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Sara Ubeda Jasanada

# Estudio de contaminantes emergentes en materiales destinados al envase alimentario

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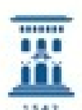

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Tesis Doctoral

# ESTUDIO DE CONTAMINANTES EMERGENTES EN MATERIALES DESTINADOS AL ENVASE ALIMENTARIO


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**UNIVERSIDAD DE ZARAGOZA**
**Escuela de Doctorado**


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# Tesis Doctoral

## Estudio de contaminantes emergentes en materiales destinados al envase alimentario

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Facultad de Ciencias
2020

Escuela de Ingeniería y Arquitectura

Departamento de Química Analítica

# *Estudio de contaminantes emergentes en materiales destinados al envase alimentario*

Memoria presentada por

SARA ÚBEDA JASANADA

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# *Estudio de contaminantes emergentes en materiales destinados al envase alimentario*

Memoria presentada por

SARA ÚBEDA JASANADA

Para optar al título de Doctor en Ciencia Analítica en Química

Dirigida por

Dra. Dª. CRISTINA NERÍN DE LA PUERTA

Dra. Dª. MARGARITA AZNAR RAMOS

La **Dra Cristina Nerín de la Puerta**, Catedrática del Departamento de Química Analítica de la Universidad de Zaragoza y la **Dra. Margarita Aznar Ramos**,

CERTIFICAN:

Que la presente memoria, titulada ***"Estudio de contaminantes emergentes en materiales destinados al envase"*** presentada por **Doña Sara Úbeda Jasanada** para optar al grado de Doctor en Ciencia Analítica en Química, ha sido realizada bajo nuestra codirección en el Instituto de Investigación en Ingeniería de Aragón (I3A) y la Escuela de Ingeniería y Arquitectura (EINA) de la Universidad de Zaragoza, de acuerdo a los objetivos presentados en el Proyecto de Tesis aprobado por el Departamento de Química Analítica. Por tanto, autorizamos su presentación para proseguir con los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

En Zaragoza, a 13 de Enero del 2020

Dra. Cristina Nerín de la Puerta | Dra. Margarita Aznar Ramos

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*A mi madre Carmen*

*A Juanan*

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# Glosario de términos

# Glosario de términos

**AA:** Adipic Acid

**AhR:** Aryl hydrocarbon Receptor

**Al o Foil:** Aluminium foil

**ANSA:** 8-Anilino-1-naphthalene sulfonic acid ammonium salt

**APCI:** Atmospheric Pressure Chemical Ionization

**APGC:** Atmospheric Pressure Gas Chromatography

**API:** Atmospheric Pressure Ionization

**AR:** Androgen Receptor

**ASAP:** Atmospheric Solids Analysis Probe

**BD:** 1,4-butanediol

**CCS:** Collision Cross-Section

**CE:** Collision Energies

**CPP:** Cast Polypropylene

**DART:** Direct Analysis in Real Time

**DCC:** Dextran-coated charcoal

**DCM:** Dichloromethane

**DEG:** Diethylene Glycol

**DESI:** Desorption Electrospray Ionization

**dHAE:** Dihydroxyalkyl Ethers

**DMSO:** Dimethyl Sulfoxide

**DMT:** Dimethyl Terephthalate

**DPG:** Dipropylene Glycol

**DVB/CAR/PDMS:** Divinylbenzene / Carboxen / Polydimethylsiloxane

**EDC:** Endocrine Disrupting Chemical

**EDI:** Estimated Daily Intake

**EFSA:** European Food Safety Authority

**EG:** Ethylene glycol

**EI:** Electron Impact

**EPS:** Expanded Polystyrene

**ESI:** Electrospray Ionization

**EtOH:** Ethanol

**FBS:** Fetal Bovine Serum

**FCM:** Food Contact Material

**FCS:** Food Contact Side

**FDA:** Food and Drug Administration

**FPSE:** Fabric Phase Sorptive Extraction

**GC:** Gas Chromatography

**GC-O:** GC-Olfactometry

**HAc o AcH:** Acetic Acid

**HD:** 1,6-Hexanediol

**HDPE:** High Density Polyethylene

**HFIP:** 1,1,1,3,3,3-Hexafluoro-2-propanol

**HFLME:** Hollow Fiber Liquid Phase Microextraction

**HPLC:** High-Performance Liquid Chromatography

**HS:** Head Space

**IAS:** Intentionally Added Substances

**IM o IMS:** Ion Mobility Spectrometry

**IPA:** Isophthalic Acid

**KI:** Kovats Index

**LA:** Lactic Acid

**LC:** Liquid Chromatography

**LDPE:** Low Density Polyethylene

**LOD:** Limit of Detection

**LOQ:** Limit of Quantification

**MALDI:** Matrix-Assisted Laser Desorption/Ionization

**MeOH:** Methanol

**MF:** Modified Frequency

**MF:** Molecular Formula

**MRM:** Multiple Reaction Monitoring

**MS:** Mass Spectrometry

**$MS^E$:** High and low energy adquisition mode in Mass Spectrometry

**NIAS:** Non-Intentionally Added Substances

**NIST:** National Institute of Standards and Technology

**NMR:** Nuclear Magnetic Resonance

**NPG:** Neopentyl Glycol

**OFAS:** Office of Food Additive Safety

**OHF:** Hydroxyflutamide

**OML:** Overall Migration Limit

**OPA:** Oriented Polyamide

**PA:** Phthalic Acid o Polyamide

**PBAT:** Poly(butylene adipate-co-terephthalate)

**PCA:** Principal Component Analysis

**PE:** Polyethylene

**PET:** Polyethylene Terephthalate

**PETr:** Polyethylene Terephthalate Recycled

**PG:** Propylene Glycol

**PHA:** Poly Hydroxyl Alkanoate

**PHB:** Poly Hydroxyl Butyrate

**PLA:** Polylactic Acid

**PP:** Polypropylene

**PS:** Polystyrene

**PU:** Polyurethane

**QqQ o TQ:** Triple Quadrupole

**QToF:** Quadrupole Time of Flight

**R1881:** Methyltrienolone

**$R^2$:** Determination Coefficient

**RSD:** Relative Standard Deviation

**SEM:** Scanning Electron Microscopy

**SIR:** Single Ion Recording

**SML:** Specific Migration Limit

**SPE:** Solid Phase Extraction

**SPME:** Solid Phase Micro Extraction

**$T_4$:** Thyroxine

**TCA:** Trichloroacetic Acid

**TCDD:** 2,3,7,8- Tetrachlorodibenzo-p-dioxin

**TPA:** Terephthalic Acid

**TTC:** Threshold of Toxicological Concern

**TTR:** Transthyretin

**UPLC:** Ultra-Performance Liquid Chromatography

**UV:** Ultraviolet

# PRESENTACIÓN

# PRESENTACIÓN

La presente tesis se titula ***"Estudio de contaminantes emergentes en materiales destinados al envase alimentario",*** y se enmarca en el campo de investigación de la seguridad del envase alimentario que está llevando a cabo el Grupo Universitario de Investigación Analítica (GUIA) del Departamento de Química Analítica de la Universidad de Zaragoza. El grupo GUIA está liderado por la Dra. Cristina Nerín de la Puerta, y está integrado en el Instituto de Investigación en Ingeniera de Aragón (I3A).

Esta tesis fue llevada a cabo gracias a la ayuda para contratos predoctorales para la formación de doctores 2016 (FPI), otorgada por el Ministerio de Economía y Competitividad a Sara Úbeda. Esta tesis está enmarcada dentro del proyecto SENEMs (AGL2015-67362-P) del Programa Estatal de Fomento de la Investigación Científica y Técnica de Excelencia. También destacar que parte de la tesis fue realizada en el Grupo de Investigación de Toxicidad Molecular y Reproductiva de la Universidad Técnica de Copenhague, durante una estancia de 3 meses.

Esta tesis estudia los compuestos de migración desde diferentes tipos de envase alimentario a diferentes simulantes alimentarios. Concretamente se centra en los compuestos conocidos como "oligómeros", potenciales migrantes de envases poliméricos. Para ello, se han evaluado diferentes tipos de envases, tanto polímeros convencionales de fuentes fósiles como biopolímeros.

Se ha realizado un estudio exhaustivo de la composición de estos materiales utilizando y optimizando tanto diversos tratamientos de muestras (disolución total y extracción) como diversas técnicas analíticas que han servido como potentes herramientas para la detección y cuantificación de los compuestos.

La memoria se ha estructurado en cinco secciones:

La sección I ofrece una introducción general del marco en el que se ha desarrollado la tesis doctoral.

La sección II expone los objetivos que se pretenden conseguir durante el desarrollo de este trabajo.

La sección III muestra el desarrollo experimental realizado, dividido en seis capítulos. Todos los capítulos son autocontenidos, es decir, incorporan inicialmente un resumen en español y en inglés del capítulo, sus objetivos detallados, una pequeña introducción, los métodos y materiales utilizados, así como resultados y conclusiones.

- ***Capítulo 1:*** Se estudia la migración global y específica de los compuestos no-volátiles procedentes de dos materiales multicapa. También se realizan estudios cinéticos y de hidrólisis de dos oligómeros de poliuretano.
- ***Capítulo 2:*** Se estudia tanto la composición como la migración de oligómeros de diferentes muestras de PET. Además, se estudia la eficacia de dos tratamientos de muestra, extracción y disolución total, para la identificación del mayor número de oligómeros.
- ***Capítulo 3:*** Se estudia la composición no volátil de muestras de PLA optimizando un tratamiento de muestra basado en la disolución total, al igual que la migración específica de dicho material.
- ***Capítulo 4:*** Se estudia el comportamiento de los oligómeros presentes en muestras de PLA tanto en la disolución como la migración.
- ***Capítulo 5:*** Se estudia tanto la composición como la migración específica de compuestos volátiles y semi-volátiles de muestras de PLA.
- ***Capítulo 6:*** Se estudia la bioaccesibilidad y la toxicidad de dos oligómeros del adhesivo de poliuretano utilizado en envases multicapa.

La memoria se cierra con las secciones IV y V, en las cuales se recoge, respectivamente, un resumen de las conclusiones más relevantes extraídas del trabajo y las publicaciones que derivan del mismo.

A continuación se muestra un esquema con la estructura de esta Tesis.

**Sección I: Introducción General**

**Sección II: Objetivos**

**Sección III: Desarrollo Experimental**

**Capítulo 1:** Overall and specific migration from multilayer high barrier food contact materials – Kinetic study

**Capítulo 2:** Determination of oligomers in virgin and recycled polyethylene terephthalate (PET) samples by UPLC-QTOF

**Capítulo 3:** Determination of non-volatile components of a biodegradable food packaging material based on polyester and polylactic acid (PLA) andits migration to food simulants

**Capítulo 4:** Migration of oligomers from a food contact biopolymer based on polylactic acid (PLA) and polyester

**Capítulo 5:** Determination of volatile compounds and their sensory impact in a biopolymer based on polylactic acid (PLA) and polyester

**Capítulo 6:** Migration studies and toxicity evaluation of cyclic polyesters oligomers from food packaging adhesives

**Sección IV: Conclusiones generales**

**Sección V: Publicaciones**

# **SECCIÓN I:** Introducción General

# INTRODUCCIÓN

## 1. Envase alimentario

Los alimentos están expuestos a la acción de factores externos, químicos, físicos y microbiológicos durante su almacenamiento y comercialización, que pueden provocar que el alimento sufra una serie de reacciones que alteren sus características nutritivas, sanitarias y/o sensoriales, y que lleven con el tiempo a la alteración y pérdida del producto. El envase alimentario tiene como misión fundamental reducir al mínimo la incidencia de los factores externos y proteger, por tanto, la integridad del producto, conservando su calidad y características nutritivas, sensoriales y sanitarias (Catalá, 2002).

En la actualidad, se dispone de un gran número de envases de muy diversos materiales y características adecuadas para cubrir la demanda que plantea el envasado de alimentos. Los materiales más usados para contacto con alimentos son el plástico, el papel y el cartón, el vidrio y el metal. En la figura 1 se muestra el porcentaje de uso de los materiales de envase de alimentos (Muncke, 2016).

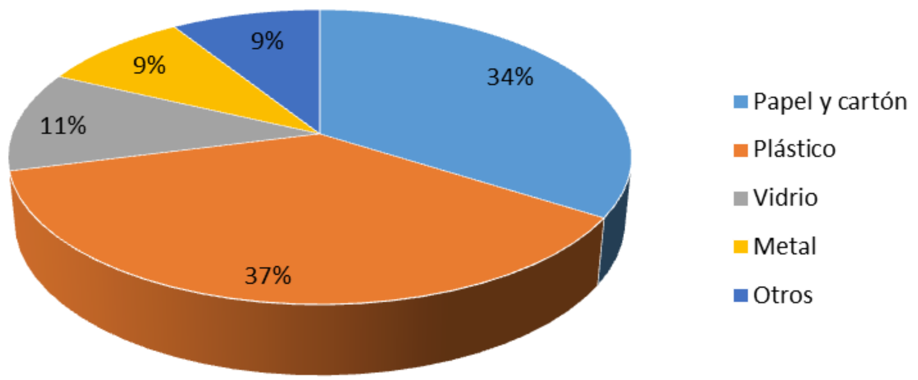



**Figura 1.** Porcentaje de uso de los materiales de envasado de alimentos-gráfico modificado (Muncke, 2016).

## 1.1 Plásticos

Entre los materiales de envase alimentario, los plásticos son los más utilizados. Los más comunes son el polietileno de baja densidad (LDPE), polipropileno (PP), tereftalato de polietileno (PET), polietileno de alta densidad (HDPE), poliestireno (PS), y poliestireno expandido (EPS) (Muncke, 2016).

El plástico es un material relativamente nuevo, pero que se ha convertido en indispensable tanto para la vida diaria como para todo tipo de industrias. Es un material barato, fácil de trabajar y moldear, y un buen aislante de electricidad y sonido, y es por ello que ofrece numerosas aplicaciones muy útiles para el ser humano. Sin embargo, también tiene desventajas como su elevado tiempo de degradación, que dará lugar a un incremento en la contaminación del medio ambiente. Además, el plástico proviene del petróleo, y para su fabricación son necesarios procesos químicos que contaminan la atmósfera y contribuyen al efecto invernadero (Muller, González-Martínez, & Chiralt, 2017).

Como resultado de todos estos inconvenientes, ha habido un gran incremento en la investigación de los procesos de reciclaje. Los plásticos reciclables son aquellos que pueden ser reutilizados de nuevo tras su uso principal, gracias a un tratamiento de reciclaje. Este proceso está limitado por la dificultad técnica del proceso o por su alto coste. Aunque hay muchos tipos de polímeros plásticos, no todos ellos pueden ser destinados al reciclaje. Uno de los polímeros más reciclados es el PET, debido a su bajo contenido de aditivos en el material virgen, además de sus características de baja difusividad y baja absorción de compuestos (Félix, Alfaro, & Nerín, 2011).

Por otro lado, como alternativa a los plásticos de origen fósil no degradables, ha aumentado el interés por los biopolímeros. Por el momento, los biopolímeros, también denominados bioplásticos, suponen un bajo porcentaje de la cantidad total de polímeros presentes en el mercado, por debajo de un 1%, respecto a los polímeros convencionales (Ashter, 2016), aunque su uso está en aumento.

## 1.2 Bioplásticos

Los biopolímeros engloban todos aquellos polímeros que son biodegradables y/o compostables, y también aquellos que provienen de fuentes renovables (biobase) (Jabeen, Majid, & Nayik, 2015). La diferencia entre un polímero biodegradable y un polímero compostable es que el primero se descompone por la acción de microorganismos en su ambiente natural, y el segundo necesita unas condiciones específicas de compostaje para que se lleve a cabo el proceso de degradación. Se considera que un polímero es compostable si al menos el 90% del polímero se degrada en un tiempo de 6 meses.

Es por ello que los bioplásticos pueden dividirse en tres categorías principales en función de su origen y de su degradabilidad (Geueke, 2014; Muller, González-Martínez, & Chiralt, 2017):

- Origen biológico y no biodegradable/compostable
- Origen biológico y biodegradable/compostable
- Origen fósil y biodegradable/compostable

Los dos biopolímeros más utilizados en el mercado son el ácido poliláctico (PLA) y el almidón, representando el 47 y 41 % respectivamente del consumo global de biopolímeros (Muller, González-Martínez, & Chiralt, 2017). Tanto el PLA como el almidón son de origen natural, y a su vez son biodegradables/compostables. El PLA es un biopolímero compostable, ya que sólo es degradable en condiciones industriales bajo control, pero no lo es en el medio ambiente natural.

## 2. Tipos de interacciones

En todos los materiales de envase hay siempre una interacción alimento/envase/entorno, que condiciona la conservación del producto envasado. Estas interacciones (figura 2) son consecuencia de mecanismos de transferencia que se manifiestan en dicho sistema, tales como (Catalá, 2002):

- Permeabilidad o paso de gases, vapores y radiaciones a través del material polimérico.
- Adsorción/Absorción por el polímero de componentes del alimento o del entorno
- Migración o transferencia de componentes y residuos de los materiales del envase a los alimentos envasados.

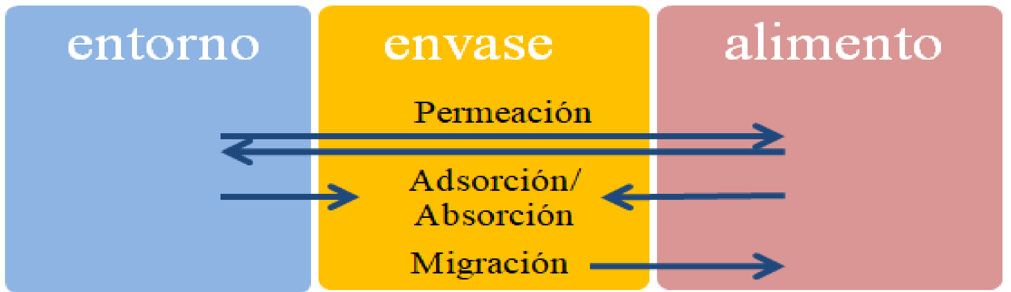



**Figura 2**. Interacciones del sistema alimento/envase/entorno.

Estas interacciones pueden ser desfavorables para la calidad y salubridad del producto, ya que pueden producir cambios en la calidad sensorial del producto como la pérdida de sabor, olores desagradables o cambios de textura, y también la contaminación del mismo.

## 3. Migración y tipos de migrantes

De las diferentes interacciones que tienen lugar, la migración es un fenómeno de gran importancia, ya que da lugar a la incorporación de sustancias no deseadas al alimento. Estas sustancias pueden alterar las características sensoriales y nutritivas de los alimentos, o bien ser tóxicas y hacer que los alimentos sean rechazables para el consumo (Muncke, 2016).

En la práctica se distinguen dos tipos de migración: global y específica. La migración global se refiere a la cantidad total de los componentes del material de envase que son transferidos al alimento, sean conocidos o no, en las condiciones de preparación y almacenamiento menos favorables. Por ello, mide la inercia química del material pero no identifica ninguna sustancia ni está relacionada con la posible toxicidad o seguridad del material. Por otro lado, la migración específica se refiere a la cantidad de cada sustancia concreta e identificable que es transferida al alimento (Muncke, 2016) y tiene claras implicaciones de toxicidad y seguridad alimentaria.

Las sustancias del material de envase que pueden migrar son los compuestos de bajo peso molecular que se encuentran en el polímero, tales como monómeros residuales, oligómeros, disolventes…; coadyuvantes de la polimerización (catalizadores, aceleradores, inhibidores…), aditivos para facilitar el procesamiento y/o modificar las características del material final; compuestos procedentes de la descomposición, degradación o bien reacciones colaterales del polímero y/o aditivos; y sustancias adyacentes al material (adhesivos, barnices y tintas de impresión). Se ha demostrado que todos los compuestos de tamaño inferior a 1000 Da tienen mayor probabilidad de migrar (Catalá, 2002).

Además, hay que tener en cuenta que la migración no se produce por igual en todos los envases en contacto con alimentos. Así, en los materiales metálicos la interacción se produce básicamente por fenómenos de corrosión e incorporación de iones metálicos. En cambio, en vidrio y materiales cerámicos la interacción es más bien un proceso de disolución química o lixiviación. Por otro lado, en los materiales poliméricos se produce la interacción mediante mecanismos de transferencia de masa a través de la matriz polimérica (Catalá, 2002).

Se han estudiado ampliamente los mecanismos básicos de la migración en los materiales poliméricos. El equilibrio y la cinética están presentes en todos estos procesos de transporte de masa, y están controlados por la difusión de los migrantes a través del envase y la partición o reparto de los migrantes entre el envase y el alimento. Los dos coeficientes que rigen el proceso de migración son (Aznar, Domeño, Nerín, & Bosetti, 2015; Vera, Aznar, Mercea, & Nerín, 2011):

- Coeficiente de partición: expresa la relación entre las concentraciones en el equilibrio de los migrantes en el alimento y en el envase.
- Coeficiente de difusión: determina la transferencia de masa debida a movimientos aleatorios de las moléculas desde las regiones con alta concentración hacia las regiones con baja concentración hasta la obtención de un equilibrio.

La migración es un proceso que depende de diferentes factores, tales como el tiempo y la temperatura de contacto, el espesor del material, la naturaleza del material, la concentración de migrante en el material, o la polaridad del migrante. Es necesario, por tanto, conocer en profundidad las condiciones de migración que pueden darse en los distintos materiales de envase, para reducir al mínimo su incidencia, seleccionando el material que permita mantener la calidad y seguridad del producto envasado durante el periodo de comercialización previsto (Catalá, 2002).

El análisis de la migración, tanto global como específica, directamente en el alimento es una tarea muy compleja. Por ello se recurre a los llamados simulantes alimentarios, es decir, sustancias que se caracterizan por poseer un poder extractivo similar a los alimentos a los que está destinado el envase. Las condiciones de temperatura y tiempo de estos ensayos están establecidos en el reglamento EU/10/2011 (EC, 2011), y dependerán de las condiciones de almacenamiento del producto envasado (Nerin, Alfaro, Aznar, & Domeño, 2013).

El envase alimentario puede contener diferentes tipos de sustancias: añadidas intencionadamente (IAS) y no añadidas intencionadamente (NIAS) que pueden migrar en ambos casos y contaminar los alimentos. Entre los IAS destacan los antioxidantes, lubricantes, retardantes de llama, absorberdores UV, catalizadores, plastificantes…

Por otro lado, los NIAS comprenden todas las sustancias que no han sido añadidas de forma intencionada durante la fabricación de los materiales en contacto con alimentos (FCM). Pueden agruparse, dependiendo de sus diferentes orígenes (figura 3a), como productos de reacción colaterales, productos de degradación de los polímeros o aditivos e impurezas presentes en las materias primas.

La mayoría de los NIAS y compuestos desconocidos deben considerarse un riesgo para la salud, porque no hay información toxicológica, y por lo tanto no

se conocen los daños adversos que pueden producir en el ser humano. Es cierto que cada vez se están identificando más NIAS, aunque también lo es que no todas esas sustancias conocidas han sido evaluadas hasta ahora (figura 3b). Muchos NIAS pueden haber sido detectados, pero sus estructuras permanecen desconocidas; por lo tanto, no se puede llegar a una conclusión sobre la seguridad. El último grupo de esta clasificación de NIAS son aquellas sustancias que permanecen completamente desconocidas, ya que no son detectados por ninguno de los métodos analíticos.

El mayor problema para identificar los NIAS es que no existen estándares comerciales con los que confirmarlos y, debido a las bajas concentraciones a las que se encuentran, se necesitan técnicas avanzadas de análisis con una alta sensibilidad. Por esta razón, podemos encontrarnos con NIAS que pueden predecirse en función del conocimiento de los procesos químicos, la experiencia del fabricante y las condiciones de uso. Estas sustancias pueden identificarse más fácilmente mediante análisis químicos específicos (targeted analysis). Sin embargo, la presencia de muchos otros NIAS no puede predecirse, y es necesario un análisis no específico (non-targeted analysis) (Figura 3c) (Geueke, 2018).

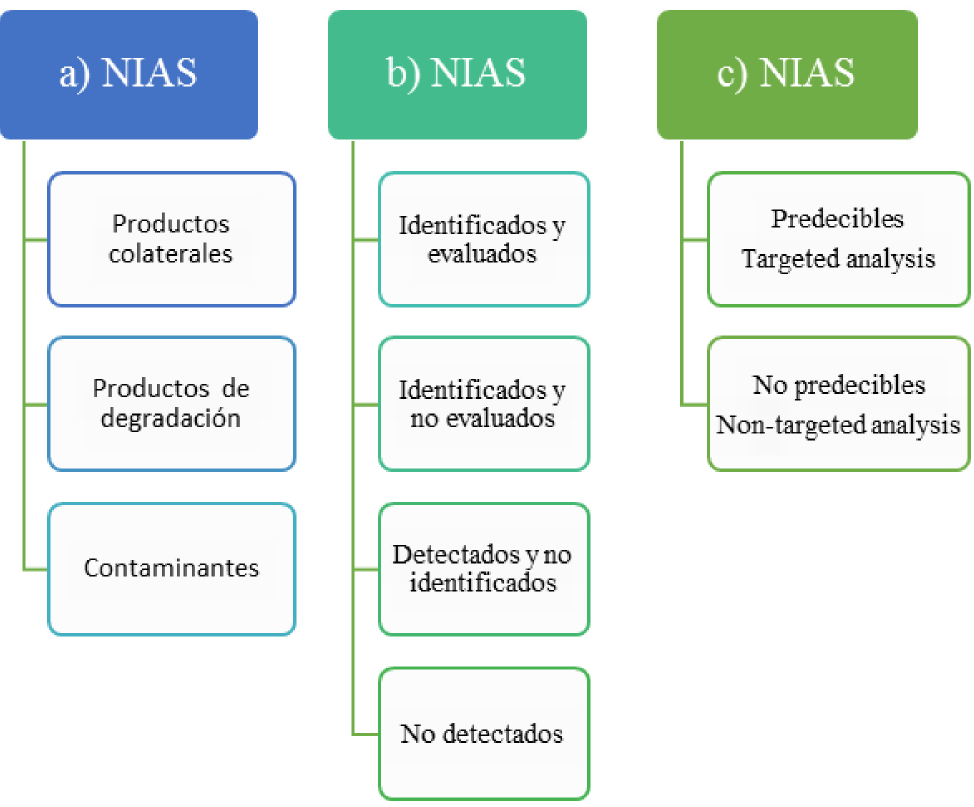



**Figura 3.** Orígenes (a) y categorías (b y c) de las sustancias añadidas no intencionadamente (NIAS)-figura modificada (Geueke, 2018).

# 4. Metodología analítica para la identificación de IAS y NIAS

Una de las tareas más difíciles cuando se quieren identificar los migrantes procedentes de una muestra desconocida, es determinar cuál es el procedimiento más apropiado. No existe un protocolo estándar, pero con frecuencia, en primer lugar, se analiza directamente el envase, ya que la concentración de los componentes desconocidos es mayor, y posteriormente se analizan las muestras de migración. De esta manera se pueden identificar los migrantes potenciales, y así facilitar el proceso de identificación en las migraciones. No obstante, este procedimiento exige un esfuerzo analítico enorme para identificar todos los migrantes potenciales. Las concentraciones de los compuestos en migración son muy bajas, y la sensibilidad de los equipos puede optimizarse mejor cuando se

busca un compuesto determinado previamente identificado (*targeted analysis*) que cuando se lleva a cabo la determinación de todos los compuestos presentes en la muestra (*non-targeted analysis*) (Nerin, Alfaro, Aznar, & Domeño, 2013). La figura 4 muestra un esquema de los diferentes procedimientos de análisis que se pueden seguir.

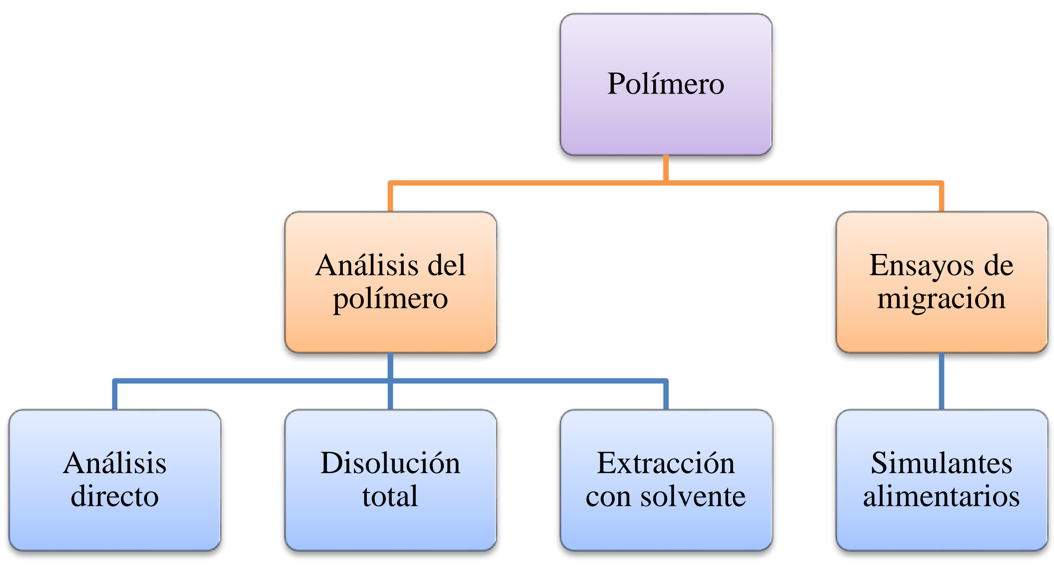



**Figura 4.** Esquema general de los procedimientos de análisis - figura modificada (Nerín, 2016).

Para el análisis de los polímeros, se pueden usar diferentes procedimientos de análisis:

a) Análisis directo. Se pueden utilizar técnicas como la desorción térmica de los polímeros con un posterior análisis por GC-MS, o técnicas de análisis directo por MS: ASAP (Atmospheric Solids Analysis Probe), DART (direct analysis in real time o DESI (Desorption electrospray ionization).

c) Disolución total del polímero y análisis por GC-MS o LC-MS.

d) Extracción con disolvente y análisis por GC-MS o LC-MS.

El análisis directo tiene la ventaja de que no necesita tratamiento de muestra previo, y sólo se suele utilizar principalmente para la confirmación de un compuesto, ya que no hay etapas previas de separación, lo que complica la interpretación de los espectros de masas. Sin embargo, las dos últimas técnicas sí que necesitan una etapa previa de disolución o extracción, pero son idóneas para la identificación de compuestos.

Tras seleccionar el procedimiento de análisis, tanto en el caso de la disolución total y extracción como en especial en los ensayos de migración, es necesario preconcentrar compuestos de interés para el análisis. Para ello, se llevan a cabo diferentes técnicas de preconcentración, tales como la concentración con corriente de nitrógeno, SPME (Solid Phase Microextraction), compuestos SPE (solid phase extraction), FPSE (fabric phase sorptive extraction) o HFLPME (hollow fiber liquid phase microextraction).

Por último, la última etapa en la determinación de compuestos es el análisis de las muestras mediante el uso de diferentes técnicas analíticas.

# 5. Técnicas de análisis

Para llevar a cabo la determinación de los analitos presentes, tanto en los extractos del polímero como en las muestras de migración, se requiere el uso combinado de técnicas instrumentales.

Por un lado, es necesaria una separación previa de los compuestos presentes en la muestra. La técnica instrumental más importante para la separación de sustancias es la cromatografía. Se trata de una técnica basada en la distribución de los componentes entre dos fases inmiscibles, una estacionaria que se mantiene fija, y otra móvil que se desplaza por la estacionaria. Según sea su constante de distribución o reparto, los compuestos tendrán tendencia a estar en una fase u otra. La cromatografía de líquidos se utiliza para el análisis de

sustancias orgánicas con pesos moleculares altos, inestabilidad térmica o baja volatilidad; y la cromatografía de gases para la separación de sustancias orgánicas volátiles o semi-volátiles.

Por otro lado, la espectrometría de masas, debido a su elevada sensibilidad y selectividad, es el detector más utilizado para la identificación de las sustancias migrantes. Es un instrumento que ioniza especies químicas y las separa basándose en su relación m/z (masa/carga). Esta técnica proporciona tanto información de la masa molecular de un compuesto como información de su estructura (Wrona & Nerín, 2019).

Dado que las disoluciones de las migraciones contienen más de un compuesto, la combinación de ambas técnicas es indispensable para la identificación y confirmación de IAS y NIAS. Estas herramientas permiten la caracterización total de las migraciones (Martínez-Bueno, Gómez Ramos, Bauer, & Fernández-Alba, 2019; Nerin, Alfaro, Aznar, & Domeño, 2013).

La figura 5 muestra un árbol de decisión para el procedimiento analítico en la identificación NIAS e IAS, dependiendo si los compuestos son volátiles o no volátiles (Nerín, 2016)

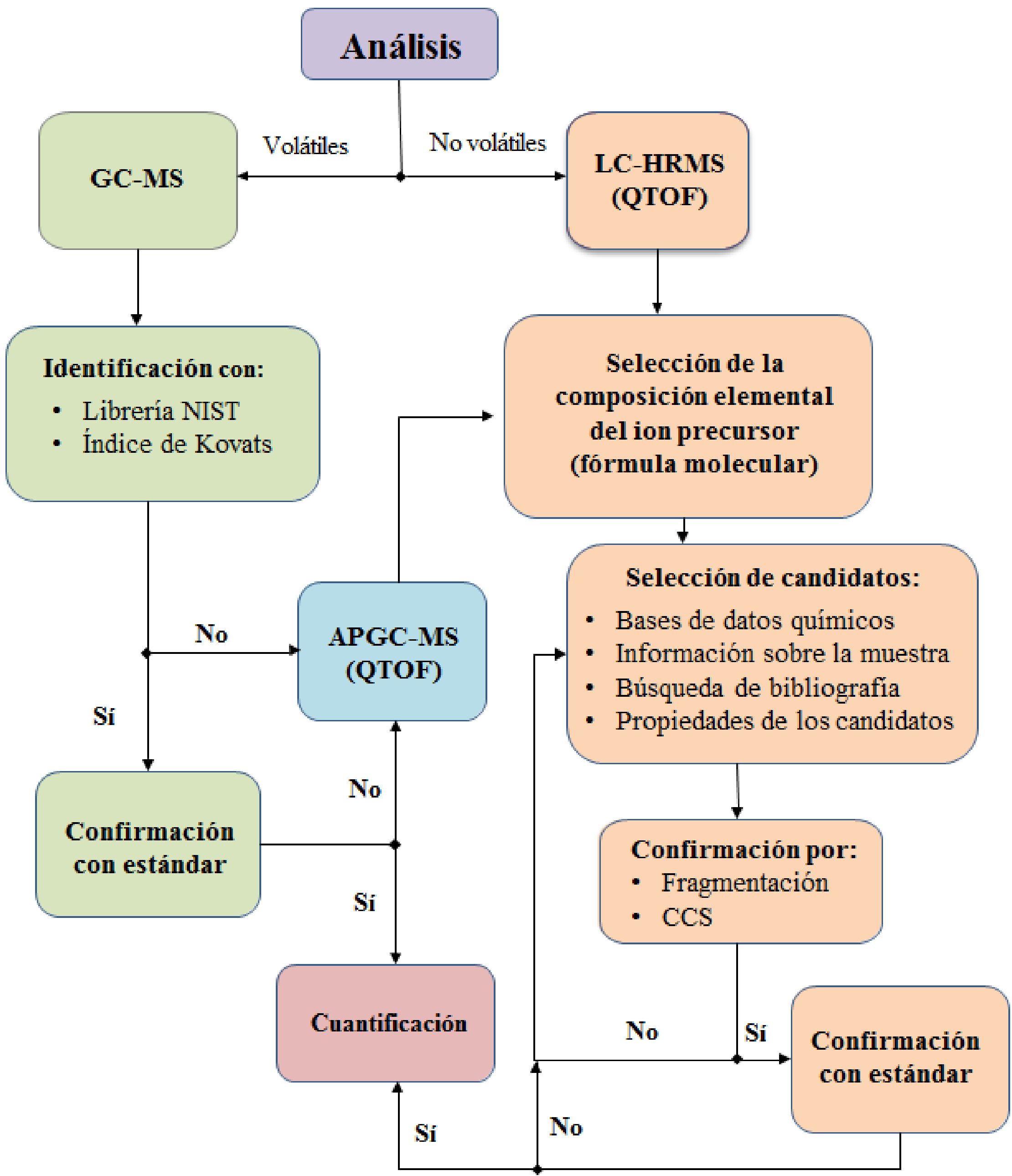



**Figura 5.** Árbol de decisión para el procedimiento analítico en identificación NIAS e IAS-figura modificada (Nerín, 2016).

## 5.1 Enfoque analítico para la identificación de compuestos volátiles

La GC–MS con impacto electrónico (EI) es la técnica más utilizada para el análisis de moléculas pequeñas en migración. Este instrumento permite la identificación de los compuestos con la ayuda de una librería de espectros de masas comercial (NIST) y con el uso de los índices de Kovats (flavornet.com, NIST). Cuando no es posible la identificación mediante GC-MS (EI), se recurre si es posible al uso de la espectrometría de masas de alta resolución (HRMS), que puede llevarse a cabo en un equipo de cromatografía de gases con ionización a presión atmosférica (APGC) acoplado a un espectrómetro de masas con analizador de tiempo de vuelo (TOF). Una vez identificado el posible candidato, la confirmación final se llevará a cabo mediante la inyección de estándares puros. El esquema general para este protocolo está descrito en la figura 5.

## 5.2 Enfoque analítico para la identificación de compuestos no volátiles

La LC-MS es la técnica más utilizada para la identificación de compuestos no volátiles. El proceso de identificación es más difícil que en GC-MS, y requiere mucho más tiempo, debido a la ausencia de bibliotecas para espectros de masas adquiridos por LC-MS. Esto se debe a que el espectro obtenido dependerá de diferentes factores, tales como el diseño del instrumento, condiciones de origen, composición de la fase móvil y componentes de muestra. Estos parámetros son difíciles de controlar entre laboratorios y también pueden variar con el tiempo en un mismo instrumento.

Se pueden usar diferentes analizadores de masas, como el triple cuadrupolo (QqQ) o analizadores de alta resolución (HRMS), entre los que se encuentra el tiempo de vuelo (TOF) o el Orbitrap. El cuadrupolo se usa con frecuencia para fines cuantitativos debido a su alta sensibilidad y selectividad.

Sin embargo, los analizadores de alta resolución se utilizan para la identificación de compuestos desconocidos, ya que se pueden recopilar espectros completos con masas muy precisas. Estas técnicas permiten la obtención de la masa exacta, tanto del ion precursor como de los fragmentos, posibilitando así su elucidación estructural. Por lo tanto, los instrumentos híbridos permiten unificar varias ventajas en un solo instrumento, como por ejemplo un tándem de cuadrupolo y tiempo de vuelo (Q-TOF), que permitirá una mejor elucidación estructural.

Para detectar el mayor número posible de compuestos deben optimizarse diferentes parámetros. La adquisición debe realizarse a baja y alta energía de colisión (CE), proporcionando así dos tipos de espectros de masas de los compuestos. Los espectros de baja energía proporcionan información sobre el ion precursor, y los espectros de alta energía proporcionan información sobre sus fragmentos. También se deben verificar varias sondas, como la ionización electrospray (ESI) o la ionización química a presión atmosférica (APCI). La primera opción para analizar muestras desconocidas es usar la sonda ESI, ya que proporciona un mayor grado de sensibilidad. Otros parámetros para controlar y aumentar la sensibilidad son el voltaje de cono (V), el voltaje del capilar o la intensidad (A) de la descarga de corona. El intervalo de masa para el análisis general se puede establecer entre 50 y 1000 Da, ya que 1000 Da es el peso molecular máximo esperado para los migrantes.

Un esquema del protocolo utilizado en la identificación de compuestos no volátiles usando LC–MS se muestra en la figura 5. En primer lugar, es importante identificar los compuestos pertenecientes únicamente a la muestra desconocida, comparando cromatogramas de la muestra y de los blancos. De esta manera se descartarán compuestos pertenecientes a los disolventes utilizados, o incluso procedentes del propio equipo. Una vez detectados los compuestos pertenecientes a la muestra, es importante determinar la composición elemental del ion precursor mediante un software generador de fórmulas empíricas. Una vez conocida la fórmula molecular, se proponen candidatos, utilizando sitios web de bases de datos químicas como [www.chemspider.com] y

[www.scifinder.com]. La selección de los candidatos propuestos se realiza basándose en la información sobre la muestra, búsqueda de bibliografía y propiedades de los candidatos. Es también de utilidad el análisis por espectrometría de movilidad iónica-espectrometría de masas (IMS-MS), que permite la determinación de los valores CCS (Collision Cross Section), relacionados con el tamaño y la forma del ion. El siguiente paso es comprobar si los fragmentos encontrados en el espectro de alta energía pertenecen al ion precursor, utilizando un software como MassFragment®. Finalmente, siempre que sea posible, el compuesto debe confirmarse por inyección del estándar puro.

## 6. Legislación general

Para garantizar la seguridad de los alimentos, hay una serie de legislaciones que los envases alimentarios deben cumplir, y que especifican el uso de sustancias permitidas en el envase y sus límites de migración.

A nivel general, existen dos reglamentos europeos que deben cumplir los materiales destinados al contacto con alimentos.

- *Reglamento (CE) 1935/2004*, de 27 de octubre de 2004, del Parlamento Europeo y del Consejo, sobre los materiales y objetos destinados a entrar en contacto con alimentos (EC, 2004).

El principio básico del presente Reglamento es que, cualquier material u objeto destinado a entrar en contacto directa o indirectamente con alimentos, ha de ser lo suficientemente inerte, para evitar que se transfieran sustancias a los alimentos en cantidades lo suficientemente grandes para poner en peligro la salud humana, o para ocasionar una modificación inaceptable de la composición de los productos alimenticios, o una alteración de las características organolépticas de éstos.

- *Reglamento (CE) 2023/2006*, de 22 de diciembre de 2006, de la Comisión, sobre buenas prácticas de fabricación de materiales y objetos destinados a entrar en contacto con alimentos (EC, 2006).

Este reglamento se aplica a todos los sectores y todas las etapas de fabricación, procesamiento y distribución de los materiales y objetos, hasta la producción de sustancias primas, ésta no inclusive.

A nivel específico, los materiales plásticos están regulados por:

- *Reglamento (UE) 10/2011*, del 14 de enero de 2011, de la Comisión, sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos (EC, 2011).

En este reglamento se define la lista positiva de compuestos que pueden utilizarse en la elaboración de plásticos para contacto alimentario y sus límites máximos de migración. Se establecen dos límites, el de migración global (OM) que se establece en 10 $mg/dm^2$ de material plástico o 60 mg $kg^{-1}$ de alimento; y el de migración específica (SML) para cada compuesto que se describe en el anexo I y se expresa en mg de sustancia por kg de alimento (mg $kg^{-1}$).

Las sustancias que no aparecen en la lista positiva del anexo I, no deberán superar el límite de 0.01 mg $kg^{-1}$en la migración, siempre y cuando se compruebe que no son carcinogénicas, mutagénicas o reprotóxicas.

En el anexo III se establecen los simulantes alimentarios que han de emplearse en los ensayos para demostrar la conformidad de materiales y objetos plásticos que aún no estén en contacto con alimentos. En él se detalla una lista de los simulantes alimentarios, donde aparece una clasificación según la descripción del alimento.

En el anexo V se describen las condiciones de contacto, tanto de temperatura como de tiempo, para los ensayos de migración con simulantes alimentarios.

En caso de encontrar en la migración compuestos que no se encuentran en la lista positiva, con concentraciones por encima de 0.01 mg $kg^{-1}$ y sobre los que no se hayan realizado estudios toxicológicos que definan su toxicidad, se puede aplicar el modelo de Umbral de Preocupación Toxicológica, o TTC por sus siglas en inglés *Threshold of Toxicological Concern* (Bolognesi, Castoldi, Crebelli, Barthélémy, Maurici, Wölfle, et al., 2017). Este modelo está basado en las reglas de Cramer, que permiten asignar de forma teórica, y en función de su estructura química, una clase de toxicidad a cada compuesto (Cramer, Ford, & Hall, 1978). En función de la clase Cramer, el límite máximo de ingesta diaria variará. Todas las sustancias químicas están clasificadas en tres clases de toxicidad, clase I (baja toxicidad), clase II (toxicidad media) y clase III (toxicidad alta) y los límites de ingesta diaria para cada clase son 1.8, 0.54 y 0.09 mg/persona/día respectivamente.

# 7. Contaminantes emergentes

Se entiende como contaminante emergente en materiales destinados al envase alimentario, todo compuesto químico o material cuya presencia en los materiales de envase no es necesariamente nueva, pero sí lo es la preocupación por las posibles consecuencias de la misma. Según la Agencia de Protección Ambiental de Estados Unidos (EPA) estos contaminante emergentes se perciben como un peligro potencial o real para la salud humana o el medio ambiente, o sus efectos sobre la salud no están establecidos (EPA, 2017).

Entre las sustancias no añadidas intencionadamente (NIAS) en los envases alimentarios llaman la atención los oligómeros; sustancias presentes en materiales poliméricos que son potencialmente migrantes. Estos compuestos, no están incluidos normalmente en ninguna base de datos y, a menudo, los estándares comerciales no están disponibles, lo que hace que su identificación y confirmación sean procesos difíciles (Nerin, Alfaro, Aznar, & Domeño, 2013).

Hoy en día, estos compuestos son considerados contaminantes emergentes y están levantando un gran interés social porque apenas hay conocimiento sobre su toxicidad.

Teniendo en cuenta la clasificación de los NIAS descrita en la figura 3, los oligómeros son productos colaterales formados durante la síntesis del polímero aunque también pueden producirse por la degradación del polímero durante la fabricación del envase. Además pueden ser sustancias predecibles que pueden ser identificadas o no, pero cuya toxicidad no ha sido evaluada.

Los oligómeros se definen como moléculas que consisten en varias unidades de monómeros. Cuando el número de unidades es elevado, la molécula resultante se denomina polímero. Por lo general, los oligómeros contienen entre dos y 40 unidades repetitivas y cuando su peso molecular es inferior a 1000 Da se consideran migrantes potenciales relevantes (EU, 2014). Los oligómeros siempre están presentes en los materiales poliméricos y por tanto es necesaria su determinación en la migración.

En función de las unidades y el tipo de monómeros que lo forman, podemos distinguir dos tipos de oligómeros (M. Hoppe, de Voogt, & Franz, 2016):

- Oligómeros formados por un solo tipo de monómeros. La denominación de estos oligómeros vendrá dada por el número de unidades monoméricas que lo forman (n=2 dímero, n=3 trímero, etc.). Estos oligómeros provienen de homopolímeros (polímeros formados por un solo tipo de unidad monomérica), tales como el PP, PE y PLA.

- Oligómeros formados por diferentes tipos de monómeros. En este caso, la unidad monomérica la constituiría el bloque de monómeros que se repite. Esta unidad monomérica puede ser de un solo tipo de bloque de monómeros como por ejemplo el caso del PET. En el PET, la unidad monomérica la constituiría el bloque TPA-EG, siendo el dímero el TPA-EG-TPA-EG y el

trímero TPA-EG-TPA-EG-TPA-EG. Estos oligómeros provienen de copolímeros (polímeros formados por diferentes tipos de unidades monoméricas), tales como el PET. La unidad monomérica también puede estar formada por varios bloques de monómeros como por ejemplo el oligómero AA-DEG-IPA-DEG del PU, que tendrá dos unidades monoméricas (AA-DEG y IPA-DEG).

La formación química de oligómeros se produce principalmente durante el proceso de polimerización, ya sea por polimerización incompleta o por degradación térmica o hidrolítica de las cadenas del polímero durante el procesamiento del material polimérico y las condiciones de uso. El oligómero resultante puede ser muy complejo y puede consistir en especies lineales, ramificadas y cíclicas que pueden tener diferentes propiedades de migración y perfiles toxicológicos (M. Hoppe, de Voogt, & Franz, 2016).

Debido al alto número de monómeros utilizados para la producción de polímeros, y a los numerosos procesos de descomposición, puede surgir una gran variedad de oligómeros presentes en los plásticos destinados para envase alimentario. Esto hace más importante la investigación de su presencia, de su potencial de migración y de los efectos toxicológicos para el ser humano.

Hay poca información sobre la toxicidad de los oligómeros, esto es debido en parte a la falta de estándares comerciales para los estudios toxicológicos. A menudo se ha asumido que los oligómeros tienen la misma toxicidad que sus monómeros iniciales, sin embargo, es evidente que los productos de reacción pueden tener diferentes propiedades (Grob, Camus, Gontard, Hoellinger, Joly, Macherey, et al., 2010; Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). Según la EFSA (EFSA, 2008), cuando el polímero se forma por la polimerización de un monómero aprobado, su falta de genotoxicidad se establece por los datos sobre el monómero, y no se requieren datos experimentales sobre el polímero en sí. Pero se ha demostrado que en algunos casos el perfil toxicológico de los productos de reacción y las sustancias de partida son diferentes, por

ejemplo en el caso de los oligómeros de estireno (Gelbke, Banton, Block, Dawkins, Leibold, Pemberton, et al., 2018). Por lo tanto, es importante estudiar la toxicidad, no solo del material de partida, sino también de los posibles oligómeros que pueden formarse.

El interés toxicológico se centra en la posibilidad de que las sustancias se absorban en el tracto gastrointestinal. Se demostró que la absorción depende de varios factores como la vía de absorción, el peso molecular, la polaridad y la forma de la molécula o la región del tracto gastrointestinal donde se absorba (Bjarnason, Macpherson, & Hollander, 1995).

La Oficina de Seguridad de Aditivos Alimentarios (OFAS) establece que los materiales oligoméricos con un peso molecular inferior a 1000 Da son importantes desde un punto de vista toxicológico, ya que podrían migrar a los alimentos y ser absorbidos en el tracto gastrointestinal (Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). Se demostró además en otros estudios que la absorción de moléculas superiores a 1000 Da era inferior al 1% (Schaefer, Ohm, & Simat, 2004) aunque estudios recientes plantean la re-evaluación de este límite por considerar que compuestos de alto peso molecular juegan un papel importante en el tracto gastrointestinal (Groh, Geueke & Muncke, 2017) .

En general, se ha demostrado que las tecnologías de espectrometría de masas acopladas a la cromatografía líquida y cromatografía gas representan el mejor enfoque analítico para la identificación y cuantificación de oligómeros en materiales plásticos destinados al envase alimentario. De hecho, la identificación de oligómeros se realiza principalmente por espectrometría de masas, ya que las masas de los posibles compuestos oligoméricos se conocen teóricamente por el conocimiento de las unidades de monómero (Maria Hoppe, de Voogt, & Franz, 2018; Martínez-Bueno, Gómez Ramos, Bauer, & Fernández-Alba, 2019). Si los monómeros de los polímeros son conocidos, los posibles oligómeros de ese material pueden ser determinados más fácilmente.

Por último, hay que tener en cuenta que el mayor problema en la cuantificación de oligómeros es la elección de un estándar apropiado, ya que la mayoría de ellos no tienen estándar comercial. Comúnmente se utilizan sustancias comerciales con similares características estructurales al oligómero. Al realizar este procedimiento, en realidad, se está realizando una semi-cuantificación del oligómero. No obstante, es necesario realizar esta aproximación para poder estimar su concentración en migración, ya que la mayoría de los oligómeros no tienen determinada ninguna toxicidad y por lo tanto, al no estar específicamente autorizados por la legislación, su límite de migración específica debería ser inferior a 0.01 mg $kg^{-1}$.

La tesis se ha centrado en la identificación de NIAS y en concreto en el estudio de oligómeros de diferentes envases alimentarios. Los materiales estudiados han sido: el polímero convencional PET, el biopolímero PLA y el envase multicapa con adhesivo PU. Por esta razón, se explicarán a continuación de forma general los materiales estudiados junto a sus respectivos tipos de oligómeros.

## 7.1 Oligómeros del tereftalato de polietileno (PET)

El tereftalato de polietileno (PET) es un poliéster formado por la polimerización de los monómeros de etilenglicol (EG) y ácido tereftálico (TPA). Este polímero es considerado uno de los más usados debido a su ligereza, resistencia a los golpes, transparencia y reciclabilidad. Se utiliza para la fabricación de botellas, películas flexibles y transparentes, bandejas de embalaje y recipientes para microondas (Awaja & Pavel, 2005; Kassouf, Maalouly, Chebib, Rutledge, & Ducruet, 2013).

Los principales NIAS que se han identificado en este material son los oligómeros cíclicos y lineales de primera y segunda serie. Los oligómeros de primera serie están compuestos por un igual número de TPA y EG (figura 6),

mientras que en los de la segunda serie, una unidad EG es sustituida por un dietielenglicol (DEG). Los oligómeros de tercera serie también pueden formarse sustituyendo dos EG por dos DEG. La segunda y tercera serie de los oligómeros surge debido a la formación del subproducto DEG durante la producción de PET. Todos estos oligómeros tienen potencial para migrar a los alimentos y por esta razón es importante llevar a cabo la identificación y cuantificación de los componentes del PET destinado a contacto con alimentos (Maria Hoppe, Fornari, de Voogt, & Franz, 2017).

**Figura 6**. Monómero del PET

## 7.2 Oligómeros del ácido poliláctico (PLA)

Durante los últimos años, el ácido poliláctico (PLA) se ha convertido en uno de los biopolímeros más atractivos para el envasado de alimentos porque tiene propiedades físico-químicas comparables a las del PET. Entre las aplicaciones de envasado destacan los vasos de yogur, botellas de agua, leche y zumo, y contenedores de comida y envoltorios (Salazar, Domenek, Plessis, & Ducruet, 2017). El PLA pertenece al grupo de los poliésteres alifáticos y por tanto sus grupos éster pueden degradarse por hidrólisis en presencia de agua o por procesos térmicos llevando a la formación de oligómeros. En la figura 7 se muestra la estructura del monómero, el dímero y el hexámero del PLA. Es importante estudiar estos oligómeros en detalle y desarrollar metodologías para su identificación y cuantificación (Dopico-García, Ares-Pernas, Otero-Canabal, Castro-López, López-Vilariño, González-Rodríguez, et al., 2013).

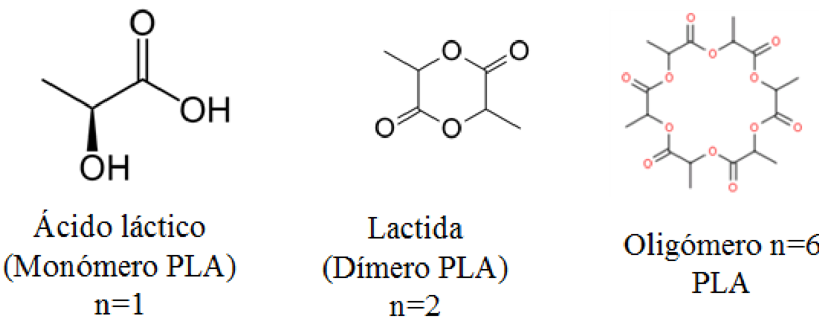



**Figura 7.** Monómero y oligómeros del PLA

## 7.3 Oligómeros del poliuretano (PU)

Entre los envases alimentarios, destacan los envases multicapa formados por diferentes capas de polímero que están unidas por una capa de adhesivo, comúnmente basado en poliuretano (figura 8). Este formato de envase multicapa ha sido adoptado por la industria para mejorar las propiedades barrera, resistencia mecánica, sellado térmico y propiedades de impresión de los sustratos, permitiendo una mejor preservación de los productos alimenticios (Engels, Pirkl, Albers, Albach, Krause, Hoffmann, et al., 2013). En estos materiales hay que tener en cuenta que la migración no sólo se produce desde capas en contacto directo con el alimento sino también desde capas internas. Por esta razón, aunque el adhesivo está en las caras internas del material, sus componentes también pueden migrar hasta el alimento debido a procesos de difusión y partición (Zhang, Kenion, Bankmann, Mezouari, & Hartman, 2018).

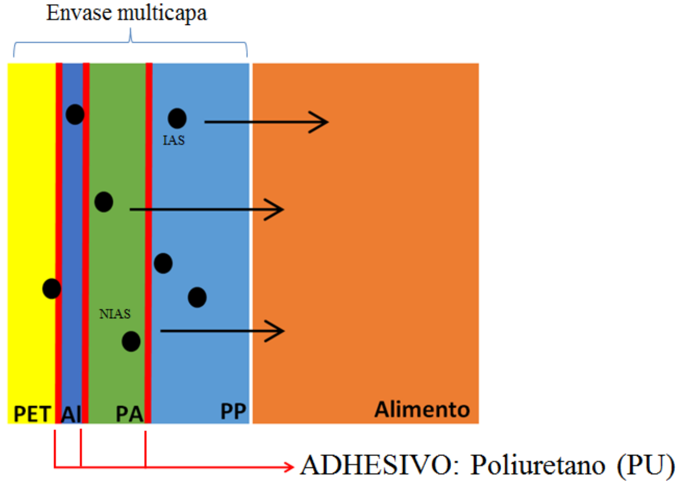
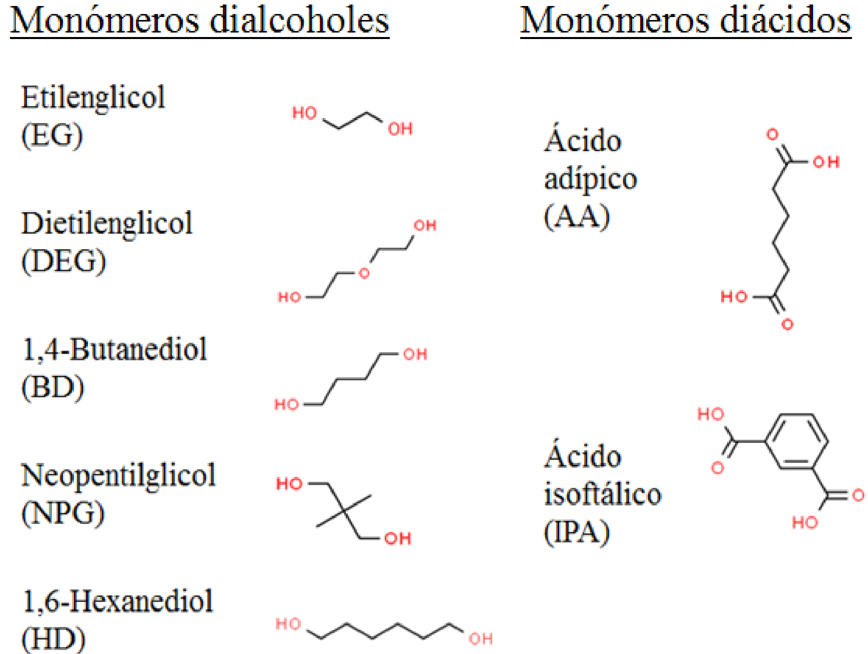



**Figura 8.** Esquema de un material multicapa

El poliuretano es un poliéster formado por la reacción entre diisocianatos y poliésteres lineales, donde estos últimos se producen por la reacción entre polialcoholes y ácidos carboxílicos aromáticos o alifáticos (tabla 1).



| Monómeros dialcoholes | Monómeros diácidos |
| --- | --- |
| Etilenglicol (EG) | Ácido adípico (AA) |
| Dietilenglicol (DEG) | |
| 1,4-Butanediol (BD) | |
| Neopentilglicol (NPG) | Ácido isoftálico (IPA) |
| 1,6-Hexanediol (HD) | |

**Tabla 1.** Monómeros utilizados en la formación de poliésteres lineales.

Cuando la última reacción no se desarrolla en condiciones de equilibrio, se favorece la formación de oligómeros cíclicos y lineales de bajo peso molecular (Brunelle, 2003) (figura 9). Estos subproductos, como se ha dicho, pueden migrar al alimento (Athenstädt, Fünfrocken, & Schmidt, 2012; Canellas, Vera, & Nerín, 2017; Félix, Isella, Bosetti, & Nerín, 2012). En el estudio realizado por Zhang et al 2018 (Zhang, Kenion, Bankmann, Mezouari, & Hartman, 2018), se identificaron 56 oligómeros diferentes procedentes de 537 muestras laminadas con PU como adhesivo.

Dialcohol Dicarboxílico Oligómero de bajo peso molecular

**Figura 9.** Reacción de formación de oligómeros de PU.

## 7.4. Oligómeros de la poliamida (PA)

Las poliamidas se usan frecuentemente para el contacto directo e indirecto con el alimento. Las aplicaciones de PA para contacto indirecto son envases multicapa, donde las películas de PA proporcionan propiedades barrera para gases y aroma y están laminadas con capas de sellado de poliolefinas. Los tipos de PA más utilizados para envase alimentario son la PA6, que se fabrica por polimerización del monómero caprolactama, y la PA66, producida por la policondensación de la hexametilendiamina y el ácido adípico. Las principales sustancias migrantes de las PA son los monómeros y oligómeros cíclicos, que se forman por reacciones secundarias, sobre todo tratamientos térmicos, durante la síntesis de estos materiales (Heimrich, Nickl, Bönsch, & Simat, 2015). La figura 10 muestra la estructura del monómero y dímero cíclico de la PA6 y la estructura del monómero cíclico PA66.

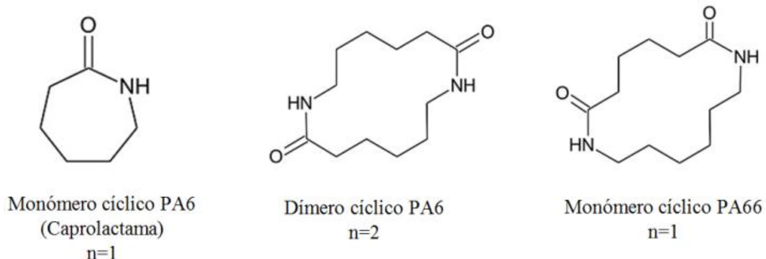



**Figura 10.** Monómero del PA6 y oligómeros de la PA6 y PA66

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# **SECCIÓN II:** Objetivos Generales

# OBJETIVOS GENERALES

El **objetivo principal** de la presente tesis ha sido el análisis de los contaminantes emergentes presentes en diferentes materiales destinados al contacto con alimentos y sus estudios de migración a diferentes simulantes alimentarios. Como contaminante emergente en materiales destinados al envase alimentario se entiende todo compuesto químico cuya presencia en los materiales de envase no es necesariamente nueva, pero sí la preocupación por las posibles consecuencias de la misma. El trabajo se ha centrado en el estudio de los oligómeros, que se definen como moléculas formadas por un número reducido de unidades del monómero. Estos compuestos se consideran compuestos no añadidos de forma intencionada, NIAS, y para la mayoría de ellos se desconoce su toxicidad para el ser humano.

Además, se ha estudiado la composición y los migrantes principales de diferentes materiales de envase, utilizando y optimizando diversos tratamientos de muestra y diversas técnicas analíticas. Se han identificado y cuantificado principalmente los compuestos no volátiles pero también se ha llevado a cabo el estudio, en determinados materiales, de los compuestos volátiles y semi-volátiles, así como de su posible impacto sensorial en el alimento envasado.

El número de materiales estudiados ha sido muy amplio con el fin de tener una visión global de los posibles contaminantes emergentes que se pueden encontrar en los alimentos envasados. En primer lugar, se han estudiado los materiales multicapa, muy frecuentes en la fabricación de envases flexibles. Estos materiales están formados por varias capas de polímeros unidas por un adhesivo de poliuretano. En segundo lugar, se ha estudiado el tereftalato de polietileno (PET), polímero convencional de origen fósil muy utilizado en la fabricación de botellas. Se ha trabajado, no solo con muestras de PET virgen, sino también con PET reciclado (PETr), dado el auge en su utilización para la mejora de la preservación del medio ambiente. Y por último, dada la importancia

en la actualidad de reducir el uso de los polímeros de origen fósil, se ha estudiado el PLA, un biopolímero de origen natural y a su vez compostable en condiciones controladas a escala industrial.

Para lograr este objetivo, se han planteado varios **objetivos específicos**:

- ✓ Desarrollo y optimización de métodos de tratamiento de muestra de los materiales de estudio previos al análisis con el fin de aumentar en los extractos a analizar, la concentración de los IAS y NIAS presentes, tanto volátiles como no volátiles. De esta forma, ha sido posible tener una mayor sensibilidad y selectividad en los posteriores análisis. Entre otras metodologías se han estudiado técnicas de disolución/precipitación o extracción y técnicas de pre-concentración tales como la microextracción en fase sólida (SPME).
- ✓ Elaboración de protocolos de análisis que han permitido la identificación y cuantificación de los oligómeros y otras sustancias presentes en los materiales estudiados. Las técnicas utilizadas han sido tanto GC-MS y APGC-QToF para la detección de los compuestos más volátiles, como UPLC-QToF, UPLC-QqQ y UPLC-IMS-QToF para la detección de los compuestos menos volátiles.
- ✓ Elaboración de una librería con los compuestos identificados en los materiales de estudio (oligómeros principalmente).
- ✓ Estudio de la cinética de migración de algunos oligómeros presentes en los materiales seleccionados.
- ✓ Evaluación del posible impacto sensorial en los alimentos de algunos de los materiales de estudio.
- ✓ Evaluación del riesgo de los materiales estudiados basada en estudios de migración con distintos simulantes alimentarios y en el Reglamento EU/10/2011 y el método de umbral de preocupación toxicológica (TTC).
- ✓ Determinación de la toxicidad de los oligómeros realizando ensayos de disrupción endocrina.

- ✓ Estimación de la bioaccesibilidad de los oligómeros mediante estudios de digestión gástrica e intestinal.

# **SECCIÓN III:** Desarrollo Experimental

**Capítulo 1:** *Overall and specific migration from multilayer high barrier food contact materials - kinetic study of cyclic polyester oligomers migration*

**Capítulo 2:** *Determination of oligomers in virgin and recycled polyethylene terephthalate (PET) samples by UPLC-QToF*

**Capítulo 3:** *Determination of non-volatile components of a biodegradable food packaging material based on polyester and polylactic acid (PLA) and its migration to food simulants*

**Capítulo 4:** *Migration of oligomers from a food contact biopolymer based on polylactic acid (PLA) and polyester*

**Capítulo 5:** *Determination of volatile compounds and their sensory impact in a biopolymer based on polylactic acid (PLA) and polyester.*

**Capítulo 6:** *Migration studies and toxicity evaluation of cyclic polyesters oligomers from food packaging adhesives*

# Capítulo 1

## *Overall and specific migration from multilayer high barrier food contact materials – Kinetic study of cyclic polyester oligomers migration*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
3. INTRODUCTION
4. MATERIAL AND METHODS
5. RESULTS AND DISCUSSION
6. CONCLUSIONS
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# 1. RESUMEN

La mayoría de los materiales multicapa usados en el envasado de alimentos tienen una capa de adhesivo de poliuretano en su estructura. Para evaluar la seguridad de estos materiales, es importante determinar tanto los compuestos añadidos intencionadamente a los adhesivos (IAS) como los no añadidos intencionadamente (NIAS). Durante la fabricación de los adhesivos de poliuretano, pueden formarse algunos subproductos, como los oligómeros de poliéster cíclicos procedentes de la reacción entre ácido di-carboxílicos y polioles. Dado que estos compuestos no están listados en el Reglamento 10/2011/EU, no deben encontrarse en la migración por encima de 0.01 mg $kg^{-1}$ de simulante. En este estudio fueron estudiados dos materiales de envasado multicapa flexibles. Se evaluó la migración a simulante A (etanol 10% v/v), simulante B (ácido acético 3% w/v) y simulante de etanol 95% v/v durante 10 días a 60 ºC. La identificación y cuantificación de los compuestos no volátiles se llevó a cabo mediante un UPLC-QToF. La mayoría de los compuestos migrantes fueron oligómeros de poliésteres cíclicos y oligómeros de la caprolactama. Además, la migración global y específica del ácido adípico-dietielenglicol (AA-DEG) y el ácido ftálico-dietilenglicol (PA-DEG) se monitorizaron a lo largo del tiempo y se analizaron por UPLC-QqQ. En la mayoría de los casos, se encontraron los valores más altos de concentración en el simulante etanol 95%. La cinética de migración global siguió un patrón similar a la cinética de migración específica.

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El principal objetivo de este capítulo fue por tanto, determinar los compuestos migrantes procedentes de dos materiales multicapa, formados por la combinación de tereftalato de polietileno (PET), poliamida (PA), polipropileno cast (CPP) y aluminio (Al) a tres simulantes alimentarios. Sus estructuras eran: [PET//PA//CPP] y [PET//Al//PA//CPP], siendo en ambos casos el CPP el material en contacto con el alimento. Otro objetivo fue estudiar la cinética de migración de dos oligómeros del PU y sus posibles hidrólisis en contacto con simulantes acuosos. Estos oligómeros tenían la estructura de AA-DEG y PA-DEG, dónde AA es ácido adípico, DEG es dietilenglicol y PA es ácido ftálico. Para conseguir estos objetivos, se detallan a continuación las tareas llevadas a cabo:

- Búsqueda de bibliografía sobre los posibles migrantes procedentes de materiales multicapa con poliuretano como adhesivo destinados al envase alimentario.

- Realización de ensayos globales y específicos de migración con tres simulantes alimentarios diferentes.

- Aprendizaje del manejo de los equipos UPLC-QToF y UPLC-QqQ.

- Identificación de los compuestos migrantes y cuantificación o semi-cuantificación de los mismos.

- Búsqueda de los compuestos migrantes en el Reglamento 10/2011/EU y evaluación del riesgo de algunos oligómeros identificados utilizando el método de umbral de preocupación toxicológica (TTC).

- Realización de ensayos cinéticos con dos ésteres cíclicos específicos del PU.

- Realización de ensayos de hidrólisis de dos oligómeros del PU.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 1.

**Esquema 1:** Diseño experimental del Capítulo 1.

# 3. INTRODUCTION

Nowadays, food packaging is widely used and its main function is minimizing the incidence of external factors and thus protecting the integrity of the product, preserving its quality and nutritional, sensory and health characteristics. Food packaging contains intentionally added substances (IAS) and it can also contain non-intentionally added substances (NIAS), coming from degradation processes and/or impurities present in the raw materials, that can migrate to food and compromise food safety. If a large amount of migrants is transferred to foodstuffs, they can reach levels that are harmful to health and that can also affect the composition and properties of food such as color, smell, taste and appearance, affecting the shelf life of the product. Therefore, it is important to control them in order to assure food quality and safety. Migrants identification is very difficult and it is necessary to use high sensitive advanced analytical techniques, especially for non-volatile compounds (Nerin, Alfaro, Aznar, & Domeño, 2013; 2012).

Most of multilayer high barrier materials used in food packaging have an adhesive layer, often based on polyurethane. Although the adhesives are not in the food contact side of the packaging, their components can also migrate to food due to diffusion and partition processes (Tehrany & Desobry, 2004). Previous works have described the presence of adhesive components in food migration (Aznar, Vera, Canellas, Nerin, Mercea, & Stormer, 2011; Vera, Aznar, Mercea, & Nerin, 2011). Polyurethane is usually produced using polyesters and di-isocyanate compounds. In addition, several additives such as antioxidants, surfactants, biocides or catalysts can be included in the adhesive to improve their properties. At the same time, linear polyesters necessary for polyurethane manufacture, are produced by reaction ofpolyols and aliphatic and/or aromatic carboxylic acids. When the reaction proceeds under equilibrium conditions, linear polyesters are produced. As the reaction continues, it can unbalance and favors the formation of short chain cyclic polyesters, commonly referred as

lactones, that are considered by-products of the polyurethanes manufacture and can migrate to food (Carrizo, Maccagnan, Félix, Nerín, & Bosetti, 2015; Nerin, Alfaro, Aznar, & Domeño, 2013; 2012). One of the most difficult tasks is the identification and quantification of these by-products, as none of the chemical database includes oligomers (Heimrich, Nickl, Bönsch, & Simat, 2015; Hoppe & Franz, 2016; Paseiro-Cerrato, MacMahon, Ridge, Noonan, & Begley, 2016).

To ensure the food safety, food packaging must comply with legislation. Food contact materials and articles, including adhesives, must comply with the Regulation (EC) 1935/2004 (EC, 2004) and must not transfer their constituents to food in quantities which could endanger human health. Regulation (EU) no. 10/2011 (EC, 2011) applies on plastic materials and articles intended to come into contact with food. This regulation includes a positive list of substances that can be present in migration at concentration values below its specific migration limit (SML). If the substances are not included in this list, their migration should not be found above 0.01 mg $kg^{-1}$ of food/simulant. This is the case of some cyclic polyester oligomers, and therefore, the determination of its migration to food is very relevant.

The first aim of this study was to identify and quantify the non-volatile compounds present in migration to three food simulants in contact with two high barrier food packaging multilayer materials.

Migration kinetic plays a critical role in the final food packaging migration and may affect the migration of oligomers. For this reason, the second important objective of this study was to evaluate the migration kinetics in the studied materials, both in overall and specific migration of two cyclic polyesters coming from polyurethane adhesives (Isella, Canellas, Bosetti, & Nerin, 2013). Finally, the last aim of the work was to study the cyclic polyesters stability in acidic medium, in order to know the variation of their concentration overtime. Liquid chromatography coupled to high resolution mass spectrometry was used

for these purposes, as it is the required powerful tool to identify non-volatile compounds.

# 4. MATERIAL AND METHODS

## 4.1. Reagents

Caprolactam (CAS 105-60-2) was purchased from Merck (Madrid, Spain). Bis(2-etilhexil) adipate 99% (CAS 103-23-1), acetyl tributyl citrate (CAS 77-90-7), 1-stearoyl-rac-glycerol (CAS 123-94-4), butyl 4-hydroxybenzoate (CAS 94-26-8), dioctyl phthalate (CAS 117-81-7), Irganox 1010 (CAS 6683-19-8) and acid acetic (CAS 64-19-7) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Cyclic esters composed by diethylene glycol (DEG) and adipic acid (AA) or phthalic acid (PA), AA-DEG and AA-DEG-PA-DEG, were synthetized and provided by an adhesives company. Ethanol was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Millipore Milli-QPLUS 185 system (Madrid, Spain). Milli-Q water (18 M·Ω·cm) was used to prepare all solutions. Methanol and water for UPLC analysis (ultra LC–MS quality) were supplied by Baker (Deventer, The Netherlands).

## 4.2. Samples

Two flexible multilayer materials used for cured meat products packaging were tested. Both were provided by the company Alico S.A. (Medellín, Colombia). Multilayers were manufactured with PET: polyethylene terephthalate (PET), aluminium foil (Al), polyamide (PA) and cast polypropylene (CPP), joint by polyurethane (PU) layers. CPP was in both cases the food contact side (FCS). Their structures were:

- Material 1: $[PET//PA//CPP]_{FCS}$

Material thickness was 105 µm and PU grammage was 2 grams per square meter of laminate ($g/m^2$) - (PET: 10; PA: 15; CPP: 80 µm).

- Material 2: $[PET//Al//PA//CPP]_{FCS}$

Material thickness was 113 µm and PU grammage was 2 grams per square meter of laminate ($g/m^2$) - (PET: 12; Foil: 9; PA: 25; CPP: 80 µm).

## 4.3. Instrumental analysis

### 4.3.1 Ultra-performance liquid chromatography (UPLC)

Chromatography was carried out in an Acquity system supplied by Waters (Milford, MA, USA). A UPLC BEH C18 column of 1.7 µm particle size (2.1 x 100 mm) from Waters was used. Chromatography was carried out at 0.3 mL $min^{-1}$ column flow and 35 ºC column temperature. Mobile phase A was water (with 0.1% v/v formic acid) and mobile phase B was methanol (with 0.1% v/v formic acid). Chromatography started at 95:5 (A:B) and changed to 5:95 (A:B) in 6 minutes and maintained two minutes. Injection volume was 10 µL.

### 4.3.2 Mass spectrometry detection with quadrupole-time-of-flight mass analyzer (MS–QToF)

A Xevo G2 QToF mass spectrometer supplied from Waters (Milford, MA, USA) was used for the analysis by MS-QToF. This system was coupled to the UPLC system with an ESI probe. The experimental instrument parameters were as follows: positive and negative ionization (ESI+ y ESI-), sensitivity mode, capillary voltage 2.5 kV, cone voltage 30 and 70 V, extraction cone 4 V, source temperature 120 ºC, desolvation temperature 450 ºC, cone gas flow 20 L $h^{-1}$, and desolvation gas flow 700 L $h^{-1}$. Acquisition was performed in $MS^E$ mode to allow using low and high collision energy (CE) in the collision cell during the same

run. The mass spectrum at low energy (CE 4 V) provides information about the precursor ion (function 1) and the mass spectrum at high energy (CE ramp: from 15 to 30 V) information about fragment ions (function 2).

### 4.3.3 Identification of compounds detected by UPLC–MS-QToF

From the spectra obtained in function 1 the elemental formula was obtained. Once the molecular formula of each accurate mass was known, it was necessary to use a database of chemical compounds in order to propose the most likely candidate. Chemspider (ChemSpider, 2014) and SciFinder (Finder, 2014) were used to obtain a list of candidates. Then, with the use of function 2, the fragmentation spectra were obtained and the proposed structures were checked through MassFragment® software from Waters. This software enabled us to evaluate and confirm whether the product ions detected in the high collision energy spectrum could be linked to the fragments generated from the chemical structures of the candidates proposed. Furthermore, when possible, the standards were purchased and the compounds were confirmed by a comparison of the retention time and mass spectrum.

### 4.3.4 Mass spectrometry detection with triple quadrupole mass analyzer (MS-QqQ)

A TQ triple quadrupole mass spectrometer supplied by Waters (Milford, MA, USA) was used for quantification purposes. The UPLC system was coupled to an ESI probe to the QqQ. The experimental instrument parameters used were as follows: positive ionization and SIR (selected ion recording) acquisition mode, capillary voltage 2.5 kV, cone voltage 30 V, extraction cone 3 V, source temperature 120 ºC, desolvation temperature 450 ºC, cone gas flow 30 L $h^{-1}$, and desolvation gas flow 650 L $h^{-1}$. The ions monitored were 217.1 [$MH^+$] for AA-DEG, 259.1 [$MNa^+$] for PA-DEG and 129.3 [$MNa^+$] for DEG. AA was measured under the same conditions but in negative mode, ion monitored was 145.05 [$H^-$].

For building the calibration curves, solutions of the cyclic ester AA-DEG standard were prepared and injected before each analysis. MassLynx v.4.1 and QuanLynx software (Waters, Milford MA, USA) were used to analyze the samples.

## 4.4. Migration test

For the migration experiments, bags made with materials described before were manufactured by thermosealing. Afterwards, they were filled with different food simulants. The materials were tested in ethanol 10% v/v (simulant A) and acetic acid 3% w/v (simulant B) as aqueous simulants and in ethanol 95% v/v as fat simulant. Meat used in this study was “cured meat” that in most cases does not require refrigeration and it has a long time storage. EU/10/2011 established that for contact times above 30 days at room temperature and below the specimen shall be tested in an accelerated test at elevated temperature for a maximum of 10 days at 60 °C. For this reason, the bags were maintained in an oven the forced convection at 60 ºC for 10 days. Simulants and test conditions used for the migration assays were chosen according to the European Regulation 10/2011. The samples were analyzed by UPLC-QToF. All the concentrations were corrected according to the rate of 6 $dm^2$ of packaging material per 1 kg of simulant, in accordance with European Regulation 10/2011 (EC 2011) .

## 4.5 Identification and quantification of migrants in food simulants

For the identification of the main migrants, migration solutions of simulant D samples were gently concentrated under a nitrogen current (x5) and they were injected in a UPLC-QToF system. For migrant quantification, calibration curves were performed with the pure standards at different concentration levels. When the standards were not available, the quantification was done with a standard with similar chemical structure.

### 4.6 Hydrolysis of AA-DEG oligomer in acetic acid 3 % (w/v) overtime

This study was carried out to see the behavior of the cyclic ester AA-DEG in acidic medium. Firstly, an aqueous solution of the cyclic ester of 1µg $g^{-1}$ was prepared. Half of dissolution was added with acetic acid to have a final 3% v/v acetic acid concentration. Three aliquots of 20 mL of this solution were placed in glass vials and introduced in the oven at 60º for 10 days. A 600 µl aliquot was taken at 1, 6, 24, 72, 144, 192 and 240 hours and analyzed by UPLC-QqQ. A parallel experiment was performed with aqueous solutions (without acetic acid) and AA-DEG at the same concentration.

### 4.7 Kinetic study

The kinetic migration study was performed for overall migration and specific migration of two cyclic esters, AA-DEG and PA-DEG. During migration test, migration aliquots were evaluated at six different times: 1, 6, 24, 72, 144, 192 and 240 hours. For overall migration, a gravimetry analysis of migration residues was performed. For specific migration, a 1 ml aliquot of migration samples was taken and analyzed by UPLC-QqQ. In all cases, three independent replicates of each sample were analyzed.

## 5. RESULTS AND DISCUSSION

### 5.1 Identification and quantification of non-volatile migrants in UPLC-QToF

Table 1 and 2 summarize the identification and quantification of compounds present in migration from $[PET//PA//CPP]_{FCS}$ and $[PET//Al//PA//CPP]_{FCS}$ materials respectively. For those compounds non listed in

the Regulation EU/10/2011 the specific migration limit (SML) is shown. Migration of compounds not listed in the Regulation must be below 10 ng $g^{-1}$. The analytical characteristics of the standards used for quantification are shown in table 3.

Many migrants were identified from both materials, both IAS such as antioxidants, plasticizers and monomers (caprolactam) and NIAS, such as oligomer esters coming from PU adhesive. In most cases, the migration values were higher in ethanol 95 % v/v than in ethanol 10% v/v or acetic acid 3% w/v.

In the migration tests from material $[PET//PA//CPP]_{FCS}$ (table 1), 17 compounds were identified, most of them were oligomers . Caprolactam, the monomer of polyamide 6 (PA 6), was detected in the three simulants but at lower concentration of that allowed in the European Regulation (15 mg $kg^{-1}$). Four caprolactam oligomers (n=2, 3, 4 and 5) were also detected in ethanol 95% v/v simulant, what reveal a higher tendency of these kind of compounds to migrate to fat simulants than to aqueous ones. Since these compounds are not present in the positive list, their migration concentration should be below 0.01 mg $kg^{-1}$. Oligomer n=4 showed the highest concentration migration values. Oligomers coming from polyurethane adhesives were also found. They were cyclic esters made up of phthalic acid (PA) and diethylene glycol (DEG) in combination 1:1 (PA-DEG) or 2:2 (PA-DEG-PA-DEG). Several additives were also found such as plasticizers (tributyl acetyl citrate, dioctyl phthalate or bis(2-etilhexil) adipate), antioxidants (oxidation products of butylhydroxytoluene, Irganox 1010 and 3,5-di-tert-butyl-4-hydroxybenzaldehyde), lubricants based on glycerol (Glycerol monotridecanoate, Glycerol monoheptadecanoate, Glycerol mononononadecanoate).

**Table 1.** Compounds detected in migration from $[PET//PA//CPP]_{FCS}$ multilayer material. Retention time (rt) and measured mass (mass), type of ion found (adduct), compound candidate, molecular formula (MF), quantification standard (QS). Migration values in different food simulants and specific migration limit (SML) according to EU/10/2011 Regulation.

| | **rt**<br>**mass** | **Adduct** | **Candidate**<br>**MF** | **QS** | **EtOH 95%**<br>**($ng\ g^{-1}$)** | **EtOH 10%**<br>**($ng\ g^{-1}$)** | **HAc 3%**<br>**($ng\ g^{-1}$)** | **SML**<br>**($mg\ kg^{-1}$)** | **Remarks** |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 2.52<br>249.1589 | $[MNa]^+$ | Caprolactam oligomer (n=2)<br>C12H22N2O2 | 1 | 271 ± 40 | <LOD | <LOD | not listed | Polyamide oligomer |
| **2** | 2.74<br>114.0918 | $[MH]^+$ | Caprolactam✓✓<br>C6H11NO | 1 | 69.9 ±14.6 | 129 ±10 | 122 ±29 | 15 | Polyamide monomer |
| **3** | 3.25<br>362.2425 | $[MNa]^+$ | Caprolactam oligomer (n=3)<br>C18H33N3O3 | 1 | 2600 ± 280 | <LOD | <LOD | not listed | Polyamide oligomer |
| **4** | 3.72<br>475.3268 | $[MNa]^+$ | Caprolactam oligomer (n=4)<br>C24H44N4O4 | 1 | 3450 ± 317 | <LOD | <LOD | not listed | Polyamide oligomer |
| **5** | 4.05<br>588.4082 | $[MNa]^+$ | Caprolactam oligomer (n=5)<br>C30H55N5O5 | 1 | 169 ± 34 | <LOD | <LOD | not listed | Polyamide oligomer |
| **6** | 4.31<br>259.0588 | $[MNa]^+$ | PA-DEG<br>C12H12O5 | 2 | 779 ± 142 | 240 ± 28 | 222 ± 39 | not listed | Polyurethane oligomer |
| **7** | 5.22<br>495.1267 | $[MNa]^+$ | PA-DEG-PA-DEG<br>C24H24O10 | 3 | 71.5 ± 7.9 | 32.3 ± 5.8 | 28.9 ± 7.3 | not listed | Polyurethane oligomer |
| **8** | 5.60<br>269.0617 | $[MNa]^+$ | Anhydride of monomethyl succinate<br>C10H14O7 | 6 | 57.7 ± 2.0 | 6.55 ± 2.89 | <LOD | not listed | |
| **9** | 6.02<br>311.2203 | $[MNa]^+$ | Glycerol monotridecanoate✓<br>C16H32O4 (EU FCM nº10) | 6 | 3.59 ± 0.11 | <LOD | <LOD | No SML | Lubricant |
| **10** | 6.38<br>233.1536 | $[M-H]^-$ | 3,5-di-tert-butyl-4-hydroxybenzaldehyde✓✓<br>C15H22O2 | 10 | 2.89 ± 0.56 | <LOD | <LOD | not listed | |
| **11** | 6.46<br>383.2782 | $[MNa]^+$ | Erythritol monopalmitate<br>C20H40O5 | 6 | 9.28 ± 0.33 | <LOD | <LOD | not listed | |
| **12** | 6.84<br>425.2158 | $[MNa]^+$ | Tributyl acetylcitrate✓✓<br>C20H34O8 | 5 | 29.8 ± 1.0 | <LOD | <LOD | 60 (G32) | Plastizicer |
| **13** | 7.19<br>679.4187 | $[MNa]^+$ | Irganox 1010✓✓<br>C73H108O12 | 9 | 1602 ± 358 | <LOD | <LOD | No SML | Antioxidant |
| **14** | 7.63<br>391.2831 | $[MH]^+$ | Bis(2-ethylhexyl) phthalate✓✓<br>C24H38O4 | 8 | 53.3 ± 3.7 | <LOD | <LOD | 1.5<br>60<br>(G32) | Plastizicer |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **15** | 7.88<br>367.2822 | $[MNa]^+$ | Glycerol monoheptadecanoate✓<br>C20H40O4 (EU FCM nº10) | 6 | 19.5 ± 3.3 | <LOD | <LOD | No SML | Lubricant |
| **16** | 8.11<br>393.2999 | $[MNa]^+$ | Bis(2-ethylhexyl) adipate✓✓<br>C22H42O4 | 4 | 47.1 ± 2.3 | <LOD | <LOD | 18<br>60 (G32) | Plastizicer |
| **17** | 8.59<br>395.3137 | $[MNa]^+$ | Glycerol mononondecanoate✓<br>C22H44O4 (EU FCM nº10) | 6 | 10.3 ±1.3 | <LOD | <LOD | No SML | Lubricant |

LOD: detection limit; PA: phthalic acid; DEG: diethylene glycol; EU FCM: European food contact number in EU/10/2011
✓confirmed by MassFragment; ✓✓confirmed by internal standard
(G32) Specific migration limit for the combination of substances present in group 32.

**Table 2.-** Compounds detected in migration from [PET//Al//PA//CPP]FCS multilayer material. Retention time (rt) and measured mass (mass), type of ion found (adduct), compound candidate, molecular formula (MF), quantification standard (QS). Migration values in different food simulants and specific migration limit (SML) according to EU/10/2011 Regulation.

| **No** | **rt**<br>**mass** | **Adduct** | **Candidate**<br>**MF** | **QS** | **EtOH 95% (ng g$^{-1}$)** | **EtOH 10% (ng g$^{-1}$)** | **HAc 3% (ng g$^{-1}$)** | **SML (mg kg$^{-1}$)** | **Remarks** |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 2.73<br>114.0922 | $[MNa]^+$ | Caprolactam✓✓<br>C6H11NO | 1 | 1300±127 | 882±44 | 1040±170 | 15 | Polyamide monomer |
| **2** | 3.00<br>257.0999 | $[MNa]^+$ | **AA-DEG +$H_2O$**<br>**C10H18O6** | 2 | <LOD | <LOD | 51.2±10.1 | not listed | PU oligomer |
| **3** | 3.23<br>362.2425 | $[MNa]^+$ | Caprolactam oligomer (n=3)<br>C18H33N3O3 | 1 | 18.4±6.2 | 20.9±4.7 | <LOD | not listed | Polyamide oligomer |
| **4** | 3.55<br>239.0905 | $[MNa]^+$ | AA-DEG✓✓<br>C10H16O5 | 2 | 759±72 | 366±38 | 57.9±11.9 | not listed | PU oligomer |
| **5** | 3.71<br>475.3269 | $[MNa]^+$ | Caprolactam oligomer (n=4)<br>C24H44N4O4 | 1 | 35.5±11.3 | 28.0±7.0 | <LOD | not listed | Polyamide oligomer |
| **6** | 4.03<br>588.4092 | $[MNa]^+$ | Caprolactam oligomer (n=5)<br>C30H55N5O5 | 1 | 31.8±10.1 | 18.8±5.1 | <LOD | not listed | Polyamide oligomer |
| **7** | 4.25<br>701.5039 | $[MNa]^+$ | Caprolactam oligomer (n=6)<br>C36H66N6O6 | 1 | 8.34±0.98 | 13.3±2.5 | <LOD | not listed | Polyamide oligomer |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **8** | 4.33 255.1207 | [MNa]+ | **AA-NPG + $H_2O$ C11H20O5** | 2 | <LOD | <LOD | 12.80 ± 1.2 | not listed | PU oligomer |
| **9** | 4.55 299.1463 | [MNa]+ | **AA-dHAE ($C_7$) +$H_2O$ C13H24O6** | 2 | <LOD | <LOD | 13.52 ± 0.68 | not listed | PU oligomer |
| **10** | 4.70 267.1210 | [MNa]+ | AA-DPG C12H20O5 | 2 | 9.65±1.19 | 17.6±0.2 | 2.65±0.52 | not listed | PU oligomer |
| **11** | 4.76 455.1890 | [MNa]+ | AA-DEG-AA-DEG C20H32O10 | 3 | 5.87±0.94 | 4.49±0.20 | <LOD | not listed | PU oligomer |
| **12** | 4.93 237.1108 | [MNa]+ | AA-NPG C11H18O4 | 2 | 1.50±0.30 | 1.98±0.70 | <LOD | not listed | PU oligomer |
| **13** | 5.10 475.1592 | [MNa]+ | AA-DEG-PA-DEG✓✓ C22H28O10Na | 3 | 12.1±2.1 | 8.87±0.41 | <LOQ | not listed | PU oligomer |
| **14** | 5.18 495.1298 | [MNa]+ | PA-DEG-PA-DEG C24H24O10 | 3 | <LOD | <LOD | <LOQ | not listed | PU oligomer |
| **15** | 5.23 281.1380 | [MNa]+ | AA- dHAE ($C_7$) C13H22O5 | 2 | 687±55 | 344±13 | 40.2±10.0 | not listed | PU oligomer |
| **16** | 5.50 445.1492 | [MNa]+ | PA-EG-AA-PG C18H20O8 | 3 | 45.5±4.8 | 34.8±0.9 | 7.99±1.36 | not listed | PU oligomer |
| **17** | 5.63 453.2098 | [MNa]+ | AA-DEG-AA-NPG C21H34O9 | 3 | 25.1±2.6 | 25.7±0.8 | <LOQ | not listed | PU oligomer |
| **18** | 5.96 473.1801 | [MNa]+ | PA-DEG-AA-NPG C23H30O9 | 3 | 60.1±6.1 | 38.8±1.6 | 7.84±1.52 | not listed | PU oligomer |
| **19** | 6.27 443.1696 | [MNa]+ | PA-EG-AA-HD C22H28O8 | 3 | 48.7±5.1 | 8.83±0.3 | <LOQ | not listed | PU oligomer |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **20** | 6.58<br>471.1994 | $[MNa]^+$ | PA-DEG-PA-BD<br>C24H32O8 | 3 | 43.3±5.6 | <LOQ | <LOQ | not listed | PU oligomer |
| **21** | 6.08<br>395.2433 | $[M-H]^-$ | Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, 3-hydroxy-2,2-bis(hydroxymethyl)propyl ester<br>C22H36O3 | 7 | 2.29±0.45 | 4.46±0.17 | <LOQ | No SML | Antioxidant |
| **22** | 6.28<br>277.1809 | $[M-H]^-$ | Irganox 1310<br>C17H26O3 | 7 | 18.1±2.6 | 9.81±0.50 | <LOQ | No SML | Antioxidant |
| **23** | 6.39<br>233.1532 | $[M-H]^-$ | 3,5-di-tert-butyl-4-hydroxybenzaldehyde✓✓<br>C15H22O2 | 10 | 6.25±1.1 | <LOD | <LOD | not listed | |
| **24** | 10.47<br>1199 | $[MNa]^+$ | Irganox 1010 ✓✓<br>C73H108O12 | 9 | 1010±142 | <LOD | <LOD | No SML | Antioxidant |

LOD: detection limit; LOQ: quantification limit ; AA: adipic acid; PA: phthalic acid; DEG: diethylene glycol; NPG: neopentilglycol; EG: ethylene glycol; dHAE: dihydroxyalkyl ether; DPG: Dypropylen glycol; PG: propylene glycol ; BD: butylene glycol; HD: hexanediol; PU: polyrethane

**Table 3.** Standards used for quantification in migration analysis, working range, determination coefficient ($R^2$) and limits of detection (LOD) and quantification (LOQ)

| No | Compound | Working range (µg g$^{-1}$) | $R^2$ | LOD (µg g$^{-1}$) | LOQ (µg g$^{-1}$) |
|---|---|---|---|---|---|
| **1** | Caprolactam | 0.21 - 6.40 | 0.9986 | 0.07 | 0.21 |
| **2** | Oligomer AA-DEG | 0.04 - 1.27 | 0.9922 | 0.01 | 0.04 |
| **3** | Oligomer AA-DEG-IPA-DEG | 0.03 - 1.70 | 0.9921 | 0.01 | 0.03 |
| **4** | Diethylhexyl adipate | 0.03 - 0.59 | 0.9921 | 0.01 | 0.03 |
| **5** | Tributy acetylcytrate | 0.04 - 0.60 | 0.9963 | 0.01 | 0.04 |
| **6** | Glycerol monostearate | 0.04 - 1.95 | 0.9907 | 0.01 | 0.04 |
| **7** | Butyl 4-hydroxybenzoate | 0.05 - 2.53 | 0.9971 | 0.02 | 0.05 |
| **8** | Dioctyl phtalate | 0.03 - 1.19 | 0.9970 | 0.01 | 0.03 |
| **9** | Irganox 1010 | 0.03 - 0.31 | 0.9984 | 0.01 | 0.03 |
| **10** | 3,5-di-tert-butyl-4-hydroxybenzaldehyde | 0.02 - 1.56 | 0.9960 | 0.004 | 0.02 |

AA: adipic acid; DEG: diethylene glycol, IPA: isophthalic acid

Table 2 shows migration from material $[PET//Al//PA//CPP]_{FCS}$. Caprolactam and its oligomers were also found in migration from this material. As it happened in the previous material, oligomer n=4 showed the highest values. This material showed a high quantity of polyurethane oligomers in migration, made up of diacids such as adipic acid (AA) or phthalic acid (PA), and diols such as diethylene glycol (DEG), neopentyl glycol (NPG), dipropylene glycol (DPG), dihydroxyalkyl ethers (dHAE), ethylene glycol (EG), propylene glycol (PG), butylene glycol (BD) or hexanediol (HD). None of them was confirmed with standard because the commercial standards were not available. It was not possible either confirmation by MassFragment since sodium adducts were formed and no fragmentation was observed. Therefore, the identification was based on the possible combination among diacid and diol compounds and taking into account their characteristic molecular mass. Only compounds with migration values above 0.01 mg kg$^{-1}$ are shown. It is remarkable that some compounds were only found in migration to acetic acid 3%, such as 3.00_257.0999, 4.33_255.1207 and 4.55_299.1463 (retention time_mass).

Figure 1 shows the chromatograms of migration solutions after 10 days at 60 ºC in acetic acid 3% w/v and ethanol 10% v/v. According to their mass, these

compounds corresponded to the cyclic oligomers AA-DEG, AA-NPG and AA-dHAE plus $H_2O$. They could be the consequence of the hydrolysis of the cyclic esters and the opening of the ring due to the acidic medium. This hypothesis is in agreement with previous studies (Carrizo, Maccagnan, Félix, Nerín, & Bosetti, 2015). Figure 2 shows the high collision energy spectra for AA-DEG (a) and its hydrolyzed form AA-DEG + $H_2O$ (b). Fragments observed successfully matched with the proposed structure. Even though opening of the ring could take place in both the ether and the ester oxygen, the first option was selected as the most likely one. The second option would produce an acid and the analysis in ESI- mode did not show any carboxylic compound. This behavior was found also for the oligomers AA-NPG and AA-dHAE ($C_7$). These results showed that the hydroxylation, and therefore the opening of the cycle, is more likely when the oligomer was made up of adipic acid rather than phthalic acid and when only 1 diacid and 1 diol composed the oligomer. Three antioxidants were also detected in migration from this material: Agidol 110, Irganox 1310 and Irganox 1010.

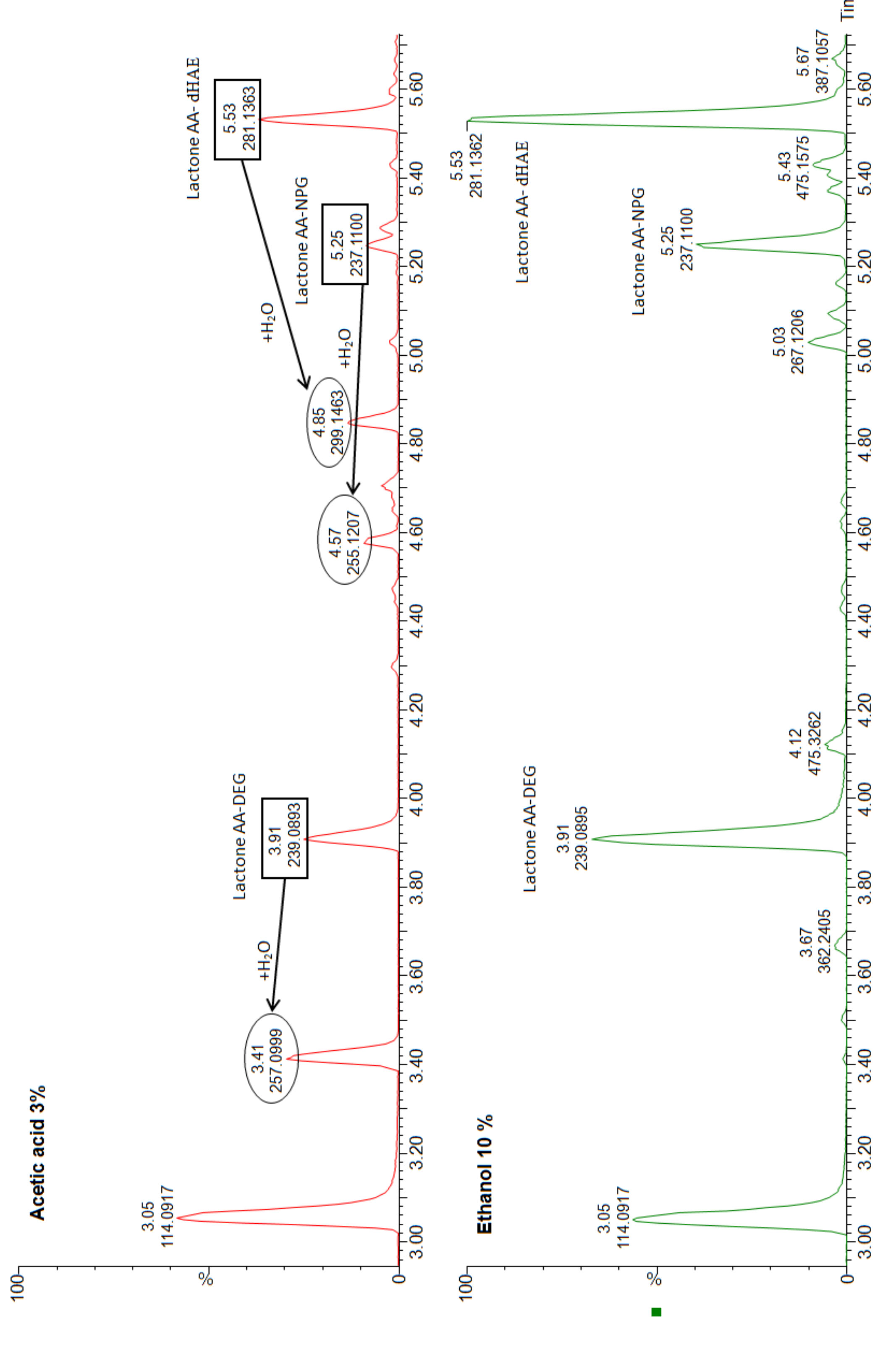


**Figure 1.** Chromatograms of migration test in acetic acid (up) and ethanol 10% (down) after 10 days at 60 °C.

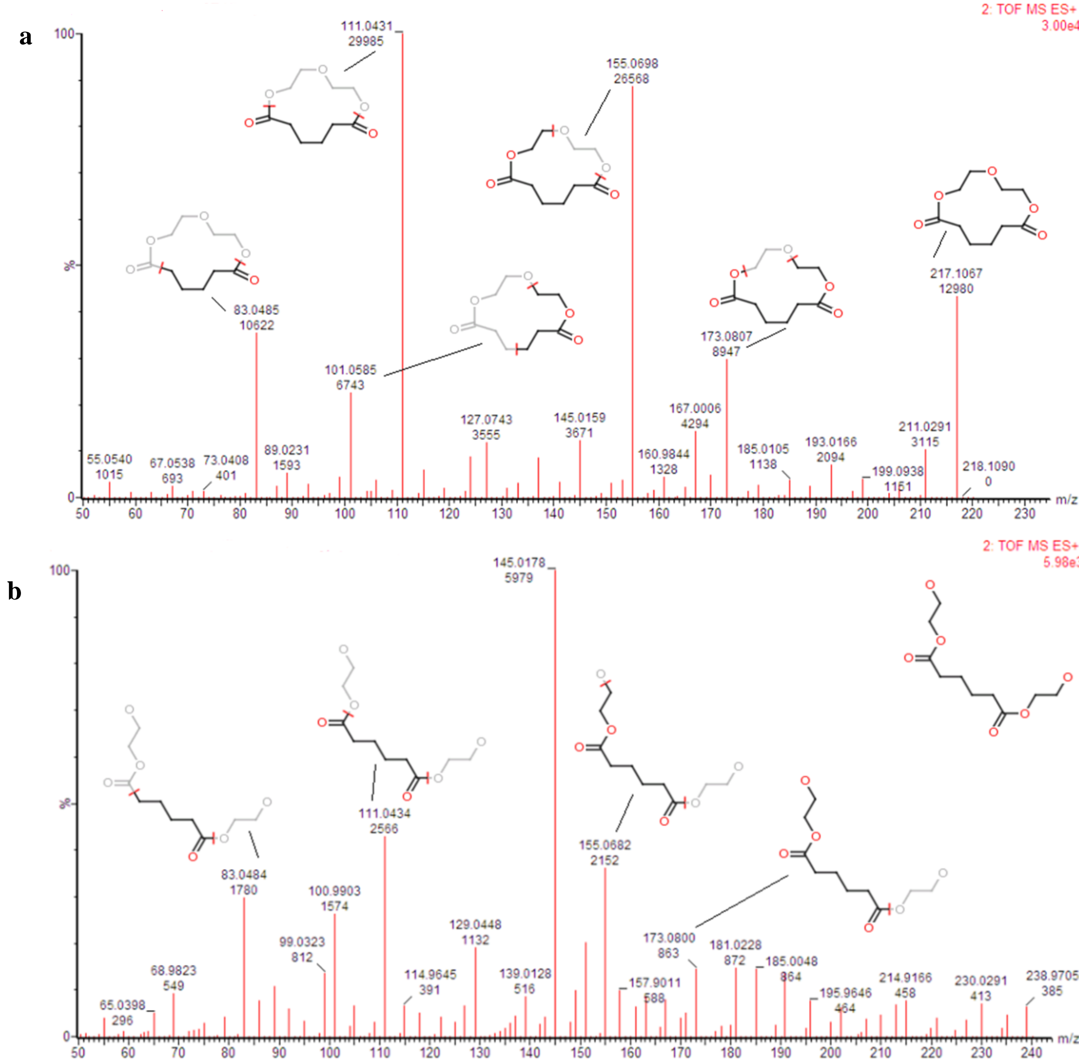


**Figure 2.** The high collision energy spectra for AA-DEG (a) and its hydrolyzed form (b) with their fragments.

## 5.2. Hydrolysis of AA-DEG oligomer in acetic acid 3 % (w/v) overtime

The analysis of AA-DEG and its hydrolyzed formed AA-DEG+$H_2O$ in the acetic acid 3% simulant over time showed a progressive reduction of the amount of lactone (figure 3). In contrast, the hydrolyzed lactone started increasing after 1 day of storage. To measure these compounds, the oligomer AA-DEG standard was used. Its working range was 0.033-2264 µg $g^{-1}$and 0.011 µg $g^{-1}$was its limits of detection (LOD).

Lactone AA-DEG decreased by 52 % (1046-506 ng $g^{-1}$) after 10 days and hydrolyzed lactone increased to 74.60 ng $g^{-1}$. On the other hand, in water medium no changes in AA-DEG concentration were observed. The injection of the acidic solutions in the UPLC-QToF, both in positive and negative mode, did not show any additional compound. In addition, the monomers that made up the lactone, adipic acid and diethylene glycol, were also analyzed by UPLC-QqQ in order to check if they were also the resulting reaction products of lactones hydrolyzation. The results showed that none of the monomers was present above the limits of detection (LOD DEG= 3 ng $g^{-1}$, LOD AA=13 ng $g^{-1}$).

Transformations of lactones to their opened form decrease its theoretical toxicity in most cases. Lactone AA-NPG belongs to class I but AA-DEG and AA-dHAE ($C_7$) belong to class III Cramer group, what means the highest theoretical toxicity, having a maximum daily intake of 0.09 mg/person/day according to Cramer. However, their opened hydrolyzed forms belong to class I, what means lower toxicity and a higher allowed daily intakes (1.8 mg/person/day).

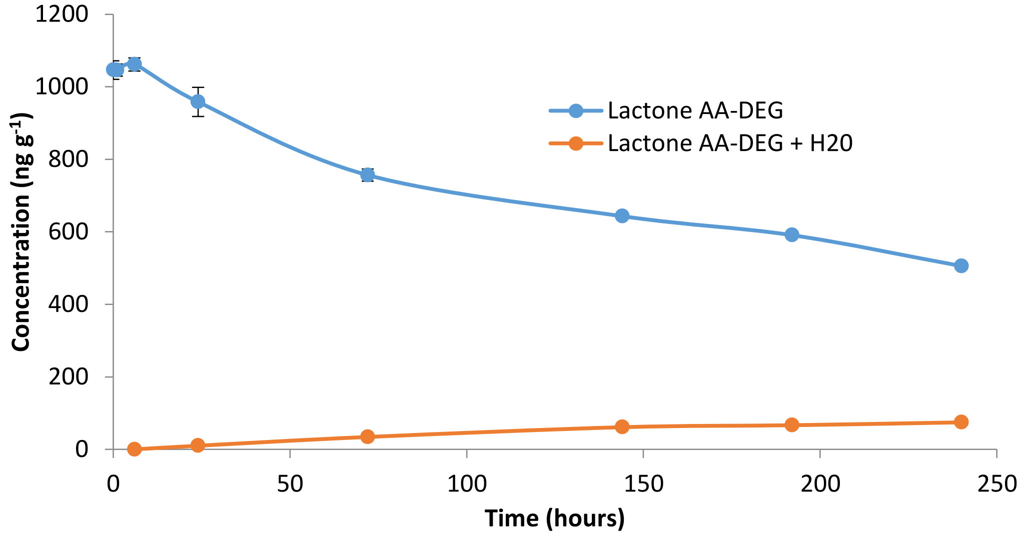


**Figure 3.** Evolution of lactone AA-DEG and hydrated lactone AA-DEG in acid medium over time.

## 5.3. Kinetic study of migration

Since kinetics play a critical role in the final food packaging migration, migration kinetic study, both specific and overall migration, were performed from both materials. Figures 4a and 4b show the results for overall migration of materials [PET//PA//CPP] and [PET//Foil//PA//CPP] respectively. In both cases overall migration was higher with ethanol 95% v/v as simulant, reaching values after 10 days of 7.5 and 6.6 µg $g^{-1}$ while for ethanol 10% v/v and acetic acid 3% w/v very similar kinetic behaviour was obtained over time, reaching values always below 5 µg $g^{-1}$. In most cases the maximum migration values and thus inertia to transfer non-volatile substances from both packaging materials was reached after 144-192 hours (days 6-8) and afterwards they remained stable. Small differences between the migration values reached between material 1 and 2 sere found, attributed mainly to the presence of aluminum foil as functional barrier. Finally, in both cases, the established limit for global migration was achieved in the Regulation of the European Commission 10/2011 of 60 mg $kg^{-1}$.

The kinetic study of specific migration was performed for the cyclic ester PA-DEG in [PET//PA//CPP] (figure 4c) and the cyclic ester AA-DEG in [PET//Al//PA//CPP] (figure 4d). Specific migration of both lactones increased over time until 144-192 hours (days 6-8) where they reached maximum values and afterwards they remained constant. It is interesting to remark that the profile of overall and specific migration is very similar, what means that no other compounds different from oligomers migrate from the materials at a significant concentration. As happened for overall migration, the simulant with the highest migration values was ethanol 95% v/v. However, in this case, ethanol 10% v/v and acetic acid 3% w/v showed different values, especially for AA-DEG. This was probably due to the hydrolyzation of the cyclic ester in acidic medium, which decreased its concentration in acetic acid over time, as it was observed in the results from section 5.2. The results showed a different kinetic pattern for this compound, since a slight decrease over time was observed.

Comparing migration of PA-DEG and AA-DEG, it can be observed that PA-DEG migration values in aqueous simulants (ethanol 10% v/v, acetic acid 3% w/v) was more similar to ethanol 95% v/v than in the case of AA-DEG, what could be attributed to the higher polarity of PA-DEG.

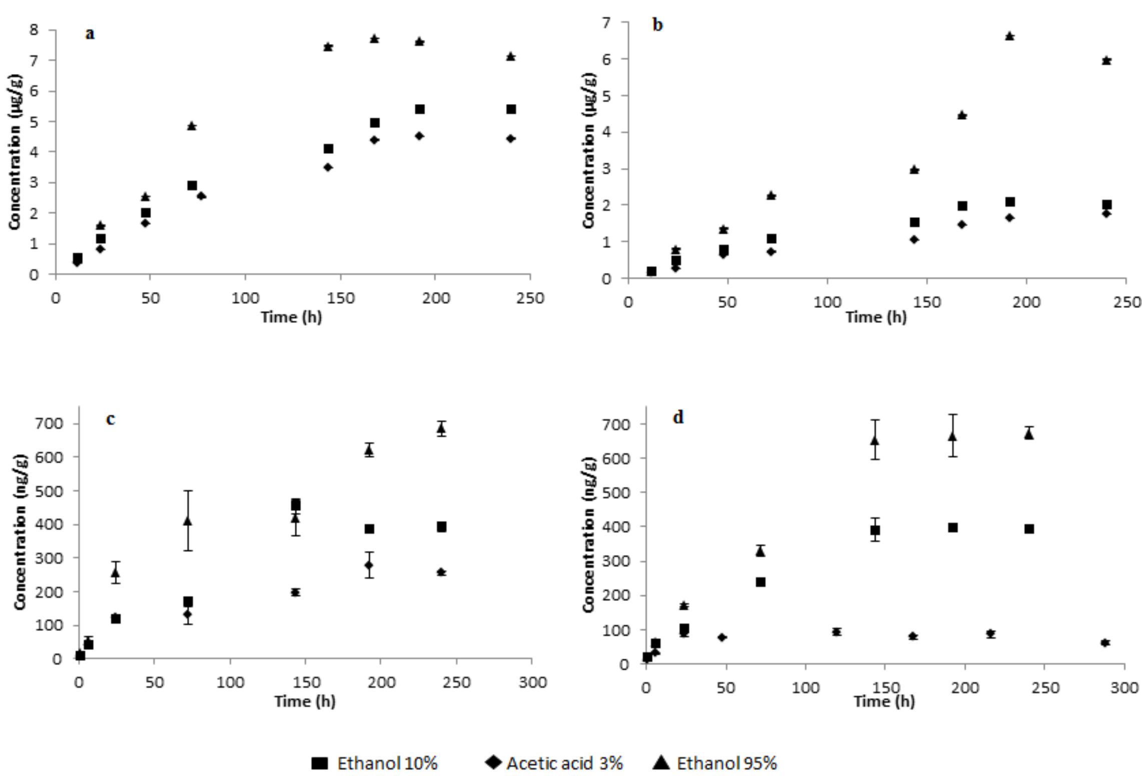


**Figure 4.** Kinetic study for overall migration of [PET//PA//CPP] material (a) and [PET//Foil//PA//CPP] material (b) and kinetic study of specific migration of the cyclic ester PA-DEG in [PET//PA//CPP] (c) and the cyclic ester AA-DEG in [PET//Al//PA//CPP] (d) in three simulants.

# 6. CONCLUSIONS

UPLC-QToF has been demonstrated to be a powerful tool for identifying compounds and NIAS migrated from the adhesives used to laminate food packaging materials. In these materials, cyclic esters coming from PU adhesives

were the main migrants in all simulants, which corroborates the migration from internal material layers and the importance of NIAS screening.

Comparison between migration values in the three simulants showed in most cases that ethanol 95% v/v was the simulant with the highest values. Ethanol 95% was used as substitute of simulant D2 (vegetal oil), proposed for food with free fats at the surface. Nevertheless, simulant D1 (ethanol 50%, v/v) that it has been also proposed for fatty food, was not evaluated in this study and it probably would provide less migration concentration values. According to the results obtained, these materials should be used more cautiously with food with free fats at the surface but probably could be safely used with lower fat content food. Finally, overall migration kinetics followed a similar pattern than specific migration kinetics, what confirms that the equilibrium was reached in both cases under the selected experimental conditions.

The results from degradation of cyclic oligomers in acidic media showed that the concentration of some cyclic oligomers coming from PU adhesives can decrease over time if they are in acidic media. The resultant reaction products showed in most cases a lower theoretical toxicity, what is very positive for health consumers safety.

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# Capítulo 2

## *Determination of oligomers in virgin and recycled polyethylene terephthalate (PET) samples by UPLC-QToF*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
3. INTRODUCTION
4. MATERIAL AND METHODS
5. RESULTS AND DISCUSSION
6. CONCLUSIONS
7. REFERENCES

# 1. RESUMEN

Un oligómero es una molécula que consiste en un número reducido de unidades de monómero. Pueden formarse durante la fabricación de un polímero y también posteriormente debido a procesos de degradación del polímero durante su almacenamiento o incluso durante su uso. Ya que los oligómeros no están incluidos en ninguna librería química, su identificación es un proceso complejo. En este trabajo, se han determinado los oligómeros presentes en 20 diferentes muestras de pellets de tereftalato de polietileno (PET). Se llevaron a cabo dos procedimientos de tratamiento de muestra, extracción con solvente y disolución total, con el fin de seleccionar el procedimiento con mayor eficiencia. El análisis fue realizado mediante UPLC-QToF. El uso de espectrometría de masas de alta resolución permitió la elucidación de estos compuestos y su correcta identificación. Los principales oligómeros identificados fueron cíclicos y lineales de primera, segunda y tercera serie. Todos ellos estaban compuestos por ácido tereftálico (TPA), dietilenglicol (DEG), y etilenglicol (EG). En ambos procedimientos los valores de concentración fueron muy diferentes. En la disolución total de las muestras de PET, la concentración de los oligómeros fue siempre, al menos, 10 veces mayor que en la extracción; algunos compuestos fueron solo detectados cuando se utilizó la disolución total. Los resultados mostraron que los oligómeros con valores de concentración más altos fueron dímeros y trímeros cíclicos y lineales de la primera y segunda serie. El oligómero con mayor valor de concentración fue $TPA_2$-EG-DEG, que fue encontrado en todas las muestras en un intervalo de concentraciones de 2493 a 19290 ng $g^{-1}$ PET. No hubo diferencias entre el PET virgen y reciclado. Los experimentos de migración fueron llevados a cabo en dos botellas de PET, y los resultados mostraron que la transferencia de la mayoría de estos oligómeros fue en simulantes alimentarios grasos (etanol 95%).

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El principal objetivo de este capítulo fue determinar los principales oligómeros, tanto cíclicos como lineales, presentes en muestras de PET y determinar las posibles diferencias en el perfil de oligómeros de muestras de PET reciclado y virgen. Para ello fue necesario previamente seleccionar el tratamiento de muestra más eficiente para la determinación de estos compuestos. Fue también un objetivo prioritario, el determinar los oligómeros de migración mayoritarios en función de la naturaleza del alimento y evaluar de esta manera el riesgo que suponen para el consumidor. Para ello, se detallan a continuación las tareas concretas que se llevaron a cabo en este capítulo:

- Búsqueda de bibliografía sobre los oligómeros presentes en muestras de PET.

- Comparación de la eficiencia de dos tratamientos de muestras, extracción y disolución total, para identificar el mayor número de oligómeros

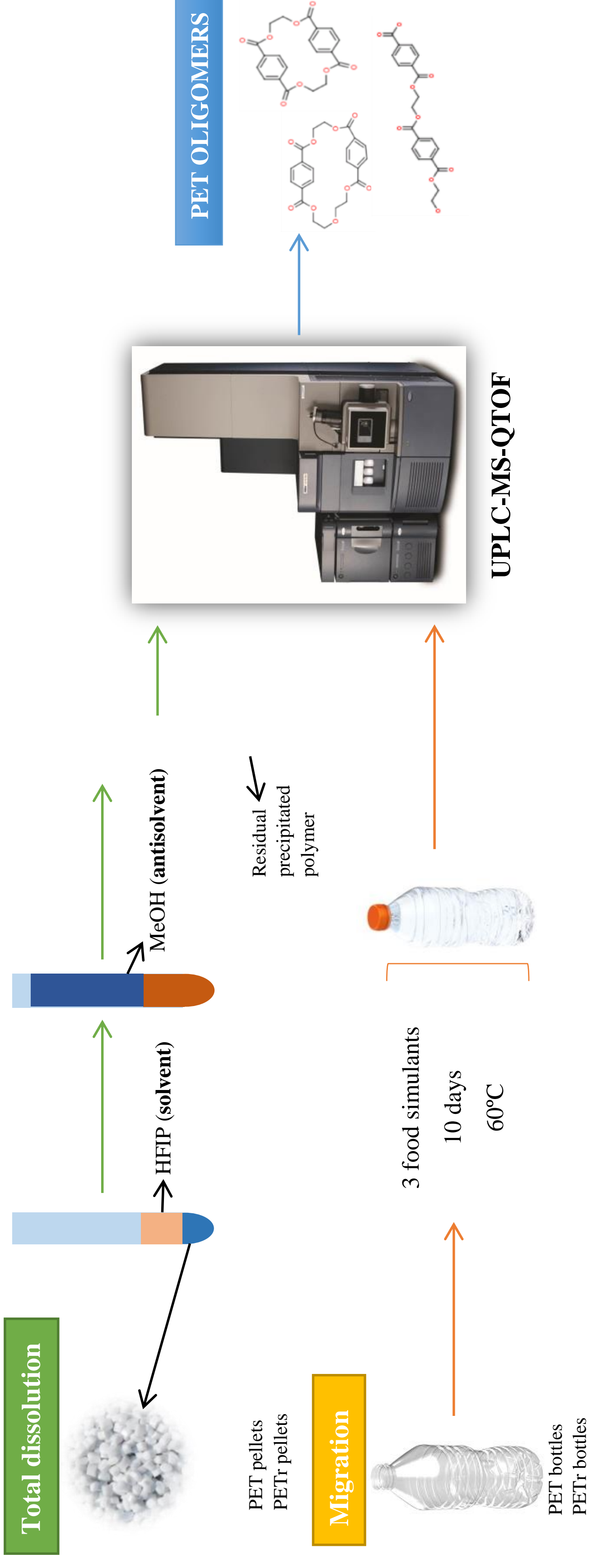
- Identificación y semi-cuantificación de los oligómeros presentes en pellets de PET.

- Estudio de los tipos de oligómeros del PET presentes en el material: serie 1, serie 2 y serie 3.

- Estudio de la distribución de los tipos de oligómeros en PET reciclado y virgen.

- Realización de ensayos de migración y determinación de los oligómeros migrantes desde botellas de PET.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 2.




**Esquema 2:** Diseño experimental del Capítulo 2

# 3. INTRODUCTION

Polyethylene terephthalate (PET) has been considered as one of the most important engineering polymers in the past two decades. It is regarded as an excellent material for many applications and is widely used for food packaging due to its physico-chemical properties such as good gas barrier properties, low diffusivity, good mechanical and thermomechanical properties, highly inert material, transparency and good processability (Begley *, Biles, Cunningham, & Piringer, 2004; Li, Wang, Lin, & Hu, 2016; Welle, 2014). In addition, it can be said that probably is the polymer with the lowest number and concentration of additives, as the pristine properties make it appropriate for many applications, especially in food contact area. Another important advantage of PET as a packaging material is the good recyclability, low diffusivity and low uptake characteristics (Awaja & Pavel, 2005; Bayer, 2002; Dutra, Freire, Nerín, Bentayeb, Rodriguez-Lafuente, Aznar, et al., 2014; Félix, Alfaro, & Nerín, 2011; Oliveira, Echegoyen, Cruz, & Nerin, 2014; Welle, 2011).

PET is manufactured by polymerization of ethylene glycol (EG) and terephthalic acid (TPA) or dimethyl terephthalate (DMT) during a polycondensation reaction. Amorphous preforms are obtained by processing the PET granules (pellets). Then, preforms are stretched by a blow-molding process to achieve bi-axially oriented bottles. Each step of this process could generate new substances that will be part of the polymer, posing a risk of unacceptable migration of these substances from PET bottles into foodstuffs in contact. They are defined as non-intentionally added substances (NIAS) and it is important to investigate their presence in each step of the manufacturing chain, in order to avoid its formation and therefore their presence in the final food contact materials (Awaja & Pavel, 2005; Dutra, Pezo, Freire, Nerín, & Reyes, 2011; Kassouf, Maalouly, Chebib, Rutledge, & Ducruet, 2013; Nerìn, Albiñana, Philo, Castle, Raffael, & Simoneau, 2003; Oliveira, Echegoyen, Cruz, & Nerin, 2014).

The most common NIAS in PET polymers are oligomers. Their identification is difficult as they are not included in any database and there are not standards available. Thus, the identification of their chemical structure has to be done based on their fragmentation mass spectra and selecting different analysis conditions. Several oligomers have been identified in this work, either cyclic or open oligomers.

The main NIAS identified in this material were cyclic oligomers from the first and second series besides the linear ones due to ring tension. The first series oligomers are composed by an equal number of terephthalic acid and ethylene glycol units, whereas in the second series, a single ethylene glycol unit is substituted by a diethylene glycol unit. Third series can also be formed, and in this case, two ethylene glycol units are replaced by two diethylene glycol units. The second and third series of oligomers arise because of the DEG formation during PET production as a by-product. All these oligomers have potential to migrate into foods and for this reason their identification and quantification in PET used for food packaging is very important (Barnes, Damant, Startin, & Castle, 1995; Begley, Dennison, & Hollifield, 1990; Hoppe, Fornari, de Voogt, & Franz, 2017; Kim & Lee, 2012; Monteiro, Nerín, & Reyes, 1996; Nasser, Lopes, Eberlin, & Monteiro, 2005; Úbeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017).

Since high concentration levels of the unknown compounds facilitate its identification, it is frequent to first analyse directly the polymer in order to identify the potential migrants. Fortunately, many of NIAS identified in the polymer will never migrate at a concentration level that could endanger the human health.

In this work, virgin and recycled PET pellets from different sources, were analysed by UPLC-QToF and the oligomers were identified and quantified to determine their oligomers profile. As sample treatment, two procedures were performed and compared: extraction with dichloromethane and total

dissolution/precipitation, using hexafluoroisopropanol as solvent and methanol as antisolvent. The results are shown and discussed.

# 4. MATERIAL AND METHODS

## 4.1. Reagents

A cyclic ester oligomer composed by diethylene glycol (DEG), adipic acid (AA) and isophtalic acid (IPA), AA-DEG-IPA-DEG (95% w), was used as standard for oligomers quantification. It was synthetized and provided by an adhesives company. Its structure and purity were confirmed by NMR. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, CAS 920-66-1) was purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Methanol and dichloromethane were purchased from Panreac (Barcelona, Spain). Methanol and water for UPLC analysis (ultra LC–MS quality) were obtained from Baker (Deventer, The Netherlands).

## 4.2 Samples

Twenty different samples of PET pellets were supplied by different manufacturing companies. Samples 01, 04, 07, 09, 15, 16 were virgin PET and samples 02, 03, 05, 06, 08, 10, 11, 12, 13, 14, 17, 18, 19, 20 were recycled PET. In order to reduce samples to powder, they were cryogenically cooled using liquid nitrogen and then ground using a knife mill under liquid nitrogen. In this way, there was an improvement in the extraction efficiency and a better sample homogeneity.

## 4.3 Sample preparation

Two different procedures were tested for sample preparation:

### 4.3.1 Total dissolution

An amount of 0.4 g of ground PET was weighed in a 20 mL glass vial and 4 mL of HFIP were added. The vial was closed with a screw cap and kept in an

oven at 40 ºC for 24 hours in order to assure the complete dissolution of the polymer. After this time, it was cooled down at room temperature and then, 8 mL of methanol were added as anti-solvent. The vial was shaken in order to guarantee a deep contact between solvent and polymer. Thus, the vial was kept at 4 ºC for 1 hour to facilitate the polymer precipitation. The mixture was centrifuged at 4000 rpm for 10 minutes and the supernatant was extracted. Subsequently, the residual precipitated polymer was washed with 1 mL of pure methanol and the liquid phases were combined and weighed. Three replicates were prepared from each sample and several procedural blanks were also analysed.

### 4.3.2 Solvent extraction

An amount of 10 g of ground PET was weighed in a 100 mL glass container and three consecutive extractions with dichloromethane (DCM) were applied under the same conditions, but with different extraction volumes: 12 mL, 10 mL and 5 mL. In all of them the vial was kept in an ultrasonic bath for 1 hour and the supernatant was collected, filtered and transferred to a glass vial. The collected extracts were mixed together, evaporated to dryness under a gentle nitrogen current at low temperature and afterwards re-dissolved in 1 mL of methanol in ultrasonic bath. Finally, they were analyzed by UPLC-QToF. Three replicates and several procedural blanks were prepared from each sample.

## 4.4 Migration Test

Only 2 out of the 20 pellet samples were also available as bottles, PET 15 and PET 20. Migration test were performed with three food simulants for 10 days at 60 ºC. Bottles were totally filled with simulant A (ethanol 10% v/v) and simulant B (acetic acid 3 % w/v) as aqueous simulants and ethanol 95 % v/v as fat simulant. Three replicates for each simulant were carried out. Simulants and test conditions used for the migration assays were chosen according to the European Regulation 10/2011 (EC, 2011). The samples were analysed by UPLC-QToF. All the concentrations were corrected according to the rate of 6 $dm^2$ of

packaging material per 1 kg of simulant, in accordance with European Regulation 10/2011.

## 4.5 Instrumental analysis

### 4.5.1 Ultra-performance liquid chromatography (UPLC) coupled mass spectrometry detection with quadrupole-time-of-flight mass analyser (MS-QToF)

The UPLC-QToF system consisted of an ACQUITY UPLC chromatograph coupled to a Xevo G2 QToF mass spectrometer from Waters (Milford, MA, USA). An UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size) from Waters was used Flow rate was 0.3 mL min$^{-1}$, injection volume 10 µL and column temperature was set at 35 ºC. Mobile phase was water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). The separation was performed using a gradient elution: initial mobile phase A/B 98/2 was changed to A/B 0/100 over 6 minutes and afterwards maintained at this rate for 2 additional minutes.

Mass spectrometer was coupled to the UPLC system with an ESI probe. The following conditions were employed: positive ionization (ESI+) and negative ionization (ESI-), sensitivity mode, capillary voltage 2.5 kV, cone voltage 30 and 70 V, extraction cone 4 V, source temperature 120 ºC, desolvation temperature 450 ºC, cone gas flow rate of 20 L h$^{-1}$, and desolvation gas flow rate of 700 L h$^{-1}$. Acquisition was performed in MSE mode to allow using low and high collision energy (CE) in the collision cell during the same run. The mass spectrum at low energy (CE 4 V) provided information about the precursor ion (function 1) and the mass spectrum at high energy (CE ramp: from 15 to 30 V) information about fragment ions (function 2).

### 4.5.2 Identification of compounds detected by UPLC–MS-QToF

A screening test of non-volatile compounds present in PET samples was carried out on the extracts obtained from total dissolution and solvent extraction. Three different acquisition conditions were used for the analysis; this allowed achieving an overall view of the potential oligomers. MassLynx (v. 4.0) software was used to acquire and process the chromatographic and MS data. With spectra from function 1, the elemental formula was obtained. Then, with the use of function 2, the fragmentation spectra were obtained and the proposed candidates were checked through MassFragment® software from Waters. This software enabled us to evaluate and confirm whether the product ions detected in the high collision energy spectrum could be linked to the fragments generated from the chemical structures of the candidates proposed.

## 5. RESULTS AND DISCUSSION

PET oligomers were quantified by external calibration using the oligomer AA-DEG-IPA-DEG as standard. The working range in the instrument was 0.03-1.70 µg $g^{-1}$ and the coefficient of determination ($R^2$) obtained in the calibration curve was 0.9994. The limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.03 µg $g^{-1}$ respectively. The LOD and LOQ were calculated as the minimum concentration whose signal was equal to 3 times and 9 times the baseline noise.

In total dissolution, the content of oligomers was calculated following equation 1:

$$[\text{Oligomer}]_{TD}\ (g / g_{PET}) = [\text{Oligomer}]_s\ x\ g_s / g_{PET} \qquad \textit{Equation 1}$$

where $[\text{Oligomer}]_s$ is the concentration in the supernatant (g $g^{-1}$) obtained from the interpolation in the calibration curve, $g_s$ are the grams of supernatant after the polymer precipitation, and $g_{PET}$ the grams of PET dissolved.

In solvent extraction, the content of oligomers was calculated according to equation 2:

$[Oligomer]_{SE}$ (g / $g_{PET}$) = $[Oligomer]_e$ *x* $g_e$ / $g_{PET}$ *Equation 2*

where $[Oligomer]_e$ is the concentration in the methanol extract (g $g^{-1}$) obtained from the interpolation in the calibration curve, $g_e$ are the grams of methanol used for the reconstitution of the extract and $g_{PET}$ the grams of PET extracted.

## 5.1 Comparison between solvent extraction and total dissolution of PET samples

Four PET samples were analysed following the two different procedures described in “Sample preparation” section to determine which procedure was more efficient to identify profile PET oligomers.

Table 1 summarizes the concentration of the four compounds detected in all the selected samples (PET 11, 12, 13, 14) and in both sample treatments, total dissolution and solvent extraction. All the compounds selected were oligomers coming from PET polymer. They were composed by terephthalic acid (TPA), diethylene glycol (DEG) and ethylene glycol (EG): $(TPA\text{-}EG)_2$, $TPA_2$-EG-DEG, $(TPA\text{-}EG)_3$ and $TPA_3$-$EG_2$-DEG.

Some of the compounds, such as $TPA_3$-$EG_2$-DEG, were only detected when total dissolution was used. In total dissolution of PET samples, the concentration of oligomers was always, at least, 10 times higher than in solvent extraction. This was expected since in total dissolution, all the compounds will be present in the final extract, while in the solvent extraction procedure; the presence of compounds is limited by the surface of PET particles in contact with the solvent, the partition coefficient between PET and the solvent used for extraction and the physico-chemical properties of the compounds. The conditions applied for total dissolution were quite mild, so, the compounds identified cannot

**Table 1** Quantification of non-volatile compounds identified in PET11, PET12, PET13, PET14 samples in total dissolution (TD) and solvent extraction (SE).

| No | rt<br>mass | Candidate<br>MF | PET11 (ng g$^{-1}$ PET) | | PET12 (ng g$^{-1}$ PET) | | PET13 (ng g$^{-1}$ PET) | | PET14 (ng g$^{-1}$ PET) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | TD | SE | TD | SE | TD | SE | TD | SE |
| **1** | 7.51<br>451.1001 | $TPA_2$-EG-DEG<br>$C_{22}H_{20}O_9$ | 16400<br>±43 | 949<br>± 46 | 13800<br>±886 | 1280<br>±22 | 16000<br>±1100 | 1257<br>±40 | 13100<br>±794 | 1158<br>±13 |
| **2** | 8.10<br>407.0730 | $(TPA\text{-}EG)_2$<br>$C_{20}H_{16}O_8$ | 996<br>±11 | 60<br>± 4 | 873<br>±35 | 79<br>±11 | 770<br>±91 | 76<br>±1 | <LOQ | <LOD |
| **3** | 8.45_8.64<br>599.1180 | $(TPA\text{-}EG)_3$<br>$C_{30}H_{24}O_{12}$ | 4810<br>±106 | 73<br>± 8 | 4510<br>±232 | 102<br>±12 | 5424<br>±42 | <LOD | 5390<br>±619 | <LOD |
| **4** | 8.49<br>643.1443 | $TPA_3$-$EG_2$-DEG<br>$C_{32}H_{28}O_{13}$ | 1880<br>±4 | <LOD | 1590<br>±169 | <LOD | 1741<br>±45 | <LOD | 1950<br>±249 | <LOD |

Retention time (rt), measured mass (mass), compound candidate and molecular formula (MF).

TPA: terephthalic acid; DEG: diethylene glycol; EG: ethylene glycol; LOD: limit of detectionnd.

be attributed to the degradation of the polymer, as usually happens at high temperature. Therefore, total dissolution is recommended as sample treatment to know the potential migrants of the polymer.

## 5.2 Identification of oligomers in PET samples by UPLC-QToF and analytical features

Twenty PET pellet samples were analysed using the total dissolution protocol described in "Total dissolution" section. Table 2 shows the 14 PET oligomers identified and quantified by UPLC-QToF (Bentayeb, Batlle, Romero, & Nerín, 2007) and their concentration range in those samples where they were detected above the limit of detection (LOD). Relative standard deviation was always below 20%. Ten cyclic and 4 linear oligomers were detected, all of them composed by terephthalic acid (TPA) and ethylene glycol (EG) or diethylene glycol (DEG). Dimers, trimers, tetramers as well as pentamers were observed. Linear structures were only detected for dimers and trimers.

This analysis revealed three series of oligomers. In the first one, made up with oligomers containing only EG and TPA ($TPA_nEG_n$), seven different oligomers were determined, both cyclic and linear: TPA-EG, $(TPA\text{-}EG)_2+H_2O$, $(TPA\text{-}EG)_2$, $(TPA\text{-}EG)_3+H_20$, $(TPA\text{-}EG)_3$, $(TPA\text{-}EG)_4$ and $(TPA\text{-}EG)_5$. In the second series, where one EG monomer is replaced by a DEG unit $(TPA_nEG_{n-1})DEG$, five oligomers were identified: $TPA_2\text{-}EG\text{-}DEG+H_2O$, $TPA_2\text{-}EG\text{-}DEG$, $TPA_3\text{-}EG_2\text{-}DEG+H_2O$, $TPA_3\text{-}EG_2\text{-}DEG$ and $TPA_4\text{-}EG_3\text{-}DEG$. Finally, in the third series, where two EG monomers are replaced by two DEG units $(TPA_nEG_{n-2})DEG_2$, only 2 cyclic oligomers were detected, $(TPA\text{-}DEG)_2$ and $TPA_4\text{-}EG_2\text{-}DEG_2$.

**Table 2** Oligomers identified in total dissolution in 20 samples of PET pellets. Retention time (rt), measured mass (mass), type of ion found (adduct), compound candidate, molecular formula (MF), range of concentration and occurrence of oligomers in PET pellets.

| No | rt | mass | Adduct | Candidate MF | Remarks | Concentration (ng $g^{-1}$ PET) | Absolute Frecuency of Occurrence | Relative Frecuency of Occurrence (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 6.79 | 469.1104 | $[MNa]^+$ | $TPA_2$-EG-DEG+$H_20$ $C_{22}H_{22}O_{10}$ | Lineal dimer 2 series | 183-7719 | 14 | 70 |
| 2 | 6.84 | 193.0498 | [MH]+ | TPA-EG $C_{10}H_8O_4$ | Cyclic monomer | 183-270 | 14 | 70 |
| 3 | 6.85 | 425.0843 | $[MNa]^+$ | $(TPA-EG)_2+H_2O$ $C_{20}H_{18}O_9$ | Lineal dimer 1series | 183-507 | 18 | 90 |
| 4 | 7.32 | 495.126 | $[MNa]^+$ | $(TPA-DEG)_2$ $C_{24}H_{24}O_{10}$ | Cyclic dimer 3 series | 685-4843 | 20 | 100 |
| 5 | 7.35 | 451.0999 | $[MNa]^+$ | $TPA_2$-EG-DEG $C_{22}H_{20}O_9$ | Cyclic dimer 2 series | 2493-19290 | 20 | 100 |
| 6 | 7.35 | 879.21 | $[MNa]^+$ | $TPA_4$-$EG_2$-$DEG_2$ $C_{44}H_{40}O_{18}$ | Cyclic tetramer 3 series | 183-1102 | 19 | 95 |
| 7 | 7.54 | 661.1524 | $[MNa]^+$ | $TPA_3$-$EG_2$-DEG+$H_20$ $C_{32}H_{30}O_{14}$ | Lineal trimer 2 series | 183-1370 | 14 | 70 |
| 8 | 7.63 | 617.1263 | $[MNa]^+$ | $(TPA-EG)_3+H_20$ $C_{30}H_{26}O_{13}$ | Lineal trimer 1 series | 183-697 | 14 | 70 |
| 9 | 7.79 | 407.0738 | $[MNa]^+$ | $(TPA-EG)_2$ $C_{20}H_{16}O_8$ | Cyclic dimer 1 series | 183-1248 | 18 | 90 |
| 10 | 8.18 | 643.1419 | $[MNa]^+$ | $TPA_3$-$EG_2$-DEG $C_{32}H_{28}O_{13}$ | Cyclic trimer 2 series | 335-2219 | 20 | 100 |
| 11 | 8.3 | 599.1158 | $[MNa]^+$ | $(TPA-EG)_3$ $C_{30}H_{24}O_{12}$ | Cyclic trimer 1 series | 661-6978 | 20 | 100 |
| 12 | 8.5 | 835.1839 | $[MNa]^+$ | $TPA_4$-$EG_3$-DEG $C_{42}H_{36}O_{17}$ | Cyclic tetramer 2 series | 183-591 | 14 | 70 |
| 13 | 8.84 | 791.1578 | $[MNa]^+$ | $(TPA-EG)_4$ $C_{40}H_{32}O_{16}$ | Cyclic tetramer 1 series | 610-848 | 20 | 100 |
| 14 | 8.95 | 983.1998 | $[MNa]^+$ | $(TPA-EG)_5$ $C_{50}H_{40}O_{20}$ | Cyclic pentamer 1 series | 183-169 | 10 | 50 |

The frequency of occurrence of all oligomers was compiled and is shown in table 2. The absolute frequency of occurrence for each oligomer is based on recording the number of PET samples that contains that oligomer. The relative frequency of occurrence of each oligomer is related to the total number of samples analysed and expressed as percentage. The cyclic dimer of second and third series, $TPA_2$-EG-DEG and $(TPA\text{-}DEG)_2$; cyclic trimer of first and second series, $(TPA\text{-}EG)_3$ and $TPA_3$-$EG_2$-DEG; and cyclic tetramer of first series, $(TPA\text{-}EG)_4$, were identified in all samples so the relative and absolute frequencies of occurrence were 100%. The oligomer with the maximum concentration value was $TPA_2$-EG-DEG, that was found in all the samples in a concentration range from 2493 to 19290 ng $g^{-1}$ PET. All PET oligomers have common fragmentation spectra which confirm the similarity of their structures (Hoppe, Fornari, de Voogt, & Franz, 2017). Their common masses are 149.0240, 193.0503, 341.0659 and 385.0918 m/z. Figure 1a shows the high collision energy spectra of a second series PET dimer, linear (up) and cyclic (down). Figure 1b shows high collision energy spectra of first series PET dimer, linear (up) and cyclic (down). Fragments observed successfully matched with the proposed structures.

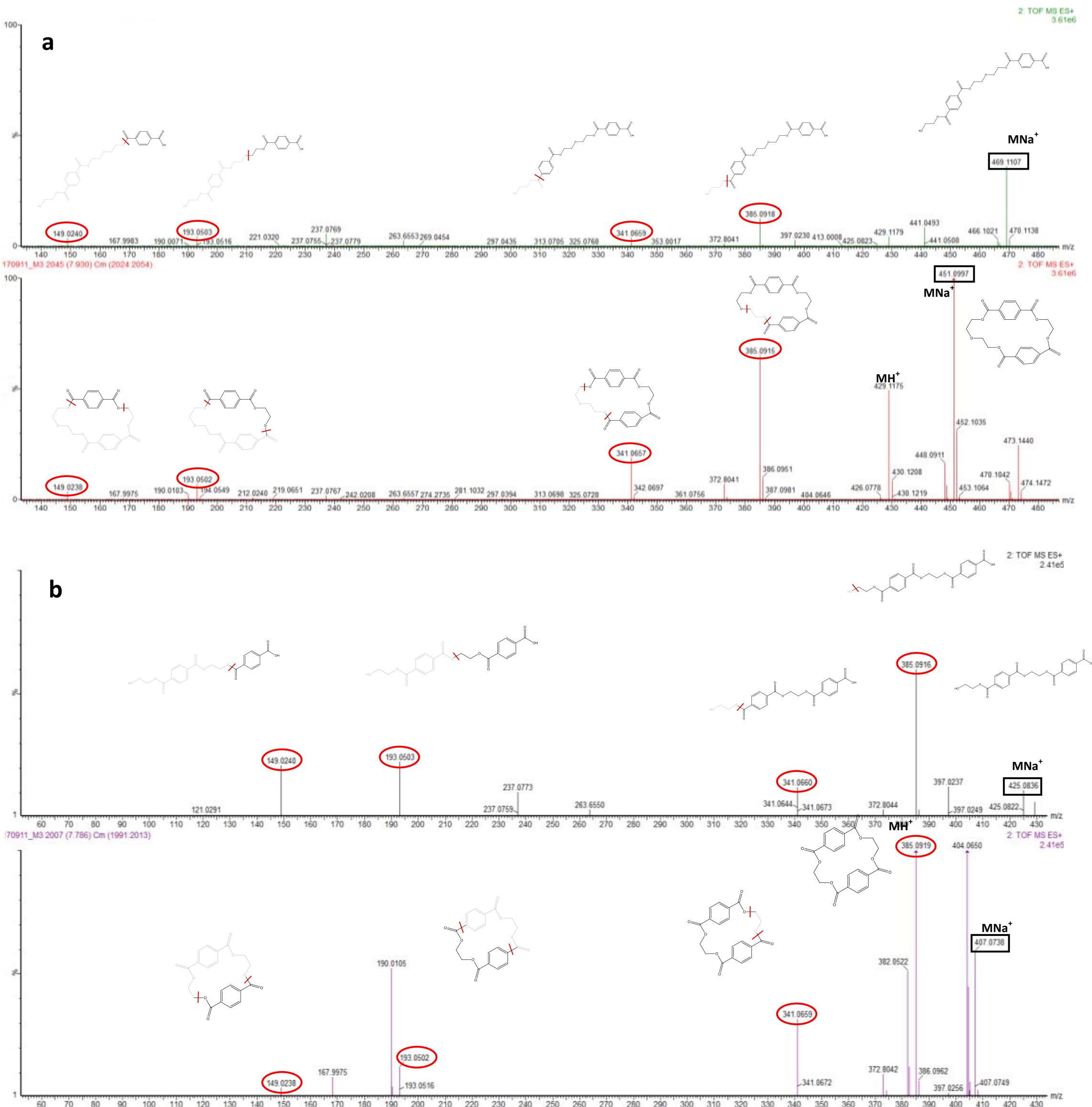



**Figure 1.** (a) High collision energy mass spectra of second the series PET dimer, lineal (up) and cyclic (down); (b) high collision energy mass spectra of the first series PET dimer, lineal (up) and cyclic (down).

As expected according to previous literature, the main compounds identified in PET samples were the cyclic and linear dimers and trimers from the first and second series, (Barnes, Damant, Startin, & Castle, 1995; Bryant & Semlyen, 1997; De A. Freire, Damant, Castle, & Reyes, 1999; Kim & Lee, 2012; Nasser, Lopes, Eberlin, & Monteiro, 2005), being the dimers from the second series the most abundant ones. The cyclic dimer from the third series was also

found at relative high concentration. It can be also highlighted that cyclic oligomers were much more abundant than the linear ones (Figure 2).

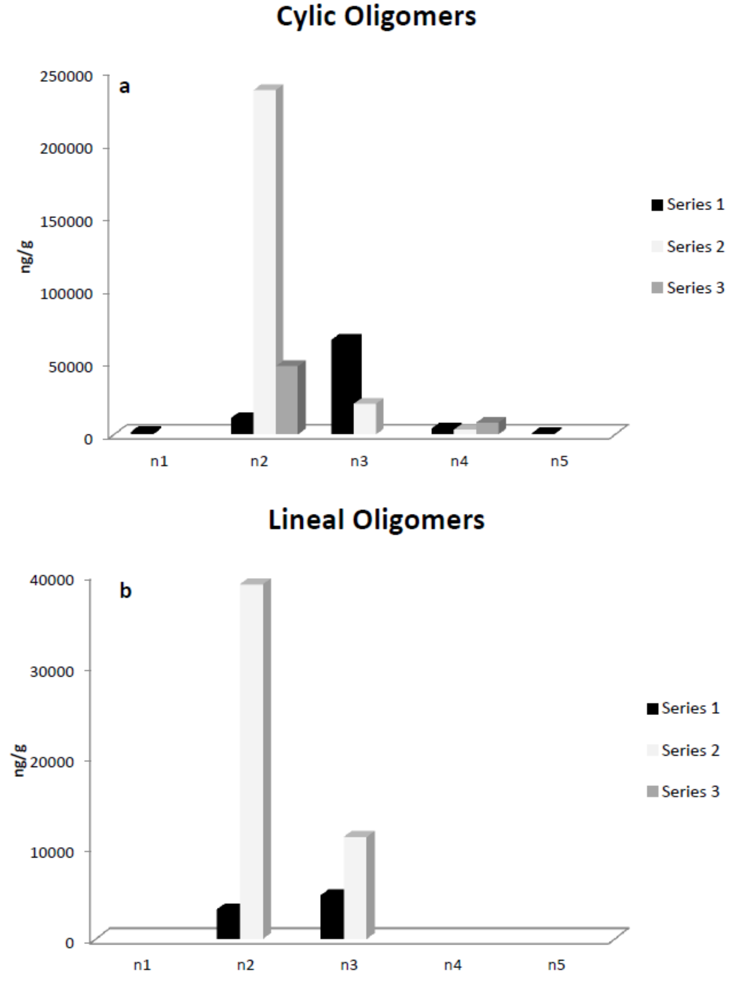



**Figure 2.** Total concentration (ng $g^{-1}$) of cyclic **(a)** and lineal **(b)** oligomers with different monomer units (n) in series 1, 2 and 3.

Furthermore, figure 3a shows the concentration of the 5 oligomers with the highest concentration in the 20 PET samples in order to see its distribution. The oligomers distribution was different in the studied samples. No visual differences in the concentration of the main oligomers was observed between virgin (V) and recycled (R) PET samples. A principal component analysis was also carried out with Unscrambler X software (Camo S. A) in order to check if there was a statistical sample grouping taking into account all the oligomers

present in the samples. PCA results did not show any aggrupation between virgin and recycled samples. A Pearson correlation analysis was also performed with the normalized concentration values. Normalized values of each variable were calculated by subtracting the average and dividing by the standard deviation. High correlation values were found between $TPA_2$-EG-DEG and $TPA_3$-$EG_2$-DEG (0.8639) $TPA_2$-EG-DEG and $TPA_3$-$EG_2$-DEG (0.9186), cyclic and linear respectively, dimer and trimer of the second series. Figure 3b and 3c show the profiles of these compounds in the different samples.

Figure 4 shows four chromatograms of dimers (a-d) and four chromatograms of trimers (e-h). Chromatograms a, b, e, and f show linear and cyclic second series respectively, figures c, d, g, and h show linear and cyclic first series respectively. Figure 4 also shows that those oligomers that contain more DEG units (2 and 3 series) eluted on the leading edge of all EG oligomers. Furthermore, linear oligomers eluted firstly than the corresponding cyclic ones (Úbeda, et al., 2017).

Other non-volatile compounds likely present in the PET samples have not been presented in this work.

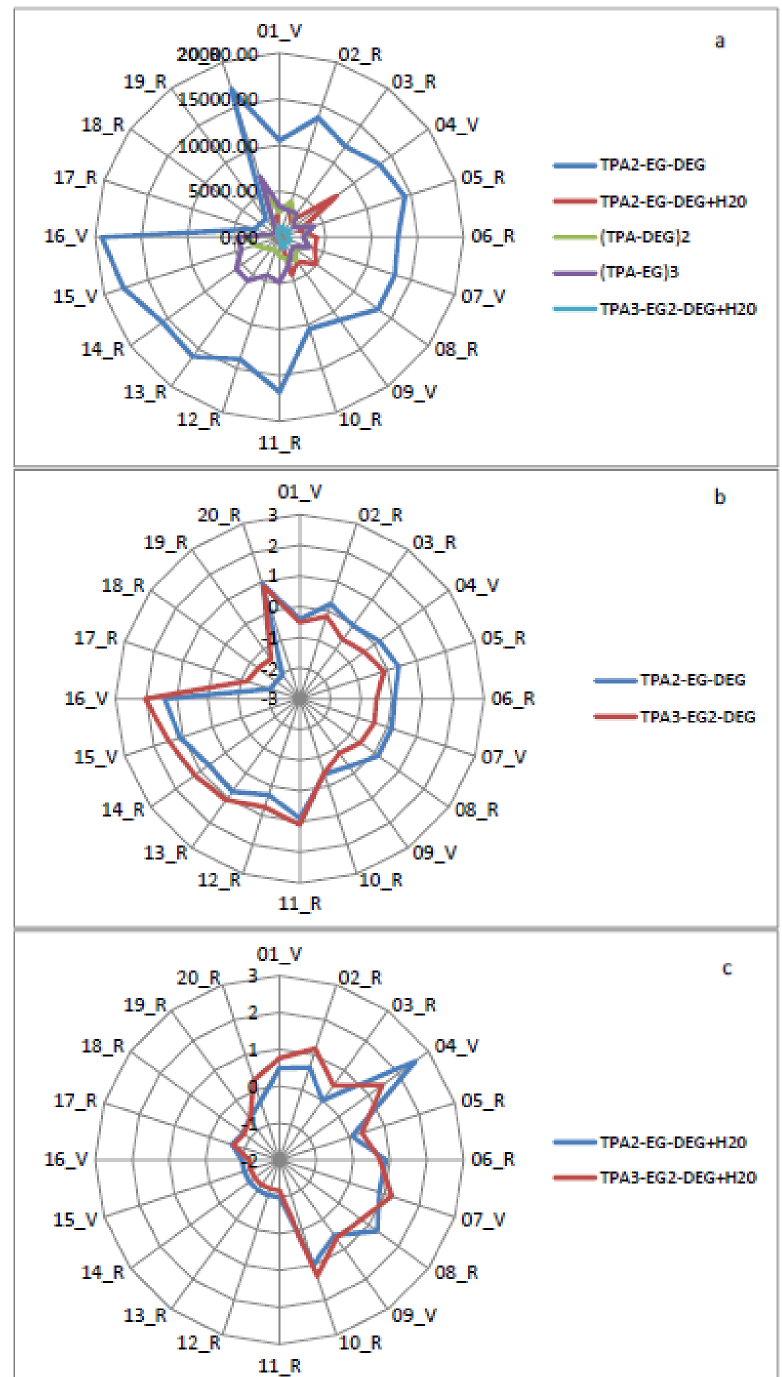



**Figure 3.** Concentration radar chart (a) ng g$^{-1}$ of the main cyclic PET oligomers in the 20 samples analysed, recycled (R) and virgin (V); (b) normalized concentration of cyclic dimer and trimer of the second series; (cb) normalized concentration of lineal dimer and trimer of the second series.

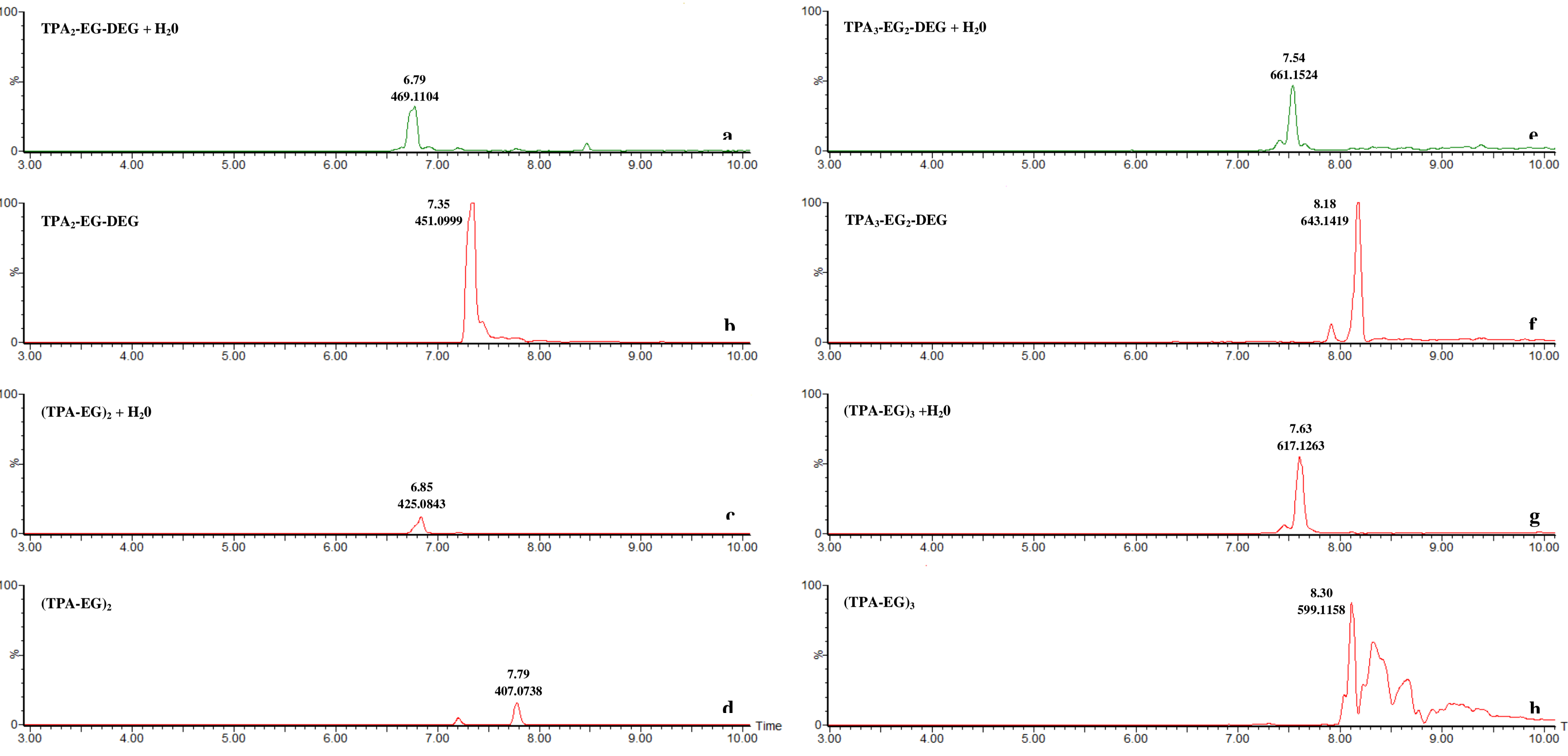



**Figure 4.** UPLC-QTOF chromatograms of the main PET dimers (a-d) and trimers (e-h).

## 5.3 Content of oligomers in migration

Table 3 shows the concentrations of the oligomers identified in migration test. None of the oligomers was found in aqueous food simulants, ethanol 10% or acetic acid 3%. Seven oligomers were identified and quantified in migration to ethanol 95%, 5 of them ($TPA_2$-EG-DEG, $(TPA\text{-}DEG)_2$, $(TPA\text{-}EG)_3$, $TPA_3$-$EG_2$-DEG and $(TPA\text{-}EG)_4$) were previously detected in pellet samples with the highest concentrations. Among the oligomers detected, $TPA_2$-EG-DEG was the most abundant in the migration solution. Since these compounds are not present in EU/10/2011, their concentration should be below 10 ng $g^{-1}$ (EC, 2011). Most of these oligomers had been previously detected in migration by other authors (Hoppe, Fornari, de Voogt, & Franz, 2017; Kim & Lee, 2012).

**Table 3.** Concentration of oligomers (ng $g^{-1}$) in migration from PET bottles to ethanol 95%.

| Samples | $(TPA\text{-}EG)_2$ | $TPA_2$-EG-DEG | $(TPA\text{-}DEG)_2$ | $(TPA\text{-}EG)_3$ | $TPA_3$-$EG_2$-DEG | $TPA_3$-$EG_2$-DEG $+H_2O$ | $(TPA\text{-}EG)_4$ |
|---|---|---|---|---|---|---|---|
| PET15 | 24.41 ± 9.00 | 275.87 ± 7.91 | 60.39 ± 11.63 | 189.65 ± 3.67 | 43.08 ± 2.68 | 43.04 ± 4.48 | <LOQ |
| PET20 | <LOD | 21.27 ± 1.35 | <LOD | 30.58 ± 1.31 | <LOQ | <LOD | 34.90 ± 1.52 |

# 6. CONCLUSIONS

Total dissolution using hexafluoroisopropanol/methanol as solvent/antisolvent system, is recommended as sample treatment in order to identify and quantify the potential migrants of PET samples. Liquid extraction from PET provided incomplete qualitative information about oligomers, and very different quantitative values. A total of 20 samples, virgin and recycled, have been analyzed and the oligomers present have been identified and quantified. Results provided a complete overview of the oligomers that can be found in PET pellets and the range of concentration. This information would allow evaluating

the suitability of PET pellets for the manufacturing of food contact materials. Fourteen oligomers composed by terephthalic acid, ethylene glycol and diethylene glycol were detected, both cyclic and linear, and their chemical structure was elucidated. Among them, 6 were of the first series, 5 of second series and 2 of third series. The oligomers detected with the highest concentration in the studied samples were the cyclic and linear dimers and trimers from the first and second series. UPLC-QToF is very useful for the determination of oligomers content in PET samples as well as the presence of non-volatile additives and other NIAS. The identified oligomers have shown to be able to migrate to food simulants in the studied samples, and this fact confirms the importance of knowing the oligomers composition in the raw material. This would allow evaluating its suitability for manufacturing the food contact materials.

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# Capítulo 3

*Determination of non-volatile components of a biodegradable food packaging material based on polyester and polylactic acid (PLA) and its migration to food simulants*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
3. INTRODUCTION
4. MATERIAL AND METHODS
5. RESULTS AND DISCUSSION
6. CONCLUSIONS
7. REFERENCES

# 1. RESUMEN

Los materiales bioplásticos se utilizan cada vez más debido a sus beneficios para la preservación del medio ambiente. Entre ellos, los materiales de envasado de alimentos con base de ácido poliláctico (PLA) son los más empleados. En este trabajo, se ha optimizado una metodología de tratamiento de muestras basada en disolución/precipitación, seleccionando finalmente diclorometano/etanol como sistema solvente/antisolvente. Los extractos obtenidos fueron analizados por UPLC-QToF, que permitió la identificación de los principales componentes no volátiles del PLA. Los resultados de recuperación fueron entre 100.9 y 114.0%. La metodología se aplicó al análisis de pellets y films de una muestra de PLA-poliéster. Se detectaron un total de 37 compuestos diferentes, donde los cuatro compuestos con intensidad más alta en muestras de pellets fueron oligómeros cíclicos procedentes de la parte del poliéster y compuestos por ácido adípico (AA), ácido ftálico (PA) y butanodiol (BD). También se realizaron experimentos de migración con 3 simulantes de alimentos: etanol al 95% (v/v), etanol al 10% (v/v) y ácido acético al 3% (w/v). Los resultados mostraron que, además de los compuestos previamente detectados en el film, nuevos compuestos provenientes de la reacción de compuestos de PLA con los simulantes estaban presentes en las disoluciones de migración.

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El principal objetivo fue determinar los principales compuestos no volátiles presentes en muestras de PLA-poliéster tanto en pellets como en film, para lo que fue necesaria la optimización de un método de disolución/precipitación. También fue un objetivo prioritario, la evaluación de la migración de estos compuestos a 3 simulantes alimentarios diferentes. Para ello, se llevaron a cabo las siguientes tareas:

- Búsqueda de bibliografía sobre la composición no volátil presente en muestras de PLA.

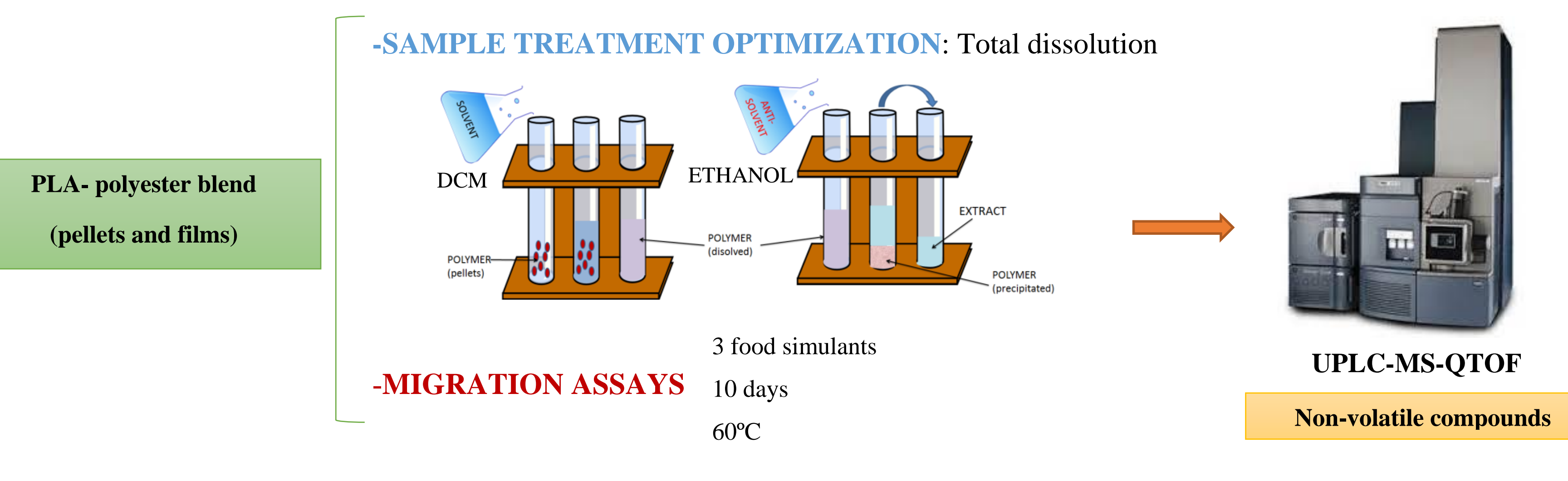
- Optimización y evaluación de un tratamiento de muestra basado en disolución/precipitación para el biopolímero PLA/poliéster.

- Identificación de los componentes no volátiles del film y pellets del PLA-poliéster.

- Evaluación del efecto del proceso de extrusión en la composición mediante la comparación entre la composición de pellets y films.

- Evaluación de la migración desde el biopolímero a diferentes simulantes alimentarios.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 3.




**Esquema 3:** Diseño experimental del Capítulo 3.

# 3. INTRODUCTION

Bioplastics encompass those plastics that are biodegradable and/or compostable and also those ones that come from renewable sources (Jabeen, Majid, & Nayik, 2015). They still account a low percentage of the polymer market, below 1 %. However, the increasing interest in preserving the environment has driven the plastic research towards the study and development of new packaging bio-materials. Poly(lactic acid) (PLA), derived from the fermentation of starch along with starch-based polymers, are the two most important commercial biodegradable polymers, representing about 47% and 41% of the total biodegradable polymer consumption (Ashter, 2016). Other bioplastics that are also being studied are those produced by the bacterial fermentation of starch and glucose such as poly hydroxyl alkanoates (PHA's) or poly hydroxyl butyrates (PHBs) (Jabeen, Majid, & Nayik, 2015).

Bioplastics have been used for different commercial applications such as disposable houseware, medical devices, consumer electronics, bags, automotive or food packaging. When these materials are used for food contact they are expected to protect food and maintain food quality, and it is important to evaluate that they don't transfer any component to food that could modify its sensory properties or imply any risk to consumers health (EU, 2004).

This work has been focused on the study of a biodegradable PLA based material intended to food packaging. PLA comes from natural sources such as maize, wheat or corn and is also fully biodegradable and compostable with the right temperature and humidity under industrial composting facilities. It has already been used in different packaging applications such as cups for beverages, bowls for salads, bags for potato chips or jars for yogurts.

PLA is a linear aliphatic polyester that is obtained 100% from the fermentation of renewable plant sources. Starch is chemically converted to dextrose and dextrose is fermented to lactic acid followed by polycondensation (Inkinen, Hakkarainen, Albertsson, & Sodergard, 2011). Other manufacturing way is the ring-opening polymerization of lactide, a cyclic dimer composed by 2 units of lactic acid (LA). This last methodology provides higher molecular weight polymers and consequently is more used (Bradley, 2010). Different techniques can be used to transform PLA pellets to the final product such as injection moulding, film extrusion or thermoforming. PLA has very good physical properties, such as mechanical and barrier properties comparable to synthetic polymers like polystyrene (PS) and polyethylene terephthalate (PET)(R. A. Auras, Harte, Selke, & Hernandez, 2003). It is suitable for food contact applications and it has a competitive cost. Nevertheless it has some drawbacks, such as its brittleness and its low resistance to oxygen permeation, that can be solved by blending PLA to other polymers, such as fossil-based polyesters, starch (Muller, Gonzalez-Martinez, & Chiralt, 2017) or PHA. For improving its properties, the addition of several plasticizing agents has also been performed, such as glycerol (Erdohan, Cam, & Turhan, 2013), acetyl tributyl citrate (Coltelli, Della Maggiore, Bertold, Signori, Bronco, & Ciardelli, 2008), tributyl citrate (Ljungberg & Wesslen, 2002) or polyethylene glycol (Choi, Choi, Han, Park, & Ha, 2013). These compounds are named intentionally added substances (IAS). In addition, there are non-intentionally added substances (NIAS) that can be present in the polymer due to different reasons, such as impurities of the raw materials or degradation and reaction process that can also migrate to food. IAS as well as NIAS can be transferred to food when the polymer is used as a food contact material and for this reason, migration tests must be performed before its use for a proper risk assessment of the material (Nerin, Alfaro, Aznar, & Domeno, 2013). Migration studies performed previously in the literature detected lactic acid, lactoyllactic acid and some small oligomers (R. Auras, Harte, & Selke, 2004) and some NIAS, volatiles (E. Canellas, Vera, & Nerin, 2017a) as well as non-volatiles such as N,N-

diethyldodecanamide, N-[(9Z)-9-octadecen-1-yl] acetamide, 1-palmitoylglycerol or glycerol stearate (Martinez-Bueno, Hernando, Ucles, Rajski, Cimmino, & Fernandez-Alba, 2017). Finally, it is important to highlight the presence and likely migration of oligomers, defined as molecules consisting of a few monomer units. The presence of oligomers and its migration to food simulants has been previously studied in different kind of polymers such as polyethylene terephthalate (PET) (Hoppe, Fornari, Voogt, & Franz, 2017) (Kim & Lee, 2012), polyurethanes (S Ubeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017) or polyamides (Heimrich, Nickl, Bönsch, & Si, 2014). In PLA, migration of oligomers (Mutsuga, Kawamura, & Tanamoto, 2008) (Bor, Alin, & Hakkarainen, 2012) (Dopico-Garcia, Ares-Pernas, Otero-Canabal, Castro-Lopez, Lopez-Vilarino, Gonzalez-Rodriguez, et al., 2013) and how factors such as pH can affect the degradation kinetics (Lazzari, Codari, Storti, Morbidelli, & Moscatelli, 2014) has also been studied by different authors. Since PLA is also used in pharmaceutical and surgical devices, oligomers presence has also been determined in these materials (Osaka, Yoshimoto, Watanabe, Takama, Murakami, Kawasaki, et al., 2008).

The main aim of this work was to develop a methodology for the determination of the main non-volatile potential migrants, including NIAS, of a biodegradable PLA-polyester blend intended for food contact and its migration to different food simulants.

# 4. MATERIAL AND METHODS

## 4.1. Reagents

Methanol and water for UPLC-MS analysis (ultra LC-MS quality) were purchased from Baker (Deventer, The Netherlands); ethanol (HPLC quality) and dichloromethane were purchased from Scharlau Chemie S.A. (Sentmenat, Spain)

and purified water was obtained with a Milli-Q 185 Plus system (Millipore, Bedford, MA, USA). Acetaminophen, caffeine, reserpine and sulfadimethoxine were purchased from Sigma Aldrich Química (Barcelona, Spain).

## 4.2. Samples

Samples in pellets and films were provided by a packaging company. They were a blend of PLA and a biodegradable fossil-based polyester. It fulfils the requirements of the European standard DIN EN 13432 for compostable and biodegradable polymers. Its mass density is 1.24-1.26 g/cm3 and its melt volume rate (190 ºC, 5 Kg) 7.0-11.0 mL/10 min

## 4.3. Optimization of sample treatment

The dissolution/precipitation methodology used in this work had already been used for the analysis of other polymer's composition (Bayer, 2002) (Begley & Hollifield, 1989) (Poulakis & Papaspyrides, 2001) (Li, Wang, Lin, & Hu, 2016) (S. Ubeda, Aznar, & Nerín, 2018).

### 4.3.1. Base sample treatment

For the optimization process, a base sample treatment was used. The steps of this treatment were as follows: 0.25 g of PLA pellets were mixed with 3 mL of the selected solvent and the mixture was placed in an ultrasound bath for 1 hour for its total dissolution. The volume of solvent was selected in order to completely cover the PLA sample. Once the sample was dissolved, the solution was placed in a vial and the antisolvent was added under magnetic agitation (500 rpm). Afterwards, the precipitated was removed and the vial placed in the freezer for 1 hour for a complete polymer precipitation. Finally, the extract was filtered

through a 0.25 µm PET, evaporated to dryness under a nitrogen current and redissolved with 1 mL of methanol/water (1/1; v/v).

### 4.3.2. Optimization and evaluation of the precipitation/redissolution process

First, different solvents were tested for being used as solvent/antisolvent systems according to PLA solubility: dichloromethane/methanol, dichloromethane/ethanol, chloroform/methanol and chloroform/ethanol. They were selected on the basis of previous experiments about PLA solubility (Kalia & Avérous, 2011) (Xiao, Wang, Yang, & Gauthier, 2010). The extracts were analyzed by UPLC-QToF.

For determining the volume of antisolvent necessary 3 different ratios solvent/antisolvent were checked, they were selected according to the literature: 1/1, 1/2 and 1/3 (Bayer, 2002) (Begley & Hollifield, 1989) (Poulakis & Papaspyrides, 2001) (Li, Wang, Lin, & Hu, 2016). Two different procedures were applied to confirm the efficiency of the treatment, the first one based on the weight of precipitate and the second one on the supernatant. Once the polymer was precipitated, the extract was removed and the polymer was dried in an oven until constant weight. While, the weight of the polymer increased around 6 times from ratio 1/1 to 1/2, no significant differences were found between 1/2 and 1/3 ($p<0.01$). For this reason, a ratio 1/2 was used. In order to confirm a complete precipitation, the extract was measured by molecular absorption spectroscopy in the visible range (400-700 nm) and compared to the blank (dichloromethane/ethanol, 1/2) to confirm the absence of light scattering. In presence of particles or colloids in the solution the incoming light would be scattered and higher absorbance would be observed. No differences were observed between the blank and the extract.

Then, the effect of performing a washing step in the precipitated polymer was checked. For this purpose, 3 mL of ethanol were added over the precipitated polymer and it was manually shaken. Then, the ethanol was removed and mixed with the previous extract, and the base sample treatment continued.

To calculate possible losses due to the evaporation process, recovery percentages as well as the possible matrix effects the following experiments were performed. For the study, a surrogate solution of acetaminophen, caffeine, reserpine and sulfadimethoxine (20 mg $kg^{-1}$) in methanol was used. These compounds were selected in order to have compounds from different chemical families and with different molecular weights that could represent different kind of sample components. The extracts were analyzed by ULPC-QqQ and the areas of the peaks were measured (A):

- Experiment 1: A solution of 1 mL methanol/water (1/1, v/v) was spiked with 100 µL of the standards solution (AE1)

- Experiment 2: A solution of DCM (1 mL) + EtOH (3 mL) was spiked with 100 µL of the standards solution, concentrated to dryness and redissolved with 1 mL of methanol/water (AE2).

- Experiment 3: 1 mL of PLA solution was precipitated using the base protocol and the final methanol/water extract (1 mL) was spiked with 100 µL of the standards solution (AE3).

- Experiment 4.1: 1 mL of PLA solution was spiked with 100 µL of the standards solution and analyzed using the base protocol (AE4.1).

- Experiment 4.2: 1 mL of PLA solution was spiked with 100 µL of the standards solution and analyzed using the base protocol with the washing step (AE4.2).

All the experiments were performed in triplicate.

## 4.4. Optimized sample treatment

This protocol was used for pellet samples as well as for films or trays. An amount of 0.25 g of sample was weighted and 3 mL of dichloromethane were added. The mixture was shaken in an ultrasound machine during 1 hour until it was dissolved. For the precipitation of the polymer, 6 mL of ethanol were added to the dissolved sample was added with 6 mL of ethanol under magnetic stirring (500 rpm, 15 min). After this time, the solvent was removed and stored in a vial. Afterwards, the precipitated polymer was washed with 3 mL of ethanol and the mixture was manually shaken. Then, the polymer was gently pressed with a glass bar and the solvent was removed and mixed to the previous extract. The final extract was filtered through a 0.25 µm PET filter, concentrated to dryness under a gentle nitrogen current and reconstituted with 1 mL of methanol/water (1/1; v/v).

## 4.5. Migration tests

Migration tests were performed by total immersion of samples into the simulants. Cut-offs of 5 x 1 cm were placed in 20 mL vials and immersed in 3 different food simulants: ethanol 10% (v/v) (simulant A), acetic acid 3% (w/v (simulant B) and ethanol 95% (v/v) (substitute of simulant D2). Vials were filled in according to the rate 6 $dm^2$ contact surface $kg^{-1}$ of simulant, established by the Regulation EU/10/2011 (EU, 2011). Afterwards, vials were placed in an oven at 60 ºC during 10 days.

All the migration experiments were performed in triplicate and according to the European Regulation for food contact materials EU/10/2011 (EU, 2011).

### 4.6. UPLC-MS analysis

Chromatography was carried out using an Acquity system and a UPLC BEH C18 column of 1.7 µm particle size (2.1 x 100 mm), both from Waters (Milford, MA, USA). Chromatography was carried out at 0.4 mL min-1 column flow, 40 ºC column temperature and using an injection volume of 10 µL. The mobile phase was composed by 2 phases, water with 0.1 % formic acid v/v/ (phase A) and methanol with 0.1 % formic acid (v/v) (phase B). Chromatography started at 98/2 phase A/phase B (1 minute), changed to 0/100 in 7 minutes and stayed at 0/100 for additional 2 minutes.

For ULPC-QToF analysis, the UPLC was connected with an ESI probe to a Xevo G2 QToF mass spectrometer also from Waters. Instrument configuration was as follows: capillary at 2.5 kV, sampling cone at 30 V, extraction cone at 4 V, source temperature at 120 ºC, desolvation temperature at 450 ºC, cone gas flow at 20L hr-1and desolvation gas flow at 500L hr-1. Acquisition was carried out in sensitivity and MSE mode. MSE mode allows the acquisition at low and high collision cell energies (CE) during the same run. Even though most of the compounds are detected at 30 V of cone voltage, analyses were also performed at 70 V to not lose any information. Data were recorded using MassLynx v4.1 software. The identification of the compounds detected was carried out following the methodology previously described by the authors (Aznar, Alfaro, Nerin, Jones, & Riches, 2016) (Nerin, Alfaro, Aznar, & Domeno, 2013).

For quantitative purposes, ULPC-QqQ was used, where the UPLC was connected to an ESI probe to a TQ mass spectrometer from Waters. Instrument configuration was as follows: capillary at 2.5 kV, sampling cone at 40 V, extraction cone at 3 V, source temperature at 120 ºC, desolvation temperature at

450 ºC, cone gas flow at 60L hr-1and desolvation gas flow at 600L hr-1. Acquisition was carried out in SIR (selected ion recording) mode and the protonated masses of the molecular ions were recorded: acetoaminophen [MH+] = 152.1, caffeine [$MH^+$] = 195.1, sulfadimethoxine [$MH^+$] = 311.1 and reserpine [$MH^+$] = 609.3.

# 5. RESULTS AND DISCUSSION

## 5.1. Optimization of PLA sample treatment by dissolution/precipitation

The four solvent/antisolvent systems studied (dichloromethane/methanol, dichloromethane/ethanol, chloroform/methanol and chloroform/ethanol) provided good results in terms of dissolution and polymer precipitation and no significant differences were found when they were analyzed by UPLC-QToF. Since these extracts were expected to be used as well in GC-MS in future experiments, solvent properties for GC-MS analysis were taken into account in the decision. Dichloromethane was selected versus chloroform since it was eluted at a shorter retention time in the GC-MS analysis and therefore, it allows detecting more compounds with high volatility. Ethanol was selected as antisolvent versus methanol, since it had a lower expansion volume and therefore it was more appropriate for GC-MS. Finally, the system dichloromethane/ethanol was selected for PLA sample analysis.

The possible compound losses due to the evaporation to dryness process were evaluated comparing the areas obtained from experiments 1 and 2. The results showed that there were no losses for the reference compounds since no significant differences were observed in the measured areas for any compound.

The calculation of matrix effect was performed with the results obtained from experiments 1 and 3 using the following equation:

Matrix effect (%) = 100 x AE3 / AE1

The results are shown in table 1. All the values obtained for the reference compounds, except for caffeine, were in the range from 80 to 120 %, which implies that no matrix effects should be considered.

The calculation of recovery data was carried out with results obtained from experiments 3, 4.1 and 4.2 using the following equation:

Recovery (%) = 100 x AE4 / AE3

The best recoveries were found when the washing step of the precipitated polymer was performed, with values between 100.9 to 114.0 % (table 1).

In order to have a better sensitivity, in the final sample treatment protocol the whole PLA solution was used, maintaining the same solvents rates than in the optimization process for polymer precipitation and the washing step.

**Table 1:** Recovery data and matrix effect for PLA-polyester sample treatment by dissolution/precipitation

| | **Acetoaminophen** | **Caffeine** | **Reserpine** | **Sulfadimethoxine** |
|---|---|---|---|---|
| Recovery Without washing (%) | 53.0 | 55.6 | 30.3 | 34.1 |
| Recovery With washing (%) | 120.4 | 113.1 | 81.3 | 79.7 |
| Matrix effect (%) | 103.4 | 131.7 | 100.9 | 114.0 |

## 5.2. Composition of PLA pellets and film

The optimized protocol was applied to the analysis of pellets and film PLA samples. Three replicates of each were dissolved and analyzed. Figure 1 shows a chromatogram of a PLA pellets extract.

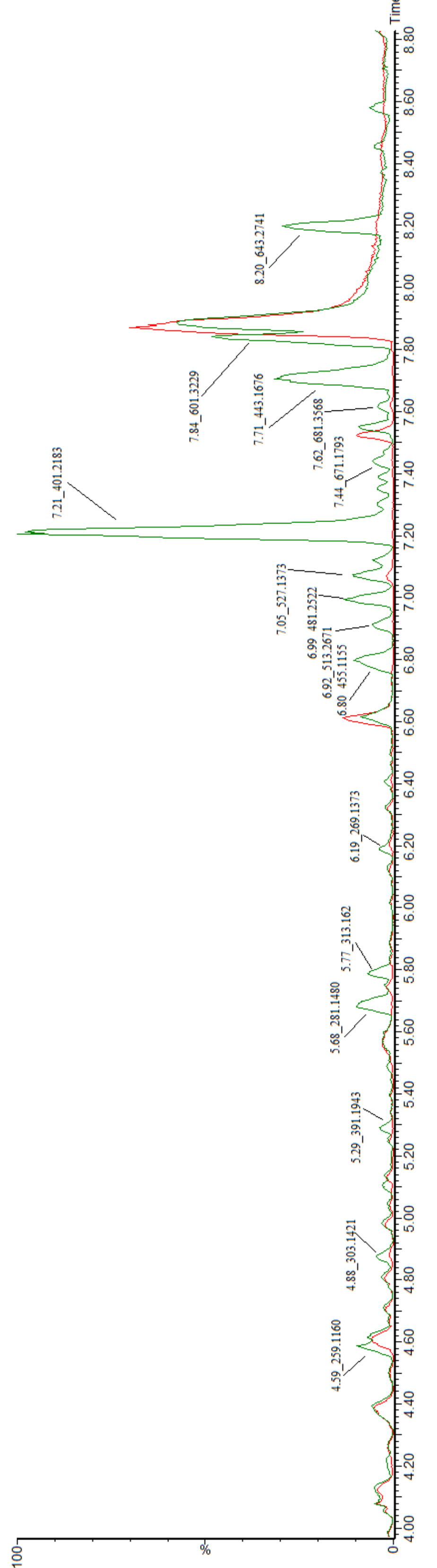



**Figure 1**. Chromatogram obtained by UPLC- QTOF analysis of PLA pellet samples overlaid with a blank sample.

Table 2 shows the main compounds found in PLA samples (pellets and film) and in migration solutions. A total of 37 different compounds were detected. Initially, 23 were detected in pellet samples and afterwards, 19 out of them were also detected in films. No new compounds were detected in films with regard to those found in pellets. This fact would mean that the extrusion process from pellets to film did not generate any new compound but some of them disappeared. With regard to migration results, 14 out of the 19 compounds found in the film were finally detected in any of the food simulants. In addition, 14 new compounds were also found, probably generated from the reaction between the components of the packaging material and the simulants.

The initial compounds screening was performed in positive mode and 30V of cone voltage, since according to previous studies it provided the best sensitivity (Aznar, Alfaro, Nerin, Jones, & Riches, 2016). Chromatograms obtained at 70 V did not show any new compound and only 14 out of the 23 detected at 30 V were detected. Among them, only 4 were detected at 70 V with a higher intensity.

**Table 2:** Compounds detected in pellets, films and migration and its areas intensity (1 high, 2 medium, 3 low) at 30V cone voltage; molecular formula; proposed candidates; remarks, main fragments in the high collision energy mass spectrum and its scores (S) obtained by MassFragmet®.

| Code rt_mass | I pellets | I film | I mig | Adduct | Molecular formula | Candidates | Remarks/Fragments (scores) |
|---|---|---|---|---|---|---|---|
| **3.60_331.0711** | nd | nd | 1 | $[MNa]^+$ | $C_{22}H_{12}O_2$ | No candidates | 167.0027, 185.0129 |
| **4.50_257.0635** | nd | nd | 2 | $[MNa]^+$ | $C_9H_{14}O_7$ | $HO-[LA]_3-H$ *(linear)* | PLA oligomer |
| **4.59_259.1160** | 2 | nd | nd | $[MNa]^+$ | $C_{10}H_{20}O_6$ | 1,3,6,9,11,14-Hexaoxacyclohexadecane CAS 74485-37-3 (Glycol) | PLA plasticizer 103.0392 (S2); 131.0706 (S2); 87.0438 (S2) |
| **4.88_303.1421** | 1 | nd | nd | $[MNa]^+$ | $C_{12}H_{24}O_7$ | Poly(trimethylolpropane adipate) CAS 28301-90-8 | Indirect additives by FDA [23] 131.0705 (S2) |
| **4.98_241.1043** | 1 | 1 | 2 | $[MNa]^+$ | $C_{10}H_{18}O_5$ | Diethylene glycol, dipropionate CAS 6942-59-2 | PLA plasticizers |
| **5.09_213.0723** | nd | nd | 2 | $[MNa]^+$ | $C_8H_{14}O_5$ | $CH_3-O-[LA]_2-CH_3$ *(linear)* | PLA oligomer |
| **5.29_391.1943** | 1 | nd | nd | $[MNa]^+$ | $C_{16}H_{32}O_9$ | No candidates | |
| **5.31_329.0840** | nd | nd | 2 | $[MNa]^+$ | $C_{12}H_{18}O_9$ | $HO-[LA]_4-H$ *(linear)* | PLA oligomer |
| **5.68_281.1480** | 2 | 2 | 1 | $[MNa]^+$ | $C_{12}H_{22}N_2O_4$ | Piperidine family | Detected by Bradley in PLA [5] Hindered piperidines are light stabilizer |
| **5.71_285.0946** | nd | nd | 2 | $[MNa]^+$ | $C_{11}H_{18}O_7$ | $CH_3-O-[LA]_3-CH_3$ *(linear)* | PLA oligomer |
| **5.77_313.162** | 2 | 2 | 2 | $[MNa]^+$ | $C_{14}H_{26}O_6$ | Bis(2-ethoxyethyl) adipate CAS 109-44-4 | Detected by Bradley in starch and cellulose [5] 111.0440 (S1); 101.0597 (S1.5); 129.0545 (S1); 73.0651 (S0.5) |
| **5.87_401.1044** | nd | nd | 2 | $[MNa]^+$ | $C_{15}H_{22}O_{11}$ | $HO-[LA]_5-H$ *(linear)* | PLA oligomer |
| **6.19_269.1373** | 1 | nd | nd | $[MNa]^+$ | $C_{12}H_{22}O_5$ | 2,2'-(oxybis((methyl-2,1-ethanediyl)-oxymethylene)) bisoxirane CAS 41638-13-5 | Indirect additives by FDA [23] 83.0493 (S2); 101.0601 (S1.5) |
| **6.24_473.1271** | nd | nd | 2 | $[MNa]^+$ | $C_{18}H_{26}O_{13}$ | $HO-[LA]_6-H$ *(linear)* | PLA oligomer |
| **6.26_357.1161** | nd | nd | 2 | $[MNa]^+$ | $C_{14}H_{22}O_9$ | $CH_3-O-[LA]_4-CH_3$ *(linear)* | PLA oligomer |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **6.40_383.0955** | 1 | 1 | nd | $[MNa]^+$ | $C_{15}H_{20}O_{10}$ | $[LA]_5$ *(cyclic)* | PLA oligomer<br>217.0704 (S2); 145.0494 (S2) |
| **6.58_545.1499** | nd | nd | 2 | [MNa]+ | $C_{21}H_{30}O_{15}$ | HO-$[LA]_7$-H *(linear)* | PLA oligomer |
| **6.61_429.1371** | nd | nd | 2 | $[MNa]^+$ | $C_{17}H_{26}O_{11}$ | $CH_3$-O-$[LA]_5$-$CH_3$ *(linear)* | PLA oligomer |
| **6.80_455.1155** | 2 | 2 | nd | $[MNa]^+$ | $C_{18}H_{24}O_{12}$ | $[LA]_6$ *(cyclic)* | PLA oligomer<br>217.0704 (S2); 145.0494 (S2); 289.0922 (S2) |
| **6.82_617.1703** | nd | nd | 2 | $[MNa]^+$ | $C_{24}H_{34}O_{17}$ | HO-$[LA]_8$-H *(linear)* | PLA oligomer |
| **6.92_513.2671** | 2 | 2 | 1 | $[MNa]^+$ | $C_{24}H_{42}O_{10}$ | Bis[1-(2-butoxyethoxy)-1-oxo-2-propanyl] adipate | Detected by Bradley [5]<br>201.1120 (S1.5); 111.0440 (S1); 101.0597 (S1.5); 129.0545 (S1) |
| **6.94_501.1603** | nd | nd | 2 | $[MNa]^+$ | $C_{20}H_{30}O_{13}$ | $CH_3$-O-$[LA]_6$-$CH_3$ *(linear)* | PLA oligomer |
| **6.99_481.2522** | 2 | 2 | 1 | [MNa]+ | $C_{22}H_{38}N_2O_8$ | Urethane dimethacrylate UDMC<br>CAS 72869-86-4 | Detected by Bradley [5]<br>129.0548 (S0.5); 241.1543 (S1.5) |
| **7.05_527.1373** | 2 | 2 | nd | $[MNa]^+$ | $C_{21}H_{28}O_{14}$ | $[LA]_7$ *(cyclic)* | PLA oligomer<br>217.0708 (S2); 289.0913 (S2); 361.1119 (S2) |
| **7.12_461.1782** | 1 | 1 | 2 | $[MNa]^+$ | $C_{22}H_{30}O_9$ | No candidates | Detected by Bradley [5] |
| **7.21_401.2183** | 3 | 3 | 3 | $[MH]^+$ | $C_{20}H_{32}O_8$ | $[AA\text{-}BD]_2$ *(cyclic)*<br>CAS 78837-87-3 | Polyester oligomer. Detected by Bradley [5]<br>201.1120 (S2);129.0547 (S2); 111.0443 (S2); 255.1580 (S2); 183.1013 (S2) |
| **7.30_599.1591** | 1 | 1 | nd | $[MNa]^+$ | $C_{24}H_{32}O_{16}$ | $[LA]_8$ *(cyclic)* | PLA oligomer<br>361.1119 (S2); 433.1345 (S2) |
| **7.32_645.1956** | nd | nd | 2 | $[MNa]^+$ | $C_{26}H_{38}O_{17}$ | $CH_3$-O-$[LA]_8$-$CH_3$ *(linear)* | PLA oligomer |
| **7.34_533.2356** | 1 | 1 | 1 | $[MNa]^+$ | $C_{26}H_{38}O_{10}$ | No candidates | 221.0818 (S2); 149.0234(S2); 167.0339 (S2); 111.0443 (2) |
| **7.44_671.1793** | 2 | 2 | nd | $[MNa]^+$ | $C_{27}H_{36}O_18$ | $[LA]_9$ *(cyclic)* | PLA oligomer<br>361.1133(S2);433.1345 (S2); 505.1565 (S2) |
| **7.45_717.2216** | nd | nd | 2 | $[MNa]^+$ | $C_{29}H_{42}O_{19}$ | $CH_3$-O-$[LA]_9$-$CH_3$ *(linear)* | PLA oligomer |
| **7.62_681.3568** | 1 | 1 | 1 | $[MNa]^+$ | $C_{38}H_{50}N_4O_6$ | No candidates | 349.1647; 381.2684 |
| **7.71_443.1676** | 3 | 3 | 3 | $[MNa]^+$ | $C_{22}H_{28}O_8$ | [PA-BD-AA-BD] *(cyclic)* | Polyester oligomer. Detected by Bradley [5]<br>221.0818 (S2); 149.0234(S2); 167.0339 (S2); 111.0443 (2) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **7.84_601.3229** | 3 | 3 | 2 | $[MH]^+$ | $C_{30}H_{48}O_{12}$ | $[AA\text{-}BD]_3$ *(cyclic)*<br>CAS 1135871-65-6 | Polyester oligomer. Detected by Bradley [5]<br>201.1124 (S2); 329.1581 (S2); 255.0549 (S2); 183.1017 (S2) |
| **8.20_643.2741** | 3 | 3 | 3 | $[MNa]^+$ | $C_{32}H_{44}O_{12}$ | [PA-BD-AA-BD-AA-BD] *(cyclic)* | Polyester oligomer. Detected by Bradley [5]<br>421.1862 (S2); 221.0818 (S2); 149.0234 (S2); 129.0548 (S2) |
| **8.45_843.3785** | 1 | 1 | 2 | $[MNa]^+$ | No formula | No candidates | 621.2953; 421.1875 |
| **8.58_663.2416** | 1 | 1 | 2 | $[MNa]^+$ | $C_{34}H_{40}O_{12}$ | No candidates | Detected by Bradley [5]<br>369.0979; 441.1561 |

nd: non detected; LA: lactic acid; AA: adipic acid; PA: phthalic acid; BD: butanediol;

The four compounds detected with the highest intensity in pellet samples were cyclic oligomers composed by adipic acid (AA), phthalic acid (PA) and butanediol (BD), which corresponded to the formula $[AA\text{-}BD]_2$ (7.21_401.2183), [PA-BD-AA-BD] (7.71_443.1676), $[AA\text{-}BD]_3$ (7.84_601.3229) and [PA-BD-AA-BD-AA-BD] (8.20_643.2741). Common fragments were observed for these structures in high collision mass spectra, such as 221.0818 and 149.0234 in those containing PA or 201.112, 255.1580 and 183.1013 in those containing only AA and BD. All masses had been previously detected by Bradley in biobased materials used for food contact applications (Bradley, 2010) but they had not been identified. Since samples were a blend of PLA and polyester, these compounds came from the polyester component. These compounds showed also the highest intensity in films and afterwards in migration experiments, which implies that the polyester part of the blend had a critical role in the risk assessment of this kind of materials.

Among the compounds detected with medium intensity in pellets it is important to highlight 3 PLA cyclic oligomers composed by lactic acid monomers (LA): $[LA]_6$, $[LA]_7$ and $[LA]_9$. $[LA]_5$ and $[LA]_8$ which were also detected but at lower intensity. In this case, even though they maintain the same intensity in films they were not detected in migration in any case, probably because they reacted with food simulants, inducing a cycle opening and formation of new compounds. Five more compounds were also detected with medium intensity such as 2 plasticizers with adipate structure (5.77_313.162 and 6.92_513.2671); a glycol (4.59_259.1160), probably also used as PLA plasticizer or a compound with piperidine structure (5.68_281.1480), probably added as light stabilizer. All of them had been previously detected by Bradley in biobased materials (Bradley, 2010) but they had not been identified.

Among the compounds with the lowest intensities, some plasticizers, PLA oligomers and compounds defined as indirect additives for food contact materials by the U.S. Food&Drug administration were also found (Administration, 2018).

Figure 2 shows the comparison of area intensity between pellets and films for those compounds detected in the PLA-polyester blend. The results showed very similar values for pellets and films. Only 4 compounds were detected just in pellets and were not present in films. These compounds were: 4.59_259.1160, 4.88_303.1421, 5.29_391.1943 and 6.19_269.1373. The Pearson correlation factor was calculated taking out these four compounds. The factor value obtained, 0.996, indicates a good correlation between pellets and film composition.

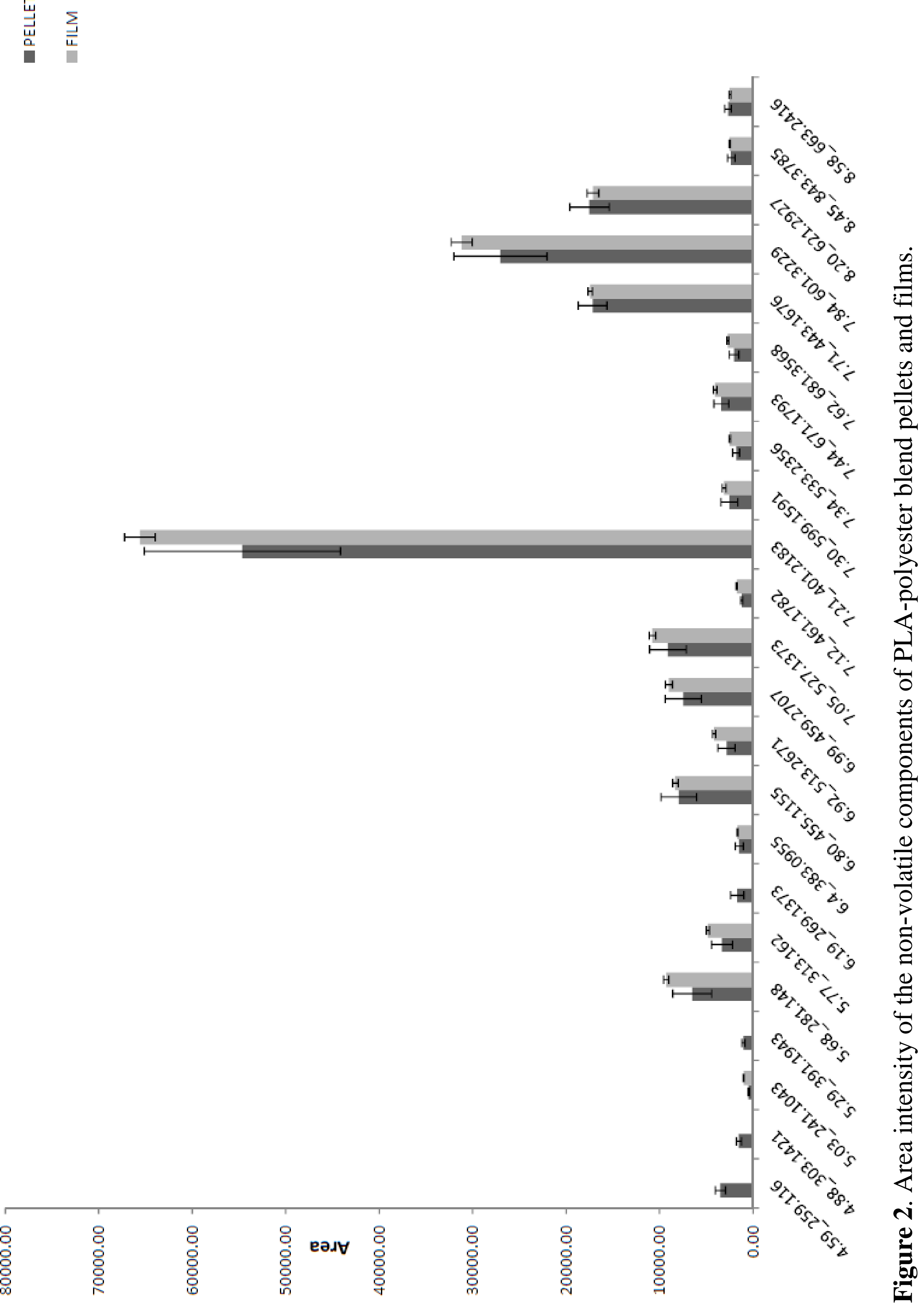



**Figure 2**. Area intensity of the non-volatile components of PLA-polyester blend pellets and films.

## 5.3. Composition of migration solutions

Migration was tested in 3 different simulants in order to predict the behavior of this material with different kinds of food, from aqueous to fat. Figure 3 shows a chromatogram of migration in ethanol 95%, ethanol 10% and acetic acid 3%. The first observation was that not all the compounds present in the film were detected in migration, as expected based on previous experience (Elena Canellas, Aznar, Nerin, & Mercea, 2010; E. Canellas, Vera, & Nerin, 2015, 2017b). A total of 19 compounds were detected initially in the film but only 14 of them were present in any of the food simulants; 13 in ethanol 95%, 9 in ethanol 10% and 5 in acetic acid 3%. None of the cyclic PLA oligomer detected in the material (6.40_383.0955, 6.80_455.1155, 7.05_527.1373, 7.30_599.1591, 7.44_671.1793) migrated to food simulants.

Figure 4 shows the area intensity of the peaks detected in migration in the different food simulants. The highest migration values were found in ethanol 95% as food simulant for two cyclic oligomers coming from the polyester part of the blend: $[AA\text{-}BD]_2$, (7.21_401.2183) and [PA-BD-AA-BD] (7.71_443.1676).

Section "a" of figure 4 shows the compounds that had been previously detected in the film (14) and "b" those only detected in migration (14). Those ones only detected in migration were probably formed as a consequence of the reaction between the PLA components and the food simulants. Six linear PLA oligomers with two hydroxyl groups corresponding to the formula HO-[LA]n-H (n= 3-8) were found as a consequence of the reaction with aqueous food simulants (ethanol 10% or acetic acid 3%). In all cases the intensity was higher in acetic acid 3% than in ethanol 10%. The compound 3.60_331.0711 was only found in acetic acid 3% but it was not possible to identify it. When PLA was in contact with food simulants with ethanol content (ethanol 95% or ethanol 10%) 7 different linear PLA oligomers with an additional $C_2H_4O$ were detected. Different structures can be proposed for these oligomers: $CH_3\text{-}O\text{-}[LA]_n\text{-}CH_3$,

$C_2H_5$-O-$[LA]_n$-H or $C_2H_5$-$[LA]_n$-OH. Since the first option was previously described by Badia et al in PLA samples (Badia, Stromberg, Ribes-Greus, & Karlsson, 2011), it was chosen as the principal candidate. In all cases the intensity of these oligomers was higher in ethanol 95% than in ethanol 10%.

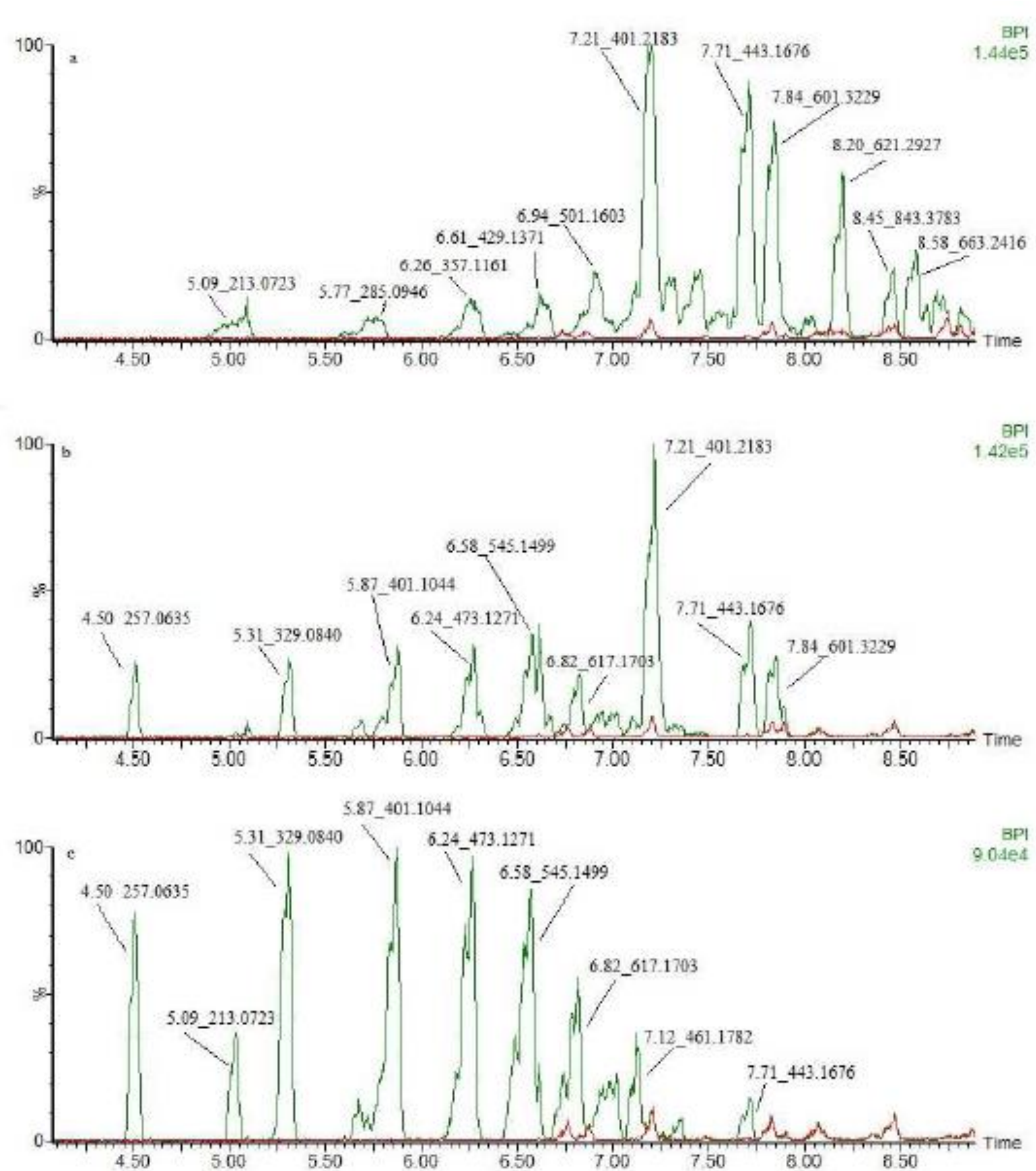



**Figure 3**. Chromatogram obtained by UPLC-QTOF analysis of migration solutions from PLA-polyester blend films to different food simulants: ethanol 95% (a), ethanol 10% (b) and acetic acid 3% (c), overlaid with blank samples.

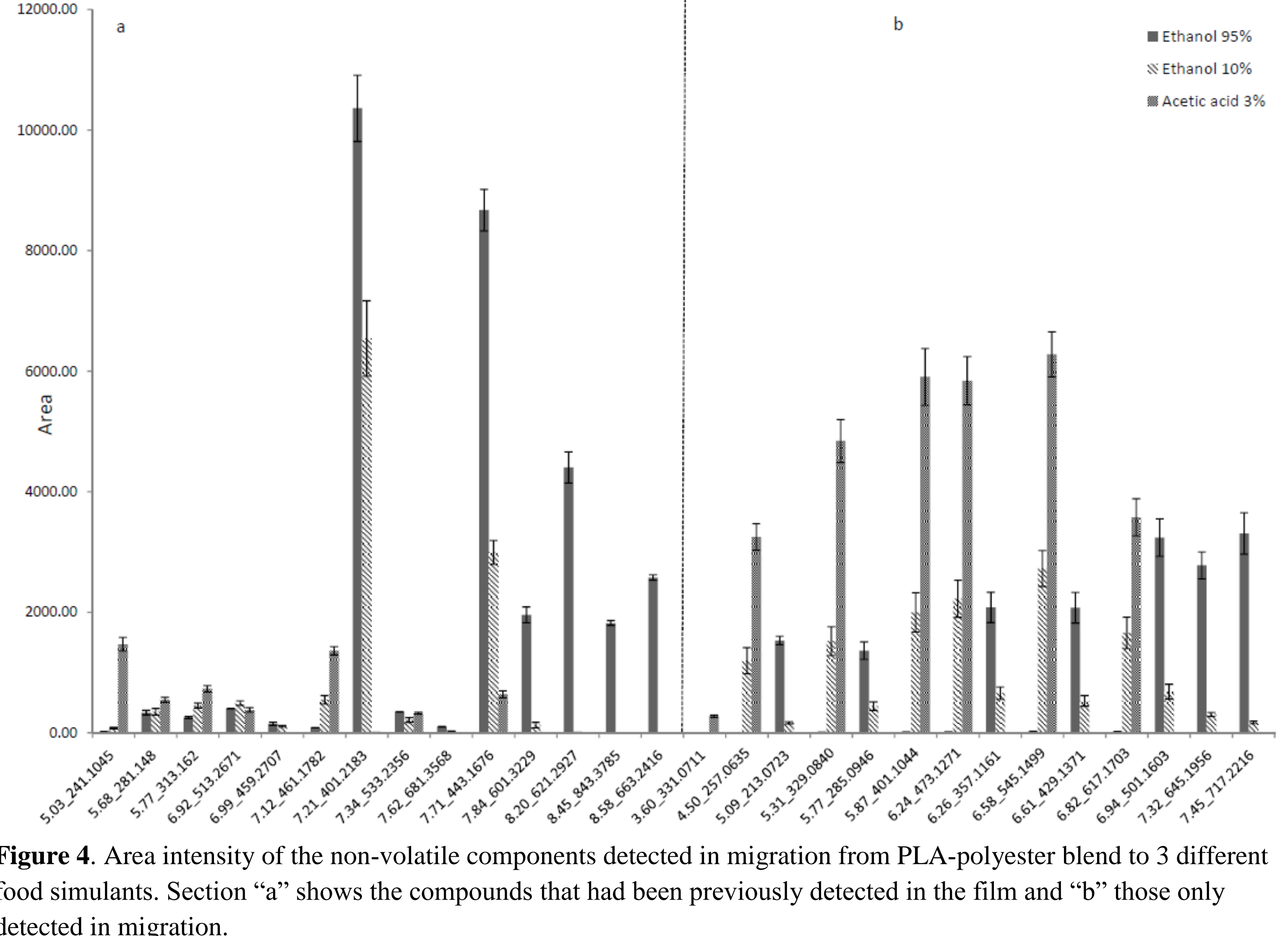



**Figure 4**. Area intensity of the non-volatile components detected in migration from PLA-polyester blend to 3 different food simulants. Section “a” shows the compounds that had been previously detected in the film and “b” those only detected in migration.

In order to determine the weight of the film components and the reaction components in migration, the relative percentage of area intensity of each detected peak related to the total area in migration was calculated. It was observed that 44.6% of the total intensity corresponded to peaks previously detected in the film and 55.4 % to peaks detected only in migration. When considering each simulant individually, different behaviors were observed. In ethanol 95%, 65.6 % of the total area corresponded to compounds already detected in the film, which would mean that these compounds have a slight higher weight in migration that the new-formed. In the case of ethanol 10%, similar results were found for each kind of compounds, 45.5 and 54.5 %. In acetic acid 3%, only a 15.4% of the area corresponded to compounds already detected in films, and 84.6 % corresponded to new-formed compounds, what would mean that in this food simulant the new-formed compounds have a very relevant role.

# 6. CONCLUSIONS

The dissolution/precipitation sample treatment developed in this work followed by the analysis of extracts by UPLC-QToF showed to be an efficient methodology for the analysis of non-volatile components present in a biodegradable PLA-polyester blend. Very similar composition was found in PLA pellets and films, since no new compounds were formed during the extrusion and only 4 disappeared during this process. The compounds with the highest intensities in PLA blend samples as well as in migration studies came from the polyester part of the blend and corresponded to cyclic oligomers composed by adipic acid, phthalic acid and butanediol. Even though several cyclic oligomers had been detected in pellets and film samples with medium intensity, they were not detected in migration solutions. In contrast, new linear oligomers formed as a consequence of a cycle opening were identified. Furthermore, some plasticizers as well as other additives were found, both in samples and migration. It is important to remark that in addition to the screening in the film samples, a

screening study in the migration solutions is necessary in order to have a comprehensive information about the migrants present in the food simulants. A future quantification of the compounds detected would allow establishing a correct risk assessment of the material.

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# Capítulo 4

*Migration of oligomers from a food contact biopolymer based on polylactic acid (PLA) and polyester*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
3. INTRODUCTION
4. MATERIAL AND METHODS
5. RESULTS AND DISCUSSION
6. CONCLUSIONS
7. REFERENCES

# 1. RESUMEN

El ácido poliláctico (PLA) es un biopolímero comúnmente utilizado en el envasado de alimentos debido a sus buenas características, similares al PET. Para evaluar la seguridad de este material, se requiere el análisis de las sustancias añadidas no intencionadamente (NIAS). Los oligómeros necesitan un estudio profundo ya que son considerados NIAS y pueden migrar a los alimentos. En este trabajo, se realizó el análisis del polímero y de la migración a diferentes simulantes alimentarios. Se aplicó un procedimiento de disolución/precipitación total en pellets y films de PLA, utilizando diclorometano/etanol como sistema solvente/antisolvente. Los ensayos de migración se llevaron a cabo con tres simulantes líquidos para simular cualquier tipo de alimento. Dado que los oligómeros no están presentes en la lista positiva del Reglamento (UE) nº 10/2011, su concentración debe estar por debajo de los 0.01 mg $kg^{-1}$ de alimento. Para el análisis se utilizó un UPLC-QToF, con y sin movilidad iónica (IM). Se identificaron treinta y nueve oligómeros del PLA formados por unidades monoméricas repetidas de [LA] ($C_3H_4O_2$) y con diferentes estructuras. Se identificaron oligómeros cíclicos con estructura [LA]  y dos grupos de oligómeros lineales, uno con un grupo hidroxilo, OH-$[LA]_n$-H, y el otro con un grupo etoxi, $CH_3$-$CH_2$-O-$[LA]_n$-H. Solo aparecieron oligómeros cíclicos en el material y no estuvieron presentes en las disoluciones de migración. Los oligómeros lineales HO-$[LA]_n$-H ya estaban presentes en los pellets/films y migraron en una mayor extensión a los simulantes de alimentos acuosos (EtOH 10% y AcH 3%). Sin embargo, oligómeros lineales $CH_3$-$CH_2$-O-$[LA]_n$-H no estaban presentes inicialmente en los pellets / films, pero se detectaron en la migración en simulantes con etanol, EtOH 95% y EtOH 10%. Además, se identificaron 5 oligómeros cíclicos procedentes del poliéster en la migración. Las disoluciones de migración con etanol al 95% y etanol al 10% también se analizaron mediante microscopía electrónica de barrido (SEM). Se observaron

microestructuras que podrían atribuirse a la migración de oligómeros y por tanto definirse como microplásticos.

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El principal objetivo de este capítulo fue determinar y caracterizar los principales oligómeros presentes en un biopolímero PLA-poliéster destinado al contacto con alimentos tanto en el mismo polímero como en la migración y conocer las posibles reacciones entre los oligómeros y los simulantes alimentarios. Para ello, se llevaron a cabo las siguientes tareas:

- Aprendizaje del manejo del equipo UPLC-IMS-QToF.

- Aprendizaje y conocimiento básico de la microscopía Electrónica de Barrido (SEM).

- Identificación y semi-cuantificación de los oligómeros presentes en el film, pellets y migración del PLA-poliéster.

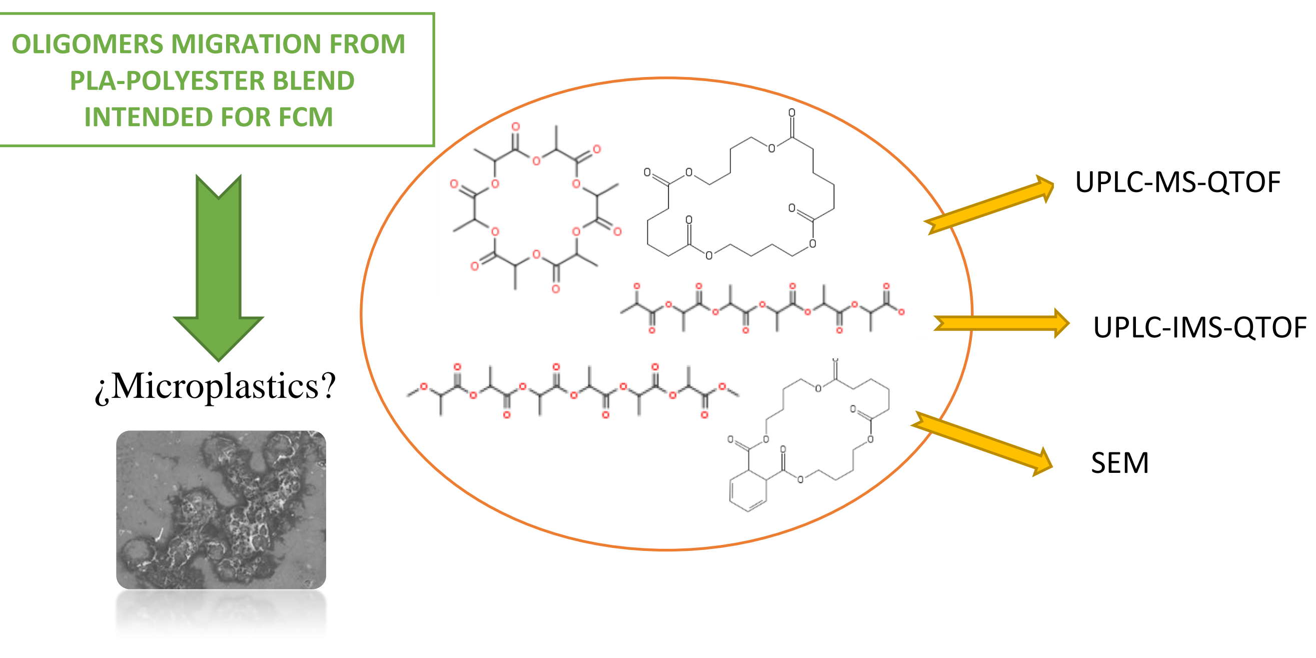
- Comparación de los oligómeros del PLA entre la composición de pellets y films para evaluar el efecto del proceso de extrusión en la composición.

- Estudio del comportamiento de los oligómeros y sus procesos de hidrólisis en el material (pellets y film) y en la migración.

- Determinación mediante espectrometría de movilidad iónica de los oligómeros principales del PLA y cálculo de sus valores CCS (Collisional Cross Section).

- Análisis SEM de las disoluciones de migración para evaluar la presencia de microplásticos.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 4.




**Esquema 4:** Diseño experimental del Capítulo 4.

# 3. INTRODUCTION

Biopolymers have been regarded as alternative materials to conventional plastics made from petroleum because they are biodegradable, renewable and abundant and its use would significantly decrease our dependence on fossil resources.

Biopolymers encompass two kinds of polymers: biobased polymers and biodegradable and/or compostable polymers. A polymer is considered biobased when it comes from renewable sources (biomass) (Geueke, 2014; Jabeen, Majid, & Nayik, 2015). A polymer is considered biodegradable when is broken down by the action of microorganisms in their natural environment since is considered compostable when at least 90% of the polymer is degraded within 6 months in an industrial composting plant (AENOR, 2001). They can thus be divided into three main categories depending on its origin and degradability: biobased and biodegradable; biobased and non-biodegradable; and fossil-based and biodegradable. Polylactic acid (PLA) is considered a biopolymer because it derives from biomass sources and, in addition, it is compostable at industrial composting plants.

PLA is aliphatic polyester whose monomer, lactic acid, derives from carbohydrates from agricultural crops such as corn, potato, and cassava (Jiang & Zhang, 2011) by bacterial fermentation and it is one of the most commonly used biopolymer produced nowadays at industrial scale.

The most frequent route used for PLA manufacturing is the ring-opening polymerization of lactide. Another route is the direct condensation of lactic acid monomers. In 1992, PLA was approved by FDA as food contact material and in 2010, PLA reached the second highest consumption volume of any bioplastic in the world (Bor, Alin, & Hakkarainen, 2012; Lazzari, Codari, Storti, Morbidelli, & Moscatelli, 2014). PLA is a thermoplastic biopolymer and easy to process with standard equipment. This fact, together with its good mechanical and barrier

properties, similar to PET (Auras, Harte, & Selke, 2004; Gandini, 2008), makes it suitable and more attractive for the manufacturing of food packaging, such as bottles, food containers and wrappers (Jamshidian, Tehrany, Imran, Jacquot, & Desobry, 2010; Salazar, Domenek, Plessis, & Ducruet, 2017). Usually, PLA is blended to a polyester to improve its mechanical properties (flexibility, viscosity, etc.) and therefore to increase its applications. It has been also found blended to starch-based biopolymers (Osorio, 2019). Biodegradable aliphatic polyesters have been used in many areas during the last years due to the environmental contamination issues.

As in all polymers, biopolymers can contain oligomers (Gómez Ramos, Lozano, & Fernández-Alba, 2019; Heimrich, Nickl, Bönsch, & Simat, 2015; Hoppe, de Voogt, & Franz, 2016; Omer, Cariou, Remaud, Guitton, Germon, Hill, et al., 2018; Ubeda, Aznar, & Nerín, 2018; Úbeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017), defined as molecules consisting of a few monomer units. They are often included under the group of non-intentionally added substances (NIAS) (Canellas, Vera, & Nerín, 2015; Nerin, Alfaro, Aznar, & Domeño, 2013; Tian, Lin, & Bayen, 2019; Vera, Canellas, & Nerín, 2018). When PLA is used as food contact material, the oligomers can be transferred into food and consequently pose a risk to the consumer, thus, migration processes of these compounds must be evaluated (Catalá & Gavara, 2002). Migration of these oligomers could be seen as microplastics coming from plastic food contact materials (Bouwmeester, Hollman, & Peters, 2015; Gigault, Halle, Baudrimont, Pascal, Gauffre, Phi, et al., 2018; Silva, Bastos, Justino, da Costa, Duarte, & Rocha-Santos, 2018). For this reason is very important to identify the kind of oligomers and to quantify them.

Thermal processing as well as moisture or other external phenomena can affect PLA stability and can lead to the formation of PLA oligomers during their manufacturing or storage. In addition, the contact with aqueous food can affect its composition, since water can hydrolytically degrade PLA leading to the formation of new oligomers (Dopico-García, Ares-Pernas, Otero-Canabal,

Castro-López, López-Vilariño, González-Rodríguez, et al., 2013; Mutsuga, Kawamura, & Tanamoto, 2008; Osaka, Yoshimoto, Watanabe, Takama, Murakami, Kawasaki, et al., 2008; Salazar, Domenek, Plessis, & Ducruet, 2017) as well as other NIAS. It is important to study these oligomers in detail and develop new methodologies for PLA analysis that allow its detection (Aznar, Ubeda, Dreolin, & Nerín, 2018). Several works related to the degradation processes of PLA by hydrolysis and how this process affects its physico-chemical properties as well as the formation of new oligomers have been described in the literature (Andersson, Hakkarainen, Inkinen, Södergård, & Albertsson, 2010; Bor, Alin, & Hakkarainen, 2012; Dopico-García, et al., 2013; Inkinen, Hakkarainen, Albertsson, & Södergård, 2011; Lazzari, Codari, Storti, Morbidelli, & Moscatelli, 2014; Mutsuga, Kawamura, & Tanamoto, 2008). For the determination of the oligomers content, different strategies have been followed. One of the most common strategies has been to transform all the oligomers in lactic acid through an alkaline hydrolysis reaction and measure the differences in the lactic content before and after the PLA hydrolysis process (Bor, Alin, & Hakkarainen, 2012; Dopico-García, et al., 2013; Mutsuga, Kawamura, & Tanamoto, 2008). The lactic acid was quantified by LC-UV as well as by LC-MS. Other works have directly analysed PLA extracts by ESI-MS with no chromatographic separation, and masses related to sodiated cyclic oligomers [23 + 72n] (Bor, Alin, & Hakkarainen, 2012) as well as linear, in the case of aqueous solutions, [23 + 18+ 72n] (Andersson, Hakkarainen, Inkinen, Södergård, & Albertsson, 2010) were observed. In the study performed by Dopico et al. (Dopico-García, et al., 2013), the surface of a PLA polymer was directly analysed by MALDI-TOF, detecting cyclic and linear oligomers in a mass range from m/z 700 to 5000 (n = 15 - 35). The linear oligomers increased when PLA was submitted to an ageing process. The study of PLA migration by LC-MS has been performed by some authors, such as Mutsuga et al., where oligomers were measured by ESI in negative mode (Mutsuga, Kawamura, & Tanamoto, 2008) but as far as the authors know, only in the work carried out by Martinez Bueno et al (Martínez-Bueno, Hernando, Uclés, Rajski, Cimmino, &

Fernández-Alba, 2017), migration extracts were analyzed by LC coupled to high resolution mass spectrometry but oligomers were not detected.

The European Regulation 10/2011 on plastic materials intended to come into contact with food established specific rules for plastic materials but oligomers are not specifically regulated. Lactic acid is included in the list of authorized monomer without restrictions. However, cyclic lactide and linear or cyclic oligomers are not included in this list. For this reason, their migration should not exceed a level of 0.01 mg $kg^{-1}$ food (EC, 2011). The lack of analytical standards and spectral database of these substances and the low migration values are an important issue that requires a great effort to assure food safety and quality. Thus, the analysis of this type of compounds needs high resolution mass spectrometry in order to perform its structural elucidation.

In this work, a dissolution/precipitation procedure with dichloromethane/ethanol was used for the analysis of the oligomer profile in PLA pellets and film samples. Migration tests were also carried out to determine the interactions of PLA oligomers when the biopolymer is in contact with different food simulants. The analyses were performed by UPLC-QToF.

# 4. MATERIAL AND METHODS

## 4.1. Reagents

A cyclic ester oligomer AA-DEG-IPA-DEG (95% w) formed by diethylene glycol (DEG), adipic acid (AA) and isophtalic acid (IPA), was used as standard for oligomers determination. It was provided by an adhesive producer. Its structure and purity were confirmed by NMR at the University of Zaragoza. Methanol (MeOH), ethanol (EtOH) and dichloromethane (DCM) were purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q

Ultramatric Wasserlab GR 216071 (Madrid, Spain). Acetic acid (CAS 64-19-7) was from Sigma-Aldrich Química S.A. (Madrid, Spain). Methanol and water for UPLC analysis (ultra LC–MS quality) were supplied by Baker (Deventer, The Netherlands).

### 4.2. Samples

Two different biopolymer samples based on PLA were supplied by 2 different packaging companies, PLA 1 and PLA 2. PLA 1 was blended to a biodegradable polyester. They were delivered in pellets as well as in films. For confidential reasons, additional information about the samples is not provided.

### 4.3. Total dissolution

Total dissolution was performed following the optimized protocol of PLA (Aznar, Ubeda, Dreolin, & Nerín, 2018). In this protocol,an amount of 0.25 g of PLA pellets or films was weighed in a 20 mL glass vial and 3 mL of DCM were added as solvent. The vial was closed with a screw cap and introduced in ultrasonic bath for 1 hour in order to guarantee a deep contact between solvent and polymer. After this time, PLA solution was taken and 6 mL of ethanol were added as antisolvent. The mixture was centrifuged at 500 rpm for 15 minutes and the supernatant was extracted. Subsequently, the residual precipitated polymer was washed with 3 mL of ethanol and the liquid phases were combined. Thus, the extract was kept at 4 ºC for 1 hour to facilitate the polymer precipitation. Finally, the solution was filtered, evaporated under a nitrogen current and the residue was redissolved with methanol/water (1:1). Three replicates were prepared from each sample and several procedural blanks were also analyzed. They were injected into a UPLC-QToF system.

## 4.4. Migration tests

For the migration experiments, PLA films were introduced in three food simulants according to the rate of 6 $dm^2$ of packaging material per 1 kg of simulant, in accordance with European Regulation 10/2011 (EC, 2011).

The materials were tested in ethanol 10% (simulant A) and acetic acid 3% (simulant B) as aqueous simulants and in ethanol 95% as fat simulant. The vials with films were maintained in an oven at 60 ºC for 10 days. Simulants and test conditions used for the migration assays were chosen according to the European Regulation 10/2011 (EC, 2011). The samples were analyzed by UPLC-QToF and also by UPLC-IMS-QToF. Three replicates of every test were analyzed.

## 4.5. Instrumental analysis

### 4.5.1. Ultra-performance liquid chromatography-mass spectrometry quadrupole time-of-flight (UPLC-QToF)

Chromatography was carried out in an AcquityTM system using an Acquity UPLC BEH C18 column (2.1 mm x 100 mm x 1.7 µm particle size), both from Waters (Milford, MA, USA). The solvents used as mobile phases for positive mode were water and methanol both with 0.1 % formic acid (solvents A1 and B1 respectively). For negative mode, water and methanol (solvents A2 and B2 respectively) were used. The column flow was 0.3 mL $min^{-1}$ and the column temperature was 40 °C. The gradient elution (12 min) was performed with mobile phase A/B variating from 98/2 % to 0/100 % over 8 min and maintained for two minutes. After that, mobile phase A/B changes to 98/2 % again to condition the column. The volume of sample injected was 10 µL.

The detector was an API source (atmospheric pressure ionization) with ESI (electrospray ionization) coupled to a mass spectrometer (Xevo G2) consisting of a quadrupole, a collision cell and a time-of-flight (QToF) detectors all supplied by Waters (Milford, MA, USA).

The electrospray probe was used in positive (ESI+) and negative (ESI−) modes as well as sensitivity analyzer mode. The mass range was from 50 to 1200 Da. The capillar voltage was 2.5 kV. The sampling cone voltage was 30 V and 70 V for positive mode and 30 V for negative mode. Source temperature was 120°C. Nitrogen was used as the desolvation gas: the flow rate was 450 L $h^{-1}$ at 400 °C. The cone gas flow rate was 20 L $h^{-1}$.

Acquisition was performed in MSE mode to allow using low and high collision energy (CE) in the collision cell during the same run. The mass spectrum at low energy (CE 4 V) provided information about the precursor ion (function 1) and the mass spectrum at high energy (CE ramp: from 15 to 30 V) information about fragment ions (function 2). The accuracy and reproducibility were guaranteed by the infusion of a LockSpray solution of leucine-enkephalin (2 ng $mL^{-1}$ in water/acetonitrile with 0.1 % formic acid) at a flow rate of 5 µL $min^{-1}$. MassLynx version 4.1 (Waters, Milford, MA, USA) was used to analyze the samples.

### 4.5.2. Identification of oligomers of PLA detected by UPLC-QToF

High-resolution mass spectrometry with a tandem quadrupole-time of flight mass spectrometer was used for the identification of non-volatile compounds. This technique provides molecular fragmentation combined with mass accuracy in order to elucidate the molecular structure that could lead to the identification of the compounds. Conditions used for the acquisition were explained in section 4.5.1.

Function 1 corresponds to the acquisition without collision energy, where the molecular formula of the oligomers based on the molecular ion can be obtained through the measurement of its accurate mass and the isotopic ratios.

Finally, with the use of function 2, the fragmentation spectra of the oligomers were obtained and the proposed candidates were checked through MassFragment® software from Waters. This software enabled us to evaluate and confirm whether the product ions detected in the high collision energy spectrum could be linked to the fragments generated from the chemical structures of the candidates proposed. Since oligomers are not included in any chemical databases and there are not standards available, it was necessary to draw the molecules in ChemDraw Ultra 12.0 to obtain a .mol document for further confirmation of unknown oligomers.

### 4.5.3. Semi-quantification of oligomers of PLA detected by UPLC-QToF

PLA oligomers were semi-quantified by calibration curve of oligomer AA-DEG-IPA-DEG as standard. The working range in the instrument was 0.03-1.30 µg $g^{-1}$ and the coefficient of determination ($R^2$) obtained in the calibration curve was 0.9999. The limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.03 µg $g^{-1}$ respectively. The LOD and LOQ were calculated as the minimum concentration whose signal was equal to 3 times and 9 times the baseline noise. Five independent replicates of solutions containing 0.01 and 0.03 µg $g^{-1}$ of the standard were injected for their calculation.

### 4.5.4. Ultra-performance liquid chromatography-ion mobility mass spectrometry quadrupole time-of-flight (UPLC-IMS-QToF)

UPLC-IMS-QToF analysis was performed in a Vion IMS QToF supplied by Waters (Milford, MA, USA). Chromatography was carried out following the same conditions described in 2.5.1. MS data were acquired in (HD)MSE mode,

in the range 50-1000 m/z. Low collision energy was set at 4 V and high collision energy at a ramp from 20 to 40 V. Source and desolvation conditions were the same as described in 2.5.1. IMS was used to measure the ions drift-time, that after a calibration process was transformed to collision cross-section values (CCS). Nitrogen was used as drift gas. Trap conditions were as follows: IMS wave velocity of 250 m/s, IMS pulse height 45 V, trap bias 40V, trap wave velocity 100 m/s, trap pulse height A 10V, trap pulse height B 5V and gate release 2 ms. Data were processed using UNIFI v1.8 software.

### 4.6. Analysis by scanning electron microscopy (SEM)

Analyses were performed with a JEOL JSM 6400 microscope, using a voltage of 15kV. In order to analyse the morphology of the samples a detector of secondary electrons was used. Some drops of the migration samples were placed on the wafer and left dry. Blanks of migration were also analysed.

## 5. RESULTS AND DISCUSSION

### 5.1. PLA oligomers identified in PLA-based biopolymer

Table 1 summarizes the oligomers identified in total dissolution extracts of PLA1 pellets and films and in migration simulants after the exposure. A total of 39 different oligomers, cyclic and linear, were identified. Among them, 24 were identified in total dissolution of pellets and film samples, 50% of them were cyclic and 50% linear. Ten out of 24 oligomers were also detected in migration together with 15 new oligomers formed in the reaction between PLA components and food simulants. All the oligomers detected in migration were linear oligomers. Most of them had been detected in previous studies, their exact mass was provided but no candidates were proposed (Bradley, 2010). These oligomers are also displayed in table 1 according to their retention time. In all cases, the ion

detected was the sodium adduct [MNa+]. All the oligomers detected contained the monomer unit [LA], that corresponded to the molecular formula $C_3H_4O_2$. They were classified in 2 groups based on their chemical structure: group 1 formed by cyclic oligomers, $[LA]_n$; and group 2 formed by linear oligomers, OH-$[LA]_n$-H and $CH_3$-$CH_2$-O-$[LA]_n$-H. The exact mass for n values ranging from n =1 to n =20 was calculated in all oligomer structures and extracted in the chromatograms, in order to check their presence in the samples. All these chemical structures or similar were previously described by Badía et al. (Badía, Strömberg, Ribes-Greus, & Karlsson, 2011). Other similar structures described by Badía were searched in the samples, such as $CH_3$-O-$[LA]_n$-H and $CH_3$-CO-O-$[LA]_n$-H but none of them was found in pellets or films.

The value of n ranged from n=5 to n=16 in $[LA]_n$, from n=3 to n=15 in OH-$[LA]_n$-H and from n=2 to n=15 in $CH_3$-$CH_2$-O-$[LA]_n$-H.

The order in the chromatogram for oligomers with the same number of monomers but different structures was as follows: firstly OH-$[LA]_n$-H, then [LA]n and finally, $CH_3$-$CH_2$-O-$[LA]_n$-H.

**Table 1**. Oligomers identified in total dissolution of PLA materials (pellets and film) and in migration to 3 food simulants: ethanol 95%, ethanol 10% and acetic acid 3%. Retention time (rt), measured mass (mass), molecular formula (MF) and number of times that monomer of PLA is repeated (n).

| rt | Mass [MNa$^+$] | MF | Oligomers | | PLA samples | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Type | n | Pellets/films | Migration EtOH 95% | Migration EtOH 10% | Migration AcH 3% |
| 4.50 | 257.0631 | $C_9H_{14}O_7$ | Linear HO-[LA]$_n$-H | 3 | | | x | x |
| 5.09 | 213.0733 | $C_8H_{14}O_5$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 2 | | x | x | |
| 5.31 | 329.0842 | $C_{12}H_{18}O_9$ | Linear HO-[LA]$_n$-H | 4 | x | x | x | x |
| 5.77 | 285.0944 | $C_{11}H_{18}O_7$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 3 | | x | x | |
| 5.87 | 401.1053 | $C_{15}H_{22}O_{11}$ | Linear HO-[LA]$_n$-H | 5 | x | x | x | x |
| 6.26 | 357.1155 | $C_{14}H_{22}O_9$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 4 | | x | x | |
| 6.27 | 473.1264 | $C_{18}H_{26}O_{13}$ | Linear HO-[LA]$_n$-H | 6 | x | x | x | x |
| 6.41 | 383.0948 | $C_{15}H_{20}O_{11}$ | Cyclic [LA]$_n$ | 5 | x | | | |
| 6.58 | 545.1475 | $C_{21}H_{30}O_{15}$ | Linear HO-[LA]$_n$-H | 7 | x | x | x | x |
| 6.61 | 429.1366 | $C_{17}H_{26}O_{11}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 5 | | x | x | |
| 6.80 | 455.1159 | $C_{18}H_{24}O_{12}$ | Cyclic [LA]$_n$ | 6 | x | | | |
| 6.82 | 617.1686 | $C_{24}H_{34}O_{17}$ | Linear HO-[LA]$_n$-H | 8 | x | x | x | x |
| 6.94 | 501.1577 | $C_{20}H_{30}O_{13}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 6 | | x | x | |
| 7.02 | 689.1897 | $C_{27}H_{38}O_{19}$ | Linear HO-[LA]$_n$-H | 9 | x | x | x | x |
| 7.07 | 527.1370 | $C_{21}H_{28}O_{14}$ | Cyclic [LA]$_n$ | 7 | x | | | |
| 7.18 | 761.2108 | $C_{30}H_{42}O_{21}$ | Linear HO-[LA]$_n$-H | 10 | x | x | x | x |
| 7.30 | 599.1581 | $C_{24}H_{32}O_{16}$ | Cyclic [LA]$_n$ | 8 | x | | | |
| 7.31 | 573.1788 | $C_{23}H_{34}O_{15}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 7 | | x | x | |
| 7.32 | 833.2319 | $C_{33}H_{44}O_{23}$ | Linear HO-[LA]$_n$-H | 11 | x | x | x | x |
| 7.32 | 645.1999 | $C_{26}H_{38}O_{17}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 8 | | x | x | |

**Continue table 1**

| rt | Mass [MNa+] | MF | Oligomers | | PLA samples | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Type | n | Pellets/films | Migration EtOH 95% | Migration EtOH 10% | Migration AcH 3% |
| 7.44 | 905.2530 | $C_{36}H_{48}O_{25}$ | Linear HO-[LA]$_n$-H | 12 | x | x | x | x |
| 7.45 | 717.2210 | $C_{29}H_{42}O_{19}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 9 | | x | x | |
| 7.53 | 977.2741 | $C_{39}H_{52}O_{27}$ | Linear HO-[LA]$_n$-H | 13 | x | x | x | x |
| 7.56 | 789.2421 | $C_{32}H_{46}O_{21}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 10 | | x | | |
| 7.58 | 743.2003 | $C_{30}H_{40}O_{20}$ | Cyclic [LA]$_n$ | 10 | x | | | |
| 7.60 | 1049.2952 | $C_{42}H_{56}O_{29}$ | Linear HO-[LA]$_n$-H | 14 | x | | | |
| 7.64 | 861.2632 | $C_{35}H_{48}O_{23}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 11 | | x | | |
| 7.72 | 1121.3163 | $C_{45}H_{60}O_{31}$ | Linear HO-[LA]$_n$-H | 15 | x | | | |
| 7.73 | 933.2843 | $C_{38}H_{52}O_{25}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 12 | | x | | |
| 7.75 | 815.2214 | $C_{33}H_{44}O_{22}$ | Cyclic [LA]$_n$ | 11 | x | | | |
| 7.80 | 1005.3054 | $C_{41}H_{56}O_{27}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 13 | | x | | |
| 7.84 | 887.2425 | $C_{36}H_{48}O_{24}$ | Cyclic [LA]$_n$ | 12 | x | | | |
| 7.88 | 1077.3265 | $C_{44}H_{60}O_{29}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 14 | | x | | |
| 7.93 | 1149.3476 | $C_{47}H_{64}O_{31}$ | Linear $CH_3$-$CH_2$-O-[LA]$_n$-H | 15 | | x | | |
| 7.98 | 959.2635 | $C_{39}H_{52}O_{26}$ | Cyclic [LA]$_n$ | 13 | x | | | |
| 8.05 | 1031.2846 | $C_{42}H_{56}O_{28}$ | Cyclic [LA]$_n$ | 14 | x | | | |
| 8.12 | 1103.3057 | $C_{45}H_{60}O_{30}$ | Cyclic [LA]$_n$ | 15 | x | | | |
| 8.16 | 1175.3268 | $C_{48}H_{64}O_{32}$ | Cyclic [LA]$_n$ | 16 | x | | | |

All PLA oligomers had common fragmentation spectra, which confirmed the similarity of their structures (Figure 1). Their common masses were 89.0600, 145.0503 and 217.0709 m/z, corresponding to the formula $C_3H_5O_3$, $C_6H_9O_4$ and $C_9H_{13}O_6$, respectively. Figure 1 shows the high collision energy spectra of the 3 different kinds of PLA oligomers for n=6: $[LA]_6$ (a), OH-$[LA]_6$-H (b) and $CH_3$-$CH_2$-O-$[LA]_6$-H (c). Fragments observed successfully matched with the proposed structures. These three masses were more intense in the cyclic oligomers than in the linear ones. In the cyclic oligomers, 217.0709 was the most intense while in the linear oligomers its intensity was similar to 145.0491.

Lactide ($C_6H_8O_4$), the monomer of PLA, was not detected in the analysis. This was due to its difficult ionization in the mass spectrometer. As the purpose of this paper was the determination of oligomers, the initial monomer was not finally included in the study.

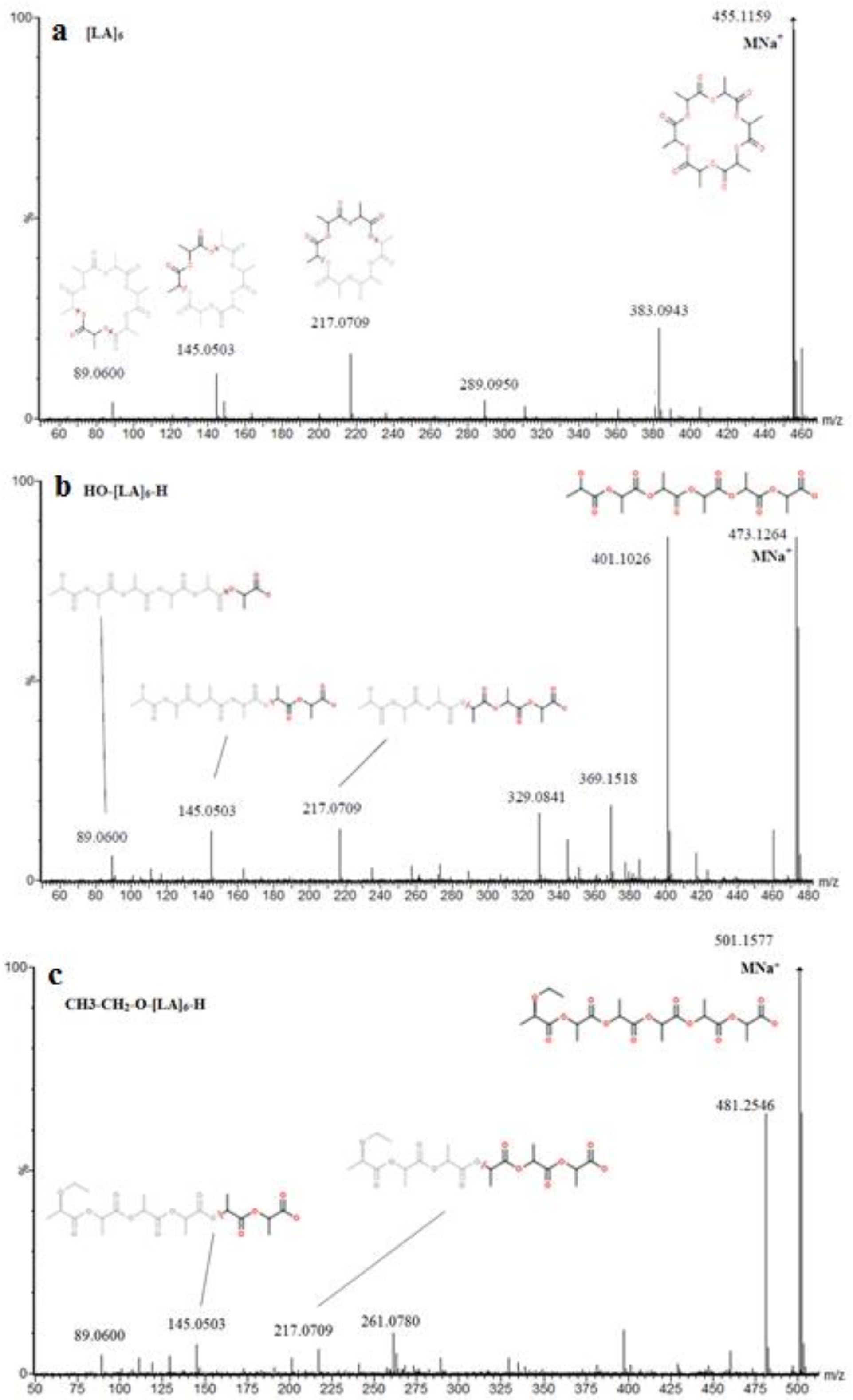



**Figure 1**. High collision energy spectra for [LA]n (a), H-[LA]n-H (b) and $CH_3$-

$CH_2$-O-[LA]n-H (c) when n=6.(Zoom x16).

## 5.2. Comparison of PLA oligomers content in pellets and films

The next aim was to compare the PLA oligomers profile in pellets and film in order to determine if the extrusion process had induced any change in PLA´s composition. The results showed that the areas of the oligomers identified in the total dissolution analysis of pellets and films were very similar. Figure 2a shows the areas of cyclic and linear oligomers in pellets vs films, and a good correlation can be observed (Pearson correlation value of 0.998). Therefore, no changes in the oligomer profile due to the manufacturing process are expected .These results are interesting as they demonstrate that applying high temperatures as those used in the film manufacturing from the pellets does not affect the composition of the material. Thus both pellets or films can be used for determining the oligomers profile of a PLA sample.

Figure 2b shows the distribution (ng $g^{-1}$) of the oligomers in the film. Only the cyclic oligomers from n=5 to 11 and the linear oligomers with HO-$[LA]_n$-H structure from n=5 to 8 were detected in the raw material, while $CH_3$-$CH_2$-O-$[LA]_n$-H were not present. The results show that the concentration values were much higher (5 to 20 times) for the cyclic oligomers than for the linear ones. This means that the cyclation of the oligomeric molecules is easier than the linear reaction between different oligomers, what results in a higher concentration of the cyclic ones. The cyclic structure of the oligomers will probably affect also their migration. The oligomer with the highest values was $[LA]_7$ followed by n= 6 and 8.

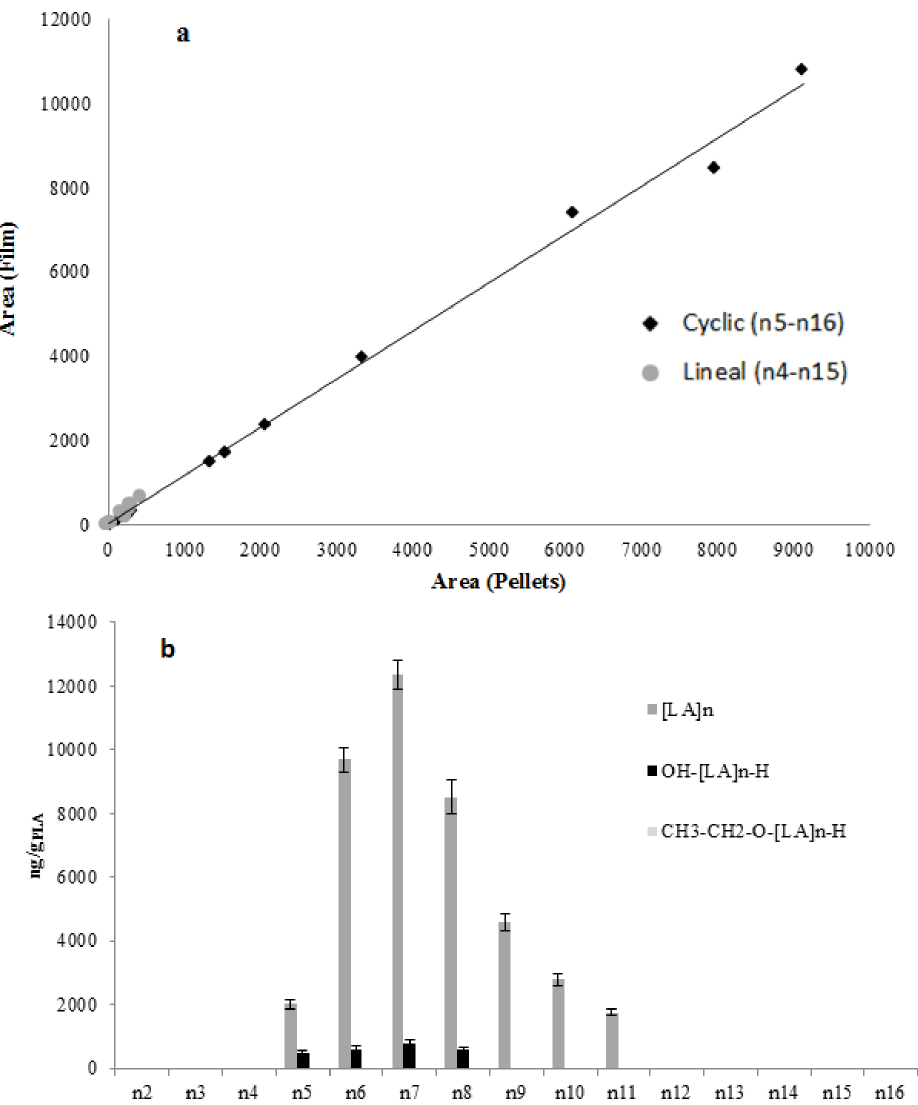



**Figure 2**. a) Representation of PLA oligomers area values of pellets vs films; b) Evolution of three types of PLA oligomers depending on the number of monomers in films.

## 5.3. Migration of PLA oligomers

Figure 3 shows the distribution (ng $g^{-1}$) of the two kinds of PLA oligomers, HO-$[LA]_n$-H (a) and $CH_3$-$CH_2$-O-$[LA]_n$-H (b), detected in migration from films to three different food simulants (EtOH 10%, AcH 3% and EtOH 95%). The values of n ranged from 3 to 16. Cyclic oligomers, which have been previously detected in total dissolution of films, were not present in any migration test.

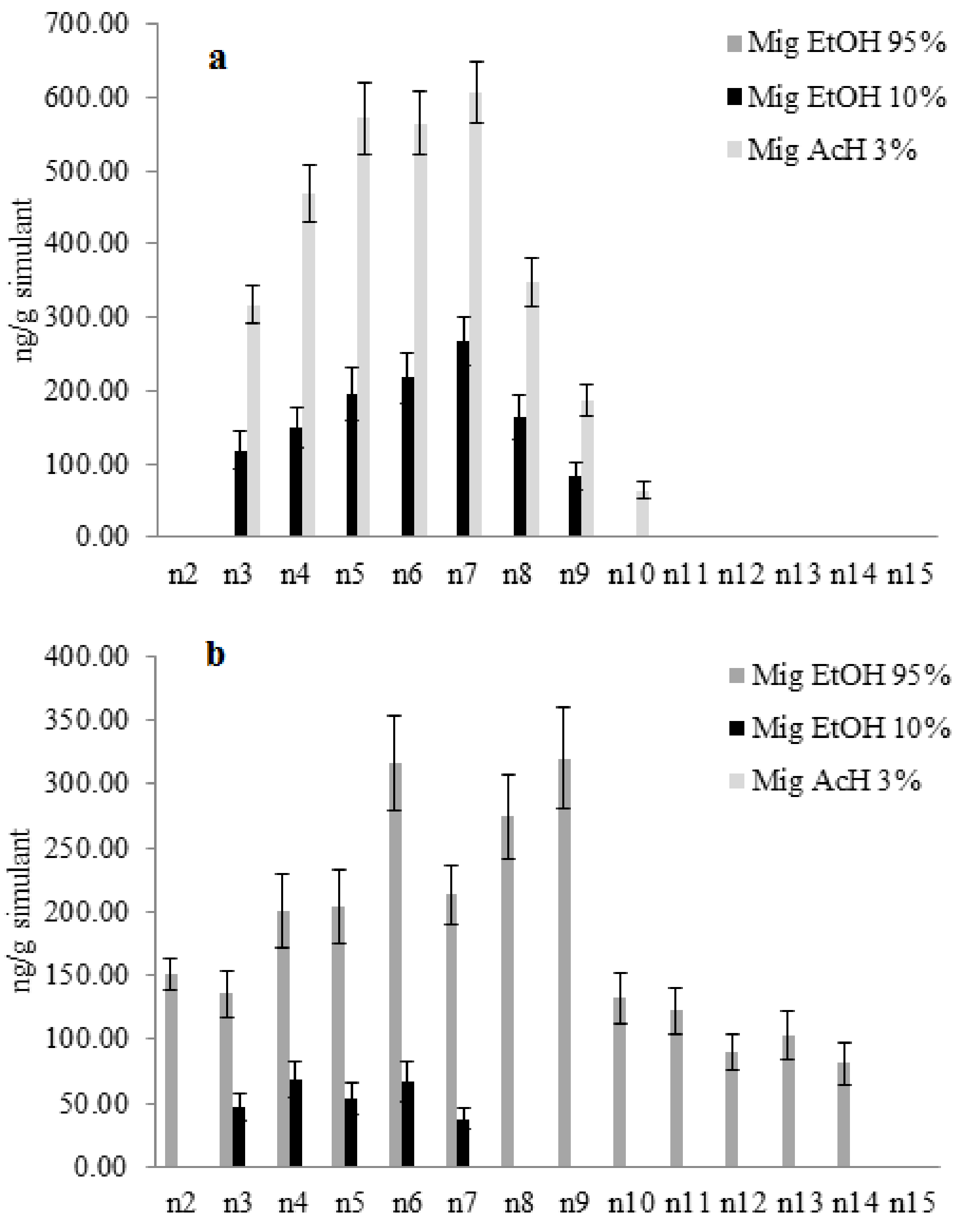
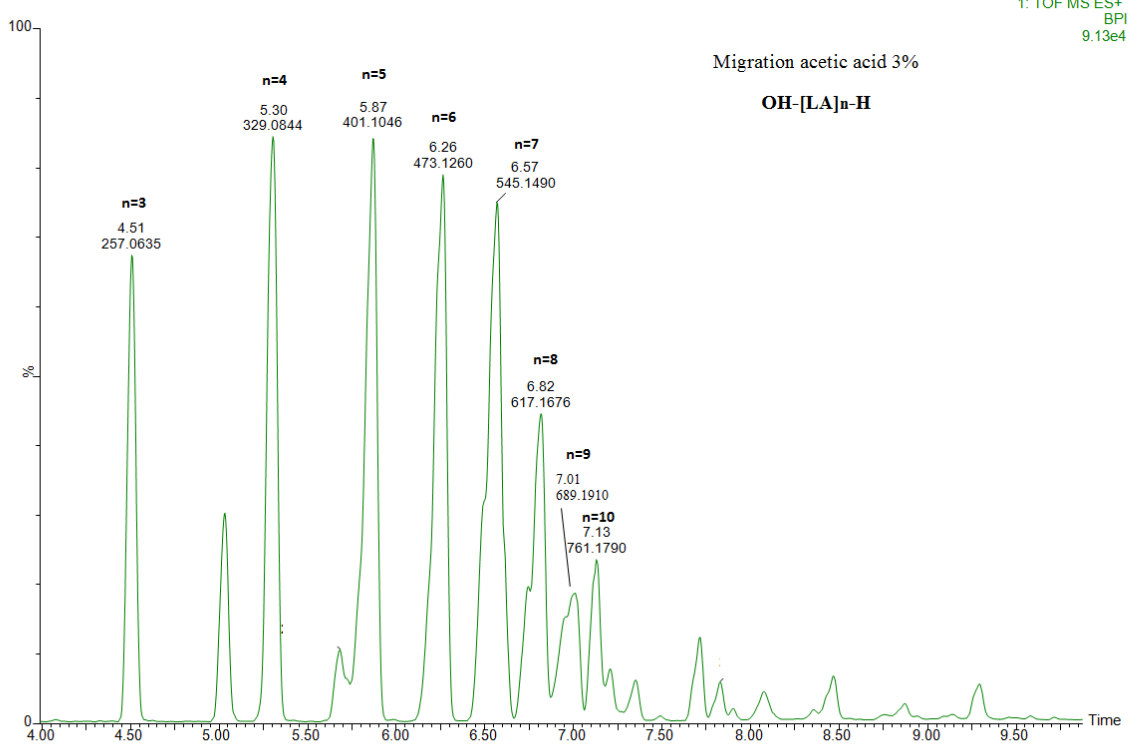



**Figure 3.** Migration of PLA oligomers: H-[LA]n-H (a) and $CH_3$-$CH_2$-O-[LA]n-H (b), in three different food simulants (Et95%, EtOH 10% and AcH 3%). (n = number of monomers).

Oligomers with HO-[LA]n-H structure were already present in the film. They migrated in a higher extension to aqueous food simulants (AcH 3% and EtOH 10%) than to fatty simulants (EtOH 95%). Chromatogram of these linear oligomers from n=3 to n=10 in 3% acetic acid follow a Gaussian profile and the distribution was very similar in the three simulants, where n=6-7 showed the highest values (Supplementary Material 1).

**Supplementary material 1.** Chromatogram of OH-[LA]n-H oligomer (function 1) in acetic acid 3% from n=3 to n=10.




$CH_3$-$CH_2$-O-$[LA]_n$-H was not initially present in the film but it was detected in migration to simulants with ethanol content, EtOH 95% and EtOH 10%. This chemical structure was selected based on the previous studies performed by Badía et al (Badía, Strömberg, Ribes-Greus, & Karlsson, 2011), where $CH_3$-O-$[LA]_n$-$CH_3$ oligomer was formed when PLA was in contact with methanol. In this case, the use of ethanol as simulant in migration test suggests that a structure $CH_3$-$CH_2$-O-$[LA]_n$-H would be more feasible. Both of them matched with the fragments found in the high collision energy mass spectra.

As it has been described, cyclic oligomers were not present in food simulants. This fact could be attributed either because they did not migrate or because they reacted with the simulants and thus the cycle was opened, forming new linear compounds. To elucidate this question, a new assay was performed. A solution of cyclic oligomers was exposed to different food simulants and stored

in an oven at 60 ºC for 10 days. Since there were not standards for these oligomers, an aliquot of a total PLA dissolution in $MeOH/H_20$ was taken and diluted five times with $MeOH/H_20$, EtOH 95%, EtOH 10% or AcH 3%. Figure 4 shows the areas of $[LA]_n$, OH-$[LA]_n$-H and $CH_3$-$CH_2$-O-$[LA]_n$-H at the initial time and after the storage time in the three food simulants. Oligomers with n values from 4 to 9 were selected for this study since they showed the highest areas. Similar profiles were observed for oligomers with different n value. The results are showed in figure 4. Figure 4a shows that the cyclic oligomers, $[LA]_n$, barely decreased when it was in contact with ethanol 95%, (Figure 4a), demostrating that they were quite stable in this simulant. In contrast, they clearly decreased in those simulants with a high water content such as EtOH 10% and AcH 3%, observing in parallel an increase of the linear oligomers in these simulants. The oligomers with OH-$[LA]_n$-H structure increased in contact with aqueous simulants (Figure 4b) while the oligomers with $CH_3$-$CH_2$-O-$[LA]_n$-H structure increased with ethanolic simulants, reaching the highest values for the simulant with the highest ethanol content, EtOH 95% (Figure 4c).

Thus, since migration of cyclic oligomers should mainly take place in ethanol 95%, and the results showed its stability in this simulant, the absence of cyclic oligomers in migration was not because they disappeared due to reaction processes between them and food simulants, but because they did not migrate. The presence of linear oligomers in migration was probably due to the interaction between the simulants and PLA, that provided with preference these oligomers due hydrolysis processes.

Migration tests were also performed from film PLA 2. But in this case, the material was clearly damaged after the study. According to EU/10/2011 (EC, 2011), if the material presents physical changes after the migration test, this material cannot be used under the tested conditions. For this reason, the concentration of oligomers is not shown in this manuscript. It is important to

highlight that the concentration of oligomers was much higher than expected, although similar oligomer profiles were observed.

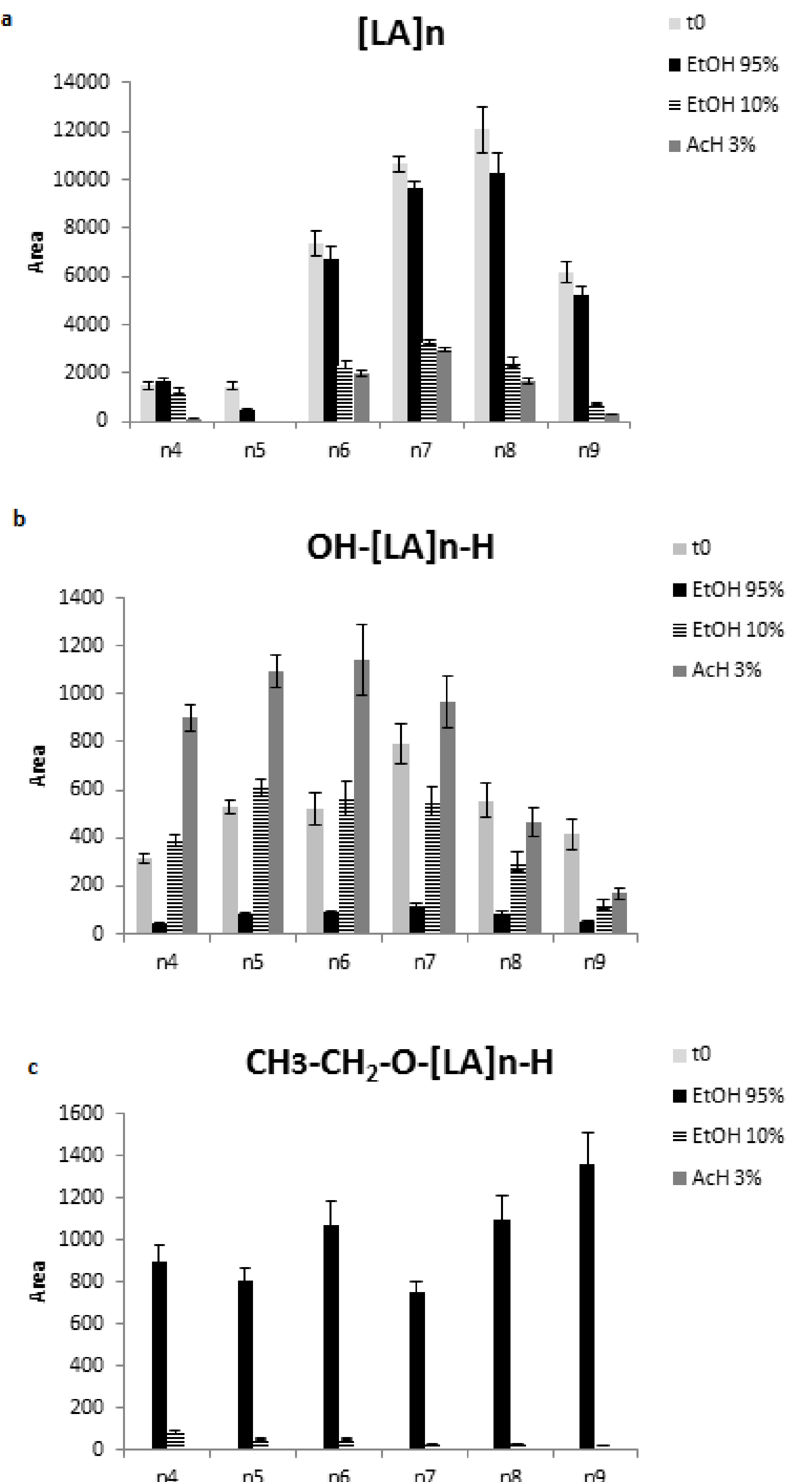


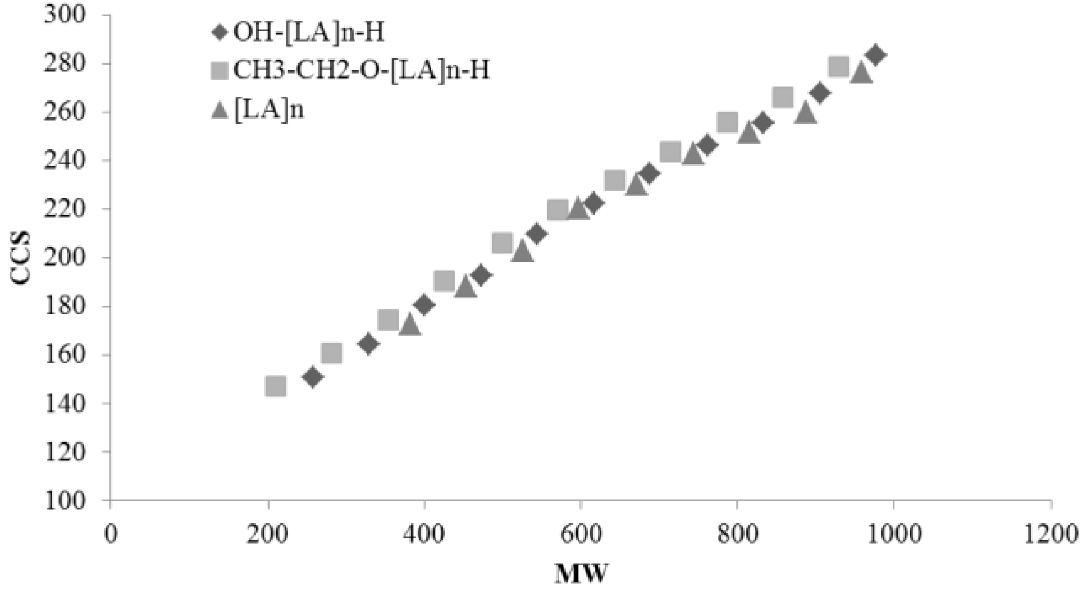

**Figure 4.** Evolution of $[LA]_n$ (a), OH-$[LA]_n$-H (b) and $CH_3$-$CH_2$-O-$[LA]_n$-H (c) at the initial time and after 10 days at 60 ºC in the three food simulants.

## 5.4. Collision cross section values of PLA oligomers

A good correlation was observed between the CCS values of the 3 kinds of oligomers versus their molecular weight (Supplementary Material 2). The correlation equations for each type of oligomers were:

- $[LA]_n$: y = 0.1763x + 107.69 ($R^2$= 0.9910)
- $OH-[LA]_n-H$: y = 0.1817x + 105.90 ($R^2$= 0.9976)
- $CH_3-CH_2-O-[LA]_n-H$: y = 0.1841x + 108.62 ($R^2$= 0.9983)

**Supplementary material 2.** CCS of the three kinds of oligomers versus their molecular weight.




According to the theory of ion mobility, the CCS values of cyclic and linear oligomers should be very different. As it can be observed, the three kinds of oligomers showed similar correlation equations, having the one corresponding

to the cyclic oligomers a slope slightly lower. Bigger differences in CCS values were expected between linear and cyclic oligomers with a similar molecular weight, as CCS depends on the tridimensional molecule structure and linear molecules are expected to have higher CCS values. Probably, the linear molecules stayed folded in the original position, maintaining their CCS similar to those of the cyclic ones and this could be the explanation of the similar behavior between the cyclic and linear oligomers.

## 5.5. Other oligomers identified in migration

In PLA 1 migration, 5 cyclic polyesters oligomers were also identified. They were composed by adipic acid (AA), phthalic acid (PA) and butanediol (BD). The structures were $[AA]_2$-$[BD]_2$ (7.21_401.2183), PA-AA-$[BD]_2$ (7.71_443.1676), $[AA]_3$-$[BD]_3$ (7.84_601.3229), PA-$[AA]_2$-$[BD]_3$ (8.20_643.2741) and PA-$[AA]_3$-$[BD]_4$ (8.45_843.3785). All masses had been detected in a previous work performed in our laboratory (Aznar, Ubeda, Dreolin, & Nerín, 2018) and their structure elucidated (supplementary material 3), except for 8.45_843.3785, which has been elucidated in this work by the first time. Figure 5 shows the concentration of these oligomers in three different simulants. Unlike PLA oligomers, cyclic oligomers from polyester migrated to food simulants and no hydrolysis to linear oligomers was observed. Migration values were especially high in the fatty food simulant rather than in aqueous simulants.

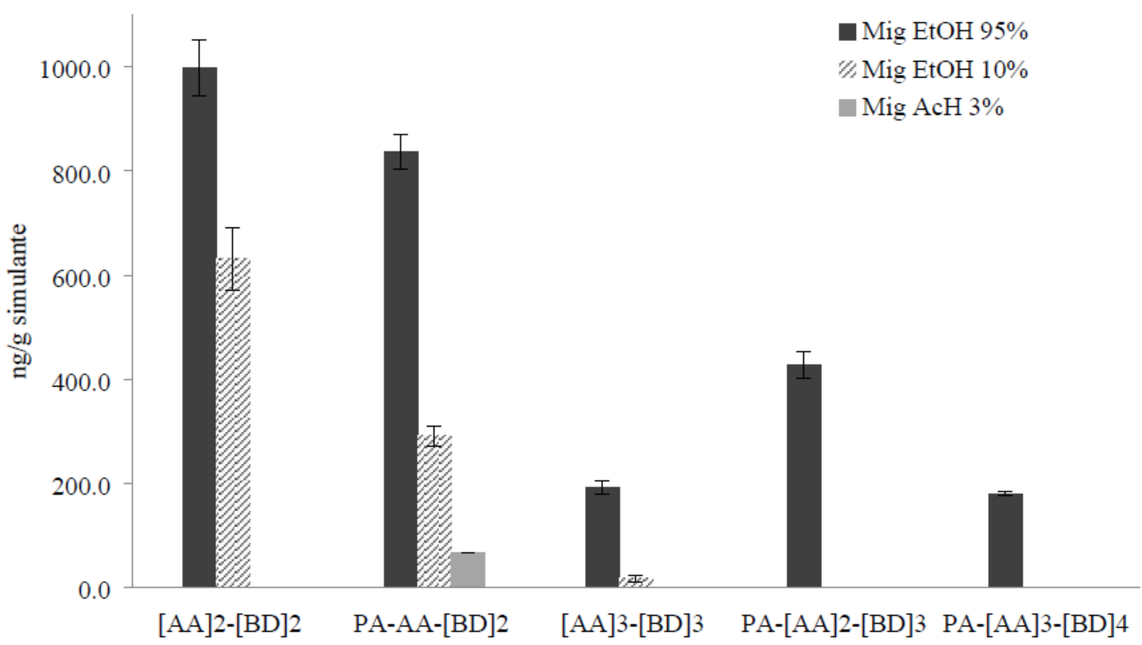
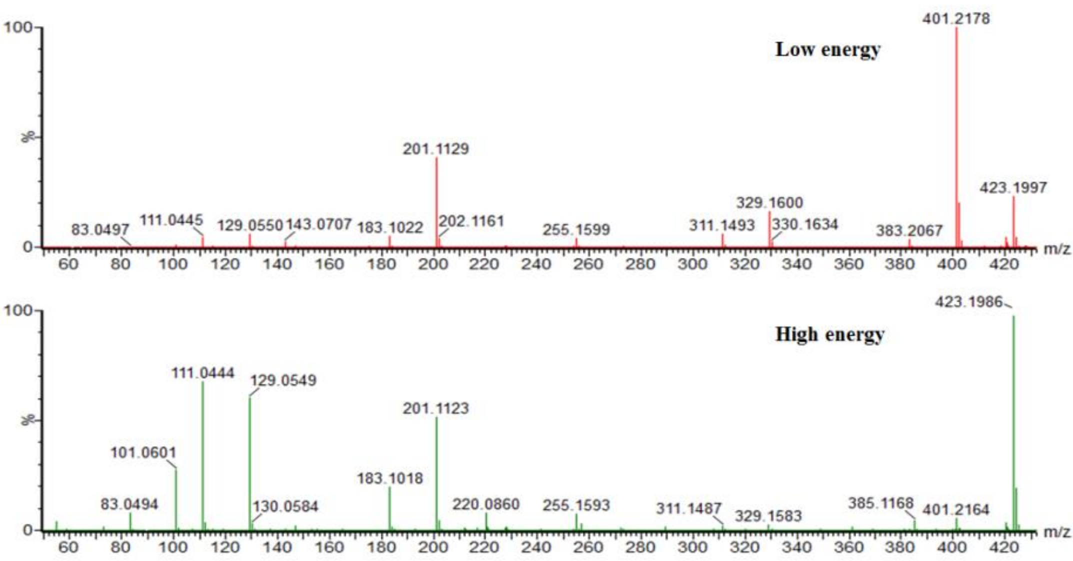



**Figure 5.** Migration of polyester oligomers in three different food simulants (Et95%, EtOH 10% and AcH 3%). (AA: adipic acid, PA: phthalic acid, BD: butanediol)

**Supplementary material 3.** Structural elucidation of 7.21_401.2183

Low and high energy mass spectra of 7.21_401.2183:




At first, elemental composition was calculated using low energy spectra. The results were:

- 401.2178_$[MH]^+$_ $C_{20}H_{32}O_8$ with 0.3 mDa error

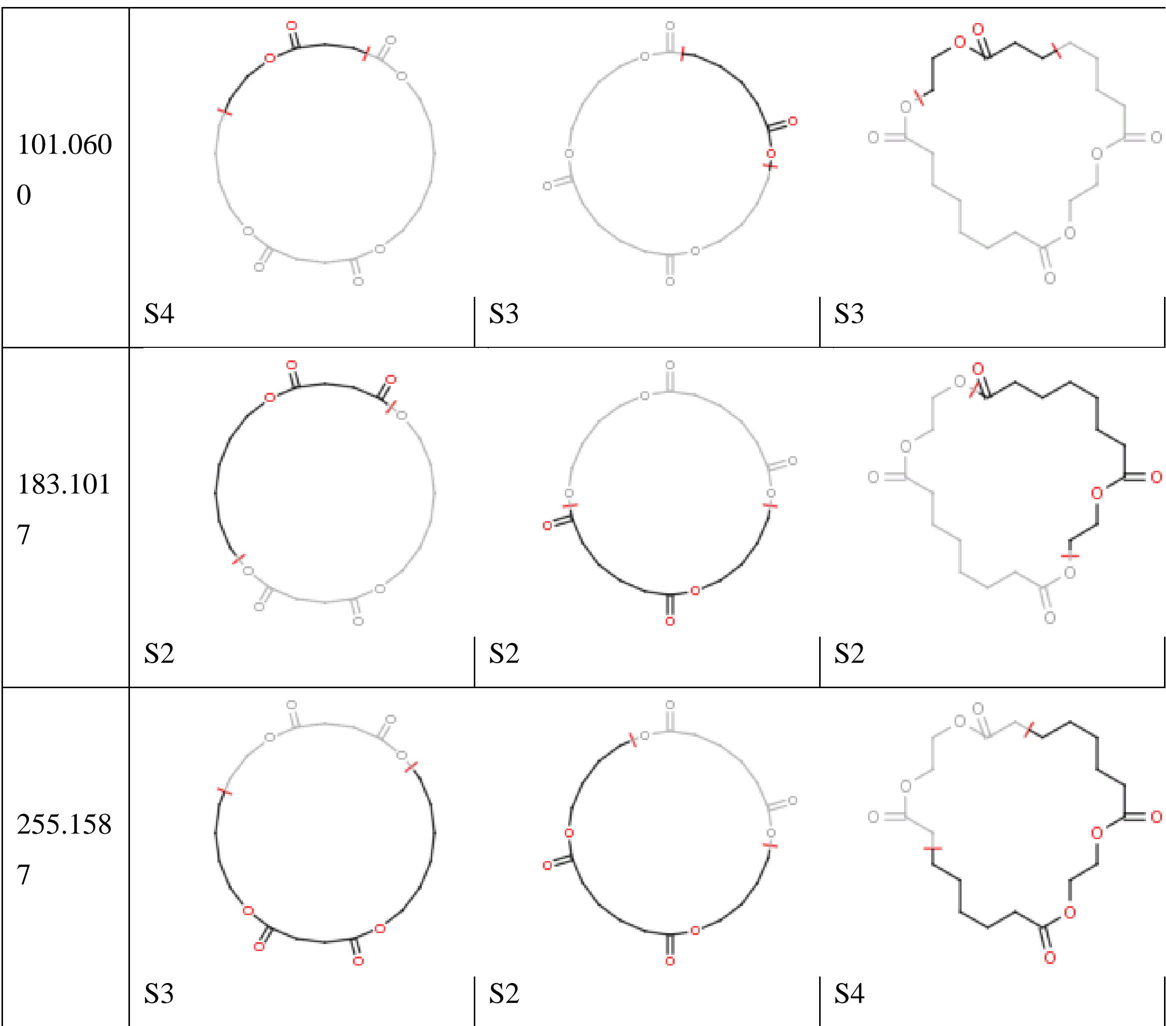
- 423.1996_[MNa]$^+$_ $C_{20}H_{32}O_8$ with 0.1 mDa error

The possible candidates for this formula were:

- [SubA-EG]$_2$ (suberic acid and etilenglycol)
- [SucA-HG]$_2$ (succinic acid and 1,6-Hexanediol)
- [AA-BD]$_2$ (adipic acid and butanediol)

Finally, high energy spectra masses were evaluated using MassFragment®. The following table describes the main fragments and the Score (S) obtained in MassFragment. S values ≤ 3 indicate that the proposal ruptures are probable.

| Fragment mass | Proposal rupture | | |
|---|---|---|---|
| | [Suc-HG]$_2$ | [AA-BD]$_2$ | [Sub-EG]$_2$ |
| 111.0443 | S10 | S2 | S5 |
| 129.0549 | S3 | S2 | S3 |
| 201.1122 | S4 | S2 | S4 |




Follow table summarizes the fragments and mass error values and scores for 7.21_401.2183:

| Mass fragment | Error (mDa) | Formula | Score MassFragment ® | | |
|---|---|---|---|---|---|
| | | | $[Suc\text{-}HG]_2$ | $[AA\text{-}BD]_2$ | $[Sub\text{-}EG]_2$ |
| 111.0443 | -0.3 | $C_6H_7O_2$ | 10 | 2 | 5 |
| 129.0549 | -0.3 | $C_6H_9O_3$ | 3 | 2 | 3 |
| 201.1122 | -0.5 | $C_{10}H_{17}O_4$ | 4 | 2 | 4 |
| 101.0600 | -0.3 | $C_5H_9O_2$ | 4 | 3 | 3 |
| 183.1017 | -0.4 | $C_{10}H_{15}O_3$ | 2 | 2 | 2 |
| 255.1587 | -0.9 | $C_{14}H_{23}O_4$ | 3 | 2 | 4 |

The final candidate was $[AA\text{-}BD]_2$.

## 5.6. Analysis of oligomers migration by SEM

Figure 6 shows the images at 2 different gains, x1500 and x8000 for a migration solution of ethanol 95% and ethanol 10%. Images show structures bigger that 1 μm, which could imply the presence of microplastics in the migration solutions (Gigault, et al., 2018). No structures were observed in blanks of migration (data not showed). The observed structures were different in ethanol 95% and ethanol 10%, what makes sense, since the oligomers that migrated to these two simulants were different. While cyclic oligomers from polyester migrated in a major extension to ethanol 95%, linear PLA oligomers were the main migrants to ethanol 10%.

The wafer with the samples of ethanol 95 % was afterwards placed in the oven at 260 ºC for 1 hour in order to see the effect of heat on these structures. Images after this period showed that they seemed to have melted, which could be attributed to a plastic nature of the structures. In any case, future studies in this research line should be done in order to confirm this hypothesis.

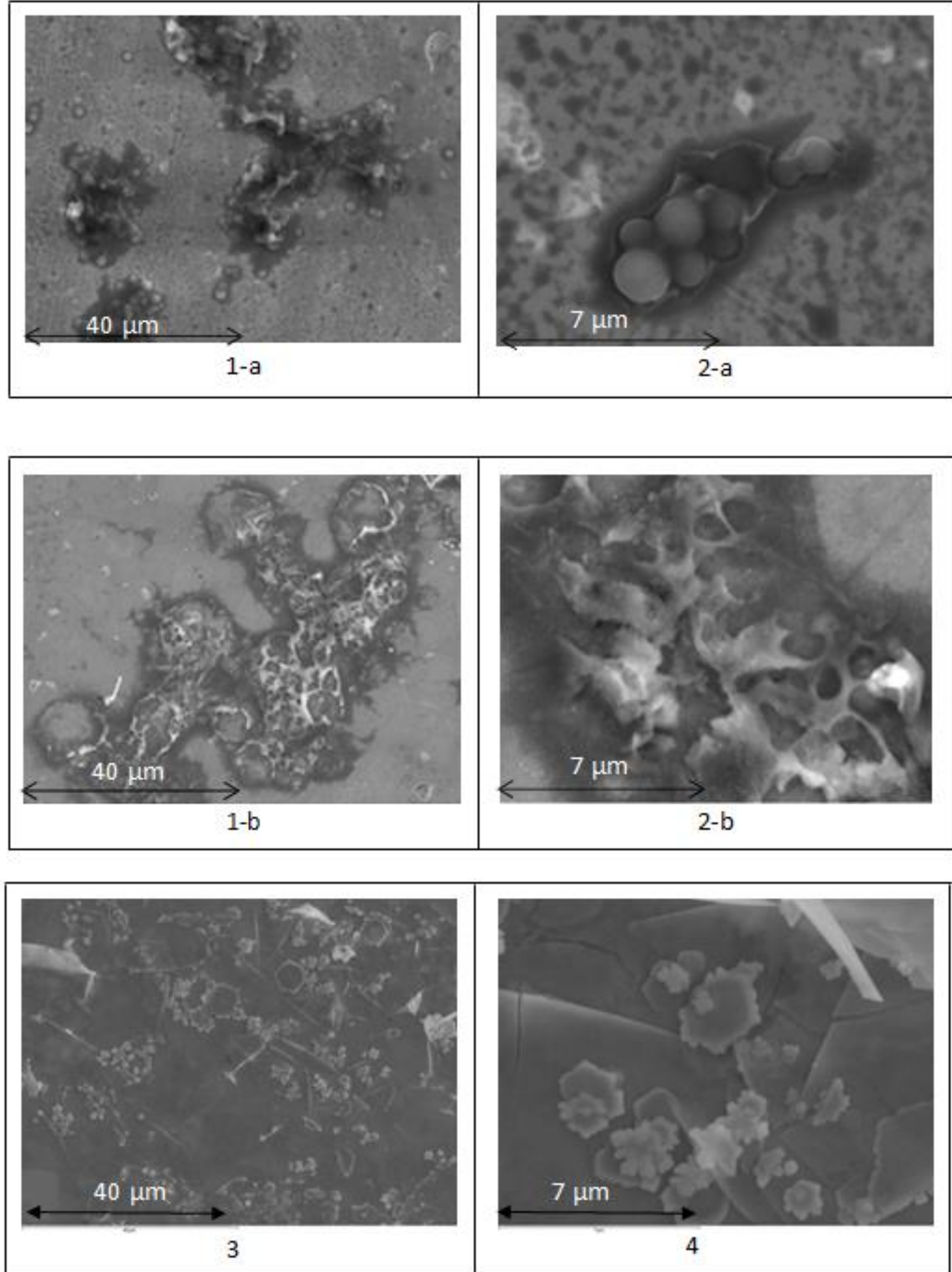



**Figure 6.** SEM images for ethanol 95% migration solution at 2 different gains, x1500 (1) and x8000 (2), before (a) and after (b) being submitted at 260 ºC for 1 hour. SEM images for ethanol 10% solution at x1500 (3) and x8000 (4) gains.

# 6. CONCLUSIONS

Identification and quantification of oligomers in food contact materials is an important and difficult task due to the little knowledge about them. Oligomers are neither included in libraries nor in chemical databases. There are not commercial standards and none knowledge of the toxicological properties of oligomers. However, oligomers migrate from the polymers in a quite wide extension and dissolve in the food or beverages, where they could be seen as microplastics.

Total dissolution/precipitation of PLA pellets and film has resulted to be a good sample treatment to determine the oligomers composition of PLA-base materials, mainly composed by cyclic oligomers, $[LA]_n$ , and linear ones such as HO-$[LA]_n$-H. Migration tests confirmed the presence of some of these oligomers in food simulants as well as new neo-formed oligomers such as, due to the reaction processes between PLA components and food simulants. What this work shows is the importance of the evaluation of migration, since the compounds present in food will depend on a great extent on the chemical reactions between packaging and food or food simulants and these neo-formed compounds could have toxic effects on consumers health. Production processes should be optimized in order to reduce and minimize as much as possible the presence of these oligomers in PLA-based materials since its possible transference to food has been demonstrated. It should be also considered that migration test were performed at temperatures close to the PLA glass transition temperature (50-60 ºC) and this fact could affect to its stability. New experiments at lower migration temperatures could demonstrate a better applicability of these materials to food packaging under refrigerated storage conditions.

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# Capítulo 5

*Determination of volatile compounds and their sensory impact in a biopolymer based on polylactic acid (PLA) and polyester*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
3. INTRODUCTION
4. MATERIAL AND METHODS
5. RESULTS AND DISCUSSION
6. CONCLUSIONS
7. REFERENCES

# 1. RESUMEN

Los materiales bioplásticos son ampliamente utilizados ya que son menos contaminantes para el medio ambiente. El ácido poliláctico (PLA) es uno de los más empleados para la fabricación de envases alimentarios. Para evaluar la seguridad de este material, en este trabajo se estudió una muestra de PLA-poliéster; su composición volátil en el polímero y en la migración a simulantes alimentarios. Los análisis se llevaron a cabo por cromatografía de gases acoplada a espectrometría de masas de impacto electrónico (GC-MS) y por cromatografía de gases a presión atmosférica acoplada a espectrometría de masas con tiempo de vuelo y cuadrupolo (APGC-QToF).

Los resultados mostraron que ambas técnicas proporcionaban información complementaria dando una información completa sobre la composición del biopolímero. Algunos compuestos como la lactida o la ciclopentanona se detectaron sólo por GC-MS, mientras que otros, como el dímero cíclico $[AA\text{-}BD]_2$ (AA: ácido adípico, BD: butanodiol) fueron detectados sólo por APGC-QToF. En la migración, se identificaron la lactida, AA-BD y $[AA\text{-}BD]_2$ en etanol al 95% mientras que en simulantes acuosos no se identificó ningún compuesto. También se realizó un estudio mediante GC-Olfatometría (GC-O). Algunos compuestos mostraron impacto sensorial en el polímero pero no en migración.

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El principal objetivo de este capítulo fue determinar los compuestos volátiles y semi-volátiles presentes en muestras de un biopolímero basado en PLA-poliéster utilizando dos técnicas, GC-MS y APGC-MS y su migración a diferentes simulantes alimentarios. Fue también un objetivo prioritario, determinar el posible impacto sensorial de los componentes del biopolímero en los alimentos envasados. A continuación se detallan las tareas que se llevaron a cabo en este capítulo:

- Búsqueda de bibliografía sobre la composición volátil en muestras de PLA.

- Optimización de un tratamiento de muestra basado en disolución/precipitación del biopolímero PLA-poliéster para determinar los compuestos volátiles.

- Aprendizaje del manejo de los equipos GC-MS y GC-MS-O con detector cuadrupolo y APGC-QToF con detector de tiempo de vuelo y cuadrupolo. Usando en los tres equipos inyección líquida y SPME.

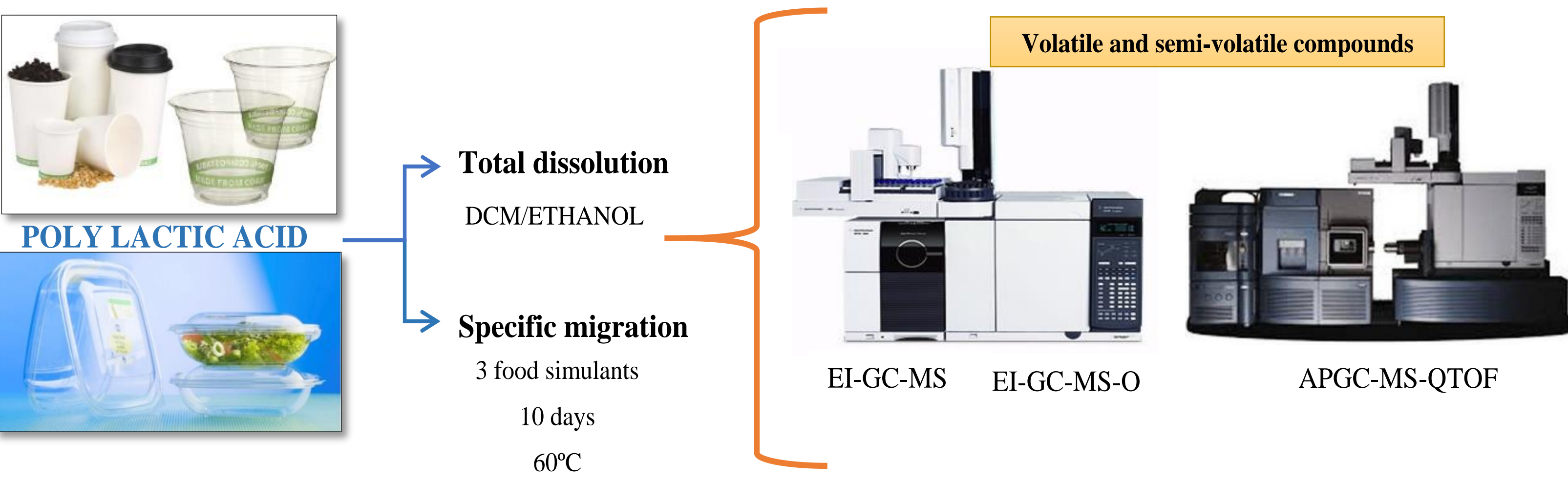
- Análisis de las muestras mediante las técnicas GC-MS y APGC-QToF y comparación de los resultados.

- Identificación de los componentes volátiles del film y pellets de PLA-poliéster.

- Estudio de los principales migrantes volátiles del PLA- poliéster.

- Estudio olfatométrico del biomaterial y de los simulantes de migración tras el contacto con el material.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 5.




**Esquema 5:** Diseño experimental del Capítulo 5.

# 3. INTRODUCTION

Biopolymers obtained from renewable resources represent an interesting and cost-effective alternative route to commodity petrochemical-based materials. One of the most common biopolymers applications is the manufacturing of food packaging materials (Auras, Harte, & Selke, 2004; Rhim, Park, & Ha, 2013). One of the most promising bio-based polyesters aimed for food packaging is polylactic acid (PLA) (Panseri, Martino, Cagnardi, Celano, Tedesco, Castrica, et al., 2018), a biopolymer chemically synthesized using monomers obtained from agro-resources like wheat, corn, and cassava. PLA can be manufactured from lactic acid, or most commonly, from its cyclic dimmer, lactide through a ring opening polymerization. At the moment, due to its availability on the market, its low price and its mechanical and barrier properties, similar to PET, PLA is one of most used bio-polyesters, particularly for the manufacturing of packaging (Avérous, 2008; Bordes, Pollet, & Avérous, 2009).

Food packaging contributes to keep food safety and quality. However, packaging materials are not inert and mass transfer from them to foodstuff can take place, affecting consumers' health. Biopolymer packaging materials contain additives to stabilize the polymer during processing or to improve its properties, such as antioxidants, ultraviolet light absorbers, slip agents and plasticizers (Hahladakis, Velis, Weber, Iacovidou, & Purnell, 2018; Lau & Wong, 2000; Llana Ruiz-Cabello, Pichardo, Jiménez-Morillo, González-Vila, Guillamon, Bermudez, et al., 2017). They are considered IAS (intentionally added substances). Other molecules may also be present in the packaging as residual monomers or low molecular weight oligomers and even non-intentionally added substances (NIAS) (Arrieta, Parres, López, & Jiménez, 2013; Kameno, Yamada, Amimoto, Amimoto, Ikeda, & Koga, 2016; Mutsuga, Kawamura, & Tanamoto, 2008; Nerin, Alfaro, Aznar, & Domeño, 2013; Ubeda, Aznar, & Nerín, 2018; Úbeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017). All these

compounds can be volatile or non-volatile compounds and they could migrate to the food in contact and affect food safety or change its sensory properties.

To ensure food safety, all food contact materials (FCM) must comply with the Regulation (EU) no. 10/2011 (EC, 2011). This legislation includes a positive list of substances that can be present in migration at concentration values below its specific migration limit (SML). If the substances are not included in this list, their migration should not be found above 0.01 mg $kg^{-1}$. Due to this low value, it is important the use of analytical methodologies with high sensitivity for identification of compounds migrating from the FCM.

The identification of volatile compounds is possible when using highly sensitive analytical methods, such as gas chromatography-mass spectrometry (GC-MS) or Atmospheric Pressure Gas Chromatography coupled to quadrupole-time of flight mass spectrometry (APGC-QToF) (Canellas, Vera, Domeño, Alfaro, & Nerín, 2012; Canellas, Vera, & Nerín, 2014). They are two powerful complementary techniques that allow the identification of volatile and semi-volatile compounds even though samples are at low level of concentration. GC–MS systems often use ionization under vacuum conditions, like electron impact ionization (EI). EI is considered as a hard ionization technique, meaning that the energy of the electrons is high enough to produce highly reproducible fragmentation patterns of small molecules. This fact allows the use of scientific libraries of mass spectra and an easy identification when the detected compound is recorded in the libraries. If it is not, the identification process becomes very complex. In contrast, chemical ionization (APCI) source and APGC are considered soft ionization techniques compared to EI, and fewer fragments are formed. When these techniques are coupled to high resolution mass spectrometry techniques, it is possible to have the exact mass of the parent ion and the structural elucidation process of the molecule is possible. Therefore, the APGC ionization process compared to EI will give us different structural information and for this reason, they are considered complementary techniques (Canellas et

al. n, 2012; Canellas et al.  , 2014; Cherta, Portolés, Pitarch, Beltran, López, Calatayud, et al., 2015; Domeño, Canellas, Alfaro, Rodriguez-Lafuente, & Nerin, 2012; Lv, Niu, Zhang, Shao, & Du, 2017; Sales, Cervera, Gil, Portolés, Pitarch, & Beltran, 2017; Stevens, Shi, & Hsu, 2013; ten Dam, Pussente, Scholl, Eppe, Schaechtele, & van Leeuwen, 2016).

Some volatile compounds present in FCM can modify the flavor composition, odor and properties of the packaged food, giving rise to different off-flavours. Aroma sorption and permeation through packaging materials can affect the sensory quality of food products (Leelaphiwat, Harte, Auras, Ong, & Chonhenchob, 2017; Martínez-Bueno, Hernando, Uclés, Rajski, Cimmino, & Fernández-Alba, 2017; Osorio, Aznar, & Nerín, 2019; Salazar, Domenek, & Ducruet, 2014; Salazar, Domenek, Plessis, & Ducruet, 2017; Vera, Canellas, & Nerín, 2014; Vera, Uliaque, Canellas, Escudero, & Nerín, 2012). For this reason, it is important to study the aroma profile of the biodegradable polymers, in order to be sure that it will not modify the sensory properties of food in contact with it.

In this study, the main volatile and semi-volatile compounds of a biodegradable material intended to food packaging were identified. The key aroma compounds of the biopolymer blend, in both, the material and the migration simulants, were also determined by GC-Olfactometry-MS (GC-O-MS). The material under study was a blend of PLA and a polyester added to facilitate the processability. The blend was analyzed by total dissolution/precipitation and migration tests were applied with three food simulants at 60 ºC for 10 days. The analysis were done by both techniques, GC-MS and APGC-QToF.in order to have a more accurate composition of volatile potential migrants. The use of these three techniques provided a more complete profile of aroma and volatile compounds present in the blend samples containing PLA.

# 4. MATERIAL AND METHODS

## 4.1. Sample characteristics

A biodegradable blend composed by a polyester with 18 % of PLA was used for this study. Information about the polyester blended to PLA was not provided by the supplier. Based on previous studies performed in our laboratory and the volatile and non-volatile substances found the attempt of poly(butylene adipate-co-terephthalate) can be considered. The samples were provided as pellets and films. The material was certified as biodegradable polymer.

Mass and bulk densities were 1.24-1.26 and 0.78 g/cm$^3$. Melt volume rate (MVR) was 7-11 mL min$^{-1}$. Melting points were 110-120 to 140-155 ºC. Permeation rates of water vapor at 38 ºC and 90 % r.h. was 600 g m$^{-2}$ d$^{-1}$. Good thermostability up to 230 ºC. Film thickness was 0.17 mm.

## 4.2. Reagents and solvents

Lactide [4511-42-6] was from Sigma–Aldrich (Madrid, Spain). A cyclic ester oligomer composed by diethylene glycol (DEG), adipic acid (AA) was used as standard for oligomer quantification. It was synthetized and provided by an adhesives company and the purity and structure were confirmed by NMR at the University of Zaragoza. Octanal, 1-octen-3-one, E-2-nonenal, sotolon, citronellal and dodecanal were purchased from Sigma Aldrich and nonanal was by Fluka.

Dichloromethane, acetic acid and ethanol (HPLC quality) were supplied by Scharlau Chemie S.A. (Sentmenat, Spain). Ultra-pure water was obtained with a Water purification System Type I Ultrapure from Wasserlab (Navarra, Spain).

## 4.3. Sample treatment

### 4.3.1 Total dissolution/precipitation procedure

The protocol used for the dissolution of PLA samples, pellets as well as films, was based on the protocol developed by Aznar (Aznar, Ubeda, Dreolin, & Nerín, 2019), applying some modifications according to the final analysis methodology.

A quantity 0.25 g of PLA was weighted, 3 mL of dichloromethane were added and the mixture was shaken in an ultrasound machine for 1 hour until it was totally dissolved. Six mL of ethanol were added to the dissolved PLA under magnetic stirring (500 rpm, 15 min) for the precipitation of the polymer. After this time, the supernatant solvent was removed and stored in a vial. Afterward, 3 mL of ethanol were added to the precipitated polymer and the mixture was manually shaken. Then, the polymer was gently squeezed with a glass bar and the solvent was removed and mixed with the previous extract. The final extract was kept in the freezer for 1 hour and finally, it was filtered through a 0.25 μm PET filter. The final dissolution was concentrated 4 folds under a nitrogen current. 2 folds concentration was alternatively applied but the chromatogram showed a lower number of compounds. Total dissolution of PLA sample was performed in triplicate. These samples were analysed by GC-MS and APGC-QToF with liquid injection.

### 4.3.2 Samples for direct analysis

The direct analysis was done on PLA blend films. They were cut in pieces of 1x1 cm2, of approximately 0.4 g, and introduced in 20 mL vials. These samples were analysed by GC-O-MS with HS-SPME injection. They were analysed in triplicate.

## 4.4. Migration test

The migration analysis was performed by total immersion of the PLA blend films in three different food simulants. Food simulants were ethanol 95%, ethanol 10 % (A) and acetic acid 3% (B). Pieces of films were cut (5x1 $cm^2$) and introduced in 20 mL vials. Then, 18 g of food simulant were added fulfilling a ratio close to 6 $dm^2$ $kg^{-1}$ of simulant according to the Regulation 10/2011. After that, these vials were kept in the oven for 10 days at 60 ºC. All the migration experiments were performed in triplicate.

Simulant ethanol 95% was analysed by liquid injection while ethanol 10% and acetic acid 3% were analysed by HS-SPME, transferring previously an aliquot of 4 mL to a 20 mL vial. Analyses were performed by GC-MS, GC-O-MS (both with electronic impact ionization) and APGC-QToF in triplicate.

## 4.5. Instrumental analysis

### 4.5.1. Sample injection

The injection was performed in splitless mode and the detector temperature was fixed at 250 ºC. Liquid injection was performed with an injection volume of 1 µL. HS-SPME injection was carried out with a DVB/CAR/PDMS fibre with medium polarity and the vials were extracted at 80 ºC during 15 minutes.

### 4.5.2. Gas Chromatography-mass spectrometry-olfactometry analysis (GC-O-MS)

The experiment was carried out using an Agilent Technologies a 7820 GC System coupled in parallel with a 5977B MSD single quadrupole mass spectrometer and a Phaser olfactory port from GL Sciences B.V. (Netherlands). The chromatographic column used was an HP-5MS (30 m length x 0.25 mm inner diameter x 0.25 µm film thickness). The carrier gas was He at 1 ml $min^{-1}$.

The oven program began with an initial temperature of 40 °C for 5 min and then temperature increased to 300 °C at 10°C $min^{-1}$.

The MS acquisition was carried out in electron impact ionization mode and the temperature of source was 230 ºC. Scan mode was used between 50 and 450 amu. NIST 14 mass spectral library was used for the identification of the compounds, a minimum of 80% of matching was required to consider a compound as identified by NIST. Finally, the standards of the compounds were analysed for confirmatory purposes.

In order to determine the sensory impact of the volatile compounds present in the sample, an olfactometry analysis was also performed in parallel. The identification of the odorants was carried out by a comparison of odors and Kovats retention indices with bibliography from Flavornet Database (www.flavornet.org) and National Institute of Standards and Technology (https://webbook.nist.gov/chemistry/). For that, a standard mixture of alkanes C8 to C21 was analysed under the same conditions as the samples and Kovats retention index were calculated. Some compounds were also confirmed by mass spectrometry, but this was not possible in some cases, since the sensitivity of the compound was higher by GC-O than by GC-MS. When the standards were commercially available they were injected to confirm compounds identification.

The olfactometry of each sample was performed by five trained GC/O sniffers. They were previously trained using the same methodology than Osorio (Osorio et al., 2019). For the analysis, they were asked to describe the aromas perceived and their intensity on a 3 point category scale (1 = weak odor; 2 = clear; and 3 = intense); half values were also allowed. The modified frequency percentage (MF %) was calculated for each odorant perceived to be able to determine the most important odorous compounds present in the sample. It was calculated with the following equation (Vera et al., 2012):

$$MF\ (\%) = \sqrt{F(\%) \times I(\%)} \qquad \text{[equation 1]}$$

where F(%) is the detection frequency of an aromatic attribute expressed as percentage and I (%) is the average intensity expressed as percentage of the maximum intensity. Odors detected with a MF (%) higher than 50 represent the most important compounds present in each sample (Wrona, Vera, Pezo, & Nerín, 2017).

### 4.5.3 Atmospheric pressure gas chromatography-mass spectrometry quadrupole-time of flight (APGC-QToF)

For the chromatographic analysis, an Agilent 6890N coupled to a CTC Analytics CombiPal autosampler was used. The capillary column used was an HP-5MS (30 m × 0.25 mm × 0.25 µm). The oven program was as follows: 60°C for 5 min, with a rate of 10 °C $min^{-1}$ up to 300°C, maintained for 5 min. The helium flow was 1.2 mL $min^{-1}$.

The detector was a quadrupole-time of flight analyser (QToF) Xevo G2 from Waters (Milford, MA, USA) coupled to an atmospheric pressure gas chromatography (APGC) source. API positive polarity and sensitivity analyser mode were selected. The corona voltage was 2.2 kV and the corona current was 0.8 A. The probe temperature was 200 ºC. The sampling cone and extraction cone voltage were 30 V and 3V respectively. The source temperature was 150 °C and the desolvation gas flow was 200 L $h^{-1}$. The mass range considered was m/z 50–550. MSE mode was selected for the acquisition; for function 2, a collision ramp energy from 15 to 40 V was used. As lockmass, column bleed with the exact mass 281.0517 was used. The transfer line settings were as follows: make up gas used was nitrogen at 400 mL $min^{-1}$ and the transfer line temperature was 350 °C. Data were collected and processed using MassLynx software from Waters.

# 5. RESULTS AND DISCUSSION

## 5.1. Volatile and semi-volatile compounds identified in PLA biopolymer by GC-MS

For the identification of compounds, the dissolved PLA samples were used. Extracts were analysed by liquid injection. Figure 1 shows a chromatogram of the extracts obtained by total dissolution of PLA blend film (1a) and pellets (1b) samples.

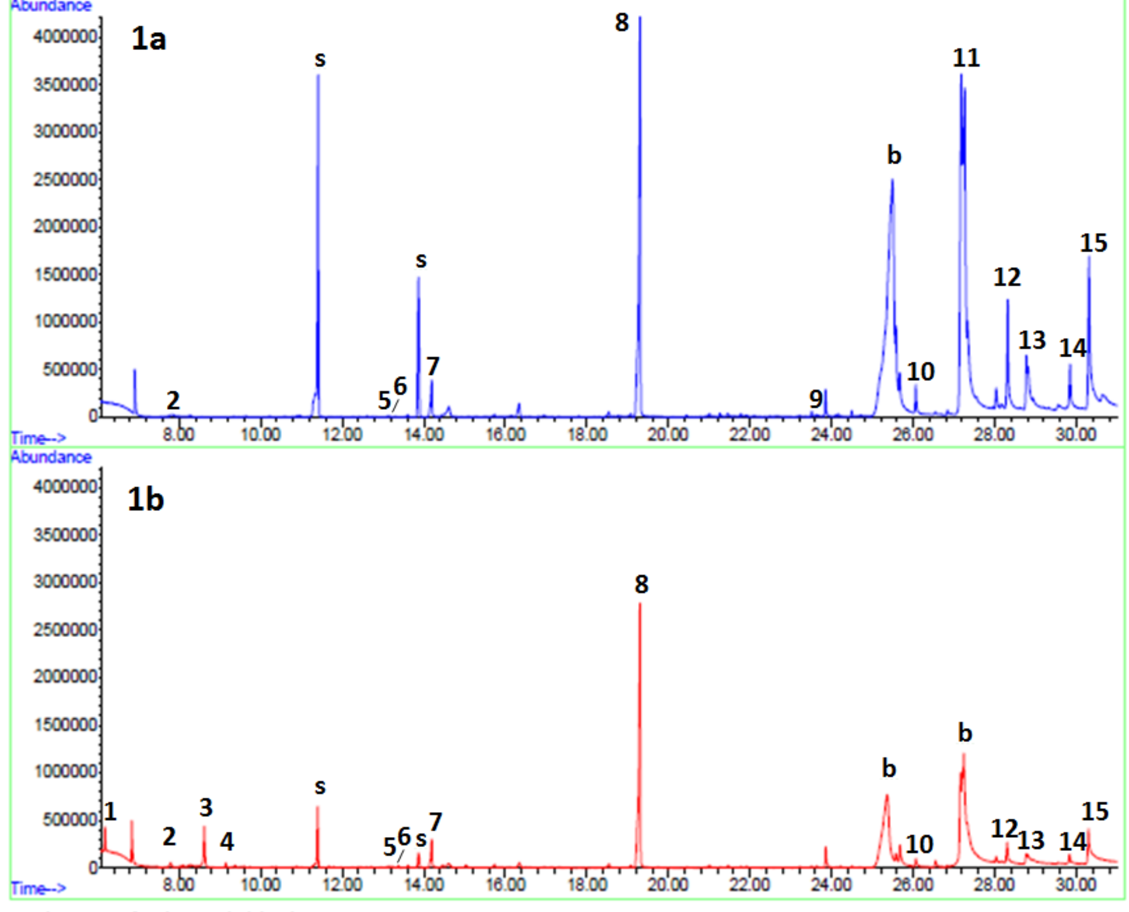



**Figure 1.** Chromatograms of PLA films (1a) and pellets (1b) dissolution extracts by GC-MS. Numbers correspond to compounds in table 1 (s: siloxane of column, b:blanck).

This methodology was carried out to facility the identification process of unknown migrants. In the material, the concentration of the compounds is much

higher than in migration. It is frequent to first analyze directly the polymer before migration assays in order to identify the potential migrants (Aznar et al., 2019; Nerin et al., 2013; Ubeda et al., 2018). Table 1 shows the main volatile compounds found in the sample. The intensity of the peaks is written in the third and fourth column for pellets and films respectively. The last column of table 1 shows the references where the compounds had been previously detected and the fragments of those compounds that were not identified. A total of 15 different volatile compounds were identified in PLA blend pellets (13 compounds) and films (12 compounds) by GC-MS with a match value higher than 80% according to NIST library. Ten compounds were common in both samples. One of the most important identified compounds was lactide, the cyclic dimer of lactic acid. It is not listed in the positive list of Regulation EU/10/2011 (EC, 2011) and therefore it is important to control it. It could come from intramolecular transesterification, unzipping depolymerization or oxidative processes (Salazar et al., 2017). Adipic acid – Butanediol (AA-BD) was the most abundant substance in both kinds of samples, pellets and films. Its mass was previously detected by Bradley in bio-based materials used for food contact materials (Bradley, 2010) but it was not identified. Samples were a blend of PLA and polyester, so this compound probably came from the polyester component of the biopolymer. Based on previous studies on the non-volatile substances found in this material, where the poly(butylene adipate-co-terephthalate) (PBAT) monomer was identified (Aznar et al., 2019), the attempt of PBAT as the polyester blended to PLA can be considered. The compounds found with medium or high intensity in the film were oleamide, glycerol 1-palmitate, erucamide, N-[(9Z)-9-octadecen-1-yl]acetamide, glycerol 1-stearate, palmitic acid and a non identified compound. The compounds glycerol 1-palmitate, N-[(9Z)-9-octadecen-1-yl]acetamide and glycerol 1-stearate were previously detected by Martínez-Bueno (Martínez-Bueno et al., 2017).

**Table 1:** Volatile and semi-volatile compounds identified in total dissolution of PLA films and pellets by GC-MS where rt is retention time and I is the intensity according to its relative abundance : I=1 (0-5%),I=2 (5-20%), I=3 (20-50%), I=4 (50-100% )

| No | rt | I pellets | I films | Candidate | CAS Number | Molecular Formula | Remarks |
|---|---|---|---|---|---|---|---|
| **1** | 6.18 | 2 | nd | 3-Penten-2-ol | 1569-50-2 | $C_{11}H_{14}O$ | Detected similar by Salazar, R. 2017 |
| **2** | 7.80 | 1 | 1 | Cyclopentanone[a] | 120-92-3 | $C_5H_8O$ | Detected similar by Salazar, R. 2017 |
| **3** | 8.62 | 2 | nd | Ethyl chloroacetate | 105-39-5 | $C_4H_7ClO_2$ | Detected similar by Salazar, R. 2017 |
| **4** | 9.15 | 1 | nd | Chloroacetaldehyde | 107-20-0 | $C_2H_3ClO$ | Detected similar by Salazar, R. 2017 |
| **5** | 13.10 | 1 | 1 | Undecane[a] | 1120-21-4 | $C_{11}H_{24}$ | |
| **6** | 13.14 | 1 | 1 | Nonanal[a] | 124-19-6 | $C_9H_{18}O$ | Detected by Salazar, R. 2017 |
| **7** | 14.20 | 2 | 2 | Dimethyl-1,4-dioxane-2,5-dione [LACTIDE][a] | 4511-42-6 | $C_6H_8O_4$ | Detected by Mutsuga, M. 2008 and Salazar, R. 2017 |
| **8** | 19.30 | 4 | 4 | 1,6-Dioxacyclododecane-7,12-dione [AA-BD] ✓ | 777-95-7 | $C_{10}H_{16}O_4$ | Detected by Bradley, 2010 |
| **9** | 23.52 | nd | 2 | Palmitic acid[a]✓ | 57-10-3 | $C_{16}H_{32}O_2$ | Detected similar by Salazar, R. 2017 |
| **10** | 26.06 | 1 | 2 | ni | | | Fragments: 55/73/99.1/141.1/168.1 |
| **11** | 27.18 | nd | 4 | Oleamide✓ | 301-02-0 | $C_{18}H_{35}NO$ | Indirect additives in FDA 2018 and EC, 2011 |
| **12** | 28.32 | 2 | 3 | Glycerol 1-palmitate✓ | 542-44-9 | $C_{19}H_{38}O_4$ | Detected by Martínez-Bueno, M.J 2017 and Bradley, 2010 |
| **13** | 28.76 | 1 | 2 | N-[(9Z)-9-octadecen-1-yl]acetamide✓ | 40165-68-2 | $C_{20}H_{39}NO$ | Detected by Martínez-Bueno, M.J 2017 |
| **14** | 29.84 | 1 | 2 | Glycerol 1-stearate[a]✓ | 123-94-4 | $C_{21}H_{42}O_4$ | Detected by Martínez-Bueno, M.J 2017 and Bradley, 2010 |
| **15** | 30.31 | 2 | 3 | Erucamide[a]✓ | 112-84-5 | $C_{22}H_{43}NO$ | Indirect additives in FDA 2018 and EC, 2011 |

nd: non detected, ni: non identified, ✓compounds detected also in APGC-MS [a] Confirmed by standard

The intensity of the following compounds increased from pellets to films, probably because they were added during film manufacturing: glycerol palmitate, that is used as emulsifier; amides, that are slip agents and antiblocking additives used in films to reduce friction resistance; stearic acid esters, which are listed as approved monomers for food contact applications in the EU regulation (EC, 2011); oleamide and erucamide, that are commonly used as slip agents and are defined as indirect additives for food contact materials by US Food and Drug Administration(FDA, 2018; EC, 2011) and an unknown compound at 26.06 minutes, whose main fragments were 55. 73, 99.1, 141.1 and 168.1. Palmitic acid, that was only identified in films, is a slip agent and it is also an approved monomer for food contact material in the EU regulation (EC, 2011). It was identified with similar structures by Salazar (Salazar et al., 2017).

Other compounds detected in films but at lower intensities were cyclopentanone, undecane and nonanal. Cyclopentanone could be explained by the radical reaction between aldehyde and ketones. Undecane could be either a compound used in the polymerization process or a degradation product from additives or processing aids. Nonanal could be produced from trans-esterification reactions. These compounds were identified with the same intensity in PLA pellets and films. On the other hand, a few compounds such as 3-penten-2-ol, ethyl chloroacetate and chloroacetaldehyde were only detected in pellets, in all cases with low intensities. 3-penten-2-ol could be formed by hydrolysis of ester groups. Ethyl chloroacetate and cloroacetaldehyde could have been produced by the reaction with dichloromethane used during the dissolution/precipitation process and acetaldehyde. Acetaldehyde was not identified, as it is difficult to detect it in GC-MS. Most of these compounds had been previously identified in PLA by Salazar (Salazar et al., 2017).

## 5.2. Volatile and semi-volatile compounds identified in the biopolymer by APGC-QToF

As in GC-MS analysis, the dissolved pellets and film of the PLA blend samples were used for the identification of compounds. The compounds identified by APGC-QToF are shown in Table 2 and Figure 2 shows the chromatograms of the analysis. The table is organized according to retention time. All the compounds were protonated [H+] during its ionization, except glycerol 1-palmitate and glycerol 1-stearate, detected without a water molecule due to a dehydration process during ionization. The intensity of these compounds is described in the table according to its abundance in the chromatogram. Fourteen compounds were identified by APGC-QToF. Except the stereamide, that was only detected in the film, all of them were detected in pellets as well as in the film.The oligomer $[AA\text{-}BD]_2$, that had not been detected previously by GC-MS, was the most intense in the chromatogram together with poly(trimethylolpropane adipate) and 2 unknown compounds, 10.57_355.0686 and 31.90_466.0538 (retention time_exact mass). AA-BD, coming from the polyester component was also detected with this instrument but with a lower intensity. They come from polyester component of the biopolymer. Poly(trimethylolpropane adipate) is a compound defined as indirect additive for food contact materials by Food and Drug Administration (FDA, 2018; EC, 2011). Other compounds found with medium intensity in the film were N,N-diethyldodecanamide, oleamide, glycerol 1-palmitate, erucamide. All of them increase their intensity in films. N,N-diethyldodecanamide was previously identified by Martínez-Bueno (Martínez-Bueno et al., 2017) and the rest were previously identified and confirmed by GC-MS. Finally, compounds with lower intensities like palmitic acid, N-[(9Z)-9-octadecen-1-yl]acetamide and glycerol 1-stearate were identified. They had also been identified by GC-MS. Stearamide, a compound used as indirect additive for food contact materials by Food and Drug Administration (FDA, 2018; EC, 2011), and the unknown compound (25.30_387.2941) were also detected with low intensity.

**Table 2:** Volatile and semi-volatile compounds identified in total dissolution of PLA films and pellets by APGC-MS where rt is retention time and I is the intensity according to its relative abundance: I=1 (0-5%),I=2 (5-20%), I=3 (20-50%), I=4 (50-100% )

| No | rt | mass | Adduct | I pellets | I films | Molecular formula | Candidate CAS number | Remarks/Fragments |
|---|---|---|---|---|---|---|---|---|
| **1** | 7.48 | 281.0519 | [MH]+ | 3 | 4 | C12H24O7 | Poly(trimethylolpropane adipate) 28301-90-8 | Detected by Aznar, M. 2019 Indirect additives in FDA 2018 |
| **2** | 10.57 | 355.0686 | [MH]+ | 4 | 4 | C15H14O10 | ni | |
| **3** | 16.25 | 201.1116 | [MH]+ | 2 | 2 | C10H16O4 | 1,6-Dioxacyclododecane-7,12-dione, [AA-BD] ✓ 777-95-7 | Detected by Aznar, M. 2019 and Bradley, 2010 |
| **4** | 21.04 | 257.2462 | [MH]+ | 2 | 2 | C16H32O2 | Palmitic acid ✓ 57-10-3 | Detected similar by Salazar, R. 2017 |
| **5** | 22.89 | 256.2642 | [MH]+ | 2 | 3 | C16H33NO | N,N-Diethyldodecanamide 222-118-3 | Detected by Martínez-Bueno, M.J 2017 |
| **6** | 24.44 | 282.2783 | [MH]+ | 3 | 3 | C18H35NO | Oleamide ✓ 301-02-0 | Indirect additives in FDA 2018 and EC, 2011 |
| **7** | 24.62 | 284.2967 | [MH]+ | nd | 2 | C18H37NO | Stearamide 110-30-5 | Indirect additives in FDA 2018 and EC, 2011 |
| **8** | 25.30 | 387.2941 | [MH]+ | 2 | 2 | C25H38O3/ C28H36N | ni | 263.9876/131.0526 |
| **9** | 25.60 | 313.2749 | $[MH]^+$-$H_20$ | 2 | 3 | C19H38O4 | Glycerol 1-palmitate✓ 542-44-9 | Detected by Martínez-Bueno, M.J 2017 and Bradley, 2010 |
| **10** | 26.09 | 310.3124 | [MH]+ | 2 | 2 | C20H39NO | N-[(9Z)-9-octadecen-1-yl]acetamide✓ 40165-68-2 | Detected by Martínez-Bueno, M.J 2017 |
| **11** | 27.16 | 341.3055 | $[MH]^+$-$H_20$ | 2 | 2 | C21H42O4 | Glycerol 1-stearate ✓ 123-94-4 | Detected by Martínez-Bueno, M.J 2017 and Bradley, 2010 |
| **12** | 27.61 | 338.3428 | [MH]+ | 2 | 3 | C22H43NO | Erucamide ✓ 112-84-5 | Indirect additives in FDA 2018 and EC, 2011 |
| **13** | 28.89 | 401.2182 | [MH]+ | 4 | 4 | C20H32O8 | 1,6,13,18-Tetraoxacyclotetracosane-7,12,19,24-tetrone, $[AA\text{-}BD]_{2,}$ 78837-87-3 | Detected by Aznar, M. 2019 and Bradley, 2010 |
| **14** | 31.90 | 466.0538 | [MH]+ | 4 | 4 | --- | ni | 421.1882 /221.0834/149.0231 |

nd: non detected, ni: non identified, ✓compounds detected in GC-MS.

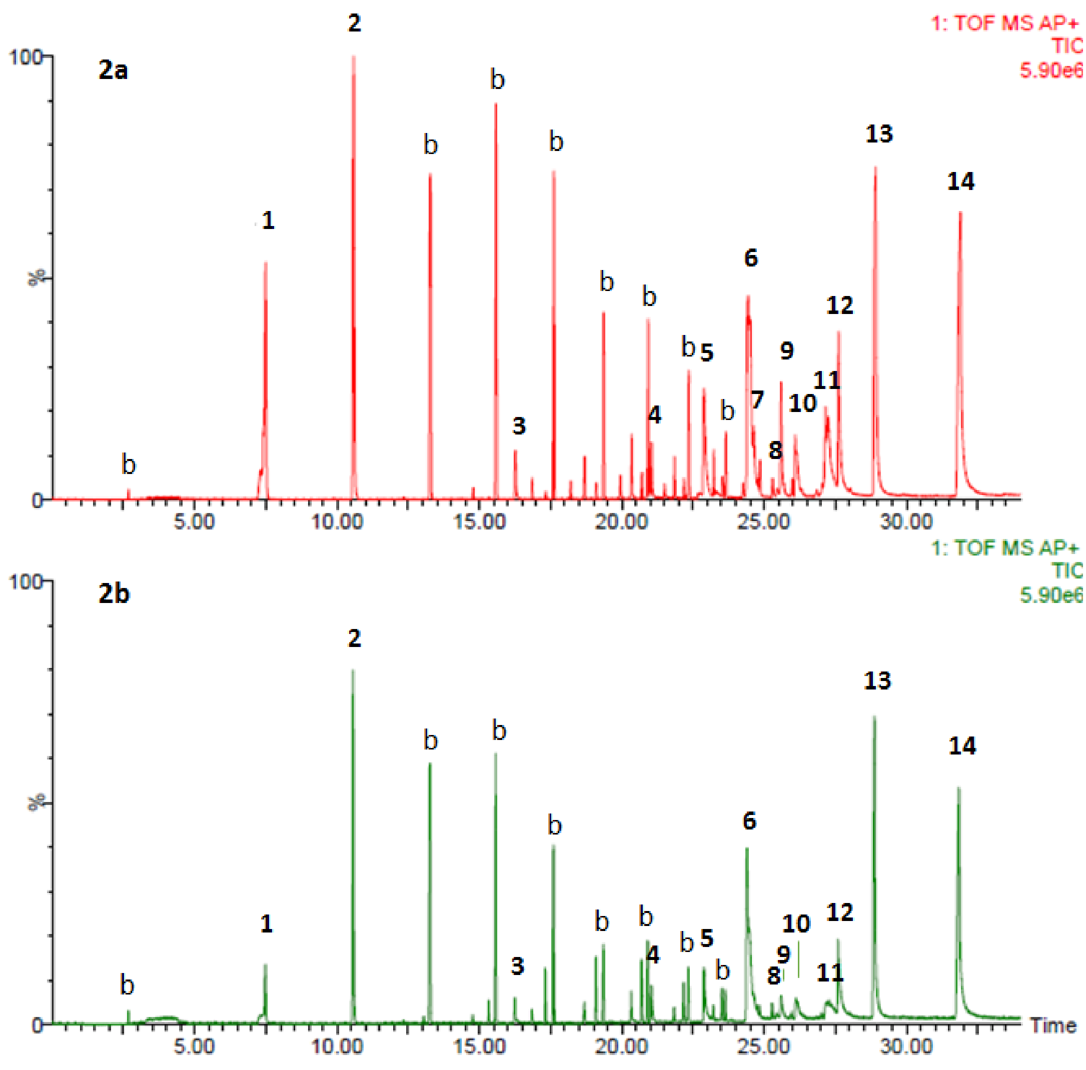



**Figure 2**. Chromatograms of PLA films (2a) and pellets (2b) dissolution extracts by APGC-QTOF. Numbers correspond to compounds in table 2 (b:blanck)

## 5.3. Comparison of APGC-MS vs. GC-MS

Seven compounds were detected by both techniques, GC-MS (with EI ionization) and APGC-QToF. They were [AA-BD] oligomer, palmitic acid, oleamide, glycerol 1-palmitate, N-[(9Z)-9-octadecen-1-yl]acetamide, glycerol 1-stearate and erucamide. Figure 3 shows the profile of these compounds in a biopolymer film sample analysed by both techniques. The areas of GC-MS divided by 100000 and the areas of APGC-MS divided by 1000 are represented

in order to compare the results obtained. The profile in both techniques was similar; oleamide and palmitic acid showed the highest and the lowest intensities respectively in both analysis. AA-BD was identified with higher intensity in GC-MS than in APGC-QToF, however, its dimer was only identified by APGC-QToF, where high resolution MS was available. Having the molecular ion in APGC spectra, makes possible the structural elucidation of the oligomers, whereas in EI the molecular ion barely appears and consequently the identification is much more difficult.

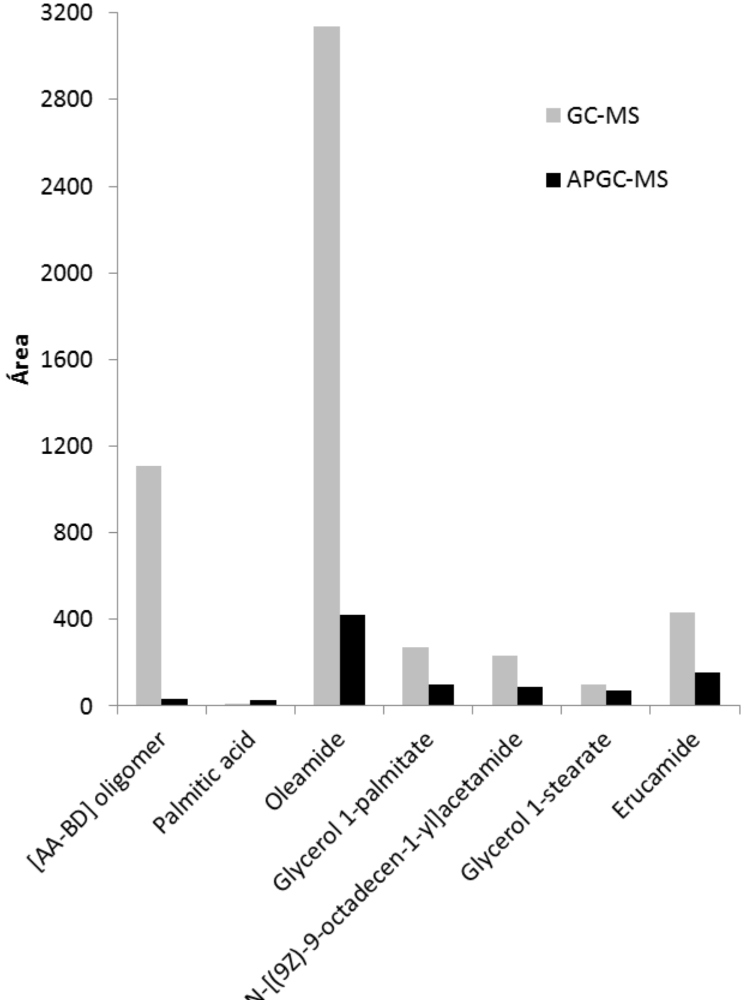



**Figure 3.** Profile of seven compounds detected by both techniques, GC-MS and APGC-QTOF.

## 5.4. Identified compounds in migration tests

After the exposure of films to the simulants during 10 days at 60 ºC, only 2 compounds were identified by GC-MS in ethanol 95% and none in ethanol 10% or acetic acid 3%. The results showed the presence of AA-BD and lactide. The concentration of AA-BD was between the LOD (310 µg $kg^{-1}$) and the LOQ (920 µg $kg^{-1}$). Due to the absence of commercial standards for AA-BD, the quantification was performed with a similar oligomer AA-DEG oligomer (adipic acid – diethylene glycol) available in the laboratory.

Similar results were obtained for lactide, whose concentration was also between the detection and quantification limits (LOD and LOQ), 540 µg $kg^{-1}$ and 1700 ppb, respectively.

Only the dimer $[AA\text{-}BD]_2$ was identified in the migration test to ethanol 95% by APGC-MS and it was quantified with AA-DEG oligomer. In this case, LOD and LOQ were 2.8 mg $kg^{-1}$ and 8.4 mg $kg^{-1}$, respectively and this compound was below LOQ. No compounds were identified in aqueous simulants, ethanol 10% and acetic acid 3%. The limit of detection of AA-DEG oligomer, used as standard, was 226 µg $kg^{-1}$.

## 5.5. Key aroma compounds in the PLA blend biopolymer and in the migration simulants after exposure

To identify the main key aroma compounds present in the PLA blend biopolymer, a direct analysis of the PLA sample by GC- O-MS was performed. Table 3 shows the odorants identified. For confirmation, retention index and aroma description were compared with data reported in the bibliography (Flavornet and NIST database). Some compounds were also confirmed by spectrum mass match in NIST library ($^{✓}$) and standard injection ($^{✓✓}$). A total of ten odorants with MF % values above 40 % were identified in PLA based biopolymer material. Most of them were ketones and aldehydes like 1-octen-3-

one, octanal, 3,5 octadienone, 3-nonenal/3,6-nonadienal, nonanal, citronellal, E-2-nonenal and dodecanal. High concentration of aldehydes tend to be very pungent and overwhelming, but low concentration can show a wide range of aromas. Other compounds identified were sotolon and α-cubebene. The compound with the highest aromatic impact was sotolon with 91.3 % of MF. Sotolon could have been formed during the pellets manufacturing process due to temperature and pressure conditions, or present in the other raw materials used for its manufacture (Osorio et al., 2019). Nonanal, 1-octen-3-one and 3-nonenal/3,6-nonadienal had MF values over 70 (87.6, 79.6 and 74.8 % MF, respectively). Finally, E-2-nonenal, citronellal and α-cubebene were detected with values above 50 %. However, the results of olfactometry of migration samples did not show any compound with MF % values above 20% in any of the food simulants. This fact means that even though the material itself presents some aromas that could be described as off-flavours, they are not expected to modify the food aroma properties, as most of them disappear during the manufacture of the polymer from pellets to film.

**Table 3:** Odorant compounds detected in PLA based biofilms. KI, experimental Kovats retention index; rt, retention time; MF, molecular formula.

| KI | rt | MF (%) | Olfactory description | Candidates | CAS No | Molecular Formula | KI Bibliography[a] | Description Bibliography[a] |
|---|---|---|---|---|---|---|---|---|
| 983 | 11.28 | 79.6 | Mushroom, cucumber, sweet | 1-octen-3-one✓✓ | 4312-99-6 | $C_8H_{14}O$ | 975-983 | Earth, mushrooms, moisture |
| 1007 | 11.74 | 46.9 | Aldehyde, fruity, sweet | octanal✓✓ | 124-13-0 | $C_8H_{16}O$ | 1000-1011 | Aldehyde, orange skin, citrus, floral |
| 1084 | 13.05 | 42.4 | Chemical, sweet | 3,5 octadienone | 38284-27-4 | $C_8H_{12}O$ | 1066-1091 | Fruit, fat, mushroom |
| 1103 | 13.36 | 74.8 | Aldehyde, mushroom, green | 3-nonenal//<br>3,6-nonadienal | 1120-21-4 | $C_{11}H_{24}$ | 1100 // 1100 | Cucumber, fat // fat, soap |
| 1111 | 13.49 | 87.6 | Aldehyde, chemical, green | nonanal✓✓ | 124-19-6 | $C_9H_{18}O$ | 1098-1112 | Fat, citrus, green |
| 1117 | 13.58 | 91.3 | Black licorice, toasted, burned | sotolon✓✓ | 28664-35-9 | $C_6H_8O_3$ | 1079-1115 | Black licorice, curry, maple syrup |
| 1155 | 14.17 | 62.2 | Sweet, flower | citronellal✓✓ | 106-23-0 | $C_{10}H_{18}O$ | 1126-1167 | Floral, sweet, citrus, green |
| 1166 | 14.34 | 67.3 | Lemon, cucumber, dried fruit | (E)-2-nonenal✓✓ | 18829-56-6 | $C_9H_{16}O$ | 1156-1165 | Fatty, cucumber, green, citrus |
| 1350 | 16.91 | 51.6 | Wet, green grass, floral | α-cubebene | 17699-14-8 | $C_{15}H_{24}$ | 1337-1360 | Herb, wax |
| 1389 | 17.42 | 40.0 | Soap | dodecanal✓✓ | 112-54-9 | $C_{12}H_{24}O$ | 1397-1417 | Soap, orange skin, citrus, wax, floral |

✓confirmed by spectrum mass-NIST; ✓✓confirmed by standard
[a]Data reported in Flavornet and NIST databases.

# 6. CONCLUSIONS

The protocol for PLA blend sample treatment based on dissolution/precipitation allowed identifying volatile and semi-volatile compounds by GC-MS (with EI ionization) and APGC-QToF. The use of both techniques provided a more complete profile of the volatile compounds present in PLA blend biopolymer. Common compounds identified by both techniques showed more sensitivity by GC-MS than APGC-QToF. However, the advantages provided by APGC, such as the presence of the molecular ion or the coupling to high resolution MS, facilitate the identification of oligomers and compounds which cannot be identified by the standard GC-MS using EI and quadrupole as detector. Then, the analysis by both techniques provides an accurate information of volatile compounds present in the material. The results of migration solutions showed that only AA-BD, $[AA\text{-}BD]_2$ and lactide were transferred to ethanol 95%, but in all cases below their limit of quantification. According to these results, at the studied conditions, these materials would be more recommended for aqueous food than for fatty food. In addition, even though some compounds present in the biopolymer showed a high sensory impact on the polymer odor, they were not detected in migration studies and therefore no changes in the sensory profile of packaged food are expected.

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# Capítulo 6

*Migration studies and toxicity evaluation of cyclic polyesters oligomers from food packaging adhesives*

1. RESUMEN
2. OBJETIVOS Y ESQUEMA DE TRABAJO
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4. MATERIALS AND METHODS
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# 1. RESUMEN

Los materiales multicapa usados en los envases alimentarios son comúnmente fabricados con una capa de adhesivo de PU, la cual puede contener oligómeros de ésteres cíclicos como migrantes potenciales. Sin embargo, poco se conoce sobre su toxicidad. En este trabajo, dos ésteres cíclicos del PU son evaluados en migración desde 20 diferentes envases multicapa. Estos oligómeros están compuestos por ácido adípico (AA), dietilenglicol (DEG) y ácido isoftálico (IPA) y sus estructuras son AA-DEG y AA-DEG-IPA-DEG. La concentración de estos compuestos en migración superó el valor máximo establecido por la regulación EU/10/2011 (10 ng $g^{-1}$). La bioaccesibilidad de ambos compuestos fue evaluada por estudios de digestión gástrica e intestinal. Los estudios mostraron que la concentración de los compuestos decrecía durante la digestión y que sus moléculas hidrolizadas aumentaban. Además, se llevaron a cabo ensayos de la actividad endocrina in vitro. Fue identificado un débil antagonismo del receptor androgénico, mientras que no se encontró actividad del receptor de arilhidrocarburo ni unión a la proteína de transporte de la hormona tiroidea.

# 2. OBJETIVOS Y ESQUEMA DE TRABAJO

El objetivo principal de este capítulo fue estudiar tanto la migración, la bioaccesibilidad y la actividad endocrina de dos oligómeros procedentes del PU utilizado como adhesivo en diferentes materiales multicapa. Estos oligómeros tenían las estructuras AA-DEG y AA-DEG-IPA-DEG (AA: ácido adípico; DEG: dietilenglicol: IPA es ácido isoftálico). Para ello, se llevaron a cabo las siguientes tareas:

- Cuantificación de dos oligómeros del PU en ensayos de migración de 20 muestras diferentes.

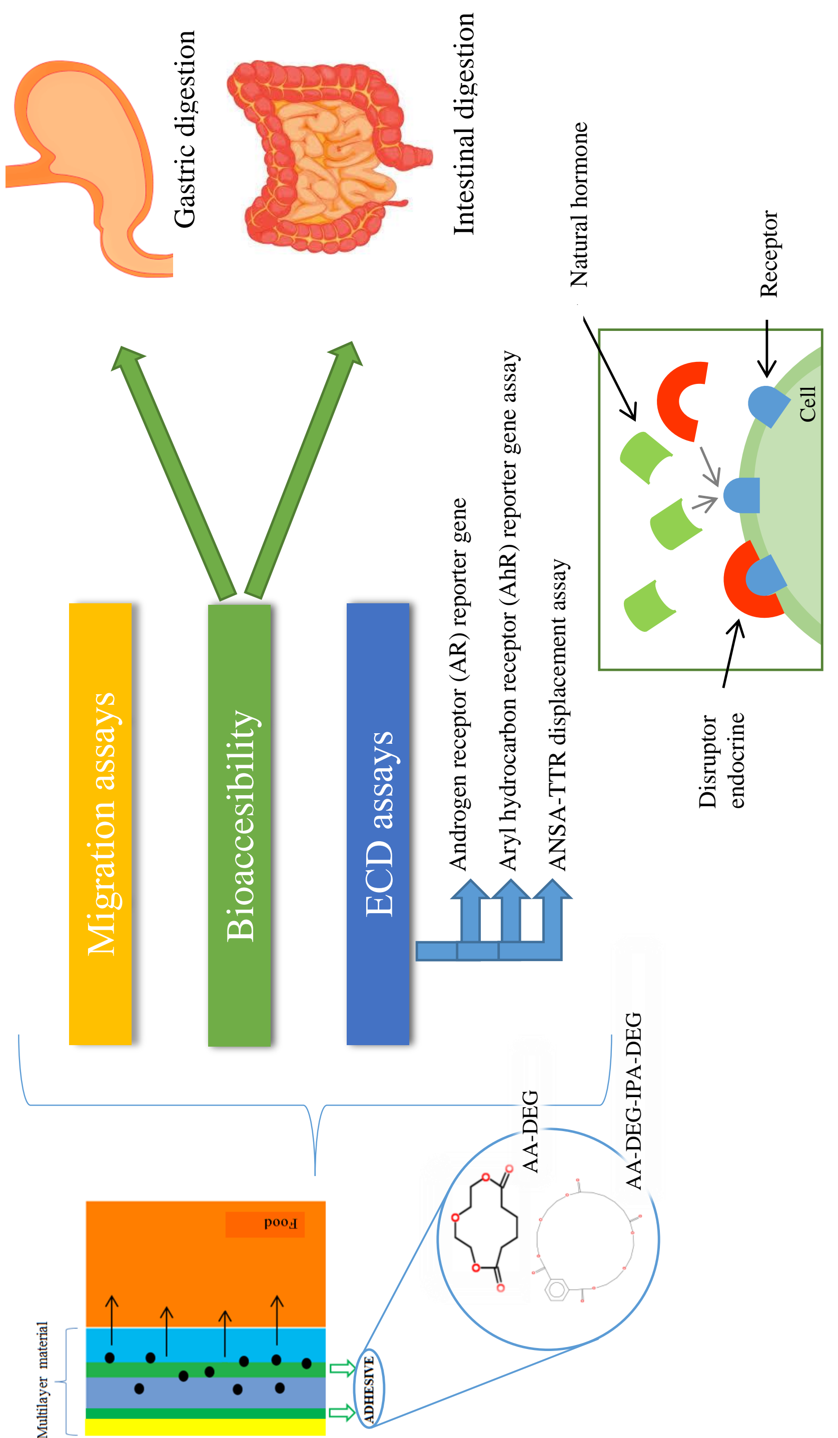
- Evaluación del riesgo de ambos oligómeros utilizando el método de umbral de preocupación toxicológica (TTC).

- Estudio de la bioaccesibilidad de los oligómeros mediante ensayos de digestión gástrica e intestinal.

- Aprendizaje de conocimientos básicos sobre estudios de toxicidad endocrina.

- Estudio de la actividad endocrina de ambos oligómeros mediante tres tipos de ensayos endocrinos.

A continuación se mostrará un esquema gráfico del trabajo realizado en el capítulo 6.




**Esquema 6:** Diseño experimental del Capítulo 6.

# 3. INTRODUCTION

Food contact materials (FCMs) protect food from external contamination and preserve the nutritional value as well as the physical and sensory quality of food. However, it is important to control the migration of compounds from packaging materials to foods, as it may lead to the transference of unwanted substances that can make food less safe for consumption or that may alter its sensory and nutritional characteristics. It is necessary, therefore, to identify the compounds that are present in the different packaging materials and that can be potential migrants (Wrona & Nerín, 2019). Substances in FCM can be intentionally or non-intentionally added (IAS and NIAS). NIAS are difficult to control, as they are often not chemically well characterized and are present at low concentration levels. This complicates its identification and therefore, advanced techniques with high sensitivity and resolution are needed (Margarita Aznar, Ubeda, Dreolin, & Nerín, 2019; Hoppe, de Voogt, & Franz, 2016; Nerin, Alfaro, Aznar, & Domeño, 2013; Pietropaolo, Albenga, Gosetti, Toson, Koster, Marin-Kuan, et al., 2018).

In the case of multilayer packaging materials, where the material is made of multiple polymer layers bonded by adhesives, migration can occur not only from the material that is in direct contact with food, but also from internal layers of the material including the adhesives. This process is due to diffusion and partition processes of the compounds between the different layers (Margarita Aznar, Vera, Canellas, Nerín, Mercea, & Störmer, 2011; Tehrany & Desobry, 2004). Due to its low temperature properties, flexibility, durability and impact resistance, polyurethane (PU) is the most commonly used adhesive for flexible multilayer structures (Heath & Cooper, 2013). PU adhesives are also used in other applications such as in the assembly of shoes, automotive interiors, windshield bonding or textile laminates (Engels, Pirkl, Albers, Albach, Krause, Hoffmann, et al., 2013). Therefore, there can be different potential exposure sources of these compounds.

PU adhesive synthesis is a reaction between di-isocyanates and linear polyester compounds, where the latter are produced by polycondensation reaction between polyols (ethylene glycol, EG; diethylene glycol, DEG; 1,4-butanediol, BD; neopentyl glycol, NPG; 1,6-hexanediol, HD) and aliphatic or aromatic carboxylic acids (adipic acid, AA; isophthalic acid, IP). When the last reaction does not proceed under equilibrium conditions, it favors the formation of short chain cyclic polyesters, so-called lactones, in addition to linear polyesters (Shrikhande, 2012). These cyclic esters can also be considered oligomers as they are formed by several monomer units. The formation of cyclic esters is undesirable from an industrial point of view as they can impair the physical properties of the material (Eceiza, Martin, de la Caba, Kortaberria, Gabilondo, Corcuera, et al., 2008; Shrikhande, 2012; Xiao & Sue, 2019; Zhang, 2014). Furthermore, from a food packaging perspective, these unwanted by-products are considered NIAS and, as demonstrated previously (Félix, Isella, Bosetti, & Nerín, 2012; Gómez Ramos, Lozano, & Fernández-Alba, 2019; Nerin, Alfaro, Aznar, & Domeño, 2013; Ubeda, Aznar, & Nerín, 2018; Úbeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017; Zhang, Kenion, Bankmann, Mezouari, & Hartman, 2018) have a high migration potential. Migration of theses oligomers could be seen as microplastics coming from plastic FCMs (Ubeda, Aznar, Alfaro, & Nerín, 2019). As they are NIAS, they are not included in any database and often commercial standards are not available, making identification and confirmation a difficult process.

There is no specific European legislation for food packaging adhesives and its components, though some countries such as Switzerland have a national legislation (Swiss-Confederation, 2013). However, when PU adhesive are used in the manufacture of multilayer plastic for FCM they are controlled by Regulations 1935/2004/EC (EC, 2004) and 10/2011/EU (EC, 2011). The Regulation states that FCM components must not be transferred into food in quantities that may harm human health. The oligomers are not specified in the Regulation

10/2011/EU (EC, 2011), thus a limit of migration to food simulants of 10 ng $g^{-1}$ should not be exceeded.

There is little information on the hazards of oligomers. This is partially due to the lack of commercial standards necessary for toxicological testing. It has often been assumed that oligomers have the same toxicity as their starting monomers and that they should therefore be covered by their toxicological evaluation (Grob, Camus, Gontard, Hoellinger, Joly, Macherey, et al., 2010; Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). However, it is evident that reaction products can have different properties. According to EFSA (EFSA, 2008), when the polymer is formed by the polymerization of an approved monomer, its lack of genotoxicity is established by the data on the monomer, and no requirement for experimental data on the polymer itself are needed such as for cyclic butylene terephthalate (EFSA, 2009). In some cases, the same toxicity results of monomers and their oligomers have been demonstrated, such as for oligomers of halocarbon 3.1 oil and chlorotrifluoroethylene trimer acid (Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). In contrast, it has been demonstrated in other cases that the toxicological profile of the reaction products and starting substances differed, such as the oligomers of styrene (Gelbke, Banton, Block, Dawkins, Leibold, Pemberton, et al., 2018). Thus, it is important to test the toxic potential, not only of the starting material, but also of the present oligomers. Initially, these tests can be done by in vitro examinations.

The safety evaluation from the Office of Food Additive Safety (OFAS) states that oligomeric materials with a molecular weight below 1000 Da are important from a toxicological point of view as they could migrate into food and be absorbed in the gut (Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). Hence, it is crucial to assess the safety of those oligomers with lower molecular weights. However, as far as the authors know, the toxicological properties are not well characterized.

In addition, foodstuff undergoes a series of processes before being absorbed into the body, such as gastric and intestinal digestions. These processes might change the concentrations of substances available to be absorbed and could even lead to the formation of new compounds. These changes may have implications for the final toxicity. Thus, it is important to study the bioaccessibility as well as the gastrointestinal degradation of the migrant compound to enhance the understanding of the chemical composition of the fraction available for absorption (M. Aznar, Gómez-Estaca, Vélez, Devesa, & Nerín, 2013).

Exogenous compounds such as endocrine disrupting chemicals (EDCs) are of special interest because they mimic, block or in other ways alter the activities of endogenous hormones. In vitro assays have been developed for a wide range of toxicological effects including induction of cytochrome P450 enzymes, androgenic activity and thyroid disruption. The binding or blocking of steroid hormone receptors like the androgen (AR) receptor by chemicals has been a significant focus for assessment of endocrine disruption potential as this receptor has got a pivotal role in development of male reproductive health (Schwartz, Christiansen, Vinggaard, Axelstad, Hass, & Svingen, 2019). Increasing attention is now being given to the ability of chemicals to disrupt the thyroid hormones system, which plays an important role in ensuring normal development of the embryonic brain (Duntas & Stathatos, 2015). Another important assay is the aryl hydrocarbon receptor (AhR) assay that – when activated – leads to increased metabolism of chemicals, drugs, and hormones and which also plays an important role in our immune defense (Esser & Rannug, 2015).

In this study, the objective was to investigate migration of two cyclic esters from multilayer packaging material based on PU adhesives, as well as to evaluate their bioaccessibility to the body. The potential formation of new compounds during gastrointestinal digestion was also evaluated. Furthermore,

the in vitro endocrine disruptive potential of both compounds was studied in assays covering androgen receptor and aryl hydrocarbon receptor activity, as well as binding to transthyretin – an important transport protein of thyroid hormones

# 4. MATERIAL AND METHODS

## 4.1. Test chemical

Two cyclic ester oligomers, AA-DEG and AA-DEG-IPA-DEG, composed of diethylene glycol (DEG), adipic acid (AA) and isophthalic acid (IPA) were tested. Test substances were supplied by a nondisclosed adhesives company. Their structures and purity were confirmed by NMR at the University of Zaragoza.

## 4.2. Samples

Twenty multilayer plastic materials mainly intended for FCM and the storage of biological fluids were tested (samples code: 1S-20S). Polyurethane was used as adhesive in the manufacture of all evaluated samples. The materials contained a combination of aluminium (Al), polyethylene terephthalate (PET), polyamide (PA), polypropylene (PP) and polyethylene (PE) and had different thickness. They were supplied by different manufacturing companies and are described in Table 1.

## 4.3. Migration test

For the migration experiments, multilayer materials were cut (10 x 10 cm2), folded in half and thermo-sealed. The internal surface of the bags was 0.64 $dm^2$. Afterwards, they were filled with different simulants. The simulants used, as well as the temperatures and times of the migration experiments were selected depending on the intended use of the material and according to EU/10/2011 (EC, 2011). Ultrapure water (Milli-Q Ultramatric Wasserlab GR 216071, Madrid,

Spain) and ethanol 10 % were used as aqueous simulants and ethanol 95 % (Panreac, Barcelona, Spain) as fat simulant. Water was used when the materials were intended for biological fluids. When samples were intended for food contact, 10% ethanol was selected for food with hydrophilic character and 95% ethanol for fat and dry food.

EU/10/2011 (EC, 2011) established that for contact times above 30 days at room temperature, materials should be tested in an accelerated test at 60 °C for a maximum of 10 days. For contact times longer than 2 days at room temperature, three days at 40 ºC was selected. For pasteurized materials, the conditions were different. In this case, bags were introduced in a stainless steel extraction cells, completing the cell space with water and maintaining the assembly for 30 min at 121 °C. This way, the ethanol is kept in liquid phase during the assay, due to the pressure exerted under these conditions by the water inside the cell. In the case of biological samples, tests were performed at 40 ºC for 3 days on the basis of its use.

Although the materials had dissimilar end use, the migration concentrations were corrected to 6 $dm^2$ of packaging material per 1 kg simulant, in accordance with European Regulation 10/2011 (EC, 2011) to compare results. Three replicates of every test were analysed. Samples were analysed by UPLC-QTOF.

## 4.4. Digestion assays

The protocol was prepared according to 2008 EFSA guide (EFSA, 2008). The experiments were carried out in three independent replicates and analysed by UPLC-QqQ (MRM mode) and UPLC-QTOF.

### 4.4.1 Gastric digestion

Gastric simulant was 0.07 M HCl (35 %, Panreac). The pH of the solution was 1.2 ± 0.1.

An aliquot of 100 µL of cyclic ester (100 µg $g^{-1}$ water) was added to 10 mL of gastric simulant (final concentration 1 µg $g^{-1}$) and afterwards heated at 37 ºC. This solution was maintained with agitation at 37 ºC for 4 h. During digestion, aliquots of 1 mL were taken at 4 different times ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$) and neutralized with 250 µL 0.02M sodium hydroxide (NaOH) (1M, Panreac) at pH 6.

### 4.4.2 Intestinal digestion

Intestinal simulant was carried out with pancreatin from porcine pancreas (Sigma Aldrich) according to 2008 EFSA Guide (EFSA, 2008).

For its preparation, 6.8 g of potassium dihydrogen orthophosphate (KH2PO4) (Pro Analyse Merck) was dissolved in 250 mL water and transferred to a 1 L volumetric flask to which 190 mL 0.2 M NaOH and 400 mL water were added and mixed briefly. Then, an amount of 10 g of pancreatin extract was introduced into a 250 mL beaker with little water to make a homogenous paste. After this, the paste was gradually diluted with small portions of water, stirring well after each dilution to give approximately 150 mL of a lump-free solution. The solution was transferred to the 1 L volumetric flask where 0.5 g of sodium taurocholate (Sigma-Aldrich) were added and shaken. Then, water was added leaving space to adjust pH to 7.5 ± 0.1 with 0.2 M NaOH.

Digestion assay was carried out adding 50 µL of 100 µg $g^{-1}$ of cyclic ester in water to 10 mL of intestinal simulant previously tempered at 37 ºC and (500 ng $g^{-1}$ final concentration). This dissolution was maintained at 37 ºC with constant agitation. During digestion, aliquots of 1 mL were taken and evaluated at 4 different time points ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). In order to precipitate the proteins present in the aliquot, 1 mL of 20 % (w) trichloroacetic acid (TCA) (Sigma-Aldrich) was added to each aliquot and then cooled on ice bath for 30 min. Successively, the solutions were centrifuged at 8000 rpm for 15 min and 1 mL of

the supernatant was filtered (PET 0.22µm) and transferred to a vial with 250 µL of 0.02 M NaOH to adjust to neutral pH.

In order to check if the addition of TCA could degrade the cyclic esters, 500 µL of cyclic ester were mixed with 500 µL of TCA and 250 µL of 0.02M NaOH and the results were compared to the cyclic esters without TCA addition. The signals were similar in both experiments and therefore it was concluded that TCA did not hydrolyse the cyclic ester.

## 4.5. Instrumentation and conditions

### 4.5.1 Ultra-performance liquid chromatography analysis (UPLC)

Chromatography was performed using an Acquity™ system with a UPLC BEH C18 column of 2.1 mm x 100 mm and 1.7 µm particle size supplied by Waters (Milford, MA, USA). The column temperature was 40 ºC and the column flow was 0.3 mL $min^{-1}$. The sample injection volume was 10 µL (QTOF) and 5 µL (QqQ). Mobile phases were water (phase A) and methanol (phase B) with 0.1% formic acid. Chromatography started at 98/10 phase A/phase B, changed to 0/100 in 7 minutes.

### 4.5.2 MS-QTOF conditions

MS-QTOF analysis was performed in a Xevo G2 mass spectrometer supplied by Waters (Milford, MA, USA). The detector consisted of an API source (atmospheric pressure ionization) with an electrospray ionization (ESI). The electrospray probe was used in positive (ESI+) and sensitivity mode. The accuracy and reproducibility of all the analyses were guaranteed by use of a LockSpray™. The mass range considered was from 50 to 1200 Da. The capillary voltage was 2.5 kV, the cone voltage was 30 V and the source temperature was 120 ºC. The desolvation gas temperature and flow were 450 ºC and 550 L h−1 respectively. The cone gas flow was 20 L $h^{-1}$

The acquisition was carried out in MSE mode with two functions; acquiring at low-energy (function 1) to obtain information about the precursor ion and at high energy (function 2) to provide information about fragment ions. The collision ramp energy was from 15 to 30 V.

MassLynx v.4.1 software (Waters, Milford MA, USA) was used to analyse the samples.

### 4.5.3 MS-QqQ conditions

MS-QqQ analysis was performed in TQ mass spectrometer from Waters (Milford, MA, USA). The UPLC system was coupled with an ESI probe to the QqQ. The electrospray probe was used in positive (ESI+) and acquisition was performed in MRM mode. The parameters used were as follow: capillary voltage was 3.5 kV, source temperature was 150 ºC, desolvation temperature 450 ºC, cone gas flow 60 L $h^{-1}$, and desolvation gas flow 600 L $h^{-1}$.

The parent ion was 217.1 [MH+] for AA-DEG and the mass transitions 217.1 → 173.1, 217.1 → 155.1 and 217.1 → 111.05 were monitored. The parent ion used for AA-DEG-IPA-DEG was 453.18 [MH+] and mass transitions 453.18 → 237.08, 453.18 → 193.05 and 453.18 → 155.07 were monitored. Cone and collision voltages were optimized from 20 to 70V. Finally, 30V cone voltage and 20V were selected as optimum values for both compounds.

MassLynx v.4.1 and QuanLynx software were used to analyse the samples.

## 4.6. In vitro endocrine activity

Stock solutions of AA-DEG and AA-DEG-IPA-DEG of 100 mM were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Copenhagen, Denmark).

### 4.6.1 Androgen receptor (AR) reporter gene assay

The potential of the test substances to affect AR activity was tested in an AR reporter gene assay using a stably transfected AR-EcoScreenTM cell line based on Chinese hamster ovary cell line (CHO). The protocol was essentially according to the OECD test guideline (Guidelines for the Testing of Chemicals, 2016). The cells contain three stably transfected constructs: a human androgen receptor expression construct, a firefly luciferase reporter construct with an androgen response element, and a renilla luciferase reporter construct. The latter is used to examine compromised cell viability.

Cells were cultured in Phenol Red Free Gibco® Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (D-MEM/F-12) supplemented with 5% fetal bovine serum (FBS), 200 µg $mL^{-1}$ zeonin, 100 µg $mL^{-1}$ hygromycin, 100 units/mL penicillin and 100 µg $mL^{-1}$ streptomycin. All medium components were supplied by InvitrogenTM, Life TechnologiesTM (Carlsbad, California, USA).

Cells were seeded in white 96-well plates (Perkin Elmer) to a final concentration of 9 x 103 cells/well in assay medium (Phenol Red Free DMEM F-12 supplemented with 5 % dextran treated FBS (DCC-FBS), 100 units/mL penicillin and 100 µg $mL^{-1}$ streptomycin). The cells were incubated overnight at 37 $^{O}$C in a humidified atmosphere of 5 % $CO_2$. Successively, medium was removed and new assay medium was added. Test substances and positive controls were added using HP D300 Digital Dispenser (Tecan Group Ltd., Zürich, Switzerland). R1881 (Perkin Elmer, Skovlunde, Denmark) and hydroxyflutamide (OHF) (Toronto Research Chemicals, Toronto, Canada) was included in all independent experiments to ascertain assay performance in agonist and antagonist mode, respectively, in concentrations ranging from 0.002-2.7 nM and 31-8000 nM, respectively. In the antagonist mode of the assay, R1881 was added to all wells at a concentration of 0.1 nM. Test chemicals were tested in concentrations of 12.5, 25, 50, 100, and 200 µM. DMSO was used as vehicle control and was kept constant in all wells (0.2%) – a non-cytotoxic

concentration (data not shown). The cells were incubated with test chemicals for 20-24 h.

Dual-Glo Luciferase Assay System from Promega Corporation (Madison, Wisconsin, USA) was used to measure firefly and renilla luciferase activity. Luminescence was measured on a LUMIstar® Galaxy luminometer (BMG LABTECH, Offenburg, Germany). 100 µL Dual-Glo® Luciferase Reagent was added to each well and the plates were placed on a horizontal shake for 10 min. The firefly luminescence was then measured. Successively, 60 µL/well of Dual-Glo® Stop & Glo® was added. After 10 minutes shaking luminescence was measured. Seven independent experiments were conducted for each test chemical and each exposure concentration was tested in triplicates within the independent experiment.

### 4.6.2 Aryl hydrocarbon receptor (AhR) reporter gene assay

The potential of the test substances to affect AhR activity was tested in an AhR reporter gene assay. The stably transfected rat hepatoma (H4IIE-CALUX) cells obtained from Dr. Michael Denison (University of California, USA) were used and the assay was performed as described previously (Rosenmai, Taxvig, Wedebye, Dybdahl, Vinggaard, Pedersen, et al., 2014).

Cells were cultured in Minimum Essential Medium alpha (MEMα) supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg $mL^{-1}$ streptomycin and 100 µg $mL^{-1}$ fungizone. Medium components were supplied by InvitrogenTM, Life Technologies TM (Carlsbad, California, USA).

Cells were seeded in white clear-bottomed 96-well plates (Corning® Inc., Corning, New York, USA) at a concentration of 22 x 103 cells/well in assay medium (MEMα supplemented with 1% FBS and 100 units/mL penicillin, 100 µg $mL^{-1}$ streptomycin and 100 µg $mL^{-1}$ fungizone). For cell viability studies, cells were seeded in black clear-bottomed 96-well plates (Corning® Inc.,

Corning, New York, USA) at a concentration of 11 x 103 cells/well in assay medium. Cells were incubated for 24 h.

Successively, medium was exchanged and test substances and controls were added manually. Test substances were tested in nine 2-fold dilutions ranging from 0.8-200 µM. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (AACN Standards) was used as a positive control and tested in concentrations ranging from 0.5-3000 pM. The vehicle was kept constant in all wells (0.2 %) – a non-cytotoxic concentration (data not shown). The cells were incubated with test chemicals for 20-24 h.

At experiment termination, cells were lysed with 25 µL/well lysis buffer (25 mM of triphosphate (Sigma Aldrich), 15 % glycerol (VWR/BB), 1 % triton X (Sigma Aldrich), 1 mM dithiothreitol (Sigma Aldrich), and 8 mM $MgCl_2$ (Sigma Aldrich)) and left on shaker table for approximately 20 min. Successively, 40 µL/well luciferin solution were injected automatically and luminescence was measured on LUMIstar® Galaxy luminometer.

Cell viability was examined by use of resazurin. At experiment termination medium was removed and 100 µL of a 5 µg $mL^{-1}$ resazurin solution (Sigma Aldrich) was added to each well. Plates were left to incubate for 3 h at 37 ºC, 5% $CO_2$, and a humidified atmosphere. Fluorescence was measured on EnSpire (Perkin Elmer) with an excitation and emission wavelength of 560 nm and 590 nm, respectively.

Three independent experiments were conducted for each test chemical with each exposure concentration in triplicates.

### 4.6.3 ANSA-TTR displacement assay

Binding of test chemicals to transthyretin (TTR) was examined in the ANSA-TTR displacement assay. The ANSA fluorophore (8-Anilino-1-naphthalene sulfonic acid ammonium salt) increases its fluorescence signal when

bound to TTR, whereas the signal is reduced when ANSA is displaced by competition with thyroid hormones or exogenous substances.

Standard solutions in 1% DMSO were mixed in a black flat bottom 96-well plate (PerkinElmer, Skovlunde, Denmark) with 0.6 µM ANSA (Sigma Aldrich) and 0.5 µM TTR (Sigma Aldrich) in PBS. Test substance concentrations were 50, 100 and 200 µM. After 2 h of incubation at 4°C, the plate was gently shaken for 10 s and fluorescence was measured (Enspire, Perkin Elmer). Negative controls only with 0.6 µM ANSA, ANSA-TTR positive controls, and T4 (thyroxine) (Sigma Aldrich) 0.156, 0.625 and 2.5 µM displacement controls were included on every plate. ANSA fluorescence was measured with excitation filter 380 ± 20 nm/emission filter 475 ± 20 nm). The experiment was repeated in three independent experiments with each exposure concentration tested in triplicates within each independent experiment.

### 4.6.4 Data processing

For AR and AhR reporter assay data, each data point within the independent experiment was normalized to the mean of the plate controls. Successively, means from independent experiments were pooled. In the ANSA-TTR displacement assay, the fluorescence from the negative control was subtracted, and data were expressed as fluorescence relative to the ANSA-TTR maximal fluorescence (positive control). Each data point was normalized against the mean of the plate control and means from the three experiments were pooled.

Kruskal-Wallis test (Dunn's post hoc test) was used to examine differences between exposed groups and controls and a p-value of $<0.05$ was perceived as statistically significant. All data processing and statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Ic, La Jolla, CA, USA).

# 5. RESULTS AND DISCUSSION

## 5.1 Migration assays by UPLC-QTOF

Cyclic esters were quantified by external calibration with AA-DEG and AA-DEG-IPA-DEG standards. The analytical parameters of UPLC-QTOF are shown in Table 2, including linearity, limit of detection (LOD) and limit of quantification (LOQ).

Table 1 summarizes the migration values (ng g-1) of both cyclic esters in 20 different samples. The concentration of the cyclic esters in migration was highly variable but AA-DEG migration values were in all cases higher than the AA-DEG-IPA-DEG values. AA-DEG oligomer was in all migration samples between 20-994 ng $g^{-1}$ except for 17S that was below of limit of migration according to legislation (10 ng g-1). However, AA-DEG-IPA-DEG oligomer was only present in concentration values between 4 and 346 ng g-1 in 8 out of the 20 samples. To clarify, the detection and quantification limits of the method were calculated and reported in Table 2 taking into account the dimension of the bags and the ratio 6dm per 1 kg simulant according to EU/10/2011.

Table 2: Analytical parameters of UPLC-QTOF and UPLC-QqQ (MRM) analysis

| Parameters | UPLC-QTOF | | UPLC-QqQ (MRM) | |
|---|---|---|---|---|
| | AA-DEG | AA-DEG-IPA-DEG | AA-DEG | AA-DEG-IPA-DEG |
| Linear range (ng $g^{-1}$) | 15-1240 | 10-1320 | 17.7-1135 | 1.1-1071 |
| $R^2$ | 0.9994 | 0.9980 | 0.9990 | 0.9999 |
| LOD (ng $g^{-1}$) | 5 | 3.3 | 5.9 | 0.4 |
| LOQ (ng $g^{-1}$) | 15 | 10 | 17.7 | 1.1 |

AA: adipic acid, DEG: diethylene glycol, IPA: isophthalic acid

Table 1: Migration values (ng $g^{-1}$) of AA-DEG and AA-DEG-IPA-DEG in different food simulants from different materials with polyurethane adhesive.

| Material code | Structure | Uses | Migration conditions / Simulant | Concentration AA-DEG (ng $g^{-1}$ simulant) | Concentration AA-DEG-PA-DEG (ng $g^{-1}$ simulant) |
|---|---|---|---|---|---|
| **1S** | PET/PE | BF | 3 days at 40 ºC / Water | 529 ± 67 | <1.0 |
| **2S** | PET/PE | BF | 3 days at 40 ºC / Water | 496 ± 40 | <1.0 |
| **3S** | PET/PE | BF | 3 days at 40 ºC / Water | 275 ± 170 | <1.0 |
| **4S** | PET/PE | BF | 3 days at 40 ºC / Water | 698 ± 4 | 56 ± 10 |
| **5S** | PET/PE | BF | 3 days at 40 ºC / Water | 566 ± 55 | 4.0 ± 0 |
| **6S** | PET/PE | BF | 3 days at 40 ºC / Water | 428 ± 70 | <3.0 |
| **7S** | PET/PE | BF | 3 days at 40 ºC / Water | 994 ± 201 | 346 ± 59 |
| **8S** | PET/PE | BF | 3 days at 40 ºC / Water | 360 ± 2 | <1.0 |
| **9S** | PET/PE | BF | 3 days at 40 ºC / Water | 530 ± 10 | <1.0 |
| **10S** | PET/PE | BF | 3 days at 40 ºC / Water | 350 ± 20 | <1.0 |
| **11S** | Unknown | FC | 10 days at 60ºC / 10% ethanol | 20 ± 5 | <1.0 |
| **12S** | cPP/Al | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 72 ± 12 | <1.0 |
| **13S** | Unknown | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 30 ± 2 | <1.0 |
| **14S** | PET/Alu/OPA/cPP | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 342 ± 10 | 98 ± 8 |
| **15S** | PET/Alu/PE | FC | 10 days at 60 ºC / 95% ethanol | 93 ± 6 | 14 ± 4 |
| **16S** | Alu/PE | FC | 10 days at 60ºC / 95% ethanol | 51 ± 12 | <1.0 |
| **17S** | OPA/cPP | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | <1.5 | <1.0 |
| **18S** | PET/Alu/PE | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 443 ± 89 | 261 ± 30 |
| **19S** | Unknown | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 535 ± 16 | 250 ± 60 |
| **20S** | PET/Al/PA/CPP | FC | 10 days at 60 ºC / 95% ethanol | 759 ± 72 | 12 ± 2.0 |

BF: biological fluids; FC: food contact. Al: aluminum; CPP: cast polypropylene; PET: polyethylene terephthalate; OPA: oriented polyamide; PE: polypropylene; PA: polyamide.

For most multilayer materials, migration of t$^{h}$e cyclic esters exceeded the migration limit established by EU/10/2011 (EC, 2011) for not-listed substances, which is 10 ng $g^{-1}$. Therefore, only the sample 17S should comply with the EU Regulation. Nevertheless, when a compound is not listed in the regulation, the Threshold of Toxicological Concern (TTC) approach can be used (EFSA, 2012). This approach assigns a theoretical toxicity class according to the compound chemical structure and Cramer rules (Cramer, Ford, & Hall, 1978). All the compounds are classified into three classes according to its toxicity: class I (low toxicity), class II (intermediate class) and class III (high toxicity), and a recommended value of maximum daily intake for each class is established (1.8, 0.54 and 0.09 mg/person/day, respectively). Toxtree software was used to estimate the theoretical toxicity of the cyclic esters. According to the TTC approach, both cyclic esters are classified as Cramer class III (high toxicity) and hence the maximum daily intake should be below 0.09 mg/person/day (Cramer, Ford, & Hall, 1978). The maximum recommended migration value according to the maximum daily intake can be calculated with the Estimated Daily Intake (EDI) equation described by FDA :

$$\text{EDI (mg/person/day)} = \text{Mig (mg·kg-1)} \times 3\ \text{kg} \times \text{CF} \qquad \textit{Equation 1}$$

where 3 kg corresponds to the total food intake per person/day and CF is the consumption factor (daily fraction of food that is expected to be in contact with the packaging material). For adhesives, CF value is 0.14. Therefore, the maximum recommended migration for these compounds according to FDA would be 214 ng $g^{-1}$.

According to EFSA (PlasticsEurope, 2014), the Estimated Daily Intake (EDI) equation is different:

$$\text{EDI (mg/person/day)} = \text{Mig (mg·kg}^{-1}\text{)} \times 1\ \text{kg} \qquad \textit{Equation 2}$$

where 1 kg corresponds to the total food eat per person/day. This equation is more restricted than the FDA equation. In this case, the maximum recommended migration for these compounds would be 90 ng $g^{-1}$.

When using the TTC approach for risk assessment, the number of multilayer packaging materials that could be used is 6 out of 20, according to FDA, and 5 out of 20, according to EFSA.

In view of these results, gastric and intestinal digestions of the cyclic esters were performed. This study made it possible to obtain knowledge on the transformation processes of these compounds inside the human body and their bioaccessibilities.

## 5.2 Digestions assays

The aim of digestion assays was to examine if cyclic esters degraded in the stomach and intestine, thus decreasing their concentration and therefore reducing the amount of cyclic esters available to be absorbed by the body. Samples resulting from the digestion assays were analysed by UPLC-QqQ (MRM). Analytical parameters of UPLC-QqQ (MRM mode) of AA-DEG and AA-DEG-IPA-DEG standards are shown in Table 2.

The results showed that digestion led to a decrease in concentration of the cyclic polyesters. Figure 1 shows the percentage values of AA-DEG and AA-DEG-IPA-DEG oligomers after gastric (1a) and intestinal (1b) digestions at different time points (t0, t1h, t2h and t4h). These data were normalized to a control with no digestion.

The amount of both cyclic oligomers progressively decreased during digestion. For AA-DEG, the final percentages of decrease were 31.2% (± 3.9) and 18.2 % (±3.5) after gastric and intestinal digestion, respectively. Gastric digestion was more effective than intestinal digestion. An overall summary of the AA-DEG digestion can be carried out taking into account that gastric digestion

occurs first and intestinal digestion happens consecutively. The digestion resulted in an overall decrease of the parent compound of 43.7% (RSD<5%). On the other hand, for AA-DEG-IPA-DEG the final decrease after each digestion was higher, reaching 53.2 % (± 2.1) for gastric and 91% (± 6.8) for intestinal digestion, with an overall decrease of 95.8 % (RSD < 5%).

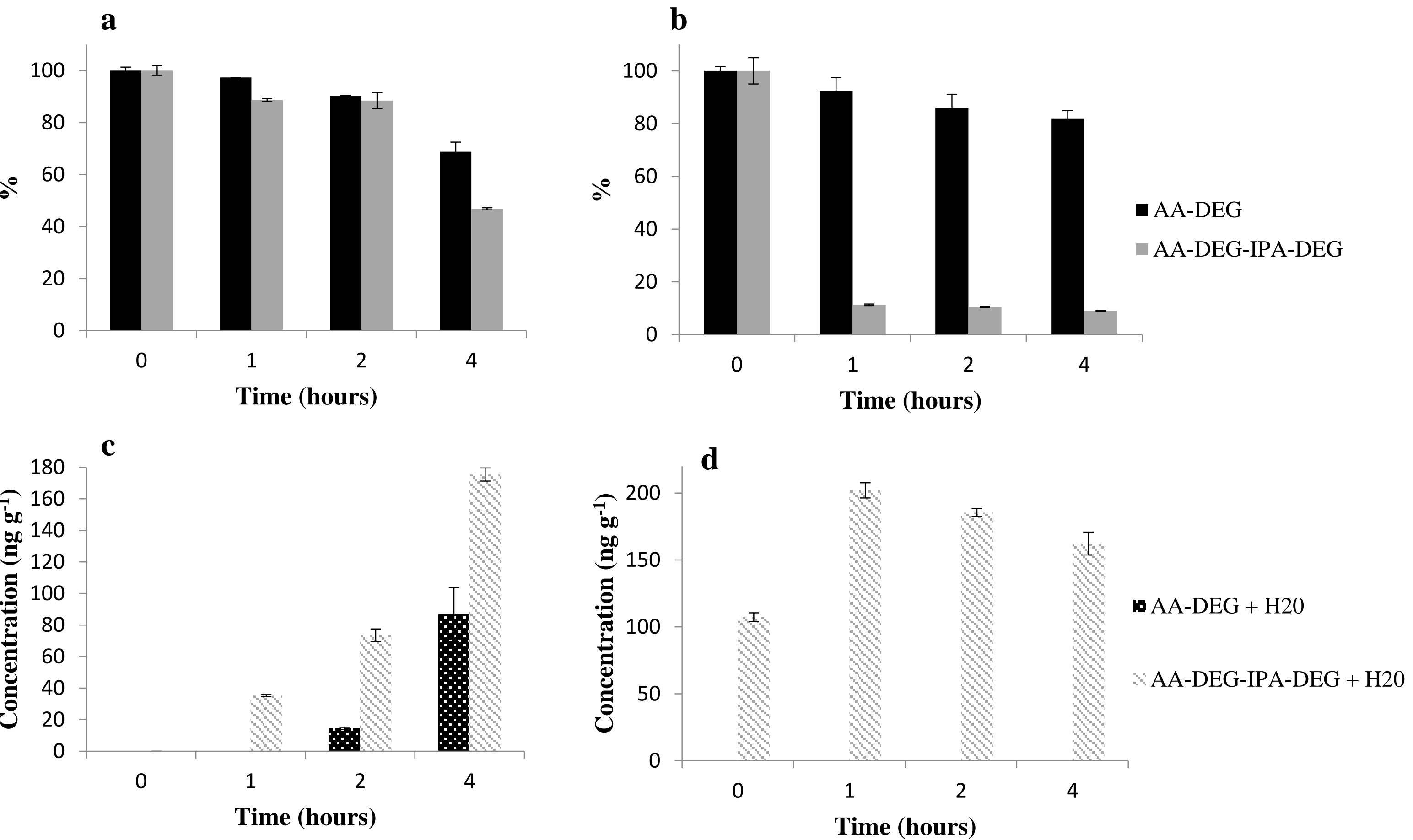



**Figure 1.** Decrease percentage evolution of AA-DEG and AA-DEG-IPA-DEG oligomers for gastric (a) and intestinal (b) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). Evolution of concentration of AA-DEG + H2O and AA-IDEG-PA-DEG + H2O oligomers for gastric (c) and intestinal (d) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$).

Digestion extracts were also analysed by UPLC-QTOF. Chromatograms showed the decrease of the oligomers peaks and, in addition, the emergence of new peaks with signals increasing with digestion. Figure 2 shows a chromatogram of a solution of AA-DEG (a) and AA-DEG-IPA-DEG (b) before (t0) and after (t4h) being submitted to a gastric digestion. In both cases a new peak could be observed. The new peaks after digestions were 5.31_257.099 and 6.50_493.167 (retention time_mass) in AA-DEG and AA-DEG-IPA-DEG respectively. According to their mass, these compounds corresponded to the cyclic esters plus a water molecule. Its formation was the consequence of the hydrolysis of the cyclic esters and the opening of the ring due to the interaction with the gastric and intestinal simulants. This hypothesis is in agreement with previous studies (Gómez Ramos, Lozano, & Fernández-Alba, 2019; Úbeda, et al., 2017). Hydrolysed molecules always eluted before the parent molecule, as other authors have stated before (Úbeda, et al., 2017). AA-DEG high energy mass spectrum has been published in our own previous studies (Úbeda, et al., 2017). Figure 3 shows high collision energy mass spectra of AA-DEG-IPA-DEG (a) and its hydrolysed form (b) with their fragments. The spectra allowed the detection of the fragments and therefore its structure elucidation. Their common masses between cyclic and linear compound were 281.1040 and 193.0503 m/z.

In intestinal digestion, the same analysis was carried out and the same new peaks were also detected.

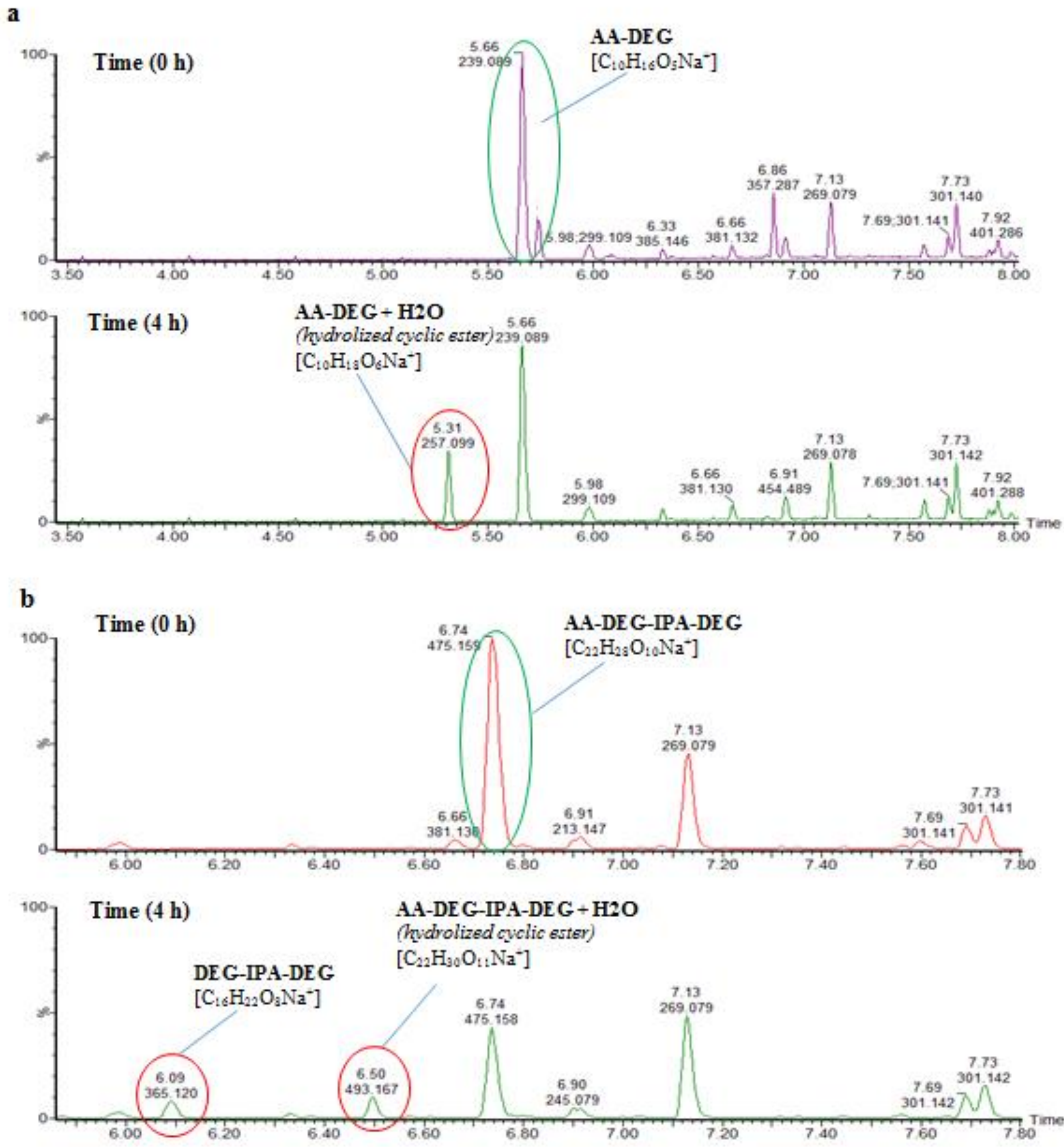



**Figure 2**. Chromatograms of AA-DEG (a) and AA-DEG-IPA-DEG (b) in gastric digestion assays at time 0 and after 4 hours by UPLC-QTOF

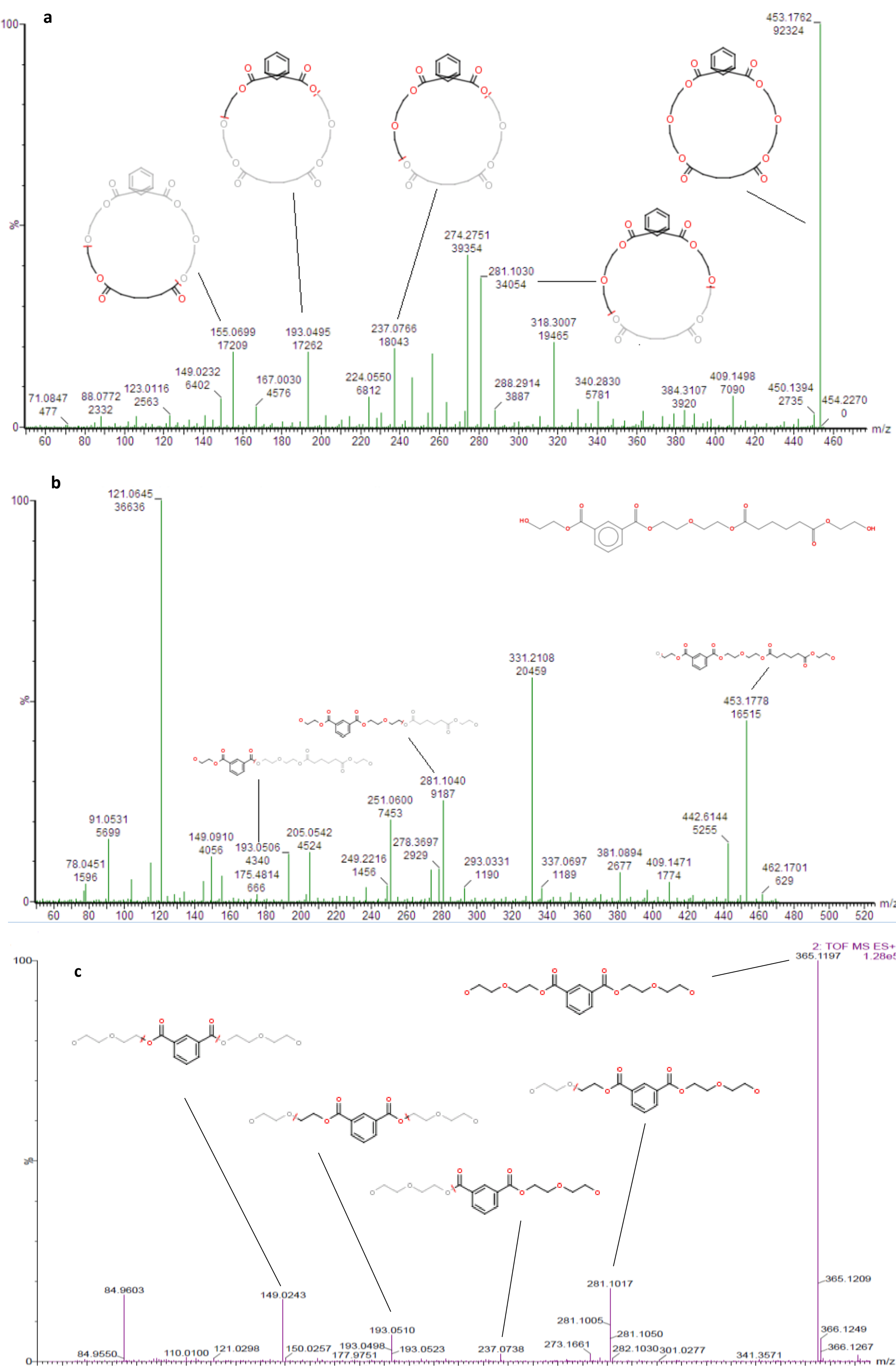



**Figure 3**. High collision energy spectra for AA-DEG-IPA-DEG (a), its hydrolysed form (b) and a fragmentation product, DEG-IPA-DEG (c)

The concentration of hydrolysed molecules in digestion assays was calculated using the cyclic oligomers as standards. Its evolution over time is shown in Figures 1c and 1d. Figure 1c shows concentration values of AA-DEG + H2O and AA-DEG-IPA-DEG + $H_2O$ during gastric digestion and Figure 1d shows concentration values of hydrolysed molecules during intestinal digestion. In gastric digestion, AA-DEG + $H_2O$ concentration increased to 86.7 ng $g^{-1}$ and AA-DEG-IPA-DEG + $H_2O$ to 175.4 ng $g^{-1}$. However, after intestinal digestion, AA-DEG + $H_2O$ concentration was below 6ng $g^{-1}$ (LOD) and AA-DEG-IPA-DEG + H2O concentration was to 162.2 ng $g^{-1}$.

It is important to highlight that the new compounds formed had lower toxicity according to Cramer rules (class I) which is a positive message. Transformations of cyclic esters to their opened form decreased their theoretical toxicity in most cases. Lower toxicity means a higher recommended daily intake (1.8 mg/person/day) and therefore, higher maximum recommended migration values, 4286 and 1800 ng $g^{-1}$ according to FDA and EFSA, respectively. According to the migration values in Table 1, all linear oligomers were below these limits and therefore no health risk for consumers would be expected.

On the other hand, the monomers (AA, DEG and TPA) were checked. The results showed that none of the monomers were present after the oligomer digestion assays.

## 5.3 In vitro endocrine assays

In the present study, AA-DEG-IPA-DEG showed a statistically significant antagonistic activity on AR at high concentrations (100 and 200 µM) with a maximum efficacy of approximately 25% decrease compared to vehicle control. AA-DEG led to a statistically significant antagonistic effect at 200 µM, however the maximum efficacy was approximately 10% compared to vehicle control (Figure 4). These effects occurred at non-cytotoxic concentrations.

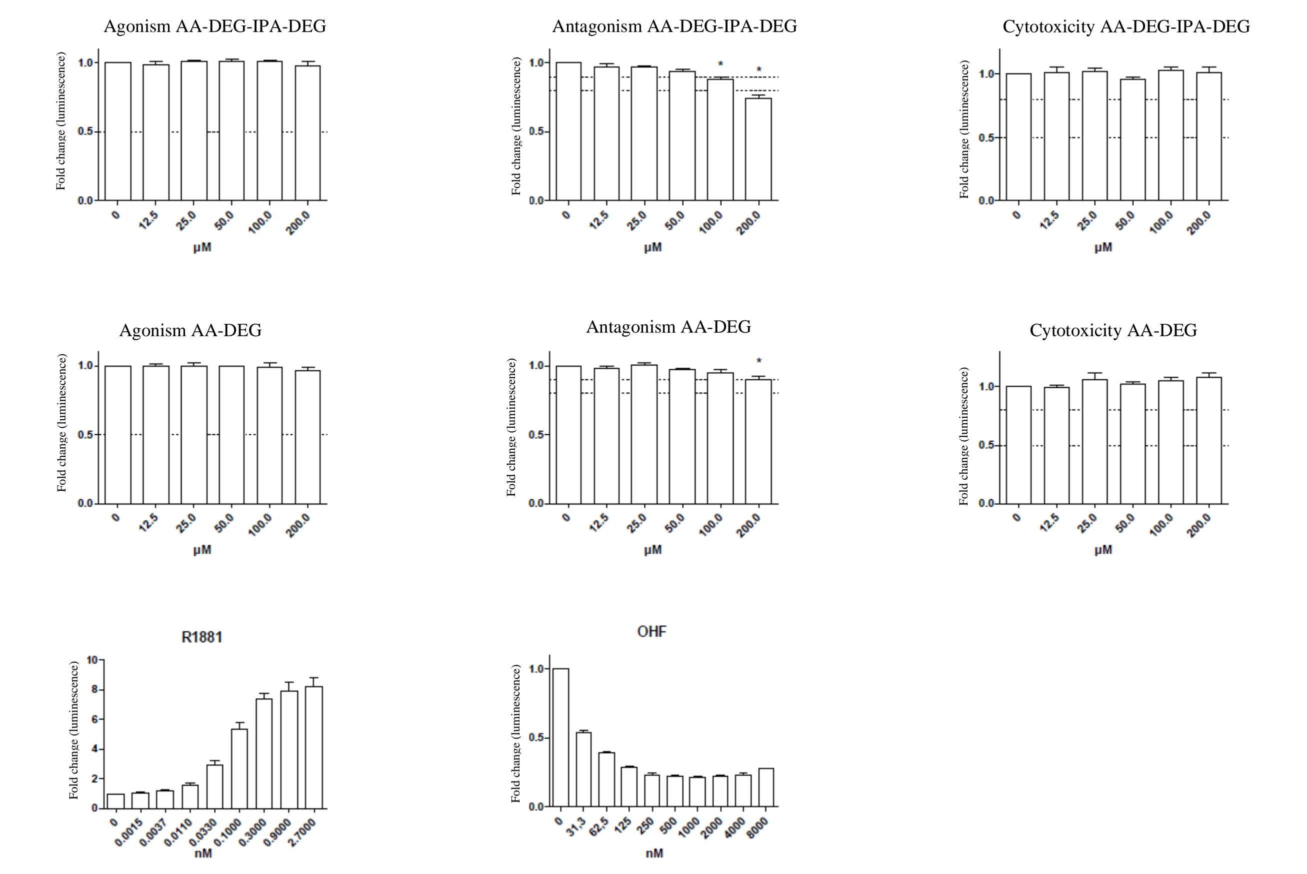
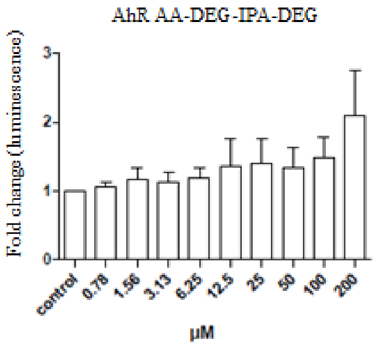
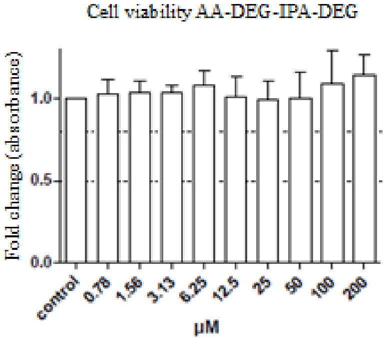


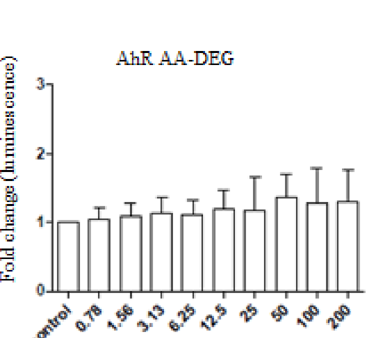

**Figure 4.** Agonism, antagonism and cytotoxicity data from the androgen receptor reporter gene assay of AA-DEG-IPA-DEG (up) and AA-DEG (down) oligomer. Data presented normalized to the vehicle control as pooled means from 7 independent experiments (mean ± SD, n=7). *indicates significant differences ($p < 0.05$).

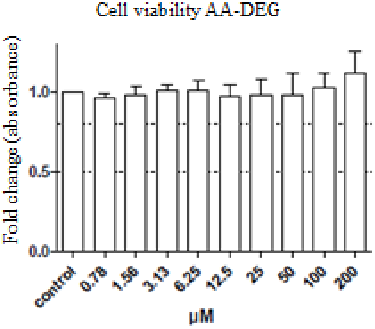
Comparatively, AA-DEG-IPA-DEG thus has greater antiandrogenic potential than AA-DEG. Neither of the test compounds exhibited any major effects in the AhR reporter gene assay (Supplementary material 1) nor the ANSA-TTR assay (Supplementary material 2).

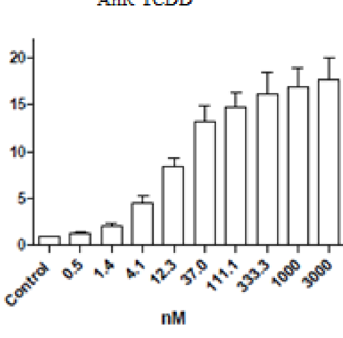
**Supplementary material 1.** Aryl hydrocarbon receptor (AhR) reporter gene assay for AA-DEG-IPA-DEG (up) and AA-DEG (down) oligomer. Data presented normalized to the vehicle control as pooled means from 3 independent experiments (mean ± SD, n=3).* indicates significant differences ($p < 0.05$).



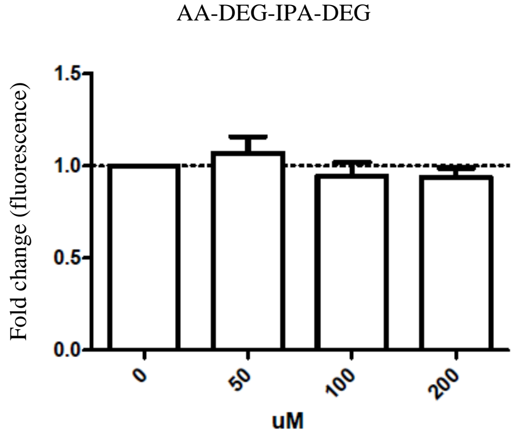



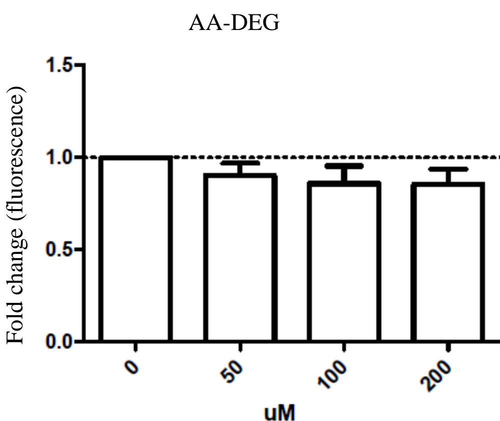



**Supplementary material 2**. ANSA-TTR displacement assay. Data presented as pooled means of the average fluorescence relative to an ANSA-TTR solution from 3 independent experiments (mean ±SD, n=3). *indicates significant differences ($p < 0.05$).




To our knowledge, this is the first time AA-DEG-IPA-DEG and AA-DEG have been tested for ability to interfere with AR, AhR, and TTR. However, the monomers DEG and IPA have been tested for AR binding both in silico and in vitro, as well as in an AR transactivation assay, but exhibited no effect (Osimitz, Welsh, Ai, & Toole, 2015). These findings could suggest that the AA moiety of the compounds play a role in the observed antiandrogenic activities.

As a next step, we preliminarily evaluated whether the metabolites of the cyclic esters exhibited any AR antagonism. The results indicated that no active metabolites were formed at concentrations up to 12.5 µM of parent compound, suggesting that the parent compounds were responsible for the activity (data not shown).

The concentrations leading to antiandrogenic activity (AA-DEG: 200 µM; AA-DEG-IPA-DEG: 100-200 µM) are greater than the migration values of the compounds under the assumption of 1 kg food intake per day containing the highest migration distributed in 5 L blood (higher migration value of AA-DEG: 994 ng $g^{-1}$=> 0.92 µM; and of AA-DEG-IPA-DEG: 346 ng $g^{-1}$ => 0.15 µM). This

suggests that the migration from a single FCM to food would not lead to a concentration that could cause inhibition of AR activity. However, humans may be exposed to oligomers from multiple FCMs simultaneously, as well as other sources, thereby increasing the exposure to these substances. In addition, multiple substances have been reported antiandrogens (Vinggaard, Niemelä, Wedebye, & Jensen, 2008), which can exert mixture effects when exposure occur simultaneously (Metzdorff, Dalgaard, Christiansen, Axelstad, Hass, Kiersgaard, et al., 2007; Orton, Ermler, Kugathas, Rosivatz, Scholze, & Kortenkamp, 2014). Therefore, a better understanding of human exposure sources as well as human levels are needed in future studies.

# 6. CONCLUSIONS

The migration values of the cyclic polyesters that are formed during PU manufacturing (AA-DEG and AA-DEG-IPA-DEG), was highly variable for the different multilayer materials studied. The PU manufacturing process together with the physico-chemical materials properties and the migration conditions could be the explanation for these differences. Besides, results showed that AA-DEG migrated more than AA-DEG-IPA-DEG, probably due to its smaller structure and the absence of the aromatic ring.

The digestion studies showed that the cyclic esters were degraded significantly after gastric and intestinal digestion, which was very positive because their bioaccessibility to the human body became lower. In addition, the new compounds formed had lower toxicity according to Cramer rules, what was also positive from a food safety and human health perspective.

The digestion processess affected the two cyclic esters differently. In the case of AA-DEG, gastric digestion influenced the most with a decrease of 31%, whereas in the case of AA-DEG-IPA-DEG, the influence of intestinal digestion was greater (decrease of 91%). Global digestion (gastric plus intestinal digestion)

was more dominant for AA-DEG-IPA-DEG than for AA-DEG. This means that the bioaccessibility of AA-DEG-IPA-DEG is expected to be lower than of AA-DEG.

Regarding to the endocrine activity, slight effects were observed on AR activity at higher test concentrations suggesting that the compounds can act as AR antagonists. When comparing the compounds, AA-DEG had lower antagonistic activity than AA-DEG-IPA-DEG. This can be hypothesized to be due to the fact that this last compound has a phthalate as part of its chemical structure. Monomers have so far shown no toxicity but their oligomers has slightly AR activity. No effect on TTR binding or AhR activity was found. It may be hypothesized that this lack of effects in vitro might be due to the large size of these molecules that may hinder accessibility to the target.

It would be interesting to perform a broader in vitro screening to expand the toxicological knowledge on these compounds.

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# **SECCIÓN IV:** Conclusiones Generales

# CONCLUSIONES GENERALES

En cada capítulo se han presentado las conclusiones derivadas del trabajo realizado. A continuación se presenta a modo de resumen final, una exposición de las conclusiones finales más relevantes.

1. Se ha demostrado que la cromatografía acoplada a la espectrometría de masas es una combinación perfecta para la identificación y cuantificación de los NIAS e IAS presentes en los materiales de envase. La espectrometría de masas con cuadrupolo/tiempo de vuelo acoplada a la separación por UPLC, UPLC-QToF, es una herramienta muy potente para realizar la determinación de compuestos no volátiles de muestras desconocidas. La espectrometría de masas de alta resolución ofrece la posibilidad de conocer la masa exacta de los analitos, permitiendo su elucidación estructural. Además, gracias a la utilización del UPLC acoplado a un triple cuadrupolo (QqQ) se ha conseguido alcanzar límites de detección muy bajos permitiendo cuantificar los analitos a nivel de trazas. Por otro lado, la GC con impacto electrónico y cuadrupolo, GC-MS(EI), y GC a presión atmosférica con cuadrupolo/tiempo de vuelo (APGC-QToF) han sido dos técnicas complementarias muy útiles para conseguir una elucidación estructural completa de los compuestos volátiles y semi-volátiles.

2. Se ha demostrado que en los polímeros estudiados, la concentración de compuestos no volátiles en pellets y en film es muy similar, lo que sugiere que durante el proceso de extrusión no se modifica la composición del polímero ni se forman compuestos de degradación no volátiles. Para la evaluación de compuestos no volátiles de un polímero se podrían utilizar por tanto ambos tipos de muestra. Sin embargo, los compuestos volátiles

mostraron diferencias en su intensidad cuando se analizaban en pellets o films, por lo que en este caso el proceso de extrusión si afecta a la composición del material.

3. Los biopolímeros son una alternativa más ecológica a los polímeros convencionales pero es necesario garantizar su seguridad cuando se utilizan para el envasado de alimentos, ya que también pueden contener compuestos que migren al alimento.

4. Se ha demostrado que aunque algunos compuestos presentes en el biopolímero de PLA muestran un alto impacto sensorial en el olor del polímero, en los estudios de migración no se detectan y, por lo tanto, no se esperan cambios en el perfil sensorial de los alimentos envasados. Esto nos indica que es necesario realizar ensayos sensoriales en las muestras de migración y no tomar conclusiones únicamente del impacto sensorial del polímero.

5. En los estudios cinéticos de migración se ha comprobado que las cinéticas de migración global y específica siguen un patrón similar. Esto indica que se podría conocer el tiempo necesario para llegar a un equilibrio de migración únicamente realizando el estudio de la migración global, un trabajo más sencillo de realizar que el de la migración específica.

6. Dentro de los NIAS presentes en los materiales de envase destinados al contacto con alimentos, es importante destacar la importancia de la determinación de los oligómeros. Esto es debido a que están siempre presentes en los materiales poliméricos y a que migran, tanto desde capas en contacto directo con el alimento como desde capas internas. Esta migración es en muchas ocasiones, en una extensión bastante amplia, y podrían verse en el alimento como microplásticos. Su identificación y cuantificación es una tarea importante y difícil debido al poco

conocimiento sobre ellos. Los oligómeros no están incluidos en bibliotecas ni en bases de datos químicos, no existen estándares comerciales y no se conocen sus propiedades toxicológicas.

7. De los tres ensayos endocrinos estudiados para los dos oligómeros cíclicos del PU (AA-DEG y AA-DEG-IPA-DEG), únicamente se observaron ligeros efectos sobre la actividad de AR a concentraciones más altas, lo que sugiere que los compuestos pueden actuar como antagonistas de AR.

8. Se ha demostrado que los oligómeros cíclicos se degradan en medio ácido formándose oligómeros lineales. Este resultado es muy positivo para la seguridad alimentaria y la salud humana ya que los oligómeros lineales suelen tener una toxicidad teórica menor que los cíclicos. Además, los estudios de digestión han mostrado que los oligómeros cíclicos se degradan significativamente después de la digestión gástrica e intestinal, por lo que su bioaccesibilidad al cuerpo humano disminuye.

9. El protocolo seleccionado para el análisis de migración de los oligómeros y otros NIAS, incluye realizar inicialmente el análisis del material mediante procedimientos de disolución total y extracción ya que es en el material donde se encuentran los analitos en mayor concentración, y será más sencilla su identificación. Posteriormente, se realizan los estudios de migración y el análisis de los simulantes. No obstante, hay que tener en cuenta que en los ensayos de migración se identifican nuevos tipos de oligómeros, especialmente lineales, debido a las reacciones entre el material con los simulantes alimentarios durante los ensayos de migración, por lo que es imprescindible estudiar con precisión las muestras de migración. Aunque el análisis del material permite una mejor identificación de los compuestos presentes ya que se encuentran a mayor concentración, hay que tener también en cuenta que exige un esfuerzo analítico muy importante y que parte de los compuestos extraídos

posteriormente no migran. Es por ello que sería necesario evaluar en cada caso la necesidad de abordar ambos estudios, en el polímero y en migración.

10. En los ensayos de migración desde diferentes muestras de un mismo tipo de polímero, las concentraciones de los oligómeros son variables. Esta diferencia de valores puede deberse a diferentes factores, como el proceso de fabricación, las propiedades fisicoquímicas de los materiales o las condiciones de migración.

11. Tras estudiar diferentes polímeros destinados al contacto con alimentos (PU, PA, PET, PLA), se ha comprobado que los oligómeros cíclicos son identificados a concentraciones más altas en los ensayos de migración con simulante etanol 95%; sin embargo, utilizando simulantes acuosos como etanol 10% y acético 3%, que conllevan procesos de hidrólisis de los enlaces éster, predominan más los oligómeros lineales. Esto nos indica que los oligómeros cíclicos estarán presentes en mayor cantidad en alimentos grasos frente a los oligómeros líneales que estarán principalmente en alimentos acuosos.

12. En los trabajos realizados con muestras de PET y PET reciclado, se ha observado que la composición de oligómeros era similar en ambos materiales, por lo que los procesos de reciclado no influyeron en la composición de estos compuestos.

13. Para que biopolímeros basados en PLA tengan las propiedades mecánicas adecuadas es necesario en muchos casos mezclarlos con otros biopolímeros. Se ha comprobado que en el biopolímero estudiado basado en PLA y un poliéster, los compuestos con mayor intensidad en la migración tenían su origen en la parte de poliéster y eran en su mayoría, oligómeros cíclicos. Este hecho sugiere que aunque la adición de estos

poliésteres al PLA pueda mejorar sus propiedades mecánicas, es necesario evaluar su efecto en la migración final al alimento.

Com se puede apreciar en estas conclusiones, tanto los análisis del material como de la migración, son necesarios para poder evaluar la idoneidad de los materiales destinados al contacto con alimentos. Los procesos de producción de los materiales poliméricos deben optimizarse para reducir y minimizar al máximo la presencia de estos oligómeros en el polímero y por tanto su posterior migración a los alimentos. Es igualmente importante minimizar la presencia de otros NIAS que pudieran suponer un riesgo para el consumidor.

En muchos casos, los oligómeros migran a los alimentos en cantidades que superan los límites establecidos en la legislación europea, por lo que es importante continuar su investigación. Además, sería necesario por parte de laboratorios de síntesis, ampliar el número de patrones disponibles, y por otro lado, sería interesante ampliar los estudios de toxicidad in vitro. Todo esto permitiría ampliar el conocimiento toxicológico de estos compuestos, que pueden ser ingeridos por el ser humano y de los que no se conocen en muchos casos sus posibles efectos en la salud.

# **SECCIÓN V:** Publicaciones

# PUBLICACIONES

**Úbeda S**, Aznar M, Vera P, Nerín C, Henríquez L, Taborda L, Restrepo C (2017) Overall and specific migration from multilayer high barrier food contact materials - kinetic study of cyclic polyester oligomers migration. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 34 (10):1784-1794. doi:10.1080/19440049.2017.1346390

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