method described by Re et al. (1999) with modifications. Those modifications are described in Llana-Ruiz-Cabello et al. (2015c). Briefly, minced film (0.5 g) was mixed with 10 ml of methanol and stirred vigorously for 48 h at 25°C. The supernatant obtained after filtration was analysed for testing films' antioxidant properties

Antimicrobial activity

Three Gram-negative and four Gram-positive bacteria were selected to assess the *in vitro* antimicrobial properties of films commonly involved in food spoilage and food diseases. Most of microorganisms were purchased from the Spanish Culture Collection (CECT) (Valencia, Spain). In addition, the antimicrobial activity of the film was evaluated in salad packaged with active films testing total aerobic bacteria, enterobacteria, yeasts and moulds.

Antimicrobial activity of the films

The dilution method was selected to evaluate the antimicrobial activity of the films following the method described by Llana-Ruiz-Cabello et al. (2015a). Briefly, different tubes containing sterile saline solution, being in contact with 50 cm^2 of active or control films, were uniformly inoculated with microbial suspensions. After 0, 1 and 3 days, the tubes were cultured on a microorganism-specific solid agar. After incubation microbial colonies were counted and data were expressed as the number of colony-forming units (CFU ml^{-1}). All determinations were performed in triplicate.

Antimicrobial activity in salad

The antimicrobial activity of the films in packaged salads was assessed after 1, 3, 5 and 7 days of packaging following the ISO 4833-2:2014 guidelines with modifications. Briefly, samples of packaged salad were homogenised, seeded and incubated at the required conditions for each microorganism according to Llana-Ruiz-Cabello et al. (2015a). The counts were carried out in a colony counter (Suntex 570 Colony Counter, Suntex Instruments Co., New Taipei City, Taiwan). The results are expressed as the log CFU g^{-1} ± SD (n = 3).

Sensory analyses

The sensory tests were carried out on days 1, 4 and 7 after packing. Smell, visual appearance and general acceptability were evaluated by an untrained panel composed of 20 judges recruited from among the staff of ITENE. Samples of salads were placed in transparent glasses, covered and identified by three-digit codes.

For the sensory test a nine-point hedonic scale was used ranged from 1 (dislike extremely) to 9 (like extremely), with 5 being neither like nor dislike. Three descriptors were assessed to determine the sensory properties: smell, visual appearance and general acceptability of the four different packaging systems (i.e., salad packaged with PLA containing 0%, 2%, 5% and 10% of OEO). The Williams method for constructing the experimental design was used for this sensory study (MacFie et al. 1989).

Packaging and storage

Several packages were produced containing 200 g of 'iceberg salad' packed in contact with the different PLA films (PLA and PLA-2%, 5% and 10% OEO). To this end, four pieces (strips) of PLA films (dimensions 10 × 12.5 cm) were introduced into the commercial PP bags to cover completely the whole surface of the PP bag facing the food with the PLA films. It this sense, lettuce was enclosed into a 'surrounding bag' made of strips of PLA in the same fraction as it would be in a conventional package (estimated at 1.9 cm^2 of film per g of salad). Then the bags were packed with an initial modified atmosphere composed by 10% O_2–10% CO_2–80% N_2.

Sample bags of 'iceberg salad', both control (with no PLA films) and samples, were stored at 4°C for 7 days, simulating commercial conditions of production, transport and commercialisation.

All developed films were stored at −20°C before their characterisation.

Statistical analysis

Experiments were performed at least in triplicate. Data were presented as means ± SD; differences between groups were consider significant at $p < 0.05$. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between factors and levels. Comparison of the means was done by Dunnet's test. The statistical analysis of the data was performed using GraphPad InStat software (GraphPad Software Inc., La Jolla, CA, USA).

For the sensory analysis, STATGRAPHICS Plus for Windows statistical software was used to calculate the analysis of variance multifactor (ANOVA) and Fisher's least significant difference (LSD) to evaluate the impact of the day and packaging material on sensory attributes. Significance differences were determined at $p < 0.05$.

Results and discussion

Content of OEO and carvacrol/thymol incorporated in the films

Figure 1 shows the TGA curves obtained for OEO, PLA and PLA containing different concentrations of OEO. The TGA curve for OEO showed a single-weight loss

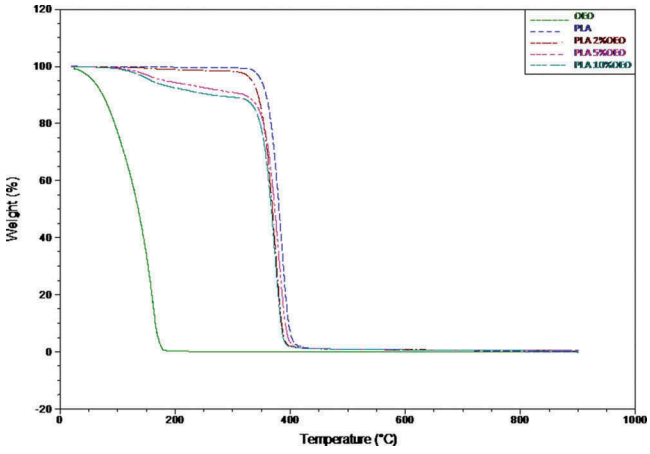

Figure 1. (colour online) TGA curves obtained for PLA and formulations with *Origanum vulgare* L. *virens* (OEO) at 2, 5% and 10% w/w.

step starting at RT up to 180°C which is related to the complete volatilisation of the different chemicals present in the OEO. On the other hand, the TGA curve for PLA film showed just one weight loss in the range 320–400°C.

The TGA curves for the PLA films containing OEO showed two weight losses which are related to the OEO and the PLA matrix. The first weight loss took place at temperatures above 150°C while the second took place at temperatures above 350°C. Considering the weight losses of OEO and PLA, the first weight loss peak provides an estimation of the overall content of OEO incorporated into the PLA after the processing of the films. For this purpose, the content of OEO was estimated as the weight lost at 200°C giving approximately 1.2%, 5.7% and 7.6% w/w of OEO in the PLA films with a nominal amount of 2%, 5% and 10% w/w, respectively. As can be observed, OEO was partially lost during the extrusion process applied to obtain the active PLA films containing 2% and 10% of OEO. Different behaviour was observed for films containing a nominal content of 5% OEO where the amount of the EO reported by TGA analysis was higher than expected. This result could be related with an erroneous over-feeding of the OEO through the lateral liquid port of the twin-screw extruder. However, despite this finding, the film with 5% OEO contained an interesting content of OEO close to this percentage (5.7%); therefore, the rest of the analysis was performed. As reported in other researches, the incorporation of EOs or some of their main constituents such as carvacrol or thymol by means of melting extrusion processes generates losses by volatility due to the high temperatures applied (Ramos et al. 2012; Llana-Ruiz-Cabello et al. 2015a). Moreover, the chemical instability of OEO under thermal treatments may result in non-intentionally added substances (NIAS). In this regard, some preliminary tests have been carried out by GC-MS-TOF and results obtained showed non-significant findings on new chemical (data not shown). Therefore, despite the fact that temperatures of 200°C were achieved during the extrusion process, OEO was successfully incorporated to the PLA films. In particular, the yield of OEO incorporation was ranged between 60% and 80%. OEO seems to reduce its volatility once mixed with the PLA matrix despite the high temperatures. OEO solubilisation into the PLA could be reducing its vapour pressure. In fact, the TGA for OEO showed that it was completely volatilised at 180°C while the TGA for the films of PLA containing OEO showed that the weight loss of OEO continues until temperatures around 200°C (data obtained from the derivative of the weight versus temperature; results not shown).

Finally, the analyses performed to determine the specific amount of carvacrol and thymol were carried out by solvent extraction and GC-MS analysis. Results showed that both active agents were present in the PLA films developed. In fact, the content of carvacrol and thymol was correlated with the amount of OEO incorporated. These contents were approximately 0.75%, 3.15% and 3.81% w/w for carvacrol and 0.07%, 0.28% and 0.36% w/w for thymol for the films with a nominal amount of OEO of 2%, 5% and 10% w/w, respectively. Moreover, the ratio between the content of carvacrol to the content of thymol remained at 10 for all the materials (Figure 2). This ratio is in agreement with the composition of the OEO declared by the supplier (i.e., 55% carvacrol and 5% thymol; Llana Ruiz-Cabello et al. 2014), thus meaning that the extrusion process did not produce selectively losses of these two molecules. In fact, carvacrol and thymol are structural isomers with similar boiling points at 232 and 237°C, respectively, and a vapour pressure of 0.023 and 0.016 mmHg, respectively (data estimated with the MPBPWIN v1.43 software provided by the USEPA).

Physical and mechanical properties

Nowadays, the transparency of the packages is a growing requirement for its commercialisation in the food sector (Pillin et al. 2006; Ojagh et al. 2010). The visibility of the food products is a factor influencing the buying decisions of consumers (Sehrawet & Kundu 2007).

Table 1 shows the light transmittance at specific wavelengths within the range of 200–800 nm, and the calculated transparency from the different PLA-OEO films. As can be observed, the light transmittance for

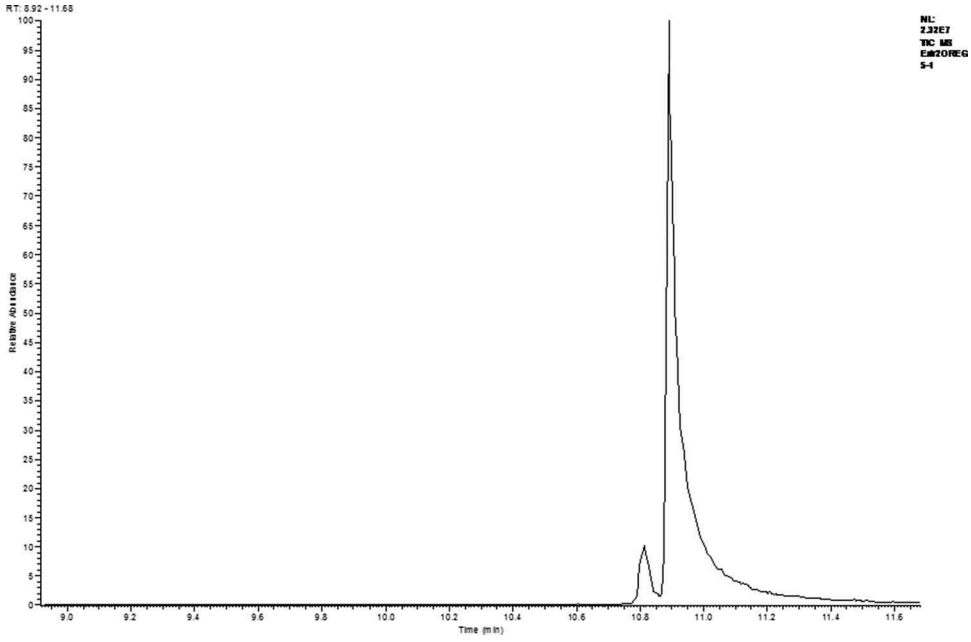

Figure 2. GC-MS chromatogram of the solvent extraction solution of the film containing 5% OEO showing the peaks for thymol and carvacrol at 10.8 and 10.9 min, respectively.

Table 1. Light transmittance (%) and transparency values of PLA films with *Origanum vulgare* L. *virens* (OEO) at various concentrations (nominal 2%, 5% and 10% w/w).

	Wavelength (nm)								
Film sample	200	280	350	400	500	600	700	800	Transparency[a]
Control	0.41	63.90	77.62	81.85	85.22	86.84	87.74	88.31	0.59 ± 0.15
PLA 2% OEO	0.00	12.94	83.21	86.38	88.78	90.07	90.82	91.26	0.56 ± 0.05
PLA 5% OEO	0.00	0.00	78.12	82.27	85.16	86.77	87.78	88.42	0.62 ± 0.03
PLA 10% OEO	0.00	0.00	76.14	81.34	85.06	86.93	88.05	88.78	0.60 ± 0.07

Note: [a]Values are mean ± SD from triplicate determinations.

the PLA films at 600 nm was 86.8%, while transparency was 0.59. These values reflect a high transparency of this film. The incorporation of OEO to the PLA films did not modified the transparency compared with the control film from a statistical point of view. The only differences between PLA films and PLA with OEO ones were observed for the light transmittance at 200 and 280 nm (UV region), thus showing that films with OEO has the capability of absorbing UV light. These results are in agreement with other researches. The transparency of sodium and calcium caseinate edible active films was not modified after incorporating carvacrol (Arrieta et al. 2013), while films based on fish gelatine and chitosan containing OEO had superior barrier capability to UV light (Hosseini et al. 2015).

Table 2 shows the mechanical properties of the different films. PLA films without OEO showed a high elastic modulus and a low elongation at break, thus indicating that these films are stiff and brittle. The addition of OEO to the PLA films decreased the tensile strength and elastic modulus while it increased the elongation at break. Moreover, the effect was related to the content of OEO incorporated to the films. The addition of OEO at a nominal 2% (actually 1.4% OEO)

Table 2. Mechanical properties of films tested containing nominal 2%, 5% and 10% of *Origanum vulgare* L. *virens* (OEO).

Sample	TS (Mpa)	EAB (%)	*E* (Gpa)	Thickness (mm)
PLA	65.42 ± 6.93	3.13 ± 2.00	3.01 ± 0.29	0.105 ± 0.002
PLA 2% OEO	55.80 ± 6.72**	2.86 ± 0.09	1.98 ± 0.43**	0.093 ± 0.003**
PLA 5% OEO	35.64 ± 4.59**	375 ± 17.24**	1.46 ± 0.24**	0.094 ± 0.006**
PLA 10% OEO	29.40 ± 3.28**	338 ± 20.41**	1.45 ± 0.26**	0.086 ± 0.004**

Notes: Values are mean ± SD. Reported values are means of at least six replicates ± SD. The significance levels observed are **$p < 0.01$ in comparison with the control group.
TS, tensile strength; EAB, elongation at break; E, Young's module.

barely provoked a modification in the mechanical properties in comparison with PLA without additives despite significant differences were found for tensile strength and elastic modulus, thus reducing the stiffness of the material while keeping brittleness. The incorporation of higher contents of OEO in the PLA films (nominal contents of 5% and 10% OEO; actually 5.7% and 7.6% OEO, respectively) generated significant modifications in the mechanical properties of the films. In particular, tensile strength and elastic modulus decreased more than a 50% while elongation at break increased by 100-fold in comparison with PLA films. Therefore, the addition of OEO at these levels generated a clear plasticising effect of the films. Materials became more flexible.

Similar results were observed for biodegradable films based on a combination of Ecoflex® and PLA containing α-tocopherol and olive leaf extracts which also showed an increase in the elongation at break and a decrease in the tensile strength (Marcos et al. 2014). In other study, the addition of thymol to PLA materials led to the modification of the elastic modulus and elongation at break (Ramos et al. 2014a). OEO as well as other EOs is liquid dispersed into the film structure and in gelatine matrix they can form oil droplets that can be easily deformed and thus enhancing film flexibility (Ahmad et al. 2012).

Finally, the thickness of active films decreased significantly in comparison with the PLA without OEO ($p < 0.01$). The incorporation of the OEO to the PLA polymer affected the fluidity of the molten mass thus making the films thinner. For instance, thickness variations around 20 µm were observed for the PLA film with the highest OEO in comparison with PLA films. Moreover, the thickness profile of the films in the transverse machine direction was less homogenous (higher SD is observed in Table 2) for the case of the PLA containing oregano due to this higher fluidity.

Thermal analysis

Table 3 shows the thermal parameters determined for the different PLA films developed. In particular, Tg, Tc and Tm are reported.

As can be observed, PLA films without OEO showed Tg, Tc and Tm values around 50, 94 and 153°C, respectively,, which are within the normal values for PLA (Belgacem & Gandini 2008). However, a carefully inspection of the DSC thermogram revealed a second melting point at 140°C that corresponds to the GS PLA™ additive. This Tm peak for GS PLA™ was found as a small peak almost overlapped the bigger peak found for PLA.

Table 3. Thermal properties of films tested containing nominal 2%, 5% and 10% of *Origanum vulgare* L. *virens* (OEO) (second heating cycle).

Sample	Tg (°C)	Tc (°C)	Tm1 (°C)	Tm2 (°C)
PLA	50 ± 0.7	94 ± 0.1	153 ± 1.4	153 ± 1.0
PLA 2% OEO	49 ± 1.2	94 ± 0.5	151 ± 2.0	152 ± 0.9
PLA 5% OEO	31 ± 0.3***	81 ± 0.8***	143 ± 1.8***	145 ± 1.1***
PLA 10% OEO	28 ± 0.8***	78 ± 0.5***	143 ± 0.9***	143 ± 1.0***

Note: Significance levels observed are ***$p < 0.001$ in comparison with the control group.
Tg, glass transition temperature; Tc, crystallisation temperature; Tm, melting temperature.

The addition of nominal 2% OEO (actually, 1.4% OEO) to the PLA films did not produce any changes in Tg, Tc and Tm. However, at nominal contents of 5% and 10% OEO significant decreases ($p < 0.001$) of Tg, Tc and Tm of the PLA films were found (over 20, 15 and 10°C less than PLA with no additives, respectively).

Similar results were obtained in other studies. For instance, the Tm and Tc of PLA materials decreased due to a plasticisation effect of the polymer after the addition of substances such as α-tocopherol and carvacrol (Jamshidian et al. 2012; Ramos et al. 2014a).

Antioxidant and antimicrobial activities

Antioxidant activities

The scavenging ability of the films containing OEO on DPPH and ABTS free radical is shown in Figure 3. Although samples containing 2% OEO did not show scavenging activity, films with 5% and 10% of OEO revealed antioxidant capacity that was significantly different from control films without OEO incorporated ($p < 0.001$) and from 2% OEO films ($p < 0.001$). Results obtained for ABTS scavenging ability were similar. No differences between control and 2% OEO films were

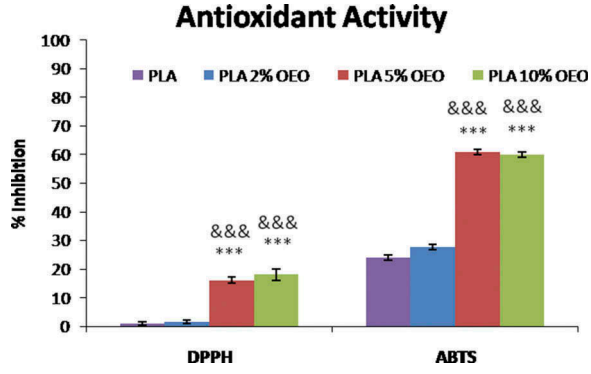

Figure 3. (colour online) Antioxidant activity of PLA films containing 2%, 5% and 10% of *Origanum vulgare* L. *virens* (OEO). Significance levels observed are ***$p < 0.001$ in comparison with their control, and &&&$p < 0.001$ when 5% or 10% OEO are compared with 2% OEO.

observed. Moreover, films containing higher amounts of OEO (5% and 10%) exhibited significant antioxidant activity in comparison with the control group and the 2% OEO group. This activity increased from 27.6% to 60.8% when the OEO concentration in samples increased from nominal contents of 2–10%. Ramos et al. (2014b) observed that the DPPH inhibition values were correlated with the EOs amount released from the films. Our results from the TGA analyses showed that the content of OEO was 5.7% and 7.6% for 5% and 10%, respectively. This fact could explain the similarities in the antioxidant activity between films containing the highest amounts of OEO.

Some authors have reported that chemical composition of EOs is responsible of their antioxidant properties (Nerín et al. 2008). In this sense, our results are in good agreement with other authors (Stamenic et al. 2014; Sarikurkcu et al. 2015; Llana-Ruiz-Cabello et al. 2015c) who reported that carvacrol and thymol, the main compounds of OEO, are considered as effective scavengers of the free radicals. Ramos et al. (2014b) have reported antioxidant activity of carvacrol and thymol from polypropylene active packaging films. In general, most of the tested EOs or their alcoholic extracts showed antioxidant activity in different *in vitro* assays including ABTS or DPPH assays (Teixeira et al. 2013; Sanches-Silva et al. 2014). Thymol antioxidant activity has been evidenced to be greater than that of carvacrol, possibly due to greater steric hindrance of the thymol phenolic group (Ramos et al. 2014b).

Antimicrobial assays

Antimicrobial activity in films. The dilution method used to quantify the bacterial reduction caused by films showed that films containing small amounts of OEO were able to reduce the growth only of *S. aureus* ($p < 0.05$ for PLA 5%) and *Y. enterocolitica* ($p < 0.01$ for PLA 2% and 5%) after 3 days of contact (Table 4). Moreover, films containing 10% OEO caused a significant decrease ($p < 0.01$) in the microbial growth of *S. enterica* after it was assayed both times. For *S. aureus*, *Y. enterocolitica*, *L. monocytogenes*, *E. faecalis* and *S. carnosus* this reduction was significant only after 3 days of contact.

Active packaging developed with natural additives, such as EOs, has been confirmed as being effective against microbial spoilage (Llana-Ruiz-Cabello et al. 2015a). Regarding OEO several authors reported its ability to inhibit the growth of different microorganism and the effectiveness of different active packaging which contains this extract (Oussalah et al. 2004; Aguirre et al. 2013; Jouki et al. 2014; Teixeira et al. 2014). Jouki et al. (2014) reported that quince seed mucilage films containing OEO can inhibit the growth of Gram-positive and -negative bacteria, even at low concentrations of OEO (2%). This fact, which was not observed in the present work, could be explained by the different matrix in which OEO is included, so that the hydrophilicity of mucilage films induced swelling of the polymer network in contact with water and led to more release of EOs. Teixeira et al. (2014) found different antimicrobial activity of fish protein films containing OEO against the same microbial strains depending on the antimicrobial method developed. They reported that OEO release could be related to possible interactions between active agent and film constituents. In addition, Oussalah et al. (2004) detected inhibition of *Pseudomonas* spp. and *E. coli* O157:H7 in meat samples packaged with milk protein-based films containing this extract. Similarly, triticale protein films containing OEO showed

Table 4. Antimicrobial activity of PLA films containing nominal 2%, 5% and 10% of *Origanum vulgare* L. *virens* (OEO).

Microorganisms/film	Day 1 (log)	Day 3 (log)
Staphylococcus aureus		
PLA	5.67 ± 0.18	5.65 ± 0.12
PLA 2% OEO	5.65 ± 0.16	5.63 ± 0.36
PLA 5% OEO	5.63 ± 0.08	5.18 ± 0.27*
PLA 10% OEO	5.58 ± 0.21	5.00 ± 0.15**
Yersinia enterocolitica		
PLA	4.92 ± 0.19	4.91 ± 0.14
PLA 2% OEO	4.90 ± 0.18	4.00 ± 0.14**
PLA 5% OEO	4.79 ± 0.23	4.36 ± 0.20**
PLA 10% OEO	4.78 ± 0.31	4.18 ± 0.37**
Listeria monocytogenes		
PLA	5.81 ± 0.21	5.81 ± 31
PLA 2% OEO	5.79 ± 0.26	5.79 ± 0.19
PLA 5% OEO	5.80 ± 0.20	5.72 ± 0.42
PLA 10% OEO	5.76 ± 0.15	4.93 ± 0.36**
Enterococcus faecalis		
PLA	6.58 ± 0.13	6.57 ± 0.16
PLA 2% OEO	6.57 ± 0.42	6.54 ± 0.37
PLA 5% OEO	6.51 ± 0.31	6.18 ± 0.27
PLA 10% OEO	6.48 ± 0.26	6.04 ± 0.36*
Salmonella enterica		
PLA	4.79 ± 0.16	4.78 ± 0.31
PLA 2% OEO	4.79 ± 0.14	4.76 ± 0.15
PLA 5% OEO	4.65 ± 0.16	4.63 ± 0.14
PLA 10% OEO	4.08 ± 0.23**	3.97 ± 0.29**
Staphylococcus carnosus		
PLA	5.96 ± 0.31	5.95 ± 0.16
PLA 2% OEO	5.96 ± 0.16	5.94 ± 0.14
PLA 5% OEO	5.96 ± 0.14	5.86 ± 0.16
PLA 10% OEO	5.93 ± 0.16	4.81 ± 0.23**
Escherichia coli O157:H7		
PLA	4.57 ± 0.23	4.56 ± 0.32
PLA 2% OEO	4.58 ± 0.36	4.55 ± 0.15
PLA 5% OEO	4.51 ± 0.48	4.51 ± 0.38
PLA 10% OEO	4.46 ± 0.16	4.51 ± 0.49

Note: Values are mean ± SD from three different experiments. The significance levels observed in comparison with the control group are *$p < 0.05$ and **$p < 0.01$. Reductions of microbial counts were calculated with respect to control.

antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus* (Aguirre et al. 2013).

Antimicrobial activity in salads. The antimicrobial activity of developed films was also assessed in the packaged salad. Figure 4 shows changes in the microbial population of the salad during storage time. The storage of Enterobacteriaceae counts increased gradually in the packaged salad at 4ºC and none of the developed films containing OEO was effective at inhibiting the growth of these microorganisms (Figure 4(a)). Similarly, developed films did not show any antimicrobial activity against aerobic bacteria (Figure 4(b)). In our work, the bacterial counts were slightly high, but according to Little et al. (1999) high levels of enterobacteria are common in raw vegetables and they should not be used to indicate the microbiological quality of lettuce.

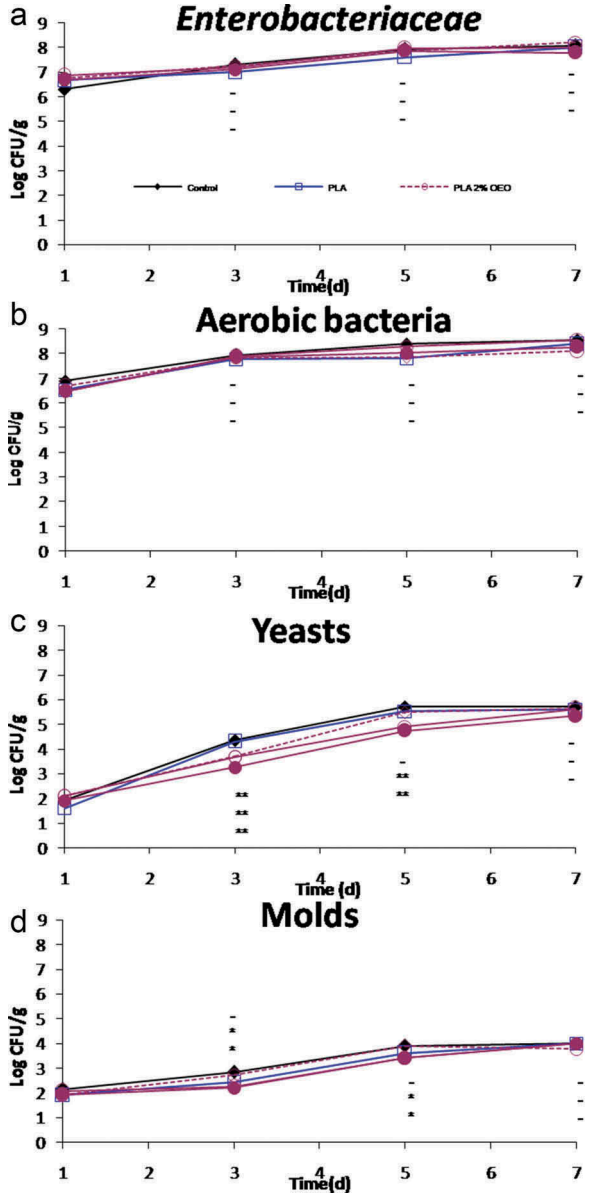

Figure 4. (colour online) Antimicrobial activity of films containing *Origanum vulgare* L. *virens* (OEO) in packaged salad along with the storage time for Enterobacteriaceae (a), aerobic bacteria (b), yeasts (c) and moulds (d). Values are mean ± SD from three different experiments. Significance levels observed are $^{*}p < 0.05$ and $^{**}p < 0.01$ in comparison with the control group.

Yeasts and moulds seemed to be more sensitive than bacteria. Moreover, this antimicrobial activity was observed in films containing higher amounts of EO. The activity against yeast was observed up to 5 days of storage, exhibiting the highest effectiveness after 3 days of storage for PLA 2%, 5% and 10% ($p < 0.01$). However, the antimicrobial activity decreased throughout the storage time (Figure 4(c)). Similar behaviour was observed for active films against moulds. In this regard, Figure 4(d) shows that films containing 5% and 10% of OEO displayed an antimicrobial activity after up to 5 days ($p < 0.05$), without any significant effect after 7 days. The decrease in this activity could be related with the volatilisation of active compounds within time. The results obtained in this section show significant reduction in yeast and moulds counts in salad packaged with OEO films. In this regards, EOs characterised by high contents of phenolic compounds, such as thymol or carvacrol, have been shown strong antimicrobial activity against yeast and moulds (Lang & Buchbauer 2012). Although lettuce is very unlikely to be contaminated by yeasts and moulds, the results obtained in the present work indicated that PLA containing OEO could be an useful packaging for other foods that are more susceptible to these microorganisms such as fruit, vegetables or cereals (Pitt & Hocking 2009).

The ability of OEO incorporated in different film matrices to reduce microbial counts in food has been reported by several authors. Khanjari et al. (2013) observed that chitosan films containing OEO reduce the total viable counts in chicken fillets and had a significant antibacterial and preservative effect. Similarly, a significant decrease in different bacterial counts (Enterobacteriaceae, lactic acid bacteria, psychrotrophic, yeast and moulds) in comparison with the control was observed in lettuce packaged in active EVOH-coated PP films containing OEO (Muriel-Galet et al. 2013). The effectiveness of these films could be related to the phenolic compounds' concentration, which depends on the variety, origin or time of harvest (Oussalah et al. 2004). Moreover, the diffusion rates of active agents could play an important role in the antimicrobial activity of these films (Jouki et al. 2014).

Sensory analyses

The use of EO in ready-to-eat food may have a significant sensory impact that could result in non-acceptance by the consumer (Muriel-Galet et al. 2012). For this reason, sensory analyses were carried out on the salads packaged with PLA containing OEO. Figure 5 shows the average scores obtained for each packaging material evaluated at each specific day and sensory descriptor (smell, visual appearance and general acceptability).

All three descriptors were scored over the threshold value of 5 in the hedonic scale for the 7 days of packing regardless of the packaging material. No significance differences were found in the ANOVA test ($p > 0.5$) for the two factors of interest: day and packaging material.

Smell was considered the most critical sensory attribute due to the aromatic character of the EO (Nedorostova et al. 2009). However, although a characteristic odour was noticed at the time of opening the packages, it was not detected at the time of evaluation of the lettuce samples by the evaluators. Only samples

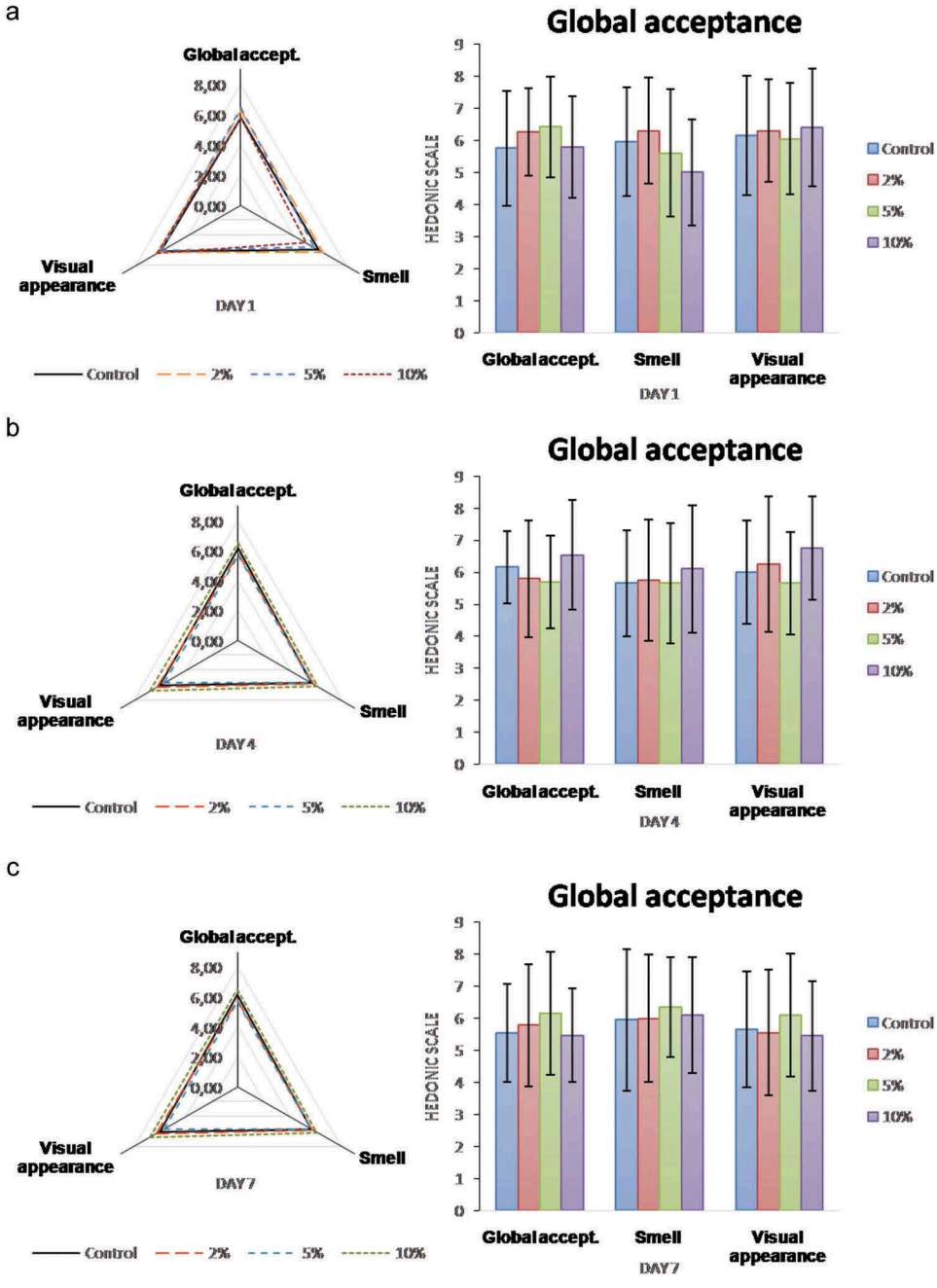

Figure 5. (colour online) Sensory scores of salad stored in control and active packaging containing 2%, 5% and 10% OEO at days 1 (a), 4 (b) and 7 (c).

packed in packages with PLA + 10% OEO at day 1 (Figure 5(a)) scored worst in comparison with the other packaging materials in terms of odour, although they were above 5 in the hedonic scale.

On the other hand, although non-significant differences were found among days, a slight decrease in the scores of visual appearance and general acceptability was observed along days. For instance, average visual appearance and general acceptance varied from 6.22 to 5.69 and from 6.10 to 5.73, respectively, from days 1 to 7.

Undesirable flavours caused by the direct addition of EOs into foods could be avoided, including these substances in active food packaging (Ramos et al. 2012). In this regard, the sensory results obtained have demonstrated that lettuce packed in active packaging materials gave similar results to the control. Therefore, although a sensory impact was expected mostly in smell due to the aromatic character of oregano essential, this was not found critical since the panellists did not perceive it.

Conclusions

The properties of films containing 2%, 5% and 10% of OEO were studied in the present work in order to confirm the usefulness of this packaging in ready-to-eat salads. The incorporation of OEO in PLA films resulted in a material exhibiting mechanical and physical properties suitable for the development of active food packaging. In addition, antioxidant effect was observed for films with 5% and 10% of OEO. The greater antimicrobial activity was observed against yeast and moulds that could be useful for other ready-to-eat food susceptible to suffer spoilage by these microorganisms. Furthermore, despite the fact that OEO exhibits great aromatic character, the sensorial study revealed no impact in the acceptability of food packaged in active film containing this EO.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Aguirre A, Borneo R, León AE. 2013. Antimicrobial, mechanical and barrier properties of triticale protein films incorporated with oreganos essential oil. Food Biosci. 1:2–9. doi:10.1016/j.fbio.2012.12.001

Ahmad M, Benjakul S, Prodpran T, Agustini TW. 2012. Physico-mechanical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils. Food Hydrocol. 28:189–199.

Arrieta MP, Peltzer MA, Garrigós MC, Jiménez A. 2013. Structure and mechanical properties of sodium and calcium caseinate edible active films with carvacrol. J Food Eng. 114:486–494.

Becerril R, Gómez-Lus R, Goñi P, López P, Nerín C. 2007. Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon oro regano against *E.coli* and *S. aureus*. Anal Bioanal Chem. 388:1003–1011.

Belgacem MN, Gandini A, eds. 2008. Monomers, polymers and composites from renewable resources. Oxford: Elsevier.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol. 94:223–253.

Commission Regulation (EU) No. 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.

Del Nobile MA, Conte A, Buonocore GG, Incoronato AL, Massaro A, Panza O. 2009. Active packaging by extrusion processing of recyclable and biodegradable polymers. J Food Eng. 93:1–6.

Fan Y, Nishida H, Shirai Y, Tokiwa Y, Endo T. 2004. Thermal degradation behaviour of poly(lactic acid) stereocomplex. Polym Degrad Stabil. 86:197–208.

Gutiérrez L, Sánchez C, Battle R, Nerín C. 2009. New antimicrobial active package for bakery products. Trends Food Sci Tecn. 20:92–99.

Gutiérrez L, Battle R, Andújar S, Sánchez C, Nerín C. 2011. Evaluation of antimicrobial active packaging to increase shelf life of gluten-free sliced bread. Packag Technol Sci. 24:485–494.

Han JH, Floros JD. 1997. Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. J Plast Film Sheet. 13:287–298.

Hosseini SF, Rezaei M, Zandi M, Farahmandghavi F. 2015. Bio-based composite edible films containing *Origanum vulgare* L. essential oil. Ind Crop Prod. 67:403–413.

Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. J Agric Food Chem. 53:1841–1856.
ISO 4833-2:2014. Microbiology of the food chain – Horizontal method for the enumeration of microorganisms- Part 2: Colony counts at 30 degrees C by the surface plating technique. (ISO 4833-2:2013).
Jamshidian M, Tehrany EA, Imran M, Akhtar MJ, Cleymand F, Desobry S. 2012. Structural, mechanical and barrier properties of active PLA-antioxidant films. J Food Eng. 110:380–389.
Jin T, Zhang H. 2008. Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. Food Saf. 73:127–134.
Jongjareonrak A, Benjakul S, Visessanguan W, Tanaka M. 2008. Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and α-tocopherol. Food Hydrocol. 22:449–458.
Jouki M, Mortazavi SA, Yazdi FT, Koocheki A. 2014. Characterization of antioxidant-antimicrobial quince seed mucilage films containing thyme essential oil. Carbohyd Polym. 99:537–546.
Khanjari A, Karabagias IK, Kontominas MG. 2013. Combined effect of N,O-carboximethyl chitosan and oregano essential oil to extend shelf life and control *Listeria monocytogenes* in raw chicken meat fillets. Food Sci Tech. 53:94–99.
Kocic-Tanackov SD, Dimic GR, Mojovic LV, Pejin JD, Tanackov IJ. 2014. Effect of caraway, basil, and oregano extracts and their binary mixtures on fungi in growth medium and on shredded cabbage. Food Sci Tech. 59:426–432.
Lang G, Buchbauer G. 2012. A review on recent research results (2008-2010) on essential oils as antimicrobials and antifungals. A review. Flavour Frag J. 27:13–39.
Little C, Roberts D, Youngs E, Louvois JD. 1999. Microbiological quality of retail imported unprepared whole lettuces: a PHLS Food Working Group study. J Food Protect. 62:325–328.
Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Pichardo S, Moreno FJ, Bermúdez JM, Aucejo S, Cameán AM. 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem Toxicol. 64:281–290.
Llana-Ruiz-Cabello M, Pichardo S, Baños A, Núñez C, Bermúdez JM, Guillamón E, Aucejo S, Cameán AM. 2015a. Characterisation and evaluation of PLA films containing an extract of *Alliums* sp. to be used in the packaging of ready-to-eat salads under controlled atmospheres. Food Sci Tech. 64:1354–1361.
Llana-Ruiz-Cabello M, Pichardo S, Maisanaba S, Puerto M, Prieto AI, Gutiérrez-Praena D, Jos A, Cameán AM. 2015b. *In vitro* evaluation of essential oils and their main compounds used in active food packaging: a review. Food Chem Toxicol. 81:9–27.
Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Puerto M, Pichardo S, Jos A, Cameán AM. 2015c. *In vitro* pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. Toxicol *In Vitro*. 29:647–656.
López P, Sánchez C, Battle R, Nerín C. 2007. Vapor-phase activities of cinnamon, Thyme, and oregano essential oils and key constituents against foodborne microorganisms. J Agr Food Chem. 55:4348–4356.
MacFie HJ, Bratchell N, Greenhoff K, Vallis LV. 1989. Designs to balance the effect of order of presentation and first-order carry-over effects in hall test. J Sensory Stud. 4:129–148.
Manso S, Pezo D, Gómez-Lus R, Nerín C. 2014. Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil. Food Control. 45:101–108.
Marcos B, Sárraga C, Castellari M, Kappen F, Schennink G, Arnau J. 2014. Development of biodegradable films with antioxidant properties based on polyesters containing α-tocopherol and olive leaf extract for food packaging applications. Food Packag Shelf Life. 1:140–150.
Muriel-Galet V, Cerisuelo JP, López-Carballo G, Lara M, Gavara R, Hernández-Muñoz P. 2012. Development of antimicrobial films for microbiological control of packaged salad. Int J Food Microbiol. 157:195–201.
Muriel-Galet V, Cerisuelo JP, López-Carballo G, Aucejo S, Gavara R, Hernández-Muñoz P. 2013. Evaluation of EVOH-coated PP films with oregano essential oil and citral to improve the shelf-life of packaged salad. Food Control. 30:137–143.
Nerín C, Tovar L, Salafranca J. 2008. Behaviour of a new antioxidant active packaging versus several oxidizable model compounds. J Food Eng. 84:313–320.
Nodorostova L, Kloucek P, Kokoska L, Stolcova M, Pulkrabek J. 2009. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control. 20:157–160.
Ojagh SM, Rezaei M, Razavi SH, Hosseini SMH. 2010. Development and avaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. Food Chem. 122:161–166.
Ortega-Nieblas MM, Robles-Burgueño MR, Acedo-Félix E, González-León A, Morales-Trejo A, Vázquez-Moreno ML. 2011. Chemical composition and antimicrobial activity of oregano (*Lippia palmeri* S. Wats) essential oil. Rev Fitotec Mex. 34:11–17.
Oussalah M, Caillet S, Salmiéri S, Saucier L, Lacroix M. 2004. Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of whole beef muscle. J Agric Food Chem. 52:5598–5605.
Pillin I, Montrelay N, Grohens Y. 2006. Thermo-mechanical characterization of plasticized PLA: Is the miscibility the only significant factor? Polymer. 47:4676–4682.
Pitt JI, Hocking AD. 2009. Fungi and food spoilage. New York, NY: Springer.
Prior RL, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agri Food Chem. 53:4290–4302.
Ramos M, Jiménez A, Peltzer M, Garrigós MC. 2012. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. J Food Eng. 109:513–519.
Ramos M, Jiménez A, Peltzer M, Garrigós MC. 2014a. Development of novel nano-biocomposite antioxidant films based on poly (lactic acid) and thymol for active packaging. Food Chem. 162:149–155.
Ramos M, Beltrán A, Peltzer M, Valente AJM, Garrigós MC. 2014b. Release and antioxidant activity of carvacrol and thymol from polypropylene active packaging films. LWT – Food Sci Tech. 58:470–477.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 26:1231–1237.

Rodríguez-Lafuente A, Battle R, Nerín C. 2007. The use of natural essential oils as antimicrobial solutions in paper packaging. Part II. Prog Org Coat. 60:33–38.

Sanches-Silva A, Costa D, Albuquerque TG, Buonocuore GG, Ramos F, Castilho MC, Machado AM, Costa HS. 2014. Trends in the use of antioxidants in active food packaging: a review. Food Addit Contam: Part A. 3:374–395.

Sarikurkcu C, Zengin G, Oskay M, Uysal S, Ceylan R, Aktumsek A. 2015. Composition, antioxidant, antimicrobial and enzyme inhibition activities of two *Origanum vulgare* subspecies (subsp. *vulgar* and subsp. *hirtum*) essential oils. Ind Crop Prod. 70:178–184.

Sehrawet M, Kundu SC. 2007. Buying behavior of rural and urban consumers in India: the impact of packaging. Int J Consum Stud. 31:630–638.

Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M. 1996. Antimicrobial and cytotoxic activities of *Origanum* Essential Oils. J Agricul Food Chem. 44:1202–1205.

Solórzano-Santos F, Miranda-Novales MG. 2012. Essential oils from aromatic herbs as antimicrobial agents. Curr Opin Biotech. 23:136–141.

Stamenic M, Vulic J, Djilas S, Misic D, Tadic V, Petrovic S, Zizovic I. 2014. Free-radical scavenging activity and antibacterial impact of Greek oregano isolates obtained by SFE. Food Chem. 165:307–315.

Teixeira B, Marques A, Ramos C, Neng NR, Nogueira JMF, Saraiva JA, Nunes ML. 2013. Chemical composition and antibacterial and antioxidant properties of comercial essential oils. Ind Crop Prod. 43:587–595.

Teixeira B, Marques A, Pires C, Ramos C, Batista I, Saraiva JA, Nunes ML. 2014. Characterization of fish protein films incorporated with essential oils of clove, garlic and oregano: Physical, antioxidant and antibacterial properties. Food Sci Tech. 59:533–539.

Wu J-H, Tung Y-T, Wang S-Y, Shyur L-F, Kuo Y-H, Chang S-T. 2005. Phenolic antioxidants from the heartwood of Acacia confuse. J Agric Food Chem. 53:5917–5921.

Wu J, Ge S, Liu H, Wang S, Chen S, Wang J, Li J, Zhang Q. 2014. Properties and antimicrobial activity of silver carp (Hypophthalmichthys molitrix) skin gelatin-chitosan films incorporated with oregano essential oil for fish preservation. Food Pack Shelf Life. 2:7–16.

CAPÍTULO 13 / CHAPTER 13

María Llana-Ruiz-Cabello, Silvia Pichardo, José M. Bermúdez, Alberto Baños, Juan J. Ariza, Enrique Guillamón, Susana Aucejo, Ana M. Cameán.

CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ACTIVE POLYPROPYLENE FILMS CONTAINING ORIGANUM AND ALLIUM EXTRACTS.

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Abstract: Cooked ham is more prone to spoilage than other meat products due to its properties, making the preservation techniques a key step in the commercialization. One of the most promising preservation strategies is the use of active packaging. Oregano essential oil (OEO) and Proallium® (an Allium extract) have previously shown to be useful in PLA active films for ready to eat salads. The present work aims to study the suitability of polypropylene (PP) films containing OEO and Proallium® in the preservation of cooked ham. Concerning the technological features of the studied material, no remarkable change in the mechanical or optical properties of PP films containing the active substances were recorded in comparison to the PP film without extracts. Both active substance showed antibacterial properties, however, Proallium® active films seemed to be more effective against Brochotrix thermosphacta than PP films containing OEO. In addition, materials containing the lowest Proallium® content exhibited the higher acceptability by the consumers in the sensory analyses, even better than the control package.

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27th April, 2017

Dear Editor,

We would be very grateful if you consider the manuscript entitled **"Characterization and antimicrobial activity of active polypropylene films containing Origanum and Allium extracts"** for its publication in "LWT - Food Science and Technology".

To the extent of our knowledge, this is the first work successfully developing active food packaging of PP with Proallium® and Oregano Essential Oil. The incorporation of the active agent in the film has been checked as well as the mechanical and physical properties. In addition, this active packaging has resulted to exert antimicrobial properties in cooked ham. The acceptability by the consumers has been also confirmed in the sensory analyses.

The authors declare that there are no conflicts of interest.

I am looking forward to receiving a positive answer from you.

Sincerely,

Silvia Pichardo

***Highlights (for review)**

Active packaging of PP with oregano and *Allium* extracts are useful for cooked ham

No change in physical properties of PP films containing active substances were found

PP-Proallium® films showed better antibacterial than PP-OEO

PP-Proallium® exhibited the highest acceptability by consumers

Characterization and antimicrobial activity of active polypropylene films containing *Origanum* and *Allium* extracts.

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Abstract

Cooked ham is more prone to spoilage than other meat products due to its properties, making the preservation techniques a key step in the commercialization. One of the most promising preservation strategies is the use of active packaging. Oregano essential oil (OEO) and Proallium® (an *Allium* extract) have previously shown to be useful in PLA active films for ready to eat salads. The present work aims to study the suitability of polypropylene (PP) films containing OEO and Proallium® in the preservation of cooked ham. Concerning the technological features of the studied material, no remarkable change in the mechanical or optical properties of PP films containing the active substances were recorded in comparison to the PP film without extracts. Both active substance showed antibacterial properties, however, Proallium® active films seemed to be more effective against *Brochotrix thermosphacta* than PP films containing OEO. In addition, materials containing the lowest Proallium® content exhibited the higher acceptability by the consumers in the sensory analyses, even better than the control package.

Keywords: polypropylene; oregano essential oil; *Allium*; active packaging

1. Introduction

Meats and meats products are good media for the growth of microorganism (Van Haute et al., 2016). Cooked ham is more prone to spoilage than other meat products due to its low salt content, pH near 6 and water activity higher than 0.945. In addition, cooked ham is usually sliced and packaged before being sold and consequently its shelf-life could decrease (Baños et al., 2012). Therefore, the preservation strategies in these foods are of great importance.

New trends in consumer demands, regarding minimally processed food or ready-to-eat fresh food, are driving a search for innovative ways to maintain food quality, freshness and safety (Appendini and Hotchkiss, 2002). One of these new developments is the active packaging, which was defined in Regulation (EC) No 450/2009. These materials include antimicrobial active films that can be classified in two categories: those constructed by using polymers inherently antimicrobial; and those that involve the direct incorporation of the antimicrobial additive into the packaging films (Suppakul, Miltz, Sonneveld & Bigger, 2003). Active food packaging systems containing antimicrobial additives are polymeric materials developed to release these active substances into the food in order to prevent microbial growth and to extend foodstuff shelf-life (Del Novile et al., 2009). Polyolefins have been used as food packaging matrices in the development of active films due to their mechanical, barrier, optical and thermal properties (Ramos, Jimenez, Pelzer & Garrigos, 2012; Gigli et al., 2014). Polypropylene (PP) is a polyolefin with low water vapor transmission, medium gas permeability, good resistance to greases and chemicals, good abrasion resistance, high temperature stability and good gloss and high clarity, all them features making it ideal for food packaging (Robertson, 2012; Ramos et al., 2012; Ramos, Beltran, Peltzer, Valente & Garrigos, 2014; Muriel-Galet et al., 2013).

Regarding active substance, several studies have shown that consumers have recently become more informed about food additives and tend to choose those with additives of natural origin rather than their synthetic analogues (Carocho, Morales & Ferreira, 2015). In this regard, essential oils (EOs) and plant extracts have been the target of extensive research because these substances have demonstrated biological properties that exhibit benefits in food (Ribeiro-Santos, Andrade, Ramos de Melo & Sanchez-Silva, 2017). Therefore, new alternatives based on aromatic plants, such as EOs, have been studied (Ramos et al., 2014). The advantages observed for active packaging containing EOs compared with direct addition of these additives to the food, such as lower amounts of compounds required or extended effects due to controlled migration from the film to the food are increasing the demand of these materials (Sanchez-Silva et al., 2014). Besides, active packaging containing EOs can avoid the denaturalization of the active substances contained in these extracts caused by food constituents when the additive is sprayed directly to foods (Sung et al., 2013). Among EOs, oregano essential oil (OEO) and its major compounds, carvacrol and thymol, have shown its usefulness as active substance in food packaging. A previous experimental study performed in our laboratory have evidence the suitability of OEO when incorporated to a matrix of polylactic acid (PLA) as antioxidant agent in ready-to-eat salads (Llana-Ruiz-Cabello et al., 2016). Muriel-Galet et al. (2013) also showed the ability of EVOH-coated PP films containing OEO to improve shelf life of packaged salad. Similarly, Ramos et al. (2012) reported the antimicrobial activity of PP films containing carvacrol and thymol against bacterial strains present in foods such as, *Escherichia coli* or *Staphylococcus aureus*. Moreover, another plant extract, Proallium®, is a commercial product based on *Allium* extract that is composed of organosulphur compounds. Garlic EO and its major organosulphur compounds have been shown antimicrobial activity when they are included in active packaging (Sung et al., 2014; Teixeira et al., 2014).

Specifically, we have previously observed that films of PLA containing Proallium® showed antimicrobial activity against bacteria, yeast and molds (Llana-Ruiz-Cabello et al., 2015a). Considering all this background, the aim of the present work was to develop new active films based on PP containing OEO and Proallium® and further characterize them though optical, mechanical and thermal properties. Moreover, the effectiveness of these films in the preservation of cooked ham was also evaluated.

2. Materials and Methods

2.1 Supplies and Chemicals

Polypropylene ISPLEN® PP 050 G2M was purchased from Repsol YPF S.A. (Madrid, Spain). Oregano essential oil was acquired from El Jarpil (Almería, Spain). Proallium® with 14.5% of PTSO (Llana-Ruiz-Cabello et al., 2015a) as active agent was provided by Domca S.L. (Alhendin, Granada, Spain).

Chemicals for the different assays were purchase from Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Ham for the *in vivo* assays was purchase in a local market (Valencia, Spain).

2.2 Film preparation

Two types of active PP films were obtained, one of them containing nominal OEO contents of 2, 5 and 8 % (w/w) and the other containing nominal Proallium® contents of 2, 3 and 4 % (w/w). Films were prepared according to Llana-Ruiz-Cabello et al. (2015a) for Proallium® and Llana-Ruiz-Cabello et al. (2016) for OEO.

2.3 Quantification of the overall content of OEO and Proallium® incorporated into PP films

112 The amount of OEO and Proallium® contained in PP films was evaluated using
113 Thermogravimetric Analysis (TGA) as described in Llana-Ruiz-Cabello et al. (2015a).
114
115 *2.4 Quantification of carvacrol, thymol and propyl thiosulphinate oxide (PTSO) incorporated*
116 *into PP films*
117 The main constituents of OEO (i.e. carvacrol and thymol) and Proallium® (i.e. PTSO)
118 incorporated into the PP films were extracted using tetrahydrofuran (THF) as solvent. The
119 quantification of carvacrol and thymol was performed following the method described by
120 Llana-Ruiz-Cabello et al. (2016) and the quantification of PTSO was carried out according to
121 Llana-Ruiz-Cabello et al. (2015a).
122
123 *2.5 Physical properties*
124 The light transmission, transparency and thickness were measured according to Llana-Ruiz-
125 Cabello et al. (2015a).
126
127 *2.6 Mechanical properties*
128 Films were conditioned for 48 h at 25 ºC and 50±5 % RH before testing their mechanical
129 properties. Tensile strength (TS), elongation at break (EAB) and elastic modulus (E) were
130 determined according to Llana-Ruiz-Cabello et al. (2015a).
131
132 *2.7 Thermal analysis*
133 A Differential Scanning Calorimeter DSC Q2000 (TA Instruments) was used to develop the
134 thermal analysis of films. Crystallisation temperature (Tc), and melting temperature (Tm) of
135 the different PP films were measured by the method described in Llana-Ruiz-Cabello et al.
136 (2015a).

The crystallinity was calculated as the ratio between the enthalpy of the melting point to the corresponding theoretical enthalpy of 100 % crystalline PP (estimated as 165 J/g for PP according to Zhu (2002) in percentage basis.

2.8 Antimicrobial activity in cooked ham

2.8.1 Model cooked ham

Freshly made cooked ham was prepared with following recipe: 75 % of pork meat was mixed with 25 % of salt brine, containing 5 % of NaCl, using an electric mixer (RM-20, MAINCA SL, Barcelona, Spain). Then, 9 % of potato starch was added. This mixture was filled in a cook-in-casings of 80 mm calibre (Cryovac, Sealed air, Elmwood Park, USA) and tempered at 4 ºC before cooking occurred at 75 ºC to a core set of 70ºC. After cooling at 5 ºC, the cooked ham sausages were cut into slides of 50 cm^2.

2.8.2 Microbial strain

Brochothrix thermosphacta, supplied by the Spanish Type Culture Collection (CECT), was grown overnight on brain heart infusion (BHI, Sharlau, Barcelona, Spain) at 28 ºC in aerobic atmosphere.

2.8.3 Experimental design

Three slides of ham were placed in polystyrene trays (135 x 180 mm, BANDESUR, Alcalá la Real, Spain) for each treatment and inoculated with a suspension of *B.thermophacta* to yield a final bacterial level of 10^5 CFU/cm^2 using a Digralsky spreader. In order to separate the slider the developed films were used as interlayer. Different trays were prepared using PP and PP containing OEO (2, 5 and 8 %) or PP containing Proallium® (2, 3 and 4 %).

161 Samples packaged were stored in polyethylene plastic bags under vacuum (Technotrip EVT-10-
162 2-CD-SC, Barcelona, Spain) and stored at 5 ºC.
163 The antimicrobial activity of PP films was evaluated after 1, 6, 12, 21, 27 and 60 days of
164 packaging by studying the evolution of *B. thermophacta* population in the cooked ham along
165 the storage time.
166
167 *2.8.4 Microbial analysis*
168 Samples of the surface of each slide of ham were taken at selected times to measure viable
169 counts of bacteria. Each slide (aprox. 14 g) were aseptically removed and homogenized with
170 125 ml of peptone water (0.1 % peptone) using a Masticator blender (IUR, Barcelona, Spain)
171 for 1 min. Then, a decimal dilution series was prepared for plating on STAA agar (Oxoid LTD,
172 Basingstoke, Hampshire, England). Finally, plates were incubated aerobically at 28 ºC for 48-72
173 h before bacterial counts were determined. Results were expressed as colony forming units
174 per square centimeter (CFU/cm2)
175
176 *2.9 Sensory analyses*
177 The sensory tests were performed on days 1, 4, 11, 18, 24, 32 and 60 after packing according
178 to the method described by Llana-Ruiz-Cabello et al. (2016).
179 Finally, the panellists were asked to answer about their willingness to purchase the product.
180
181 *2.10 Packaging and storage*
182 Several packages were produced containing three slices of “sliced ham” (approximately, 15
183 grams per slice) packed in contact with the different PP films containing OEO and Proallium®.
184 For this purpose, 4 pieces of dimensions 8 x 17 cm of each PP film were used in total per
185 package; 2 of them inserted between the three slices of ham like they were separators and

186 another two pieces of PP films used to cover the slices of ham at the top and at the bottom.
187 The sandwich of PP films and sliced ham was vacuum packed into commercial bags of
188 PP/polyamide using a Multivac Vacuum Chamber Machine (Germany) at 150 mbar.
189 Bags of "ham", both control and samples were stored at 4 ºC for 7 days, simulating commercial
190 conditions of production, transport and commercialization. All developed films were stored at
191 -20 ºC before their characterization.
192
193 *2.11 Statistical analysis*
194 All measurements were performed at least in triplicate. Results were presented as means ± SD;
195 significant differences were consider when $p<0.05$. The analysis of variance (ANOVA) was used
196 to evaluate the significance in the difference between factors and levels. Comparison of the
197 means was done by Dunnet's test. The statistical analysis of the data was performed using
198 GraphPad InStat software (GraphPad Software Inc., La Jolla, CA, USA).
199 For the sensory analysis STATGRAPHICS Plus for Windows statistical software was used to
200 calculate the analysis of variance multifactor (ANOVA) and Fisher's least significant difference
201 (LSD) to evaluate the impact of the day and packaging material on sensory attributes.
202 Significant differences were determined at $p<0.05$.
203
204 **3. Results and Discussion**
205 *3.1 Content of OEO and Proallium® and their main constituents incorporated in the films*
206 The TGA curves obtained for OEO, Proallium®, PP and PP films containing different
207 concentrations of OEO and Proallium® are observed in Figure 1. Both, OEO and Proallium®
208 showed a lower thermal stability than the PP films. OEO, which is mainly composed by a
209 mixture of different volatile substances, showed a gradual loss of mass from room
210 temperature up to 180 ºC. Proallium® also showed a gradual loss of mass occurring at

211 temperatures around 150 ºC as consequence of the volatiles which represent almost an 80% of
212 the Proallium® composition. The other 20% of the chemical composition of Proallium® is lost in
213 a second degradation step occurring between 350 and 450 ºC. On the other hand, the TGA
214 curve for PP film showed a degradation step at temperatures from 400 to 500 ºC.
215 The TGA curves for the PP films containing OEO and Proallium® showed two weight loss steps.
216 The first one occurring from 100º C up to 250 ºC corresponds mainly to the loss of mass
217 related with the OEO and Proallium® incorporated into the PP films. Therefore, the
218 measurement of the weight lost at 250 ºC provides an estimation of the overall content of
219 OEO incorporated into the PP during the manufacturing of the films.
220 In relation to this, the contents of OEO and Proallium® incorporated in the materials are found
221 in table 1. The yield of incorporation of OEO into the PP films by melting extrusion achieved an
222 average value near an 80%, which can be considered quite good attending to the volatile
223 nature of the OEO and the temperatures of the process (Ramos et al., 2012). The yield of
224 incorporation for Proallium® was 85% except for the incorporation of 4% w/w Proallium®
225 which was 70%. These results are in agreement with those previously obtained for OEO and
226 Proallium® included in another plastic matrix, PLA, with weight losses around 70-80% (Llana-
227 Ruiz-Cabello et al., 2015a, 2016). Similarly, other authors have evidence similar behaviour
228 explaining that the losses observed are also due to the volatility during the extrusion process
229 (Altiok, Altiok & Tihminlioglu, 2010; Ramos et al., 2012).
230 On the other hand, the analysis of the main constituents of OEO and Proallium® detected that
231 the higher the amount of active agent incorporated, the higher the content of active
232 components. The values obtained are shown in table 1. In relation to the overall OEO content
233 into the PP films, carvacrol represents the 60%-75% of the total while thymol represents the
234 7%-9%. Considering the composition of the OEO declared by the supplier which is 55%
235 carvacrol and 5% thymol (Llana-Ruiz-Cabello et al., 2017), this means that the extrusion

process led to a concentration of these two molecules probably due to the higher selective losses of the more volatile substances of the OEO (e.g. α-pinene, α- and β-terpinene or ρ-cymene) during the process. In fact, these substances show vapour pressure values from 1 to almost 5 mmHg while carvacrol and thymol have a vapour pressure around 0.02 mmHg.

Finally, concerning the content of PTSO in the PP films containing Proallium®, the results evidenced that PTSO represents almost a 10% of the overall Proallium® incorporated to the films. This value is slightly lower than the 14.5% of PTSO content declared by the supplier for Proallium®-SO-DMC (Llana-Ruiz-Cabello et al., 2015b) thus pointing out towards a selective loss of this active agent in comparison with other components of Proallium® such as propylenglycol.

3.2 Physical properties

Consumers require transparent packages in order to evaluate the aspect of the food product to take the most adequate buying decision (Sehrawet & Kundu, 2007). Table 2 shows the transparency values. PP films not containing OEO and Proallium® showed a transparency value of 1.56. This value shows a moderate transparency of the PP films. In particular, transparency parameter was highly affected by the addition of high contents of OEO. Hence, the incorporation of 8% of OEO decreased the transparency in a 40%. On the other hand, Proallium® affected in a higher extend the transparency of the films than the incorporation of OEO. In this case, films containing 2% Proallium® had transparency values similar to films containing 8% OEO. This finding were also observed when these active agents were incorporated into PLA, Proallium® exhibiting changes in the transparency when included in PLA but OEO had no effect (Llana-Ruiz-Cabello et al., 2015a, 2016a).

260 The thickness of the PP films was ranged between 80 and 97 µm. Although, the addition of
261 OEO and Proallium® to PLA (Llana-Ruiz.Cabello, et al. 2015a, 2016a) was found to decrease the
262 thickness of the films due to the plastification of the polymeric matrix, in the case of PP such
263 effect was not significant from a statistical point of view. Only the film containing the highest
264 amount of OEO had a significant decrease of the thickness.
265
266 *3.3 Mechanical properties*
267 Table 2 shows the mechanical properties of the different active films in terms of tensile
268 strength, Young's modulus and elongation at break. Mechanically, PP is a semi-rigid and
269 though material. In relation to this, Young's modulus gives a measurement of the stiffness
270 (rigidity). While increasing the content of OEO from 2% to 8% did not produce a significant
271 variation in Young's modulus, the incorporation of different amounts of Proallium® provoked a
272 significant variation on this parameter. Anyway, PP films containing these both extracts were
273 less resistant to deformation than PP films thus becoming materials more flexible.
274 On the other hand, the incorporation of OEO and Proallium® significantly decreased the tensile
275 strength. Elongation at break was also affected by the incorporation of the OEO and especially
276 with Proallium® although non-significant statistical differences with regards PP films were
277 actually found. Therefore, considering tensile strength and elongation at break, the
278 incorporation of OEO and Proallium® produced films that were less strong due to a
279 plasticisation effect of both extracts.
280 Similar results were obtained by Ramos et al. (2012) who observed that the incorporation of
281 carvacrol and thymol to PP materials provoked a clear decrease in Young's modulus and tensile
282 strength, and a slightly increase in elongation at break. However, EVOH-coated PP films
283 containing OEO exhibited an increase in the tensile resistance of PP films and a reduction in
284 the elongation at break (Muriel-Galet et al., 2013). Nevertheless, clear of evidence of the

285 plasticisation effect of EO when incorporated to the plastic matrix have been observed by
286 many authors (Altioket al., 2010; Ahmad, Benjakul, Prodpran & Agustini, 2012; Marcos et al.,
287 2014; Llana-Ruiz-Cabello et al., 2015a,2016a).
288
289 *3.4 Thermal properties*
290 Table 3 shows the crystallization (T_c) and melting temperatures (T_m) as well as their
291 corresponding enthalpies (ΔH_c and ΔH_m) and the degree of crystallinity (in %) for all the PP
292 films developed.
293 The PP films incorporating OEO decreased all the thermal parameters in comparison with neat
294 PP films. The enthalpies related to the crystallization and melting point also decrease as the
295 content of OEO incorporated into the PP films increases. Crystallinity degree significantly
296 decreased for the PP films containing the highest OEO levels (i.e. 5% and 8%). Similar results
297 were obtained by Ramos et al. (2012) who observed that the incorporation of carvacrol and
298 thymol to PP materials provoked a clear decrease in crystallinity and melting enthalpy while
299 the other thermal parameters were slightly affected.
300 In the case of Proallium®, the addition of higher contents of Proallium® further decreased T_c up
301 to 114 ºC but not T_m. The enthalpy related to crystallization decreased with the addition of
302 Proallium®. On the other hand, the enthalpy related to the melting point and the crystallinity
303 degrees were not significantly affected by the incorporation of Proallium® as it happened with
304 OEO.
305
306 *3.5 Antimicrobial activity in cooked ham*
307 *Brochotrix thermosphacta* is a ubiquitous microorganism throughout the meat production
308 chain, from animals to food and this microorganism can dominate the spoilage microbiota at
309 the expense of other genera (Remenant, Jaffrès, Dousset, Pilet & Zagorec, 2015). *B.*

310 *thermosphacta* is responsible to spoilage meat products, including cooked ham, developing a
311 disgusting, sour-sweet odor associated with acetoin production (Pin, García de Fernando &
312 Ordóñez, 2002).
313 In order to evaluate the antimicrobial activity of developed films against this microorganism,
314 samples were subjected to microbial analysis on the 1^{st}, 6^{th}, 12^{th}, 21^{st}, 27^{th} and 60^{th} day of
315 refrigerated storage. Results of microbial counts of *B*. *thermophacta* in cooked ham are shown
316 in table 4.
317 For control samples no changes in the bacterial counts were observed along the storage time.
318 The development of *B*. *thermophacta* could be stopped due to the vacuum-packed process
319 because the growth of this microorganism is influenced by available oxygen remaining in the
320 package (Remenant et al., 2015). The shelf life of sliced cooked hams packed in a modified
321 atmosphere or vacuum is limited to between 3-6 weeks (Leroy, Vasilopoulos, Van Hemelryck,
322 Falony & De Vuyst, 2009). In the present study, the active packaging containing Proallium® was
323 able to eliminate the content of *B*. *thermophacta* from cooked ham after 8 weeks of storage.
324 Moreover, PP-OEO films also decreased the microbial counts of this microorganism. Therefore,
325 the use of these interlayer films suppose a novel tool that in combination with other hurdles,
326 such as refrigeration or vacuum packaging, could extend the shelf life of meat products.
327 The ability of OEO, and its major compounds, incorporated in different film matrices to reduce
328 microbial counts in food has been reported by several authors. Ramos et al. (2012) reported
329 that PP films containing 8 % of carvacrol and thymol and films containing an 8 % mixture of
330 both substances showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia*
331 *coli*. Antimicrobial activity was observed in salad packaged in EVOH-coated PP films containing
332 OEO (Muriel-Galet et al., 2013) and polylactic acid (PLA) films containing OEO (Llana-Ruiz-
333 Cabello et al., 2016).
334

335 *3.6 Sensory analyses*

336 Figure 2 shows the median scores for each sensory descriptor at the different storage times
337 and packaging material as well as the box and whisker plot for the scores obtained along
338 storage time for each packaging material.

339 Smell was in general the lowest scored descriptor in comparison to the others and thus the
340 most critical one (Figure 2A). The panellists detected in most of the cases the inherent smell of
341 the oregano and *Allium* extracts and they evaluated the smell as something unexpected. In
342 fact, general acceptability and purchase intention were highly correlated to the smell.

343 Multifactor ANOVA showed a significant impact of both, packaging materials and days of
344 storage ($p < 0.05$) on the sensory perception of the smell. Ham in the control package was
345 above the threshold value of 5 from day 1 to day 11. From that point smell was slightly below
346 the threshold up to day 60. The active materials containing OEO were significantly below the
347 scores given to the control package. Materials containing 3% and 4% Proallium® were below
348 the control package although non-significant differences were found. The best results for the
349 smell were obtained for materials containing 2% of Proallium®. This material was scored above
350 5 for the entire storage time.

351 Figure 2B shows the median scores for visual appearance. In this case, this sensory attribute
352 was above or very close to the threshold value for the different materials and storage time.
353 ANOVA identified the storage time as non-significant ($p > 0.05$). In the case of the packaging
354 materials, just the material containing 2%OEO was significantly different from the other
355 materials, scoring the worst. Surprisingly, visual appearance was better evaluated than smell,
356 but scarcely influenced in the purchase intention of consumers.

357 General acceptability showed a similar trend to the smell (Figure 2C). ANOVA showed a
358 significant impact of the storage time and packaging material ($p < 0.05$). Acceptability of the
359 ham packaged in the control material decreased from day 1 to day 11 and remained close or

above the threshold up to day 60. The active materials containing OEO and Proallium® showed values in general below the control package although with very few significant differences. The exemption was PP containing 2% Proallium®, which scored the best and above the threshold for the entire storage.

Finally, Table 5 shows the percentage of panellists willing to purchase or consume the ham packaged into the different materials and storage time. The best results were obtained for the control material and the PP containing 2% Proallium®. Purchase intention was higher than 50% for both materials and along the entire storage time. Materials containing OEO were in general below 50% including 0% purchase intention at some specific days.

4. Conclusions

Polypropylene (PP) films containing Proallium® or OEO showed similar thermical and mechanical properties than PP. Moreover, Proallium® active films seemed to be more effective against *B. thermosphacta* than PP films containing OEO. Regarding the sensorial analyses, materials containing the lowest Proallium® percentage was highly accepted by the consumers, even better than the control package. However, oregano flavour was noticed as strange to the ham and have a negative impact in purchase intention.

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384 **6. References**

385 Ahmad, M., Benjakul, S., Prodpran, T. & Agustini, TW. (2012). Physico-mechanical and
386 antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated
387 with essential oils. *Food Hydrocolloids, 28,* 189–199.

388 Altiok, D., Altiok, E., & Tihminlioglu, F. (2010). Physical, antibacterial and antioxidant properties
389 of chitosan films incorporated with thyme oil for potential wound healing applications. *Journal*
390 *of materials Science: Materials in Medicine*, 21, 2227-2236.

391 Appendini P, Hotchkiss JH. (2002). Review of antimicrobial food packaging. *Innovative Food*
392 *Science & Emerging Technologies,* 3: 113-126.

393 Baños, A., Ananou, S., Martínez-Bueno, M., Gálvez, A., Maqueda, M. & Valdivia, E. (2012).
394 Prevention of spoilage by enterocin AS-48 combined with chemical preservatives, under
395 vacuum, or modified atmosphere in cooked ham model. *Food Control, 24*, 15-22.

396 Carocho, M., Morales, P. & Ferreira, I.C.F.R. (2015). Natural food additives: *Quo vadis?*. *Trends*
397 *in Food Science & Technology, 45,* 284-295.

398 Commission Regulation (EC) No 450/2009 of 29 May 2009 on active and intelligent materials
399 and articles intended to come into contact with food.

400 De Novile, M.A., Conte, A., Buonocore, G.G., Incoronato, A.L., Massaro, A. & Panza, O. (2009).
401 Active packaging by extrusion processing of recyclable and biodegradable. *Journal of Food*
402 *Engineering, 93,* 1-6.

403 Gigli, M., Lotti, N., Gazzano, M., Siracusa, V., Finelli, L., Munari, A. & Rosa, M.D. (2014).
404 Biodegradable aliphatic copolyesters containing PEG-like sequences for sustainable food
405 packaging applications. *Polymer Degradation and Stability, 105,* 96-106.

406 Leroy, F., Vasilopoulos, C., Van Hemelryck, S., Falony, G., De Vuyst, L. (2009). Volatile analysis
407 of spoiled, artisan-type, modified-atmosphere-packed cooked ham stored under different
408 temperatures. *Food Microbiology, 26,* 94–102.

409 Llana-Ruiz-Cabello, M., Pichardo, S., Baños, A., Núñez, C., Bermúdez, J.M., Guillamón, E.,
410 Aucejo, S. & Cameán, A.M. (2015a). Characterisation and evaluation of PLA films containing an
411 extract of *Allium* spp. to be used in the packaging of ready-to-eat salads under controlled
412 atmospheres. *LWT-Food Science and Technology, 64,* 1354-1361.
413 Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Moreno, F.J., Baños, A.,
414 Nuñez, C., Guillamón, E. & Cameán, A.M (2015b) Acute toxicological studies of the main
415 organosulfur compound derived from Allium sp. intended to be used in active food packaging.
416 *Food and Chemical Toxicology, 82,* 1–11.
417 Llana-Ruiz-Cabello, M., Pichardo, S., Bermúdez, J.M., Baños, A., Núñez, C., Guillamón, E.,
418 Aucejo, S. & Cameán A.M. (2016). Development of PLA films containing oregano essential oil
419 (*Origanum vulgare* L *virens*) intended to be use in food packaging. *Food Additives &*
420 *Contaminants: Part A, 33,* 1374-1386.
421 Llana-Ruiz-Cabello, M. Maisanaba, S., Puerto, M., Pichardo, S., Jos, A., Moyano, R. & Cameán,
422 A.M. (2017) A subchronic 90-day oral toxicity study of Origanum vulgare essential oil in rats.
423 *Food and Chemical Toxicology, 101,* 36-47.
424 Marcos, B., Sárraga, C., Castellari, M., Kappen, F., Schennink, G. & Arnau, J. (2014).
425 Development of biodegradable films with antioxidant properties based on polyesters
426 containing α-tocopherol and olive leaf extract for food packaging applications. *Food Packaging*
427 *Shelf Life, 1,* 140–150.
428 Muriel-Galet, V., Cerisuelo, J.P., López-Carballo, G., Aucejo, S., Gavara, R. & Hernández-Muñoz,
429 P. (2013). Evaluation of EVOH-coated PP films with oregano essential oil and citral to imrpove
430 the shelf-life of packaged salad. *Food Control, 30,* 137-143.
431 Pin, C., García de Fernando, G.D., & Ordóñez, J.A., (2002). Effect of Modified Atmosphere
432 Composition on the Metabolism of Glucose by Brochothrix thermosphacta. *Applied and*
433 *Environmental Microbiology, 68*, 4441–4447.

Ramos, M., Jiménez, A., Peltzer, M. & Garrigós MC. (2012). Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *Journal of Food Engineering, 109,* 513-519.

Ramos, M., Beltrán, A., Peltzer, M., Valente, A.J.M. & Garrigós, M.C. (2014). Release and antioxidant activity of carvacrol and thymol from polypropylene packaging films. *LWT-Food Science and Technology, 58,* 470-477.

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M.F. & Zagorec; M. (2015). Bacterial spoilers of food : Behaviour, fitness and functional properties. *Food Microbiology, 45,* 45-53.

Ribeiro-Santos, R., Andrade, M., Ramos de Melo, N. & Sanches-Silva, A. (2017). Use of essential oils in active food packaging: Recent advances and future trends. *Trends in Food Science & Technology, 61,* 132-140.

Robertson, G.L. (2012). Food Packaging: Principles and practice. Boca Raton FL, CRC Press Taylor and Francis Group.

Sanches-Silva, A., Costa, D., Albuquerque, T. G., Buonocore, G. G., Ramos, F., Castilho, M. C., et al. (2014). Trends in the use of natural antioxidants in active food packaging: a review. *Food Additives and Contaminants: Part A, 31(3),* 374-395.

Sehrawet, M. & Kundu, S.C. (2007). Buying behavior of rural and urban consumers in India: the impact of packaging. *International Journal of Consumer Studies, 31,* 630–638.

Sung, S.Y., Sin, L.T., Tee, T.T., Bee, S.T., Rahmat, A.R., Rahman, W.A.W.A., Tan, A.C. & Vikhraman, M. (2013). Antimicrobial agents for food packaging applications. *Trends in Food Science & Technology, 33,* 110-123.

Sung, S. Y., Sin, L. T., Tee, T. T., Bee, S. T., Rahmat, A. R., & Rahmat, W. A. W. A. (2014). Control of bacteria growth on ready-to-eat beef loaves by antimicrobial plastic packaging incorporated with garlic oil. *Food Control, 39,* 214-221.

458 Suppakul, P., Miltz, M., Sonneveld, K. & Bigger, S.W. (2003). Active packaging technologies with
459 an emphasis on antimicrobial packaging and its application. *Concise Reviews and Hypotheses in*
460 *Food Science, 68,* 408-420.
461 Teixeira, B., Marques, A., Pires, C., Ramos, C., Batista, I., Saraiva, J. A., et al. (2014).
462 Characterization of fish protein films incorporated with essential oils of clove, garlic and
463 origanum: physical, antioxidant and antibacterial properties. *Food Science and Technology, 59,*
464 533-539.
465 Van Haute, S., Raes, K., Van der Meeren, P. & Sampers, I. (2016). The effect of cinnamon,
466 oregano and thyme essential oils in marinade on the microbial shelf life of fish and meat
467 products. *Food Control, 68,* 30-39.
468 Zhu, L. (2002). Chapter 10 - Thermal analysis of polymer films. In Z. D. C. Stephen (Ed.),
469 Handbook of Thermal Analysis and Calorimetry (Vol. Volume 3, pp. 355-407): Elsevier Science
470 B.V.
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Figure caption

Figure 1. TGA curves obtained for OEO, Proallium, PP film and PP containing OEO and Proallium®.

Figure 2. Sensory analysis measured as smell (A), visual appearance (B) and general acceptability (C) of cooked ham in control and active packaging containing OEO (2, 5, 8 %) or Proallium® (2, 3 4 %). Pro: Proallium®.

Table caption

Table 1. Content of OEO, Proallium® and their main compounds incorporated into the PP films.

Table 2. Thickness, transparency values and mechanical properties of PP films incorporated with OEO or Proallium® at various concentrations. TS: Tensile strength. EAB: Elongation at break. E: Young´s module. Values are given as mean ± SD from triplicate determinations for thickness and transparency and at least 6 replicates for mechanical properties. Figures within each column with a common letter at the superscript mean that they are not significantly different at 95% of confidence level.

Table 3. Thermal properties of PP films incorporating OEO and Proallium® (2nd heating cycle). Tc: Crystallization temperature. ΔHc: crystallizing enthalpy. Tm: Melting temperature. ΔHm: melting enthalpy. Figures within each column with a common letter at the superscript mean that they are not significantly different at 95% of confidence level.

Table 4. Antimicrobial activity against B. thermosphacta of films containing OEO and Proallium® in cooked ham. Values are given as the mean ± SD from three different experiments. * significantly different from the control ($P<0.05$); ** highly significantly different from the control ($P<0.01$).

Table 5. Percentage of panellists willing to purchase or consume the ham packaged into the different materials and storage time.

Table 1

Film sample	Overall content (%)	Carvacrol content (%)	Thymol content (%)	PTSO content (%)
PP 2% OEO	1.6 ± 0.3	1.10 ± 0.10	0.132 ± 0.011	
PP 5% OEO	3.9 ± 0.2	2.9 ± 0.3	0.33 ± 0.02	
PP 8% OEO	5.84 ± 0.11	4.2 ± 0.3	0.420 ± 0.011	
PP 2% Proallium	1.680 ± 0.014			0.183 ± 0.011
PP 3% Proallium	2.56 ± 0.06			0.196 ± 0.015
PP 4% Proallium	2.82 ± 0.10			0.25 ± 0.06

Table 1. Content of OEO, Proallium and main substances incorporated into the PP films

Table 2

Film sample	Thickness (μm)	Transparency	Tensile Strength (MPa)	Young's modulus (GPa)	Elongation at break (%)
PP	90 ± 3^{bc}	1.56 ± 0.10^{a}	42 ± 6^{cd}	1.43 ± 0.24^{d}	500 ± 140^{ab}
PP 2% OEO	86 ± 2^{ab}	2.14 ± 0.03^{b}	38 ± 4^{bc}	0.83 ± 0.10^{b}	490 ± 80^{ab}
PP 5% OEO	88.3 ± 1.2^{b}	2.16 ± 0.07^{b}	38 ± 4^{b}	0.85 ± 0.04^{b}	560 ± 100^{b}
PP 8% OEO	80 ± 2^{a}	3.03 ± 0.12^{c}	30 ± 4^{a}	0.80 ± 0.12^{ab}	530 ± 140^{ab}
PP 2% Proallium	97 ± 5^{c}	2.91 ± 0.18^{cd}	45 ± 6^{d}	1.28 ± 0.21^{c}	510 ± 180^{ab}
PP 3% Proallium	86 ± 6^{ab}	3.26 ± 0.19^{de}	37 ± 3^{b}	0.89 ± 0.14^{b}	420 ± 70^{a}
PP 4% Proallium	89 ± 7^{b}	3.4 ± 0.3^{e}	36 ± 3^{b}	0.68 ± 0.13^{a}	420 ± 70^{a}

Table 3

Film sample	Tc (ºC)	ΔHc (J/g)	Tm (ºC)	ΔHm (J/g)	Crystallinity (%)
PP	120.5 ± 0.7^{d}	107.7 ± 0.3^{d}	162.5 ± 0.7^{c}	113.9 ± 0.4^{b}	69.0 ± 0.2^{b}
PP 2% OEO	120.0 ± 0.0^{d}	101.2 ± 0.4^{bc}	161.0 ± 0.0^{b}	109.9 ± 4.5^{b}	66.6 ± 0.8^{b}
PP 5% OEO	117.0 ± 0.0^{c}	94.4 ± 0.1^{a}	161.0 ± 0.0^{b}	100.8 ± 1.3^{a}	61.1 ± 2.0^{a}
PP 8% OEO	115.5 ± 0.7^{b}	95.0 ± 2.5^{a}	159.5 ± 0.7^{a}	99.8 ± 3.7^{a}	60.5 ± 0.9^{a}
PP 2% Proallium	115.5 ± 0.7^{b}	103.0 ± 2.0^{c}	160.0 ± 0.0^{ab}	112.1 ± 1.3^{b}	67.9 ± 2.7^{b}
PP 3% Proallium	114.5 ± 0.7^{ab}	99.6 ± 1.7^{b}	160.5 ± 0.7^{ab}	114.2 ± 3.3^{b}	69.2 ± 0.8^{b}
PP 4% Proallium	114.0 ± 0.0^{a}	100.3 ± 0.9^{bc}	161.0 ± 0.0^{b}	111.3 ± 1.4^{b}	67.5 ± 2.2^{b}

Table 4

	Day 0	Day 1	Day 6	Day 12	Day 21	Day 27	Day 60
CONTROL	4.7 ± 0.0	4.8 ± 0.2	4.7 ± 0.2	4.8 ± 0.07	4.6 ± 0.2	4.7 ± 0.2	4.8 ± 0.0
OEO 2 %	4.7 ± 0.0	4.4 ± 0.1	3.5 ± 0.8*	2.9 ± 0.1**	2.7 ± 0.2**	2.3 ± 0.1**	2.3 ± 0.1**
OEO 5 %	4.7 ± 0.0	3.8 ± 0.2*	2.9 ± 0.1**	1.6 ± 0.6**	2.0 ± 0.1**	1.6 ± 0.0**	1.8 ± 0.0**
OEO 8 %	4.7 ± 0.0	2.6 ± 0.2**	1.7 ± 0.1**	1.2 ± 0.1**	1.1 ± 0.1**	1.0 ± 0.0**	1.2 ± 0.0**
P 2 %	4.7 ± 0.0	2.4 ± 0.4**	1.8 ± 0.1**	1.5 ± 0.3**	1.5 ± 0.1**	1.4 ± 0.1**	0.0 ± 0.0**
P 3 %	4.7 ± 0.0	1.7 ± 0.1**	1.5 ± 0.2**	1.2 ± 0.3**	1.2 ± 0.2**	1.0 ± 0.0**	0.0 ± 0.0**
P 4 %	4.7 ± 0.0	1.4 ± 0.1**	1.2 ± 0.2**	1.0 ± 0**	0.9 ± 0.2**	0.0 ± 0.0**	0.0 ± 0.0**

Table 5

Day	Control	2%OEO	5%OEO	8%OEO	2%Pro	3%Pro	4%Pro
1	83	50	50	67	83	83	83
4	89	67	0	50	63	67	80
11	61	33	50	17	67	50	67
18	61	17	0	0	83	67	83
24	56	17	33	33	100	33	17
32	67	50	0	33	100	50	33
60	67	33	33	50	50	50	17

Figure 1

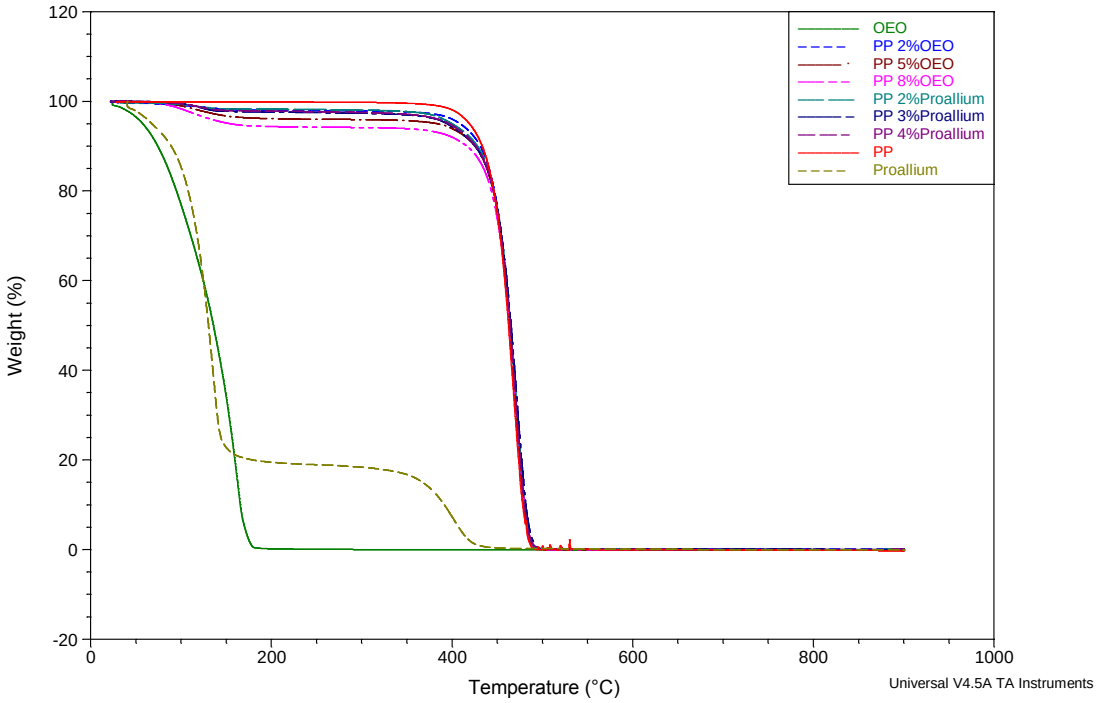
OEO
PP 2%OEO
PP 5%OEO
PP 8%OEO
PP 2%Proallium
PP 3%Proallium
PP 4%Proallium
PP
Proallium
Weight (%)
Temperature (°C)
120
100
80
60
40
20
0
-20
0
200
400
600
800
1000
Universal V4.5A TA Instruments

Figure 2

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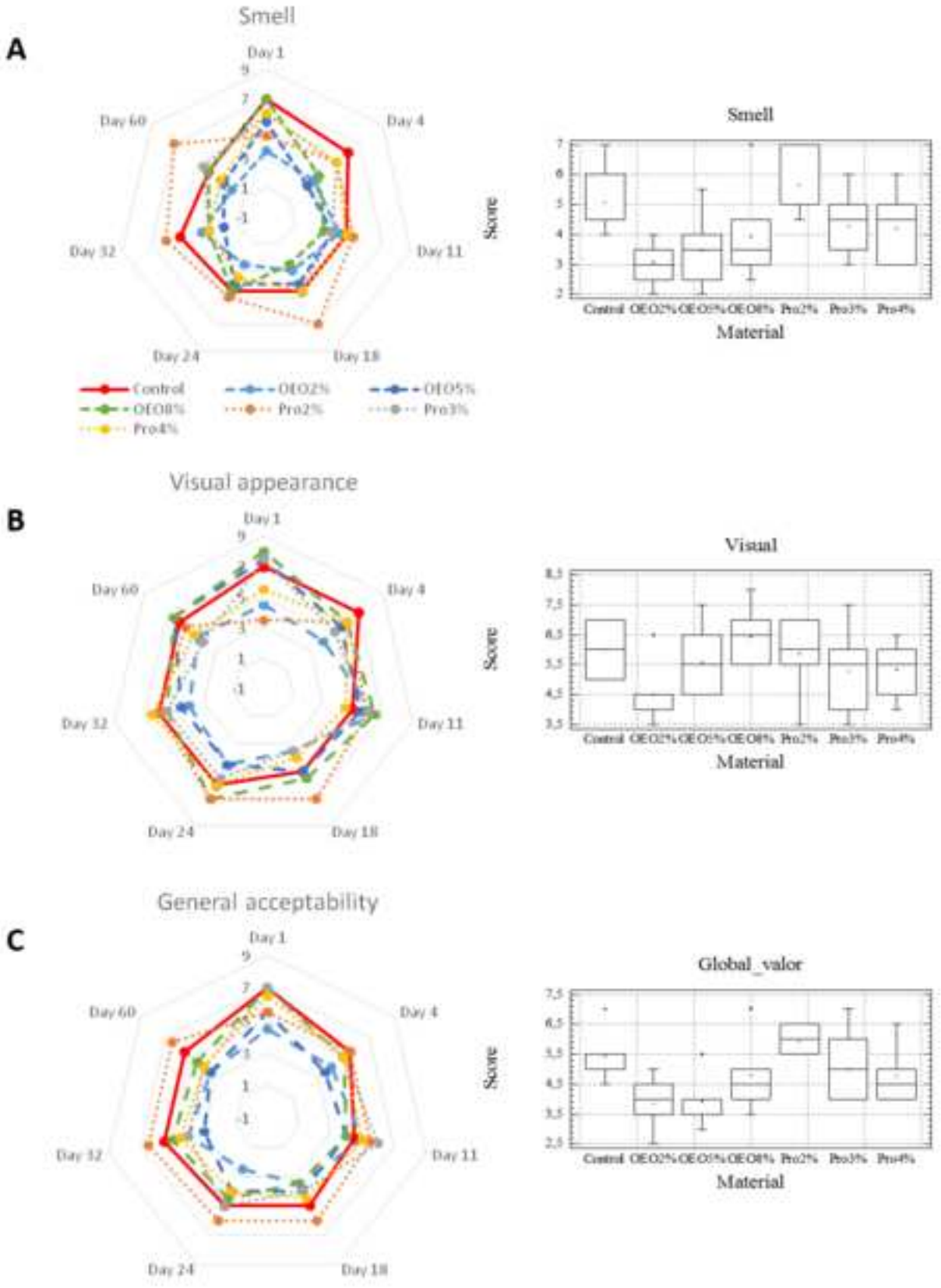

CAPÍTULO 14 / CHAPTER 14

María Llana-Ruiz-Cabello, Silvia Pichardo, Nicasio T. Jiménez-Morillo, Paloma Abad, Enrique Guillamón, Francisco J. González-Vila, Ana M. Cameán, Jose A. González-Pérez

CHARACTERISATION OF A BIO-BASED PACKAGING CONTAINING A NATURAL ADDITIVE FROM ALLIUM SPP. USING ANALYTICAL PYROLYSIS AND CARBON STABLE ISOTOPES.

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Characterisation of a bio-based packaging containing a natural additive from *Allium* spp. using analytical pyrolysis and carbon stable isotopes

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ABSTRACT

Analytical pyrolysis (Py-GC/MS) was used to fingerprint a polylactic acid (PLA) with polybutylene succinate (PBS) (950 g Kg^{-1}:50 g Kg^{-1}) film extruded with variable quantities (0, 20, 50 and 65 g Kg^{-1}) of a commercial additive (Proallium®) prepared from *Allium* spp. extracts. The carbon isotopic signature($\delta^{13}C$) was used to estimate the amount of additive to be incorporated into the casting of the active biopolymer. The main PLA:PBS pyrolysis products were lactide enantiomers and monomer units, in addition to structures from the PBS fraction. The pyrolysis of the plant extract additive, apart from a number of organosulphur compounds derived from the active ingredients, released oligomers of polyethylene glycol, alkyl ethers and a conspicuous peak of oleic acid, 3-hydroxypropyl ester compatible with a polyoxyethylene sorbitan monooleate surfactant. The sulphur compound, propyl sulphide, was a diagnostic peak with which to trace the additive in the polymer. In fact, a correlation coefficient of value 0.997R^2 ($p < 0.001$) was found between the chromatographic area of this peak and the amount of additive in the bioplastic. The real amount of additive incorporated in the bioplastic was estimated through a mass-balance equation, taking advantage of the differential $\delta^{13}C$ signatures between the polymeric base and the additive.

1. Introduction

Environmental, economic and safety challenges motivate a shift towards safer materials for food packaging. Due to considerations related to the use of non-biodegradable petrochemical-based plastics such us polyolefins, polyesters, polyamides, etc., the food and packaging industries show an increasing interest in the development of healthier and more environmentally-friendly materials. New packaging is made of biopolymers based on polysaccharides, proteins and lipids which are generally biodegradable, and have low toxicity [1]. Some of these materials are synthesised chemically from naturally-derived monomers such us lactic acid [2]. Polylactic acid (PLA) is one of these bioplastics derived from fermented plant starch, mainly from corn, and it is becoming one of the alternatives to the traditional petroleum-based plastics [3]. PLA can be processed with a large number of techniques. PLA is commercially and largely available (large-scale production) in a wide range of grades. It has a reasonable price and some remarkable properties to fulfil different applications. Moreover its useful optical, physical and mechanical characteristics make PLA an attractive polymer to develop food contact materials [4,5]. PLA can be tuned to satisfy different applications because it can vary from soft and elastic materials to stiff and high strength materials [6]. However, the brittleness and stiffness of PLA can be major drawbacks for some of these applications. A possible strategy to decrease the brittleness is to make a blend between PLA and others polymers. In this sense, polybutylene succinate (PBS) has demonstrated its ability to increase the bulk crystallinity of pure PLA [7]. Moreover, when PLA is extruded in combination with plasticizers, such as PBS, it becomes thermoplastic, mouldable and an amorphous material with an excellent oxygen barrier characteristic [4].

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Market globalisation results in increased distribution distances and longer storage times. These factors, together with the consumer demands of ready-to-eat products, encourages the food industry to develop new packaging systems [8]. In addition, the current global demand for minimally-processed and preservative-free food stuffs has promoted the use of new bioactive packaging techniques and ingredients from natural sources which are gaining increasing attention from the industry. One possibility to progress in quality and safety of packed products is to include additives that are able to improve polymer barrier properties by creating a bioactive material [9]. A bioactive polymer is designed to deliberately incorporate components that would release into or absorb substances from the packaged food or the environment surrounding the food [10].

In this regard, active packaging is an innovative food industry concept that combines different advances in an effort to enhance the safety of consumer demand for fresh-like, safe products [11]. In this sense, the use of natural extracts as functional ingredients is gaining attention due to their preservative action, aimed at avoiding lipid deterioration, oxidation and spoilage by microorganisms [12,13]. Moreover, the incorporation of natural extracts such as oregano or onion and garlic-based essential oil in food-packaging films allows the controlled release of active substances which reduce undesirable flavours caused by direct addition into food [14–16].

Recently, we have produced a biofilm containing a commercial natural extract (Proallium®) which was developed by the food industry [17]. This extract has been obtained from different species of *Allium* spp. (garlic and onion), and is composed of a blend of flavouring substances including disulphides, thiosulfinates and thiosulfonates, and a tiosulfinate oxide named propiltiosulfinate oxide (PTSO) for which the antimicrobial properties have been previously demonstrated [16,18]. This is an interesting substance in order to develop food contact materials (FCM) that can be used in active packaging to improve the shelf life of perishable vegetables. The biofilm manufacture includes extrusion, a widely-used technique in the plastics industry that involves high temperatures that limits its use in manufacturing polymeric materials containing volatile substances, mainly due to a lack of understanding of the behaviour of these materials in the extruder [19]. Therefore, due to the volatility of Proallium® and the temperature conditions used for the active bio-plastic, it is necessary to ascertain that the compound, as well as its active components, remains in the desired quantities within the final plastic film.

In this work, direct analytical pyrolysis (Py-GC/MS) was used for a detailed characterisation or "fingerprinting" of Proallium® and its detection within a bio-based 950 g kg^{-1} polylactic acid plastic (PLA) extruded with 50 g kg^{-1} polybutylene succinate (PBS) to ameliorate crystallinity. Also added mixtures of Proallium® extract (20, 50 and 65 g Kg^{-1} in dry weight) extruded into the bio-plastic to conform an active-package were studied. Carbon stable isotopic ($\delta^{13}C$) analysis was used to quantify the amount of the plant extract additive in the bioplastic.

2. Materials and methods

2.1. Supplies and chemicals

In this work, polylactic acid (PLA) extrusion-grade (2003D) was purchased in pellets from NatureWorks LLC (Minnetonka, USA). PLA 2003D is a transparent, thermoformable and high molecular weight biopolymer specifically designed for use in fresh food packaging. Moreover, polybutylene succinate (PBS) GS PlaTM FD92WD purchased from Mitshubishi Chemical Corporation (Tokyo, Japan) was used due to its flexibility and moldability. Commercial Proallium® (L14/7), extract obtained from *Allium* spp. with a PTSO concentration of 14.5%, was supplied by the manufacturer DOMCA S.A. (Alhendín, Granada, Spain). Chemicals for the different assays were purchased from Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

2.2. Film preparation

The different active PLA films were obtained by melt blending in a twin-screw extruder (DSE 20–40D, Brabender, Germany). Different concentrations (20, 50 and 65 g Kg^{-1} which correspond to 2, 5 and 6.5% w/w, respectively) of Proallium® were fed into the barrel trough via the lateral liquid port at L/D 10 in order to reduce possible volatility and degradation losses. Barrel temperatures were set at 200–205 °C working at a screw speed of 70 min^{-1}. In the control films the additive Proallium® was not added. The average thickness of the final films was adjusted to 80 μm (320 Gauge).

2.3. Analytical pyrolysis (Py-GC/MS)

Conventional chromatographic techniques for the study of polymers is restricted due to their high molecular mass and low volatility. Analytical pyrolysis is defined as the thermochemical decomposition of organic materials at elevated temperatures in the absence of oxygen [20]. The products of pyrolysis (pyrolysate) are amenable to chromatographic separation which, when combined with an appropriate detector i.e., mass spectrometry (Py-GC/MS), yields valid fingerprint information about the molecular structure and even of complex mixtures of natural and synthetic macromolecular substances [21]. This technique is commonly used in polymer science for the characterisation of synthetic plastics and additives [22–26] and bio-based polymers, including polylactic acid [27–32] and polybutylene succinate [32,33] plastics. Pyrolytic techniques have well-known additional advantages such as the requirement of small sample sizes and little to no sample preparation, thus providing convenience for inexpensive and relatively rapid analyses.

Direct pyrolysis-gas chromatography–mass spectrometry (Py-GC/MS) analysis was performed using a double-shot Pyrolyzer® (Frontier Laboratories, model 2020i) attached to a GC/MS system Agilent 6890N. Samples of 0.5 mg were placed in small crucible capsules and introduced into a preheated micro-furnace at 500 °C for 1 min. The evolved gases were transferred into the GC/MS for analysis. The gas chromatograph was equipped with a low polar fused silica (5% phenyl-methylpolysiloxane) capillary column Agilent J&W HP–5 ms Ultra Inert, of 30 m × 250 μm × 0.25 μm film thickness The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min^{-1}, from 100 °C to 300 °C at 10 °C min^{-1}, and stabilised at 300 °C for 10 min using a heating rate of 20 °C min^{-1} in the scan modus. The carrier gas used was helium at a controlled flow of 1 mL min^{-1}. The detector was an Agilent 5973 mass selective detector, and mass spectra were acquired at 70 eV ionising energy. Compound assignment was achieved via single-ion monitoring for various homologous series, via low-resolution mass spectrometry, and via comparison with published and stored (NIST and Wiley libraries) data.

2.4. Carbon stable isotope analysis ($\delta^{13}C$ IRMS)

Differences in the stable carbon isotope ($\delta^{13}C$ IRMS) composition are a valuable tool for food traceability/fraud detection [34–38]. A well-known large differential $\delta^{13}C$ value is between plants that are differing in their photosynthetic pathways. Most terrestrial plants are C3 plants with $\delta^{13}C$ values ranging from −24 to −34‰, whereas many aquatic, desert and salt marsh plants as well as tropical grasses have the C4 pathway and $\delta^{13}C$ values ranging from −6 to −19‰ [39]. Nowadays, lactic acid that is the main

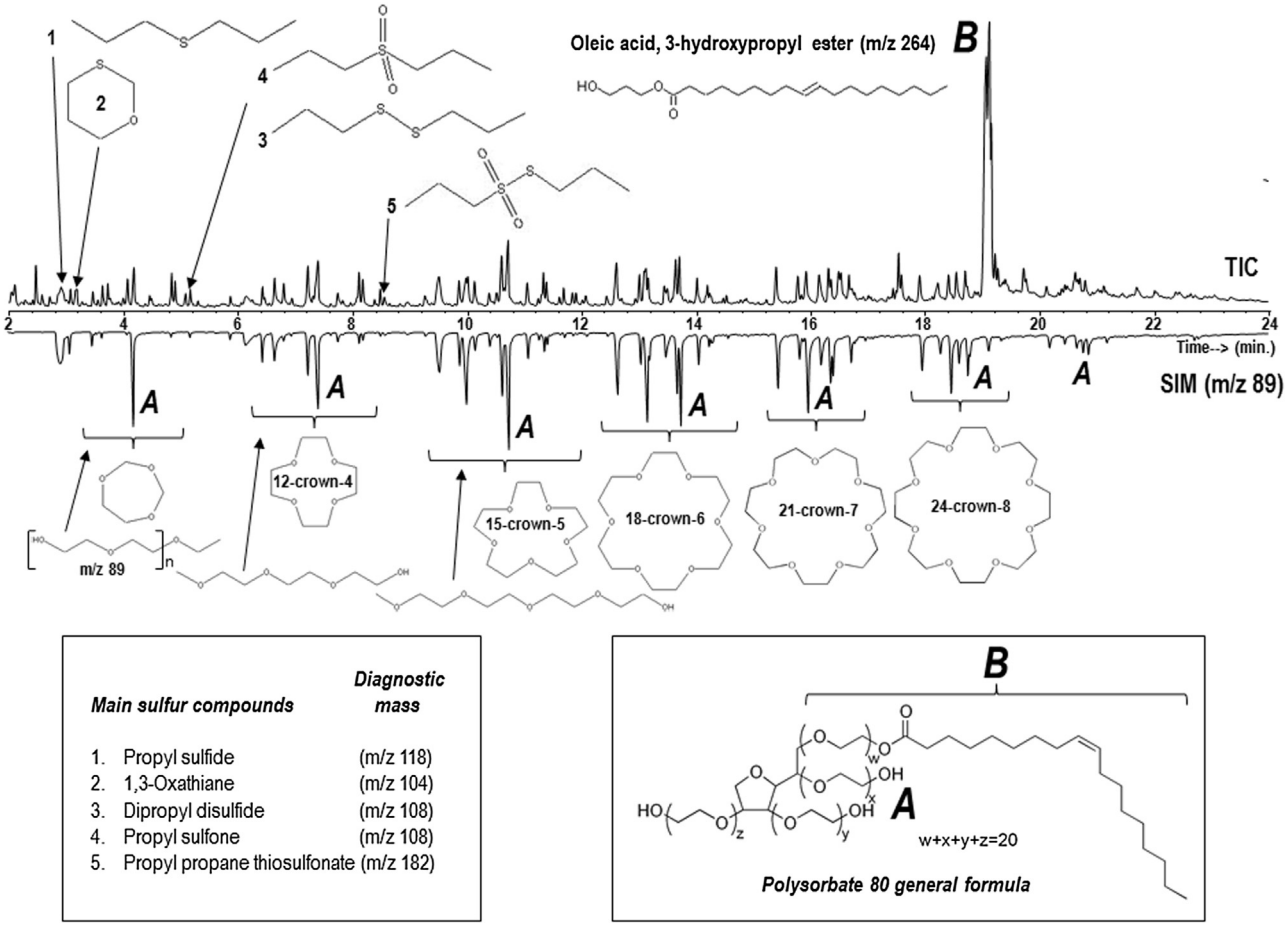

Fig. 1. Food additive Proallium® Total current ion chromatogram (TIC) with an indication of the main pyrolysis products released at 500 °C and single current ion chromatogram (SIM) for fragment with mass to charge ratio *m*/*z* 89.

feedstock for PLA based polymers is largely obtained by a biological fermentation of starch, usually using corn as raw material [40]. Corn is a C4 plant and is therefore bearing a heavier C stable isotope signature (enriched in the C^{13} isotope) than that of the added Proallium® extract obtained from C3 plants such as *Allium* spp. This fact makes IRMS a suitable technique to assess the presence of this additive in PLA based plastics.

The stable carbon isotopic signature ($\delta^{13}C$) was analysed using a Flash 2000 HT/IRMS system (Thermo Scientific, Bremen, Germany). The micro-analyser was coupled via a ConFlo IV interface unit to a continuous flow Delta V Advantage isotope ratio mass spectrometer (IRMS) (Thermo Scientific, Bremen, Germany) (EA-IRMS). The combustion furnace was set at 1020 °C and sample size was 1.00 mg wrapped in tin foil. Isotopic ratios are reported as parts per thousand (‰) deviations from appropriate standards recognised by the International Atomic Energy Agency (IAEA) [41]. The standard deviation of bulk $\delta^{13}C$ was typically less than ±0.05‰. Finally, the proportion of additive (Proallium®) in the bioplastic was calculated using a mass balance equation as described by Bernoux et al. [42].

Proportion of additive in bio-plastic = 100* (A–B)/(C–B)

A: $\delta^{13}C$ bioplastic with additive

B: $\delta^{13}C$ bioplastic

C: $\delta^{13}C$ additive

3. Results and discussion

Biodegradable polymers can now compete with non-biodegradable thermoplastics in different fields (packaging, textile, biomedical, etc.). Among these biopolyesters, PLA is one of the most promising biopolymer [43]. Due to concerns about the environmental and health, consumers are avoiding the use of petroleum-based conventional packaging, which takes hundreds of years to decompose, and food products containing synthetic additives or preservatives [17,44].

Additives designed to replace synthetic preservatives in food packaging as FCM are also novel approaches in the food industry. Proallium AP® is a commercial *Allium* extract that has been previously evidenced as antioxidant and antimicrobial when included in active food packaging containing lettuce [17]. In addition Proallium AP® appears to be safe after subchronic administration to Sprague-Dawley rats at doses up to 400 mg/kg/day [45]. Moreover, no damage has been reported for organosulfur compounds present in the extract that did not induce any significant adverse effects in Caco-2 cells and in the Ames test [15]. Similarly, no toxic effects were recorded in human cell lines or in rats exposed to the major component of Proallium AP®, propyl thiosulfinate oxide (PTSO), at the concentrations intended to be used in the food packaging [16].

3.1. Analytical pyrolysis of Proallium® additive

Total Ion Chromatogram (TIC) of the pyrolysis products (Pyrogram) release at 500 °C from the commercial plant extract (Proallium®), with an indication to the chemical identities of the main pyrolysis products is depicted in Fig. 1. The pyrogram shows a typical mixture of oligomers of polyethylene glycol and its alkyl ethers including cycled crown ethers (A), this pattern is neater when searching for the molecular diagnostic fragment at *m*/*z* 89. Also, a conspicuous peak of Oleic acid, 3-hydroxypropyl ester (B)

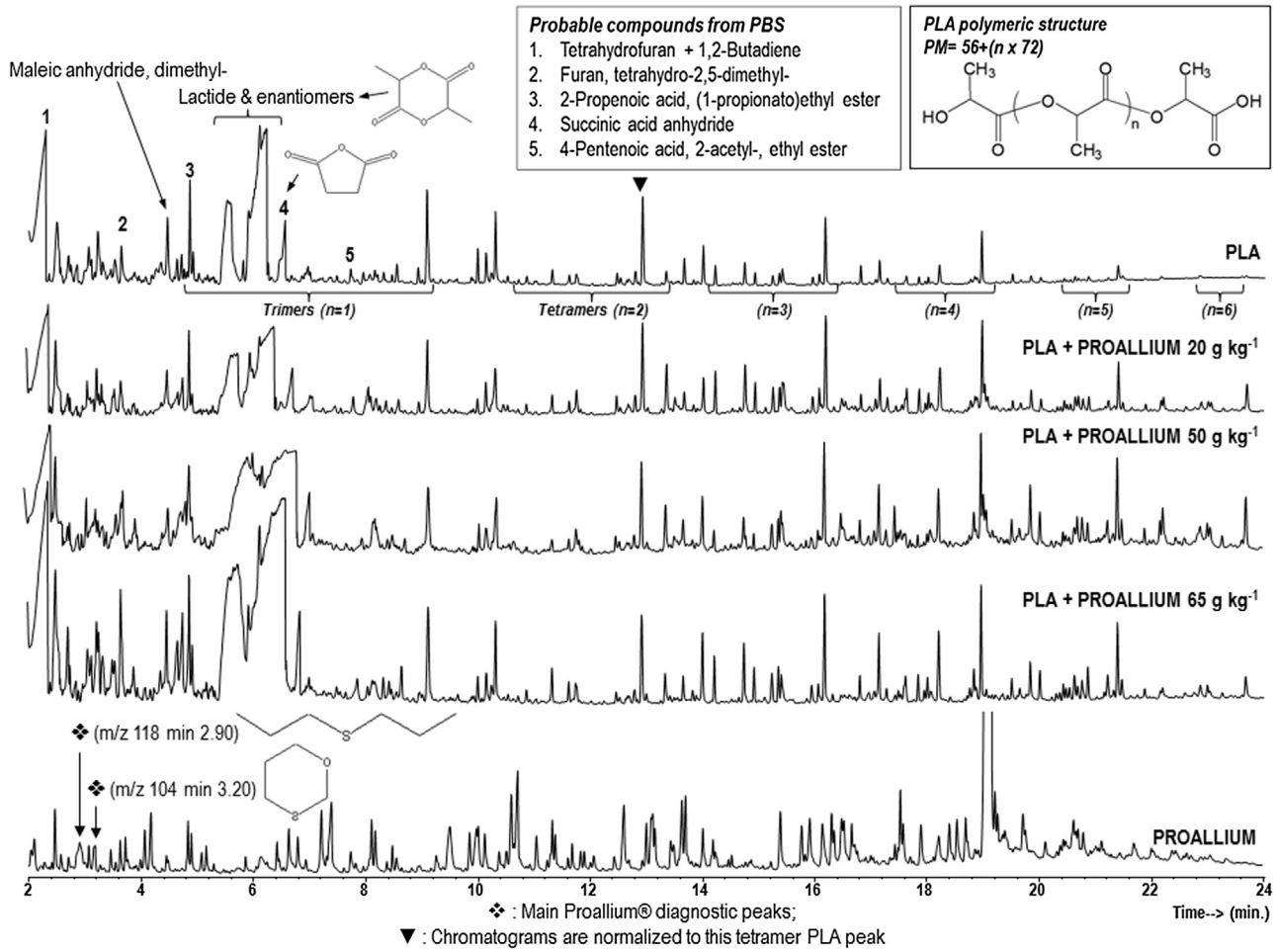

Fig. 2. Total current ion chromatograms (TIC) of biodegradable polymer blend PLA:PBS (950 g Kg^{-1}:50 g Kg^{-1}), of Proallium® additive and of the admixtures of additive in PLA (20, 50 and 65 g Kg^{-1}). The main polymeric PLA units and the probable PBS-derived compounds are also depicted, as well as the proposed main Proallium® diagnostic peaks. *m*/*z*: mass to charge ratio.

is observed at min c.19. The pyrolysis chromatograms obtained are compatible with a Polyoxyethylene sorbitan monooleate surfactant type Tween 80, where the polyethers (A) are pyrolysis products from the hydrophilic part (polyethoxylated sorbitan) and the oleic acid derivative (B) from the lipophilic group of the surfactant.

In addition, a number of sulphur-derived compounds were detected, probably derived from the Proallium® active ingredients from *Allium* spp. extract, including Propyl sulphide (*m*/*z* 118 at min 2.9) (1), 1,3-oxathiane (*m*/*z* 104 at min 3.2) (2), Dipropyl disulphide (*m*/*z* 108, 150 at min. 5.1) (3), Propyl sulfone (*m*/*z* 108, 150 at min 5.2) (4) and Propyl propane thiosulfonate (*m*/*z* 118, 182 at min 8.5) (5). Although the latter compound is known to be one of the major active ingredients of Proallium®, under the pyrolysis conditions used here it was only found in low quantity. This could be due to a thermal conversion during pyrolysis into the other main S compounds found. However, some of these compounds could be used as tracers to estimate the status of the additive in the bioplastic.

3.2. Analytical pyrolysis of PLA and PLA with Proallium® additive

In Fig. 2, the PLA and Proallium® total ion chromatograms are depicted together with the bio-based active film manufactured with Proallium® additive admixtures (20, 50 and 65 g Kg-1). The pyrolytic behaviour of a PLA biodegradable film from the same batch as the one used here (PLA:PBS (950 g Kg^{-1}:50 g Kg^{-1})) has been previously analysed and discussed in detail [28]. In summary, the main pyrolysis products released are: a broad peak at min. 6 that corresponds to lactide (di-ester of lactic acid) and enatiomeric forms, followed by cyclic oligomers with molecular mass following the polymer formula PM = 56 + (*n* × 72). In addition, a number of other peaks labelled **1** to **5** in the TIC trace were identical to those previously identified in PBS pyrolysates [29] that are probably derived from the PBS fraction in the plastic blend used for enhancing PLA crystallinity.

However, in the total current ion chromatograms (TIC) it was not possible to detect any peak clearly derived from the additive even in the films with the highest Proallium® doses (i.e., 65 g kg^{-1}). Therefore, in order to detect diagnostic/marker peaks we had to search for specific ions within the mass pyrogram and the organosulfur compound, Propyl sulphide, was found to be a good marker with which the chromatographic signal was proportional to the amount of additive within the polymer matrix (Fig. 3). In fact, a linear correlation coefficient better than 0.990R^2 value ($p < 0.001$) was found between the chromatographic area of this peak and the amount of Proallium® additive added to the biodegradable plastic to extrude the active film (Fig. 3 insert).

3.3. Estimation of the real amount of Proallium® additive in the bioplastic using carbon isotopic signature ($\delta^{13}C$)

Bulk $\delta^{13}C$ measurements on both PLA biopolymer and Proallium® additive revealed contrasting signatures between both

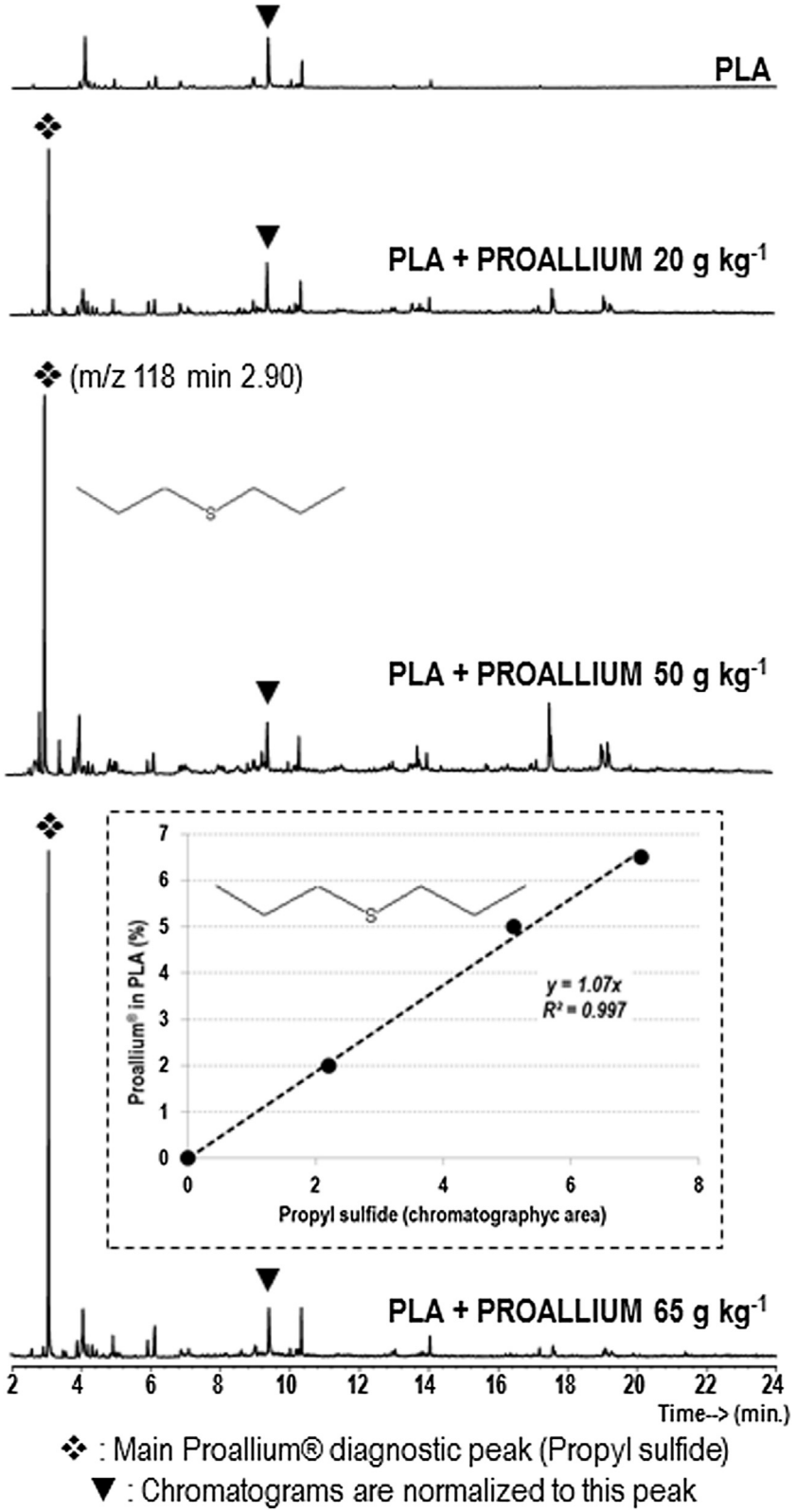

Fig. 3. Single current ion chromatogram (SIM) for fragment with mass to charge ratio *m*/*z* 118 in the biodegradable polymer blend PLA and admixtures of Proallium® additive (20, 50 and 65 g Kg^{-1}). Relation between main Proallium® pyrolysis diagnostic peak (Propyl sulphide peak at min. 2.90) and the percentage of added Proallium® in the PLA is shown in the insert.

materials, reflecting a different biogenic origin. The Proallium® carbon signature was light ($\delta^{13}C = -28.9 \pm 0.07‰$) as corresponds to a C3 plant extract (i.e., *Allium* spp), whereas the PLA biopolymers have a heavier carbon signature ($\delta^{13}C = -10.7 \pm 0.63‰$), indicative of a main C4 vegetation origin, probably corn starch.

In addition, a consistent $\delta^{13}C$ shift was observed in the PLA film extruded with variable Proallium® contents, becoming more nega-

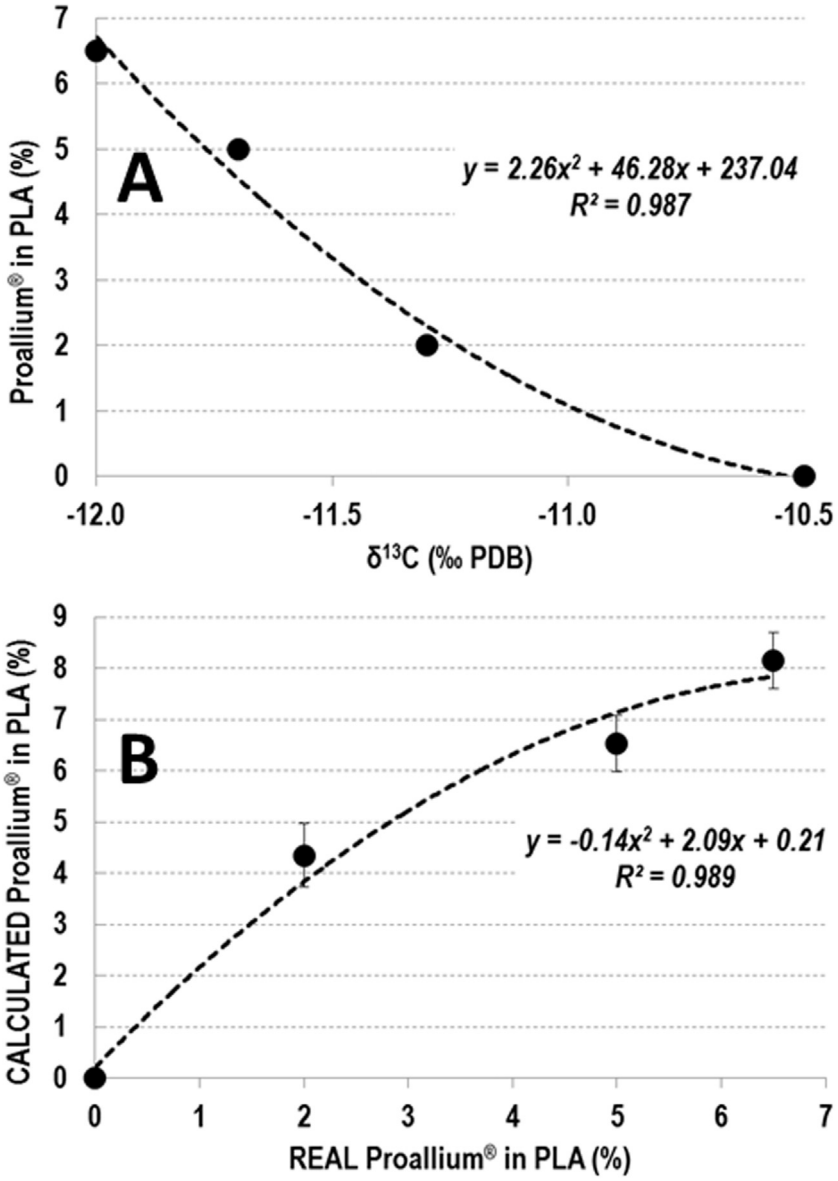

Fig. 4. Estimation of the amount of additive in the PLA, based in shifts in the carbon isotopic signature ($\delta^{13}C$). (A) Relation between $\delta^{13}C$ signature and said Proallium® added; (B) Relation between said Proallium® added and the calculated values based in a mass balance relation. Error bars indicate the mean STD (n = 3).

tive with increasing dosage (Fig. 4a). This carbon isotopic signature parallels the contribution from the Proallium® light carbon within the heavier carbon PLA matrix. Using a mass balance equation, where the proportion of C3 and C4 vegetation-derived materials are inferred from averaged $\delta^{13}C$ values, we were able to assess the amount of additive that was ultimately and effectively incorporated in the final casting of active-biopolymer (Fig. 4b). Although the estimation of the content of Proallium® additive in the bioplastic was highly correlated with the real concentration, the differences in the isotopic signatures overestimate the real quantity of additive in the biopolymer.

4. Conclusions

A detailed characterisation or "fingerprinting" of an *Allium* extract (Proallium®), and its detection within a bio-based PLA film to conform an active packaging, has been developed by analytical pyrolysis (Py-GC/MS). The sulphur compound, propyl sulphide, was found a diagnostic peak with which to trace the additive in the polymer, and a correlation coefficient of 0.997R^2 value ($p < 0.001$) was found between the chromatographic area of this peak and the amount of additive in the bioplastic. Our results indicate that analytical pyrolysis (Py-GC/MS) is a valuable tool that can provide rapid and accurate information about the origin, composition, quality and even a precise fingerprinting of additives, contained not only in active packaging made with biogenic polymers, but also when present in a wide variety of other natural or synthetic matrices.

Shifts in stable isotopic signature can be used to monitor the real contents of additives that are effectively present in a plastic matrix when the two components have a distinct origin reflected in different $\delta^{13}C$ values. However, the bulk IRMS technique used here was found to overestimates real concentration of the additive in the bioplastic and will need further research.

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References

[1] B. Teixeira, A. Marques, C. Pires, C. Ramos, I. Batista, J.A. Saraiva, M.L. Nunes, Characterization of fish protein films incorporated with essential oils of clove, garlic and origanum: physical, antioxidant and antibacterial properties, Food Sci. Technol. 59 (2014) 533–539.
[2] M. Arancibia, B. Giménez, M.E. López-Caballero, M.C. Gómez-Guillén, P. Montero, Release of cinnamon essential oil from polysaccharide bilayer films and its use for microbial growth inhibition in chilled shrimps, LWT Food Sci. Technol. 59 (2014) 989–995.
[3] L. Matuana, O. Faruk, C.A. Diaz, Cell morphology of extrusion foamed poly(lactic acid) using endothermic chemical foaming agent, Bioresour. Technol. 100 (2009) 5947–5954.
[4] V. Siracusa, P. Rocculi, S. Romani, M.D. Rosa, Biodegradable polymers for food packaging: a review, Trends Food Sci. Technol. 19 (2008) 634–643.
[5] E. Mascheroni, V. Guillard, F. Nalin, L. Mora, L. Piergiovanni, Diffusivity of propolis compounds in polylactic acid polymer for the development of anti-microbial packaging films, J. Food Eng. 98 (2010) 294–301.
[6] L. Avérous, Polylactic acid: synthesis, properties and applications, in: M.N. Belgacem, A. Gandini (Eds.), Monomers, Polymers and Composites from Renewable Resources, Elsevier, Oxford, 2008, pp. 433–450.
[7] J.W. Park, S.S. Im, Phase behaviour and morphology in blends of poly(L-lactic acid) and Poly(butylenes succinate), J. Appl. Polym. Sci. 86 (2002) 647–655.
[8] L. Vermeiren, F. Devlieghere, M. van Beest, N. de Kruijf, J. Debevere, Developments in the active packaging of food, Trends Food Sci. Technol. 10 (1999) 77–86.
[9] S. Salmieri, F. Islam, R.A. Khan, F.M. Hossain, H.M.M. Ibrahim, C. Miao, W.Y. Hamad, M. Lacroix, Antimicrobial nanocomposite films made of poly(lactic acid)–cellulose nanocrystals (PLA–CNC) in food applications: part A—effect of nisin release on the inactivation of Listeria monocytogenes in ham, Cellulose 21 (2014) 1837–1850.
[10] P. López, C. Sánchez, R. Batlle, C. Nerín, Development of flexible antimicrobial films using essential oils as active agents, J. Agric. Food Chem. 55 (2007) 8814–8824.
[11] S.R. Mupalla, S.R. Kanatt, A. Sharma, Carboxymethyl cellulose-polyvinyl alcohol films with clove oil for active packaging of ground chicken meat, Food Packag. Shelf Life 2 (2014) 51–58.
[12] G. Saccheti, S. Maietti, M. Muzzoli, M. Scaglianti, S. Manfredini, M. Radice, R. Bruni, Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods, Food Chem. 91 (2005) 621–632.
[13] M. Llana-Ruiz-Cabello, S. Pichardo, S. Maisanaba, M. Puerto, A.I. Pieto, D. Gutiérrez-Praena, A. Jos, A.M. Cameán, In vitro toxicological evaluation of essential oils and their main compounds used in active food packaging: a review, Food Chem. Toxicol. 81 (2015) 9–27.
[14] M. Llana-Ruiz-Cabello, D. Gutiérrez-Praena, S. Pichardo, F.J. Moreno, J.M. Bermúdez, S. Aucejo, A.M. Cameán, Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2, Food Chem. Toxicol. 64 (2014) 281–290.
[15] M. Llana-Ruiz-Cabello, S. Maisanaba, D. Gutiérrez-Praena, A.I. Prieto, S. Pichardo, A. Jos, F.J. Moreno, A.M. Cameán, Cytotoxic and mutagenic in vitro assessment of two organosulfur compound derived from onion to be used in the food industry, Food Chem. 166 (2015) 423–431.
[16] M. Llana-Ruiz-Cabello, D. Gutiérrez-Praena, M. Puerto, S. Pichardo, F.J. Moreno, A. Baños, C. Nuñez, E. Guillamón, A.M. Cameán, Acute toxicological studies of the main organosulfur compound derived from Allium sp. intended to be used in active food packaging, Food Chem. Toxicol. 82 (2015) 1–11.
[17] M. Llana-Ruiz-Cabello, S. Pichardo, A. Baños, C. Nuñez, J.M. Bermudez, E. Guillamón, S. Aucejo, A.M. Cameán, Characterisation and evaluation of PLA films containing an extract of *Allium* spp: to be used in the packaging of ready-to-eat salads under controlled atmospheres, LWT-Food Sci. Technol. 64 (2015) 1354–1361.
[18] R. Ruiz, M.P. García, A. Lara, L.A. Rubio, Garlic derivates (PTS and PTS-O) differently affect the ecology of swine faecal microbiota *in vitro*, Vet. Microbiol. 144 (2010) 110–117.
[19] Z.A. Nur Hanani, E. Beatty, Y.H. Roos, M.A. Morris, J.P. Kerry, Manufacture and characterization of gelatin films derived from beef: pork and fish sources using twin screw extrusion, J. Food Eng. 113 (2012) 606–614.
[20] S.C. Moldoveanu, Analytical pyrolysis of natural organic polymers, techniques and instrumentation, in: S.C. Moldoveanu (Ed.), Analytical Chemistry, Vol. 20, Elsevier, Amsterdam, 1998.
[21] J.A. González-Pérez, F.J. González-Vila, G. Almendros, H. Knicker, J.M. de La Rosa, Z. Hernández, Revisiting structural insights provided by analytical pyrolysis about humic substances and related bio-and geopolymers, in: J. Xu, et al. (Eds.), Functions of Natural Organic Matter in Changing Environment, Zhejiang University Press and Springer Science, 2013, pp. 1–5.
[22] M. Blazsó, Recent trends in analytical and applied pyrolysis of polymers, J. Anal. Appl. Pyrolysis 39 (1997) 1–25.
[23] S. Tsuge, H. Ohtani, Structural characterization of polymeric materials by pyrolysis-GC/MS, Polym. Degrad. Stab. 58 (1997) 109–130.
[24] J.K. Haken, Pyrolysis gas chromatography of synthetic polymers—a bibliography, J. Chromatogr. A 825 (1998) 171–187.
[25] S. Tsuge, H. Ohtani, C. Watanabe, Pyrolysis-GC/MS Data Book of Synthetic Polymers, Elsevier, Oxford, 2011.
[26] J.A. González-Pérez, N.T. Jiménez-Morillo, J.M. de La Rosa, G. Almendros, F.J. González-Vila, Pyrolysis-gas chromatography-Isotope ratio mass spectrometry (Py-GC-C/TC-IRMS) of polyethylene, J. Chromatogr. A 1388 (2015) 236–243.
[27] M.P. Arrieta, F. Parres, J. López, A. Jiménez, Development of a novel pyrolysis-gas chromatography/mass spectrometry method for the analysis of poly(lactic acid) thermal degradation products Pyr bio-platics, J. Anal. Appl. Pyrolysis 101 (2013) 150–155.
[28] E. Vuorinen, M. Hakkarainen, Method development for the analysis of biodegradable polymers, Int. J. Metrol. Qual. Eng. 1 (2010) 29–32.
[29] Y. Aoyagi, K. Yamashita, Y. Doi, Thermal degradation of poly[(R)-3-hydroxybutyrate], poly[(-caprolactone]: and poly[(S)-lactide], Polym. Degrad. Stab. 76 (2002) 53–59.
[30] C. Westphal, C. Perrot, S. Karlsson, Py-GC/MS as a means to predict degree of degradation by giving microstructural changes modelled on LDPE and PLA, Polym. Degrad. Stab. 73 (2001) 281–287.
[31] F.D. Kopinke, K. Mackenzie, Mechanistic aspects of the thermal degradation of poly(lactic acid) and poly(β-hydroxybutyric acid), J. Anal. Appl. Pyrolysis 40–41 (1997) 43–53.
[32] M. Llana-Ruiz-Cabello, S. Pichardo, N.T. Jiménez-Morillo, J.M. Bermúdez, S. Aucejo, F.J. González-Vila, A.M. Cameán, J.A. González-Pérez, Molecular characterization of a bio-based active package containing *Origanum vulgare* L. essential oil using analytical pyrolysis (Py-GC/MS), J. Sci. Food Agric. (2015), http://dx.doi.org/10.1002/jsfa.7502, in press.
[33] D.G. Papageorgiou, E. Roumeli, K. Chrissafis, C. Lioutas, K. Triantafyllidis, D. Bikiaris, A.R. Boccaccini, Thermal degradation kinetics and decomposition mechanism of PBS nanocomposites with silica-nanotubes and strontium hydroxyapatite nanorods, Phys. Chem. Chem. Phys. 16 (2014) 4830–4842.
[34] J.W. White, K. Winters, Honey protein as an international standard for stable isotope ratio detection of adulteration of honey, J. AOAC Int. 72 (1989) 907–911.
[35] S. Kelly, I. Parker, M. Sharman, J. Dennis, I. Goodall, Assessing the authenticity of single seed vegetable oils using fatty acid stable carbon isotope ratios, Food Chem. 59 (1997) 181–186 ($^{13}C/^{12}C$).
[36] A. Rossmann, Determination of stable isotope ratios in food analysis, Food Rev. Int. 17 (2001) 347–381.
[37] R. Karoui, J. De Baerdemaeker, A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products, Food Chem. 102 (2007) 621–640.
[38] J.A. González-Pérez, N.T. Jiménez-Morillo, J.M. de La Rosa, G. Almendros, F.J. González-Vila, Compound specific isotopic signature of carbohydrate pyrolysis products from C3 and C4 plants, J. Sci. Food Agric. 96 (2015) 948–953 (Py-CSIA).
[39] P. Deines, The isotopic composition of reduced organic carbon, in: P. Fritz, J. Fontes (Eds.), Handbook of Environmental Isotope Geochemistry, Vol. 1, Elsevier, New York, 1980, pp. 329–406.
[40] J. Lunt, Large-scale production: properties and commercial applications of polylactic acid polymers, Polym. Degrad. Stab. 59 (1998) 145–152.
[41] S. Valkiers, M. Varlam, K. Ruße, M. Berglund, P. Taylor, J. Wang, M.J.T. Milton, P. De Biévre, Preparation of synthetic isotope mixtures for the calibration of carbon and oxygen isotope ratiomeasurements (in carbon dioxide) to the SI, Int. J. Mass spectrom. 264 (2007) 10–21.
[42] M. Bernoux, C.C. Cerri, C. Neill, J.F.L. de Moraes, The use of stable carbon isotopes for estimating soil organic matter turnover rates, Geoderma 82 (1998) 43–58.
[43] L. Avérous, Polylactic acid: synthesis, properties and applications, in: M.N. Belgacem, A. Gandini (Eds.), Monomers, Polymers and Composites from Renewable Resources, Elsevier, Oxford, 2008, pp. 433–450.
[44] F. Debiagi, R.K.T. Kobayashi, G. Nakazato, L.A. Panagio, S. Mali, Biodegradable active packaging based on cassava bagasse: polyvinyl alcohol and essential oils, Ind. Crops Prod. 52 (2014) 664–670.
[45] P. Mellado-García, M. Puerto, S. Pichardo, M. Llana-Ruiz-Cabello, R. Moyano, A. Blanco, A. Jos, A.M. Cameán, Toxicological evaluation of an *Allium*-based commercial product in a 90-day feeding study in Sprague-Dawley rats, Food Chem. Toxicol. 90 (2016) 18–29.

CAPÍTULO 15 / CHAPTER 15

María Llana-Ruiz-Cabello, Silvia Pichardo, Nicasio T. Jiménez-Morillo, José M. Bermúdez, Susana Aucejo, Francisco J. González-Vila, Ana M. Cameán, Jose A. González-Pérez

MOLECULAR CHARACTERIZATION OF A BIO-BASED ACTIVE PACKAGING CONTAINING ORIGANUM VULGARE L. ESSENTIAL OIL USING PYROLYSIS GAS CHROMATOGRAPHY-MASS SPECTROMETRY.

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Molecular characterisation of a bio-based active packaging containing *Origanum vulgare* L. essential oil using pyrolysis gas chromatography–mass spectrometry

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Abstract

BACKGROUND: Environmental, economic and safety challenges motivate shift towards safer materials for food packaging. New bioactive packaging techniques, i.e. addition of essential plant oils (EOs), are gaining attention by creating barriers to protect products from spoilage. Analytical pyrolysis gas chromatography–mass spectrometry (Py-GC-MS) was used to fingerprint a bioactive polylactic acid (PLA) with polybutylene succinate (PBS) (950 g kg^{-1}:50 g kg^{-1}) film extruded with variable quantities (0, 20, 50 and 100 g kg^{-1}) of *Origanum vulgare* EO.

RESULTS: Main PLA:PBS pyrolysis products were lactide enantiomers and monomer units from the major PLA fraction and succinic acid anhydride from the PBS fraction. Oregano EO pyrolysis released cymene, terpinene and thymol/carvacrol peaks as diagnostic peaks for EO. In fact, linear correlation coefficients better than 0.950 R^2 value ($P < 0.001$) were found between the chromatographic area of the diagnostic peaks and the amount of oregano EO in the bioplastic.

CONCLUSION: The pyrolytic behaviour of a bio-based active package polymer including EO is studied in detail. Identified diagnostic compounds provide a tool to monitor the quantity of EO incorporated into the PLA:PBS polymeric matrix. Analytical pyrolysis is proposed as a rapid technique for the identification and quantification of additives within bio-based plastic matrices.

Keywords: oregano essential oil; polylactic acid; polybutylene succinate; pyrolysis gas chromatography–mass spectrometry; active packaging

INTRODUCTION

Traditionally, materials used for food packaging include a variety of petrochemical-based polymers, such as polyolefins, polyesters, polyamides, etc., because of their high specific strength and durability, ease of processing and their availability at low cost. However, today, environmental, economic and safety concerns have motivated scientists and producers to explore the possibilities of using more environmentally safe biodegradable materials. [1,2] In this sense, polylactic acid (PLA) is one of the most widely used bio-based materials in many applications, including the food packaging industry mainly used for improving the shelf life of perishable products.[3]

Chemically, PLA is an aliphatic polyester made up of lactic acid (2-hydroxypropionic acid) building blocks and is ultimately derived from renewable plant sources, such as starch and sugar.[4] The success of PLA as a food-package alternative is due to its low toxicity, high biodegradability and biocompatible thermoplastic, with high-strength, high-modulus and a good processability.[5] However, a drawback of PLA is its medium gas barrier properties. Combining PLA with other polymers,[6] or adding nanomaterials such as cellulose nanocrystals or nanowhiskers[3,7,8] may reduce such a problem, thus improving the characteristics of PLA-based packages.

Another possibility to improve the quality and safety of packed products is to include additives able to ameliorate polymer barrier properties by creating a bioactive material;[7] a polymer designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food.[9–11]

Due to the current global demand for minimally processed and preservative-free foodstuffs, new bioactive packaging techniques and ingredients from natural sources are gaining

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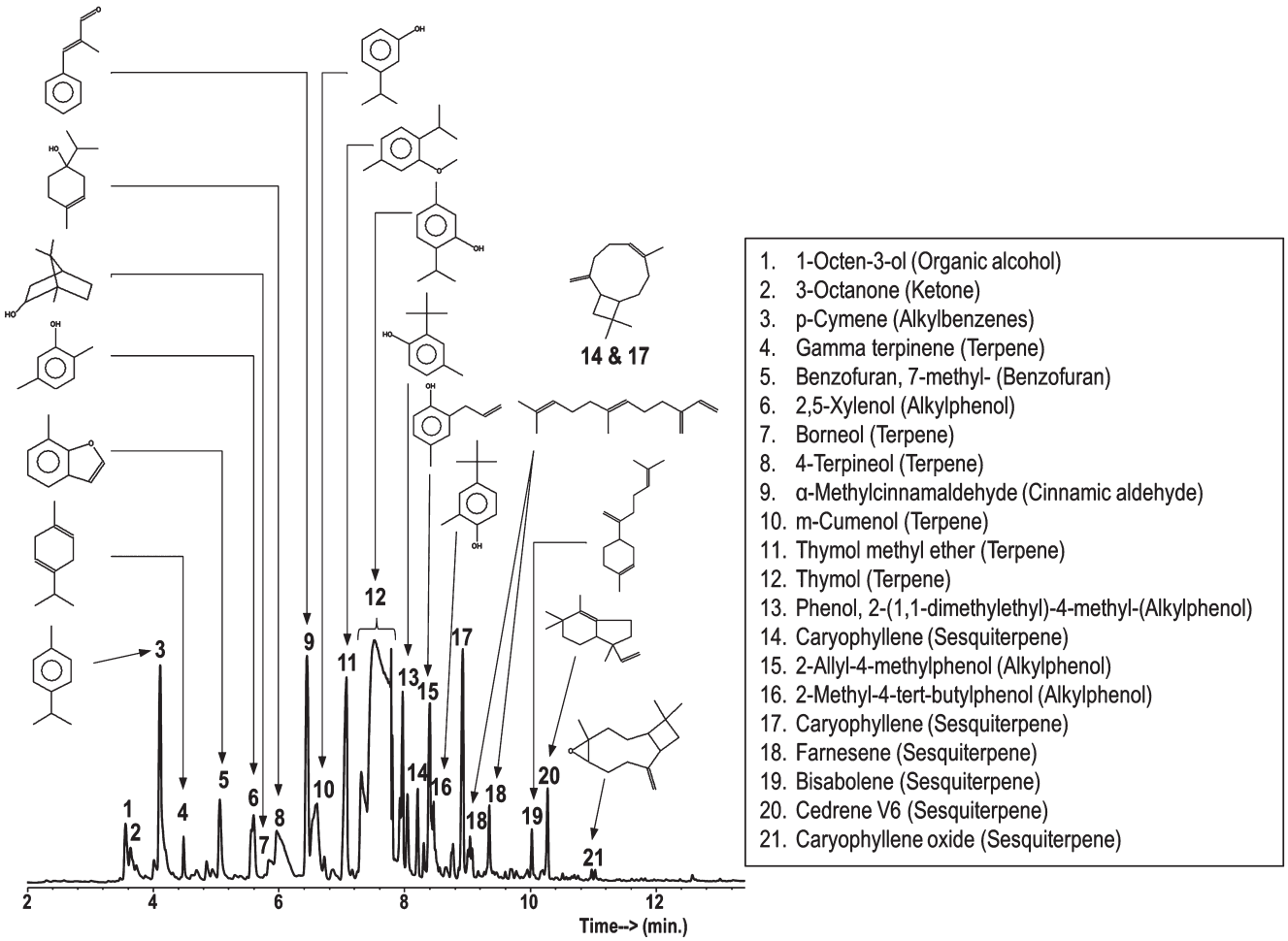

Figure 1. Total ion chromatogram (Py-GC-MS TIC) with an indication of the main pyrolysis products released at 500°C from oregano EO.

attention from the industry because these are perceived by consumers as low-health-risk materials. Therefore bioactive additives derived from essential oils (EOs), i.e. antimicrobial and antioxidants, are being increasingly used in food packaging.[12] Many of such EOs are plant secondary metabolites which have been extensively studied as natural food preservatives. Oregano essential oil is classified as 'generally recognised as safe' (GRAS) by the US Food and Drug Administration (FDA) and classified as a food additive by European Union (EU) legislation,[13,14] has been studied for its antimicrobial and antioxidant properties.[10,15] In fact, the monoterpenes carvacrol, thymol, cymene and terpinene, main constituents of oregano EO,[16,17] have been proven to have antimicrobial[18,19] and antioxidant properties.[20,21] In line with this, recent work revealed that carvacrol and thymol were able to protect Caco-2 cells against induced oxidative stress acting as an antioxidant agent *in vitro*.[22]

The use of EOs as food additives is sometimes limited due to unacceptable organoleptic properties.[14] However, the incorporation of EOs in food-packaging films allows the controlled release of active substances and reducing undesirable flavours caused by direct addition of EOs into food.[23,24] Due to the volatility of EOs and the conditions of film preparation, usually at elevated temperatures, it is necessary to ensure that the active compounds of oregano EO remain in the desired quantities in the final manufactured polymer. Analytical pyrolysis, defined as the thermochemical decomposition of organic materials at elevated temperatures in the absence of oxygen,[25] is a useful tool for the direct characterisation of polymers and additives within the polymer matrix. The products of pyrolysis (pyrolysate) are amenable to chromatographic separation and when combined with a mass spectrometry detector (Py-GC-MS), yields molecular information about the structure of complex mixtures of natural and synthetic macromolecular substances.[26] Other well-known advantages of the technique include the requirement of small sample sizes and little to no sample preparation needs. This makes analytical pyrolysis a convenient method for inexpensive and relatively rapid analyses of synthetic[27–30] and bio-based polymers including polylactic acid[31–36] and polybutylene succinate[37] plastics.

In this work direct analytical pyrolysis (Py-GC-MS) was used for a detailed characterisation ('fingerprinting) of both, an oregano EO and a bio-based 950 g kg^{-1} polylactic acid (PLA) plastic extruded with 50 g kg^{-1} polybutylene succinate (PBS) to ameliorate crystallinity. Also bio-plastic films extruded with add mixtures of essential oil (20, 50 and 100 g kg^{-1} in dry weight) were studied.

MATERIALS AND METHODS

Supplies and chemicals

The polymers used in this work were: polylactic acid (PLA) extrusion-grade (2003D) purchased in pellets from NatureWorks LLC (Minnetonka, MN, USA) and polybutylene succinate (PBS) GS Pla™ FD92WD purchased from Mitsubishi Chemical Corporation

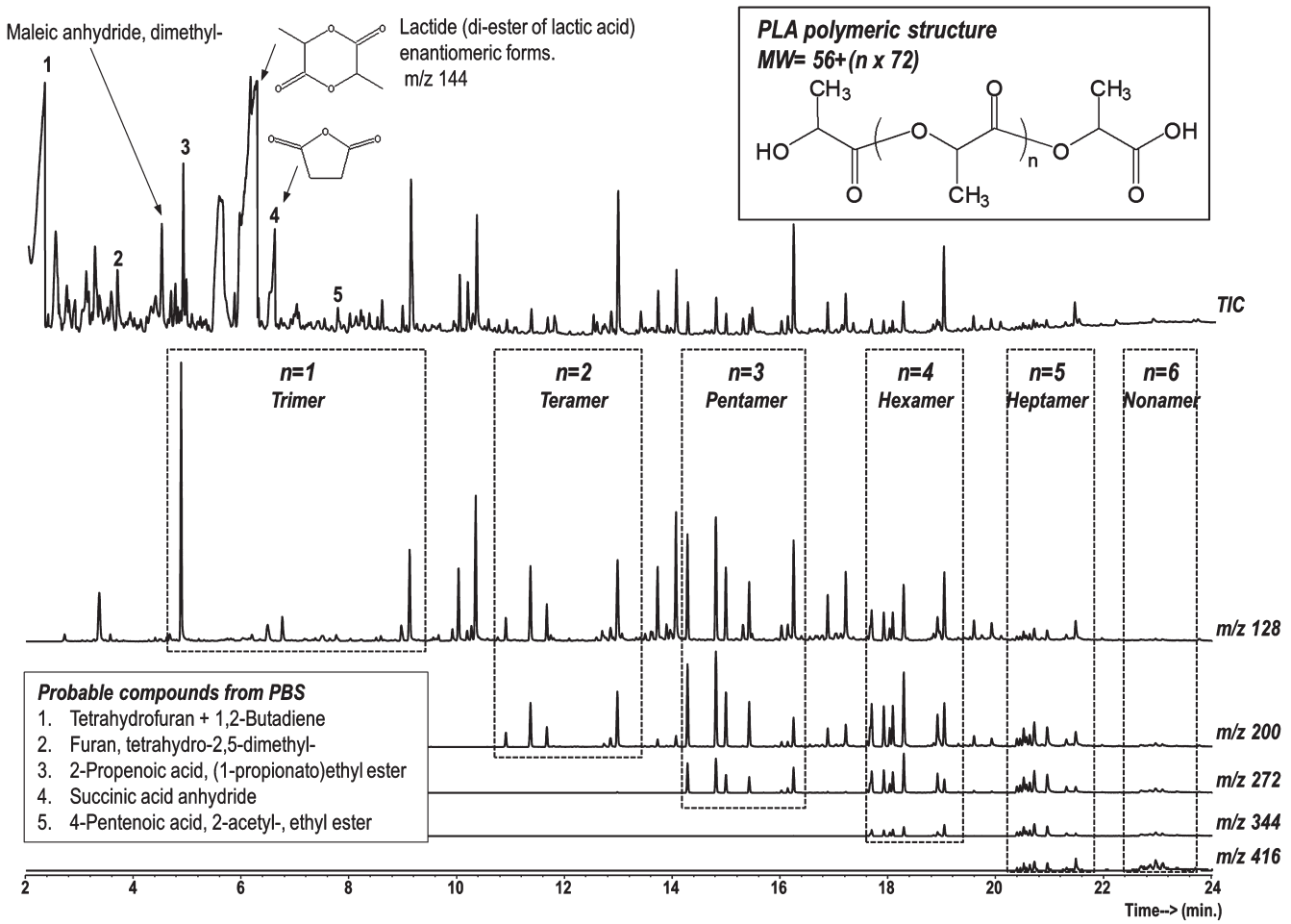

Figure 2. Total ion and selected ion monitoring chromatograms (Py-GC-MS TIC and SIM) of a biodegradable polymer blend PLA:PBS (950 g kg^{-1}:50 g kg^{-1}) with an indication of the main PLA polymeric units and probable PBS derived compounds. *m/z*, selected ion mass to charge ratio.

(Tokyo, Japan). Oregano essential oil (EO) was obtained from El Jarpil® (Almería, Spain).

Film preparation

The different active PLA films were obtained by melt blending in a twin-screw extruder (DSE 20-40D; Brabender, Duisburg, Germany). Different concentrations (20, 50 and 100 g kg^{-1} which correspond to 2, 5 and 10% w/w, respectively) of oregano EO were fed into the barrel trough the lateral liquid port at L/D 10 in order to reduce possible volatility and degradation losses. Barrel temperatures were set at 200–205°C working at a screw speed of 70 min^{-1}. A control film was extruded in the same manner but with no oregano EO added. The average thickness of the final films was 80 µm (c. 315 gauges).

Analytical pyrolysis (Py-GC-MS)

Direct pyrolysis gas chromatography–mass spectrometry (Py-GC-MS) analysis was performed using a double-shot pyrolyser F-Labs model 2020i (Frontier Laboratories, Fukushima, Japan) attached to a GC-MS system Agilent 6890N (Agilent Technologies Inc., Santa Clara, CA, USA). Samples (0.5 mg) were placed in small crucible capsules and introduced into a preheated micro-furnace at 500°C for 1 min. The evolved gases were transferred into the GC-MS for analysis. The gas chromatograph was equipped with a low polar-fused silica (5% phenylmethylpolysiloxane) capillary column J&W HP-5ms Ultra Inert, of 30 m × 250 µm × 0.25 µm film thickness. The oven temperature was held at 50°C for 1 min and then increased to 100°C at 30°C min^{-1}, from 100°C to 300°C at 10°C min^{-1}, and stabilised at 300°C for 10 min. The carrier gas used was helium at a controlled flow of 1 cm^3 min^{-1}. The detector was an Agilent 5973 (Agilent Technologies Inc.) mass selective detector, and mass spectra were acquired at 70 eV ionising energy. Compound assignment was achieved by single-ion monitoring for various homologous series, low-resolution mass spectrometry, and via comparison with published and stored (NIST05 and WILEY7N libraries) data.

The Pearson correlation coefficient was used to assess the significance of the EO added to the plastic and the chromatographic peak areas of EO derived peaks. The analysis was conducted using the PEARSON function in MS Excel 2010 software.

RESULTS AND DISCUSSION

Figure 1 shows the total ion chromatogram of the pyrolysis products (pyrogram) release at 500°C from oregano EO, with an indication to the chemical identities of the main pyrolysis products. These are a typical mixture of aromatic and hydroaromatic structures dominated by monoterpenes and sesquiterpenes with a conspicuous broad peak (at 7–8 min) (peak 12) that corresponds to a mixture of thymol [phenol, 5-methyl-2-(1-methylethyl)-] with the isomer carvacrol [phenol, 2-methyl-5-(1-methylethyl)-]

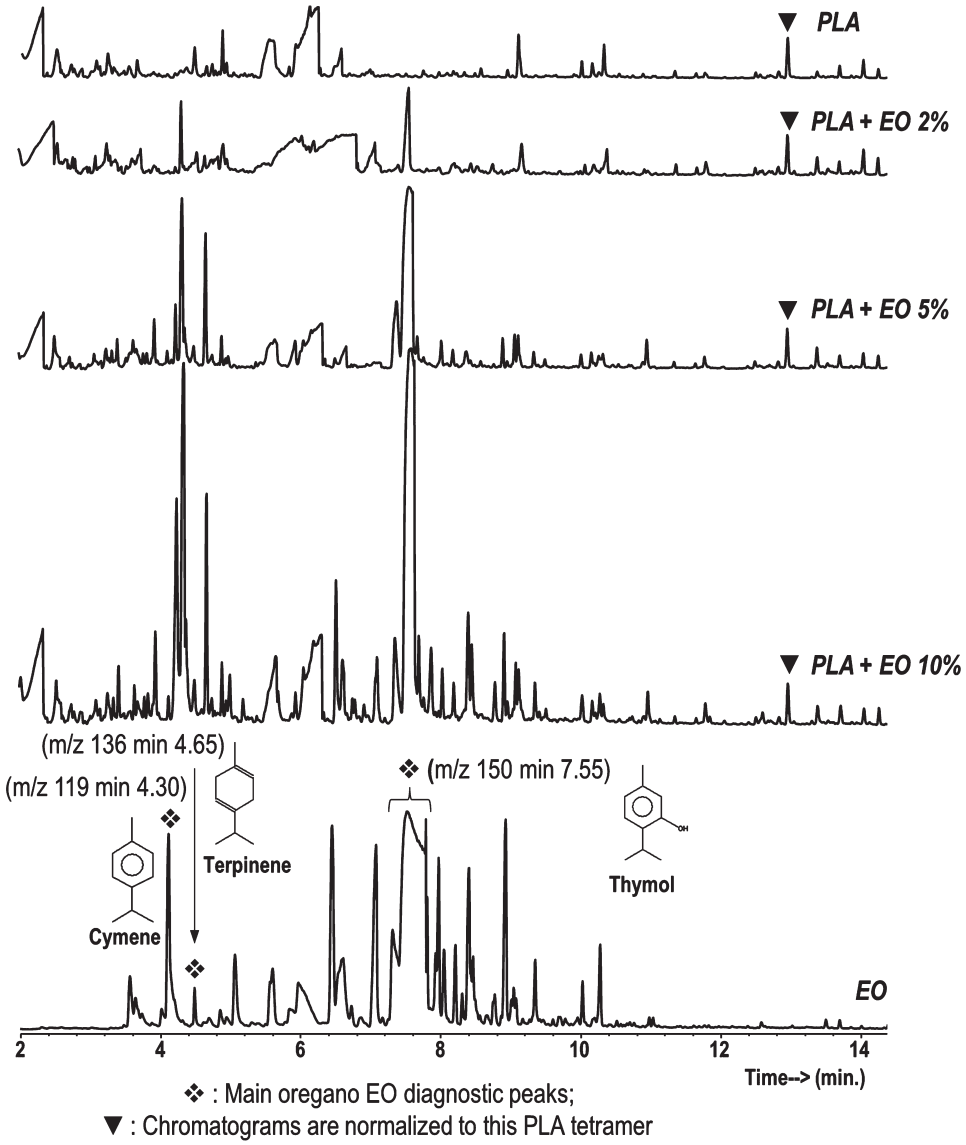

Figure 3. PLA and oregano EO total ion chromatograms (Py-GC-MS TIC) and of added mixtures of 20, 50 and 100 g kg^{-1} which correspond to 2, 5 and 10% w/w, respectively oregano EO in PLA.

which, under the chromatographic conditions used, could not be resolved. Other major pyrolysis products included the alkylbenzene *p*-cymene (peak 3), other terpenes such as γ-terpinene, (peak 4), terpineol (peak 8), α-methylcinnamaldehyde, (peak 9), thymol/carvacrol methyl ester (peak 11) and a number of known sesquiterpenes, i.e. caryophyllene (peaks 14 and 17), farnesene (peak 18), bisabolene (peak 19) and cedrene (peak 20).

A detailed pyrogram of the PLA:PBS (950 g kg^{-1}:50 g kg^{-1}) biodegradable film is shown in Fig. 2. The main pyrolysis products were a broad peak at 6 min that corresponds to lactide (di-ester of lactic acid or 1,4-dioxane-2,5-dione, 3,6-dimethyl-) and their enantiomeric forms. Besides, cyclic oligomers were clearly detected in the PLA pyrolysates when searching for specific ions following the polymer general formula: $MW = 56 + (n \times 72)$, where MW is molecular weight. Under the chromatographic conditions used up to nine monomer units with a maximum molecular weight of 488 Da were detected. These findings are in line with previous pyrolysis and PLA thermal degradation studies.[36] In addition, a number of other peaks, tetrahydrofuran and 1,2-butadiene (peak 1), furan, tetrahydro-2,5-dimethyl (peak 2), 2-propenoic acid, (1-propionato)ethyl ester (peak 3), succinic acid anhydride (peak 4) and 4-pentenoic acid, 2-acetyl-, ethyl ester (peak 5), observed in the total ion chromatogram trace were identical to those previously identified in PBS pyrolysates,[37] i.e. they most probably derive from the minor PBS fraction present in the biodegradable plastic blend used for enhancing PLA crystallinity.

In Fig. 3, the PLA and oregano EO total ion chromatograms are depicted together with the bio-based active film manufactured with oregano EO add mixtures (20, 50 and 100 g kg^{-1}) in the biodegradable PLA. Conspicuous peaks, obviously derived from the added oregano EO are clearly visible in the active film even in that with the lowest EO doses (20 g kg^{-1}). These peaks corresponded to the major oregano EO terpene thymol/carvacrol mixture (at approx. 7.55 min), the alkyl benzene cymene (at 4.30 min) and, less apparent mainly at lower EO doses, a third peak corresponding to the terpene terpinene (at 4.65 min). These three peaks can be considered as diagnostic/marker peaks to trace the added oregano EO within the bioplastic matrix. In fact, Pearson linear

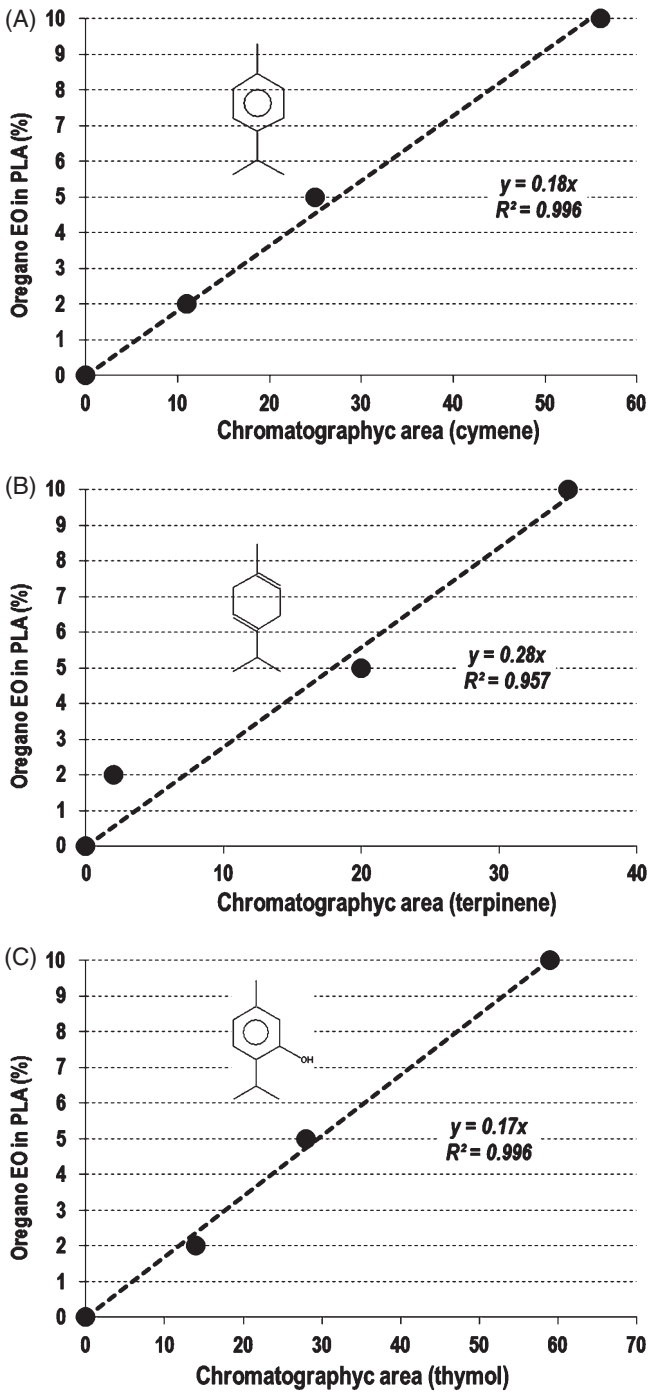

Figure 4. Relation between the main oregano EO diagnostic peaks and the percentage of added EO in the PLA. (A) Cymene peak at 4.30 min; (B) terpinene peak at 4.65 min; and (C) carvacrol/thymol peak at 7.55 min.

correlation coefficients of better than 0.950R^2 value ($P < 0.001$) were found between the chromatographic area of these three main marker peaks and the amount of oregano EO (in g kg^{-1}) added to the biodegradable plastic to extrude the active film (Fig. 4).

Although the primary use of EOs in the food industry is as flavourings, these oils also represent a source of natural food preservatives. Many studies have demonstrated the potent antimicrobial and antioxidant activities of oregano EO[16,17,20,38] and its use is increasing as a natural component of many foodstuffs and also of non-edible materials of use in the food industry, i.e. plastic films used in bio-active packaging. Previous results[31] and those described here indicate that analytical pyrolysis (Py-GC-MS) can provide rapid and accurate information about the composition, quality and even a precise fingerprinting of EOs contained in active packages made with biogenic polymers like PLA:PBS. It is also foreseen that the technique will be of use to study other EOs and additives included in a wide variety of other natural or synthetic polymeric matrices.

ACKNOWLEDGEMENTS

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REFERENCES

1 Bohlman GM, General characteristics, processability, industrial applications and market evolution of biodegradable polymers, in *Handbook of Biodegradable Polymers*, 1st edition, ed. by Bastioli C. Rapra Technology Ltd, Shropshire, UK, pp. 183–218 (2005).
2 Debiagi F, Kobayashi RKT, Nakazato G, Panagio LA and Mali S, Biodegradable active packaging based on cassava bagasse, polyvinyl alcohol and essential oils. *Ind Crop Prod* **52**:664–670 (2014).
3 Fortunati E, Peltzer M, Armentano I, Torre L, Jimenez A and Kenny JM, Effects of modified cellulose nanocrystals on the barrier and migration properties of PLA nano-biocomposites. *Carbohydr Polym* **90**:948–956 (2012).
4 Lim LT, Auras R and Rubino M, Processing technologies for poly(lactic acid). *Prog Polym Sci* **33**:820–852 (2008).
5 Rhim J-W, Mohanty AK, Singh SP and Ng PKW, Effect of the processing methods on the performance of polylactide films: Thermocompression versus solvent casting. *J Appl Polym Sci* **101**:3736–3742 (2006).
6 Jamshidian M, Tehrany EA, Imran M, Jacquot M and Desobry S, Poly-lactic acid: production, applications, nanocomposites, and release studies. *Compr Rev Food Sci F* **9**:523–571 (2010).
7 Salmieri S, Islam F, Khan RA, Hossain FM, Ibrahim HMM, Miao C *et al.*, Antimicrobial nanocomposite films made of poly(lactic acid)–cellulose nanocrystals (PLA–CNC) in food applications: part A – effect of nisin release on the inactivation of *Listeria monocytogenes* in ham. *Cellulose* **21**:1837–1850 (2014).
8 Cabedo L, Feijoo JL, Villanueva MP, Lagaron JM and Gimenez E, Optimization of biodegradable nanocomposites based on PLA/PCL blends for food packaging applications. *Macromol Symp* **233**:191–197 (2006).
9 European Commission, Regulation (EC) No. 1935/2004 of the European Parliament and the Council of 27 October 2004, on materials and articles intended to come into contact with food and repealing Directives 80/509/EEC and 89/109/EEC. *Off J Eur Comm* **338**:4–17 (2004).
10 López P, Sánchez C, Batlle R and Nerín C, Development of flexible antimicrobial films using essential oils as active agents. *J Agric Food Chem* **55**:8814–8824 (2007).
11 Salmieri S, Islam F, Khan RA, Hossain FM, Ibrahim HMM, Miao C *et al.*, Antimicrobial nanocomposite films made of poly(lactic acid)–cellulose nanocrystals (PLA–CNC) in food applications: part B – effect of oregano essential oil release on the inactivation of *Listeria monocytogenes* in mixed vegetables. *Cellulose* **21**:4271–4285 (2014).
12 Llana-Ruiz-Cabello M, Maisanaba S, Puerto M, Prieto AI, Pichardo S, Jos A *et al.*, Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. *Food Chem Toxicol* **72**:122–128 (2014).
13 Manso S, Pezo D, Gómez-Lus R and Nerín C, Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil. *Food Control* **45**:101–108 (2014).

14 Muriel-Galet V, Cran MJ, Bigger SW, Hernández-Muñóz P and Gavara R, Antioxidant and antimicrobial properties of ethylene vinyl alcohol copolymer films based on the release of oregano essential oil and green tea extract components. *J Food Eng* **149**:9–16 (2015).
15 Sánchez-González L, Vargas M, González-Martínez C, Chiralt A and Cháfer M, Use of essential oils in bioactive edible coatings. *Food Eng Rev* **3**:1–16 (2011).
16 Lambert RJW, Skandamis PN, Coote PJ and Nychas G-JE, A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* **91**:453–462 (2001).
17 Burt S, Essential oils: Their antibacterial properties and potential applications in foods. *Int J Food Microbiol* **94**:223–253 (2004).
18 Zivanovic S, Chi S and Draughon AF, Antimicrobial activity of chitosan films enriched with essential oils. *J Food Sci* **70**:M45–M51 (2005).
19 Ozkalp B, Sevgi F, Ozcan M and Ozcan MM, The antibacterial activity of essential oil of oregano (*Origanum vulgare* L.). *J Food Agric Environ* **8**:272–274 (2010).
20 Kulisic T, Radonic A, Katalinic V and Milos M, Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chem* **85**:633–640 (2004).
21 Amorati R, Foti MC and Valgimigli L, Antioxidant activity of essential oils. *J Agric Food Chem* **61**:10835–10847 (2013).
22 Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Puerto M, Pichardo S, Jos A and Cameán AM, *In vitro* pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. *Toxicol In Vitro* **29**:647–656 (2015).
23 Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Pichardo S, Moreno FJ, Bermúdez JM, Aucejo S *et al.*, Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. *Food Chem Toxicol* **64**:281–290 (2014).
24 Ramos M, Jiménez A, Peltzer M and Garrigós MC, Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *J Food Eng* **109**:513–519 (2012).
25 Irwin WJ, Analytical pyrolysis – A comprehensive guide, in Chromatographic Science, *Series 22*, ed. by Cazes J. Marcel Dekker, New York, Chapter 6 (1982).
26 González-Pérez JA, González-Vila FJ, Almendros G, Knicker H, De La Rosa JM and Hernández Z, Revisiting structural insights provided by analytical pyrolysis about humic substances and related bio- and geopolymers, in *Functions of Natural Organic Matter in a Changing Environment*, ed. by Xu J, Wu J and He Y. Zhejiang University Press and Springer Science + Business Media, Dordrecht, pp. 3–6 (2013).
27 Blazsó M, Recent trends in analytical and applied pyrolysis of polymers. *J Anal Appl Pyrolysis* **39**:1–25(1997).
28 Tsuge S and Ohtani H, Structural characterization of polymeric materials by pyrolysis-GC/MS. *Polym Degrad Stab* **58**:109–130 (1997).
29 Haken JK, Pyrolysis gas chromatography of synthetic polymers – A bibliography. *J Chromatogr A* **825**:171–187 (1998).
30 González-Pérez JA, Jiménez-Morillo NT, de La Rosa JM, Almendros G and González-Vila FJ, Pyrolysis-gas chromatography–isotope ratio mass spectrometry of polyethylene. *J Chromatogr A* **1388**:236–243 (2015).
31 Arrieta MP, López J, Ferrándiz Sand Peltzer MA, Characterization of PLA–limonene blends for food packaging applications. *Polym Test* **32**:760–768 (2013).
32 Arrieta MP, Parres F, López J and Jiménez A, Development of a novel pyrolysis-gas chromatography/mass spectrometry method for the analysis of poly(lactic acid) thermal degradation products. *J Anal Appl Pyrolysis* **101**:150–155 (2013).
33 Vuorinen E and Hakkarainen M, Method development for the analysis of biodegradable polymers. *Int J Metrol Qual Eng* **1**:29–32 (2010).
34 Aoyagi Y, Yamashita K and Doi Y, Thermal degradation of poly[(*R*)-3-hydroxybutyrate], poly[ε-caprolactone], and poly[(*S*)-lactide]. *Polym Degrad Stab* **76**:53–59 (2002).
35 Westphal C, Perrot C and Karlsson S, Py-GC/MS as a means to predict degree of degradation by giving microstructural changes modelled on LDPE and PLA. *Polym Degrad Stab* **73**:281–287 (2001).
36 Kopinke F-D and Mackenzie K, Mechanistic aspects of the thermal degradation of poly(lactic acid) and poly(β-hydroxybutyric acid). *J Anal Appl Pyrolysis* **40–41**:43–53 (1997).
37 Papageorgiou DG, Roumeli E, Chrissafis K, Lioutas C, Triantafyllidis K, Bikiaris D *et al.*, Thermal degradation kinetics and decomposition mechanism of PBSu nanocomposites with silica-nanotubes and strontium hydroxyapatite nanorods. *Phys Chem Chem Phys* **16**:4830–4842 (2014).
38 Bakkali F, Averbeck S, Averbeck D and Idaomar M, Biological effects of essential oils – A review. *Food Chem Toxicol* **46**:446–475 (2008).

CAPÍTULO 16 / CHAPTER 16

María Llana-Ruíz-Cabello, Silvia Pichardo, Nicasio T. Jiménez-Morillo, Francisco J. González-Vila, Enrique Guillamón, José M. Bermúdez, Susana Aucejo, Ana M. Cameán, Jose A. González-Pérez

MONITORING NATURAL ADDITIVES IN POLYLACTIC ACID ACTIVE FOOD PACKAGES. PYROLYSIS-GAS CHROMATOGRAPHY-ISOTOPE RATIO MASS SPECTROMETRY ANALYSIS.

Journal of Chromatrography A. (en revisión/under revision), ***2017***

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Manuscript Draft

Manuscript Number:

Title: Monitoring natural additives in polylactic acid active food packages. Pyrolysis-gas chromatography-isotope ratio mass spectrometry analysis

Article Type: VSI: SECyTA 2016

Keywords: Bioplastics; Analytical pyrolysis; Compound-specific isotope analysis; Carbon isotopes

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COVER LETTER

Monitoring natural additives in polylactic acid active food packages. Pyrolysis-gas chromatography–isotope ratio mass spectrometry analysis

By

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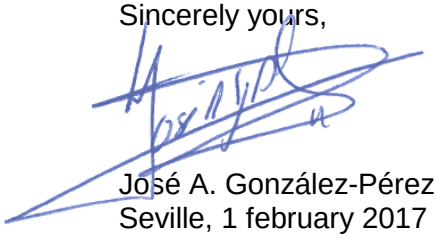
Submited for consideration at:
The Journal of Chromatography A, SECyTA 2016 special issue

Dear Editor,

The MS we are presenting deals with the application of a relative novel and powerful analytical pyrolysis hyphenized technique (Py-CSIA) applied to the study of polylactic acid (PLA) based film extruded with variable quantities of a natural plant extract or oregano essential oil for use in active food packaging. Chemical structural information of pyrolysates was first determined by analytical pyrolysis (Py-GC/MS) and direct $\delta^{13}C$ of in specific compounds was obtained by coupling a pyrolysis unit to a gas chromatograph connected to a continuous flow IRMS unit (Py-GC-(FID)-EA-IRMS). Using this Py-CSIA device it was possible to directly trace the occurrence of natural additives with depleted $\delta^{13}C$ values produced by C3 photosystem vegetation, within the naturally isotopically enriched components from the bio-plastic backbone, derived from corn (C4 vegetation) starch. This is the first application of Py-CSIA to the characterization of a bio-plastic and is shown as a promising tool to study such materials, providing not only a fingerprinting, but also valuable information about the origin of the materials and allowing the traceability of additives minimizing sample preparation.

We really hope that this contribution is of interest and acceptable for publication in the Journal of Chromatography A.

Sincerely yours,



José A. González-Pérez
Seville, 1 february 2017

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HIGHLIGHTS FOR REVIEW:

- Accurate and rapid determination of isotope composition ($\delta^{13}C$) in PLA and additives
- The technique can detect isotopically distinct additives within a bio-polymer matrix with a minimum of sample preparation
- First report of Py-CSIA application to the direct characterization of a bio-plastic

Monitoring natural additives in polylactic acid active food packages. Pyrolysis-gas chromatography–isotope ratio mass spectrometry analysis

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Abstract

Compound-specific isotope analysis (CSIA) usually requires preparative steps (pretreatments, derivatization) to get amenable chromatographic analytes from bulk geological, biological or synthetic materials. Analytical pyrolysis (Py-GC/MS) can help to overcome preparative manipulation of samples. In this communication we describe the results obtained by hyphenating analytical pyrolysis (Py-GC) with carbon isotope-ratio mass spectrometry (IRMS) for the analysis of an extruded bio-plastic based on polylactic acid (PLA) with variable quantities of a natural plant extract or oregano essential oil for its use in active food packaging. The chemical structural information of pyrolysates was first determined by conventional analytical pyrolysis and the direct measure of $\delta^{13}C$ in specific compounds was carried out by coupling a pyrolysis unit to a gas chromatograph connected to a continuous flow IRMS unit (Py-GC-(FID)-EA-IRMS). Using this Py-CSIA device it was possible to directly trace the occurrence of natural additives with depleted $\delta^{13}C$ values produced by C3 photosystem vegetation, within the naturally isotopically enriched components from the bio-plastic backbone, derived from corn (C4 vegetation) starch. This is the first application of Py-CSIA to the characterization of a bio-plastic and is shown as a promising tool to study such materials, providing not only a fingerprinting, but also valuable information about the origin of the materials, allowing the traceability of additives and minimizing sample preparation.

Keywords

Bioplastics; Analytical pyrolysis; Compound-specific isotope analysis; Carbon isotopes

1. Introduction

Due to increasing environmental concern, the study of new materials within a perspective of eco-design or sustainable development is a strategy that is currently applied in the food packaging industry [1]. In this regard, biodegradable polymers can be considered as environmentally safe alternatives to petroleum-based conventional packaging which takes hundreds of years to decompose [2].

Polylactic acid (PLA) is emerging as an important green polymeric alternative to petroleum-based packaging due to its biodegradability, biocompatibility and process ability [3]. PLA is an aliphatic polyester made primarily from renewable agricultural resources (corn) following the fermentation of starch and further condensation of lactic acid [4]. Hence, considering that PLA is classified as GRAS (Generally Recognized As Safe) by the American Food and Drug Administration (FDA) and is authorized by the European Commission (Commission Regulation No 10/2011), this polymer is an excellent candidate for producing a commercial compostable food packaging material [5]. Moreover, PLA exhibited desirable features for food packaging such as good mechanical and light barrier properties and is easily processed by injection, molding, blow molding, thermoforming or extrusion [6].

Active food contact materials were defined in Regulation No 1935/2004 of the Europe Parliament [7] and of the Council as “materials that are intended to extend the shelf-life or to maintain or improve the condition of packaged food”. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food”. In order to produce these materials, PLA incorporated with several substances such as the bacteriocin nisin, vitamin E (α-tocopherol) or as copolymer with polyethylene glycol have been developed [8]. Similarly, natural extracts and essential oils (EOs) can be incorporated into PLA to develop active food packaging to extend shelf-life of perishable product due to the antimicrobial or antioxidant properties of these substances. In this sense, recently a commercial product based on *Allium* extract (Proallium-SO-DMC®) or oregano essential oil were incorporated by extrusion into PLA to develop active food materials with the aim of improving the shelf life of ready-to-eat salads [5,

9]. However, due to its high volatility several authors reported that losses of essential oils were to be expected during the fabrication or storage of the active film or preformed packages [10, 11].

In order to assess that active packaging still contains effective concentrations of natural extracts after manufacture, previous thermogravimetric analysis has been applied [9]. However, it is desirable to explore other more accurate and informative techniques which to confirm the above fact.

Isotope ratio mass spectrometry (IRMS) is being extensively used to trace the origin of biogenic materials and to enlighten relevant scientific and technical questions for food science and the industry such is traceability and fraud detection [12–15]. The stable carbon isotopic composition ($\delta^{13}C$) of plants depends on carbon fixation process such as the C3 or C4 cycle. Most plants, including *Origanum* sp. and *Allium* sp., utilize the C3 photosynthetic pathway to assimilate CO_2. The $\delta^{13}C$ value of these C3 plants generally ranges from −24 to −30 ‰. However, corn is a tropical herb and a representative plant with C4 type photosystem known to have an enriched $\delta^{13}C$ generally between −6 and −19‰ [16]. Thus, these differences in carbon isotopic composition between corn and essential oils can be used to detect and trace additives into bio-based polymeric matrices, such as polylactic acid (PLA) manufactured from C4 plants products.

While no or little sample preparation is required for bulk isotopic analyses, for the compound-specific isotope analysis (CSIA) variant, intermediate multi-step preparative procedures are required in most cases prior to chromatographic analysis i.e., compounds must generally be first isolated from bulk sample materials, such as polymers, soils, sediments, or biological tissues. Non-volatile organic compounds usually require derivatization i.e. silylation, alkylation, acylation, esterification or other methods in order to enhance its volatility and improve chromatographic separation [17–19]. However, all these pretreatments may lead to artifacts formation, un-accuracies or misleading results.

Conventional analytical pyrolysis (Py-GC/MS) is a well-established technique that can help overcome preparative manipulation of samples; requires too small sample size with little or no preparation, thus being convenient for inexpensive and relatively rapid routine analyses. The technique has been proved to be a

rapid and inexpensive technique for the characterisation of synthetic polymers and additives [20–24] and also for bio-based polymers, including PLA [25–30] and polybutylene succinate (PBS) [30,31] plastics.

Recently we have effectively hyphenated pyrolysis (Py-GC) with light stable isotopes (C, H, N) IRMS (Py-GC–C/HT–IRMS). Early work demonstrated that pyrolysis process does not produce appreciable fractionation of stable isotopes and therefore the pyrolysis products can be considered isotopically representative of the starting material [32–34]. This technique allows on-line quantification of stable isotope proportions in chromatographically separated products released by pyrolysis and has been successfully applied to the study of widely different natural and industrial samples e.g., dyed polyethylene, sucrose from different origins [15, 24] or speleothems [35].

In this work we describe the results obtained by using carbon Py-CSIA for the analysis of a polylactic acid (PLA:PBS) based film extruded with variable quantities of natural plant extracts or essential oils for use in active food packaging.

2. Material and methods

2.1. Bio-polymer and additives

The plastic films studied in this work were made of polylactic acid (PLA) with polybutylene succinate (PBS) (950 g kg^{-1}:50 g kg^{-1}) extruded with variable quantities of oregano essential oil (EO) or of the commercial additive (Proallium®) prepared from Allium spp. extracts.

The PLA extrusion-grade (2003D) was purchased in pellets from NatureWorks LLC (Minnetonka, MN, USA) and the PBS, GS Pla™ FD92WD from Mitshubishi Chemical Corporation (Tokyo, Japan).

Oregano essential oil (EO) was obtained from El Jarpil® (Almería, Spain). Commercial Proallium® (L14/7), extract obtained from *Allium* spp. was supplied by

the manufacturer DOMCA S.A. (Alhendín, Granada, Spain). Chemicals for the different assays were purchased from Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

The different active PLA films were obtained by melt blending in a twin-screw extruder (DSE 20-40D; Brabender, Duisburg, Germany). Different concentrations (20, 50 and 100 g kg^{-1} which correspond to 2, 5 and 10% w/w, respectively) of oregano EO and (20, 50 and 65 g kg^{-1} which correspond to 2, 5 and 6.5% w/w, respectively) of Proallium® were fed into the barrel trough the lateral liquid port at L/D 10 in order to reduce possible volatility and degradation losses. Barrel temperatures were set at 200–205 ºC working at a screw speed of 70 min^{-1}. A control film was extruded in the same manner but with no oregano EO or Proallium® added. The average thickness of the final films was 80 µm (315 Gauge).

2.2. Bulk C stable isotopic analysis (IRMS)

Bulk isotopic signature of carbon ($\delta^{13}C$) was analysed using a Flash 2000 HT (C, N, S) combustion (C) and (H, O) pyrolysis (TC) elemental micro-analyser coupled via a ConFlo IV interface unit to a continuous flow Delta V Advantage isotope ratio mass spectrometer (IRMS) (Thermo Scientific, Bremen, Germany) (C/TC-IRMS). Isotopic ratios are reported as parts per thousand (‰) deviations from appropriate standards recognized by the International Atomic Energy Agency (IAEA) [36]. The standard deviation of bulk $\delta^{13}C$ was typically less than ± 0.05‰.

The proportion of additive in the bioplastic was calculated using a mass balance equation as described in [37].

Proportion of additive in bio-plastic = 100 x (A–B)/(C–B)
A: $\delta^{13}C$ bioplastic with additive
B: $\delta^{13}C$ bioplastic
C: $\delta^{13}C$ additive

2.3. Conventional analytical pyrolysis (Py-GC/MS)

In order to obtain molecular information and unambiguously characterize the main pyrolysis products, direct pyrolysis-gas chromatography–mass spectrometry (Py-GC/MS) was performed using a double-shot pyrolyzer (Frontier Laboratories, model 2020i) attached to a GC/MS system Agilent 6890N. Samples (0.5 mg) were placed in small crucible capsules and introduced into a preheated micro-furnace at (500 °C) for 1 min. The volatile pyrolysates were then directly injected into the GC/MS for analysis. The gas chromatograph was equipped with a low polar-fused silica (5%-phenyl-methylpolysiloxane) capillary column (Agilent J&W HP-5ms Ultra Inert, of 30 m × 250 µm × 0.25 µm film thickness. The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min^{-1}, from 100 °C to 300 °C at 10 °C min^{-1}, and stabilized at 300 °C for 10 min with a total analysis time of 32 min. The carrier gas was helium at a controlled flow of 1 mL min^{-1}. The detector consisted of an Agilent 5973 mass selective detector and mass spectra were acquired at 70 eV ionizing energy. Compound assignment was achieved by single-ion monitoring (SIM) for the major homologous series and by comparison with published data reported in the literature or stored in digital libraries (NIST and Wiley libraries).

2.4. Pyrolysis compound specific carbon isotope analysis (Py-CSIA)

Direct pyrolysis compounds specific isotope analysis (Py-CSIA) of carbon ($\delta^{13}C$) was carried out by coupling a double-shot pyrolyzer (Frontier Laboratories, model 3030D) attached to a GC/FID Trace GC Ultra system. At the end of the chromatographic column and to locate specific peaks within the chromatogram, the flux is divided and 10% diverted to a flame ionization detector (GC/FID) and 90% to a GC-Isolink II System equipped with a micro-reactor for combustion set at 1000 °C (EA) and coupled via a ConFlo IV universal interface unit to a continuous flow Delta V Advantage isotope ratio mass spectrometer (IRMS) (Thermo Scientific, Bremen, Germany) (Py-GC-(FID)-C-IRMS).

Samples of 1–2 mg in weight were placed in small stainless steel crucible capsules and introduced into a preheated micro-furnace at 500 °C for 1 min. The evolved gases were then directly injected into the GC/FID/IRMS system for analysis. The gas chromatograph was equipped with a low polar-polarity fused silica (5%-phenyl-methylpolysiloxane) capillary column Agilent J&W HP-5ms Ultra Inert, of 30 m × 250 µm × 0.25 µm film thickness. The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min^{-1}, from 100 °C to 300 °C at 10 °C min^{-1}, and stabilized at 300 °C for 10 min using a heating rate of 20 °C min^{-1}. The carrier gas was helium at a controlled flow of 1 mL min^{-1}.

Isotopic ratios are reported as parts per thousand (‰) deviations from appropriate standards recognized by the IAEA [36]. The standard deviation of compound specific $\delta^{13}C$ was typically less than ± 0.1‰.

Structural features of specific peaks were inferred by comparing and matching the mass spectra obtained by conventional Py-GC/MS with the Py-GC/FID and Py-GC/IRMS chromatograms obtained using the same column type and identical chromatographic conditions.

3. Results and discussion

3.1. Polylactic based active food packages structure as seen by analytical pyrolysis (Py-GC/MS)

Detailed structural study of the polymer (PLA:PBS) alone or with variable quantities of the additives within the film matrix using conventional analytical pyrolysis (Py-GC/MS) has been recently described in detail [30, 38]. In summary, the main PLA:PBS pyrolysis products were lactide enantiomers and monomer units from the major PLA fraction and succinic acid anhydride from the PBS fraction. Oregano EO pyrolysis released cymene, terpinene and carvacrol (mixture of isomers carvacrol/thymol) peaks as major diagnostic

peaks. Proallium® commercial additive main pyrolysis products, were oligomers of polyethyleneglycol, alkyl ethers and oleic acid, 3-hydroxypropyl ester compatible with a polysorbate surfactant and the sulphur compound, propyl sulphide that was a diagnostic peak for tracing the additive in the polymer.

Analytical pyrolysis was sensitive in detecting the increasing amounts of additive in the in the plastic. When comparing the chromatographic area of the main diagnostic peaks -both for OE and Proallium®- with the amount of additive added to the bioplastic, good correlations with coefficient values better than 0.950 R^2 ($P < 0.001$) were always found.

Examples of the chromatograms obtained by direct pyrolysis of the biopolymer (PLA:PBS) and of films containing additives are shown in Fig.1. Labels on peaks corresponds to compounds listed in Table 1.

3.2 PLA bulk isotopic signature

The biopolymer carbon isotope composition was found clearly ^{13}C-enriched ($\delta^{13}C = -10.7 \pm 0.63‰$) indicative of a main C4 vegetation origin, probably from corn starch. On the other hand, both additives showed lighter $\delta^{13}C$ signatures (Proallium® −28.9 ± 0.07‰; oregano OE −28.2 ± 0.05‰) reflecting biogenic origin from C3 photosystem vegetation, distinct from that of the biopolymer.

Consistent $\delta^{13}C$ shifts were observed in the PLA:PBS film extruded with variable quantities of additive, becoming more negative (^{13}C-depleted) with increasing dosage. This carbon isotopic content parallels the contribution from the additive light carbon within the heavier carbon PLA:PBS matrix and fits with correlations coefficient values better than 0.980 R^2 to a linear model in the case of oregano EO and to a quadratic model for Proallium® (Fig. 2A).

From the carbon isotope composition of the mixtures we calculated the real amount of the additive included in the bioplastic. Using a simple mass balance equation, we were able to assess the amount of additive that was ultimately and effectively incorporated in the final casting of the active-biopolymer. The estimation of the content of the two additives in the bioplastic was highly correlated with the alleged concentration (Fig. 2B). However, the IRMS

measurements overestimate the declared quantity of additive in the biopolymer in *c*. 20%.

3.3. PLA compound specific carbon isotope analysis (Py-CSIA)

Due to the differential C isotope composition between the biopolymer (^{13}C-enriched) and the additives (^{13}C-depleted), it was possible to detect specific compounds of the additive within the polymer matrix using direct Py-CSIA, as well as to estimate their $\delta^{13}C$ values. In Fig. 3 scatter plots of chromatographic retention time vs C isotope composition of selected peaks are shown for pure PLA:PBS film and additives (Proallium® and oregano EO) as well as for active food packaging film including variable quantities of the additives.

The stable C isotope composition of specific bio-plastic compounds released by direct pyrolysis was consistent with the film bulk values and indicative of a C4 vegetation (corn) origin. The $\delta^{13}C$ values ranged from −7.7 and −19.9‰, with an average stable C isotope composition for lactide enantiomers significantly heavier (L1-L3: $\delta^{13}C$ = −9.2‰ ± 1.56) than for cyclopentanones (C1-C2: $\delta^{13}C$ = −14.2‰ ± 2.11) and these heavier than for larger polymeric units ($\delta^{13}C$ = −17.2‰ ± 1.71).

This simple data representation of chromatographic retention time vs $\delta^{13}C$ allows us —at a first sight— to detect the compounds from the additive present in the film as ^{13}C-depleted outliers within the heavier compounds from the bio-plastic (Fig. 3). Specifically, for oregano EO, even at the lower concentration used (2%) it was possible to identify up to three specific marker compounds (A-C): the alkylbenzene cymene (A: $\delta^{13}C$ = −26.7‰ ± 2.52) and the monoterpenes terpinene (B: $\delta^{13}C$ = −27.1‰ ± 0.13) and carvacrol (C: $\delta^{13}C$ = −27.5‰ ± 1.80). For Proallium® specific marker compounds were detected only when the additive concentration was 5% or higher: two unknown structures (P1: $\delta^{13}C$ = −23.3‰ ± 3.32; P3: $\delta^{13}C$ = −24.4‰ ± 1.70) and butyl valerate (P2: $\delta^{13}C$ = −24.1‰ ± 3.55) (Table 1 and Fig. 1).

4. Conclusions

Using pyrolysis-gas chromatography—isotope ratio mass spectrometry (Py-CSIA) we were able to obtain, in addition to a comprehensive molecular fingerprinting, accurate isotopic signatures ($\delta^{13}C$) for specific polylactic acid based bio-plastic pyrolytic fragments (cyclopentanones, lactide enantiomers and polymeric units) as well as for specific markers of natural products added (oreagano EO: cymene, terpinene and carvacrol; Proallium®: two unidentified structures and butyl valerate). These values were consistent with measured bulk $\delta^{13}C$ values. A simple data representation of chromatographic retention time vs $\delta^{13}C$ of peaks allows detecting compounds from isotopically distinct additives contained within the bio-polymer. Py-CSIA is found a valuable technique to detect and trace natural additives in plastic films produced from corn starch with a minimum need for sample preparation.

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References

[1] A.C. Souza, G.E.O. Goto, J.A. Mainardi, A.C.V. Coelho, C.C. Tadini, Cassava starch composite films incorporated with cinnamon essential oil: Antimicrobial activity, microstructure, mechanical and barrier properties, LWT-Food Sci. Technol. 54 (2013) 346–52.

[2] F. Debiagi, R.K.T. Kobayashi, G. Nakazato, L.A. Panagio, S. Mali, Biodegradable active packaging based on cassava bagasse, polyvinyl alcohol and essential oils, Ind. Crop. Prod. 52 (2014) 664–670.

[3] A.K. Mohanti, M. Misra, G. Hinrichsen, Biofibres, biodegradable polymers and biocomposites: An overview, Macromol. Mater. Eng. 276-277 (2000) 1–24.

[4] M.A. Del Nobile, A. Conte, G.G. Buonocore, A.L. Incoronato, A. Massaro, O. Panza, Active packaging by extrusion processing of recyclable and biodegradable polymers, J. Food Eng. 93 (2009) 1–6.

[5] M. Llana-Ruiz-Cabello, S. Pichardo, A. Baños, C. Núñez, J.M. Bermúdez, E. Guillamón, S. Aucejo, A.M. Cameán, Characterisation and evaluation of PLA films containing an extract of *Alliums* sp. to be used in the packaging of ready-to-eat salads under controlled atmospheres, LWT-Food Sci. Technol. 64 (2015) 1354–1361.

[6] N.P. Mahalik, A.N. Nambiar, Trends in foor packaging and manufacturing systems and technology, Trends Food Sci. Technol. 21 (2010) 117–128.

[7] Regulation (ec) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC.

[8] Y. Byun, Y.T. Kim, S. Whiteside, Characterization of an antioxidant polylactic acid (PLA) film prepared with α-tocopherol, BHT and polyethylene glycol using film cast extruder, J. Food Eng. 100 (2010) 239–244.

[9] M. Llana-Ruiz-Cabello, S. Pichardo, J.M. Bermúdez, A. Baños, C. Núnez, E. Guillamón, S. Aucejo, A.M. Camean, Development of PLA films containing

oregano essential oil (*Origanum vulgare* L. virens) intended for use in food packaging, Food Addit. Contam. Part A-Chem. 33 (2016) 1374–1386.

[10] S. Maisanaba, M. Llana-Ruiz-Cabello, D. Gutiérez-Praena, S.Pichardo, M. Puerto, A.I. Prieto, A. Jos A.M. Cameán, New advances in active packaging incorporated with essential oils or their main components for food preservation, Food Rev Int. 33 (2017) 447–515.

[11] M. Ramos, A. Jiménez, M. Peltzer, M.C. Garrigós, Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging, J. Food Eng. 109 (2012) 513–519.

[12] J.W. White, K. Winters, Honey protein as an international standard for stable isotope ratio detection of adulteration of honey, J. AOAC Int. 72 (1989) 907–911.

[13] S. Kelly, I. Parker, M. Sharman, J. Dennis, I. Goodall, Assessing the authenticity of single seed vegetable oils using fatty acid stable carbon isotope ratios, Food Chem. 59 (1997) 181–186.

[14] A. Rossmann, Determination of stable isotope ratios in food analysis, FoodRev. Int. 17 (2001) 347–381.

[15] J.A. González-Pérez, N.T. Jiménez-Morillo, J.M. de la Rosa Arranz, G. Almendros, F.J. González-Vila, Compound specific isotopic signature of carbohydrate pyrolysis products from C3 and C4 plants (Py-CSIA), J. Sci. Food Agric. 96 (2016) 948–953.

[16] P. Deines, The isotopic composition of reduced organic carbon, in: P. Fritz, J.Fontes (Eds.), Handbook of Environmental Isotope Geochemistry, Vol. 1, Elsevier, New York, 1980, pp. 329–406.

[17] W. Meier-Augenstein, Applied gas chromatography coupled to isotope ratio mass spectrometry, J. Chrom. A. (1999) 842, 351–371.

[18] M. Elsner, M.A. Jochmann, T.B. Hofstetter, D. Hunkeler, A. Bernstein, T.C. Schmidt, A. Schimmelmann, Current challenges in compound-specific stable isotope analysis of environmental organic contaminants, Anal. Bioanal. Chem. 403 (2012) 2471–2491.

[19] N. Ivdra, S. Herrero-Martín, A. Fischer, Validation of user- and environmentally friendly extraction and clean-up methods for compound-specific stable carbon isotope analysis of organochlorine pesticides and their metabolites in soils, J. Chrom. A. (2014) 36–45.

[20] M. Blazsó, Recent trends in analytical and applied pyrolysis of polymers, J. Anal. Appl. Pyrolysis 39 (1997) 1–25.

[21] S. Tsuge, H. Ohtani, Structural characterization of polymeric materials bypyrolysis-GC/MS, Polym. Degrad. Stab. 58 (1997) 109–130.

[22] J.K. Haken, Pyrolysis gas chromatography of synthetic polymers—a bibliography, J. Chromatogr. A, 825 (1998) 171–187.

[23] S. Tsuge, H. Ohtani, C. Watanabe, Pyrolysis-GC/MS Data Book of Synthetic Polymers, Elsevier, Oxford, 2011.

[24] J.A. González-Pérez, N.T. Jiménez-Morillo, J.M. de la Rosa, G. Almendros, F.J.González-Vila, Pyrolysis-gas chromatography–isotope ratio mass spectrometry of polyethylene, J. Chromatogr. A 1388 (2015) 234–236.

[25] M.P. Arrieta, F. Parres, J. López, A. Jiménez, Development of a novel pyrolysis-gas chromatography/mass spectrometry method for the analysis of poly(lactic acid) thermal degradation products Pyr bio-platics, J. Anal. Appl. Pyrolysis 101 (2013) 150–155.

[26] E. Vuorinen, M. Hakkarainen, Method development for the analysis of biodegradable polymers, Int. J. Metrol. Qual. Eng. 1 (2010) 29–32.

[27] Y. Aoyagi, K. Yamashita, Y. Doi, Thermal degradation of poly[(R)-3-hydroxybutyrate], poly[(-caprolactone)] and poly[(S)-lactide], Polym. Degrad. Stab. 76 (2002) 53–59.

[28] C. Westphal, C. Perrot, S. Karlsson, Py-GC/MS as a means to predict degree of degradation by giving microstructural changes modelled on LDPE and PLA, Polym. Degrad. Stab. 73 (2001) 281–287.

[29] F.D. Kopinke, K. Mackenzie, Mechanistic aspects of the thermal degradation ofpoly(lactic acid) and poly(β-hydroxybutyric acid), J. Anal. Appl. Pyrolysis40–41 (1997) 43–53.

[30] M. Llana-Ruíz-Cabello, S. Pichardo, N.T. Jiménez-Morillo, J.M. Bermúdez, S. Aucejo, F.J. González-Vila, A.M. Cameán, J.A. González-Pérez, Molecular characterization of a bio-based active packaging containing *Origanum vulgare* L. essential oil using pyrolysis gas chromatography-mass spectrometry (Py-GC/MS), J. Sci. Food Agric. 96 (2016) 3207–3212.

[31] D.G. Papageorgiou, E. Roumeli, K. Chrissafis, C. Lioutas, K. Triantafyllidis, D.Bikiaris, A.R. Boccaccini, Thermal degradation kinetics and decomposition mechanism of PBS nanocomposites with silica-nanotubes and strontium hydroxyapatite nanorods, Phys. Chem. Chem. Phys. 16 (2014) 4830–4842.

[32] M.A. Goñi, T.I. Eglinton, Analysis of kerogens and kerogen precursors by flashpyrolysis in combination with isotope-ratio-monitoring gaschromatography–mass spectrometry (irm-GC-MS), J. High Resolut. Chrom. 17 (1994) 476–488.

[33] T.N. Corso, J.T. Brenna, High-precision position-specific isotope analysis, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1049–1053.

[34] S. Steinbeiss, C.M. Schmidt, K. Heide, G. Gleixner, ^{13}C values of pyrolysis products from cellulose and lignin represent the isotope content of their precursors, J. Anal. Appl. Pyrolysis. 75 (2006) 19–26.

[35] A.Z. Miller, J.M. de la Rosa, N.T. Jiménez-Morillo, M.F.C. Pereira, J.A. González-Pérez, J.M. Calaforra, C. Saiz-Jimenez, Analytical pyrolysis and stable isotope analyses reveal past environmental changes in coralloid speleothems from Easter Island (Chile). J. Chromatogr. A 1461 (2016) 144–15.

[36] S. Valkiers, M. Varlam, K. Rußе, M. Berglund, P. Taylor, J. Wang, M. Milton, P. De Bièvre, Quantification of the degree-of-isotopic-equilibrium of carbon and oxygen isotopes in mixtures of CO_2 gases, Int. J. Mass Spectrom. 263 (2007) 195–203.

[37] M. Bernoux, C.C. Cerri, C. Neill, J.F.L. de Moraes, The use of stable carbon isotopes for estimating soil organic matter turnover rates, Geoderma 82 (1998) 43–58.

[38] M. Llana-Ruíz-Cabello, S. Pichardo, N.T. Jiménez-Morillo, P. Abad, E. Guillamón, F.J. González-Vila, A.M. Cameán, J.A. González-Pérez, Characterisation of a bio-based packaging containing a natural additive from *Allium* spp. using analytical pyrolysis and carbon stable isotopes, J. Anal. Appl. Pyrol. 120 (2016) 334–340.

Figure captions:

Fig. 1. Examples of the chromatograms obtained by direct pyrolysis (Py-GC/MS) of the biopolymer (PLA:PBS) and of films containing additives. Labels on peaks corresponds to compounds listed in Table 1.

Fig. 2. Estimation of the additive in the PLA based in shifts in carbon isotopic composition ($\delta^{13}C$). (A) Relation between $\delta^{13}C$ and additive added; (B) Relation between declared additive in bioplastic and the calculated values based in a mass balance relation. Error bars indicate the mean STD (n = 3).

Fig. 3. Py-CSIA analysis; scatter plots of chromatographic retention time vs carbon isotopic composition ($\delta^{13}C$) of selected peaks for pure PLA:PBS biopolymer film and pure additives (Proallium® and oregano EO) as well as for biopolymer film including variable quantities of the additives. Labels on dots corresponds to compounds listed in Table 1.

Figure 1

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FIGURE 1:

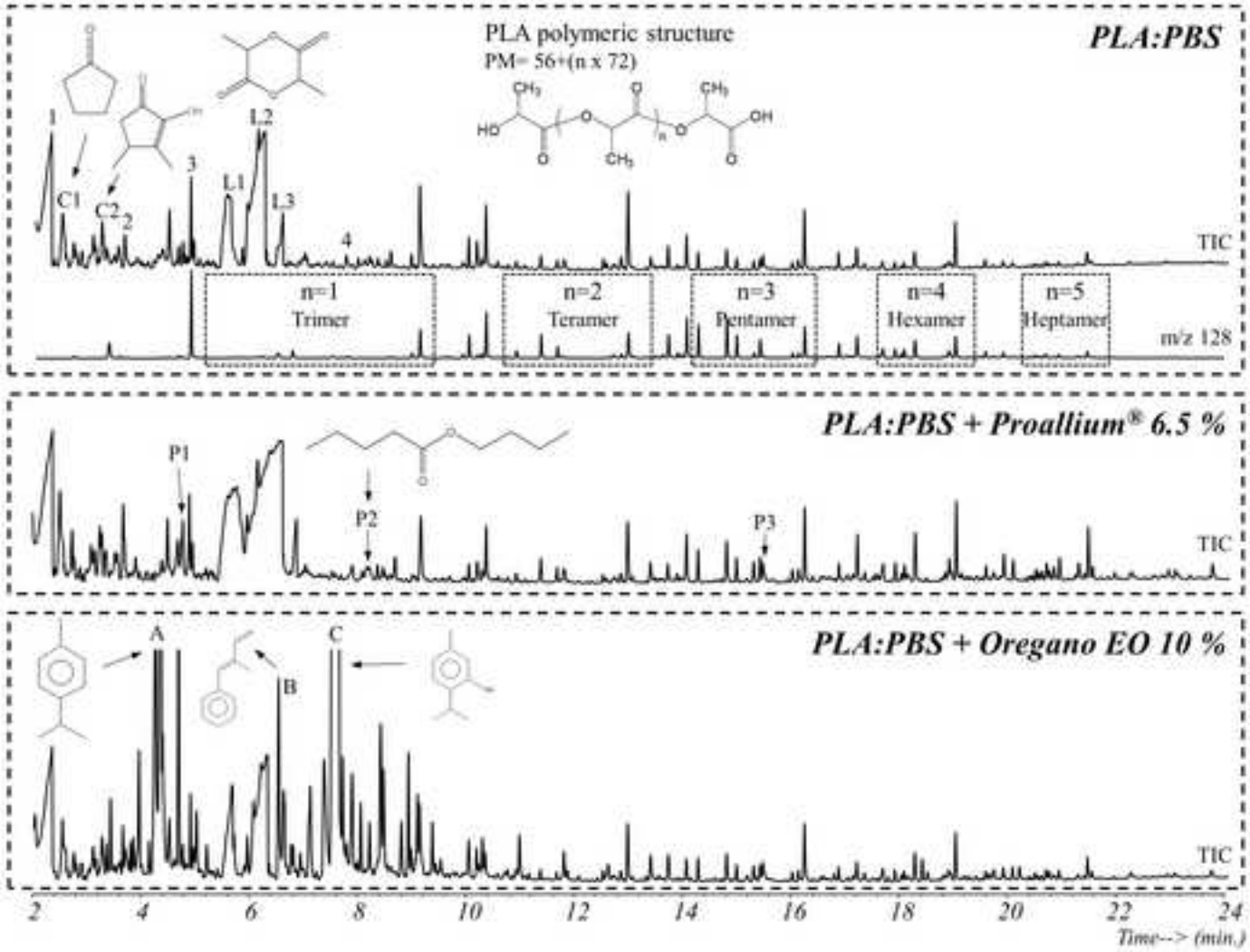

Figure 2
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FIGURE 2:

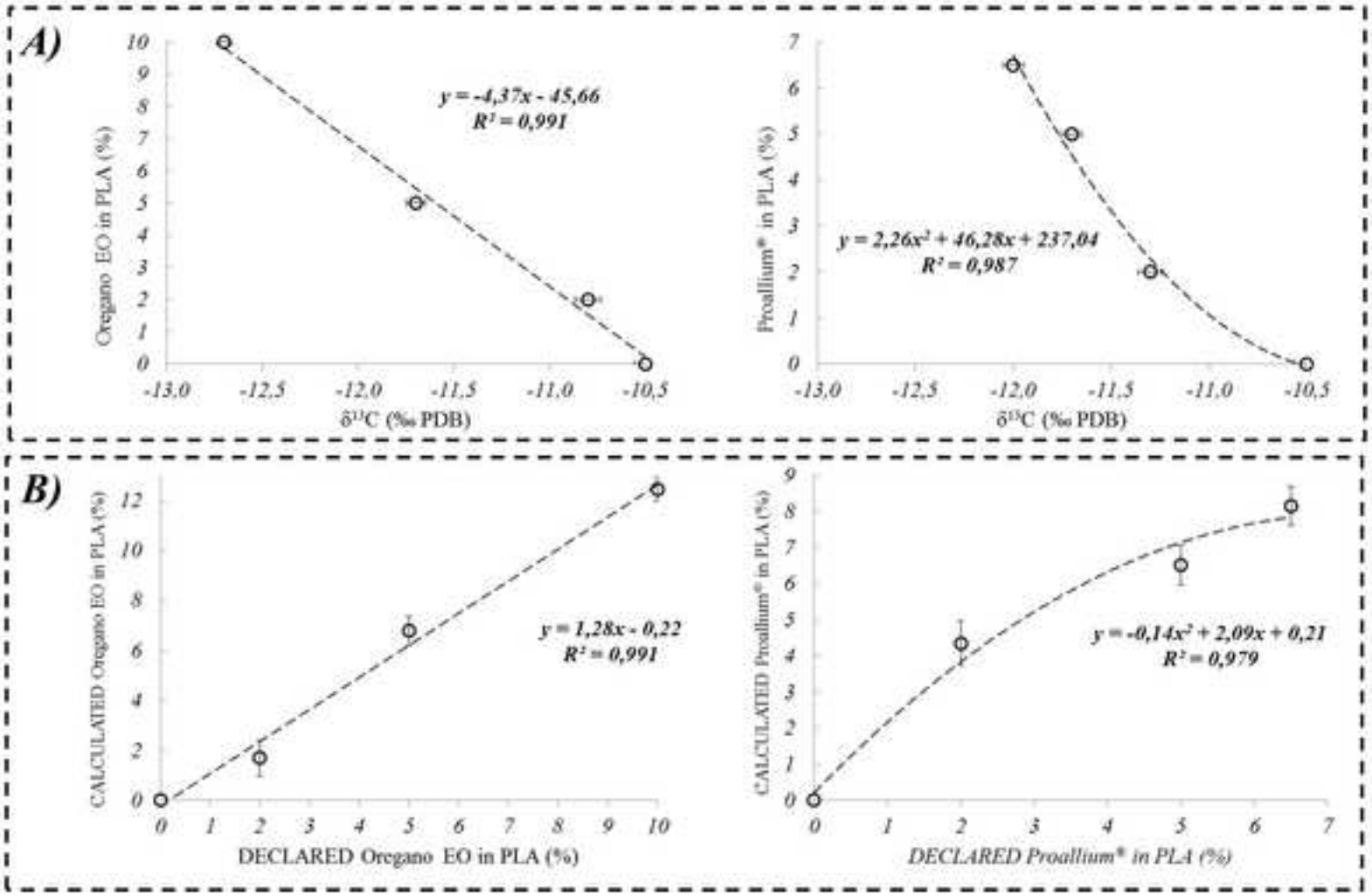

Figure 3

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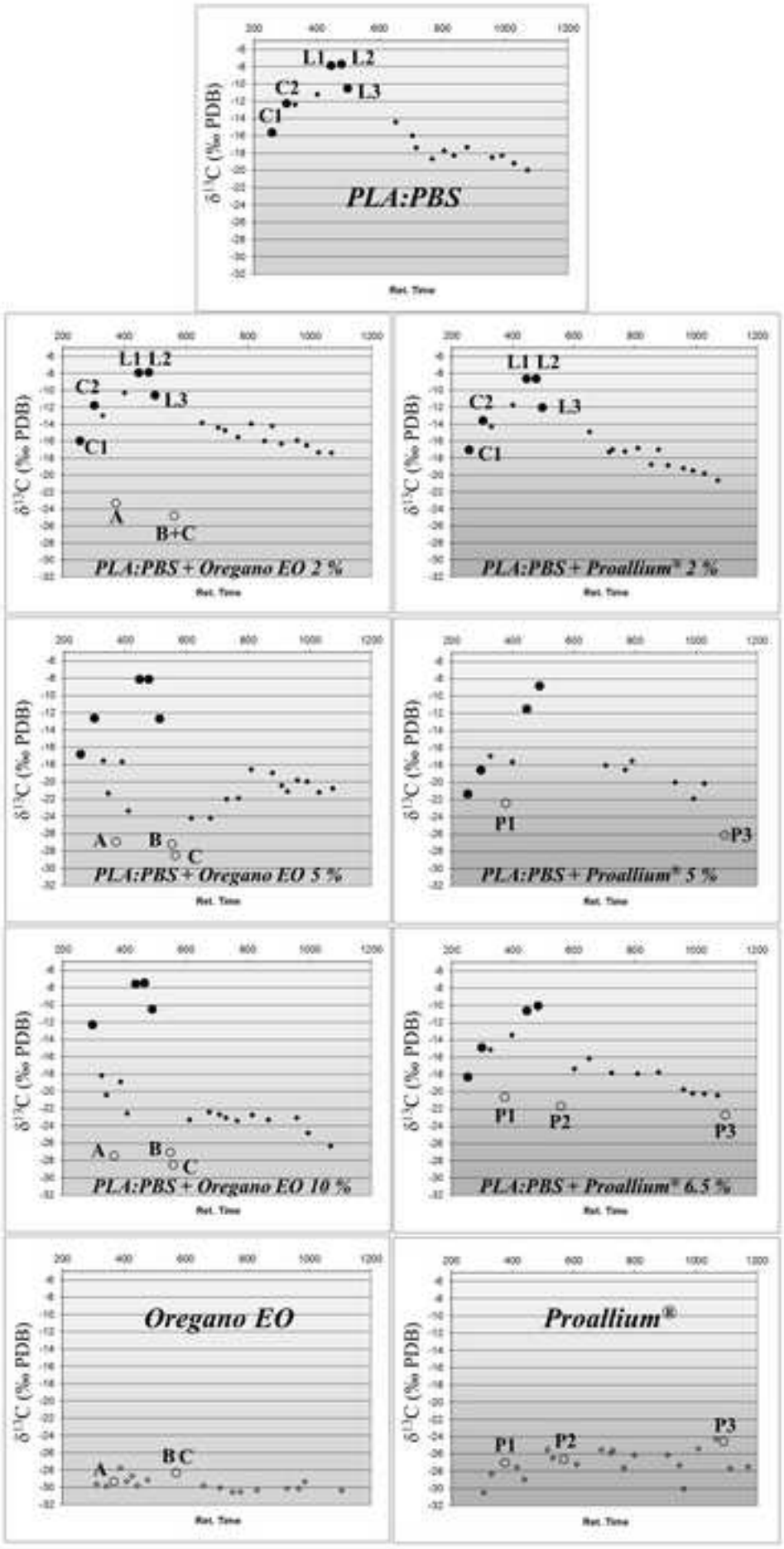

Table 1

Table 1. Main compounds identified by Py-GC/MS in the biopolymer (PLA:PBS), in the additives and in the films containing additives (main marker compounds).

	PLA main marker compounds
C1	Cyclopentanone
C2	2-Cyclopenten-1-one, 2-hydroxy-3,4-dimethyl-
L1-L3	Lactide enantiomeric forms
	PBS probable compounds
1	Tetrahydrofuran + 1,2-Butadiene
2	Furan, tetrahydro-2,5-dimethyl-
3	2-Propenoic acid, (1-propionato)ethyl ester
4	4-Pentenoic acid, 2-acetyl-, ethyl ester
	Proallium® main marker compounds
P1	Unknown (min 4.94 m/z 57, 101, 130)
P2	Pentanoic acid, butyl ester (min 8.20 m/z 57, 85,103, 158) [Butyl valerate]
P3	Unknown (min 15.42 m/z 129, 157, 187)
	Oregano EO main marker compounds
A	Benzene, 1-methyl-4-(1-methylethyl)- [Cymene]
B	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- [Terpinene]
C	Phenol, 5-methyl-2-(1-methylethyl)- [Carvacrol]

CAPÍTULO 17 / CHAPTER 17

María Llana-Ruíz-Cabello, María Puerto, Silvia Pichardo, Francisco J. González-Vila, José M. Bermúdez, Susana Aucejo, Ana M. Cameán, José A. González-Pérez

EFFECT OF AN ACTIVE BIO-PACKAGE IN THE PRESERVATION OF NUTRITIONALLY IMPORTANT PHYTOCONSTITUENTS IN READY-TO-EAT LETTUCE.

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Abstract: Natural preservatives are being added to plastic materials used in new food packages to improve foodstuff preservation. Oregano essential oil (OEO) have benefits for active packaging due to the bioactivity of main components, carvacrol and thymol. Here, the effect of a bioactive package made of polylactic acid (PLA)/polybutylene succinate (PBS) with OEO in the chemical composition of iceberg lettuce and in main bioactive components is evaluated. Analytical pyrolysis (Py-GC/MS) was used to detect changes in compounds with dietetic value such are phytosterols (PHSTs) and polyunsaturated fatty acids (PUFAs) after 1, 4 and 8 days of packaged in PLA/PBS (95:5%) films containing different OEO concentrations (2-10%).
After Py-GC/MS, lettuce showed the typical mixture of substances with known biogenic precursors, mainly polysaccharides, lignin, polypeptides and lipids including fatty acids and PHSTs. Lettuce packaged in films without OEO experimented a decrease in compounds with nutritional value, such as PUFAs and PHSTs, along the packaging time. The addition of OEO into the films favoured the preservation of these bioactive components during the shelf life of the packed food. In this regard, lettuce packaged in films containing 5 and 10% OEO still maintained the relative PUFAs and PHSTs content after 8 days of storage.

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COVER LETTER

Effect of an active bio-package in the preservation of nutritionally important phytoconstituents in ready-to-eat lettuce

By

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Submited for consideration at:
Journal of Food Composition and Analysis

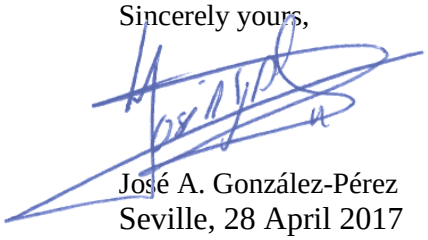
Dear Editor,

The MS we are presenting deals with the beneficial effect of a bioactive package made of polylactic acid (PLA)/polybutylene succinate (PBS) with oregano essential oil (OEO) in the preservation of iceberg lettuce main bioactive components. Analytical pyrolysis (Py-GC/MS) was used to detect changes in compounds with dietetic value such are phytosterols (PHSTs) and polyunsaturated fatty acids (PUFAs) after 1, 4 and 8 days of packaged in PLA/PBS (95:5%) films containing different OEO concentrations (2-10%).

Whereas, lettuce packaged in films without OEO experimented a decrease in bioactive compounds such as PUFAs and PHSTs, the addition of OEO into the films favoured the preservation of these components during the shelf life of the packed food. In this regard, lettuce packaged in films containing 5 and 10% OEO still maintained the relative PUFAs and PHSTs content after 8 days of storage.

We really hope that this contribution is of interest and acceptable for publication in the Journal of Food Composition and Analysis.

Sincerely yours,



José A. González-Pérez
Seville, 28 April 2017

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Effect of an active bio-package in the preservation of nutritionally important phytoconstituents in ready-to-eat lettuce.

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Abstract

Natural preservatives are being added to plastic materials used in new food packages to improve foodstuff preservation. Oregano essential oil (OEO) have benefits for active packaging due to the bioactivity of main components, carvacrol and thymol. Here, the effect of a bioactive package made of polylactic acid (PLA)/polybutylene succinate (PBS) with OEO in the chemical composition of iceberg lettuce and in main bioactive components is evaluated. Analytical pyrolysis (Py-GC/MS) was used to detect changes in compounds with dietetic value such are phytosterols (PHSTs) and polyunsaturated fatty acids (PUFAs) after 1, 4 and 8 days of packaged in PLA/PBS (95:5%) films containing different OEO concentrations (2-10%).

After Py-GC/MS, lettuce showed the typical mixture of substances with known biogenic precursors, mainly polysaccharides, lignin, polypeptides and lipids including fatty acids and PHSTs. Lettuce packaged in films without OEO experimented a decrease in compounds with nutritional value, such as PUFAs and PHSTs, along the packaging time. The addition of OEO into the films favoured the preservation of these bioactive components during the shelf life of the packed food. In this regard, lettuce packaged in films containing 5 and 10% OEO still maintained the relative PUFAs and PHSTs content after 8 days of storage.

Keywords: Lettuce; Carvacrol; Thymol; Phytosterols; PUFAs; Analytical pyrolysis

1. Introduction

The market of ready-to-eat leaf vegetables is rapidly growing at a global scale providing consumers with appealing products, rich in healthy and beneficial bioactive compounds. Among the most relevant nutritious components in leafy vegetables are phytosterols (Kim et al., 2015) and unsaturated/polyunsaturated fatty acids (Saini et al., 2016). Lettuce is known to contain high quantities of dietary phytosterols (PHSTs) (Kaliora et al., 2015) and of polyunsaturated fatty acids (PUFAs) (Saini et al., 2014).

Both, PHSTs and PUFAs are relevant bioactive components of vegetables known to have positive effects on health when included in the diets. Plant sterols are able to diminish serum low-density lipoprotein (LDL) cholesterol levels and thus protect against cardiovascular diseases (Moreau et al., 2002; Weststrate and Meijer, 1998). Dietary PUFAs like α-linolenic acid (ALA) have also many beneficial effects in the control of chronic diseases i.e. inhibition of synthesis of vasoaggressive low-density lipoprotein (LDL) and acceleration of its elimination, reduction of blood pressure, prevention of cardiovascular disease and cancer (Abedi and Ali Sahari, 2014 and references therein). This has led to the development of functional foods enriched in such bioactive components like plant sterols and PUFAs (Lagarda et al., 2006; Volker et al., 2005).

There is also interest in providing the industry with effective means for food preservation and of its nutritious beneficial properties. New trends are focusing in the development of active packaging, which can interact with the product or its environment and then improve food conservation. In general, active packaging containing essential oils (EOs) are developed to improve the shelf life of food and to avoid the undesirable flavours caused by direct addition of these substances (Gutiérrez

et al., 2009). In this sense, oregano essential oil (OEO) is being included in these new food packaging materials due to its bioactive properties (Jouki et al., 2014a; Wu et al., 2014) that are related to its content in bioactive monoterpenes, sesquiterpenes and phenolic compounds (Ortega-Nieblas et al., 2011).

On the other hand, the effect of bio-based packaged in shelf-life and its effect in the chemical composition of the food that contain, provides information regarding the usefulness of these new materials. Therefore, different films containing OEO has been found useful in reducing the microbial counts of several microorganisms in food stuffs i.e. cooked salmon (Tammineni et al., 2013), cheese (Otero et al., 2014), chicken breast (Fernández-Pan et al., 2014), rainbow trout (Jouki et al., 2014b) and also in lettuce (Llana-Ruiz-Cabello et al., 2016a). Moreover, OEO and films incorporated with this EO have shown different antioxidant activities related with the retardation of lipid peroxidation through their potent radical scavenging activity derived from their composition in carvacrol and thymol (Maisanaba et al., 2017). In fact, in a previous work we found that carvacrol, thymol, and their mixture (10:1) at low concentrations exert protective role against induced oxidative stress on Caco-2 cell lines system model (Llana-Ruiz-Cabello et al., 2015).

Such antimicrobial and antioxidant properties of additives in bio-plastics improve food conservation and consumer acceptance and this, desirably should include the preservation of the nutritional profile of the packed food. As far as we know there is no information available regarding the benefits of food packaged in active films in relation with the conservation of specific compounds with dietetic value i.e. PHSTs and PUFAs.

Analytical pyrolysis is a toll providing a direct fingerprinting and precise information about composition, quality and additives in foods and packages. The products of

pyrolysis are amenable to chromatographic separation and when combined with a mass spectrometry detector (Py-GC-MS), yields molecular information about the structure of complex mixtures of natural and synthetic macromolecular substances (González-Pérez et al., 2007; 2015). Other well-known advantages of the technique are the requirement of small sample sizes and little or no sample preparation. This technique has been used with success to detect EOs added to synthetic and bio-based polymers (Llana-Ruiz-Cabello et al., 2016b). Major plant lipid components such are sterols and fatty acids are also easily detected by direct analytical pyrolysis (Py-GC/MS) of biomass (González-Vila et al., 2001; 2009; Schnitzer et al., 2006).

In previous works, a polylactic acid (PLA) polybutylene succinate (PBS) (95:5) film containing OEO was developed (Llana-Ruiz-Cabello et al., 2016a), its antioxidant properties evaluated (Llana-Ruiz-Cabello et al., 2015) and the ability of this material to reduce microbial counts of yeasts and molds in ready-to-eat salad confirmed (Llana-Ruiz-Cabello et al., 2016a).

In this work we use a detailed Py-GC/MS analysis performed to detect changes in food composition of the relevant dietetic compounds mono, di and polyunsaturated fatty acids (PUFAs) and PHSTs, in iceberg lettuce (*Lactuca sativa*) after 1, 4 and 8 days packaged in PLA/PBS (95:5) films containing different quantities of OEO (0, 2, 5 and 10 %).

2. Materials and Methods

2.1.Bio-polymer and additives

Plastic films were made of a mixture of polylactic acid (PLA) with polybutylene succinate (PBS) (950 g kg^{-1}:50 g kg^{-1}) and extruded with variable quantities of oregano essential oil (EO). The EO was obtained from El Jarpil® (Almería, Spain), PLA (2003D extr. grade) was purchased from NatureWorks LLC (Minnetonka, MN, USA) and PBS (GS PlaTM FD92WD) from Mitshubishi Chemical Corporation (Tokyo, Japan). Chemicals for the different assays were purchased from Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

The active PLA films were obtained by melt blending in a twin-screw extruder (DSE 20-40D; Brabender, Duisburg, Germany). Different concentrations (0, 2, 5 and 10% w/w) of OEO and were fed into the barrel at L/D 10. Barrel temperatures were set at 200–205 °C working at a screw speed of 70 min^{-1}. Final average thickness of the final films was 80 μm (315 Gauge).

2.2.Analytical pyrolysis (Py-GC/MS)

Direct pyrolysis-gas chromatography–mass spectrometry (Py-GC/MS) was performed using a double-shot pyrolyzer (Frontier Laboratories, model 2020i) attached to a GC/MS system Agilent 6890N. Samples (0.5 mg dry lettuce biomass) were placed in crucible deactivated steel pyrolysis capsules and introduced into a preheated micro-furnace at (500 °C) for 1 min. The volatile pyrolysates were then directly injected into the GC/MS for analysis. The gas chromatograph was equipped with a low polar-fused silica (5%-phenyl-methylpolysiloxane) capillary column (Agilent J&W HP-5ms Ultra Inert, of 30 m × 250 μm × 0.25 μm film thickness. The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min^{-1}, from 100 °C to 300 °C at 10

°C min-1, and stabilized at 300 °C for 10 min with a total analysis time of 32 min. The carrier gas was He at a controlled flow of 1 mL min^{-1}. The detector consisted of an Agilent 5973 mass selective detector and mass spectra were acquired at 70 eV ionizing energy. Compound assignment was achieved by single-ion monitoring (SIM) for the major homologous series and by comparison with published data reported in the literature or stored in digital libraries (NIST and Wiley libraries).

3. Results and discussion

3.1.Lettuce Py-GC/MS fingerprint

The analytical pyrolysis of lettuce produced typical biomass chromatograms. A detailed pyrolysis fingerprint of lettuce is depicted in Fig. 1 and the identified compounds can be found in Table 1. The first part of the chromatogram (min 2 to 14) is dominated by pyrolysis products from lignocellulose that represent c. 43 % of the total chromatographic area and included products from the major polysaccharide component (38 %) i.e. furan [1,6,8,10,17,18] and cyclopentane [2,4,7,11,15] derivatives and methoxyphenols [13,21,25,26] from the polyphenolic lignin domain (5 %). Most long chain lipids, mainly alkane/alkene doublets [33-36, 38] and fatty acids (FA) [29-32] are eluted in the central section of the chromatogram (min 14 to 23), with a major prominent peak of palmitic acid [29] (c. min 14.8) and an oleic complex cluster that include the polyunsaturated (PUFAs) linoleic [30] and linolenic [31] acids, the monounsaturated oleic [co-eluted] and saturated FA stearic [32] acids. The last section of the chromatogram (min 23 to 28) is dominated by triterpenes, plant sterols known as PHSTs. Other compounds identified in the pyrograms from iceberg lettuce included: aromatic structures of unknown origin (ARO), alkyl benzenes [3,22], phenol [9] and methyl phenols [12]; compounds with nitrogen (N) probably derived from the

protein/polypeptide domain, nitriles [16, 19], hydrazones [28] and the heterocyclic pyrroles [5] and indoles [20,24]. Also a small proportion (1 %) of methylated FAs (FAME) were identified [37, 39] probably derived from the pyrolysis of epicuticular waxes.

Compounds with a particular dietetic interest found in the lettuce chromatograms were the bioactive PUFAs included in the oleic domain (c. min 16.5) that represented c. 4 % of total chromatographic area and the PHSTs, eluted at the end of the chromatogram that represents c. 8 % of total chromatographic area. The chemical structures of these compounds as well as their mass spectra are in Fig. 2. The preservation of these compounds during the shelf life of the packed lettuce was considered as the main target for this study.

3.2.Lettuce decay with time

The evolution of lettuce pyrolysis fingerprint packed in PLA/PBS bioplastic without OEO is shown in Fig. 3. A conspicuous disappearance of peaks of particular dietetic interest: oleic and PUFAs complex as well as of PHSTs, can be observed at a first sight in the chromatogram. This confirms that iceberg lettuce rapidly and progressively lost relevant dietetic compounds during conservation time when packed in our bioplastic (PLA/PBS) without any OAO additive.

When analysing the evolution of the lettuce pyrogram fingerprint with time and packed in the PLA/PBS bioplastic without (OEO 0%) or with variable concentrations of the OEO additive (2, 5, 10 %), the preservation of the peaks corresponding to compounds of particular dietetic interest is also evident (Fig. 4). In this regard, even when the lettuce is packed in bioplastic with the minimum OEO content (OEO 2%) the relative

content of PHST is preserved and when packaged in 5 and 10% OEO containing films, both PUFAs and PHST relative contents are preserved even after 8 days of storage.

Comparing the relative abundance of the oleic complex (peaks 30-32) and of the major PHSTs (stigmasterol and sitosterol, peaks 43 and 44 respectively) relative to a common chromatographic peak allow us to compare the evolution of compounds with dietetic interest with time and with the different concentrations of OEO added to the package bioplastic. The evolution of dietetic relevant compounds is shown in Fig. 5 relative to toluene (peak 3), this compound is usually found with relative high abundance in biomass pyrolysis.

Therefore, lettuce packaged in films with high concentrations of OEO (5% and 10%) maintained the values of oleic complex nutrients at levels similar to those observed for the first day of storage (Fig. 5A). In addition, values of PHSTs experimented an increased in lettuce packaged in films containing OEO (Fig. 5B), this may reflect the occurrence of a selective preservation of PHSTs with time.

Conclusions

Analytical pyrolysis was found useful in characterizing lettuce composition and particularly in tracing the evolution with time of dietetic relevant components like the bioactive PUFAs and PHSTs. Using this technique, we were able to evaluate the effect of an active film bio-package (PLA/PBS) containing variable quantities of OEO in the conservation of these specific dietetic compounds in packed food. The use of active bio-packages containing OEO, allowed appropriate preservation of both PHSTs and PUFAs relative contents during the shelf life (8 days) of the packed food.

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References

Abedi, E., Sahari, M.A. (2014). Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. *Food Science and Nutrition, 2,* 443–463.

Fernández-Pan, I., Carrión-Granda, X., Maté, J.I. (2014). Antimicrobial efficiency of edible coatings on the preservation of chicken breast fillets. *Food Control, 36,* 69–75.

González-Pérez, J.A., Arbelo, C.D., González-Vila, F.J., Rodríguez Rodríguez, A., Almendros, G., Armas, C.M., Polvillo, O. (2007). Molecular features of organic matter in diagnostic horizons from andosols as seen by analytical pyrolysis. *Journal of Analytical and Applied Pyrolysis, 80,* 369–382.

González-Pérez, J.A., Jiménez-Morillo, N.T., de la Rosa, J.M., Almendros, G., González-Vila, F.J. (2015). Pyrolysis-gas chromatography-isotope ratio mass spectrometry of polyethylene. *Journal of Chromatography A, 1388,* 236–243.

González-Vila, F.J., Tinoco, P., Almendros, G., Martín, F. (2001). Pyrolysis-GC-MS analysis of the formation and degradation stages of charred residues from lignocellulosic biomass. *Journal of Agriculture and Food Chemistry, 49,* 1128–1131.

González-Vila, F.J., González-Pérez, J.A., Akdi, K., Gómis, M.D., Pérez-Barrera, F., Verdejo, T. (2009). Assessing the efficiency of urban waste biocomposting by analytical pyrolysis (Py–GC/MS). *Bioresource Technology, 100,* 1304–1309.

Gutiérrez, L., Sánchez, C., Battle, R., Nerín, C. (2009). New antimicrobial active package for bakery products. *Trends in Food Science and Tecnology, 20,* 92–99.

227 Jouki, M., Mortazavi, S.A., Yazdi, F.T., Koocheki, A. (2014a). Characterization of
228 antioxidant-antimicrobial quince seed mucilage films containing thyme essential oil.
229 *Carbohydrate Polymers, 99*, 537–546.

230 Jouki, M., Yazdi, F.T., Mortazavi, S.A., Koocheki, A., Khazaei, N. (2014b). Effect of
231 quince seed mucilage edible films incorporated with oregano or thyme essential oil on
232 shelf life extension of refrigerated rainbow trout fillets. *International Journal of Food*
233 *Microbiology, 174*, 88–97.

234 Kaliora, A.C., Batzaki, C., Christe, M.G., Kalogeropoulos, N. (2015). Nutritional
235 evaluation and functional properties of traditional composite salad dishes. *LWT-Food*
236 *Science and Technology, 62*, 775–782.

237 Kim, T.J., Lee, K.B., Baek, S.-A., Choi, J., Ha, S.-H., Lim, S.-H., Park, S.-Y., Yeo, Y.,
238 Park, S.U., Kim, J.K. (2015). Determination of lipophilic metabolites for species
239 discrimination and quality assessment of nine leafy vegetables. Journal of the Korean
240 Society for Applied Biological Chemistry, *58*, 909–918.

241 Lagarda, M.J., García-Llantas, G., Farré, R. (2006). Analysis of phytosterols in foods.
242 Journal of Pharmaceutical and Biomedical Analysis, *41*, 1486–1496.

243 Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Jos, A.,
244 Cameán, A.M. (2015). *In vitro* pro-oxidant/antioxidant role of carvacrol, thymol and
245 their mixture in the intestinal Caco-2 cell line. *Toxicology in Vitro, 29*, 647–656.

246 Llana-Ruiz-Cabello, M., Pichardo, S., Bermúdez, J.M., Baños, A., Núñez, C.,
247 Guillamón, E., Aucejo, S., Cameán, A.M. (2016a). Development of PLA films
248 containing oregano essential oil (*Origanum vulgare* L. *virens*) intended for use in food
249 packaging. *Food Additives and Contaminants: Part A, 33*, 1374–1386.

Llana-Ruiz-Cabello, M., Pichardo, S., Jiménez-Morillo, N.T., Bermúdez, J.M., Aucejo, S., González-Vila, F.J., Cameán, A.M., González-Pérez, J.A. (2016b). Molecular characterisation of a bio-based active packaging containing *Origanum vulgare* L. essential oil using pyrolysis gas chromatography-mass spectrometry. *Journal of the Science of Food and Agriculture, 96,* 3207–3212.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, M., Prieto, A.I., Jos, A., Cameán, A.M. (2017). New advances in active packaging incorporated with essential oils or their main components for food preservation. *Food Reviews International*, *33*, 447–515.

Moreau, R.A., Whitaker, B.D., Hicks, K.B. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research, *41*, 457-500.

Ortega-Nieblas, M.M., Robles-Burgueño, M.R., Acedo-Félix, E., González-León, A., Morales-Trejo, A., Vázquez-Moreno, M.L. (2011). Chemical composition and antimicrobial activity of oregano (*Lippia palmeri* S. Wats) essential oil. Revista Fitotecnia Mexicana, *34*, 11–17.

Otero, V., Becerril, R., Santos, J.A., Rodríguez-Calleja, J.M., Nerín, C., García-López, M.-L. (2014). Evaluation of two antimicrobial packaging films against Escherichia coli O157: H7strains *in vitro* and during storage of Spanish ripened sheep cheese (Zamorano). *Food Control*, *42*, 296–302.

Saini, R.K., Shetty, N.P., Giridhar, P. (2014). GC-FID/MS Analysis of Fatty Acids in Indian Cultivars of *Moringa oleifera*: Potential Sources of PUFA. Journal of the American Oil Chemists' Society, *91*, 1029–1034.

Saini, R.K., Shang, X.M., Choi, J.H., Kim, D., Keum, Y.-S. (2016). Characterization of nutritionally important phytoconstituents in minimally processed ready-to-eat baby-leaf vegetables using HPLC–DAD and GC–MS. Journal of Food Measurement and Characterization, *2*, 341–349.

Schnitzer, M., McArthur, D.F.E., Schulten, H.-R., Kozak, L.M., Huang, P.M. (2006). Long-term cultivation effects on the quantity and quality of organic matter in selected Canadian prairie soils. *Geoderma, 130*, 141–156.

Tammineni, N., Ünlü, G., Min, S.C., (2013). Development of antimicrobial potato peel waste-based edible films with oregano essential oil to inhibit *Listeria monocytogenes* on cold-smoked salmon. International Journal of Food Science & Technology, *48*, 211–214.

Volker, D.H., Weng, X., Quaggiotto, P. (2005). Bioavailability of long-chain omega-3 polyunsaturated fatty acid enriched luncheon meats. Nutrition & Dietetics, *62*, 130–137.

Weststrate, J.A., Meijer, G.W. (1998). Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. European Journal of Clinical Nutrition, *52*, 334–343.

Wu, J., Ge, S., Liu, H., Wang, S., Chen, S., Wang, J., Li, J., Zhang, Q. (2014). Properties and antimicrobial activity of silver carp (*Hypophthalmichthys molitrix*) skin gelatin-chitosan films incorporated with oregano essential oil for fish preservation. Food Packaging and Shelf Life, *2*, 7–16.

FIGURE CAPTIONS:

Figure 1.- Fresh lettuce fingerprinting (Py-GC/MS at 500 °C), with an indication of the relative contribution of the main compound families. Numbers on peaks corresponds to the major compounds identified and listed in Table 1.

Figure 2.- Chemical structure and mass spectra of the main compounds with dietetic value detected by direct analytical pyrolysis of lettuce (Py-GC/MS at 500 °C). The mass spectra correspond to those obtained in our instrument.

Figure 3.- Evolution of lettuce fingerprinting (Py-GC/MS at 500 °C) with time (1, 4 and 8 days) packed in PLA/PBS bioplastic (95:5) film without OEO. Numbers on peaks corresponds to the major compounds identified and listed in Table 1.

Figure 4.- Evolution of lettuce fingerprinting (Py-GC/MS at 500 °C) with time (1, 4 and 8 days) packed in PLA/PBS bioplastic (95:5) film casted with variable concentrations of OEO (0, 2, 5, 10 % w/w).

Figure 5.- Evolution of the relative abundance (relative to toluene, methylbenzene) of the main compounds with dietetic value in lettuce with conservation time (1, 4 and 8 days) when packed in PLA/PBS (95:5) film containing different quantities of OEO (0, 2, 5 and 10%).

TABLE CAPTIONS:

Table 1.- Lettuce Py-GC/MS (500 °C) fingerprinting. Major compounds identified with indication of the retention time (Ret Time min.) and the relative abundance (Ab % total chromatographic area) and type of biogenic compound.

Highlights

- Analytical pyrolysis was useful in tracing nutritious PUFAs and PHSTs in lettuce.
- Lettuce packed in PLA/PBS film experimented PUFAs and PHSTs decrease with time.
- Using active PLA/PBS film containing OEO allowed preservation during shelf life.

Table 1.

Ref	Ret Time	Ab	Compound	Type
1	2.01	3.29	2,5-Dimethylfuran	PS
2	2.23	5.35	Cyclopentene, 1-methyl-	PS
3	2.35	7.06	Toluene	ARO
4	2.47	2.88	Cyclopentane-1,2-diol	PS
5	2.71	7.38	1H-Pyrrole, 1-methyl-	N
6	3.31	2.82	2(5H)-Furanone	PS
7	3.39	3.82	2-Hydroxy-2-cyclopenten-1-one	PS
8	3.72	2.59	5 Methyl furfural	PS
9	3.85	4.40	Phenol	ARO
10	4.05	2.77	Benzofuran	PS
11	4.34	4.98	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	PS
12	4.58	1.46	Phenol, 2-methyl-	ARO
12	4.80	2.39	Phenol, 4-methyl-	ARO
13	4.99	1.81	Phenol, 2-methoxy-	LIG
14	5.08	1.50	Ethanol, 2-butoxy-	LIP
15	5.32	2.86	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	PS
16	5.55	1.72	Benzyl nitrile	N
17	6.22	2.40	5-Hydroxymethyldihydrofuran-2-one	PS
18	6.63	4.02	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	PS
19	6.76	1.07	Benzenepropanenitrile	N
20	7.46	3.96	Indole	N
21	7.66	2.03	2-Methoxy-4-vinylphenol	LIG
22	8.18	1.38	3,5-Dihydroxytoluene	ARO
23	8.49	1.44	2-Pentylcyclopentanone	LIP
24	8.59	0.95	1H-Indole, 3-methyl-	N
25	8.76	0.74	Vanillin	LIG
26	9.32	0.45	Phenol, 2-methoxy-5-(1-propenyl)-, (E)-	LIG
27	12.27	0.32	Cyclohexene, 1,5,5-trimethyl-6-acetylmethyl-	LIP
28	12.41	0.51	Furfural phenylhydrazone	N
29	14.81	3.81	n-Hexadecanoic acid	FA
30	16.44	1.98	9,12-Octadecadienoic acid (Z,Z)-	FA
31	16.50	1.79	9,12,15-Octadecatrienoic acid (Z,Z,Z)-	FA
32	16.67	0.53	n-Octedecanoic acid	FA
33	17.85	0.31	Alk	LIP
34	18.04	0.57	ALK	LIP
35	19.42	1.86	ALk	LIP
36	20.96	1.09	Alk	LIP
37	21.20	0.51	Tetracosanoic acid, methyl ester	FAME
38	22.38	1.08	Alk	LIP
39	22.60	0.38	Hexacosanoic acid, methyl ester	FAME
40	23.06	0.23	Sitosterol acetate	PHST
41	23.68	1.06	Stigmasterol acetate	PHST
42	24.16	1.34	Stigmastan-3,5-diene	PHST
43	25.79	2.29	Stigmasterol	PHST
44	26.46	2.84	Sitosterol	PHST

ARO: aromatics unspecific; FA: fatty acid; FAME: fatty acid methyl ester; LIG: methoxyphenol from lignin; LIP: lipid; N: Nitrogen compound; PHST: phytosterol

Figure 1

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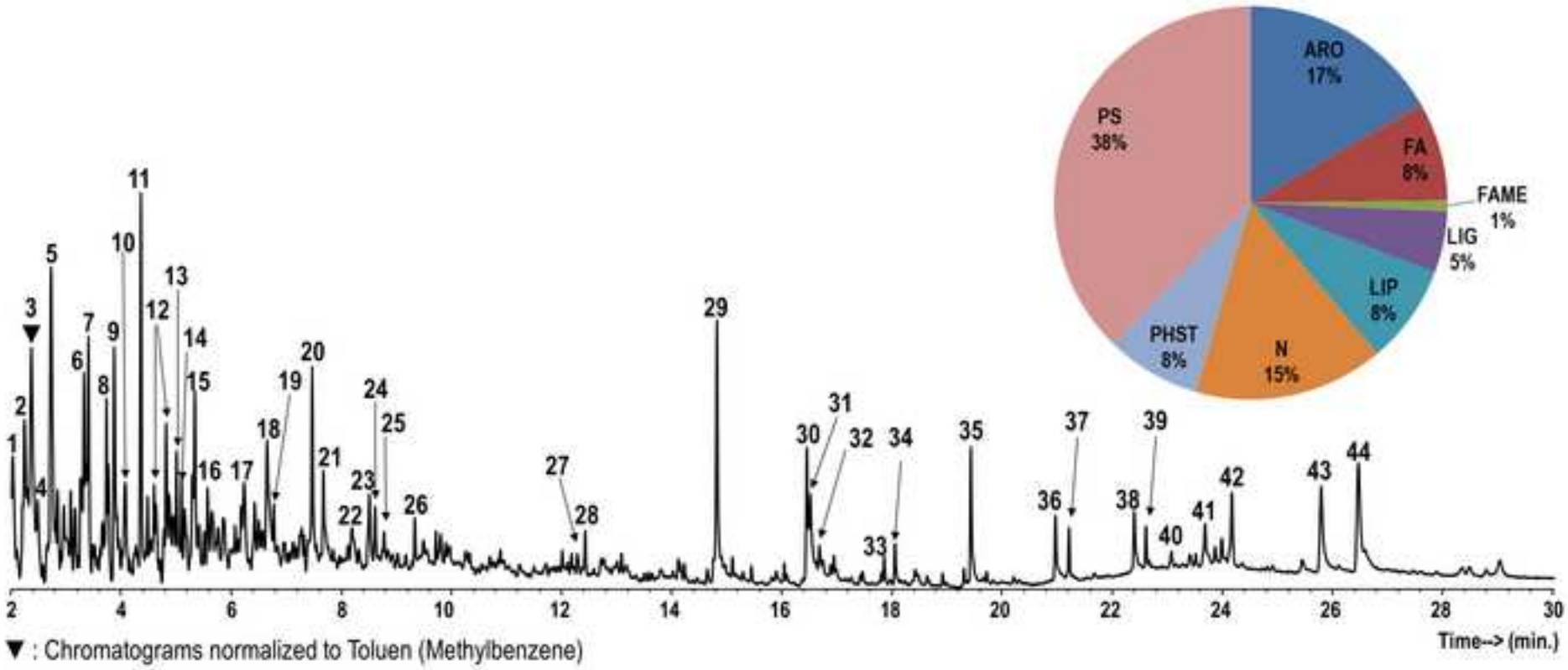

Figure 2

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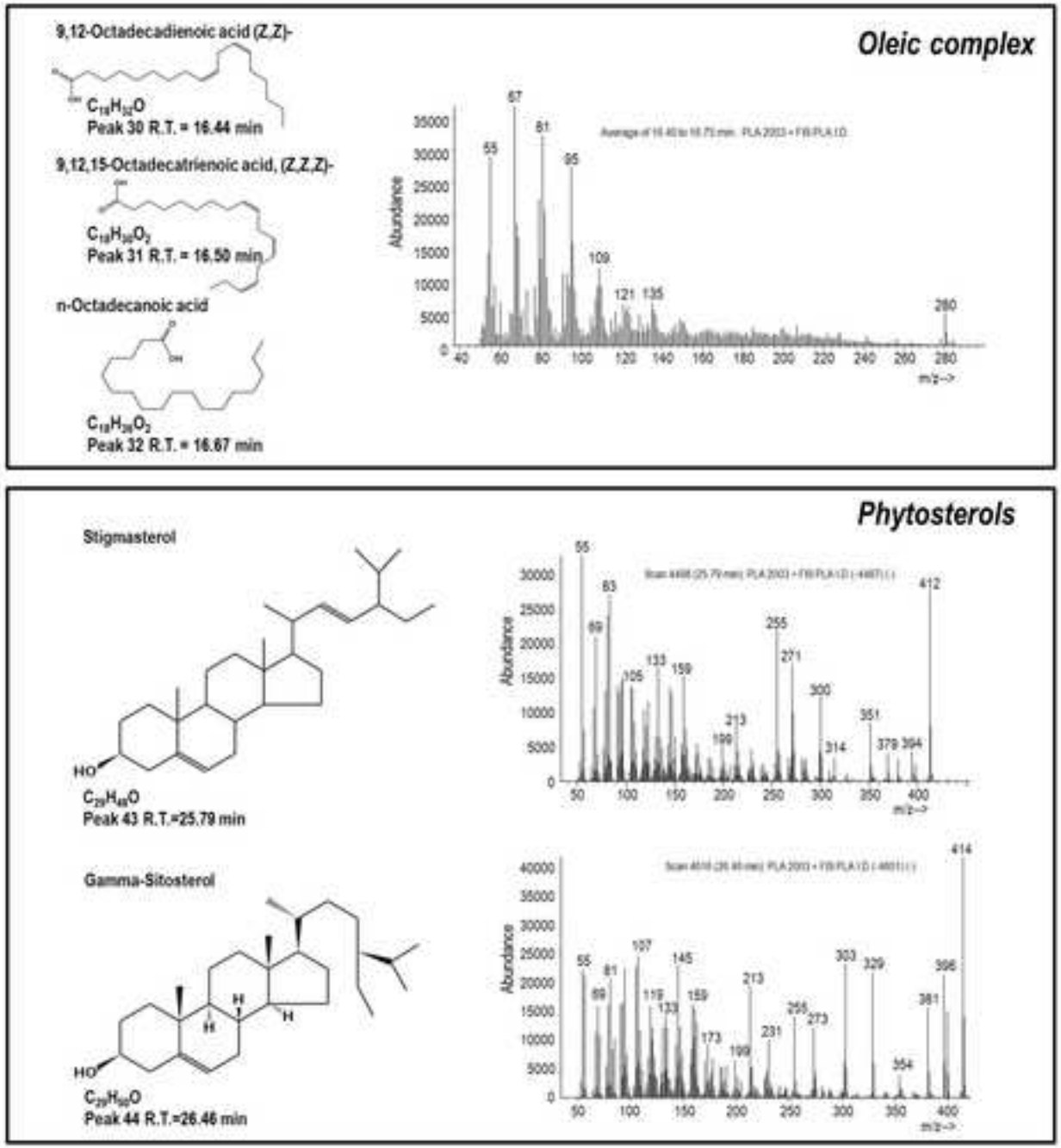

Figure 3

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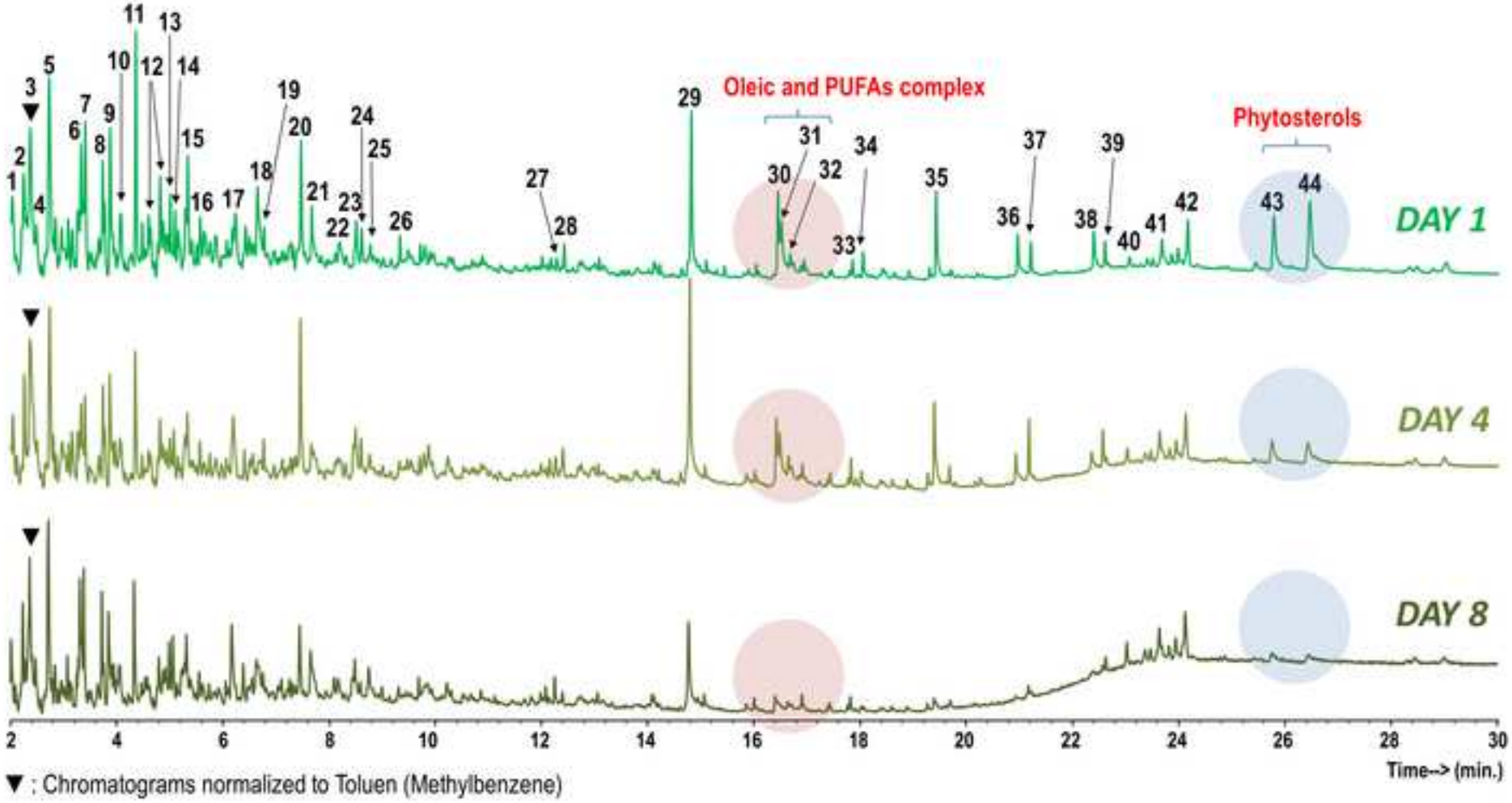

Figure 4

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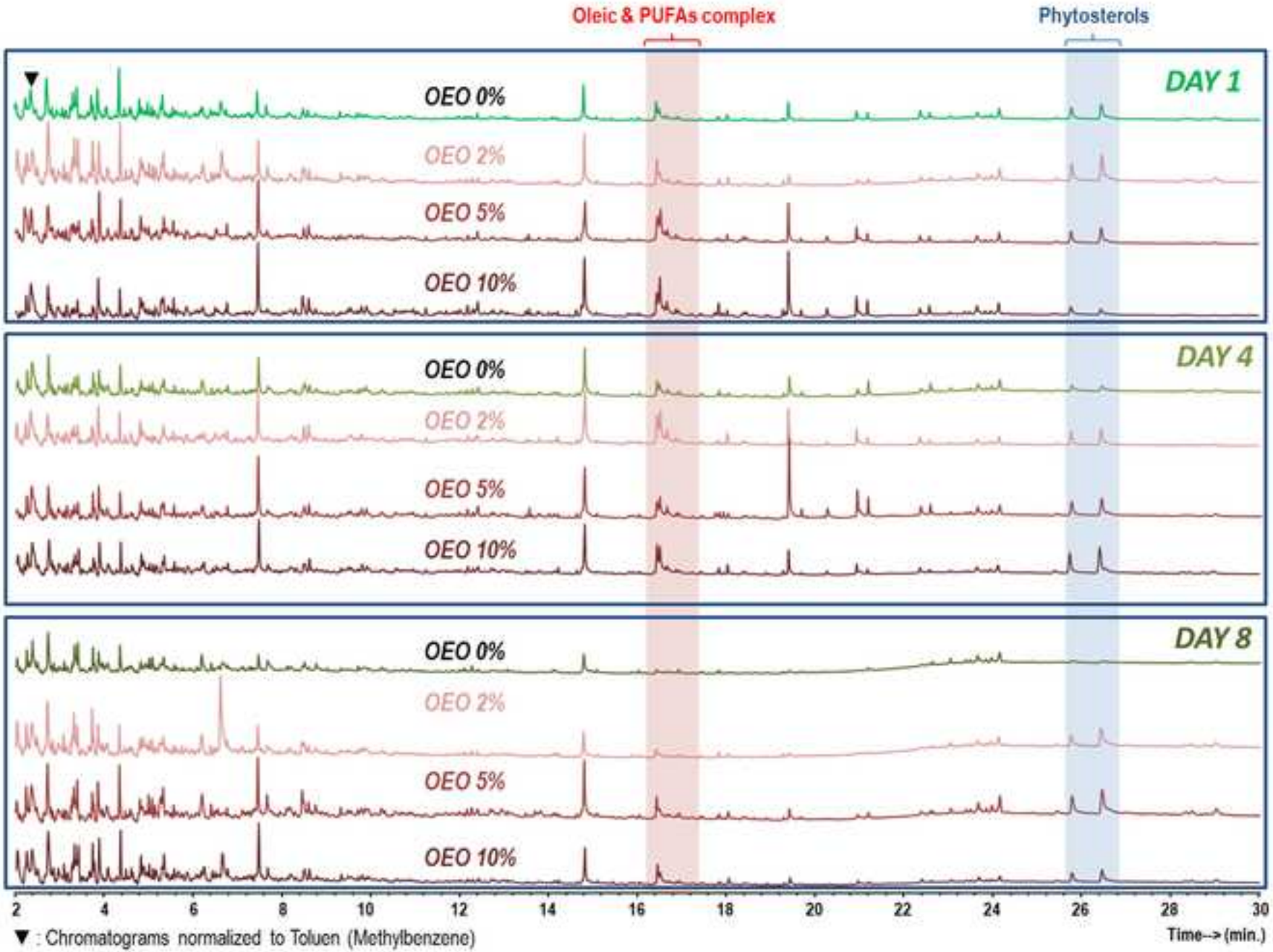

Figure 5

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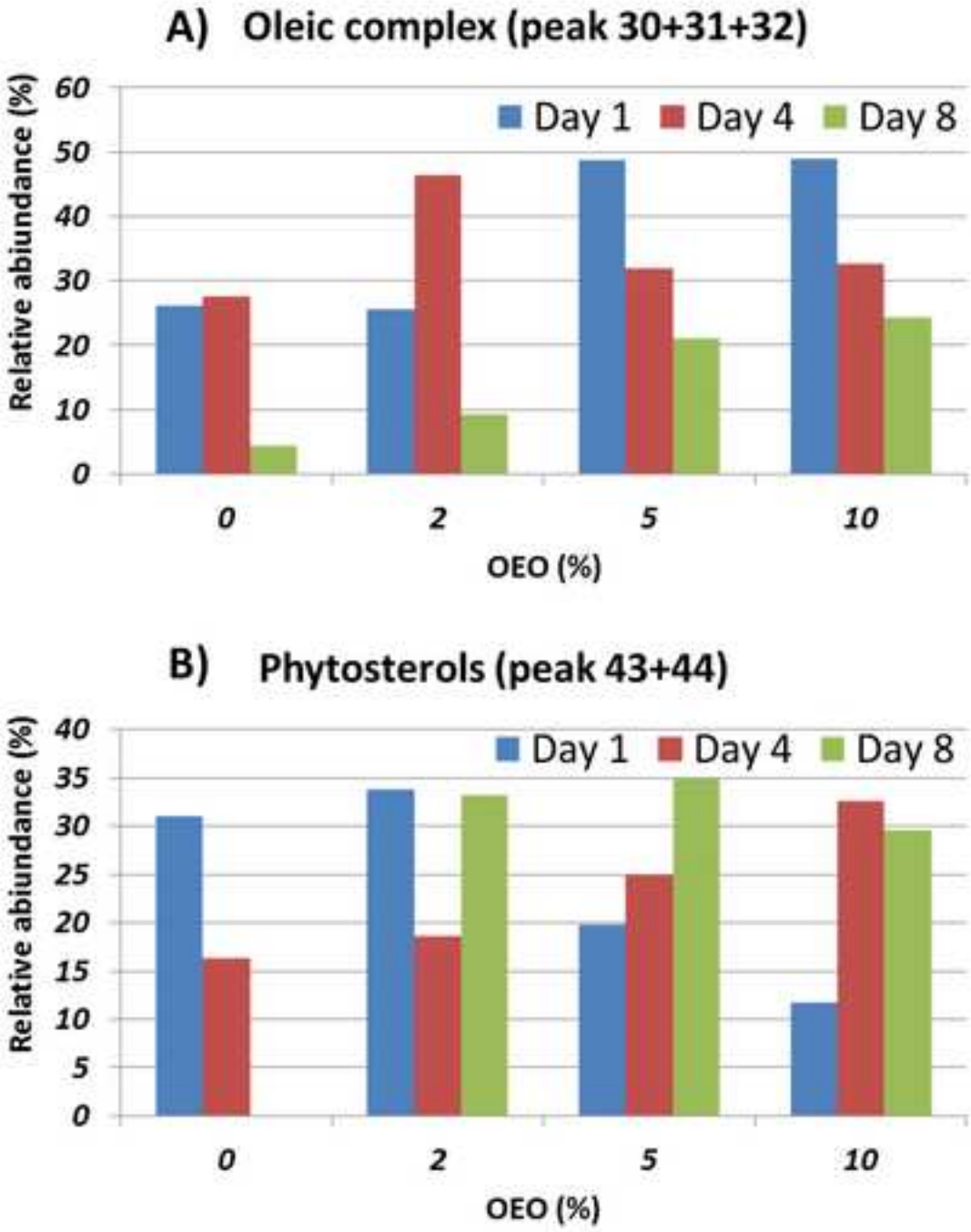

V. RESULTADOS Y DISCUSIÓN GENERAL / RESULTS & GENERAL DISCUSSION

Son varios factores que han hecho que la industria alimentaria se interese por los AEs como potenciales ingredientes tecnológicos; entre los que destacamos el historial de seguridad derivado de su uso tradicional, sus propiedades biológicas y la percepción positiva que los consumidores tienen de ellos (Saccheti et al., 2005). En la presente tesis doctoral se ha pretendido evaluar el perfil toxicológico de alguno de estos aceites y de sus compuestos mayoritarios, así como evaluar el diseño de materiales de envasado activo y su funcionalidad.

1. ESTUDIOS TOXICOLÓGICOS *IN VITRO* DE COMPONENTES MAYORITARIOS DE LOS ACEITES ESENCIALES

En la presente tesis doctoral comenzamos la evaluación de los componentes de los aceites con los estudios de citotoxicidad *in vitro*; ya que permiten definir la toxicidad basal de estas sustancias y seleccionar un rango de concentraciones adecuadas para completar la batería de ensayos *in vitro* necesaria para la evaluación de riesgo que requiere la EFSA.

Los ensayos de citotoxicidad se han llevado a cabo en líneas celulares del sistema digestivo (Caco-2 y HepG2) valorando distintos biomarcadores como son: captación de rojo neutro (RN), reducción de la sal MTS (MTS) y contenido proteico total (PT).

En el caso de los compuestos sulfurados, los resultados obtenidos no mostraron diferencias con respecto a los controles para los compuestos mayoritarios del aceite de cebolla, DPS (0-200 µM), DPDS (0-200 µM) ni para la mezcla de ambos en proporción 1:1. Sin embargo, se observaron efectos citotóxicos, en ambas líneas celulares, en el caso de PTSO (0- 500 µM). Los estudios llevados a cabo por otros autores muestran gran variabilidad en los resultados obtenidos; sin embargo, en general parece que la presencia de grupos alilos o sulfóxidos incrementan la actividad citotóxica de los compuestos sulfurados (Xiao et al., 2004; Merhi et al., 2008). Cuando las células expuestas a estas sustancias fueron observadas en busca de alteraciones ultraestructurales, aparecieron lesiones típicas de células en proceso de degradación, como condensación de la cromatina o vacuolas autofágicas, para todas las sustancias y a concentraciones en las que los biomarcadores de citotoxicidad no habían indicado daño. Este hecho, nos hace pensar que el estudio morfológico es más sensible para detectar toxicidad en comparación con los ensayos de citotoxicidad llevados a cabo. Sin embargo, la complejidad del método y la necesidad de investigadores expertos en morfología hacen que esta técnica sea menos utilizada.

Con respecto a los compuestos fenólicos mayoritarios del AEO, que resultaron ser carvacrol (56 %) y timol (5%), se evaluó la toxicidad basal de ambos, en rangos de concentración de 0-2500 µM y 0-250 µM, respectivamente, y una mezcla de ambos en proporción 10:1, respetando la relación real de los dos compuestos en el aceite objeto de estudio, *Origanum vulgare* L. *virens*. Los resultados mostraron que sólo carvacrol y la mezcla de ambos compuestos produjeron actividad citotóxica dependiente de la concentración y el tiempo. Por su parte, el incremento de toxicidad observado para la mezcla, en comparación con la exposición individual a carvacrol, podría deberse a que los compuestos minoritarios presentes en los aceites podrían intervenir en la actividad final de las mezclas al modular el efecto ejercido por los compuestos mayoritarios (Bakkali et al., 2008). Son muchos los autores que han estudiado la citotoxicidad del AEO y de sus componentes mayoritarios, y los resultados obtenidos son variados y contradictorios. En el caso de timol, Garcia et al. (2006) no observaron citotoxicidad en neuronas corticales de ratón (0-1 mM), mientras que otros autores sí observaron afectación de células HepG2 (0,25-2,20 mM) (Stammati et al., 1999) o Caco-2 (0-900 µM) (Horváthová et al., 2006). Para carvacrol, la situación es similar e incluso existen publicaciones que incluyen resultados diferentes en función de la línea celular utilizada como modelo (Savini et al., 2009; Yin et al., 2012). Además de las concentraciones de exposición utilizadas, todas estas discrepancias pueden estar relacionadas con las diferencias inherentes a cada modelo experimental o la inestabilidad de algunas de las líneas celulares que provocan diferentes respuestas en función del laboratorio (Kirkland et al., 2007).

Respecto al estudio morfológico, en las células expuestas, tanto a carvacrol como a la mezcla de carvacrol y timol, las células mostraron alteraciones ultraestructurales en mitocondrias, aparato de golgi y en el retículo endoplasmático rugoso. Además, de nuevo esta técnica resultó ser más sensible que los ensayos de citotoxicidad al mostrar daño en las células expuestas a timol. En general, todas las células mostraron un aumento en la formación de gotas lipídicas que en las concentraciones más altas ya están fusionadas vacuolizando el citoplasma e indicando procesos de muerte celular. Con idea de determinar si éste daño estaba produciendo apoptosis o necrosis en las células se llevó a cabo la determinación del proceso de muerte celular mediante citometría de flujo utilizando dos sondas marcadoras, anexina V y yoduro de propidio (IP). Este aspecto ha sido ampliamente estudiado para el AEO y sus componentes. Tanto el AEO como su componente mayoritario, carvacrol, han mostrado capacidad para inducir necrosis y apoptosis en multitud de líneas celulares entre las que se incluyen las utilizadas en la presente tesis doctoral, Caco-2 (Dusan et al., 2006) y HepG2 (Yin et al., 2012). Para timol, en la bibliografía consultada con respecto al estudio de la capacidad para inducir necrosis y apoptosis, los resultados son, de nuevo,

contradictorios. Estos trabajos indican que, en los casos positivos, esta inducción es menor que la observada para el AEO y para carvacrol (Dusan et al., 2006). En la presente tesis doctoral, tras 24 horas de exposición a carvacrol y a la mezcla de éste con timol, se observó inducción de ambos procesos. Sin embargo, y aunque el estudio morfológico mostró alteraciones en la cromatina de las células expuestas a timol, no se observaron daños significativos en estas células tras realizar el ensayo con anexina V/IP.

A continuación, se llevó a cabo el estudio de mecanismos de acción tóxica evaluando *in vitro* la posible inducción de estrés oxidativo, analizando la producción de ERO y las modificaciones en el contenido de GSH. Varios autores han informado de la capacidad antioxidante del aceite esencial de cebolla, y de los compuestos mayoritarios del mismo (DPS y DPDS) (García et al. 2009; Ye et al., 2013). Estos resultados coinciden con los obtenidos en la presente tesis doctoral en los que observamos una disminución en los niveles de ERO tras la exposición de las células a DPS, DPDS y la mezcla de ambos, siendo mayor la reducción en este último caso. Esta actividad de eliminación de radicales libres parece no estar relacionada con GSH, ya que los valores de esta sustancia permanecen inalterados a lo largo del ensayo.

El AEO, y con él sus componentes mayoritarios, pretenden ser utilizados en envasado activo por sus propiedades antioxidantes, entre otras. Por este motivo, es importante evaluar tanto la capacidad antioxidante de esta sustancia (mecanismo de acción), como los efectos tóxicos relacionados con este efecto (mecanismo de acción tóxica). Para estudiar el primero de los mecanismos llevamos a cabo dos métodos sencillos, DPPH (2,2-difenil-1-picrilhidrazilo) y ABTS (2,20 -azinobis-(ácido 3-etil-benzotiazolin-6-sulfonico), que permiten valorar la capacidad de las sustancias para eliminar ERO del medio. Varios autores han informado de la gran habilidad del AEO y sus componentes para eliminar radicales libres del medio tras realizar estos mismos ensayos (Ündeger et al., 2009; Aristale et al., 2010; Slamenova et al., 2013). Sin embargo, nuestros resultados mostraron una actividad moderada al respecto para carvacrol, timol y la mezcla de ambos, resultados que coinciden con los propuestos por Kulisic et al. (2004), que observaron un efecto antioxidante del AEO, carvacrol y timol menor al producido por el ácido ascórbico utilizado como patrón. En cuanto al mecanismo de acción tóxica, los resultados han revelado que carvacrol y la mezcla de éste con timol, incrementan los niveles de ERO en células expuestas, a la vez que disminuyen los niveles de GSH, ambas de manera dependiente a la concentración. En ambos casos, la mezcla produce un mayor efecto que el compuesto individual. Los resultados consultados en bibliografía al respecto revelan información similar tras exposición a carvacrol en el caso de células V79 (Ündeger et al., 2009) y células H1299 (Ozkan y Erdogan, 2012). Estos mismos autores revelaron, sin embargo, efectos similares en el caso de timol, lo cual

contrasta con la ausencia de actividad prooxidante obtenida en la presente tesis doctoral. De acuerdo con Ferguson (2011), es habitual que los compuestos fenólicos presenten actividad pro o antioxidante en función de las concentraciones utilizadas. De hecho, aunque hemos observado que carvacrol, al igual que la mezcla, son capaz de inducir estrés oxidativo, también han mostrado capacidad para proteger y revertir el daño inducido por peróxido de hidrógeno. Así, los resultados de ese estudio indican que tanto carvacrol, como timol y la mezcla de ambos, tienen la capacidad de proteger a las células del daño producido por el agente oxidante (peróxido de hidrógeno) y para revertir el daño inducido por la misma sustancia. Estos resultados se consolidan al relacionarlos con los propuestos por Horváthová et al. (2006) y Aherne et al. (2007) que mostraron la habilidad del AEO y sus componentes mayoritarios contra el daño inducido en el ADN de células HepG2 y Caco-2, respectivamente, tras la exposición a peróxido de hidrógeno.

Por último, respetando los requerimientos de la EFSA para la presentación de solicitudes de nuevas sustancias activas (EFSA, 2016), evaluamos la capacidad genotóxica *in vitro* de las sustancias objeto de estudio mediante dos ensayos, el test de Ames y el ensayo cometa, estándar y modificado con enzimas. Los resultados obtenidos para DPS, DPDS y la mezcla de ambos en el test de Ames mostraron ausencia de actividad mutagénica tanto en presencia como en ausencia de fracción metabólica S9 para todas las cepas de *Salmonella thyphimurium* estudiadas (TA 97, 98, 100, 102 y 104). Estos resultados coinciden con los propuestos por Shon et al. (2004) para el aceite de cebolla. Además, los compuestos DPS y DPDS se han relacionado con inhibición de estadios tempranos y tardíos de carcinogénesis (Guyonnet et al., 1999) y con actividad antimutagénica (Guyonnet et al., 2001).

Con respecto a carvacrol y timol, existen pocos estudios que evalúen su potencial mutagénico y los resultados propuestos son contradictorios (Azizan y Blevins, 1995; De Vicenzi et al., 2004; Ipek et al., 2005). Además, muchos de estos estudios se llevaron a cabo sin seguir los principios de la guía propuesta por la OCDE (OCDE 471, 1997). Nuestros resultados indican que las concentraciones de timol utilizadas (0-250 µM) no produjeron colonias revertientes en ninguna de las cepas de *S. thyphimurium* en presencia ni en ausencia de S9. Sin embargo, carvacrol ha mostrado ser un agente mutagénico para las cepas TA 97 y TA 98. Además, sus metabolitos, evaluados tras la adición de S9, muestran también actividad mutagénica en la cepa TA98. Estos resultados coinciden con los obtenidos por Ipek et al. (2005) para el AE de *Origanum onites*.

Tras observar resultados positivos para el estudio de mutagenicidad, decidimos llevar a cabo el ensayo cometa para evaluar el potencial genotóxico de carvacrol y timol debido a la implicación que podría tener con el desarrollo de procesos cancerígenos. En el ensayo cometa

estándar, ninguna de las dos sustancias produjo roturas en el ADN. En general, los estudios de genotoxicidad que se han llevado a cabo con estas sustancias han generado una gran variabilidad de resultados. Hay autores que han observado resultados positivos tras desarrollar el ensayo cometa estándar en linfocitos humanos (Aydin et al., 2005), células N2a (Aydin et al., 2014); mientras que otros informaron de la ausencia de genotoxicidad de estas mismas sustancias en células HepG2 o Caco-2 e incluso efecto protector frente a agresiones por peróxido de hidrógeno (Horváthová et al., 2006; Slamenová et al., 2007). Teniendo en cuenta que las roturas en el ADN pueden producirse por la oxidación de las bases, la incubación con enzimas de restricción como las utilizadas en la presente tesis doctoral (Endo-III y FPG) aumenta la sensibilidad del método (Collins et al., 2004) ya que estas enzimas, son capaces de convertir bases oxidadas en roturas detectables mediante electroforesis (Gielazyn et al., 2013). Los resultados de nuestro estudio indicaron para el cometa modificado que, tras incubar con las enzimas (Endo-III y FPG), carvacrol era capaz de producir daño oxidativo dirigido fundamentalmente a la bases púricas, ya que fueron los núcleos tratados con FPG los que mostraron este hecho. Resultados contrastan con los obtenidos por Ündeger et al. (2009) en células V79 expuestas a carvacrol y timol, y en las que no se observaron daños adicionales tras la incubación con FPG.

La disparidad de resultados observada en nuestros estudios de genotoxicidad *in vitro* llevados a cabo para estas sustancias nos invitan a evaluar este aspecto mediante ensayos *in vivo*, que aclaren la situación y permitan obtener resultados más claros y extrapolables al ser humano, siguiendo así las recomendaciones de la EFSA.

2. ESTUDIOS TOXICOLÓGICOS *IN VIVO* DE ACEITES ESENCIALES Y SUS COMPONENTES MAYORITARIOS

Tras analizar los resultados *in vitro* obtenidos en la presente tesis doctoral y los publicados en bibliografía en cuanto a carvacrol, consideramos de gran importancia llevar a cabo un estudio en roedores para confirmar su potencial genotóxico. Según nuestro conocimiento, este trabajo constituye la primera evaluación de la genotoxicidad de carvacrol a través de la combinación de los ensayos MN y cometa en ratas. El protocolo desarrollado fue propuesto por Bowen et al. (2011), tras la recomendación de la EFSA de disminuir el número de individuos utilizados en la evaluación de riesgo de los ingredientes tecnológicos. Los órganos en los que llevamos a cabo la evaluación de la genotoxicidad mediante el ensayo cometa fueron seleccionados de acuerdo con la guía 489 de la OCDE, que indica que el estómago es un órgano interesante a evaluar por ser el de primer contacto tras la administración por vía oral y el hígado por ser el principal órgano de

biotransformación de xenobióticos. Además, incorporamos enzimas de restricción (Endo-III y FPG) para aumentar la sensibilidad del ensayo cometa y detectar oxidación de bases pirimidínicas y púricas, respectivamente, debido a que los compuestos fenólicos presentes en el AEO, entre ellos carvacrol, han mostrado efectos antioxidantes o prooxidantes en función de la concentración utilizada (Samec et al., 2015).

En el ensayo de MN, llevado a cabo en médula ósea, y en el ensayo cometa, desarrollado en células de estómago e hígado de ratas Wistar expuestas a carvacrol (81-810 mg/kg pc/día), los resultados obtenidos mostraron ausencia de potencial genotóxico al no producirse un incremento de las células micronucleadas, ni observarse roturas en el ADN. Además, sorprende observar ausencia de daño oxidativo en el ADN tras el tratamiento con las enzimas cuando los resultados *in vitro* han mostrado un aumento significativo de la producción de ERO tras la exposición a carvacrol. Para explicar este hecho, y teniendo en cuenta que el ensayo cometa únicamente nos muestra la situación concreta de la célula en un instante determinado, pensamos que es posible que se hubieran activado mecanismos de reparación del ADN, como son los sistemas de reparación por escisión de bases o de nucleótidos, para eliminar el daño oxidativo (Azqueta et al., 2009).

Los resultados obtenidos en la presente tesis doctoral contrastan con el único estudio *in vivo* que hemos encontrado en bibliografía llevado a cabo por Azirak y Recunzogullari (2008). Estos autores informaron de la aparición de alteraciones genotóxicas, tras llevar a cabo el test de aberraciones cromosómicas, en ratas expuestas a concentraciones de carvacrol de hasta 70 mg/kg pc/día. Esta discordancia puede deberse a la diferente sensibilidad entre los métodos utilizados y en este sentido, el test de MN ha mostrado ser un método más adecuado debido al alto número de células analizables, la simplicidad del método, las posibilidades de automatización y la mayor capacidad para detectar sustancias aneugénicas (Lorge et al., 2006). Por otro lado, la ausencia de genotoxicidad observada *in vivo* en la presente tesis doctoral concuerda con los resultados obtenidos *in vitro* por otros autores en distintas líneas celulares como HepG2 (Melusova et al., 2014; Horváthová et al., 2006), linfocitos humanos (Türked y Aydin, 2013), células V79 (Ündeger et al., 2009) o células de linfoma de ratón (Maisanaba et al., 2015), mientras que contrasta con los obtenido *in vitro* en la presente tesis.

Siguiendo las recomendaciones de la OCDE (OCDE 489), se llevó a cabo el estudio de alteraciones histopatológicas en las ratas expuestas a carvacrol y, en contraste con los resultados obtenidos *in vitro*, no se observaron cambios significativos con respecto a los animales control en ninguna de las dosis de exposición en estómago, hígado ni pulmón. Todas estas diferencias entre

los resultados obtenidos *in vitro* e *in vivo* corroboran la importancia de confirmar la toxicidad obtenida *in vitro*, ya que pueden obtenerse falsos positivos debido a las deficiencias en los sistemas de metabolización de xenobióticos o en los mecanismos de reparación del material genético (Kirkland et al., 2007; EFSA, 2011).

Por otro lado, los estudios sobre genotoxicidad y toxicidad oral subcrónica *in vivo* desarrollados para AEO son muy escasos o casi inexistentes. Debido a que no encontramos en la bibliografía ningún trabajo que evaluara el AEO, decidimos llevar a cabo el estudio de toxicidad oral subcrónica a 90 días. Además, respetando la propuesta que hacen la EFSA y la OCDE en sus últimas guías sobre asociar ensayos de genotoxicidad a estudios subcrónicos para así disminuir el número de animales utilizados en los estudios, evaluamos la genotoxicidad *in vivo* del AEO en ratas wistar tras 90 días de exposición. Para lo cual, administramos diferentes dosis de AEO (50, 100 y 200 mg/kg pc/día) incorporado en gelatina neutra. Los resultados obtenidos del estudio histopatológico, bioquímico y hematológico mostraron alguna diferencia en la amplitud de distribución eritrocitaria y en los valores de glucosa. Sin embargo, estas variaciones se han considerado irrelevantes biológicamente por haberse presentado aisladas y por no seguir ningún patrón dependiente de sexo, dosis o tiempo de exposición. Por lo demás, no se observaron diferencias con respecto a los animales control por lo que propusimos como NOAEL la máxima dosis ensayada de 200 mg/kg pc/día. Aunque no hemos encontrado estudios previos sobre el AEO, los valores de NOAEL observados para otros AEs no difieren mucho del propuesto en la presente tesis doctoral para el AEO. Así, para los aceites de cúrcuma (Liju et al., 2013) y jengibre (Jeena et al., 2014) los valores propuestos fueron 500 y 400 mg/kg pc/día, respectivamente y valores similares se han publicado también para los aceites de menta peperina (460 mg/kg pc/día) (Escobar et al., 2015) o para el extracto de aliáceas Proallium® (400 mg/kg pc/día) (Mellado-García et al., 2016). Tras el sacrifico de los animales, tomamos muestras de médula ósea y de estómago, hígado y sangre periférica para llevar a cabo los ensayos de MN y cometa, respectivamente. En estos ensayos se observa una ausencia total de células micronucleadas y de daño en el ADN, tanto en forma de roturas como oxidación de sus bases. Estos resultados coinciden con la ausencia de toxicidad observada en el estudio de 90 días. Además, los resultados obtenidos para carvacrol muestran también ausencia de genotoxicidad y teniendo en cuenta que la EFSA en su guía para la evaluación de productos botánicos permite focalizar el estudio toxicológico en los componentes mayoritarios cuando éstos estén caracterizados, todas estas ausencias de genotoxicidad indicarían la seguridad del AEO como activo alimentario.

Según los protocolos de la OCDE, para que los resultados negativos obtenidos *in vivo* tengan validez, es necesario confirmar mediante alguna técnica analítica que los órganos

estudiados han estado expuestos al tóxico. En el caso de MN, la disminución del ratio entre eritrocitos policromáticos y normocromaticos observado en la sangre de los animales expuestos es suficiente para confirmar exposición de la médula ósea (OCDE 474); sin embargo, en el caso de cometa, analizamos los tejidos diana en busca del compuesto mediante Py-GC/MS para confirmar este hecho. Utilizamos esta técnica porque había pocos precedentes en el análisis de esta sustancia en tejidos biológicos y ha resultado ser de mucha utilidad. Las diferencias en las curvas dosis-respuesta, lineal solo en el caso de estómago, pueden indicar que se produce una acumulación de carvacrol como consecuencia de la saturación de los mecanismos de metabolización del mismo. Sin embargo, nos ha resultado complicado explicar este hecho porque el metabolismo de esta sustancia no ha sido estudiado en profundidad. Sería interesante investigar su toxicocinética para confirmar la seguridad del carvacrol.

3. DESARROLLO Y CARACTERIZACIÓN DE ENVASES ACTIVOS CON ACEITES ESENCIALES

Como se ha mencionado anteriormente, la adición directa de AEs en la superficie de los alimentos puede impactar negativamente en las características organoléptica de los mismos (Goñi et al., 2009; Negi, 2012) y el desarrollo de envases activos constituye una alternativa para controlar este hecho.

En los materiales desarrollados observamos una variación entre las cantidades de activo añadidas en el proceso de fabricación y las cantidades finales cuantificadas en los nuevos plásticos mediante TGA de entre un 20 y un 40 %. Algunos autores han relacionado esta pérdida con la volatilidad de los AEs y las altas temperaturas que se alcanzan en el proceso de extrusión (Altiok et al., 2010; Ramos et al., 2012).

En cuanto a las propiedades físico-mecánicas, la transparencia de los films constituye un factor influyente en las decisiones de compra, ya que compromete la visibilidad del producto (Sehrawet y Kundu, 2007). Este parámetro no se vió afectado tras la adición de AEO, mientras que Proallium® mostró capacidad para disminuir el paso de luz a través de los envases que lo contenían. Esta modificación que impide el paso de la luz, no se materializó en un aumento de la opacidad del film detectable a simple vista, por lo que no se espera repercusión en el interés de los consumidores por este material. En cualquier caso, se podría estudiar en detalle este fenómeno, ya que un aumento de la opacidad podría significar que el envase ejerciera un efecto de fotoprotección (Souza et al., 2009). Estos resultados coinciden con la disminución de transparencia observada por otros autores tras adicionar AEs a distintas matrices poliméricas

(Hosseini et al., 2009; Norajit et al., 2010; Siripatrawan y Harte, 2010; Pires et al., 2013) y puede convertirse en un fenómeno deseable para proteger de la luz a alimentos fotosensibles (Wambura et al. 2011).

En cuanto a las propiedades mecánicas, de manera general la adición de AEO y Proallium® produjo en los films un aumento de la plasticidad al afectar parámetros como el módulo elástico o la elongación a rotura. El aumento de la flexibilidad de los plásticos ha sido relacionado con la formación de gotas lipídicas, líquidas a temperatura ambiente, que facilitan la deformación del material (Ahmad et al., 2012). Este comportamiento parece ser habitual ya que ha sido observado en PLA tras incorporarle palmitato de ascorbilo, α-tocoferol o hidroxitolueno butilado) (Byun et al., 2010; Jamshidiam et al., 2012) y también, tras adicionarle α-tocoferol y un extracto de aceite de oliva (Marcos et al., 2014).

Una vez confirmado que los materiales diseñados presentaban propiedades físico-mecánicas compatibles con el envasado activo alimentario, se continuó con la evaluación de la funcionalidad de los mismos.

Los films desarrollados con base de PLA y Proallium® no mostraron actividad antioxidante tras llevar a cabo los métodos ABTS y DPPH. Estos resultados no concuerdan con los expuesto por Nerin et al. (2008) que comentaron que la actividad de los AEs está relacionada con la composición química que presenten. Teniendo en cuenta el alto contenido de organosulfurado (PTSO) en el preparado Proallium®, cabría esperar que el film presentara actividad antioxidante, tal y como habían mostrado otros materiales de envasado a los que se habían incorporado extractos de aliáceas (Teixeira et al., 2014). Sin embargo, nuestros films no mostraron esta actividad, resultados que estarían apoyados por la afirmación de Benkeblia y Lanzotti (2007) sobre el error de asociar actividad antioxidante a la sola presencia de organosulfurados en una mezcla de aliáceas. En el caso de los materiales desarrollados con AEO, se observó un incremento de la actividad antioxidante en los materiales con mayor porcentaje de activo (5 y 10 %). Estos resultados sí mostraron concordancia con multitud de autores que han informado de las propiedades antioxidantes del AEO y de sus componentes mayoritarios (carvacrol y timol) (Stamenic et al., 2014; Sarikurkcu et al., 2015), así como de materiales a base de PP que los contenían (Ramos et al., 2014).

En cuanto a la actividad antimicrobiana de los films, la evaluación para los materiales desarrollados se llevó a cabo *in vitro* e *in vivo* en lechugas de cuarta gama para los plásticos con base de PLA y en jamón cocido para los desarrollados con base de PP. En la evaluación *in vitro* de los materiales con base de PLA, los resultados mostraron que tanto los plásticos con Proallium®

como aquellos con AEO, ejercieron actividad antimicrobiana frente una amplia batería de microorganismos que incluían bacterias Gram + y Gram -, así como mohos y levaduras. Sin embargo, observamos diferencias en la potencia antimicrobiana entre ambos activos puesto que los films con Proallium® (5 y 6,5 %) ejercieron actividad desde el primer día de contacto, mientras que los plásticos con AEO (10 %), solo mostraron actividad a partir del tercer día de contacto. Nuestros resultados coinciden con otros estudios que sugieren la utilidad de estos envases activos que incorporan AEs, y sus compuestos mayoritarios, como envases antimicrobianos (Pires et al., 2013; Jouki et al., 2014; Sung et al., 2014; Teixeira et al., 2014).

Una vez evaluada la funcionalidad *in vitro* de los envases, quisimos valorar su actividad en modelos alimentarios reales. Tal como se ha comentado anteriormente, los materiales con base de PLA fueron evaluados en lechuga de cuarta gama. De nuevo, los plásticos con Proallium® mostraron mayor actividad antimicrobiana que los desarrollados con AEO. Ambos fueron útiles contra mohos y levaduras pero solo los primeros consiguieron hacer frente a enterobacterias y bacterias aerobias.

Por otro lado, la efectividad de los materiales diseñados con base de PP fue evaluada en jamón cocido frente a *Brochotrix thermosphacta,* un microorganismo que se encuentra a lo largo de toda la cadena de producción de alimentos cárnicos y es un contaminante habitual de este tipo de productos (Remenant et al., 2015). Durante los 60 días que duró el ensayo, los envases con Proallium® exhibieron, de nuevo, mayor actividad antimicrobiana que los films con AEO, aunque ambos disminuyeron en gran medida la presencia del microorganismo en el jamón.

Todos estos resultados, que ponen de manifiesto la utilidad de las dos sustancias frente a microorganismos presentes en alimentos, coinciden con distintos estudios llevados a cabo en los últimos años en los que envases activos que incorporan extractos naturales ejercen una función antimicrobiana en distintos modelos alimentarios. Así, Sung et al. (2014) informaron de la capacidad antimicrobiana de envases con aceite de ajo en carne de ternera y distintos envases diseñados con AEO han mostrado actividad antimicrobiana en filetes de pollo (Khanjari et al., 2013), lechuga (Muriel-Galet et al., 2013) o carne de caballo (Lorenzo et al., 2014).

Patrignani et al. (2015) comentaron que la aplicabilidad de estos envases podría ser limitada debido al intenso aroma que podrían transferir al alimento envasado. Es cierto, que las cantidades de activo que se requieren en el envasado activo son menores a las necesarias cuando se utilizan AEs como aditivos directos sobre alimentos (Salgado et al., 2013); sin embargo, debido al intenso aroma de estas sustancias, es posible que se produzca rechazo por parte de los consumidores a la hora de seleccionar alimentos cuya organoléptica haya variado debido a la

composición del envase (Muriel-Galet et al., 2012). Para estudiar este aspecto llevamos a cabo un análisis sensorial, tanto en lechugas como en jamón cocido, para valorar el efecto que tendría el aroma de los activos en la percepción del consumidor. De los parámetros evaluados (olor, apariencia visual, aceptabilidad general del alimentos e intención de compra), el olor del producto final es el más crítico (Nedorostova et al., 2009). Los resultados mostraron que cuando un olor no es habitual en un alimento concreto se detecta como inesperado y eso disminuye la intención de compra del producto. Es lo que se deduce de los resultados obtenidos en la presente tesis doctoral para los films con AEO. Mientras que en la lechuga, el olor a orégano fue advertido por los panelistas pero no considerado como crítico, en el jamón cocido el mismo olor disminuyó la intención de compra al ser reconocido como un aroma extraño en ese producto.

Teniendo en cuenta todos estos resultados, sería imprescindible seleccionar combinaciones entre matriz polimérica, AE y alimento que fueran tecnológicamente viables, funcionales y en las que la mezcla de aromas no fuese desagradable o extraña para los consumidores.

4. PIRÓLISIS ANALÍTICA Y ESPECTROMETRÍA DE MASAS DE RELACIÓN ISOTÓPICA COMO TÉCNICAS DE DETERMINACIÓN Y CUANTIFICACIÓN DE ADITIVOS NATURALES PRESENTES EN MATRICES DE ÁCIDO POLILÁCTICO (PLA).

La pirolisis es un método que transforma, mediante degradación térmica en ausencia de oxígeno, compuestos complejos no volátiles en una mezcla de fragmentos volátiles (Moldoveanu, 1998). La rotura de los enlaces, que se produce por acción del calor, se produce de manera predecible y reproducible, permitiendo conocer la molécula original a través de estos fragmentos, conocidos como pirolizados, que se separan por cromatografía gaseosa (GC) y se identifican mediante espectrometría de masa (MS) (González-Pérez et al., 2013).

En la presente tesis doctoral utilizamos la pirolisis analítica (Py-GC/MS) para confirmar la presencia de Proallium® y AEO en las matrices de PLA y para cuantificar la cantidad presente tras el proceso de fabricación de los nuevos materiales por extrusión. La primera determinación que se llevó a cabo fue para el propio PLA, y los resultados obtenidos mostraron estar en consonancia con lo descrito previamente por Kopinke y Mackenzie (1997) que indicaron que los pirolizados principales eran la lactida, del propio ácido láctico, y sus enantiómeros.

El pirograma obtenido para el Proallium® mostró una mezcla de compuestos relacionados con el polietilenglicol, utilizado en este producto como “soporte” para vehiculizar el activo

(Mellado-Garcia et al., 2016) y un gran número de compuestos sulfurados, entre los que se incluía el compuesto activo de esta preparación comercial, PTSO. Cabe destacar que los valores de PTSO encontrados en el preparado de aliáceas eran bastante inferiores a lo esperado, lo que puede deberse a una conversión térmica de este compuesto en otros sulfurados durante el aumento de temperatura ligado a la técnica de Py-GC/MS. Con respecto al pirograma de AEO, los resultados mostraron un perfil típico de compuestos aromáticos e hidroaromáticos.

Una vez analizadas la matriz y los activos llevamos a cabo la pirolisis en los materiales desarrollados. En el caso de los films con Proallium®, se utilizó el propilsulfuro como pico diagnóstico y se confirmó una relación lineal entre el área observada en el pirograma y las concentraciones de activo añadidas. Del mismo modo, los materiales con AEO proporcionaron pirogramas en los que eran visibles varios picos relacionados directamente con compuestos fenólicos del aceite. Estos picos, pertenecientes a la mezcla de carvacrol y timol, cimeno y terpineno, fueron detectados en todos los materiales que incorporaban AEO, incluso aquellos con la concentración más baja (2%). Tras utilizar estos picos como picos diagnóstico pudimos confirmar, de nuevo, una relación lineal entre las áreas de los mismos y las concentraciones teóricas añadidas al film. Tras comparar los resultados obtenidos mediante esta técnica con los obtenidos mediante TGA, concluimos que la Py-GC/MS es una técnica adecuada para determinar activos naturales en matrices poliméricas.

Por último, quisimos valorar también la utilidad de la espectrometría de masas de relación isotópica (IRMS) como alternativa a TGA para la determinación de activos. Esta técnica ha sido ampliamente utilizada para conocer el origen de sustancias naturales y ha sido empleada en aspectos relacionados con la seguridad alimentaria, como trazabilidad y detección de fraude (Rossmann, 2001). Tras llevar a cabo IRMS para Proallium® y AEO, obtuvimos valores de forma isotópica de carbono ($\delta^{13}C$) de -28,9 ± 0,07 ‰ y -28,2 ± 0,05 ‰, respectivamente, lo que indica que son plantas que pertenecen al grupo conocido como C3 por presentar valores de $\delta^{13}C$ comprendidos entre -24 y -30 ‰. Por otro lado, al analizar el PLA, procedente del maíz, obtuvimos unos valores de $\delta^{13}C$ de -10,7 ± 0,63 ‰, lo que indica que el maíz es una planta C4, con valores de entre -6 y -19 %. Estos valores de $\delta^{13}C$, descritos por Deines (1980) nos permiten diferenciar el origen de las sustancias y analizar el contenido de activos en la matriz al relacionar la proporción de cada sustancia presente en las muestras. De esta forma, calculamos la concentración de activo remanente en cada uno de los materiales desarrollados y obtuvimos una buena correlación entre el contenido estimado y el calculado.

Los resultados obtenidos en la presente tesis doctoral constituyen un avance en el conocimiento de la seguridad asociada al uso de AEs y sus componentes mayoritarios en envasado activo, y en la evaluación de la funcionalidad de estos nuevos materiales.

VI. CONCLUSIONES / CONCLUSIONS

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones:

PRIMERA. La revisión de la mutagenicidad y genotoxicidad *in vitro* reportada para aceites esenciales (AEs) indica: (i) un menor número de estudios para AEs con respecto a los componentes mayoritarios; (ii) los estudios llevados a cabo no siguen en todos los casos las recomendaciones de la EFSA, (iii) se hace necesario reevaluar estas sustancias de acuerdo a los protocolos de la OCDE; (iv) en general, se ha observado ausencia de actividad mutagénica y genotóxica *in vitro* para los AEs y sus compuestos mayoritarios; (v) la respuesta genotóxica, en los casos en los que ha sido detectada, se ve afectada por el modelo experimental, rango de concentraciones o la activación metabólica.

SEGUNDA. Se ha demostrado *in vitro,* en la línea celular Caco-2, ausencia de actividad citotóxica, de alteraciones morfológicas, y de potencial mutagénico para los compuestos dipropil sulfuro (DPS) y dipropil disulfuro (DPDS) y para su mezcla, en proporción 1:1, hasta concentraciones de 200 µM. Además, se ha observado capacidad de estas sustancias para eliminar ERO del medio celular, siendo mayor esta capacidad en el caso de la mezcla.

TERCERA. El compuesto organosulfurado propil propano tiosulfonato (PTSO) ha producido daño citotóxico y alteraciones morfológicas en las líneas celulares HepG2 y Caco-2. Sin embargo, estos daños se observaron a concentraciones más altas de las propuestas para envasado alimentario. Además, PTSO mostró capacidad para proteger frente a daño oxidativo y actividad antimicrobiana. Por último, la dosis máxima tolerable (DMT) de PTSO en ratas wistar se estableció en 55 mg/kg peso corporal (pc)/día.

CUARTA. Se ha comprobado actividad citotóxica de carvacrol y de la mezcla de este con timol en células Caco-2, experimentando además alteraciones morfológicas, entre las que destacan daño mitocondrial, degeneración lipídica y alteraciones en la cromatina que desembocan en procesos de apoptosis y muerte por necrosis. En el caso de timol, no se ha observado disminución de la viabilidad ni procesos de necrosis o apoptosis, aunque sí se apreciaron alteraciones morfológicas a esas mismas concentraciones.

QUINTA. La evaluación del potencial mutagénico mediante el test de Ames de carvacrol y timol, ha demostrado la capacidad de carvacrol para inducir el aumento de colonias revertientes de *Salmonella thyphimurium* en presencia y ausencia de activación metabólica. Además, carvacrol ha demostrado capacidad para oxidar bases púricas del ADN en células Caco-2. En ambos ensayos, se obtuvieron resultados negativos en el caso de timol.

SEXTA. Se ha demostrado que a altas concentraciones (2500 µM), tanto carvacrol como timol y la mezcla de ambos presentan la capacidad de eliminar radicales libres mediante los métodos DPPH y ABTS. Sin embargo, a concentraciones en torno a 150-460 µM, carvacrol y la mezcla de ambos compuestos, producen estrés oxidativo en células Caco-2. Por otro lado, cuando son utilizados a concentraciones bajas (hasta 100 µM), tanto carvacrol como timol y su mezcla protegen del daño oxidativo producido por peróxido de hidrógeno.

SÉPTIMA. Se ha comprobado mediante el ensayo combinado de micronúcleos (MN) y cometa *in vivo* la ausencia de actividad genotóxica en médula ósea, estómago e hígado, incluida ausencia de

daño oxidativo en el ADN, de carvacrol (81-810 mg/kg pc/día) en ratas wistar. Además, la pirolisis analítica (Py-GC/MS) ha demostrado ser una técnica útil en la determinación y cuantificación de estos compuestos en tejidos biológicos.

OCTAVA. El ensayo combinado de MN y cometa *in vivo* en ratas wistar expuestas durante 90 días a AEO (0-200 mg/kg pc/día), ha demostrado ausencia de actividad genotóxica en médula ósea, estómago, hígado y sangre periférica, incluida ausencia de daño oxidativo en el ADN.

NOVENA. El ensayo de toxicidad subcrónica de 90 días en ratas wistar expuestas a AEO (0-200 mg/kg pc/día) ha puesto de manifiesto la ausencia de alteraciones en peso corporal, consumo de agua y alimento, así como en los parámetros bioquímicos y hematológicos evaluados. Además, no se observaron cambios histopatológicos en los órganos investigados.

DÉCIMA. Los materiales activos desarrollados con base de ácido poliláctico (PLA) y Proallium® o AEO han mostrado propiedades físico-mecánicas compatibles con el envasado alimentario. Solo los films con AEO mostraron actividad antioxidante, mientras que ambos exhibieron actividad antimicrobiana, especialmente frente a mohos y levaduras. La evaluación sensorial mostró que la adición de AEO a los plásticos no impactaba negativamente en la aceptabilidad del consumidor.

UNDÉCIMA. La adición de AEO y Proallium® a matrices de polipropileno (PP) para desarrollar materiales activos ha demostrado modificar levemente las propiedades térmicas y mecánicas originales del PP. Además, los materiales desarrollados con Proallium® mostraron mayor capacidad antimicrobiana frente a *Brochotrix thermosphacta* que los desarrollados con AEO.

DÉCIMOSEGUNDA. Se ha demostrado que la Py-GC/MS es una técnica útil para la identificación de aditivos naturales (Proallium® y OEO) incorporados en matrices de PLA que además proporciona información sobre el origen y la composición de los mismos. Además, se ha confirmado su utilidad para cuantificar la presencia de estos aditivos en los plásticos diseñados.

DÉCIMOTERCERA. Se ha comprobado que la firma isotópica de carbono puede ser utilizada para monitorizar los contenidos reales de estos activos (Proallium® y AEO) en matrices plásticas (PLA) cuando ambas sustancias presentan distinto origen y esto queda reflejado en diferentes valores de δC^{13}.

DÉCIMOCUARTA. La Py-GC/MS ha demostrado ser una técnica útil en la caracterización de componentes de lechugas, concretamente en la trazabilidad de compuestos bioactivos como ácidos grasos poliinsaturados (PUFA) o fitosteroles (PHSTs). Además, esta técnica ha permitido confirmar que el uso de films de PLA con AEO mantiene los contenidos iniciales de estos nutrientes (PUFAs y PHTSs) durante toda la vida útil de la lechuga envasada (8 días).

DÉCIMOQUINTA. Los resultados preliminares demuestran que la adición conjunta de Proallium® y AEO en distintas proporciones (75:25, 50:50 y 25:75) a matrices de PLA produce materiales compatibles con el envasado alimentario. Estos materiales presentan actividad antimicrobiana, que resulta ser más intensa frente a mohos y levaduras. Los estudios sensoriales revelan que los films con una proporción mayor de Proallium® (75%) presentan los mejores valores frente a aceptabilidad e intención de compra.

DÉCIMOSEXTA. Resultados preliminares indican que la mezcla carvacrol:PTSO a concentraciones superiores a la proporción 127:92.5 µM es citotóxica para las células HepG2 y Caco-2. Además, en el estudio morfológico se han observado alteraciones en la cromatina y en las membranas plasmáticas de las células HepG2 expuestas y vacuolización del citoplasma o segregación nuclear en las células Caco-2.

CONCLUSIÓN GENERAL: La ausencia de toxicidad observada, *in vitro* e *in vivo*, a las concentraciones que se prevén en envasado activo, unido a las propiedades funcionales que han demostrado en distintas matrices alimentarias, al ser incorporados a polímeros plásticos, convierten a estos aditivos (AEO y Proallium®) en sustancias interesantes para el desarrollo de este tipo de materiales

The following conclusions were drawn from the results obtaine during the development of the present Doctoral Thesis:

FIRST. Regarding *in vitro* mutagenicity and genotoxicity review reported of essential oils (EOs): (i) fewer studies have been found on EOs in comparison to their main compounds; (ii) the studies recommended by the EFSA to evaluate the genotoxic potential has not always been followed; (iii) it is necessary to perform complete studies according to OECD protocols; (iv) in general, EOs and their main compounds have been reported to be not mutagenic/genotoxic; (v) the genotoxic response, when observed, may be affected by the experimental model chosen, the range of concentrations assayed or the metabolic activation.

SECOND. After *in vitro* Caco-2 exposure, no significant cytotoxicity, no morphological changes and no mutagenic potential were recorded for dipropyl sulphide (DPS), dipropyl disulphide (DPDS) and its mixture, in the proportion 1:1, up to 200 µM. Moreover, the ROS scavenger activity was observed for both organosulphur compounds, with a higher activity in the case of the mixture.

THIRD. Propyl propane thiosulphonate (PTSO) has produced cytotoxic and morphological changes in the human HepG2 and Caco-2. However, these damage were only observed at concentrations higher than those intended to be used in food packaging. Moreover, PTSO has been exhibited a protective role against an induced oxidative situation and it have exhibited antimicrobial activity. Finally, maximum tolerated dose (MTD) in wistar rats has been set at 55 mg/kg body weight (bw)/day.

FORTH. Cytotoxic activity has been confirmed in Caco-2 cells exposed to carvacrol and its mixture with thymol. Moreover, ultrastructural alterations were described highlighting mitochondrial damage, lipid degeneration and chromatin condensation, as well as cell death via apoptosis and necrosis. In contrast, while thymol did not show decreased in the cell viability nor apoptosis or necrosis, ultraestructural damage was observed for the same concentrations.

FIFTH. The mutagenic potential of carvacrol and thymol has been evaluated by the Ames test, and an increased in revertant colonies of *Salmonella thyphimurium* for carvacrol was demonstrated, also in the presence of metabolic activation. Moreover, carvacrol was also observed to induce purine DNA-oxidative effects in Caco-2 cells. In both assays, negative results were observed for thymol.

SIXTH. At high concentrations (2500 µM), carvacrol, thymol and their mixture showed scavenging activity in DPPH and ABTS methods. However, carvacrol and its mixure with thymol, induced oxidative stress in Caco-2 cells (150-460 µM). Moreover, at low concentrations up to 100 µM, all exposures assayed presented a protective role against an induced oxidative stress.

SEVENTH. The combined micronucleus (MN)-comet assay protocol demonstrates that carvacrol (81-810 mg/kg bw/day) did not induce genotoxic effects in bone marrow, stomach and liver cells of wistar rats. Moreover, no oxidative damage was observed in DNA. Finally, analytical pyrolysis (Py-GC/MS) has demonstrated to be a useful technique to identify and quantify these compounds in biological tissues.

EIGHTH. Oral subchronical exposure (90 days) to OEO (0-200 mg/kg bw/day) did not induce genotoxic effects in bone marrow, stomach, liver and blood of wistar rats in the combined MN-comet assay protocol). Moreover, no oxidative damage was observed in DNA.

NINTH. In the subchronic assay in wistar rats exposed during 90 days to OEO (0-200 mg/kg bw/day) an absence of changes in body weight, food and water consumption have been observed, as well as on biochemical and haematological parameters. Moreover, no remarkable change was observed at the histopathological level in the tissues evaluated.

TENTH. Films developed including Proallium® or OEO in polylactic acid (PLA) matrices showed physical and mechanical properties suitable for the development of food packaging. Although only PLA-OEO films exhibited antioxidant properties, films developed with OEO or Proallium® produced antimicrobial activity, being yeast and molds the most sensitive microorganisms. The sensorial study revealed no negative impact in the acceptability of food packaged in PLA-OEO films.

ELEVENTH. Polypropylene (PP) films containing Proallium® or OEO showed similar thermical and mechanical properties than PP. Moreover, Proallium® active films seemed to be more effective against *Brochotrix thermosphacta* than PP films containing OEO.

TWELFTH. Our results indicated that Py-GC/MS is a valuable tool that can provide rapid and accurate information about the presence, origin and composition of these additives (Proallium® and OEO) contained in PLA films. Moreover, this technique seems to be useful to quantify these additives in PLA matrices.

THIRTEENTH. Stable isotopic signature showed to be useful to monitor the real content of natural additives (Proallium® and OEO) in polymeric matrices (PLA) when the two components have a different origin reflected in different δC^{13}.

FOURTEENTH. Py-GC/MS was found useful in characterizing of the lettuce composition and particularly in tracing the evolution within time of dietetic components such as bioactive polyunsaturated fatty acid (PUFAs) and phytosterols (PHSTs). Moreover, this technique has demonstrated that the use of PLA-OEO films allowed appropriate preservation of both PUFAs and PHSTs relative contents during shelf life (8 days) of lettuce.

FIFTEENTH. Preliminary results suggest that PLA films containing different proportions of Proallium® and OEO (75:25, 50:50 y 25:75) presented physical and mechanical properties suitable for the development of food packaging. Moreover, these materials exhibited antimicrobial activity, mainly against yeast and molds. In addition, packaging materials containing the highest content of Proallium® showed the best values from a sensory point of view, with the highest values for global acceptability and purchase intention.

SIXTEENTH. Preliminary results of cytotoxicity endpoints indicated that carvacrol-PTSO mixtures induced cytotoxic effects in HepG2 and Caco-2 cells. Moreover, morphological study showed alterations in the chromatin and plasmatic membranes for HepG2 cells exposed to the mixture and vacuolization of the cytoplasm and nucleolar segregation for Caco-2 cells.

GENERAL CONCLUSION: The absence of toxicity observed, in vitro and in vivo, at concentrations anticipated in active packaging, coupled with the functional properties demonstrated in different food matrices, when incorporated into plastic polymers, convert these additives (AEO and Proallium®) in interesting substances in the development of these packaging materials.

VII. OTROS MÉRITOS / OTHER MERITS

Aceites esenciales con aplicación en envases activos: Una revisión.

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Resumen

Las nuevas tendencias en la industria alimentaria y las logísticas en las cadenas de distribución de alimentos han hecho necesaria la aparición de un nuevo tipo de envase que permita cubrir tanto las necesidades de los consumidores, enfocadas a alimentos más sanos y naturales, como las derivadas de la globalización de la alimentación, que necesita alimentos que permanezcan con sus cualidades inalteradas durante períodos de tiempo más prolongados. En este sentido empiezan a valorarse y a desarrollarse los envases activos que, aunque ya están siendo utilizados en Japón o Estados Unidos, la legislación europea, más restrictiva, ha retrasado su incorporación al mercado comunitario por demandar estudios que aseguren la utilidad e inocuidad de estos nuevos materiales. Entre los envases activos destacamos aquellos que incorporan aceites esenciales naturales y sus componentes en diversas estructuras poliméricas para beneficiarse de las propiedades antioxidantes y antibacterianas que presentan estos extractos naturales y aumentar así la durabilidad de los alimentos.

La legislación europea obliga a estudiar detenidamente la seguridad asociada al uso de estos envases y la utilidad real ya que, aunque los aceites presenten propiedades interesantes para la conservación de alimentos, los componentes activos deben ejercer su acción incluso sin estar en contacto directo con el producto. El objetivo de este trabajo de revisión es mostrar el estado en el que se encuentran estas investigaciones y cómo los prototipos de envases alimentarios con aceites esenciales en su composición están demostrando ser eficaces y seguros para el consumidor.

Palabras clave: envase activo, aceite esencial, antioxidantes, antimicrobianos

Abstract

Changes in retail and distribution practices such a new trends in consumer demands and internationalization of markets, resulting in an increased distribution distances and longer storage times, are putting huge demands on the food packaging industries. In the last decades one of the most innovative developments of food packaging is the “active packaging”. In Japan or USA active packaging concept are already being successfully applied. In Europe, the development and application of active packaging is limited because of legislative restrictions. Essential oils are rich in compounds which exhibit a wide range of biological effects, including antioxidant and antibacterial properties. The incorporation of essential oils in polymeric matrices provides antioxidant or antibacterial packaging films which improves shelf life of perishable products. To be on European market, each system should comply with the legislation to ensure effectiveness and safety associated with their use. The objective of this review is to provide a state of the art about the use of essential oils in active packaging.

Índice

1. INTRODUCCIÓN

El desarrollo de nuevos envases con una perspectiva más ecológica y sostenible empieza a convertirse en una filosofía compartida por las empresas de la industria alimentaria (Tharanathan, 2003). La presión ejercida por los consumidores, cada vez más conscientes de los problemas derivados del uso de aditivos sintéticos en los alimentos, unida a la necesidad de gestionar los subproductos de las industrias y buscar fuentes renovables para la creación de polímeros, hace que empiecen a desarrollarse envases con compuestos naturales. En este sentido, se trabaja con derivados procedentes de la industria del maíz o la soja y con compuestos biodegradables y biocompatibles como el acido láctico, que incorporen en su estructura sustancias capaces de ejercer acciones antibacterianas y antioxidantes con la finalidad de aumentar la vida útil de alimentos perecederos (Kerry y col., 2006).

El concepto de vida útil de un alimento fue definido por primera vez en la legislación europea, en el Reglamento 2073/2005, de 15 de noviembre de 2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios, como el periodo anterior a la fecha de duración mínima o a la "fecha de caducidad", tal como se definen, respectivamente, en los artículos 9 y 10 de la Directiva 2000/13/CE.

La vida útil de un alimento, está determinada por tres factores (Sadaka y col., 2013):

1. Factores intrínsecos: características propias del alimento como son el pH, actividad de agua, contenido en nutrientes, potencial redox, estructura biológica, etc. Muchos de estos factores pueden controlarse a través de una elección selectiva de los materiales e ingredientes que constituyen el alimento, así como de los procesos de preparación.

2. Factores extrínsecos: hacen referencia a las condiciones ambientales en las que se encuentra el alimento durante la distribución y el almacenamiento del mismo: temperatura, humedad relativa, composición del aire. Estos factores desencadenan reacciones de degradación del alimento durante su vida útil.

3. Los envases alimentarios, al poner en contacto los alimentos con el ambiente externo, influyen directamente en la repercusión de los factores extrínsecos, y por tanto, indirectamente en el desarrollo de reacciones de degradación.

La globalización de la alimentación, con cadenas de distribución cada vez más complejas ponen el foco de atención de la industria alimentaria en el desarrollo de envases capaces de transportar alimentos perecederos a través de largas distancias sin que se vean comprometidas sus características nutritivas y organolépticas (Vermeiren y col. 1999). Es en esta línea en la que la conjunción de polímero biodegradable y aceite esencial toma una fuerza mayor. La nueva generación de envases, los envases activos, permite que los alimentos puedan estar en una atmósfera natural, generada por aceites esenciales, con propiedades antibacterianas y antioxidantes que aumenten su vida útil. Este nuevo concepto de envase en el que se fuerza la interacción del mismo con el alimento plantea una serie de retos, en relación a la evaluación de la seguridad, en comparación con el envasado tradicional que buscaba materiales inertes que actuasen como mera barrera frente al ambiente externo (Danielli y col., 2008). La nueva normativa comunitaria intenta agilizar y sistematizar los procedimientos de presentación de solicitudes de autorización de sustancias activas, la evaluación de los riesgos inherentes a estos materiales y las normas relativas al etiquetado. No obstante, la industria está utilizando tímidamente estos nuevos materiales, probablemente debido a factores socio-económicos, preferencia de los consumidores y dudas sobre la implementación (Chaves Sánchez, 2011).

2. LOS ACEITES ESENCIALES EN LA INDUSTRIA ALIMENTARIA

El concepto de envasado activo no es tan novedoso como pudiera parecer. Las tradiciones ancestrales de las zonas tropicales del planeta nos muestran que en diferentes regiones de África, Asia o Sudamérica las hojas de los árboles han sido y continúan siendo utilizadas para envolver, cocinar y conservar los alimentos beneficiándose así de las propiedades antioxidantes, antibacterianas o aromáticas que confiere el contacto de las sustancias naturales de las hojas con los alimentos (Danielli y col., 2008).

Estos conocimientos han facilitado la búsqueda de sustancias activas naturales, que respondan a las demandas de la población, y sean capaces de aumentar la vida útil de los alimentos para cubrir las necesidades de las cadenas de distribución. Los aceites

esenciales y extractos de diferentes géneros de especies vegetales se han convertido así en un recurso a estudiar en el desarrollo de envases activos.

Los aceites esenciales son productos del metabolismo secundario de diversos géneros vegetales, sintetizados en estructuras glandulares (Bajpai y col., 2012). Se obtienen mediante multitud de técnicas, siendo las más sencillas presión, destilación de vapor o extracción mediante disolventes orgánicos (Stammati y col., 1999). El método utilizado para la extracción determinará la pureza y puede afectar a la composición final. La actividad biológica de los aceites esenciales está directamente relacionada con su composición y se debe tanto a los componentes mayoritarios como a los minoritarios, que actúan de forma sinérgica (Lahlou, 2004). Los aceites esenciales se han utilizado tradicionalmente con diferentes propósitos y aunque sus propiedades antioxidantes y antibacterianas han sido demostradas a lo largo de los años, no existe una sistematización de cómo la composición química, fundamentalmente terpenos y ácidos fenólicos, tanto alifáticos como aromáticos de bajo peso molecular, afecta a dichas propiedades.

Muchos de estos aceites y sus componentes principales están recogidos en la Decisión de la Comisión 2002/113/EC como aromatizantes permitidos en la Unión Europea, por ejemplo el timol, linalol, carvacrol, limoneno o eugenol, para los que no se considera que exista riesgo para la salud del consumidor (Hyldgaard y col., 2012). En Estados Unidos, existen aceites esenciales (orégano, timo, mostaza o albahaca) que ya están clasificados como Generally Recognized as Safe (GRAS) por la Food and Drugs Administration (FDA) (López y col., 2007, Zhou y col., 2006) por lo tanto, además de las aplicaciones conocidas como aromatizantes se han desarrollado aplicaciones como conservantes en alimentación por las propiedades antioxidantes y antibacterianas que presentan (Dusan y col., 2006).

En la Unión Europea, los aceites esenciales se utilizan actualmente en la industria farmacéutica, cosmética y alimentaria. Alguno de los componentes individuales de los aceites son utilizados como aromatizantes alimentarios, tanto si son extraídos directamente de las plantas como los sintetizados en laboratorio. Ya existen comercializados algunos conservantes, de adición directa sobre el alimento, que presentan aceites esenciales en su composición. Entre ellos encontramos:“DMC Base

Natural", un conservante alimentario producido por DOMCA S.A en Granada, España, constituido al 50% por aceites esenciales de diferentes especies; y también "Protecta One" y "Protecta Two" que son mezclas de extractos de hierbas reconocidas por la FDA como GRAS (Burt, 2004).

A pesar de que se ha demostrado el potencial antioxidante y antibacteriano de los aceites esenciales, y sus componentes en estudios *in vitro*, el uso como conservantes se ve limitado por la necesidad de añadir altas concentraciones de las sustancias al alimento para conseguir el fin deseado. Esto se debe a diversos factores entre los que destacan la multitud de interacciones que se producen entre las sustancias activas y la propia composición del alimento: materia grasa, almidón o proteínas, o la variación de la capacidad antimicrobiana y antioxidante del aceite o de sus componentes en presencia de diferentes condiciones de pH o temperatura. Además del inconveniente asociado a las altas concentraciones que son necesarias para cumplir estos objetivos se une que los componentes volátiles generan intensos aromas que modifican las propiedades organolépticas de los alimentos, provocando rechazo por parte del consumidor (Hyldgaard y col., 2012).

Alcanzar concentraciones efectivas sin añadir altas concentraciones de estas sustancias ha llevado a la industria alimentaria a desarrollar diversos métodos de envasado activo en los que el aceite no es añadido como un ingrediente directo del alimento sino como un componente del envase. Para ello existen varias opciones: encapsular los aceites en sacos que permitan una liberación prolongada al espacio de cabeza del envase, incorporarlos a polímeros plásticos o biodegradables para que migren lentamente al espacio de cabeza o a la superficie del alimento, etc. La ventaja de incorporar los aceites a las películas de recubrimiento es que se consigue retrasar la difusión de los componentes activos a la superficie del alimento, manteniéndolos en el espacio de cabeza y extendiendo así la vida útil del producto (Sánchez-González y col., 2011).

Como consecuencia de la naturaleza misma de estos envases activos, en los que se produce migración de las sustancias al espacio de cabeza y se genera inevitablemente contacto con el alimento, es necesario realizar una evaluación del riego exhaustiva para salvaguardar la salud del consumidor y cumplir así con el requisito de evaluación de la

seguridad exigido por el Reglamento 1935/2004 sobre los materiales y objetos destinados a entrar en contacto con alimentos.

3. ACEITES ESENCIALES UTILIZADOS EN ENVASES ACTIVOS POR SUS PROPIEDADES ANTIMICROBIANAS.

La selección del aceite esencial a incorporar en el envase no se hace únicamente en base a la actividad específica frente a los patógenos. Además intervienen otra serie de factores como la resistencia de los microorganismos a estas sustancias, la naturaleza química de los alimentos y el aceite, condiciones de almacenamiento y distribución del producto terminado, características organolépticas, toxicidad de las sustancias antimicrobianas, etc. Todos estos factores deben ser estudiados meticulosamente para conseguir un envase activo efectivo y seguro para el consumidor (Sadaka y col., 2012).

Según Coma (2008), los envases activos antimicrobianos pueden clasificarse en:

1. Envases activos con adición de saquitos o bolsitas
2. Agentes bioactivos dispersos en el envase
3. Agentes bioactivos como recubrimiento del material de envasado
4. Macromoléculas antibacterianas con propiedades formadoras de película
5. Recubrimiento bioactivo comestible

Son muchos los estudios que demuestran la capacidad antibacteriana de los aceites esenciales en el control de patógenos que intervienen en la degradación de los alimentos. En este sentido, Teixeira y col. (2012) determinaron, mediante el método de difusión en agar, la concentración inhibitoria mínima (CMI) del aceite esencial de menta frente a diversas cepas bacterianas (*Salmonella typhimurium, Escherichia coli, Listeria innocua, Listeria monocytogenes, Pseudomonas putida*, etc.), obteniendo resultados que demuestran la capacidad antibacteriana de este aceite. Así mismo, frente a *Salmonella*, uno de los patógenos alimentarios más conocidos, muchos aceites esenciales (entre ellos menta, tomillo o naranja) han demostrado ser eficaces en el control de su proliferación (Bajpai y col., 2012). En la misma línea, Dusan y col. (2006)

mostraron cómo los aceites esenciales de canela, clavo, orégano y timo produjeron inhibición del crecimiento de cepas de *Escherichia coli*. En la actualidad, el interés de la industria camina hacia el desarrollo de envases que contengan en su estructura aceites esenciales para evitar las alteraciones organolépticas derivadas de la adición directa (Coma, 2008). Por ello es fundamental comprobar la eficacia de estos envases en su liberación gradual de sustancia activa.

Uno de los grandes inconvenientes del uso de aceites esenciales en el envasado deriva de sus propiedades aromatizantes, que pueden alterar el aroma y el sabor de los alimentos. Sin embargo, la adición de concentraciones bajas de aceite esencial de canela y orégano a plásticos de recubrimiento ha mostrado eficacia antimicrobiana frente a *Escherichia coli* y *Staphylococcus aureus*, disminuyendo el riesgo de alteraciones organolépticas (Becerril y col., 2007).

3.1 Aceites esenciales con propiedades antimicrobianas utilizados en matrices poliméricas de origen mineral.

Los primeros estudios que muestran la incorporación de extractos naturales de plantas a matrices poliméricas convencionales, derivadas del petróleo, datan de 1998, cuando Lee y col. (1998) desarrollaron un polietileno de baja densidad (LDPE) que incluía un agente antimicrobiano de amplio espectro, el zumo de pomelo. El film mostró capacidad de inhibir el crecimiento de *Staphylococcus aureus* y *Escherichia coli*. Cuando se utilizó para el envasado de lechuga y soja, el film inhibió la tasa de crecimiento de las bacterias lácticas. Lee y col. (1998) mostraron así, por primera vez, cómo la adición de sustancias naturales activas podía proporcionar a los envases propiedades para mejorar la conservación de los alimentos. Otros autores estudiaron, utilizando métodos turbidimétricos, la actividad antimicrobiana frente a *Pseudomonas spp*. de envases activos de LDPE con extracto de limón o timol, componente principal del aceite esencial de timo, sin encontrar evidencias de que se afectasen las cinéticas de crecimiento de los microorganismos. Los mismos autores apuntaron a las altas temperaturas necesarias para la fabricación de los envases como factor responsable de la ineficacia antimicrobiana (Del Novile y col., 2009).

Diversos envases activos desarrollados a partir de polipropileno (PP) han mostrado propiedades antimicrobianas. Los aceites esenciales de orégano y canela, incorporados a estas matrices, han sido estudiados para evaluar su acción cuando son liberados al espacio de cabeza del envase, frente a una amplia gama de microorganismos. Ambos aceites mostraron una gran actividad frente a levaduras y mohos. Sin embargo, solo el aceite esencial de canela inhibió o redujo considerablemente el crecimiento de bacterias gram-positivas como *Bacillus cereus, Staphylococcus aureus* o *Listeria monocytogenes* (Lopez y col., 2007). La actividad antifúngica del aceite esencial de canela, en envases de polietileno tereftalato y PP, frente a mohos de crecimiento espontáneo en la fruta como *Monilica frutícola, Penicillium expansus* o *Rhizopus spp*., fue reafirmada por Montero-Prado y col. (2011). Los componentes mayoritarios del aceite esencial de orégano, carvacrol y timol, han sido incorporados a polímeros de PP para evaluar la actividad antimicrobiana por contacto directo con el alimento mediante la técnica de difusión en agar. Los resultados mostraron que el timol es más efectivo que el carvacrol frente a bacterias gram-positivas como *Staphylococcus aureus*. Sin embargo, no se observó actividad en ninguno de los casos frente a gram-negativas como *Escherichia coli* (Ramos y col., 2012).

Muriel-Galet y col. (2012) estudiaron los efectos antibacterianos del aceite esencial de oregano incorporado a copolimeros de PP y etilenvinil alcohol (EVOH) mediante la técnica de difusión en agar y determinaron que estos films eran más efectivos frente a bacterias gram-negativas (*E. coli* o *Salmonella entérica*). Se necesitaron concentraciones superiores del aceite en el envase para conseguir una zona de refracción del crecimiento para *L. monocytogenes*. En el mismo estudio se evaluó la capacidad *in vivo* del film para proteger la lechuga frente a microflora habitual de los vegetales (bacterias aerobias, enterobacterias, levaduras y mohos o bacterias psicótrofas). Los resultados mostraron actividad frente a bacterias aerobias psicotrofas en las primeras etapas del almacenamiento, cuando las concentraciones de los agentes activos en el espacio de cabeza del envase son máximas, y frente a enterobacterias, solo a las concentraciones más altas (10% de extracto en el film). Frente a bacterias patógenas (*E. coli, S. entérica* o *L. monocytogenes*), sólo los envases con un 10% de aceite esencial mostraron ser efectivos.

Además de ensayos frente a microorganismos, Licciardello y col. (2013) han estudiado el efecto repelente frente a insectos de envases de PP con aceites esenciales de orégano, romero o hierba de limón frente a la cucaracha roja de la harina (*Tribolium castaneum*) obteniendo resultados muy prometedores de entre un 53 y un 87 % de poder repelente.

Otra matriz polimérica altamente utilizada en el ámbito alimentario es el polietileno (PE). López y col. (2007) evaluaron la actividad antimicrobiana de envases de PE/EVOH con aceites esenciales de orégano, clavo y canela y mostraron que los hongos y levaduras eran los organismos más sensibles. En este mismo estudio se compararon las propiedades antibacterianas entre envases cuya matriz estaba constituida por PP o PE/EVOH y se determinó que la efectividad de los envases constituidos por polímeros de PP era mayor.

El aceite esencial de canela también ha sido evaluado como antifúngico en envases a partir de polietileno (PET). Manso y col. (2013) estudiaron la capacidad de estos envases de inhibir el crecimiento de *Aspergillus flavus* sin entrar en contacto directo con el alimento (agar) y la estabilidad de las propiedades antifúngicas de los materiales de envasado tras haber sido almacenados durante dos meses a 4ºC. Se realizaron ensayos a corto (doce días a 25ºC) y largo plazo (dos meses a 25ºC). Para los envases que contenían de 4 a 8% de aceite esencial se observó inhibición completa del crecimiento del microorganismo. Se repitió el mismo ensayo para los materiales que habían quedado almacenados durante dos meses a 4ºC y los resultados obtenidos fueron similares, por lo que se demostró la estabilidad de la capacidad antimicrobiana de estos envases.

3.2 Aceites esenciales con propiedades antimicrobianas utilizados en matrices poliméricas obtenidas de fuentes renovables. Biopolímeros.

Para reducir los problemas de contaminación y el consumo de fuentes no renovables de materiales, se ha impuesto el desarrollo de materiales compatibles con el medio ambiente, procedentes de fuentes renovables y biodegradables (Takala y col., 2013). Estos nuevos materiales son denominados biopolímeros, y se clasifican en (Valero-Valdivieso, 2013):

1. Polímeros extraídos de biomasa (polisacáridos, proteínas o lípidos)

2. Polímeros sintetizados a partir de monómeros de biomasa (celulosa bacteriana o polihidroxi-alcanoatos)

3. Polímeros producidos por microorganismos naturales o modificados genéticamente.

Uno de los polisacáridos utilizados en el desarrollo de envases alimentarios es el almidón de yuca. Souza y col. (2013) evaluaron la acción de un envase diseñado a partir de este polímero polisacarídico y aceite esencial de canela, y del propio aceite mediante el método de difusión en agar por contacto directo del film, o en su caso de discos impregnados en aceite esencial, con el alimento (agar) frente a dos especies de hongos que crecen de forma recurrente en el pan (*Penicillium commune* y *Eurotium amstelodami)*. La CMI del aceite se estableció en 2 g/100 g para *P. commune* y 0.5 g/100 g para *E. amstelodami*. La mayor sensibilidad a la acción del aceite incorporado al envase la mostró *E. amstelodami* con un porcentaje de inhibición del 91% para films con 0.8 g/100 g de aceite esencial de canela, mientras que para el mismo prototipo, la inhibición para *P. commune* alcanzó solo un 25%.

En esta misma línea, el mucílago que se obtiene a partir de semillas de membrillo ha sido utilizado para elaborar un envase activo que incorpora aceite esencial de timo (Jouki y col., 2014). Se estudió la capacidad antimicrobiana de este envase frente a una amplia variedad de bacterias gram-positivas y gram-negativas y se observó actividad frente a todas ellas en envases que contenían desde un 1% de aceite de timo. Las especies más sensibles a la acción de estos envases fueron *Shewanella putrefaciens, L. monocytogenes* y *Staphylococcus aureus*, mientras que *Pseudomonas aeruginosa* mostró la máxima resistencia.

El chitosan es otro biopolímero polisacarídico estudiado por sus posibles propiedades antimicrobianas, tras adicionarle aceite esencial de canela. La actividad antifúngica de estos envases fue determinada tanto *in vitro* como *in vivo* por Perdones y col. (2014). Frente a *Aspergillus niger*, *Botrytis cinerea* y *Rhizopus stolonifer*, los resultados del ensayo de difusión en agar *in vitro* mostraron inhibiciones significativas, para todas las especies, en envases que contenían concentraciones superiores al 0,25% de aceite esencial; siendo *B. cenerea* es organismo más sensible. El ensayo *in vivo* se realizó en fresas frescas que fueron inoculadas con *Rhizopus stolonifer* y almacenadas bajo

diferentes condiciones: 10ºC y 70% humedad relativa (HR) o 20ºC y 80% HR. En ambos casos se observó un aumento de vida media del producto en envases con 0.5 % y 0.25% de aceite de canela, respectivamente.

Los biopolímeros producidos por síntesis química utilizan monómeros biológicos de fuentes renovables. Es el caso del polímero de ácido poliláctico (PLA), que se genera a partir de monómeros de ácido láctico obtenidos de materias vegetales como el maíz o el arroz (Rasal y col., 2010). Aún existen pocos estudios en los que se evalúe la acción antimicrobiana producida por adición de aceites esenciales a estos polímeros. No obstante, Del Novile y col. (2009) estudiaron la efectividad de nuevos materiales biocompatibles, entre ellos el PLA, con presencia de extracto de limón o timol, como envases activos antibacterianos y los resultados mostraron que podrían ser utilizados para envasado alimentario.

Es interesante destacar que muchos de los envases activos antimicrobianos que se están desarrollando, además de ser biodegradables y biocompatibles son, además, comestibles. Un ejemplo de estos son los evaluados por Avilés-Sosa y col. (2012), diseñados a partir de almidón, chitosan o amaranto con incorporación de aceites esencial de orégano, canela o hierba de limón. Los estudios antimicrobianos se realizaron para cada combinación de matriz y aceite frente a *Aspergillus niger* y *Penicillium digitatum*. Los prototipos con matriz de amaranto mostraron la mínima capacidad inhibitoria frente a *A. niger*, necesitando altas concentraciones de aceite esencial (2% para canela y 4% para orégano) para producir efectos significativos. En el caso del aceite de hierba de limón no se observó efecto alguno frente a *A. niger*. Sin embargo, las capacidades antifúngicas más completas, afectando a ambos microorganismos, las presentaron los envases a base de chitosan con concentraciones bajas de los aceites de canela y orégano.

Las proteínas del suero constituyen otra de las matrices poliméricas extraidas a partir de biomasa. Tras incorporarse a este polímero aceite esencial de canela, se desarrolló un envase activo cuyas propiedades antimicrobianas fueron evaluadas frente a seis especies diferentes: *Lactobacillus lactis, Pseudomonas putida, Streptococcus agalactiae, Escherichia coli, Listeria monocytogenes, Bacillus subtilis* y *Candida albicans*. Los resultados de los ensayos de difusión en agar mostraron actividad frente a la mayoría de

la cepas, siendo la más sensible *Bacillus subtilis* y, *Streptococcus agalactiae,* la más resistente (Bahram y col., 2013).

Listeria monocytogenes es uno de los patógenos más recurrentes en la contaminación de alimentos. La acción de un envase comestible obtenido a partir de piel de patata que incorpora aceite esencial de orégano ha sido evaluada en el envasado de salmón ahumado. Tras inocular el salmón con el cultivo bacteriano y almacenar a 4ºC durante 28 días, las muestras fueron digeridas en agua de peptona e inoculadas a una placa de agar. El conteo de las placas, después de la incubación, mostró menor número de colonias de *Listeria* en las muestras de salmón envasadas con el envase activo, en relación a los controles, envasados solo en la matriz polimérica (Tammineni y col., 2013).

4. ACEITES ESENCIALES UTILIZADOS EN ENVASES ACTIVOS POR SUS PROPIEDADES ANTIOXIDANTES.

Con el objetivo de evitar la peroxidación lipídica (LPO) y con ello el deterioro de los alimentos, la adición de sustancias antioxidantes constituye una opción para la industria alimentaria. La seguridad derivada del uso de antioxidantes sintéticos comienza a cuestionarse y los consumidores se muestran preocupados por el uso de éstos en los alimentos. En respuesta a esta situación, los antioxidantes naturales, seguros y aceptados por los consumidores, se convierten en una alternativa poderosa. Muchos aceites esenciales han mostrado propiedades antioxidantes, principalmente los aceites de orégano y romero (Olmedo y col., 2013), lavanda o menta (Djenane y col., 2012).

Los estudios de envases activos con aceites esenciales comienzan a sucederse en una carrera por demostrar la seguridad y utilidad de este tipo de envasado. Las propiedades antioxidantes pretenden adaptarse a cualquier tipo de alimento utilizando una metodología que permita conseguir envases eficaces y seguros.

4.1 **Aceites esenciales con propiedades antioxidantes utilizados en matrices poliméricas de mineral.**

Bolumar y col. (2011) prepararon un prototipo de envase activo de LDPE cubierto por una solución etanólica de aceite de romero. Con este film envasaron al vacio empanadillas de carne de pollo que sometieron a altas presiones (800MPa) durante diez minutos para forzar la LPO. El grado de oxidación de las muestras fue medido a lo largo de todo el período de almacenaje (25 días), mediante el ensayo de sustancias reactivas al acido tiobarbitúrico (TBARS). Los resultados mostraron que la LPO sufrida por esta carne era menor que su análoga envasada solo en LDPE.

En esta misma línea, Nerín y col. (2008) estudiaron la capacidad antioxidante de un envase activo a base de PP que incorporaba en su estructura extracto de romero. Las propiedades antioxidantes del envase fueron medidas utilizando sustancias patrones fácilmente oxidables (ácido ascórbico, hierro (II) y ácidos grasos). Los resultados mostraron que el envase era efectivo y prevenía la oxidación de estas sustancias. Asimismo, se estudió la eficacia de envases de PP con extracto de romero en la protección de la oxidación de un producto cárnico. Los resultados mostraron que el nivel de protección frente a LPO aportado por el extracto de romero adicionado directamente sobre el alimento y el producido por el envase activo era similar, lo que parece indicar que este envasado realmente va a suponer una alternativa a la adición directa de conservantes (Nerín y col. 2006).

El aceite esencial de canela incorporado a películas de polietileno ftereftalato (PET) constituye otro de los envase activos en desarrollo y su capacidad antioxidante en el envasado de melocotones ha sido comprobada. El aceite esencial de canela es capaz de inhibir la lipooxigenasa, disminuyendo así el proceso de peroxidación y contribuyendo a aumentar las expectativas puestas en esta alternativa de envasado. Además, en este estudio se observó que las propiedades organolépticas de los melocotones se mantenían intactas y que no aparecía ningún sabor residual a canela, que constituye una de las limitaciones del uso de sustancias tan aromáticas en los envases (Montero-Prado y col., 2011).

Pezo y col. (2007) estudiaron la capacidad antioxidante de diversos prototipos de envases, que presentaban aceite esencial como componente activo, con el objetivo de evaluar si existe una correlación entre dicha capacidad de la sustancia independiente y la que presenta el envase una vez terminado. Mostraron que existe una intensa relación

entre ambas en muchos casos. Sin embargo, para otras muestras del mismo ensayo, se obtuvieron resultados contrarios con bajas tasas de correlación. La conclusión de su estudio fue que la determinación del potencial antioxidante de un envase activo debe hacerse sobre el envase final desarrollado, ya que medir el potencial de la sustancia independiente puede falsear los resultados.

López-de-Dicastillo y col. (2012) estudiaron el efecto antioxidante de un envase diseñado a partir de EVOH con extracto de té verde destinado al envasado de sardina fresca eviscerada. Tras envasarlas en contacto con el film activo, fueron almacenadas a 4ºC durante 13 días. El valor de la LPO fue evaluado mediante el método TBARS y los resultados mostraron que los envases activos con té verde fueron más eficientes en las primeras etapas del almacenaje. Sin embargo, tras siete días de conservación, la concentración de peróxidos aumentó bruscamente.

4.2 **Aceites esenciales con propiedades antioxidantes utilizados en matrices poliméricas obtenidas de fuentes renovables. Biopolímeros.**

El descubrimiento de los biopolímeros también ha permitido desarrollar materiales antioxidantes biodegradables y biocompatibles. Diversos envases constituídos por matrices poliméricas naturales que incorporan aceites esenciales han sido estudiadas por las propiedades citadas anteriormente. En este sentido, un envase con matriz de chitosan, que incorpora aceite esencial de canela, ha sido evaluado mediante el ensayo de capacidad antioxidante equivalente a Trolox (vitamina E) mostrando mayor capacidad antioxidante (envase con 1% de aceite) que su análogo sin aceite esencial (Perdones y col., 2014). El aceite esencial de timo también ha sido estudiado como potencial sustancia antioxidante en envases diseñados a partir de mucílago de semillas de membrillo mediante un ensayo de captación de radicales libres (DPPH). Los resultados mostraron actividades del 18,39% para envases sin aceite. No obstante, a medida que se incorporaron cantidades de aceite esencial, correspondientes al 1, 1,5 y 2%, los valores aumentaron hasta 30,11%, 37,29% y 43,13% respectivamente, demostrando el aumento de capacidad de captación de radicales libres y por tanto, una mayor actividad antioxidante (Jouki y col., 2014).

Los estudios sobre envases activos antioxidantes comestibles, que incorporan aceites esenciales, comienzan a sucederse en un intento por acelerar su evaluación. En este sentido, un ejemplo serían los fabricados a partir de semillas de soja y girasol. Salgado y col. (2013) estudiaron la incorporación a estos biopolímeros de aceite esencial de clavo y obtuvieron resultados muy positivos en cuanto a capacidad antioxidante *in vitro*. Los envases producidos a partir del mismo biopolímero y con presencia de aceite esencial de tomillo o menta también comienzan a ser una opción para la industria (Salarbashi y col., 2013). Así mismo, un envase con matriz de pectina y aceite esencial de canela incorporado, ha sido evaluado por su capacidad antioxidante. Los resultados del ensayo de captación de radicales libres DPPH mostraron actividad antioxidante, máxima para la mayor concentración de aceite en el envase (36,1 g/L). En el mismo estudio se cuantificó el contenido en flavonoides y compuestos fenólicos (sustancias antioxidantes presentes en la fruta) de melocotones frescos cortados que habían sido envasados en este film durante 15 días a 5ºC, obteniéndose concentraciones superiores a las observadas en fruta envasada únicamente en la matriz de pectina. La fruta contenida en el envase activo mostró un mayor poder antioxidante, tras realizarle el ensayo DPPH, en las primeras etapas de conservación (0-5 días) (Ayala-Zavala y col., 2012).

5. SITUACIÓN LEGISLATIVA.

La situación de la legislación relativa a los envases activos varía en función del país, que desarrolla políticas más o menos restrictivas. Si comparamos la situación de la Unión Europea (UE) con Japón, Estados Unidos o Australia, se observa que la incorporación al mercado de estos envases está siendo mucho más lenta. Este desfase parece estar provocado por una política poco flexible que no camina a la velocidad que lo hacen las innovaciones tecnológicas. Además los recelos de las industrias, que deberían aplicar los cambios y la actitud, más conservadora, de los consumidores ralentiza el avance de la salida al mercado de esta nueva tecnología (Danielli y col., 2008).

En la Figura 2 se representa el marco legislativo europeo en materia de materiales en contacto con alimentos.

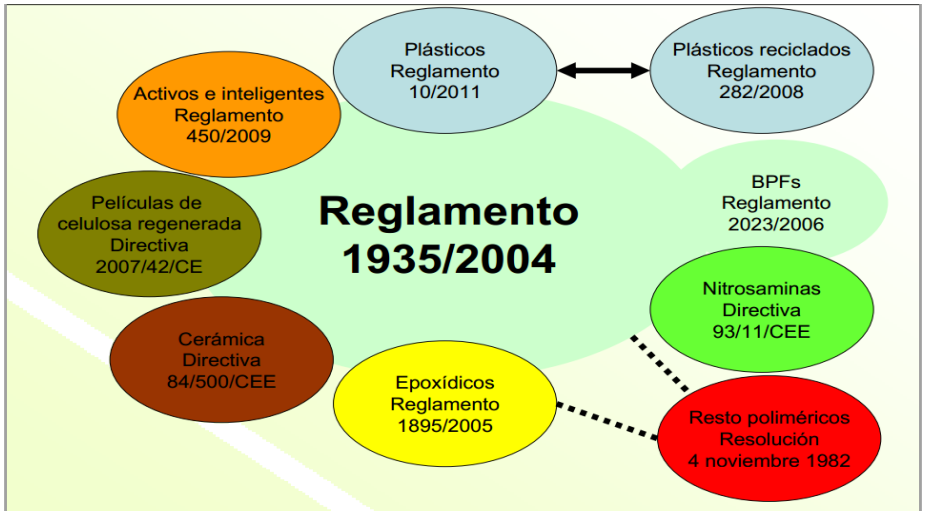

Figura 2: Marco legislativo de los materiales en contacto con alimentos en la UE (Teruel Muñoz, 2011)

Los envases activos, al igual que todos los materiales y objetos que están destinado a entrar en contacto con alimentos deben cumplir el Reglamento (CE) Nº 1935/2004 (UE, 2004), cuya finalidad es garantizar un elevado nivel de protección de la salud y de los intereses de los consumidores, así como garantizar el funcionamiento del mercado de estos productos. En el artículo 3 de dicho reglamento se detallan los requisitos generales que deben cumplir los materiales activos:

"Deberán estar fabricados de conformidad con las buenas prácticas de fabricación para que, en las condiciones normales o previsibles de empleo, no transfieran sus componentes a los alimentos en cantidades que puedan:

- representar un peligro para la salud humana

-provocar una modificación inaceptable de la composición de los alimentos

-provocar una alteración de las características organolépticas de los alimentos.

El etiquetado, la publicidad y la presentación de los materiales no deberá inducir a error a los consumidores."

En el Artículo 4 del mismo Reglamento, aparecen unos requisitos especiales para los materiales y objetos activos:

“Podrán ocasionar modificaciones de la composición o de las características organolépticas de los alimentos a condición de que dichas modificaciones cumplan las disposiciones comunitarias o nacionales aplicables a los alimentos (aditivos, aromas, enzimas)

Las sustancias liberadas en los alimentos o en su entorno deberán autorizarse y utilizarse conforme a las disposiciones comunitarias correspondientes, actualmente el el Reglamento 450/2009 el que marca el proceso de autorización.

Los materiales y objetos activos no ocasionarán modificaciones de la composición ni de las características organolépticas de los alimentos, por ejemplo enmascarando su deterioro, que puedan inducir a error a los consumidores.

Deberán llevar el etiquetado adecuado que permita al consumidor identificar las partes no comestibles.”

A partir de la publicación del Reglamento (CE) Nº 450/2009 existen una serie de requisitos específicos relativos a la composición, etiquetado y declaración de este tipo de envases:

“Únicamente las sustancias incluidas en la lista comunitaria de sustancias autorizadas podrán utilizarse en componentes de los materiales activos a excepción de:

- Las sustancias activas liberadas y las sustancias añadidas o incorporadas por métodos de implantación o inmovilización, siempre que cumplan con las disposiciones aplicables a los alimentos y con el Reglamento 1935/2004.

- Las sustancias que no estén en contacto directo con el alimento y separadas de él por una barrera funcional, siempre que la migración no exceda de 0.01mg/kg y no sean sustancias mutagénicas, carcinógenas, toxicas o con tamaño de partícula que presente propiedades diferentes a las de tamaño mayor” (AESAN 2010).

Además de este reglamento marco, existe normativa diversa (reglamentos, directivas o resoluciones) que regulan de manera más concreta determinados grupos de materiales; como el Reglamento (UE) Nº 10/2011 sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos o las directivas dedicadas a cerámica o nitrosaminas.

6. CONCLUSIONES

Los resultados de los estudios recogidos en esta revisión son muy alentadores en relación al uso de aceites esenciales en la fabricación de envases activos. Los investigadores demuestran, que las capacidades antioxidantes y antibacterianas de estos aceites son transferidas de manera efectiva a los polímeros de envasado, y que éstos mantienen dichas propiedades a lo largo del tiempo, mejorando los perfiles de perdurabilidad de los alimentos perecederos sin necesidad de adicionar directamente sobre los mismos las sustancias activas. Con esto se responde tanto a las demandas de los consumidores, preocupados por el uso de conservantes sintéticos, como a las de la industria, con necesidad de transportar alimentos a través de largas distancias sin que se vean comprometidas sus características nutricionales y organolépticas.

La publicación de más estudios que corroboren los resultados ya obtenidos harán avanzar la legislación, y con ella la incorporación al mercado europeo del envasado activo con aceites esenciales naturales.

BIBLIOGRAFÍA

Avilés-Sosa R, Palou E, Jiménez Munguía MT, Nevárez-Moorillón GV, Navarro Cruz AR, López-Malo A. Antifungical activity by vapor contact of esencial oils added to amaranth, chitosan, or starch edible films. Int J Food Microbiol 2012; 153, 66-72.

Ayala-Zavala JF, Silva-Espinoza BA, Cruz-Valenzuela MR, Leyva JM, Ortega-Ramírez LA, Carrazco-Lugo DK, Pérez-Carlón JJ, Melgarejo-Flores BG, González-Aguilar GA, Miranda MRA. Pectin-cinnamon leaf oil coating add antioxidant and antibacterial properties to fresh-cut peach. Flavour Flag J 2012; 28, 39-45.

Bahram S, Rezaei M, Soltani M, Kamali A, Ojagh SM, Adollahi M. Whey protein concentrate edible film activated with cinnamon essential oil. J Food Process Pres (Artículo en prensa).

Bajpai VK, Baek KH, Kang SC. Control of *Salmonella* in food by using essential oils: A review. Food Res Int 2012; 45, 722-734.

Becerril R, Gómez-lus R, Goñi P, López P. Combination of anlytical and microbiolical techniques to study the antimicrobial activity of a new active food packaging containing cinnamon oregano against *E. coli* and *S. aureus*. Anal Bioanal Chem 2007; 388, 1003-1011.

Bolumar T, Andersen ML, Orlien V. Antioxidant active packaging for chicken meat processed by high pressure treatment. Food Chem 2011; 129, 1406-1412.

Burst S. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol 2004; 94, 223-253.

Chaves Sánchez JA. Envases actives e inteligentes. En: Cameán Fernandez AM, García Parrilla MC (Eds) Temas de interés en Seguridad Alimentaria, Padilla Libros Editores y Libreros, Sevilla. pp. 55-88.

Coma V. Bioactive packaging technologies for extended shelf life of meat-based products. Meat Sci 2008; 78, 90-103.

Comité Científico de la AESAN. Informe científico del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) en relación a los envases activos e inteligentes. 2010;13(11):89-105.

Danielli D, Gontard N, Spyropoulos D, Zondervan-van den Beuken E, Tobback P. Trends Food Sci Tech 2008; 19, s103-s112.

Decisión de la Comisión de 23 de enero de 2002 por la que se modifica la Decisión 1999/217/CE por lo que respecta al repertorio de sustancias aromatizantes utilizadas en o sobre los productos alimenticios.

Del Novile MA, Conte A, Buonocore GG, Incoronato AL, Massaro A, Panza O. Active packaging by extrusion processing of reciclable and biodegradable polymers. J Food Eng 2009; 93, 1-6.

Directiva 2000/13/CE del Parlamento europeo y del Consejo, de 20 de marzo de 2000, relativa a la aproximación de las legislaciones de los estados miembros en materia de etiquetado, presentación y publicidad de los productos alimentarios.

Djenane D, Aïder M, Yangüela J, Idir L, Gómez D, Roncalés P. Antioxidant and antibacterial effects of *Lavandula* and *Mentha* essential oils in minced beef inoculated with *E. coli* 0157:H7 and *S. aureus* during storage at abuse refrigeration temperature. Meat Sci 2012; 92, 667-674.

Dusan F, Marián S, Katarina D, Dobroslava B. Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. Toxicol in Vitro 2006; 20, 1435-1445.

Hyldgaard M, Mygind T, Meyer RL. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front microbial 2012; 3, 12.

Jouki M, Mortazavi SA, Yazdi FT, Koocheki A. Characterization of antioxidant-antibacterial quince seed mucilage films containing thyme essential oil. Carbohyd polim 2014; 99, 537-546.

Kerry JP, O´Grady MN, Hogan SA. Past, currrent and potencial utilisation of active and intelligent packaging systems for meat and muscle-based product: A review. Meat Sci 2006; 74, 113-130.

Lahlou M. Methods to study the phytochemistry and bioactivity of essential oils. Phytother Res 2004; 18, 435-448.

Lee DS, Hwang YI, Cho SH. Developing antimicrobial packaging film for curled lettuce and soybean. Food Sci Biotechnol 1998; 7, 117-121.

Licciardello F, Muratone G, Suma P, Russo A, Nerín C. Effectiveness of a novel insect-repellent food packaging incorporating essential oils against the red flour beetle (*Tribolium castaneum*). Innov Food Sci Emerg 2013; 19, 173-180.

López P, Sánchez C, Batlle R, Nerín C. Development of flexible antimicrobial films using essential oils as active agents. J Agric Food Chem 2007; 55, 8814-8824.

López-de-Dicastillo C, Gómez-Estaca J, Catalá R, Gavara R, Hernández Muñoz P. Active antioxidant packaging films: Development and effect on lipid stability of bridned sardines. Food Chem 2012; 131, 1376-1384.

Manso S, Cacho-Nerín F, Becerril R, Nerín C. Combined analytical and antimicrobiological tools to study the effect on *Aspergillus flavus* of cinnamon essential oil contained in food packaging. Food Control 2013; 13, 370-378.

Montero-Prado P, Rodriguez-Lafuente A, Nerín C. Active label-based packaging to extend shelf-life of "Calanda" peach fruit: Changes in fruit quality and enzymatic activity. Postharvest Biol Tec 2011; 60, 211-219.

Muriel-Galet V, Cerisuelo JP, López-Carballo G, Lara M. Developments of antimicrobial films for microbiological control of packaged salad. Int J Food Microbiol 2012; 157, 195-201.

Nerín C, Tovar L, Djenane D, Camo J, Salafranca J, Beltrán JA, Roncalés P. Stabilization of beef meat by a new active packaging containing natural antioxidants. J Agric Food Chem 2006; 54, 7840-7846.

Nerín C, Tovar L, Salafranca J. Behaviour of a new antioxidant active film versus oxidizable model compounds. J Food Eng 2008; 84, 313-320.

Olmedo RH, Nepote V, Grosso NR. Preservation of sensory and chemical properties in flavoured cheese prepared with cream cheese base using oregano and rosmary essential oils. Food Sci Technol 2013; 56, 409-417.

Perdones A, Vargas M, Atarés L, Chiralt A. Physical, antioxidant and antimicrobial properties of chitosan-cinnamon leaf oil films as affected by oleic acid. Food Hydrocolloid 2014; 36, 256-264.

Pezo D, Salafranca J, Nerín C. Determination of the antioxidant capacity of active food packagings by in situ gas-phase hydroxyl radical generation and high-performance liquid chromatography-fluorescence detection. J Chromatogr A 2008; 1178, 126-133.

Ramos M, Jiménez A, Peltzer M, Garrigós MC. Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for packaging. J Food Nutr 2012; 109, 513-519.

Rasal RM, Janorkar AV, Hirt DE. Poly(lactic acid) modifications. Prog Polym Sci 2010; 35, 338-356.

Reglamento (CE) nº 1935/2004 del Parlamento europeo y del Consejo, de 27 de octubre de 2004, sobre los materiales y objetos destinados a entrar en contacto con los alimentos.

Reglamento (CE) nº 2073/2005 de la Comisión, de 15 de noviembre de 2005, relativo a los criterios microbiológicos aplicables a los productos alimentarios.

Reglamento (CE) nº 450/2009 de la Comisión, de 29 de mayo de 2009, sobre materiales y objetos actives e inteligentes destinados a entrar en contacto con alimentos.

Reglamento (CE) nº 10/2011 de la Comisión, de 14 de enero de 2011, sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos.

Sadaka F, Nguimjeu C, Brachais C-H, Vroman I, Tighzert L, Couvercelle J-P. Review on antimicrobial packaging containing essential oils and their active biomolecules. Innov Food Sci Emerg 2013 (Artículo en prensa) doi: 10.1016/j.ifset.

Salarbashi D, Tajik S, Ghasemlou M, Shojaee-Aliabadi S, Noghabi MS, Khaksar R. Characterization of soluble soybean polysaccharide film incorporated essential oil intended for food packaging. Carbohyd polym 2013; 98, 1127-1136.

Salgado PR, López-Caballero E, Gómez-Guillén MC, Mauri AN, Montero MP. Sunflower protein films incorporated with clove essential oil have potential application for the preservation of fish patties. Food Hydrocolloid 2013; 33, 74-84.

Sánchez-González L, Vargas M, González-Martínez C, Chiralt A, Cháfer M. Use of essential oils in bioactive edible coating: a review. Food Eng Rev 2011; 3, 1-16.

Souza AC, Goto GEO, Mainardi JA, Coelho ACV, Tadini CC. Cassava starch composite films incorporated with cinnamon essential oil: Antimicrobial activity, microstructure, mechanical and barrier properties. LWT-Food Sci Technol 2013; 54, 346-352.

Stammati A, Bonsi P, Zucco F, Moezelaar R, Alakomi HL, von Wright A. Toxicity of selected plant volatiles in microbial and mammalian shot-term. Food Chem Toxicol 1999; 37, 813-823.

Takala PN, Dang Vu K, Salmieri S, Khan RA, Lacroix M. Antibacterial effects of biodegradable active packaging on the growth of *Escherichia coli*, *Salmonella tphimurium* and *Listeria monocytogenes* in fresh broccoli stored at 4ºC. LWT-Food Sci Technol 2013; 53, 499-506.

Tammineni N, Ünlü G, Min SC. Development of antimicrobial potato peel wast-based edible films with oregano essential oil to inhibit *Listeria monocytogenes* on cold-smoked salmon. Food Sci Technol 2013; 48, 211-214.

Teixera B, Marques A, Ramos C, Batista I, Serrano C, Matos O, R.Neng N, Nogueira J MF, Saraiva JA, Nunes ML. European pennyroyal (*Mentha pulegium*) from Portugal: Chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil. Ind Crop Prod 2012; 36, 81-87.

Teruel Muñoz V. Jornada sobre materiales en contacto con alimentos. Nota de prensa de la AESAN 2011.

Tharanathan RN. Biodegradable films and composite coatings: past, present and future. Trends Food Sci Tech 2003;14, 71-78.

Valero-Valdivieso MF, Ortegón Y, Uscategui Y. Biopolymers: Progress and prospects. DYNA 2013; 181, 171-180.

Vermeiren L, Devlieghere F, van Beest M, de Kruijf N, Debevere J. Developments in the active packaging of food. Trends Food Sci Tech 1999; 10, 77-86.

Zhou F, Baoping JI, Zhang H, Jiang H, Yang Z, Li J, Li J, Yan W. The antibacterial effect of cinnamaldehide, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella typhimurium*. J Food Safety 2007; 27, 124-133.

VIII. REFERENCIAS / REFERENCES

Aeschbach, R., Löliger, J., Scott, B.C., Murcia, A., Butler, J., Halliwell, B., & Aruoma, O.I. (1994). Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food and Chemical Toxicology* 32, 31–36.

Aherne, S.A., Kerry, J.P., & O'Brien, N.M. (2007). Effects of plant extracts on antioxidant status and oxidant-induced stress in Caco-2 cells. *British Journal of Nutrition* 97, 321–328.

Akalin, G., & Incesu, Z. (2011). The effects of carvacrol on apoptosis of H-ras and N-ras transformed cell lines. *Turkish Journal of Pharmaceutical Sciences,* 8 (2), 105–116.

Aristatile, B., Al-Numair, K.S., Al-Assaf, A.H., Veeramani, C., & Pugalendi, K.V. (2010). Protective effect of carvacrol on oxidative stress and cellular DNA damage induced by UVB irradiation in human peripheral lymphocytes. *Journal of Biochemical and Molecular Toxicology* 29, 497-508.

Arunasree, K.M. (2010). Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB231. *Phytomedicine* 17, 581–588.

Avila-Sosa, R., Palou, E., Jiménez-Munguía, M.T., Nevarez-Moorillon, G.V., Navarro-Cruz, A.R., & Lopez-Malo, A. (2012). Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *International Journal of Food Microbiology* 153, 66-72.

Aydin, S., Basaran, A.A., & Basaran, N. (2005). Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. *Journal of Agriculture and Food Chemistry* 53, 1299–1305.

Aydin, E., Türkez, H., & Keles, M.S. (2014). The effect of carvacrol on healthy neurons and N2a cancer cells: some biochemical, anticancerogenicity and genotoxicity studies. *Cytotechnology* 66, 149–157.

Azeredo, H.M.C. (2009). Nanocomposites for food packaging applications. *Food Research International* 42, 1240-1253.

Azirak, S., & Rencuzogullari, E. (2008). The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells. *Environmental Toxicology* 23, 728-735.

Azizan, A., & Blevins, R.D. (1995). Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay. *Archives of Environmental Contamination and Toxicology* 28, 248–258.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N., & Omar, A.K.M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering* 117, 426-436.

Aznar, M., Rodríguez-Lafuente, A., Alfaro, P., & Nerín C. (2012). UPLC-Q-TOF-MS analysis of non-volatile migrants from new active packaging materials. *Analytical and Bioanalytical Chemistry* 404, 1945-1957.

Azqueta, A., Shaposhnikov, S., & Collins, A.R. (2009). DNA oxidation: investigating its key role in environmental mutagenesis with comet assay. *Mutation Research - Genetic Toxicology and Environmental,* 674, 101-108.

Bajpai, V.K., Baek, K.H., & Kang, S.C. (2012). Control of *Salmonella* in food by using essential oils: A review. *Food Research International* 45, 722-734.

Bakkali, F.,Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils – a review. *Food and Chemical Toxicology* 46, 446–475.

Baltrop, J.A., Owen, T.C., Cory, A.H., & Cory, J.G. (1991). 5-((3-Carboxyphenyl)-3-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)) tetrazolium, inner salt (MTS) and related analogs of 2-(4,5- dimethylthiazolyl)-2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell-viability indicators. *Bioorganic & Medicinal Chemistry Letters,* 1, 611.

Barlow, S.M. (2009). Risk assessment of food-contact materials: past experience and future challenges. *Food Additives and Contaminants* 26, 1526-1533.

Battini, D., Calzavara, M., Persona, A., & Sgarbossa, F. (2016). Sustainable packaging development for fresh food supply chains. *Packaging Technology and Science* 29, 25-43.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *LWT-Food Science and Technology* 37(2), 263–268.

Benkeblia, N., & Lanzotti, V. (2007). *Allium* thiosulphinates: chemistry, biological properties and their potential utilization in food preservation. *Food* 1, 193-201.

Binderup, M.L., Pedersen, G.A., Vinggaard, A.M., Rasmussen, E.S., Rosenquist, H., & Cederberg, T. (2002). Toxicity testing and chemical analyses of recycled fibre-based paper for food contact. *Food Additives and Contaminants* 19, 13-28.

Borenfreund, E., & Puerner, J.A. (1984). A simple quantitative procedure using monolayer culture for cytotoxicity assays. *Journal of Tissue Culture Methods,* 9, 7–9.

Bowen, D.E., Whitwell, J.H., Lillford, L., Henderson, D., Kidd, D., McGarry, S., Pearce, G., Beevers, C., & Kirkland, D.J. (2011). Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 722, 7-19.

Burt, S. (2004). Essential oils: their antibacterial properties and applications in food-a review. *International Journal of Food Microbiology* 94, 223-253.

Byun, Y., Kim, Y.T., & Whiteside, S. (2010). Characterization of an antioxidant polylactic acid (PLA) film prepared with α-tocopherol, BHT and polyethylene glycol using film cast extruder. *Journal of Food Engineering* 100, 239-244.

Carocho, M., Barreiro, M.F., Morales, P., & Ferreira, I.C.F.R. (2014). Adding molecules to food, pros and cons: a review of synthetic and natural food additives. *Comprehensive Reviews in Food Science and Food Safety* 13 377-399.

Chieng, B.W., Ibrahim, N.A., Then, Y.Y., & Loo, Y.Y. (2014). Epoxidized vegetable oils plasticized poly(lactic acid) biocomposites: mechanical, thermal and morphology properties. *Molecules* 19, 16024-16038.

Coma, V. (2008). Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Science* 78, 90-103.

Committee of Experts on Flavouring Substances Council of Europe. (1981). Flavouring substances and natural sources of flavourings. 3ª Ed.

Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S., van Benthem, J., & Vanparys, P. (2008). ECVAM retrospective validation of the *in vitro* micronucleus test (MNT). *Mutagenesis* 271, 271-283.

Collins, A.R., Duthie, S.J., & Dobson, V.L. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14, 1733-1735.

Collins, A.R., Azqueta, A., Brunborg, G., Gaivao, I., Giovanelli, L., Kruszewski, M., Smith, C.C., & Stetina, R. (2008). The comet assay: topical issues. *Mutagenesis* 23, 143-151.

Corzo-Martínez, M., Corzo, N., & Villamiel, M. (2007). Biological properties of onions and garlic. *Trends in Food Science & Technology* 18, 609-625.

Dainelli, D., Gontard, N., Spyropoulos, D., Zondervan-van den Beuken, E., & Tobback, P. (2008). Active and intelligent food packaging: legal aspects and safety concerns. *Trends Food Science and Technology* 19, s103-s112.

De Vincenzi, M., Stammati, A., De Vincenzi, A., & Silano, M. (2004). Constituents of aromatic plants: carvacrol. *Fitoterapia* 75, 801–804.

Deb, D.D., Parimala, G., Devi, S.S., & Chakraborty, T., (2011). Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. *Chemico-Biological Interactions* 193, 97–106.

De Martino, A., Filomeni, G., Aquilano, K., Ciriolo, M. R., & Rotilio, G. (2006). Effects of water garlic extracts on cell cycle and viability of HepG2 hepatoma cells. *Journal of Nutritional Biochemistry* 17, 742–749.

Deines, P. (1980). The isotopic composition of reduced organic carbon. In: P. Fritz, J. Fontes (Eds.), Handbook of Environmental Isotope Geochemistry, Vol. 1, Elsevier, New York, pp. 329–406.

Dobkowski, Z. (2006). Thermal analysis techniques for characterization of polymer materials. *Polymer Degradation and Stability* 91, 488-493.

Drumright, R.E., Gruber, P.R., & Henton, D.E. (2000). Polylactic acid technology. *Advanced Materials* 12(23)-1841-1846.

Dundar, E., Gurlek-Olgun, E., Isuksoy, S., Kurkçuoglu, M., Baser, K.H.C., & Bal, C. (2008). The effects of intra-rectal and intra-peritoneal application of *Origanum onites* L. essential oil on 2,4,6-trinitrobenzenesulfonic acid-induced colitis in the rat. *Experimental and Toxicologic Pathology* 59, 399–408.

Dusan, F., Marián, S., Katarína, D., & Dobroslava, B. (2006). Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. *Toxicology in Vitro* 20, 1435–1445.

EFSA, European Food Safety Authority. (2009). Guidelines on submission of a dossier for safety evaluation by the EFSA of active or intelligent substances present in active and intelligent materials and articles intended to come into contact with food. *EFSA Journal* 1208.

EFSA, European Food Safety Authority. (2009b). Guidance on safety assessment of botanicals and botanical preparations intended to be use as ingredients in food supplements. *EFSA Journal* 1249.

EFSA, European Food Safety Authority. (2010). Scientific opinion on the use of oregano and lemon balm extracts as a food additive. *EFSA Journal* 1514.

EFSA, European Food Safety Authority. (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA Journal* 2379.

EFSA, European Food Safety Authority. (2016). Recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials. *EFSA Journal* 4357.

Escobar, F.M., Cariddi, L.N., Sabini, M.C., Reinoso, E., Sutil, S.B., Torres, C.V., Zanon, S.M., & Sabini, L.I. (2012). Lack of cytotoxic and genotoxic effects of *Minthostachys verticillata* essential oil: Studies *in vitro* and *in vivo*. *Food and Chemical Toxicology* 50, 3062–3067.

Escobar, F.M., Sabini, M.C., Cariddi, L.N., Sabini, L.I., Manas, F., Cristofolini, A., Bagnis, G., Gallucci, M.N., & Cavaglieri, L.R. (2015). Safety assessment of essential oil from *Minthostachys verticillata* (Griseb.) Epling (peperina): 90-days oral subchronic toxicity study in rats. *Regulatory Toxicology and Pharmacology* 71, 1-7.

European Commission (EC) (2001). Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorization.

Ferguson, L.R. (2001). Role of plant polyphenols in genomic stability. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 475, 89–111.

Fukuda, K (2015). Food safety in a globalized world. *Bull World Health Organ* 93, 212.

García, D.A., Bujons, J., Vale, C., & Suñol, C. (2006). Allosteric positive interaction of thymol with the GABA receptor in primary cultures of mouse cortical neurons. *Neuropharmacology* 50, 25–35.

García, A., Morales, P., Arranz, N., Delgado, M.E., Rafter, J., & Haza, A.I. (2009). Antiapoptotic effects of dietary antioxidants towards N-nitrosopiperidine and N-nitrosodibutylamine- induced apoptosis in HL-60 and HepG2 cells. *Journal of Applied Toxicology* 29, 403–413.

Gómez-Estaca, J., López de Lacey, A., López-Caballero, M.E., Gómez-Guillén, M.C., & Montero, P. (2010). Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. *Food Microbiology* 27, 889-896.

González-Pérez, J.A., González-Vila, F.J., Almendros, G., Knicker, H., de La Rosa, J,M., & Hernández, Z. (2013). Revisiting structural insights provided by analytical pyrolysis about humic substances and related bio-and geopolymers. In: J. Xu, et al. (Eds.), Functions of Natural Organic Matter in Changing Environment, Zhejiang University Press and Springer Science, pp. 1–5.

Goñi, P., López, P., Sánchez, C., Gómez-Luz, R., Becerril, R., & Nerín, C. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chemistry* 116(4), 982-989.

Gosslau, A. (2016). Assessment of food toxicology. *Food Science and Human Wellness* 5, 103-115.

Grunert, K.G. (2005). Food quality and safety: consumer perception and demand. *European Review of Agricultural Economics* 32, 369-391.

Guyonnet, D., Siess, M. H., Le Bon, A. M., & Suschetet, M. (1999). Modulation of phase II enzymes by organosulfur compounds from *Allium* vegetables in rat tissues. *Toxicology and Applied Pharmacology* 154, 50–58.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M. H., & Le Bon, A. M. (2000). Liver subcellular fractions from rats treated by organosulfur compounds from Allium modulate mutagen activation. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 466(1), 17-26.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M. H., & Le Bon, A. M. (2001). Antimutagenic activity of organosulfur compounds from *Allium* is associated with phase II enzyme induction. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 495, 135–145.

He, L., Mo, H., Hadisusilu, S., Quresni, A.A., & Elson, C.E. (1997). Isoprenoids suppress the growth of murine B16 melanomas *in vitro* and *in vivo*. *The Journal of Nutritional Biochemistry* 127, 668–674.

Horváthová, E., Šramková, M., Lábaj, J., & Slamenˇová, D. (2006). Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured *in vitro*. *Neuroendocrinology Letters* 27, 44–47.

Hosseini, M.H., Razavi, S.H., & Mousavi, M.A. (2009). Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. *Journal of Food Processing and Preservation* 33, 727-743.

Hosseini, S. F., Rezaei, M., Zandi, M., & Farahmandghavi, F. (2015). Bio-based compositeedible films containing *Origanum vulgare* L. essential oil. *Industrial Crops and Products* 67, 403-413.

Hsu, S.S., Lin, K.L., Chou, C.T., Chiang, A.J., Liang, W.Z., Chang, H.T., Tsai, J.Y., Liao, W.C., Huang, F.D., Huang, J.K., Chen, I.S., Liu, S.I., Kuo, C.C., & Jan, C.R. (2011). Effect of thymol on Ca^{2+} homeostasis and viability in human glioblastoma cells. European Journal of Pharmacology 670, 85–91.

Ipek, E., Tüylü, B.A., & Zeytinoglu, H. (2004). Effects of carvacrol on sister chromatid exchanges in human lymphocyte culture. *Cytotechnology* 43, 145–148.

Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B.A., Kurkcuoglu, M., Can Baser, K.H. (2005). Genototoxicity and antigenotoxicity of *Origanum* oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. *Food Chemistry* 93, 551–556.

Jamshidiam, M., Tehrany, E.A., Imran, M., Jacquot, M., & Desobry, S. (2010). Poly-lactic acid: production, applications, nanocomposites, and release studies. *Comprehensive Reviews in Food Science and Food Safety* 9, 552-571.

Jamshidiam, M., Tehrany, E.A., Imran, M., Akhtar, M.J., Cleymand, .F, & Desobry, S. (2012). Structural, mechanical and barrier properties of active PLA-antioxidant films. *Journal of Food Engineering* 110, 380-389.

Jeena, K., Liju, V.B., Kuttan, R. (2014). A preliminary 13-week oral toxicity study of ginger oil in male and female wistar rats. *International Journal of Toxicology* 30, 662-670.

Jouki, M., Mortazavi, S.A., Yazdi, F.T., & Koocheki, A. (2014). Characterization of antioxidant-antimicrobial quince seed mucilage films containing thyme essential oil. *Carbohydrate Polymers* 99, 537-546.

Khanjari, A., Karabagias, I.K., & Kontominas, M.G. (2013). Combined effect of N,O-carboximethyl chitosan and oregano essential oil to extend shelf life and control *Listeria monocytogenes* in raw chicken meat fillets. *Food Science and Technology* 53, 94-99.

Khazaiei, S., Esa, N.M., Ramachandran, V., Hamid, R., Pandurangan, A.K., Etemad, A., & Ismail, P. (2017). *In vitro* antiproliferative and apoptosis inducing effect of *Allium atraviolaceum* bulb extract on breast, cervical, and liver cancer cells. *Frontiers in Pharmacology* 8, article 5.

Kirkland, D., Pfuhler, S., Tweats, D., Aardema, M., Crovi, R., Darroudi, F., Elhajouji, A., et al. (2007). How to reduce false positive results when *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal test: report of an ECVAM Workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 628, 31-55.

Kirkland, D., Zeiger, E., Madia, F., & Corvi, R. (2014). Can *in vitro* mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or *in vivo* genotoxic activity? II. Construction and analysis of a consolidated database. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 775-776, 69-80.

Kirsch-Volders, M., Vanhauwaert, A., Eichenlaub-Ritter, U., & Decordier, I. (2003). Indirect mechanism of genotoxicity. *Toxicology Letters* 140-141, 63-74.

Knights, J. (2010).Flavour legislation. In: A.J. Taylor and R.S.T.Linforth, Food Flavour Technology. Wiley-Blackwell, United Kingdom, pp. 24-48.

Kopinke, F.D., & Mackezie, K. (1997). Mechanical aspects of the thermal degradation of poli(lactic acid) and poly(β- hydroxybutyric acid). *Journal of Analytical and Applied Pyrolysis* 40-41, 43-53.

Koster, S., Rennen, M., Leeman, W., Houben, G., Muilwijk, B., Acker, F., & Krul, L. (2014). A novel safety assessment strategy fon non-intentionally added substances (NIAS) in carton food contact materials. *Food Additives & Contaminants: Part A* 31, 422-443.

Kotler, P., & Keller, K. (2006). Marketing management (12th ed.). Upper Saddle River, NJ: Pearson.

Krochta, J.M., & Johnston, C.D.M. (1997). Edible and biodegradable films: challenges and opportunities. *Food Technology* 51(2), 61-74.

Krochta, J.M. (2002). Proteins as raw materials for films and coatings: Definitions,current status, and opportunities. In GENNADIOS, A. Protein-based films and coatings, Boca Raton, pp. 21-61.

Kulisic, T., Radonic, A., Katalinic, V., & Milos, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry* 85, 633–640

Lanzotti, V. (2006). The analysis of onion and garlic. *Journal of Chromatography A* 1112, 3–22.

Libro Blanco de Seguridad Alimentaria. (2000). Comisión Europea.

Liju, V. B., Jeena, K., & Kuttan, R. (2013). Acute and subchronic toxicity as well as mutagenic evaluation of essential oil from turmeric (*Curcuma longa* L). *Food and Chemical Toxicology* 53, 52-61.

Liolios, C.C., Gortzi, O., Lalas, S., Tsaknis, J., & Chinou, I. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and *in vitro* antimicrobial activity. *Food Chemistry* 112, 77–83.

Lorenzo, J.M., Battle, R., & Gómez, M. (2014). Extension of the shelf-life of foal meat with two antioxidant active packaging systems. *Food Science and Technology* 59, 181-188.

Lorge, E., Thybaud, V., Aardema, M.J., Oliver, J., Wakata, A., Lorenzon, G., & Marzin, D. (2006). SFTG international collaborative study on *in vitro* micronucleus test I. General conditions and overall conclusions of the study. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 607, 13-36.

Mahadevan, B., Snyder, R.D., Waters, M.D., Benz, R.D., Kemper, R.A., Tice, R.R., & Richard, A.M. (2011). Genetic toxicology in the 21th century: reflections and future directions. *Environmetal and Molecular Mutagenesis* 52, 339-354.

Maisanaba, S., Prieto, A.I., Puerto, M., Gutierrez-Praena, D., Demir, E., Marcos, R., & Cameán, A.M. (2015). *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 784-785, 37-44.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, M., Prieto, A.I., Jos, A., & Cameán, A.M. (2017). New advances in active packaging incorporated with essential oils or their main components for food preservation. *Food Reviews International* 33, 447-515.

Marcos B, Sárraga C, Castellari M, Kappen F, Schennink G, Arnau J. (2014). Development of biodegradable films with antioxidant properties based on polyesters containing α-tocopherol and olive leaf extract for food packaging applications. Food Packaging and Shelf Life 1, 140-150.

Mehdi, S.J., Ahmad, A., Irshad, M., Manzoor, N., & Rizvi, M.M.A. (2011). Cytotoxic effect of Carvacrol on human cervical cancer cells. *Biology and Medicine* 3 (2), 307–312.

Merhi, F., Auger, J., Rendu, F., & Bauvois, B. (2008). *Allium* compounds, dipropyl and dimethyl thiosulfinates as antiproliferative and differentiating agents of human acute myeloid leukemia cell lines. *Biologics* 2(4), 885–895.

Mellado-Garcia, P., Puerto, M., Pichardo, S., Llana-Ruiz-Cabello, M., Moyano, R., Blanco, A., Jos, A., & Cameán, A.M. (2016). Toxicological evaluation of an *Allium*-based comercial product in a 90-day feeding study in Sragues-Dawley rats. *Food and Chemical Toxicology* 90, 18-29.

Melusová, M., Jantová, S., & Horváthová, E. (2014). Carvacrol and Rosemary oil at higher concentrations induce apoptosis in human hepatoma HepG2 cells. *Interdisciplinary Toxicology* 7, 189-194.

Moldoveanu, S.C. (1998). Analytical pyrolysis of natural organic polymers, techniques and instrumentation. In: S.C. Moldoveanu (Ed.), Analytical Chemistry, Vol. 20, Elsevier, Amsterdam.

Muriel-Galet, V., Cerisuelo, J.P., López-Carballo, G., Lara, M., Gavara, R., & Hernández-Muñoz, P. (2012). Development of antimicrobial films for microbial control of packaged salad. *International Journal of Food Microbiology* 157, 195-201.

Muriel-Galet, V., Cerisuelo, J.P., López-Carballo, G., Aucejo, S., Gavara, R., & Hernández-Muñoz, P. (2013). Evaluation of EVOH-coated PP films with oregano essential oil and citral to improve the shelf-life of packaged salad. *Food Control* 30, 137-143.

Negi, P.S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Microbiology* 156(1), 7-17.

Németh-Zámboriné, E. (2016). Natural variability of essential oil components. In: K.H Can Baser, G. Buchbauer (Eds.), Essential oils: Science, technology, and applications. CRC Press, Boca Raton, pp.87-126.

Nerín, C., Tovar, L., Djenani, D., Camo, J., Salafranca, J., Beltran, L.S., & Roncalés, P. (2006). Stabilization of beef meat by a new active packaging containing natural antioxidants. *Journal of Agricultural and Food Chemical* 54, 7840-7846.

Nerin, C., Tovar, L., & Salafranca, J. (2008). Behaviour of a new antioxidant active packaging versus several oxidizable model compounds. *Journal of Food Engineering* 84, 313-320.

Nodorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., & Pulkrabek, J. (2009). Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. *Food Control* 20, 157-160.

Norajit, K., Kim, K. M., & Ryu, G. H. (2010). Comparative studies on the characterization and antioxidant properties of biodegradable alginate films containing ginseng extract. *Journal of Food Engineering* 98(3), 377-384.

OECD, 1997. Guideline for the Testing of Chemicals 471: Bacterial Reverse Mutation Test, pp. 1-11.

OECD 1998. Guideline for the Testing of Chemicals 408: Repeated Dose 90-day Oral Toxicity Study in Rodents, pp 1-10.

OECD 2014. Guideline for the Testing of Chemicals 487: *In Vitro* Mammalian Cell Micronucleus Test, pp 1-23.

OECD, 2014. Guideline for the Testing of Chemical 489: *In Vivo* Mammalian Alkaline Comet Assay, pp. 1-25.

OCDE, 2014b. Guideline for the Testing of Chemical 474: Mammalian erythrocyte micronucleus test, pp 1-21.

Ozkan, A.,& Erdogan, A. (2011). A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. *Turkish Journal of Biology* 35, 735–742

Patrignani, F., Siroli, L., Serrazanetti, D.I., Gardini, F., & Lanciotti, R. (2015). Innovative strategies based on the use of essential oils and their components to improve safety, shelf-life and quality of minimally processed fruits and vegetables. *Trends in Food Science and Technology* 46, 311-319.

Peinado, M.J., Ruiz, R., Echávarri, A., & Rubio, L.A. (2012). Garlic derivative PTS-O is effective against broiler pathogens *in vivo*. *Poultry Science* 91, 2148–2157.

Peinado, M.J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L.A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology* 181, 87–92.

Pires, C., Ramos, C., Teixeira, G., Batista, I., Mendes, R., Nunes, L., et al. (2011). Characterization of biodegradable films prepared with hake proteins and thyme oil. *Journal of Food Engineering* 105, 422-428.

Pires, C., Ramos, C., Teixeira, B., Batista, I., Nunes, M.L., & Marques, A. (2013). Hake proteins edible films incorporated with essential oils: physical, mechanical,antioxidant and antibacterial properties. *Food Hydrocolloids* 30, 224-231.

Ponce, A.G., Roura, S.I., del Valle, C.E., & Moreira, M.R. (2008). Antimicrobial andantioxidant activities of edible coatings enriched with natural plant extracts:*in vitro* and *in vivo* studies. *Postharvest Biology and Technology* 49, 294-300.

Ramos, M., Jiménez, A., Peltzer, M., Garrogós, M.C. (2012). Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *Journal of Food Engineering* 109, 513-519.

Ramos, O.L., Reinas, I., Silva, S.I., Fernandes, J.C., Cerqueira, M.A., Pereira, R.N., Vicente, A.A., Poças, M.F., Pintado, M.E., & Malcata, F.X. (2013). Effect of whey protein purity and glicerol content upon physical properties of edible films manufactures therefrom. *Food Hydrocolloids* 30, 110-122.

Reglamento (CE) (2002). Nº 178/2002 del Parlamento Europeo y del Consejo de 28 de enero de 2002 por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y sefijan procedimientos relativos a la seguridad alimentaria. Diario Oficial de la Unión Europea: DO L 31/1 de 01.02.2002.

Reglamento (CE) (2004). Nº 1935/2004 del Parlamento Europeo y del Consejo, de 27 de octubre de 2004, sobre los materiales y objetos destinados a entrar en contacto con alimentos y por el que se derogan las Directivas 80/590/CEE y 89/109/CEE.

Reglamento (CE) (2005). Nº 2073/2005 DE LA COMISIÓN de 15 de noviembre de 2005 relativo a los criterios microbiológicos aplicables a los productos alimenticios

Reglamento (CE) (2008) Nº 1331/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, por el que se establece un procedimiento de autorización común para los aditivos, las enzimas y los aromas alimentarios

Reglamento (CE) (2008b). Nº 1334/2008 del Parlamento Europeo y del Consejode 16 de diciembre de 2008sobre los aromas y determinados ingredientes alimentarios con propiedades aromatizantes utilizados en los alimentos y por el que se modifican el Reglamento (CEE) n o 1601/91 del Consejo, los Reglamentos (CE) n o 2232/96 y (CE) n o 110/2008 y la Directiva 2000/13/CE

Reglamento (CE) (2009). Nº 450/2009 de la Comisión de 29 de mayo de 2009 sobre materiales y objetos activos en inteligentes destinados a entrar en contacto con alimentos.

Reglamento (UE) (2011). Nº 10/2011 de la Comisión de 14 de enero de 2011 sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos.

Reglamento (UE) (2011b). No 234/2011 de la Comisión de 10 de marzo de 2011 de ejecución del Reglamento (CE) no 1331/2008 del Parlamento Europeo y del Consejo, por el quese establece un procedimiento de autorización común para los aditivos, las enzimas y los aromasalimentarios

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M.F., & Zagorec, M. (2015). Bacterial spoilers of food: Behaviour, fitness and functional properties. *Food Microbiology* 45, 45-53.

Restuccia, D., Spizzirri, U.G., Parisi, O.I., Cirillo, G., Curcio, M., Lemma, F., Puoci, F., Vinci, G., & Picci, N. (2010). New EU regulations aspects and global market of active and intelligent packaging for food industry applications. *Food Control* 21 1425-1435.

Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical and Biological Medicine* 49, 1603–1616.

Robertson, G.L. (2016). Structure and related properties of plastic polymers. In Taylor and Francis (Eds.), Food packaging: Principles and Practice, Third Edition. CRC, Boca Ratón, FL.

Romeilah, R.M., Fayed, S.A., & Mahmoud, G.I. (2010). Chemical compositions, antiviral and antioxidant activities of seven essential oils. *Journal of Applied Sciences Research* 6(1), 50–62.

Rossmann, A. (2001). Determination of stable isotope ratios in food analysis. *Food Reviews International* 17, 347–381.

Ruiz-Navajas, Y., Viuda-Martos, M., Sendra, E., Perez-Alvarez, J. A., & Fernández-López, J. (2013). *In vitro* antibacterial and antioxidant properties of chitosan edible films incorporated with *Thymus moroderi* or *Thymus piperella* essential oils. *Food Control*, 30, 386-392.

Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., et al. (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chemistry* 91, 621–632.

Salgado, P.R., López-Caballero, M.E., Gómez-Guillén, M.C., Mauri, A.N., & Montero, M.P. (2013). Sunflower protein films incorporates with clove essential oil have potential application for the preservation of fish patties. *Food Hydrocolloids* 33, 74-84.

Samec, D., Durgo, K., Grúz, J., Kremer, D., Kosalec, I., Piljac-Zegarac, J., & SalopekSondi, B. (2015). Genetic and phytochemical variability of six *Teucrium arduini* L. populations and their antioxidant/prooxidant behavior examined by biochemical, macromoleculate- and cell-based approaches. *Food Chemistry* 186, 298-305.

Sarikurkcu, C., Zengin, G., Oskay, M., Uysal, S., Ceylan, R., & Aktumsek, A. (2015). Composition, antioxidant, antimicrobial and enzyme inhibition activities of two *Origanum vulgare* subspecies (subsp. *Vulgar* and subsp. *Hirtum*) essential oils. *Industrial Crops and Products* 70, 178-184

Savini, I., Arnone, R., Catani, M.V., & Avigliano, L. (2009). *Origanum vulgare* induces apoptosis in human colon cancer Caco2 cells. *Nutrition and Cancer* 61 (3), 381–389.

Sehrawet, M., & Kundu, S.C. (2007). Buying behavior of rural and urban consumers in India: the impact of packaging. *International Journal of Consumer Studies* 31, 630-638.

Shaath, N.A., & Flores, F.B. (1998) Egyptian onion oil. *Developments in Food Science* 40: 443–453.

Shin, H.A., Cha, Y.Y., Park, M.S., Kim, J.M., & Lim, Y.C. (2010). Diallyl sulfide induces growth inhibition and apoptosis of anaplastic thyroid cancer cells by mitochondrial signaling pathway. *Oral Oncology* 46, e15–e18.

Shon, M.Y., Choi, S.D., Kahng, G.G., Nam, S.H., & Sung, N.K. (2004). Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food and Chemical Toxicology* 42, 659–666.

Shukla, Y., & Kalra, N. (2007). Cancer chemoprevention with garlic and its constituents. *Cancer Letters* 247, 167–181.

Siracusa, V., Rocculi, P., Romani, S., & Dalla Rosa, M. (2008). Biodegradable polymers for food packaging: a review. *Trends in Food Science & Technology* 19, 634-643.

Siripatrawan, U., & Harte, B. R. (2010). Physical properties and antioxidant activity ofan active film from chitosan incorporated with green tea extract. *Food Hydrocolloids* 24, 770-775.

Slamenová, D., Horváthová, E., Sramková, M., & Marsálková, L. (2007). DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured in vitro. *Neoplasma* 54, 108–112.

Souza, A.C., Goto, G.E.O., Mainardi, J.A., Coelho, A.C.V., & Tadini, C.C. (2013). Cassava starch composite films incorporated with cinnamon essential oil: Antimicrobial activity, microstructure, mechanical and barrier properties. *LWT - Food Science and Technology* 54(2), 346-352.

Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., & von Wright, A. (1999). Toxicity of selected plant volatiles in microbial and mammalian short-term assays. *Food and Chemical Toxicology* 37, 813–823.

Stamenic, M., Vulic, J., Djilas, S., Misic, D., Tadic, V., Petrovic, S., & Zizovic, I. (2014). Free-radical scavenging activity and antibacterial impact of Greek oregano isolates obtained by SFE. *Food Chemistry* 165, 307-315.

Sundaram, S.G., & Milner, J.A. (1996). Diallyl disulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice. *The Journal of Nutrition* 126, 1355–1361.

Sung, S.Y., Sin, L.T., Tee, T.T., Bee, S.T., Rahmat, A.R., & Rahmat, W.A.W.A. (2014). Control of bacterial growth on ready-to-eat beef loaves by antimicrobial plastic packaging incorporated with garlic oil. *Food Control* 39, 214-221.

Takahashi, M., & Shibamoto, T. (2008). Chemical compositions and antioxidant/antiinflammatory activities of steam distillate from freeze-dried onion (Allium cepa L.) sprout. *Journal of Agricultural and Food Chemistry* 56, 10462–10467.

Teixeira, B., Marques, A., Pires, C., Ramos, C., Batista, I., Saraiva, J.A. et al. (2014). Characterization of fish protein films incorporated wit essential oils of clove, garlic and origanum: physical, antioxidant and antibacterial properties. *Food Science and Technology* 59, 533-539.

Tsai, C.Y., Wang, C.C., Lai, T.Y., Tsu, T.Y., Wang, C.H., Liang, H.Y., et al. (2013). Antioxidant effects of diallyl trisulfide on high glucose-induced apoptosis are mediated by the PI3K/Akt-dependent activation of Nrf2 in cardiomyocytes. *International Journal of Cardiology* 168, 1286–1297.

Türkez, H., & Aydin, E. (2013). Investigation of cytotoxic, genotoxic and oxidative properties of carvacrol in human blood cells. *Toxicology and Industrial Health* 1-9.

Ündeger, U., Basaran, A., Degen, G.H., & Basaran, N. (2009). Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. *Food and Chemical Toxicology* 47, 2037–2043.

Wambura, P., Yang, W., & Mwakatage, N. (2011). Effects of sonication and edible coating containing rosemary and tea extracts on reduction of peanut lipid oxidative rancidity. *Food and Bioprocess Technology* 4, 107–115.

Xiao, D., Choi, S., Johnson, D.E., Vogel, V.G., Johnson, C.S., Trump, D.L., et al. (2004). Diallyl trisulfide induced apoptosis in human prostate cancer cells involves cJun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. *Oncogene* 23, 5594–5606.

Yahyaoui, M., Gordobil, O., Herrera-Díaz, R., Abderrabba, M., & Labidi, J. (2016). Developments of novel antimicrobial films based on poly(lactic acid) and essential oils. *Reactive and Functional Polymers* 109, 1-8.

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., & Raneva, V.G. (1999). Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. *Food Chemistry* 64, 59–66.

Ye, C.H., Dai, D.H., & Hu, W.L. (2013). Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.). *Food Control* 30, 48–53.

Yin, Q., Yan, F., Zu, X.Y., Wu, Y., Wu, X., Liao, M., Deng, S., Yin, L., & Zhuang, Y. (2012). Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. *Cytotechnology* 64 (1), 43–51.

Zani, F., Masimo, G., Benvenuti, S., Biachi, A., Albasini, A., Melegari, M., Vampa, G., Belloti, A., & Mazza, P. (1991). Studies on the genotoxic properties of essential oils with *Bacillus subtilis* rec-assay and *Salmonella*/microsome reversion assay. Planta Medica 57, 237–241.

Zeytinoglu, H., Incesu, Z., & Baser, K.H.C. (2003). Inhibition of DNA synthesis by carvacrol in mouse myoblast cells bearing a human N-ras oncogene. *Phytomedicine* 10, 292–299.