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Departamento de Química Analítica, Nutrición y Bromatología

Área de Nutrición y Bromatología

Desarrollo de Métodos Analíticos para el Estudio en Alimentos de Fenómenos de Oxidación Lipídica y Migración provenientes del Material de Envase

Memoria para optar al grado de doctor

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Santiago de Compostela, Octubre 2004

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INFORMA: Que Dña Ana Teresa Sanches Silva presenta el trabajo titulado "DESARROLLO DE MÉTODOS ANALÍTICOS PARA EL ESTUDIO EN ALIMENTOS DE FENÓMENOS DE OXIDACIÓN LIPÍDICA Y MIGRACIÓN PROVENIENTES DEL MATERIAL DE ENVASE" realizado bajo la dirección de la Dra. Julia López Hernández, del Dr. Perfecto Paseiro Losada y de la Dra. Maria Conceição Barreto Oliveira Castilho, en los laboratorios de Bromatología de la Facultad de Farmacia de la Universidad de Santiago de Compostela.

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Santiago de Compostela, Octubre 2004

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AUTORIZAN a Dña Ana Teresa Sanches Silva a presentar la Tesis titulada "DESARROLLO DE MÉTODOS ANALÍTICOS PARA EL ESTUDIO EN ALIMENTOS DE FENÓMENOS DE OXIDACIÓN LIPÍDICA Y MIGRACIÓN PROVENIENTES DEL MATERIAL DE ENVASE" para optar al Grado de Doctor, la cual ha sido realizada bajo nuestra dirección en los en laboratorios de Bromatología de la Facultad de Farmacia de la Universidad de Santiago de Compostela

Y para que así conste, se expide la presente en Santiago de Compostela en Octubre de 2004.

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ABREVIATURAS

%	Tanto por ciento
d.i.	Diámetro interno
FAMEs	Fatty acids methyl esters (Ésteres metílicos de ácidos grasos)
FCM	Food Contact Materials (Materiales en Contacto con Alimentos)
FID	Flame ionization detector (Detector de ionización de llama)
HCl	Ácido chlorídrico
HPLC	High Performance Liquid Chromatography (Cromatografia de Líquidos de Alta Resolución)
LC-MS	Liquid chromatography- Mass Spectrometry (Cromatografia de gases-Espectrometria de Masas)
NaOH	Hidróxido de sodio
Nx	Extracción normalizada
r^2	Coeficiente de correlación
rpm	Revoluciones por minuto
UV	Ultravioleta

No se incluyen las abreviaturas correspondientes al Sistema Internacional de Unidades (Decreto 1257/74, de 25 de abril de 1974, publicado en el BOE nº 110 de 08/5/1974).

RESUMEN

La conservación de los alimentos es imprescindible para evitar las alteraciones naturales, la proliferación y la contaminación por microorganismos.

Desde hace más de diez mil años existen métodos de conservación que se han ido perfeccionando (curado, ahumado, salazón, escabechado, refrigeración y la aplicación de calor mediante el cocinado de los alimentos). El gran desarrollo de la industria alimentaria ha supuesto un notable avance en lo que se refiere a la conservación y ha puesto a disposición de los consumidores nuevas técnicas de conservación (pasteurización, liofilización y ultracongelación).

Una de las causas más comunes de deterioro de los alimentos durante el almacenamiento es la oxidación lipídica. En los últimos años este fenómeno ha suscitado un gran interés por parte de los investigadores y de la opinión pública debido a su potencial influencia en el proceso de envejecimiento y en varias enfermedades como la arteriosclerosis y el cáncer.

Los principales cambios estructurales de los alimentos son causados por el proceso de oxidación lipídica, lo que provoca la alteración de sus constituyentes y origina la formación de sabores y olores desagradables. Además de los cambios en las calidades sensoriales, el valor nutritivo, la aceptabilidad y la seguridad también disminuyen.

La presente tesis dedica la mayoría de sus capítulos al estudio de la influencia de las condiciones de almacenamiento del producto envasado (ej.: tiempo, luz, atmósfera, temperatura) en componentes nutritivos susceptibles a la oxidación lipídica (ácidos grasos), así como al estudio del perfil aromático obtenido en distintas condiciones de almacenamiento y a la búsqueda de una herramienta analítica que permita la detección precoz y la evaluación de la extensión de los procesos de oxidación lipídica.

Este trabajo ha permitido la elaboración de siete artículos, actualmente publicados o pendientes de publicación en revistas internacionales de reconocido prestigio.

El primer capítulo consiste en una breve introducción a los principales temas estudiados: oxidación lipídica y migración de sustancias de los plásticos hacía los alimentos.

En el segundo capítulo, trabajo publicado en Journal of Chromatography A (1032, 2004, 7-15) se ha llevado a cabo una comparación entre un método de cromatografía líquida de alta resolución (HPLC) y un método de cromatografía de gases (GC), para la separación y cuantificación de los ácidos grasos en patata fritas envasadas comercialmente. El método de HPLC consistió en cuatro pasos: saponificación (que se realiza con 1 ml de NaOH 0.5 N en etanol al 96%, seguida por una centrifugación a 6000 rpm durante 5 min); extracción con solvente (en la cual se adiciona 1 ml de HCl 0.6 N al sobrenadante del paso de saponificación); cambio de solvente (las muestras son evaporadas a sequedad con nitrógeno y redisueltas con metanol) y el último paso consiste en el análisis por HPLC. Se utilizaron dos fases móviles para generar un gradiente. La solución A: 25% acetonitrilo/ 75% agua y la solución B: acetonitrilo puro. El ácido acético (0,12% v/v) fue añadido a ambas fases móviles. El gradiente de elución comenzó con el 50% de cada solución y finalizó con 100% de la solución B, 40 minutos más tarde. El flujo fue de 1.2 mL min^{-1} hasta el minuto 6 para después aumentar a 2 ml min^{-1}. El análisis ha sido realizado con un sistema HPLC acoplado a un detector ultravioleta (UV) y con una columna de Extrasil ODS 2 a 60ºC. Para identificar los ácidos grasos se han utilizado el tiempo de retención y el espectro de absorción y además, se efectúo una confirmación por cromatografía líquida-espectrometria de masas (LC-MS).

En el método de cromatografía gaseosa, los ácidos grasos se extrajeron y derivatizaron en un solo paso. Las muestras se trataron con 2 ml de tolueno y 3 ml de HCl metanólico al 5%. Después de 2 horas en un baño de agua (70ºC), se añadieron a los tubos, 5 ml de una solución de K_2CO_3 al 6% y 1 ml de tolueno y se mezcló el contenido en un vortex. A continuación, la fase orgánica se secó con Na_2SO_4 y se filtró. Finalmente se inyectó 1µl de la solución en el cromatógrafo de gases equipado con un detector de ionización de llama (FID), con una columna capilar Supelcowax y con un inyector split-splitless (1:30). Se ha utilizado helio como gas portador a un flujo de 1.2 ml min^{-1} y el horno se programó de la siguiente forma: 1 min a 160ºC, después a 3.5ºC min^{-1} hasta 230ºC, temperatura que fue mantenida durante 14 min.

Los ácidos grasos, que contenían 18 átomos del carbono (ácido oleico, linoleico y linolénico), han sido cuantificados por regresión linear. Los métodos han sido evaluados respecto a su límite de detección, repetibilidad y recuperación. Al final, el método más conveniente para la cuantificación de los ácidos grasos de las patatas fritas se eligió en función de todas las ventajas y desventajas inherentes que presentaba.

El contenido en ácidos grasos calculado con el método de cromatografía líquida presenta una buena correlación cuando es comparado con los correspondientes valores del método de cromatografía gaseosa ($P>0.05$). El contenido en ácidos grasos disminuye significativamente ($P<0.05$) después de un periodo de tres meses de almacenamiento.

En el tercer capítulo, que ha sido publicado en Chromatographia (58, 2003, 1-5), se estudia el perfil de ácidos grasos de ocho marcas de patatas fritas envasadas comercialmente. El perfil ha sido evaluado y comparado, mediante la determinación de catorce ácidos grasos utilizando el método de cromatografía de gases propuesto inicialmente.

Los resultados indicaron que ninguna marca podría ser considerada representativa de las patatas fritas en general. Sin embargo, las patatas presentan un perfil similar de ácidos grasos cuando son fritas en el mismo aceite. Las distintas marcas fueron clasificadas en tres grupos de acuerdo con el ácido graso mayoritario (ácido oleico, ácido linoleico y ácido palmítico) para a continuación identificar el tipo de aceite utilizado en el proceso de fritura de cada una.

Las patatas fritas envasadas comercialmente se almacenaron a temperatura ambiente y en la oscuridad, para evaluar los cambios en el perfil durante tres meses. A lo largo de este periodo de tiempo, el contenido en ácidos grasos mayoritarios no sufrió cambios considerables.

Aunque la oxidación lipídica ha sido ampliamente estudiada, son escasos los trabajos que han investigado el efecto de las condiciones de almacenamiento sobre este fenómeno. Por lo que en el cuarto capítulo, publicado en Analytica Chimica Acta (524, 2004, 185-189) se ha evaluado la influencia de la atmósfera del interior del envase y de la temperatura de almacenamiento en la oxidación de las patatas fritas. El perfil de los ésteres metílicos de los ácidos grasos (Fatty Acids Methyl Esters - FAMEs) ha sido estudiado por el método de cromatografía gaseosa propuesto anteriormente.

Por un lado, han sido almacenadas a temperatura ambiente (~18-20ºC) y a 40ºC, en la oscuridad, cuatro marcas de patatas, fritas en diferentes aceites, para evaluar el efecto de la temperatura sobre los cambios en el perfil de los FAMEs. Se ha verificado que a temperatura ambiente no hubo cambios significativos ($P<0.05$) en el perfil de ácidos grasos, mientras que a 40ºC se produjo una disminución significativa de los ácidos poliinsaturados para todos los tipos de patatas fritas, a excepción de las que presentaban un menor contenido en grasa.

Por otro lado, se almacenó un único tipo de patatas (fritas en aceite de oliva) a temperatura ambiente, en la oscuridad, bajo diferentes atmósferas: aire, vacío, absorbedor de oxígeno y nitrógeno. La atmósfera modificada ha sido utilizada con el propósito de saber cual era, entre las cuatro estudiadas, la más eficaz para evitar o retrasar la oxidación lipídica durante el almacenamiento. Los mejores resultados se obtuvieron con el absorbedor de oxígeno, ya que redujo a niveles insignificantes la concentración residual del mismo, retrasando los fenómenos oxidativos y aumentando la vida útil del alimento.

Además, teniendo en cuenta que el mecanismo de oxidación lipídica se caracteriza por una cadena de reacciones donde los radicales libres atacan las posiciones adyacentes a los dobles enlaces, la luz es uno de los factores que contribuye a la formación de radicales libres. De esta forma la luz es otro importante parámetro a tener en cuenta cuando se evalúa la oxidación lipídica.

En el quinto capítulo, el cual ha sido publicado en Analytica Chimica Acta 524 (2004) 191-200), se estudia la influencia de la luz en la inducción de la oxidación de las patatas fritas almacenadas. Los ácidos grasos son convertidos en sus FAMEs utilizando un método en el cual la extracción y metilación se producen simultáneamente, ahorrando trabajo y tiempo de análisis.

El perfil de ácidos grasos de las patatas fritas almacenadas en la oscuridad no sufrió cambios considerables, mientras que las almacenadas en presencia de luz natural presentaron cambios muy significativos, principalmente en los ácidos grasos poliinsaturados.

En relación a los contenidos en ésteres metílicos de ácidos grasos, se ha verificado una disminución en ambas condiciones de almacenamiento, aunque es más evidente para las muestras almacenadas en presencia de luz. Al cabo de los tres meses de almacenamiento se

detectó la aparición de nuevos compuestos, que muy posiblemente son responsables del olor y sabor desagradables. Estos, fueron identificados por cromatografía gaseosa con detector de masas y se verificó que la mayoría de los compuestos correspondían a ácidos grasos de cadena corta y aldehídos, es decir, productos resultantes de la oxidación lipídica. Se concluye por tanto, que la luz es un factor que contribuye de forma determinante en el proceso de oxidación lipídica.

Con el objeto de identificar los compuestos volátiles, con bajo umbral olfativo, formados en el proceso de oxidación lipídica, se ha utilizado una novedosa técnica, la microextracción en fase sólida (SPME), que evita los inconvenientes de las técnicas más comunes (destilación, extracción con solventes, extracción con fluidos supercríticos, extracción en fase sólida, técnicas de espacio de cabeza estático y dinámico y trampas de materiales adsorbentes). Este trabajo corresponde al sexto capítulo que ha sido aceptado para su publicación en la revista Journal of Chromatography A.

El método SPME permite la extracción y pre-concentración simultáneas y ha sido utilizado anteriormente en otras aplicaciones, además del estudio de los compuestos volátiles en alimentos. Se basa en la adsorción de los analitos directamente desde las muestras en una fibra cubierta de sílica fundida. A continuación los analitos son desorbidos en el inyector del cromatógrafo de gases y separados en una columna capilar. Como resultado se obtiene un cromatograma que se puede considerar como una "huella dactilar", ya que permite determinar los compuestos responsables de los sabores y olores desagradables.

La extracción ha sido llevada a cabo por el método de espacio de cabeza, durante 20 min y después de un tiempo de equilibrio de 5 min. A continuación, la fibra (DVB/PDMS/DVB) se introduce en el bloque de inyección durante 3 min a 260ºC. La separación se efectuó en una

columna DB-5 (30 m x 0.25 mm d.i., 1 µm de espesor) usando el siguiente programa de temperaturas: se inicia el programa a 40ºC durante 1 min, a continuación comienza el gradiente de temperatura a razón de 20ºC min^{-1} hasta alcanzar los 120ºC, manteniendo esta temperatura 8 min, posteriormente se continua con este gradiente hasta los 260ºC, temperatura que se mantiene por 2 min. La detección se llevó a cabo con el detector selectivo de masas en el modo barrido completo (full scan) en el rango de masas 35-290. Muchas de las sustancias formadas durante el proceso de oxidación lipídica fueron identificadas. La mayoría pertenece a las siguientes familias: cetonas, aldehídos, alcoholes y ácidos carboxílicos. En este trabajo se discuten las vías de formación de las sustancias identificadas.

Estudiado el amplio rango de compuestos volátiles que se forman como consecuencia del proceso de oxidación lipídica, responsable del deterioro de los alimentos con alto contenido en grasas, se ha considerado la posibilidad de encontrar una sustancia que permitiera indicar el estado de oxidación de las patatas fritas. En el capítulo séptimo, publicado en la revista Journal of Chromatography A (1046 (2004) 75-81), se ha seleccionado el hexanal para evaluar su idoneidad como indicador de este proceso.

Numerosos métodos de extracción y pre-concentración de volátiles se han utilizado en el pasado para determinar el hexanal. En este trabajo se han comparado tres de estos métodos: microextracción en fase sólida (SPME), extracción en fase sólida (SPE) con mini-columnas impregnadas con el agente derivatizante y derivatización directa de la muestra. Siendo este último el que presenta mayores ventajas. El método propuesto es sencillo y rápido.

Se ha empleado como agente derivatizante la 2,4-dinitrofenilhidrazona (DNPH). A continuación, se ha llevado a cabo el

análisis cromatográfico (HPLC) empleando una columna Tracer Extrasil ODS2 5m (25 x 0.4 cm) y metanol -agua (75:25 v/v) como fase móvil a un flujo de 1.5 mL min^{-1}. El límite de detección para el hexanal ha sido de 9 ng ml^{-1}. En este trabajo también se presentan los parámetros de validación del método.

Los resultados indicaron que en las patatas fritas almacenadas en la oscuridad no se produjeron cambios en el contenido de hexanal, mientras que en las almacenadas a la luz se produjo un aumento significativo del mismo. Se verifica así, que el hexanal es un buen indicador del proceso de oxidación lipídica ya que aumenta durante el almacenamiento indicando una alteración de las propiedades sensoriales.

El octavo capítulo se ha dedicado al estudio de las migraciones de sustancias del material de envase hacía los alimentos. Durante las últimas décadas la preocupación por temas como la seguridad de los alimentos, la nutrición, los aditivos y el etiquetado del producto ha tenido una demanda creciente por parte de los consumidores. Dentro de este contexto, el envasado de los alimentos es una técnica fundamental para conservar la calidad de los mismos, reducir al mínimo su deterioro y limitar el uso de aditivos. Los materiales destinados a entrar en contacto con los alimentos (Food Contact Materials, FCM), como los plásticos, cumplen diversas funciones de gran importancia: contienen los alimentos, protegen del deterioro químico y físico, y proporcionan un medio práctico para informar a los consumidores sobre los productos. Asimismo, el envase preserva la forma y la textura del alimento que contiene, evita que pierda sabor o aroma, prolonga el tiempo de almacenamiento y regula el contenido de agua o humedad del alimento. En algunos casos, el material seleccionado puede llegar incluso a mejorar la calidad nutricional del producto.

Sin embargo hay una característica muy importante, y que se debe exigir a todos los envases, y es que no se produzcan interacciones con el contenido del mismo. Las modernas técnicas de envasado, con la utilización de nuevos materiales han solucionado muchos problemas de higiene pero plantean otros nuevos. Hay que asegurarse de que los materiales utilizados no sean tóxicos o susceptibles de interaccionar con los alimentos que van a contener. Por esto, un importante aspecto relacionado con la salud pública que preocupa a la Unión Europea es la exposición del consumidor a sustancias químicas indeseables en la dieta.

Para caracterizar el fenómeno de la migración se distingue por un lado la migración global, que se refiere a la suma de todos los componentes del envase que se transfieren al alimento y por otro la migración específica, que representa la cantidad de una sustancia concreta e identificable presente en el material que se transfiere al alimento bajo ciertas condiciones.

Por este motivo en la Unión Europea, existe legislación de obligado cumplimiento, que define los límites máximos de migración global y migración específica, para envases plásticos en contacto con alimentos. Las investigaciones científicas relacionadas con la migración potencial y con el comportamiento de los materiales de envasado han demostrado que la difusión y la migración pueden ser fenómenos previsibles y en principio descritos matemáticamente. Sin embargo, la escasa información relativa al comportamiento de muchas sustancias potencialmente migrantes y la creciente importancia que tiene la seguridad alimentaria, han suscitado el interés de la Unión Europea en lo referente a los materiales plásticos en contacto con alimentos. Entre los proyectos que la Unión Europea ha financiado relativos a este tema se encuentra el "Foodmigrosure", que está siendo desarrollado en varios centros europeos de investigación. El principal objetivo de este proyecto es proporcionar

una herramienta novedosa y económica para la estimación de la exposición del consumidor a sustancias migrantes que provengan de los materiales en contacto con alimentos. Esta herramienta está basada en un modelo de migración físico-química que describe matemáticamente los procesos de migración desde los plásticos a alimentos reales bajo cualquier condición de contacto previsible.

Incluido en este proyecto, que ha financiado la beca para la realización de esta tesis, ha sido desarrollado un método analítico, ya aceptada para su publicación en J. Separation Science, que permite evaluar los niveles de migración de triclosan, sustancia incluida en el plástico empleado para envasar alimentos.

El triclosan es un agente antimicrobiano de amplio espectro, incorporado en una amplia gama de productos, incluyendo cosméticos, jabones, cremas dentales, desodorizantes y plásticos para la cocina y utensilios de mesa. Cuando es añadido a plásticos, previene el crecimiento de bacterias gram-negativas y gram-positivas en la superficie del material, aumentando la vida útil de los polímeros.

Incluido en este contexto, el proyecto europeo Foodmigrosure ha seleccionado el triclosan como modelo para estudiar procesos de transporte y partición en alimentos. En el capítulo octavo se describe el procedimiento de extracción, así como el método de HPLC con detector UV usado para la cuantificación del triclosan. El método ha sido validado en cuatro alimentos seleccionados como representativos del amplio rango de complejidad de las matrices alimentarias: zumo de naranja (alimento acuoso, ácido y con contenido moderado en carbohidratos); pechuga de pollo (alimento sólido con elevado contenido en proteínas), queso gouda y patatas fritas (alimentos sólidos con elevado contenido en lípidos).

Las muestras fueron preparadas según un procedimiento simple y rápido, que consistió en dos pasos. Primero, extracción con un solvente

orgánico (hexano) seguido por un paso de cambio de solvente. El extracto final se disuelve en acetonitrilo al 90%, un solvente compatible con la técnica cromatográfica HPLC.

El método es capaz de detectar hasta 25 ng ml^{-1} de triclosan. La linearidad obtenida ha sido excelente ($R^2 > 0.9999$) lo que demuestra que el método es válido para la cuantificación de triclosan. Las recuperaciones del método, calculadas a tres niveles (0.5, 1 y 5 μg ml^{-1}) han sido satisfactorias en todos los casos. La identidad de triclosan ha sido confirmada por cromatografía líquida y cromatografía de gases ambas acopladas a detectores de masas.

I - INTRODUCCIÓN

I-1. Oxidación lipídica

La oxidación lipídica es una alteración que consiste principalmente en la degradación de la estructura básica de las moléculas de lípidos, que contribuyen a la textura y sabor de los alimentos. Así, es responsable de la disminución del valor nutritivo, modificación de propiedades sensoriales, aceptabilidad y seguridad. En realidad cuando el proceso se encuentra en fases muy avanzadas, pueden aparecer compuestos tóxicos capaces de poner en riesgo la salud de los consumidores.

Aunque las cantidades de lípidos que intervienen en estas reacciones suelen ser muy pequeñas, los compuestos formados presentan unas características organolépticas muy notorias y al mismo tiempo se detectan a bajas concentraciones. La conservación y vida útil de los alimentos son por tanto, dependientes de la formación de volátiles con olor desagradable siendo los ácidos grasos insaturados los que presentan mayor susceptibilidad al proceso de oxidación.

El mecanismo de oxidación lipídica se caracteriza por cuatro etapas principales: iniciación, propagación, ramificación o autooxidación y terminación.

En la iniciación hay formación de radicales libres en presencia de un agente prooxidante (calor, luz, iones metálicos, etc).

La propagación se caracteriza por una cadena de reacciones. En una primera fase el oxígeno reacciona rápidamente con radicales alquilo libres (R´.) originando radicales peroxilos (R´COO.). Éstos reaccionan con más moléculas de lípidos produciendo hidroperóxidos (R'OOH).

A continuación podrá producirse un aumento geométrico en radicales libres proveniente de la descomposición de hidroperoxidos.

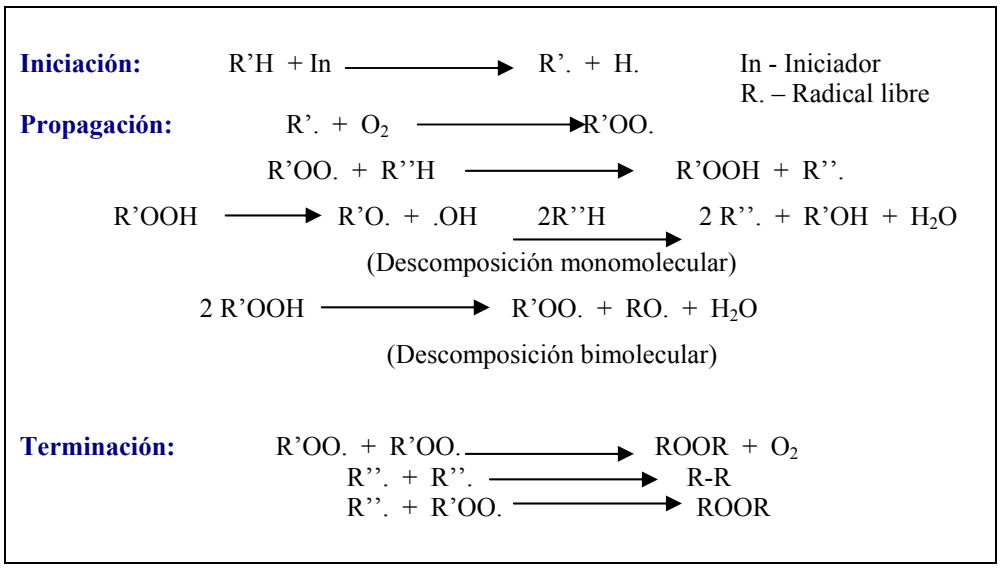

Figura I-1: Mecanismo de la oxidación lipídica (autooxidación o enranciamiento oxidativo de los ácidos grasos).

En la última etapa los radicales peróxidos reaccionan unos con otros para anularse entre sí y originan productos estables.

La composición de los alimentos es de extrema importancia en el fenómeno de la oxidación lipídica. Los alimentos que tienen mayor porcentaje de lípidos insaturados son más susceptibles a la oxidación lipídica que los que están constituidos por ácidos grasos saturados. El grado de instauración de la fracción lipídica de un alimento es, por tanto, un importante factor de su estabilidad.

En este trabajo fueron seleccionadas las patatas fritas para evaluar el grado de oxidación lipídica porque tienen un elevado porcentaje de lípidos, es decir, son susceptibles a la oxidación lipídica y no plantean problemas microbiológicos.

I-2. Envases y migración

Los alimentos están expuestos a la acción de diversos factores (químicos, físicos y microbiológicos) que originan una serie de reacciones las cuales modifican la composición y características del producto. La alteración microbiológica, oxidación lipídica, desnaturalización de proteínas, los pardeamientos y la destrucción de vitaminas son algunas de las reacciones que conducen a la alteración nutritiva, sanitaria y/o sensorial del producto. Al interponerse entre el alimento y su entorno, el envase de los alimentos es fundamental para conservar la calidad de los mismos, reducir al mínimo su deterioro y limitar el uso de aditivos. El envase cumple diversas funciones de gran importancia: contiene los alimentos, protege del deterioro químico y físico, y proporciona un medio práctico para informar a los consumidores sobre los productos. Asimismo, el envase preserva la forma y la textura del alimento que contiene, evita que pierda sabor o aroma, prolonga el tiempo de almacenamiento y regula el contenido de agua o humedad del alimento. En algunos casos, el material seleccionado para el envase puede llegar incluso a mejorar la calidad nutricional del producto. El envase permite a los fabricantes ofrecer información sobre las características del producto, su contenido nutricional y su composición.

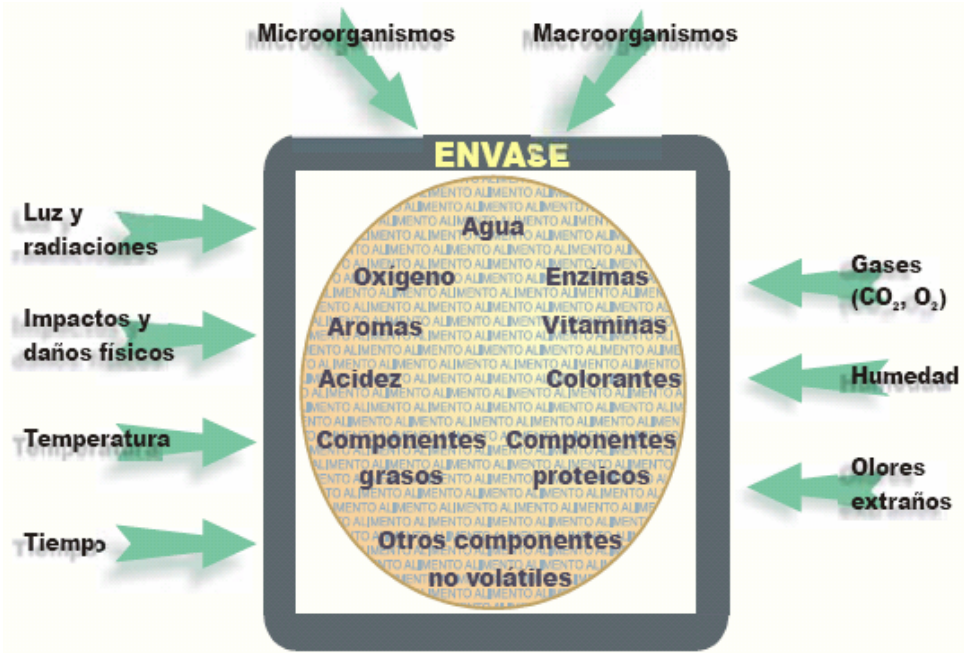

Figura I-2: Sistema alimento/envase/entorno (imagen tomada de la página web: http://www.ua.es/univerano/cursos2004/nutricion/Rafael%20GavaraConf.pdf)

De las interacciones que tienen lugar en el sistema alimento/envase/entorno, la migración de residuos y aditivos del material de envase es la que afecta más a la calidad y seguridad de los alimentos envasados. Las modernas técnicas de envasado, con la utilización de nuevos materiales han solucionado muchos problemas de higiene pero plantean otros nuevos. Por esto, un importante aspecto relacionado con la salud pública y que ha suscitado el interés de la Unión Europea es la exposición del consumidor a sustancias químicas indeseables en los alimentos, provenientes del material de envase. La Comisión Europea ha decidido financiar el proyecto Foodmigrosure ("Modelling migration from plastics into foodstuffs as a novel and cost efficient tool for estimation of consumer exposure from food contact materials"- QLK1-CT2002-2390),

como parte del programa prioritario "Quality of Life and Management of Living Resources". El principal objetivo de este proyecto es establecer un modelo de migración físico-química que pueda describir matemáticamente los procesos de migración desde los plásticos hacia los alimentos. Integrado en el plan de trabajo del mismo, se ha desarrollado un método que se expone en el último capítulo de esta tesis.

I-2.1- Conceptos básicos relacionados con la migración

La migración se define como la "transferencia de masa de una fuente externa al producto envasado por un proceso submicroscópico", lo que origina alteración de las características físico-químicas y mecánicas del material de envase, por pérdida de componentes del mismo, y cambios en la composición del producto envasado, lo que puede modificar su calidad y aceptación comercial.

Se distinguen *migración global* y *migración específica*. La primera se refiere a la cantidad total de componentes del envase que se transfieren al alimento, mientras que el término migración específica indica la cantidad de una sustancia concreta e identificable que es transferida al alimento bajo ciertas condiciones. También se puede distinguir el término *migración potencial* lo cual representa la cantidad máxima de una sustancia presente en un material, que podría ser transferida al alimento envasado.

I-2.2. Migración en plásticos

La interacción entre el envase y el alimento envasado depende del material de envase. Así, en los materiales metálicos se caracteriza por

fenómenos de corrosión, y disolución de los iones metálicos en el alimento, mientras que en los materiales cerámicos y vidrio se define como un proceso de disolución química o lixiviación que también produce la incorporación de elementos del material en el alimento. Con relación a los materiales poliméricos (plásticos y derivados celulósicos), la interacción se hace mediante la transferencia de masa a través de la matriz polimérica, produciendo la incorporación de los componentes, aditivos y residuos en los alimentos. La transferencia de masa es resultado de tres fenómenos: *permeabilidad* (paso de gases, vapores y radiaciones a través del material polimérico), *sorción* de componentes del alimento por el polímero y *migración* de compuestos del polímero al alimento.

Los plásticos son materiales con propiedades visco-elásticas, formados por una mezcla de polímeros con otras sustancias. Los polímeros base son moléculas de elevado peso molecular, inertes y de solubilidad limitada, por lo que tienen también una migración limitada. Sin embargo, son los compuestos de bajo peso molecular que se encuentran en la matriz polimérica los que presentan mayor probabilidad de migrar. Estos incluyen: residuos (monómeros, oligómeros, disolventes) y coadyuvantes de la polimerización (catalizadores, aceleradores, inhibidores), aditivos, compuestos resultantes de la degradación del polímero y/o aditivos y sustancias adyacentes al material (adhesivos, barnices y tintas de impresión).

En el presente trabajo se ha utilizado como principal alimento de estudio la patata frita envasada comercialmente.

I-3. La patata

Después de los cereales convencionales (trigo, maíz y arroz) el cultivo de la patata es el más extendido en el mundo. Su fácil cultivo, excelente adaptabilidad y valor nutritivo, la hacen producto de mil recetas y guarnición de muchos platos. Este tubérculo americano entró a Europa por la puerta de atrás, para luego convertirse en uno de los alimentos más consumidos.

I-3.1. Origen

Botánicamente la patata pertenece a la familia de las Solanáceas (Solanaceae) grupo este en que se encuentra también el tabaco (otra planta americana), entre otras muchas. El nombre científico de la patata es *Solanum tuberosum*, su centro de origen se halla en el sur del Perú, Bolivia o norte de Argentina. Se señala como centro de origen de una especie cultivada a aquella localidad o región donde se encuentran los antecesores silvestres más directos y la mayor diversidad específica, es decir donde se encuentra la mayor cantidad de especies del mismo género, para este caso, el género Solanum.

I-3.2. Su llegada a Europa

Los conquistadores de América de regreso a España, por el año 1560, trajeron las patatas y después estas se dieron a conocer a toda Europa. Sin embargo, su aspecto exterior causaba repugnancia por lo que les cerraron las puertas de la cocina.

Un farmacéutico francés, Antoine Parmentier, se interesó por este tubérculo y descubrió su valor alimenticio, echando por tierra falsos prejuicios y propició su cultivo al descubrir su rendimiento y alto valor nutritivo. Este estudioso profetizó que las patatas con el tiempo se convertirían en el pan de los pobres y en manjar de los ricos y la historia le hizo justicia. A finales del siglo XVIII, la patata ya se conocía y se cultivaba en todas partes. En España como "patata", en Finlandia como "peruna", en Alemania como "kartoffel", en Francia como "pomme de terre", en Irlanda como "murphy", en Italia como "tartuffolo" y en los Países Bajos como "aardappel" (manzana de la tierra). Sin embargo no se convirtió en un producto de consumo a gran escala en Europa hasta principios del siglo XIX.

En el siglo XIX una plaga arrasó la cosecha de patata y trajo como consecuencia numerosas muertes por hambre. Nuevas técnicas de hibridación han dado, al cabo de los años, patatas más resistentes a los parásitos y enfermedades. Los nuevos métodos de cultivo, la selección de la tierra para la siembra, variedades, tecnologías, etc., han contribuido a mejorar los rendimientos de la producción, la forma, la piel, el gusto, los calibres y las cualidades culinarias.

Hoy en día, iniciado el siglo XXI, la patata representa el 50% de toda la producción mundial de tubérculos y raíces. A la larga, el resultado fue el gran boom europeo de la patata, servida en todas sus presentaciones: frita, en purés, al vapor, horneada, salteada, en tortillas, etc.

I-3.3. Composición nutricional/Valor Nutritivo

La patata es una hortaliza que debido a su composición es difícil de encuadrar, se encuentra entre el grupo de las verduras y el de los cereales. El valor calórico de la patata no es elevado, 75-80 Kcal./100g cuando están cocidas y hasta las 253 Kcal cuando están fritas. Entre los nutrientes que presenta se destacan: los glúcidos (20%); prótidos (2%); fibra (2%) y agua (80%). La principal propiedad de la patata es su contenido en almidón. Casi no tiene materia grasa.

Al ser consumida como alimento básico también aporta una proporción alta de folato, vitamina C (sobre todo cuando es de nueva cosecha), tiamina (B_1), potasio y hierro, a la vez que contribuye de manera importante a la ingesta de proteínas. De hecho, si se consume suficiente cantidad de patatas como para satisfacer las necesidades energéticas se obtendrá un volumen equilibrado de nutrientes, con la excepción de las vitaminas liposolubles. Entre los fitoquímicos presentes en las patatas están las cumarinas y antoxantinas. Debe evitarse el consumo de patatas de color verdoso, ya que contienen glicoalcaloides tóxicos.

I-3.4. La patata Frita

En la dieta humana, la patata puede ser preparada varias formas. La opción más sana y mejor de tomar patata es asada o cocida. La patata frita aumenta el porcentaje de grasa y es más difícil de digerir, aún así, es muy sabrosa, pero no siempre recomendable, y más si lleva mucha sal, ya que aumenta la absorción de los líquidos.

Figura I-3 – Patatas fritas: (imagen tomada de la página web: www.racetothetop.org/.../ module7/page_1.htm)

Las patatas fritas fueron cocinadas por primera vez en 1853 por un cocinero indo-americano llamado George Crum, tras ser devueltas a cocina varias veces por uno de sus clientes más exigentes, por lo que decidió hacer freír las patatas más finas hasta la fecha, en aceite. Este exigente magnate ferroviario llamado Cornelius Vanderbilt, quedó maravillado de la finura de la patata y del exquisito manjar. Las patatas fueron todo un éxito. La industria de la comida rápida que incluye patatas en su elaboración, ha experimentado un gran auge entre los países en desarrollo, por lo que se ha ampliado su consumo.

Lo cierto es que las patatas fritas de bolsa son hoy en día uno de los snacks más vendidos en todo el mundo. Existen infinidad de sabores, formas, texturas y calidades.

De ahí que la presente tesis haya seleccionado como principal alimento de estudio la patata frita, principalmente debido a tres características: su elevado consumo, bajo riesgo de contaminación microbiológica y elevado contenido en grasa (lo que influye en el proceso de oxidación lipídica).

I.4- Objetivos del trabajo

1- Optimización, validación y aplicación de métodos analíticos para la determinación de componentes nutritivos y organolépticos, relacionados con procesos de oxidación lipídica y de migración desde el material de envase.

2- Evaluación de la influencia de las condiciones de envasado/almacenamiento en la alteración o aparición de dichos compuestos.

La presente tesis dedica la mayoría de sus capítulos al estudio de la influencia de las condiciones del almacenamiento del producto envasado (ej.: tiempo, luz, atmósfera, temperatura) en componentes nutritivos susceptibles a la oxidación lipídica (ácidos grasos), así como al estudio del perfil aromático obtenido en distintas condiciones de almacenamiento y a la búsqueda de una herramienta analítica que permita la detección precoz y la evaluación de la extensión de los procesos de oxidación lipídica. El último capítulo se ha dedicado al desarrollo de un método analítico que permite evaluar los niveles de migración de un antibacteriano (triclosan) incluido en el plástico para envasar alimentos. Este trabajo forma parte del proyecto Foodmigrosure, el cual ha financiado la beca para la realización de la esta tesis.

Los resultados del presente estudio han sido objeto de una serie de artículos publicados en revistas internacionales y comunicaciones a congresos internacionales.

Artículos en revistas:

1- "Gas Chromatographic Determination of Glycerides in Potato Crisps Fried in Different Oils". Chromatographia, 58 (2003) 1-5.

2- "Comparison Between HPLC and GC Methods For Fatty Acids Identification and Quantification in Potato Crisps". Journal of Chromatography A, 1032 (2004) 7-15.

3- "Modified Atmosphere Packaging and Temperature Effect on Potato Crisps Oxidation During Storage". Analytica Chimica Acta, 524 (2004) 185-189.

4- "Study of the Light Effect on Fatty Acids of Potato Crisps Using a Gas Chromatographic Method". Analytica Chimica Acta, 524 (2004) 191-200.

5- "Profiling Flavor Compounds of Potato Crisps During Storage Using Solid Phase Microextraction". Journal of Chromatography A, *in press* (aceptado para publicación el 4 de mayo de 2004).

6- "Determination of Hexanal as Indicator of the Lipidic Oxidation State in Potato Crisps using Gas Chromatography and High Performance Liquid Chromatography". Journal of Chromatography A, 1046 (2004) 75-81.

7- "Determination of Triclosan in Foodstuffs", Journal of Separation Science *in press*.

Comunicaciones a congresos:

1- "Gas Chromatographic Determination of Glycerides in Potato Crisps Fried in Different Oils". International Symposium on Separation and Characterization of Natural and Synthetic Macromolecules, Amsterdam, Holanda, 5-7 Febrero, 2003.

2- "Comparison Between HPLC and GC Methods For Fatty Acids Identification and Quantification in Potato Crisps". 27th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Nice, Francia, 15-19 Junio 2003.

3- "Modified Atmosphere Packaging and Temperature Effect on Potato Crisps Oxidation During Storage". VII International Symposium on Analytical Methodology in the Environmental Field, XII Meeting of the Spanish Society of Analytical Chemistry (VIII ISAMEF/ SEQA 2003), A Coruña, España, 21-24 Octubre, 2003.

4- "Study of the Light Effect on Fatty Acids of Potato Crisps Using a Gas Chromatographic Method". VII International Symposium on Analytical Methodology in the Environmental Field, XII Meeting of the Spanish Society of Analytical Chemistry (VIII ISAMEF/ SEQA 2003), A Coruña, España, 21-24 Octubre, 2003.

5- "Determination of Hexanal as Indicator of the Lipidic Oxidation State in Potato Crisps using Gas Chromatography and High Performance Liquid Chromatography". VII International Symposium on Analytical Methodology in the Environmental Field, XII Meeting of the Spanish Society of Analytical Chemistry (VIII ISAMEF/ SEQA 2003), A Coruña, España, 21-24 Octubre, 2003.

6- "Identification of Substances Formed During Lipid Oxidation by GC-MS". Congresso Nacional dos Farmacêuticos 2003, Lisboa, Portugal, 27-29 Noviembre, 2003.

7- "Profiling Flavor Compounds of Potato Crisps During Storage Using Solid Phase Microextraction". Eighth International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Brugge, Belgica, 4-6 Febrero, 2004.

8- "Determination of Triclosan in Foodstuffs". Eighth International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Brugge, Belgica, 4-6 Febrero, 2004.

9- "Food Contact Plastic Materials: Development of Analytical Methods and Study of Migration Kinetics". Congreso Internacional de Seguridad Alimentaria, Reynosa, México, 13-15 Octubre, 2004.

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II- COMPARISON BETWEEN HPLC AND GC METHODS FOR FATTY ACIDS IDENTIFICATION AND QUANTIFICATION IN POTATO CRISPS

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Abstract

A reverse-phase high performance liquid chromatographic (RP-HLPC) method was compared with a gas-chromatography flame ionization detection (GC-FID) method for determining fatty acids in potato crisps.

Different extraction procedures were used. Fatty acids were quantified by linear regression. Both methods presented good precision (RSD ≤ 5.88 %) and recovery (≥ 82.31 %). The precision using HPLC method was slightly better than for GC-FID method.

There was good agreement between the fatty acid composition of potato crisps analyzed by both methods. For most purposes the HPLC method would be better. However, when more fatty acids need to be analyzed, GC is a more suitable method.

Keywords: HPLC, GC, Methods comparison, Fatty acids, Potato crisps.

II-1. Introduction

Snack food such as potato crisps are an important example of the remarkable industry and consumer demand for more stable food products with increased shelf life. This is because food products with high fat content are highly susceptible to oxidation [1].

A good starting point for evaluating this phenomenon is the analysis of the most critical factors involved in the process. Light is known to be an important factor in the oxidation mechanism as it is closely involved in the formation of free radicals [2]. This paper will evaluate the changes in fatty acid (FA) content after a three-month storage period in the presence of light.

A considerable amount of research has been devoted to the development of methods for fatty acids analysis. In the past few decades they have advanced considerably. Conventional methods (e.g. gravimetric, spectrophotometric, volumetric, colorimetric) are gradually falling out of use as efficient methods are being introduced.

The greater complexity of food samples has prompted newer technical procedures such as supercritical fluid chromatography (SFC), chiral chromatography, silver ion chromatography; stable carbon isotope ration analysis (SCIRA), nuclear resonance spectrometry (NMR), near-infrared spectroscopy (NIR), Fourier transform infrared spectroscopy (FT-IR), FT-Raman spectroscopy and capillary electrophoresis [3-4]. Nevertheless, GC and HPLC chromatographic procedures are still the most extensively used [5].

The major advantages of HPLC over GC are lower temperatures during analysis, which reduces the risk of isomerization of double bonds, and the possibility of collecting fractions for further investigations [3, 6].

HPLC analysis of fatty acids can be carried out with refractive index (RI), ultraviolet absorption (UV), fluorescence (F), electrochemical and evaporative light-scatterring (ELSD) detection.

RI detection is a universal technique, very suitable for quantitative analysis when thermostable cells and isocratic elution are used, once it is affected by temperature and flow programming. This detector is used in the IUPAC method, although its poor sensitivity and inadequacy for analysis of complex mixtures [4, 7-8].

ELSD is stable, sensitive, easy to use, does not present baseline drifts and is not affected by changes in mobile phase composition or temperature. However, it is not common equipment in a control lab [4, 7-8].

F or UV detectors, offer the advantage of sensitivity. They are able to analyze at nanogram levels. Due to the lack of a chromophore in FA molecules, chromatographic derivatization step is essential when F detector is employed. Nevertheless, with UV detector this step can be avoided as we describe in the HPLC method developed in this paper [4, 9].

Although the application of HPLC to fatty acid analysis has increased over the last decade, GC is still the most widely used technique [10]. This well established procedure coupled with flame ionization detection (GC-FID) is very efficient and rapid when complex mixtures with broad molecular ranges are analyzed [7].

The purpose of this paper was to compare a RP-HPLC-UV without derivatization step and a GC- FID method for fatty acids separation and quantification in potato crisps. Moreover, the proposed methodologies were discussed for routine use in terms of sample preparation, time analysis, drawbacks and validation parameters.

II-2. Experimental

II-2.1. Sampling

Potato crisps fried in olive oil, according to the package information, were bought in a supermarket.

GC and HPLC analysis were performed on the same day: immediately after being purchased (fresh potato crisps) and following a three-month storage period exposed to natural light (oxidized potato crisps).

Packages were sealed under a nitrogen atmosphere after each analysis to avoid the oxidative effect of air.

II-2.2. Reagents and Analytical Standards

All reagents were of analytical quality. Methanol, ethanol, HCl, n-hexane, acetonitrile and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Toluene and sodium sulfate were from Sigma Aldrich (Madrid, Spain). Ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, EEUU).

The analytical standards: oleic acid (18:1 n-9); linoleic acid (18:2 n-9, 12); linolenic acid (18:3 n-9, 12, 15) and their methyl esters were from Sigma Aldrich (Madrid, Spain). Stock solutions for HPLC analysis were prepared in methanol, while for GC analysis were diluted in hexane. All were stored at 4ºC and remained stable for at least two weeks.

Methanolic HCl was prepared by slow addition of HCl to methanol (5:95, v/v), with constant stirring. 0.5 N NaOH in ethanol/water (94:4, v/v) was obtained by dissolving the NaOH in water and then diluting with ethanol until the desired concentration was achieved.

II-2.3. Calibration curves

A standard mixture of the fatty acids was prepared in methanol for HPLC analysis and in hexane for GC analysis. The concentration of the standard solution for the HPLC method was: 5.7 (18:1 n-9), 3.2 (18:2 n-9,12), 0.116 (18:3 n-9, 12, 15) mg /100 ml while for the GC analysis (expressed as mg / 50 ml) was: 250.0; 150.0 and 10.0 for 18:1 n-9, 18:2 n-9,12 and 18:3 n-9, 12, 15, respectively. Both standard mixtures had the sample fatty acid profile.

Calibration curves were constructed using diluted solutions with a range concentration such that the concentration of fatty acids in the sample lay in the middle of the range. The curves consisted of a plot of peak area vs. concentration.

II-2.4. Sample Preparation

II-2.4.1. HPLC

Samples were prepared according to a modification of the method described by Li et al [6].

In a 15 ml glass tube, about 0.1 g of potato crisps was added to 1 ml 0.5N NaOH. The mixture was centrifuged (Eba 12 centrifuge, Hettich, Kirchlengern, Germany) at 5500 rpm for 5 min after vortex-mixing (Autovortex SA6, Stuart Scientific, Redhill, UK) and one night at room temperature. The supernatant was transferred to another tube and 1 ml 0.6 N HCl was added. After 1 min on vortex, the solution was allowed to settle for 30 min.

Afterwards, samples were evaporated to dryness under a N_2 stream. Residue was re-dissolved with 10 ml of methanol and 20 µl of the solution

were analyzed by HPLC. Samples were stored at 4ºC in the dark until analysed.

II-2.4.2. GC

The method was similar to the one reported previously by Sukhija and Palmquist [11]. It presented a single step that combines lipid extraction and transesterification. 2 ml of toluene and 3 ml of freshly prepared methanolic HCl was added to 0.1 g of sample.

After 2 h in a water bath (70ºC) under a nitrogen atmosphere, 5 ml of a 6% K_2CO_3 solution and 1 ml of toluene were added to the tubes and it content mixed in a vortex. Following a centrifugation at 1100 rpm (5 min), the organic phase was dried with sodium sulfate and filtered by a Millipore 0.45 μm.

A 1 μl aliquot was injected into the GC. Prior to injection, the samples were maintained at 4ºC in the dark. Both sample preparation procedures were performed in a light protected laboratory with the temperature maintained under control.

II-2.5. Apparatus and Chromatographic conditions

II- 2.5.1. HPLC-UV

The HPLC system (Hewlett-Packard, CA, USA) equipped with a HP 1100 quaternary pump, an HP 1100 degassing device, a 20 μl injection loop (Rheodyne, Cotati, CA), a column thermostatting system (Spectra-Physics 8792, San Jose, CA, USA), an UV HP1100 detector set at 195 nm and HP ChemStation® chromatography software.

Chromatographic separation was performed with a Tracer Extrasil ODS2 column (25 x 0.4 cm I.D., 5 μm particle size) at 60ºC. Mobile

phases were: A- acetonitrile/water (25:75, v/v); B- acetonitrile. 0.12% of acetic acid was added to both mobile phases. Mobile phases were filtered prior to use. Gradient program started with 50% of each mobile phase and changed linearly to 15%A/85% B within 6 min, then returned to initial proportion until 20 min. Flow rate was 1.2 ml/min until 6 min and then increased to 2.0 ml/min to allow a correct column cleaning. Fatty acid identification was carried out not only on basis of retention time but also with respect to mass spectra (acquired by LC-MS) and UV spectra (acquired by spectrophotometry).

II-2.5.2. LC-MS

A Spectra Physics series P200 liquid chromatograph equipped with a Rheodyne loop (50 µl) and a mass detector (Fisons VG Platform) (VG Biotech, Altrincham, UK) was used to identify fatty acids. Single-ion recording (SIR) was obtained every second with a scan delay time of 0.01s. The column and mobile phase was the same as in HPLC-UV analysis (flow rate 1ml/min). Detector operated under the following conditions: negative atmospheric pressure chemical ionization mode (APCI-); probe temperature 500ºC; source temperature 130ºC, cone voltage -30 V, electron multiplier voltage 700 V, drying gas nitrogen at 425 l/h, APCI sheet gas nitrogen at 175 l/h.

II-2.5.3. UV-visible spectrometry

A UV scan from 190 nm to 400 nm was performed with a Cary 3E UV-Visible double - beam spectrophotometer. The software Cary Win UV was used for data acquisition.

II-2.5.4. GC-FID

A Fisons 8000 series gas chromatograph comprised with a flame ionization detector (FID 80) and an AS 800 autosampler (Manchester, UK) was used.

The GC was fitted with a fused silica capillary column with polyethyleneglycol phase Supelcowax 10 (30 m x 0.32 mm I.D. x 0.25 μm film thickness) (Supelco, Madrid, Spain). A 50 cm desactivated fused silica column (0.32 mm I.D.) was used to protect the analytical column. Helium was used as carrier gas with a flow rate of 1.2 ml/min.

The injection port and detector temperatures were set at 240ºC and 260ºC respectively. The column temperature was programmed as follows: 1 min at 160ºC, ramp at 3.5ºC/min to 230ºC, isothermal at 230ºC for 14 min. Injection was performed in the split mode with a split ratio of 1:30.

The software Chrom-Card for windows® (version 1.18) was used for data processing.

II-2.5.5. Statistical analysis

Data were statistically analysed using the SPSS software package, (version 11.0). Differences between HPLC and GC and among potato crisps were determined using t-test for related and independent samples ($P < 0.05$).

II-3. Results and Discussion

II-3.1. Sample Preparation

The method developed to prepare samples for RP-HPLC analysis avoids derivatization, this way it is possible to use free fatty acids for

further analysis or purposes. The first step uses 0.5 N sodium hydroxide in a solution of ethanol/water (94:4, v/v) as saponification reagent. Ethanol dissolves the sodium salts of fatty acids. The second step, extraction by 0.6 N HCl, separates fatty acids from salts and other water-soluble impurities, avoiding HPLC column contamination. Finally, FA are transferred from their sodium salt to their free acid forms (dissolved in methanol) [6]. Czauderna et al [3] used a similar sample preparation procedure, which employed 2 M NaOH and 4 M HCl. However, this method requires subsequent derivatization with dibromacetophenone in the presence of triethylamine.

In the past, the preparation of food samples prior to GC-FID analysis consisted in two steps: lipid extraction and transesterification. Lipid extraction was usually carried out with mixtures of organic solvents like chloroform-methanol (Bligh and Dyer and Folch et al procedures) or automatically with Soxhlet or Goldfish apparatus [12]. These approaches are laborious, time consuming, expensive and require large sample amounts and large reagent volumes [13, 14]. In order of overcome these main drawbacks, new methods have been developed such as “in situ” procedures [15-17]. These consist of one single step for extraction and methylation (which reduces analysis time, cost and work) as described in the experimental section.

Methyl esters can be prepared with alkaline, acid or alkaline and acid catalysis as in the American Oil Chemists’ Society (AOCS) official method [4, 18]. Diazomethane can also be used for free fatty acid although its short shelf life and the handling care required are a major limitation [13].

Despite advantages of alkaline catalysts (e.g. short reaction time and room temperature) an acid catalyst has been selected (methanolic

HCl) since this reagent derivatizes free and linked acids with little risk of saponification [13].

Ulberth et al [17] studied the influence of water in the transesterification reaction and concluded that a moisture content of 40.7% did not interfere with the process. Thus, potato crisp samples do not represent a problem.

Toluene was used as solvent because of its effectiveness and toxicity when compared to other solvents (benzene, hexane, tetrahydrofuran and chloroform) [13].

The amount of sample used is a critical factor during the extraction [14]. If the sample exceeds the capacity of the solvent, false contents may be determined. Moreover, a sample too small in size may yield a considerable variation coefficient. In order to avoid these problems distinct quantities of sample where tested (0.1-0.5 g). 0.1 g was considered to be the most suitable amount to produce quantitative results.

II-3.2. Chromatographic analysis

During method development several chromatographic conditions were assayed in order to optimize the mobile phase, flow rate and column temperature.

Various mobile phases initial proportions (A = acetonitrile/water; B = acetonitrile) were tested: 100%A/0%B; 75%A/25%B; 50%A/50%B; 0%A/100%B. The best results were obtained starting the mobile phase gradient with 50% A/50% B. Acetonitrile was used instead of methanol because several authors have reported that acetonitrile reduces retention time of unsaturated components and seems to be more effective on account of its lower viscosity [19].

Glacial acetic acid was added to both mobiles phases to suppress the ionization of fatty acids [19]. Moreover, it is easily removed by evaporation when fatty acid fractions are further used. Carboxylic group absorbance of acetic acid does not affect the chromatogram [6].

Several flow rates were tried between 0.8 and 2 ml/min. Column was thermostatted in a range of temperature from 25ºC to 65ºC at 10ºC intervals. The optimal conditions for achieving a good chromatographic resolution were 1.2 ml/min and 60ºC.

As been reported by Li et al the selected wavelength for HPLC-UV was 195 nm [6]. The highest absorbance found when an UV scanning was carried out using a FA standard was 205 nm. Nevertheless, 195 nm was selected in order to minimize possible interferences.

In preliminary studies carried out in this lab, the GC method was optimized assaying several split ratios (1:30; 1:50 and 1:100), and the effect of a pre-column in the peaks resolution was also evaluated.

With respect to peaks identification, in GC method FAME (fatty acid methyl esters) were identified only by comparing of their retention time (t_r) with standards, whereas in HPLC method a confirmation by LC-MS (figure II-1) and UV spectrophotometry was necessary because different FA may be eluted simultaneously [6].

In order to achieve the best conditions in LC-MS, several assays were performed. SIR mode was selected due to a higher sensitivity in relation to full scan mode (figure II-1.A-C). Several probe temperatures (200, 350 and 500ºC) and cone voltages (10, 30 and 60V) were evaluated. Best response was achieved with 500ºC and 30V. Characteristic masses (m/z) were 281, 279 and 277 for oleic, linoleic and linolenic acids, respectively (figure II-1.D-F). They corresponded to the ionization form $[M-H]^-$ of each molecule. According to Baty et al [9], one of the difficulties of FA analysis by HPLC is the separation of FA with the same

effective carbon number (like palmitoleic, linoleic and myristic acids). However, this does not affect current analysis once the three FA analyzed belong to the same carbon series. Thus, peaks were fully resolved, sharp and without shoulders.

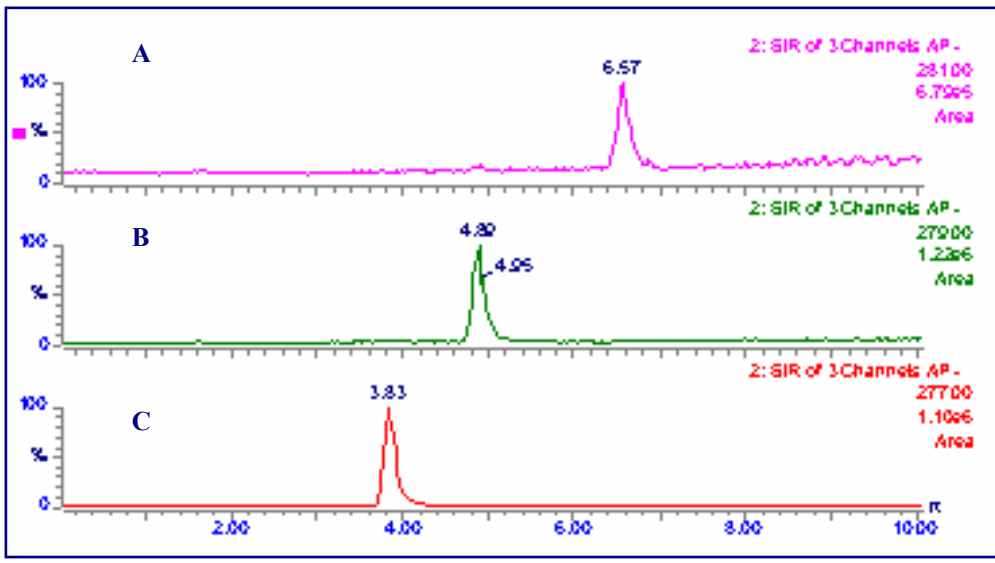
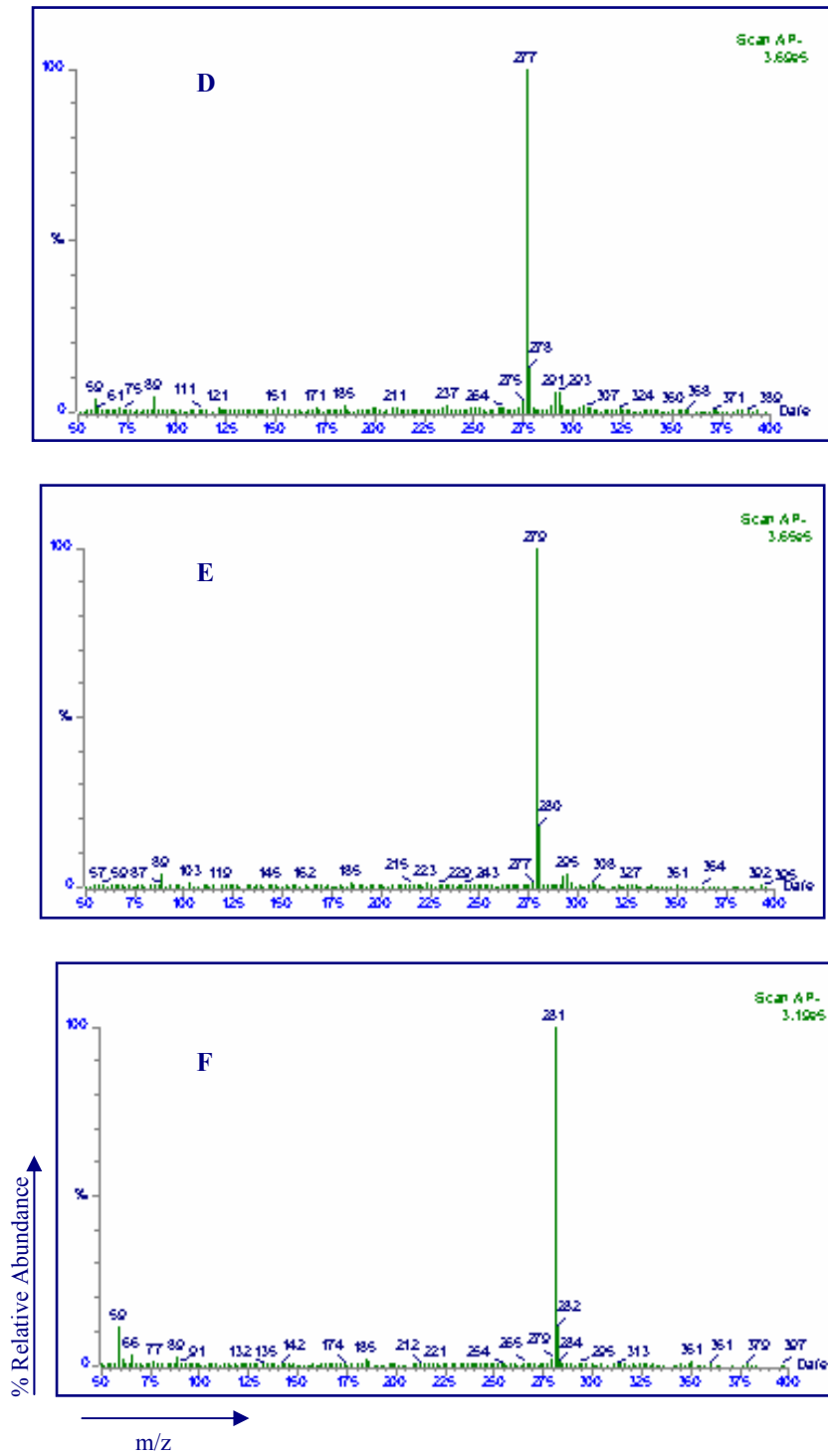


Figure II-1 - LC chromatogram with MS detection (SIR mode) for the m/z 281 (A), 279 (B) and 277 (C). Full scan mass spectra of linolenic (D), linoleic (E) and oleic (F) acids.

Figure II-1 (continuation)



Regarding elution order of FA, both methods are based on chain length and degree of unsaturation. FA retention times increase with carbon number for saturated and unsaturated FA. Within the same series, in HPLC, FA are eluted from the highest degree of unsaturation to the lowest (t_R18:1 n-9: 3.96 min; t_R18:2 n-9, 12: 2.87 min; t_R18:3 n-9, 12, 15: 2.25 min) and in GC, using a polar column like Supelcowax 10, FA are eluted in the opposite order (t_R18:1 n-9: 16.91 min; t_R18:2 n-9, 12: 17.82 min; t_R18:3 n-9, 12, 15: 19.08 min) (figure II-2 and II-3) [8, 20].

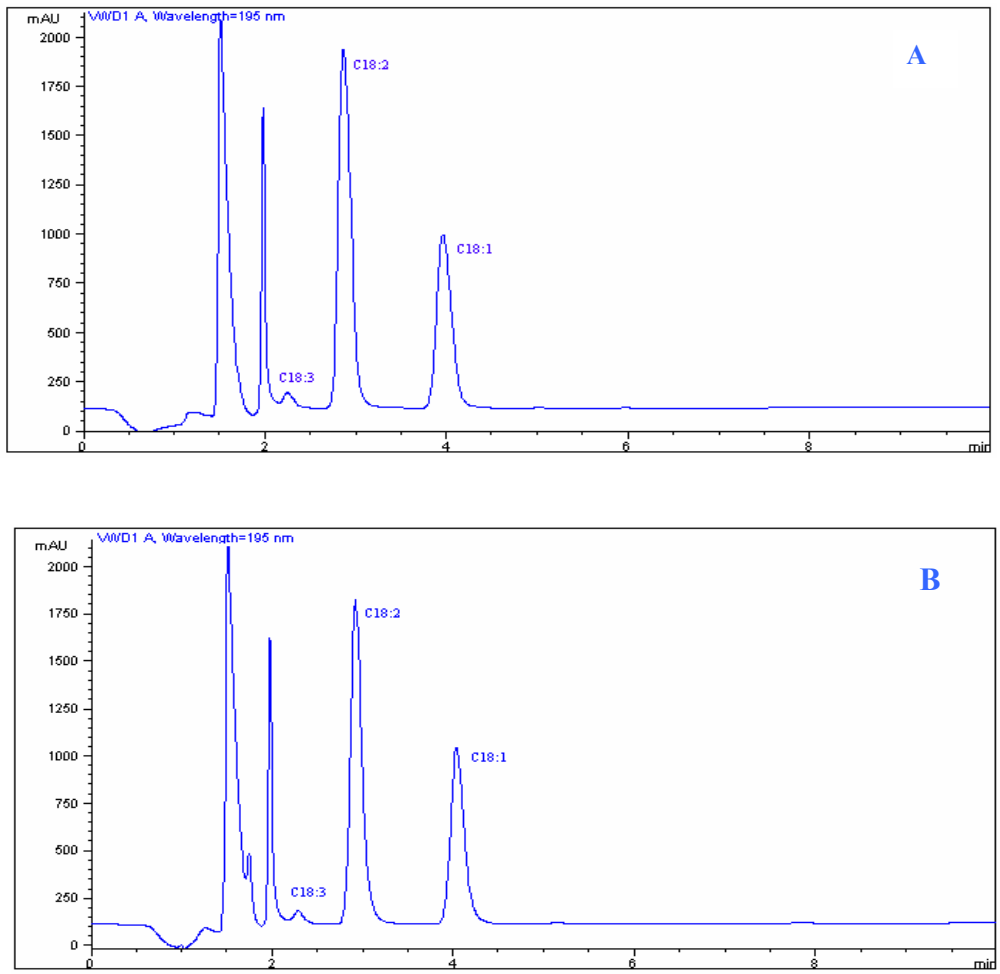


Figure II-2 - HPLC chromatograms of fatty acids: A- Fresh potato crisps; B- Oxidized potato crisps.

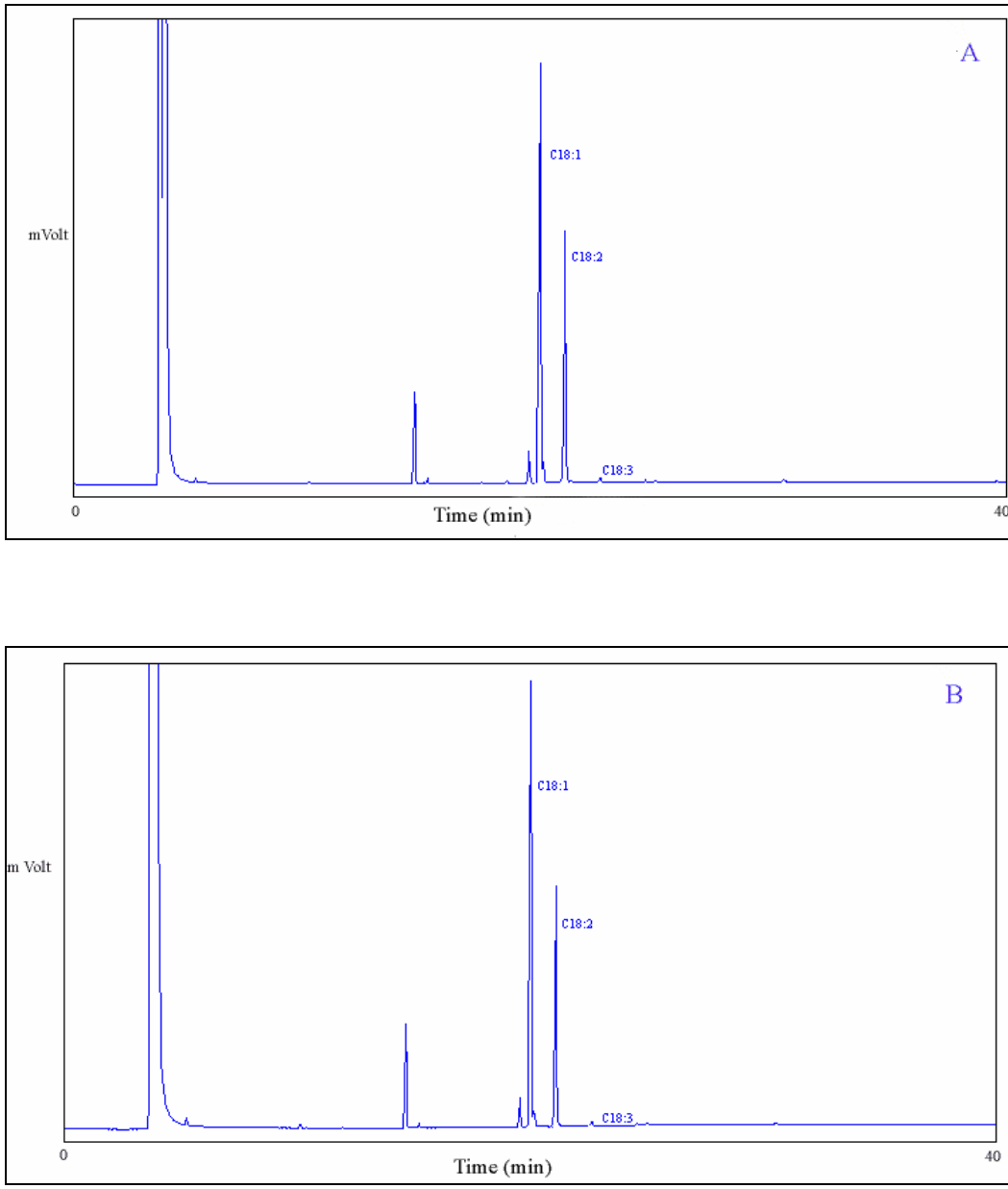


Figure II-3 - GC chromatograms of fatty acids: A- Fresh potato crisps; B- Oxidized potato crisps.

Regarding analysis time, sample preparation is much more time consuming in HPLC than in GC method. Nevertheless this disadvantage

was largely compensated by run time (20 min in HPLC compared with 35 min in GC), precision and sensitivity, as further described, for HPLC method.

II-3.3. Methods validation

Calibration curves were linear over the concentration range of 0.85-259.9 μg/ml using HPLC and 2.0-5000.0 μg/ml using GC (table II-1). The calibration data for both methods also presented high correlation coefficients (≥ 0.9980), indicating suitability for FA quantification.

Six determinations of the same sample were performed using the same reagents and apparatus to evaluate method precision on the basis of the relative standard deviation (RSD) of potato crisps.

Both methods presented good precision (table I). Method precision for HPLC (≤ 4.93%) was slightly better than for GC-FID (≤ 5.60%), except for 18:3 n-9, 12, 15.

The Guidelines of the American Chemical Society (ACS) [21] were used to determine detection limits (table I) (defined as the signal three times the height of the noise level). HPLC method (LOD ≤ 0.74 μg/ml) was more sensitive than GC method (LOD ≤ 5.00 μg/ml) for all compounds studied. Limit detection values using HPLC method are lower than those obtained by Li et al [6], whose work has used a similar extraction procedure. Regarding the method proposed by Czauderna et al [3], it presented better LOD than the HPLC method here proposed, however, on the other hand it requires a derivatization step and has a long analysis time. Recovery was tested for both methods using standard addition procedure.

Table II-1 - Comparison of methods validation parameters.

	18:1 n-9		*18:2 n-9, 12*		*18:3 n-9, 12, 15*	
	GC	**HPLC**	**GC**	**HPLC**	**GC**	**HPLC**
Precision (%)	5.60	4.93	5.16	3.87	5.58	5.88
Recovery (%)	82.31	108.92	83.80	97.09	95.30	101.30
LOD (µg/ml)	5.00	0.01	3.00	0.23	1.00	0.74
Linearity	y=1082x+40671	y= 14.375x+8.526	y= 1053.8x+23920	y= 39.767x+8.6848	y= 677.4x+381.82	y= 52.36x+36.958
$\mathbf{r^2}$	0.9980	0.9999	0.9980	0.9999	0.9999	0.9990
range (µg/ml)	50.0-5000.0	1.3-259.9	30.0-3000.0	1.17-235.0	2.0-200.0	0.85-29.6

Six samples of potato crisps were spiked before extraction (with the same amount as the expected in the sample). Mean recoveries, listed in table II-1, were always satisfactory and higher than 82.31%.

II-3.4. Correlation between results obtained using both methods

Two independent groups (fresh potato crisps and potato crisps stored for 3 months) were compared with respect to the fatty acids contents obtained by HPLC and GC methods (table II-2).

Table II-2 - Fatty acid content of potato crisps (g/100g ± SD; n = 4) determined by HPLC and GC methods.

Method	Potato crisps	18:1 n-9	18:2 n-9, 12	18:3 n-9, 12, 15
GC	**Fresh**	10.564 ± 0.640	5.478 ± 0.323	0.163 ± 0.007
	Oxidized	5.022 ± 0.803	2.698 ± 0.405	0.079 ± 0.010
HPLC	**Fresh**	10.080 ± 0.591	5.353 ± 0.194	0.148 ± 0.015
	Oxidized	5.457 ± 0.248	2.820 ±0.269	0.0783 ± 0.147

The comparison of these two methods depends not only on the sample preparation but also on chromatographic analysis. A factor calculated as MW_{FA}/MW_{FAME} was used to convert FAME in its FA. FA contents determined by HPLC showed a remarkably good correlation with the corresponding calculated values from GC method, since results were not significantly different ($P > 0.05$).

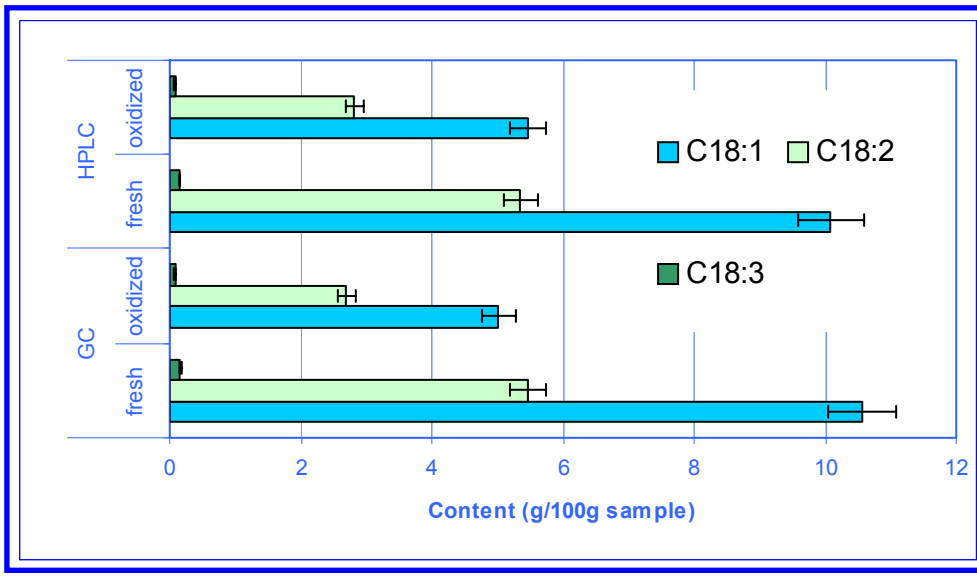


Figure II-4 - Comparison of FA content (g/100g) of fresh and oxidized potato crisps.

II-3.5. Fatty acids content of potato crisps

According to the fatty acids profile of potato crisps (figures 2 and 3), 18:1 n-9 was the predominating FA detected in the potato crisps fried in olive oil. This is in agreement with the results obtained by Pantzaris [22]. 18:1 n-9, 18:2 n-9, 12, 18:3 n-9, 12, 15 fatty acids were chosen due to their nutritional value [23] as well as for the particular interest they contribute to this work. Unsaturated fatty acids are more prone to attack by free radicals [24], thus their content allows (to some extent) the evaluation of the lipid oxidation state. In fact, the content of the three FA evaluated decreased significantly ($P < 0.05$) after a three months period storage in the presence of light (figure II-4). However, the most affected was linolenic acid because it has three double bonds.

The HPLC method is sensitive and precise and may be considered as a good alternative analytical tool for the routine determination of 18:1 n-9, 18:2 n-9, 12 and 18:3 n-9, 12, 15 in potato crisps. However, when a more complete study of FA profile is required, GC is a more suitable method, because HPLC may present peaks co-elution.

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III- GAS CHROMATOGRAPHIC DETERMINATION OF GLYCERIDES IN POTATO CRISPS FRIED IN DIFFERENT OILS

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Abstract

Glycerides in eight commercial potato crisps were identified after conversion to fatty acids. The profiles were analysed and compared, with regard to 14 fatty acids, by use of a gas chromatographic method. Results showed that the fatty acid profile is quite variable among brands and depends on the type of oil used in the frying process.

To provide a means of identifying the frying oil correctly, brands were classified into three groups depending on the main fatty acid present. After the first analysis samples were stored at room temperature in the dark and analysed again 1 and 3 months later**.**

Keywords: Gas chromatography; Lipid oxidation; Fatty acid profile; Potato crisps.

III-1. Introduction

Potato crisps are one of the most widely consumed snack foods [1]. Their high lipid content makes them very susceptible to oxidation, which results in loss of functional, sensory, and nutritive value, and affects the safety of the fried foods [2, 3]. By minimizing oxidation, therefore, shelf life can be improved [4, 5].

The industrial frying process occurs at high temperature for a short time and results in a crunchy golden product with a pleasant flavour which is improved by addition of a small amount of salt. The frying process has several disadvantages, however; it causes hydrolytic degradation (owing to water loss); oxidative degradation (owing to reaction of atmospheric oxygen with lipid molecules at the surface), and thermal degradation (because of the high temperature). This last problem is important because it is results in a decrease in nutritive value, because of the loss of polyunsaturated fatty acids (PUFA), which are essential in the human diet [6–9].

The most important factor affecting the oxidation of fried food is the type of oil used for frying, to be specific its fatty acid composition. Indeed, the fatty acid profile found in the frying oil is quite similar to that in the potato crisps, disregarding slight changes occurring during the frying process. By analysis of the fatty acid profile of potato crisps it is, therefore, possible to determine the type of oil used for frying.

Fatty acid analysis has evolved remarkably in the last few decades. Many methods have been developed, for example gravimetric [10–12], IR spectrophotometric [13], volumetric [14], and colorimetric [15] methods. Nowadays, however, chromatographic methods are most commonly used [16] - HPLC [17, 18] and, especially, GC with flame ionization detection (FID) [19–24]. Several methods for preparation of samples for GC–FID

analysis are reported in the literature. Classical methods based on lipid extraction with chloroform–methanol, in Soxhlet or Goldfisch apparatus, or saponification with KOH followed by transesterification to form FAME, and subsequent GC, are not easy tasks. These approaches are time-consuming, laborious, cumbersome, expensive, require handling of highly toxic solvents (chloroform), and are susceptible to errors resulting from incomplete removal of solvents and peroxidation of polyunsaturated FA during extraction and/or ester preparation [23, 25, 26]. The need for a rapid, economic, and easy method that could at least partly overcome these deficiencies has prompted the development of new techniques. The most outstanding of these involve use of microwaves and supercritical fluids (SF) to perform lipid extraction, and 'in situ' procedures.

This report describes continued efforts to obtain more information about the composition and stability of fried food [27–39]. The purpose of the work was to apply an 'in situ' procedure (combining extraction and methylation in one step) for determination of glycerides in potato crisps, by gas chromatography, after conversion into fatty acids. The kind of oil used is identified on the basis of the main fatty acid and other characteristic fatty acids present. Storage stability was evaluated under normal storage conditions and after three months.

III-2. Experimental

III-2.1. Reagents and Lipid Standards

Myristic acid methyl ester ($C_{14:0}$), myristoleic acid methyl ester ($C_{14:1}$), palmitic acid methyl ester ($C_{16:0}$), palmitoleic acid methyl ester ($C_{16:1}$), heptadecanoic acid methyl ester ($C_{17:0}$), heptadecenoic acid methyl ester ($C_{17:1}$), stearic acid methyl ester ($C_{18:0}$), oleic acid methyl ester (C

$_{18:1}$), linoleic acid methyl ester ($C_{18:2}$), linolenic acid methyl ester ($C_{18:3}$), arachidic acid methyl ester ($C_{20:0}$), eicosenoic acid methyl ester ($C_{20:1}$), behenic acid methyl ester ($C_{22:0}$), and lignoceric acid methyl ester ($C_{24:0}$) were from Sigma (Madrid, Spain). Methanol, HCl, and n-hexane were from Merck (Germany). Toluene was from Sigma Aldrich (EEUU). All reagents were analytical grade.

III-2.2. Sampling

Potato crisps were purchased from a local supermarket. After immediate analysis they were stored in the dark, at room temperature (20 ± 2ºC), and analysed again 1 and 3 months later (before expiry of the date by which they should have been consumed). Each sample was analysed by duplicate by GC. After each analysis the potato crisp package was sealed under a nitrogen atmosphere to minimize the effect of oxygen on the oxidative process.

III-2.3. One-Step Methylation–Extraction Method

Each sample (approx. 0.5 g) was weighed in 15-ml Pyrex tubes with PTFE-lined screw caps and toluene (2 ml) and 5% methanolic HCl (freshly prepared, 3 ml) were added. After filling the vial headspace with nitrogen and carefully mixing the contents the tubes were placed in a water bath (70ºC) for 2 h. K_2CO_3 solution (6%, 5 ml) and toluene (1 ml) were added after cooling the tubes. The tubes were vortex-mixed and immediately centrifuged at 1100 rpm for 5 min (Hettich) to separate the phases. The organic phase was dried with a small quantity of Na_2SO_4, filtered through a 0.45-µm Millipore filter, and 1 µL was injected into the GC–FID.

III-2.4. Gas Chromatographic Analysis

Gas chromatography was performed with a Fisons (Manchester, UK) 8000 series instrument equipped with a flame ionisation detector (FID; 260ºC), an AS 800 auto sampler and a split–splitless injector. Fatty acid methyl esters (FAME) were separated on a 30 m, 0.32 mm i.d., 0.25 µm film thickness Supelcowax 10 column from Supelco (Madrid, Spain). Helium was used as carrier gas; the flow rate was 1.2 ml min^{-1}. The injector temperature was 240 ºC and the split ratio was 1:30 (this was selected after investigation of 1:50 and 1:100 split ratios). The column temperature was held at 160 ºC for 1 min during injection then increased at 3.5 ºC min^{-1} to 230 ºC which was held for 14 min. Because systematic use of this column resulted, after analysis of approximately 60 samples, in loss of resolution (calculated for C18:0 and C18:1), a 50-cm precolumn of 0.32 mm i.d. deactivated fused silica was used between the injector and the analytical column, with the aim of improving column life. Use of the precolumn also resulted in increased resolution.

The software used to process peak areas was Chrom-Card for Windows (version 1.18) and FAME from samples were identified by comparison of their retention times (t_R) with those of standards. Statistical analysis of results was performed by use of SPSS for Windows (version 11.0).

III-3. Results and Discussion

III-3.1. Sample Preparation

The ‘in situ’ procedure described in this paper saves time, effort, and cost. It requires minimal amounts of sample and reduces the use of

reagents and solvents, and waste disposal. This is an important advance in fatty acids analysis [23, 24, 40].

Two major concerns in ‘in situ reaction’ or ‘one-step extraction–methylation’ are dissolution of the lipids to achieve rapid and complete reaction. Methanol is a good choice because a one-phase system is formed and prevents interference from water, in the presence of which long-chain saturated fatty acids tend to precipitate, and react much more slowly. (Potato crisps usually contain 1.0–1.5% water; this amount does not affect the process) [41].

Methods for derivatization of fatty acids, include esterification (conversion of free fatty acids to esters) and transesterification (conversion of acylglycerols to fatty acid esters). Transesterification is usually used because most fatty acids in biological samples are present as triacylglycerols and phospholipids. Although many transesterification reagents are available, none is ideal. In this work a method of transesterification that used an acid-catalysed reagent was used. Although this procedure requires heating and takes longer than basic catalysis, it derivatizes all fatty acids (free or linked) and is less affected by water, reducing the risk of saponification of the esters. Because of literature reports of favourable results from use of methanolic HCl, and of the properties of this reagent [25], compared with other acid catalysis reagents, e.g. methanolic H_2SO_4, methanolic HCl was selected in our experiment. This method has already been applied to green beans [42], fish [26], and processed food [25]. Results revealed that it was quantitatively equivalent to the traditional approach, with the advantage of being much easier.

Toluene is recommended as solvent by most researchers and has been chosen on the basis of better results than are obtained with pentane,

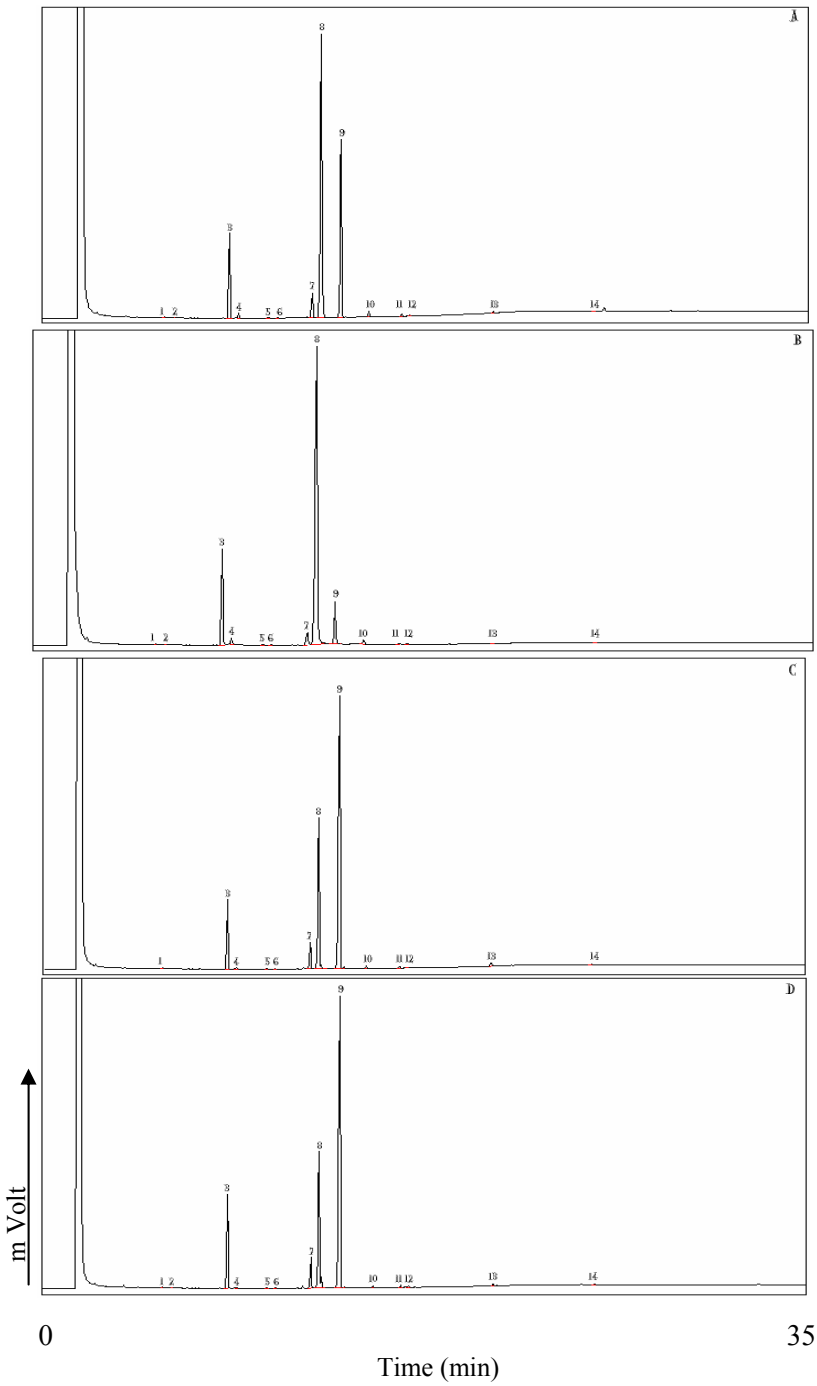
hexane, isooctane, or chloroform when extracting FAME; although its extraction power is the same as benzene, it is less toxic [23, 26].

III-3.2. The Effect of the Fat Used in the Frying Process

Figure III-1 shows chromatograms obtained from each of the eight brands analysed. Table III-1 shows the relative amounts (% area) of the 14 FAME identified and Figure III-2 depicts the % area of polyunsaturated, monounsaturated, and saturated fatty acids. There were significant differences ($P < 0.05$) among the FAME profiles of the different brands of potato crisp studied, except between brands 4 and 5. These results clearly indicate that non-brand data can be regarded as representative of potato crisps in general. Common FAME profiles were usually obtained from potato crisps fried with the same oil [43].

With the aim of enabling easier identification of the oil used to fry the crisps they were classified into three groups according to the major fatty acid present (Table III-2). The first group (comprising brands 1, 2, and 8) has C18:1 as the major fatty acid. Of the fatty acids present in brands 1 and 2 more than 50% was C18:1 (54 and 71%, respectively) whereas brand 8 contained 37% C18:1. The FAME profiles from brands 1 and 2 were identical with that of olive oil [44, 45], which is in accordance with information on the package.

Figure III-1



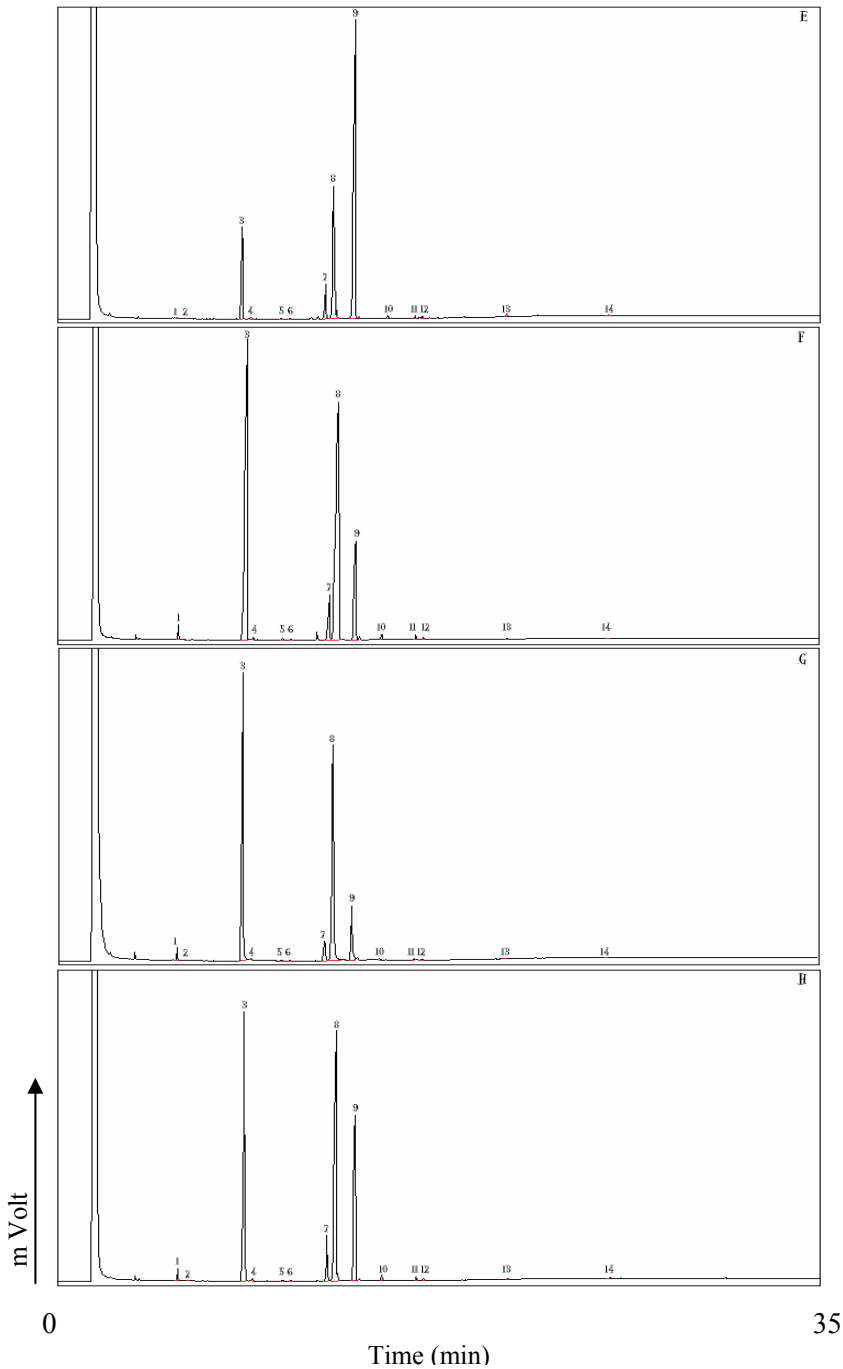


Figure III-1- Chromatograms of different potato crisps brands (A-H), this order corresponds to brands numbers from 1 to 8 of others figures. 1- $C_{14:0}$; 2- $C_{14:1}$; 3- $C_{16:0}$; 4- $C_{16:1}$; 5-$C_{17:0}$; 6- $C_{17:1}$; 7-$C_{18:0}$; 8- $C_{18:1}$; 9- $C_{18:2}$; 10- $C_{18:3}$; 11- $C_{20:0}$; 12- $C_{20:1}$; 13- $C_{22:0}$; 14- $C_{24:0}$.

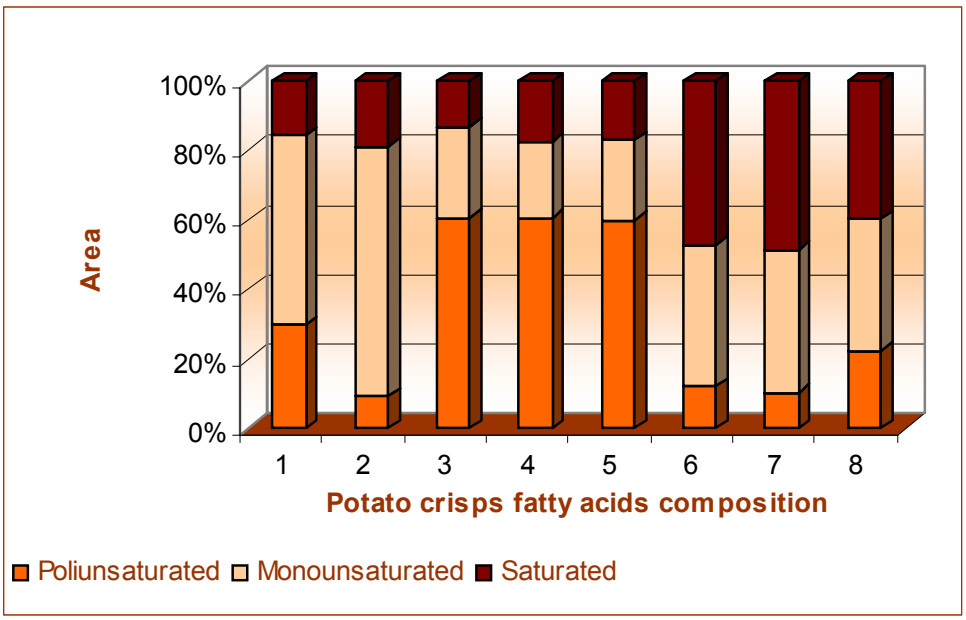


Figure III-2 - Potato crisps composition on fatty acids of different degrees of saturation.

These two brands also contain levels of $C_{16:1}$ (0.525 and 0.886%) several times higher than those of other brands. $C_{16:1}$ can therefore be used as an indicator of the use of olive oil in the frying process. Brand 8 is a new kind of potato crisp made from potato paste. Its composition is quite different from normal – it contained not only potato, fat, and salt but also cornflour, starch, maltodextrin, dextrose, and emulsifier. This brand has a shelf life of 1 year, whereas those of the others never exceed 6 months, because of the fatty acid profile obtained as a result of use of a blend of vegetable oils. Pangloli [46] reported that potato crisps fried in a 40:60 blend of palm olein oil and sunflower oil were more stable to oxidation during storage than crisps fried in sunflower oil, without sacrificing potato crisp flavour. This could also reduce the cost of the product, because palm olein oil is cheaper.

Table III-1 - Analysis of 14 fatty acids in commercial potato crisps.

	brand 1		brand 2		brand 3		brand 4		brand 5		brand 6		brand 7		brand 8	
	mean[a]	SD[b]	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
$C_{14:0}$	0.074	0.016	0.073	0.009	0.109	0.006	0.083	0.003	0.081	0.005	1.031	0.048	1.271	0.030	0.839	0.023
$C_{14:1}$	0.002	0.001	0.001	0.001	nd[c]		0.001	0.000	0.001	0.000	nd		0.003	0.003	0.001	0.000
$C_{16:0}$	11.243	0.377	15.330	0.075	7.773	0.118	12.369	0.247	12.132	0.410	41.378	0.362	42.973	0.209	33.898	0.360
$C_{16:1}$	**0.525**	0.018	**0.886**	0.020	0.129	0.005	0.086	0.007	0.090	0.008	0.156	0.002	0.127	0.033	0.153	0.012
$C_{17:0}$	0.065	0.006	0.077	0.004	0.049	0.002	0.101	0.002	0.099	0.003	0.095	0.000	0.094	0.003	0.089	0.000
$C_{17:1}$	0.068	0.003	0.104	0.004	0.025	0.001	0.053	0.002	0.050	0.005	0.026	0.000	0.021	0.003	0.029	0.002
$C_{18:0}$	3.485	0.052	3.070	0.129	4.115	0.028	4.074	0.052	4.061	0.062	4.233	0.043	3.947	0.200	4.377	0.036
$C_{18:1}$	53.733	0.403	70.734	0.535	25.926	0.382	21.828	0.045	22.672	0.179	40.473	0.267	40.720	0.382	37.640	0.179
$C_{18:2}$	29.037	0.182	8.366	0.203	60.289	0.388	53.014	0.076	52.769	0.177	11.554	0.070	10.160	0.380	21.801	0.338
$C_{18:3}$	0.826	0.089	0.805	0.079	0.409	0.075	**7.465**	0.093	**7.067**	0.117	0.476	0.006	0.317	0.128	0.547	0.008
$C_{20:0}$	0.352	0.038	0.312	0.055	0.286	0.003	0.342	0.023	0.345	0.015	0.341	0.018	0.237	0.011	0.350	0.017
$C_{20:1}$	0.173	0.011	0.168	0.032	0.109	0.002	0.149	0.003	0.153	0.006	0.115	0.004	0.067	0.017	0.133	0.007
$C_{22:0}$	0.287	0.031	0.043	0.025	0.567	0.016	0.311	0.025	0.340	0.044	0.054	0.000	0.037	0.008	0.067	0.002
$C_{24:0}$	0.129	0.018	0.032	0.004	0.213	0.015	0.124	0.011	0.140	0.047	0.067	0.004	0.027	0.026	0.077	0.008

[a] area %; n=4
[b] standard deviation
[c] not detected

Table III-2 - Classification of potato crisps brands in 3 groups according the major fatty acid present.

Group	*Brand*	*Major fatty acid*
1	1, 2, 8	$C_{18:1}$
2	3, 4, 5	$C_{18:2}$
3	6, 7	$C_{16:0}$

Brands 3, 4, and 5 formed a second group; in these the major fatty acid was $C_{18:2}$ and the frying medium could have been sunflower oil or soybean oil [44, 45]. By determination of the amount of $C_{18:3}$ it was possible to determine which of these two oils was used for each brand. In brands 4 and 5 the amount of $C_{18:3}$ was approximately 7% whereas in brand 3 it was lower than 1%; it is therefore possible to conclude that the frying medium used for brands 4 and 5 (with small differences between them) was soybean oil whereas that used for brand 3 was sunflower oil.

Finally, group 3 comprised brands 6 and 7 in which the major fatty acid was $C_{16:0}$, a saturated fatty acid associated with the health hazard of cardiovascular disease. The FAME profile is indicative of palm oil as frying medium [44, 45].

The lack of information on the crisp packages is quite amazing. In general, only for potato crisps fried in olive oil is detailed information given about the kind of oil used; for others it is limited to ‘‘vegetable oil’’ or ‘‘mixture of fats’’. According with many authors, potato crisps fried in oils rich in unsaturated fatty acids have a better impact on human health by helping to reduce blood cholesterol and LDL levels and contributing to the development of biological membranes, although a diet rich in

polyunsaturated fatty acids can also reduce HDL, which protect against cardiovascular disease and recent studies have revealed that monounsaturated fats do not reduce HDL but increase them and also increase apolipoprotein A-I, which has an important antiatherogenic role. A diet containing monounsaturated fats can therefore be recommended for prevention of cardiovascular problems [45, 47].

The outcome of this work is, therefore, that brands 1 and 2, fried in olive oil, have the best effect on human health, because of the high $C_{18:1}$ content. Olive oil also contains natural polyphenols which can reduce lipid oxidation rate and improve product shelf life, so addition of antioxidants, for example rosemary extract, sage extract, citric acid or ascorbic acid, to the frying oil can be avoided [2, 48, 49]. Despite the advantages of olive oil, consumption of large amounts of this type of product is not recommended because of the high fat content.

III-3.3. Effect of Storage on the FAME Profile of Potato Crisps

Potato crisp containers were stored at room temperature, in darkness, to evaluate profile changes during 3 months. Over this period $C_{16:0}$; $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ content did not change (Figure 3), except for minor fluctuations. C18:3, with three double bonds, is, of the fatty acids studied in this work, the most susceptible to oxidation. Because it is the first to change, $C_{18:3}$ can be used as an indicator of the early stage of oxidation.

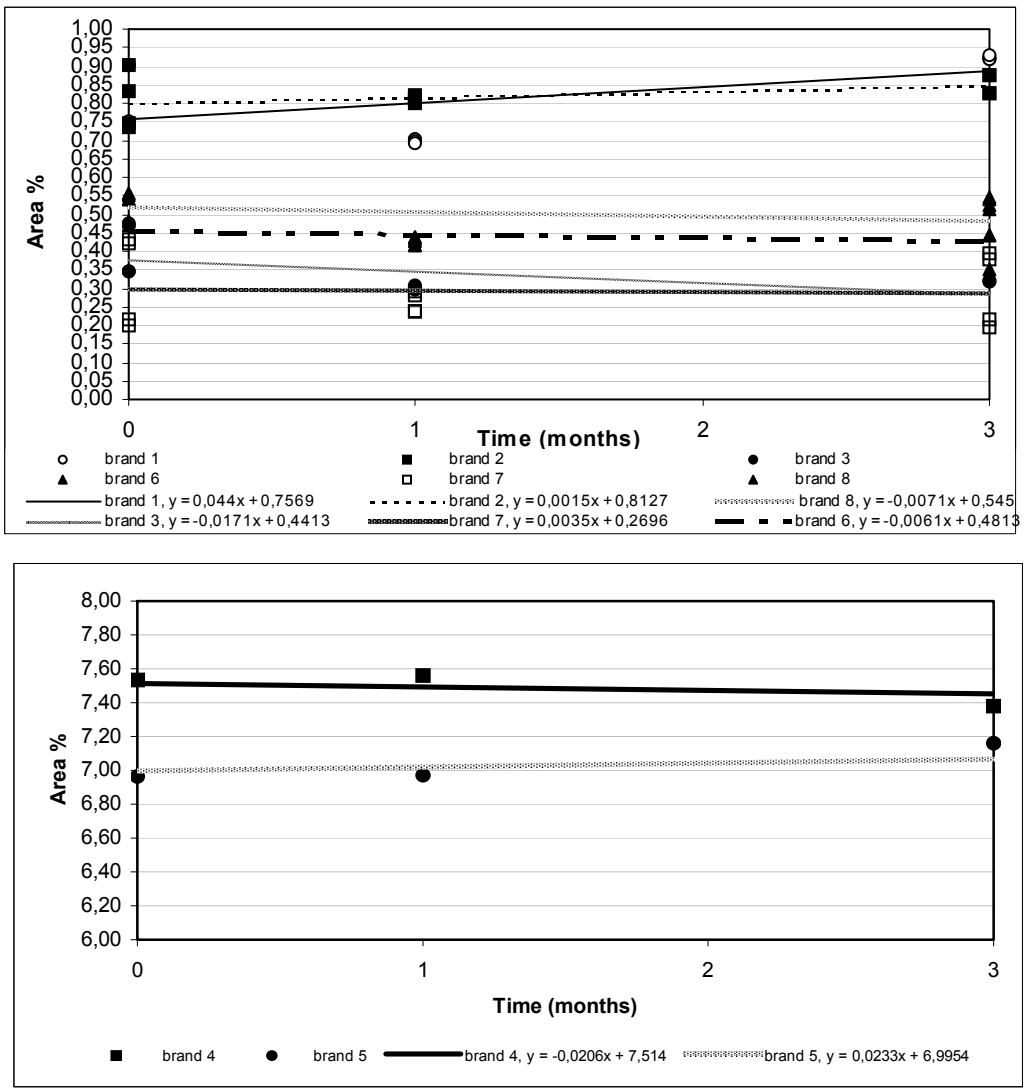


Figure III-3- Fatty acids changes during 3 months storage on normal conditions: A-Brands 1-3, 6-8; B- Brands 4, 5.

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IV- MODIFIED ATMOSPHERE PACKAGING AND TEMPERATURE EFFECT ON POTATO CRISPS OXIDATION DURING STORAGE

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Abstract

Evaluation of lipid oxidation in potato crisps has been studied extensively. Most times, the study was done on potato crisps recently prepared by deep-fat frying, comparing different frying oils. In the present work, an extraction method that involves extraction and derivatization of free fatty acids to fatty acids methyl esters (FAMEs) in a single step followed by gas chromatography is used. In one hand, four potato crisps brands, fried in different oils, were stored at room temperature and at 40ºC, in the dark, to evaluate the effect of temperature on the fatty acids. On the other hand, a single kind of potato crisps (fried in olive oil), was stored at room temperature, in darkness, under different atmospheres: air, vacuum, oxygen scavenger, and nitrogen. The most effective conditions in order to avoid or at least delay lipid oxidation were storage at room temperature with an oxygen scavenger.

Keywords: One-step extraction, Modified atmosphere packaging, Temperature, Lipid oxidation, Potato crisps.

IV-1. Introduction

In the past few years the consumer demand for food products with high quality has increased. In this context, food packaging has an outstanding importance once protects the food from microbial and chemical contamination, as well as from oxygen, water vapour and light [1]. The atmosphere surrounding the food constantly change as volatile compounds and moisture are formed during metabolic processes. In line with these facts, modified atmosphere packaging (MAP), defined by Ahvenainen et al [2] as a method where the normal gas atmosphere is changed by a package headspace as close as possible to optimal conditions, have been a challenge for scientific research due to contribute to an extend of shelf life of packed products. Moreover, the barrier properties of the polymeric material will further influence the movement of gases and moisture into and out the package. Despite the advantages of MAP, like improvement of product presentation and reduction of the need of food additives, this method has several drawbacks, such as problems with leak detection and environmental impact [2]. MAP can be a passive (before sealing) or an active operation (during storage), using a gas absorber or emitter. The latter is a recent technological innovation which involves the use of chemical scavengers (e.g. ethylene, oxygen, water) or which can release a specific gas (carbon dioxide, or microbial inhibitors such as ethanol or sulphur dioxide) [3].

Passive MAP technique has already been applied to many food products like: fruits (e.g. apples; peaches; cherries) [4-6]; vegetables (e.g. lettuce, carrots; vegetables for soup; potatoes) [7-10]; meat (e.g. cooked pork; ground beef; turkey breast fillets; cooked pork sausage) [11-14]; fish (e.g. salmon) [15]; cheese (e.g. Mozzarella) [16-17]; hazelnuts [18]; shrimp [19]; olive [20]. However, in the majority of the papers results

have been evaluated in a microbiological and not in a nutritional perspective.

The major purpose of this paper is the evaluation of the influence of storage temperature and headspace package atmosphere, on the induction of lipid oxidation in packed potato crisps. The aim of studying the influence of the temperature is to observe if high temperature influences the fatty acid profile. With respect to the atmosphere package conditions, the main reason for using a modified atmosphere inside the package container is to achieve the atmosphere medium that can better avoid or delay lipid oxidation during storage.

IV-2. Experimental

IV-2.1. Sampling

Potato crisps were purchased from a local supermarket. In order to evaluate the effect of temperature on FAMEs profile, four potato crisps brands were stored at room temperature and at 40º C, in the dark, after an analysis conducted on day 1 (day they were bought).

Brands selected pretend to represent different kinds of potato crisps once they had different characteristics: according the package information: brand 1 (fried in olive oil); brand 2 (fried in vegetable oil); brand 3 (with 30% less of fat); brand 4 (made from potato paste).

To evaluate the effect of modified atmosphere package (MAP), a single kind of potato crisps (brand 1) was stored at room temperature, in darkness, under different atmospheres: intact (market sample) atmosphere, air, vacuum, oxygen scavenger (Ageless; Mitsubishi gas chemical Co. Japan) and nitrogen. A semiautomatic packaging machine (Audiovac VM

101 HG, Rovebloc, Weesp, The Netherlands) was used to flush potato crisps packages with nitrogen, air and vacuum.

Analyses were carried out on the same day they were bought (immediately analysis) and 1 and 3 months later (before validity expiration) to evaluate the effect of the two storage conditions. Each sample was extracted twice and each extraction analyzed by duplicated in the GC. Barrier containers of potato crisps stored under different temperatures were, after each analysis, sealed under a nitrogen atmosphere to minimize the effect of oxygen in the oxidation process.

IV-2.2. Reagents

Lipid standards (fatty acid methyl esters) (CAS number in brackets) were obtained from Sigma (Madrid, Spain): myristic acid methyl ester [124-10-7], myristoleic acid methyl ester [56219-06-8], palmitic acid methyl ester [112-39-0], palmitoleic acid methyl ester [1120-25-8], heptadecanoic acid methyl ester [1731-92-6], heptadecenoic acid methyl ester [75190-82-8], stearic acid methyl ester [112-61-8], oleic acid methyl ester [112-62-9], linoleic acid methyl ester [112-63-9], linolenic acid methyl ester [301-00-8], arachidic acid methyl ester [1120-28-1], eicosenoic acid methyl ester [17735-94-3], behenic acid methyl ester [929-77-1], lignoceric acid methyl ester [2442-49-1].

Individual standard solutions were prepared in hexane. A global standard solution containing all the methyl esters was prepared with an aliquot of each individual standard solution and subsequent dilution with hexane. This solution had a similar fatty acid profile when compared with samples. All solutions were stored at 4ºC.

All reagents and solvents were analytical grade. Toluene was purchased from Sigma Aldrich (EEUU) while methanol, hydrochloric acid (HCl) and n-hexane were from Merck (Darmsstadt, Germany).

IV-2.3. Extraction Procedure

Extraction was carried out according to Sukhija and Palmquist [21]. In brief, about 0.5 g of each sample was weighted in 15 ml Pyrex tubes with a PTFE-lined screw cap. Then, 2 ml of toluene and 3 ml of 5% methanolic HCl (freshly prepared) were added. After filling the vial headspace with nitrogen and carefully mix the content, the tubes were placed in a water bath (70ºC) during 2 h. Afterwards, 5 ml of a 6% K_2CO_3 solution and 1 ml of toluene were added to the cool tubes. As soon as tubes were mixed by vortex they were submitted to a centrifugation at 1100 rpm for 5 min (Hettich). As result, phases were separated. Thus, organic phase was dried, with a small quantity of Na_2SO_4, and filtered through by a Millipore 0.45 μm. Finally, 1 μL was injected in a gas chromatograph.

IV-2.4. Gas Chromatographic analysis

Gas chromatography was performed with a Fisons – 8000 series (Manchester, UK) instrument equipped with a flame ionization detector (FID; 260ºC), an auto sampler (AS 800) and a split-splitless injector.

Fatty acid methyl esters (FAMEs) were separated using a 30 m x 0.32 mm i. d. x 0.25 μm film thickness Supelcowax 10 column from Supelco (Madrid, Spain).

Helium was used as carrier gas flowing at 1.2 ml/min. The injector used was at 240ºC and the split-ration selected was 1:30. The column temperature was held at 160ºC for 1 min during injection, and then

increased at 3.5ºC/min to 230ºC which was held for 14 min. In order to improve column life as well as peaks resolution, a pre-column (deactivated fused silica 0.32 mm i.d.) was used between injection point and the analytical column.

The software used to process peak areas was Chrom-Card for Windows (version 1.18) and FAMEs from samples were identified by comparison of their retention time (tr) with standards.

IV-2.5. Statistics

Data were subjected to statistical analysis using the software package, SPPS (version 11.0). Analysis of variance (ANOVA) and a t-test were used to determine differences among potato crisps brands and storage times with raw data.

IV-3. Results and Discussion

IV-3.1. The effect of atmosphere package on lipid oxidation

Study of potato crisps fatty acids (FA) profile during a 3 months storage indicated that oxidative changes occurred and the extension of those changes was affected by the within-package environment.

Potato crisps stored with an air atmosphere presented, after 3 months, the lowest level of polyunsaturated FA while those stored with an oxygen absorbent the highest level ($P< 0.05$) (figure IV-1-C). Potato crisps stored under nitrogen and vacuum atmospheres presented intermediate values which not differ significantly between them ($P>0.05$) (figure IV-1-C). Air is known to be a pro-oxidant agent, consequently the results achieved with this atmosphere were expected [23]. Previous studies concluded that a high oxygen MAP was correlated with increased

thiobarbituric acid (TBA) number during storage and consequently with lipid oxidation and formation of off-flavours [11-12].

In spite of the lack of information of the atmosphere used inside the potato crisps package, many packages, bought on the same day as the others, was maintained closed and stored on the same conditions. The aim was to try to identify which was the original atmosphere inside the package. After 1-month of storage the intact package (market sample) was analyzed and results revealed that the FA profile was similar ($P>0.05$) to that obtain using an air atmosphere, which supports the idea that the package was flushed with air.

According to Subramaniam [23], snacks, in general, present three main requirements when stored: protection against moisture (usually they have between 1 and 1.5% of moisture when packed, but if these levels increase to 4-5%, the product becomes unacceptable), light and oxygen [23]. In this work potato crisps were stored in opaque containers in order to minimize the influence of this factor. Barrier properties of the polymeric film constitute an important parameter to take in account when MAP is employed. Several works have studied the effect of the type of the packaged film on food stability [24-28] that may be taken into account once influence the within-package atmosphere. In the present study barrier properties of the film used were unknown. However, once all potato crisps were packed with the same film, the study of the influence of the atmosphere within was feasible.

Regarding the FA profile during storage (figure IV-1) the negative influence of oxygen became evident, mainly in the polyunsaturated FA profile, due to present a greater number of double bonds, which makes them more susceptible to lipid oxidation [29]. Table IV-1 shows the profile of 14 fatty acids in potato crisps stored under five atmospheres.

Therefore, the use of a modified atmosphere seemed to be a good alternative in order to improve product shelf life.

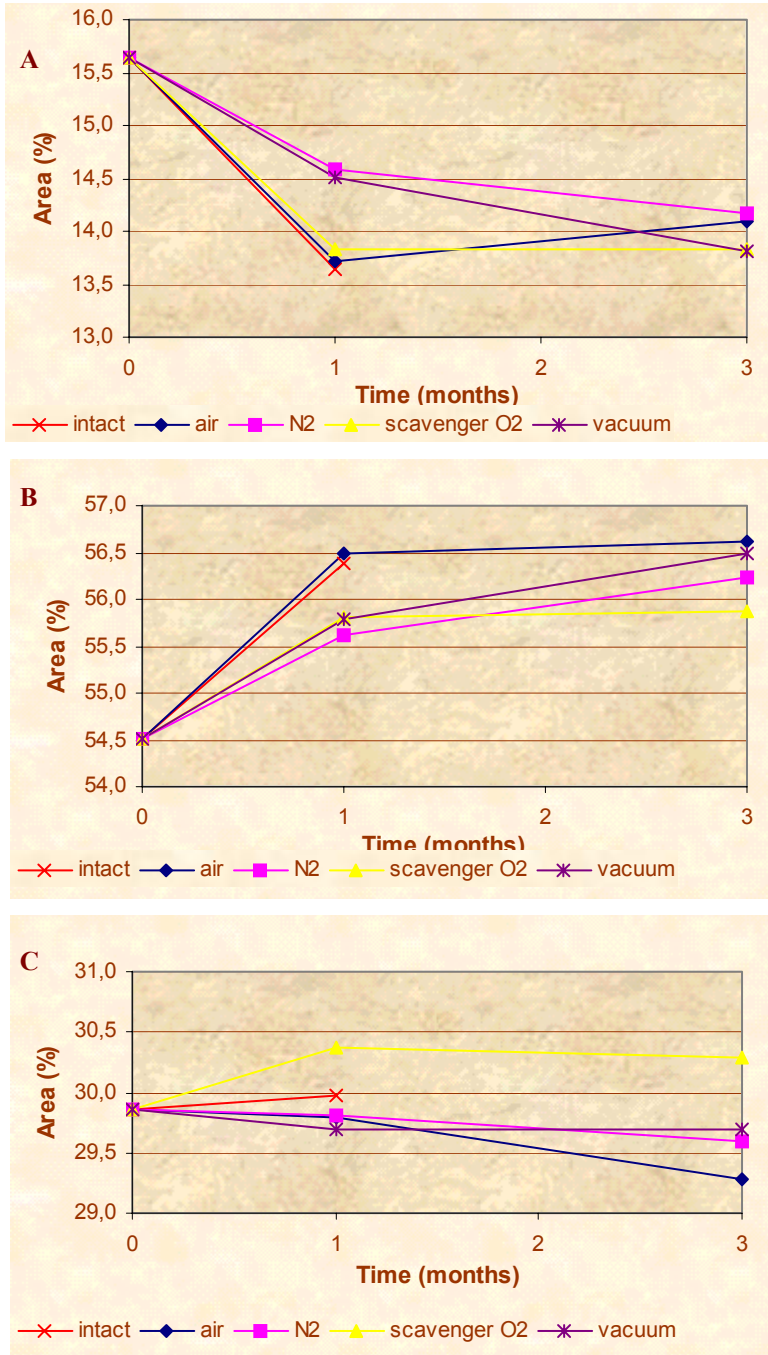


Figure IV-1- Fatty acids profile of potato crisps stored under different atmospheres: **A-** Saturated FA; **B-** Monounsaturated FA; **C-** Polyunsaturated FA.

A nitrogen atmosphere inside the package decreased FA changes, and in consequence, oxidation. This was more relevant in the first month as can be observed in figure IV-1-B. Nevertheless some authors consider that this atmosphere can just be applicable to high quality potato crisps due to the gas price and the high air volume of the package [23].

A vacuum 'atmosphere' also provides good results when FA profile is evaluated, however it presents an important disadvantage: product deformation [2].

An alternative and innovative packaging method is the active package with oxygen absorbents, which decreases the residual oxygen concentration to insignificant levels (<0.1% is easily achieved) [2, 30, 31].

Nowadays there are many companies producing oxygen absorbents. The system used in this study consists in a sachet, which contains a finely divided powdered iron that under appropriate humidity conditions uses oxygen to form non-toxic iron oxidant [26].

Oxygen scavengers present several disadvantages such as high cost or the possibility of sachets consumption (ingestion). Nevertheless potato crisps stored with oxygen absorbers presented minor changes after 3 months compared with the other three atmospheres. This way, the oxygen scavenger was the most effective solution (between those studied in this work) to obtain an within package atmosphere for potato crisps that not only decreases the addiction of chemical preservatives but also hold up the oxidative process, increasing, in this way, the product shelf life.

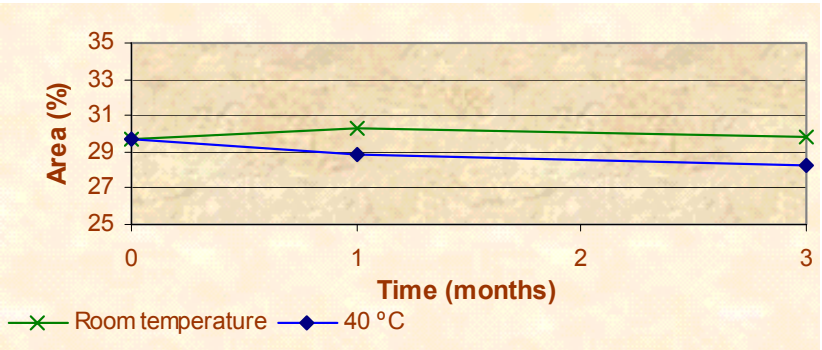
More research is needed to determine the best MAP to store snack food like potato crisps and when should the crisps should be discarded.

IV-3.2. The effect of storage temperature

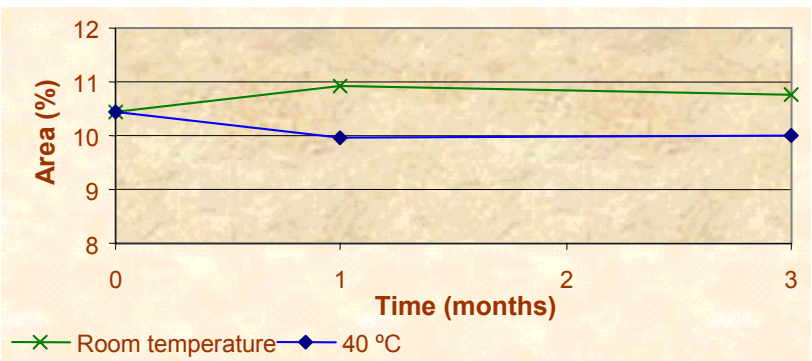
Figure IV-2 represents the polyunsaturated fatty acid profile during a 3-month storage for the four types of potato crisps and figure IV-3 shows the GC-FID chromatograms. 40ºC temperature induce lipid oxidation yielding a decrease of polyunsaturated fatty acids. These changes are generally significant for all types of potato crisps except for potato crisps with lower fat content (30% less).

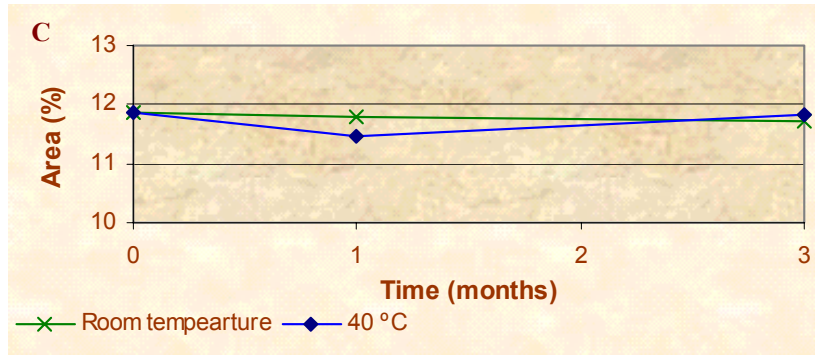
Figure IV-2

A



B





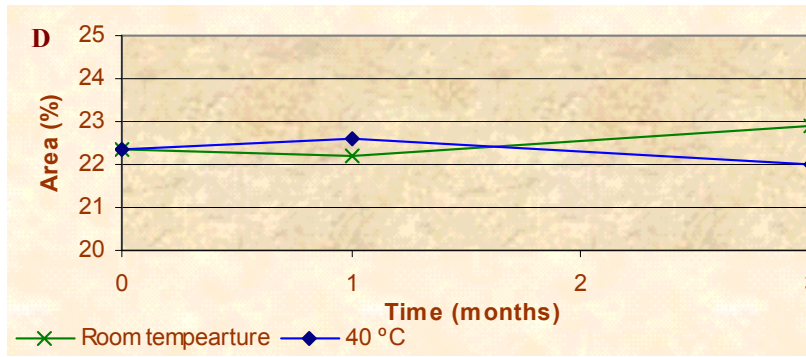


Figure IV-2- Polyunsaturated changes during a 3-month storage at room temperature and 40ºC. **A-** Potato crisps fried with olive oil; **B-** Potato crisps fried with vegetable oil; **C-** Potato crisps with less 30% of fat; **D-** potato crisps made from potato paste.

In brief, temperature is a parameter that affects oxidation, so should be controlled in order to avoid or at least delay this destructive chemical process.

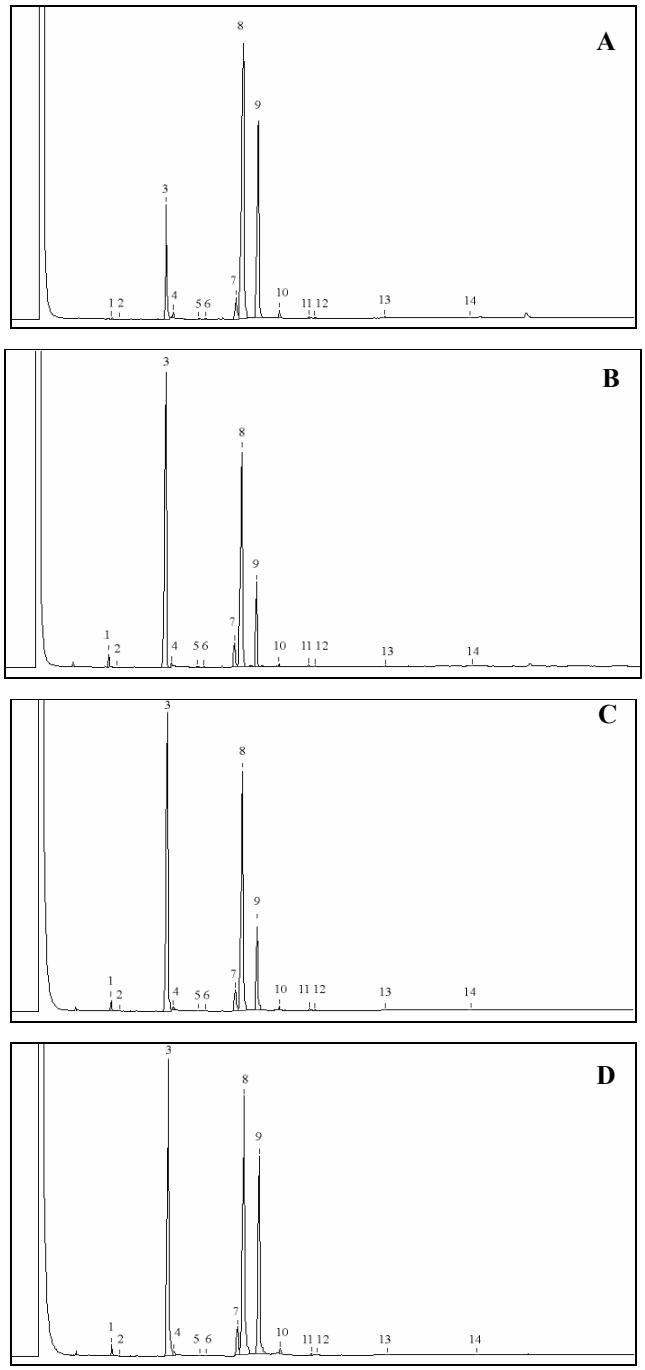


Figure IV-3 - GC-FID chromatograms of potato crisps: **A-** Potato crisps fried with olive oil; **B-** Potato crisps fried with vegetable oil; **C-** Potato crisps with less 30% of fat; **D-** Potato crisps made from potato paste.

Table IV-1: Analysis of 14 fatty acids in potato crisps stored under five atmospheres.

Time	Month 0		Month 3									
Atmosphere			Market sample		Air		N2		Scavenger O2		Vacuum	
FA	Mean[a]	SD[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$C_{14:0}$	0,07	0,02	0,05	0,00	0,04	0,00	0,05	0,00	0,05	0,00	0,05	0,00
$C_{14:1}$	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
$C_{16:0}$	11,24	0,38	10,53	0,04	10,34	0,18	10,46	0,00	10,20	0,12	10,00	0,06
$C_{16:1}$	0,52	0,02	0,45	0,01	0,44	0,00	0,45	0,00	0,44	0,01	0,44	0,00
$C_{17:0}$	0,07	0,01	0,05	0,01	0,06	0,01	0,06	0,00	0,05	0,00	0,06	0,01
$C_{17:1}$	0,07	0,00	0,06	0,00	0,06	0,00	0,06	0,00	0,06	0,00	0,06	0,00
$C_{18:0}$	3,49	0,05	3,27	0,03	3,22	0,03	3,21	0,00	3,14	0,01	3,25	0,01
$C_{18:1}$	53,73	0,40	55,17	0,57	56,01	0,01	55,61	0,27	55,27	0,30	55,88	0,18
$C_{18:2}$	29,04	0,18	29,36	0,46	28,89	0,23	29,14	0,21	29,73	0,33	29,26	0,10
$C_{18:3}$	0,83	0,09	0,54	0,11	0,40	0,01	0,45	0,06	0,56	0,09	0,43	0,00
$C_{20:0}$	0,35	0,04	0,22	0,00	0,21	0,00	0,20	0,00	0,19	0,00	0,16	0,01
$C_{20:1}$	0,17	0,01	0,11	0,00	0,11	0,00	0,11	0,00	0,10	0,00	0,11	0,00
$C_{22:0}$	0,29	0,03	0,10	0,00	0,14	0,00	0,13	0,00	0,14	0,01	0,22	0,00
$C_{24:0}$	0,13	0,02	0,08	0,02	0,09	0,00	0,08	0,00	0,06	0,02	0,08	0,00

[a] area %; n=4

[b] standard deviation

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V- STUDY OF THE LIGHT EFFECT ON FATTY ACIDS OF POTATO CRISPS USING A GAS CHROMATOGRAPHIC METHOD

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Abstract

A storage study was carried out to evaluate the behavior of fatty acids of potato crisps. Samples were stored in the darkness and under natural light.

A one step extraction-methylation method followed by gas chromatographic determination was used to analyze and compare profiles (% area) and contents (g/100g sample). Quantification was carried out using nonadecanoic acid as internal standard. The profile of potato crisps stored in the darkness has not shown considerable variation, while the one of crisps exposed to natural light has decreased significantly. Regarding FAMEs content, there was a decrease under both conditions, which was more evident in crisps stored under light. In spite of greatly contribute for the development of lipid oxidation, light should be avoided during potato crisps storage.

Keywords: Fatty acids, Gas chromatography, Light, Potato Crisps.

V-1. Introduction

Lipids are one of the most important constituents of food, besides proteins and carbohydrates [1]. In human body, they are lipossoluble vitamins transporters, essential fatty acid sources and the main energetic container, while in food they are responsible for texture and flavour [2].

Recently they have been a centre of attention from scientific research once their main degradative reaction (lipid oxidation) products were related with the aging process and pathologies like arteriosclerosis [3-4].

The oxidation of fats in foodstuffs leads to an abnormal taste and smell, once the compounds formed have a low odour threshold. Besides changes on sensorial qualities, also decrease nutritive value, aside from acceptability and safety. Indeed, when the process arise an advanced stage, it may appear toxic compounds able to put consumers' life in risk [5-9].

Lipid oxidation mechanism involves a chain of reactions where free-radicals attack adjacent positions of double bonds. Light is one of the factors that can lead to free-radicals formation together with oxygen or high temperatures. Hydroperoxides yielded are tasteless, colorless and odorless and will further originate a wide range of breakdown products. This way, evaluating parameters that influence lipid oxidation, will help to understand better and control this chemical process in food.

Food composition has great importance on lipid oxidation. Food with high content of unsaturated fatty acids is the most susceptible to lipid oxidation. Thus, fatty acids can greatly influence on food stability [10].

The evaluation of the state of lipid oxidation can be achieved determining a large number of substances, like peroxides and hydroperoxides [11]. Most of the methods are simple, rapid and based on titrations (for determination of the peroxide value or the iodine value, for example) or

spectrophotometric measurements (such as anisidine value or thiobarbituric acid test) [12-13]. Many papers focused on lipid oxidation were just based on some of these methods, according to the Americans Oil Chemists' Society [14-17].

Profiling fatty acids is another way of evaluating the lipid oxidation state. Several methods are available for fatty acid analysis (volumetric, gravimetric, colorimetric or spectrophotometric) [18-21]. However, chromatographic methods are the most widely used, mainly gas chromatography [22-24].

In the present study an one-step methylation extraction procedure followed by a separation, detection and quantification of fatty acid methyl esters with two gas chromatographic techniques (one coupled with a flame ionization detector and the other with a mass selective detector) is applied to potato crisps. The purpose is to monitor fatty acids changes and the formation of new compounds resulting from lipid oxidation during the storage in the darkness and under natural light. Potato crisps were selected due to their high fat content and minor risk of presenting microbiological contamination.

V-2. Experimental

V-2.1. Sampling

Potato crisps were purchased in a local supermarket. With the aim of studying the effect of light, just bought packages were analyzed and then stored in two different conditions until follow analyses (1 and 3 months later, before the end of their commercialization period). One group was stored in the dark, at room temperature, simulating normal storage conditions. The other group was exposed to natural light during storage.

Crisps were packed with a transparent film and, according to package information, were fried in olive oil. Immediately after each analysis potato packages were filled with a nitrogen atmosphere and sealed.

V-2.2. Reagents and analytical standards

The fatty acid methyl esters studied were (CAS number in brackets): myristic acid methyl ester [124-10-7], myristoleic acid methyl ester [56219-06-8], palmitic acid methyl ester [112-39-0], palmitoleic acid methyl ester [1120-25-8], heptadecanoic acid methyl ester [1731-92-6], heptadecenoic acid methyl ester [75190-82-8], stearic acid methyl ester [112-61-8], oleic acid methyl ester [112-62-9], linoleic acid methyl ester [112-63-9], linolenic acid methyl ester [301-00-8], arachidic acid methyl ester [1120-28-1], eicosenoic acid methyl ester [17735-94-3], behenic acid methyl ester [929-77-1], lignoceric acid methyl ester [2442-49-1]. Nonadecanoic acid methyl ester [1731-94-8] was used as internal standard. They were all supplied by Sigma (Madrid, Spain).

Individual standard solutions were prepared in hexane. A global standard solution containing all the methyl esters was prepared with an aliquot of each individual standard solution and subsequent dilution with hexane. This solution had a similar fatty acid profile when compared with samples. All solutions were stored at 4ºC.

All reagents were analytical grade. Methanol, HCl and n-hexane were from Merck (Darmsstadt, Germany) while toluene was supplied by Sigma Aldrich (Madrid, Spain).

V-2.3. Extraction Procedure

Samples were obtained according a method essentially similar to the one described by Sukhija and Palmquist [25]. About 20 g crisps were crushed in a blender homogeniser, approximately 0.5 g of sample was weighted in tubes with a PTFE- lined screw cap. Then, 2 ml of toluene and 3 ml of 5% of methanolic HCL were added. The tubes were closed with a nitrogen atmosphere and placed in a water bath (70ºC, 2h). Afterwards, 5 ml of a 6% K_2CO_3 solution and 1 ml of toluene were added. The tubes were vortex mixed and centrifuged (1100 rpm during 5 min). As result, phases were separated. Thus, organic phase was dried, with a small amount of Na_2SO_4 and then filtered by a Millipore 0.45 μm. Finally, 1 μl was injected in the gas chromatograph.

V-2.4. Gas Chromatographic Analysis

V-2.4.1. GC-FID

A Fisons 8000 series gas chromatograph (Manchester, UK) coupled with a flame ionization detector, an autosampler (AS 800) and a split/splitless injector was used to perform all GC analyses.

Fatty acid methyl esters were separated in a Supelcowax 10 column (30 m x 0.32 mm i. d. x 0.25 μm film thickness) from Supelco (Madrid, Spain). A desactivated fused silica pre-column (50 cm x 0.32 mm i. d.) was used between the injector and the analytical column to increase peaks resolution and to improve column life. Helium was used as carrier gas at a flow rate of 1.2 ml/min. The injector temperature was 240ºC and the split ratio was 1:30. The oven temperature was initially 160ºC for 1 min, ramped at 3.5ºC/min up to 230ºC and then held at 230ºC for 5 min. The detector temperature was 260ºC.

Peaks were processed using the software Chrom-card for Windows (version 1.18). The identification was performed by comparison of their retention times (tr) with those of standards.

V-2.4.2. GC-MS

A Fisons GC 8000 series Gas Chromatograph (Manchester, UK) equipped with a programmed split/splitless injector and a mass-selective detector MD800 was used to identify new peaks.

A cross-link 5 % phenyl methyl siloxane (30 m x 0.25 mm i.d. x 1µm film thickness) DB-5 column (DB J&W Scientific, CA, USA) was employed. Helium was used as carrier gas at a head pressure of 70 Kpa. The oven temperature, injector and detector temperatures were the same as for GC-FID.

The mass spectra were obtained using a mass selective detector (MD 800, Manchester, UK) by electronic impact at 70 eV and collecting data at a rate of scan 0.45 s^{-1} over the m/z range of 35-300. The software used to process peak areas was Masslab (version 1.4). The confirmation was performed by comparison of the mass spectra with that in Wiley Spectrometry Library and with the retention time of a standard injected in same conditions.

V-2.5. Statistical analysis

Results are expressed as means ± S.D. of n experiments. One-way analysis of variance (ANOVA) and t-test (SPSS version 11.5) were used to indicate significant differences in the levels of fatty acids methyl esters (FAME) among potato crisps. The P-value was considered to be statistically significant when $P< 0.05$.

V-3. Results and Discussion

V-3.1. Sample Preparation

The sample preparation method applied is an "in situ" procedure which consists in extraction and simultaneous methylation reaction. It is fast, simple, convenient and low cost. This way, it overcomes the major drawbacks of methods which consist of lipid extraction followed by transesterification [26-28].

The catalytic medium chosen for the methylation reaction was acidic, although basic catalysts (such as sodium methoxide) require a shorter reaction time and lower work temperature. The major advantage of the use of an acid catalyst is the derivatization of all fatty acids (free and linked). Many solvents, like hexane, tetrahydrofuran, benzene, toluene or chloroform have been used in "in situ" acid based methods. Favorable resulted were reported for benzene and toluene [29]. In this paper toluene was chosen due to it lower toxicity. The most frequently used reagents in acid catalysis are HCl, H_2SO_4 and BF_3 in methanol. Regarding BF_3 although having high esterifying power, presents several drawbacks (it is expensive, toxic, unstable, prone to form artifacts and greatly affected by water). HCl is less affected by water than H_2SO_4. Nevertheless the later was chosen due to it longer stability and easy and safe preparation. Moreover, the water content of crisps did not influence the transesterification reaction [29]. According to preliminary assays, temperature (70ºC) and time (2h) conditions selected were convenient in order to obtain quantitative results.

V-3.2. Fatty acids methyl esters profile/content

During a 3-month storage period (fig. V-1) the fatty acid profile has not changed ($P>0.05$) in potato crisps stored in the dark, while under light storage conditions has changed significantly ($P< 0.05$).

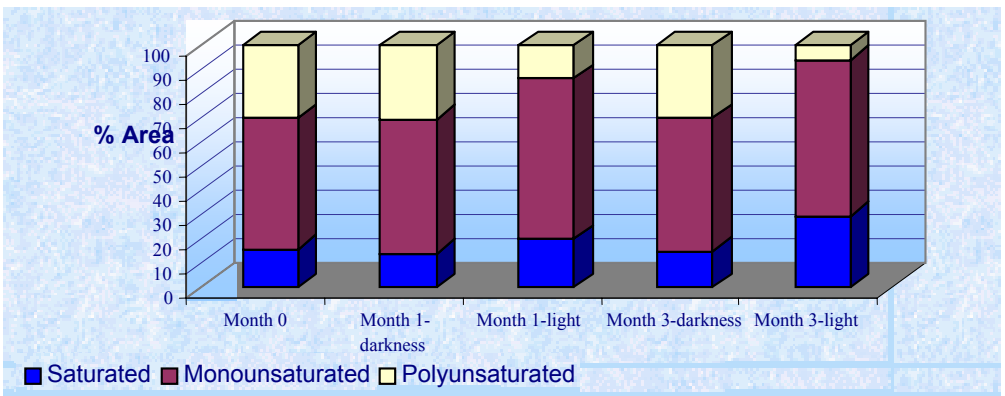


Figure V-1- Fatty acid profile of potato crisps at two different storage conditions: lightness and darkness.

The gas chromatographic separation of the FAMEs in potato crisps is presented in figure V-2. Good resolution was achieved, peaks were sharp and without shoulders. Oleic acid was the major fatty acid present (>50%) and $C_{16:1}$ level was higher than 0.5%. This profile was identical to the one found in olive oil, which confirms the package information (see Experimental).

As expected, polyunsaturated FAMEs (fig. V-3) were those which showed the main changes. The decrease of polyunsaturated fraction was mainly due to the oxidation into primary and secondary oxidation products. During a three-month storage they decreased from 29.86% to 5.83%. This decrease was more evident during the first month ($y= 16.161\ x + 29.863$; $a_l=$

-16.161) than during the second and third months (y= -3.935 x + 17.637; a_2= -3.935; a_2<a_1).

Figure V-2

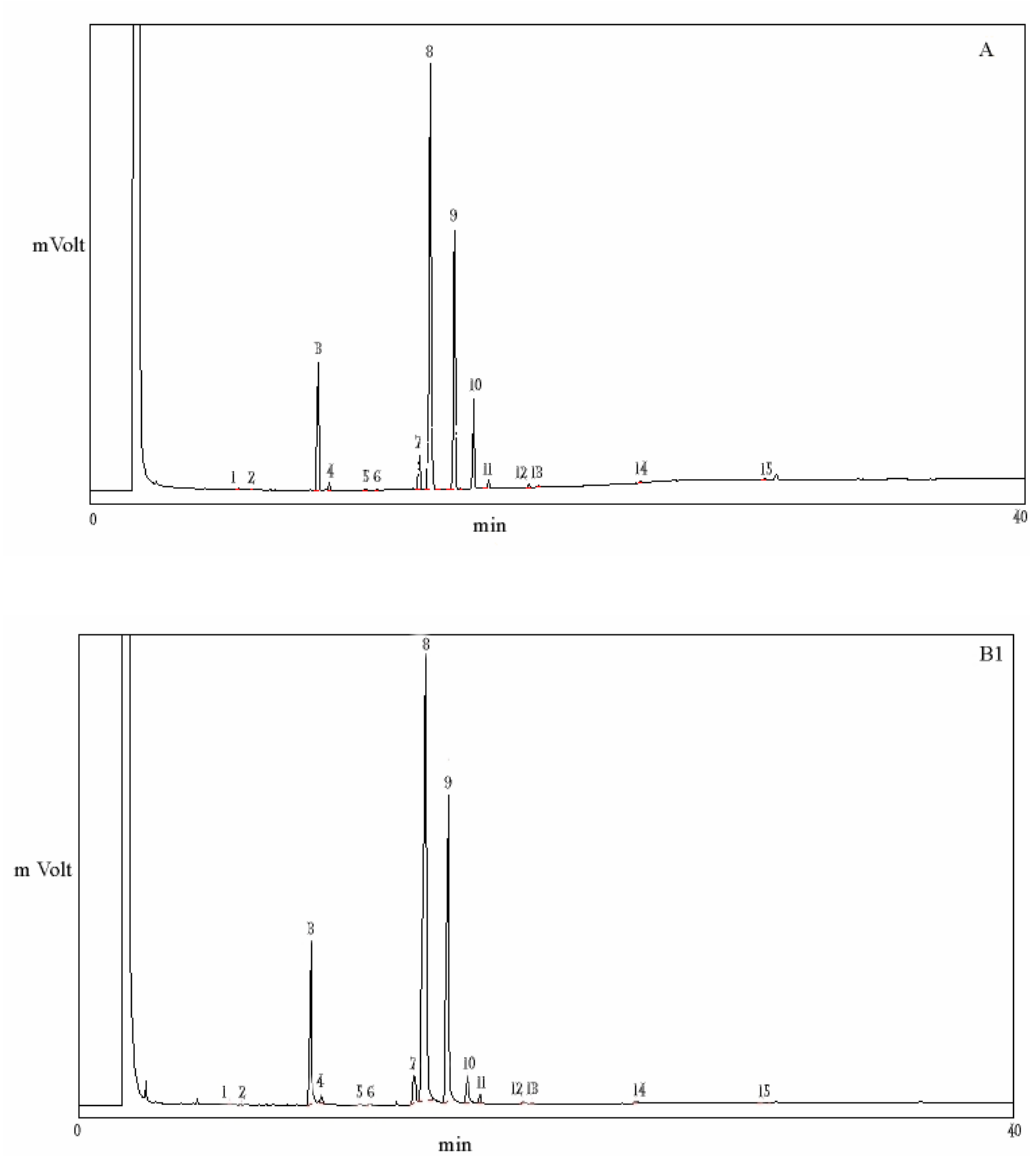
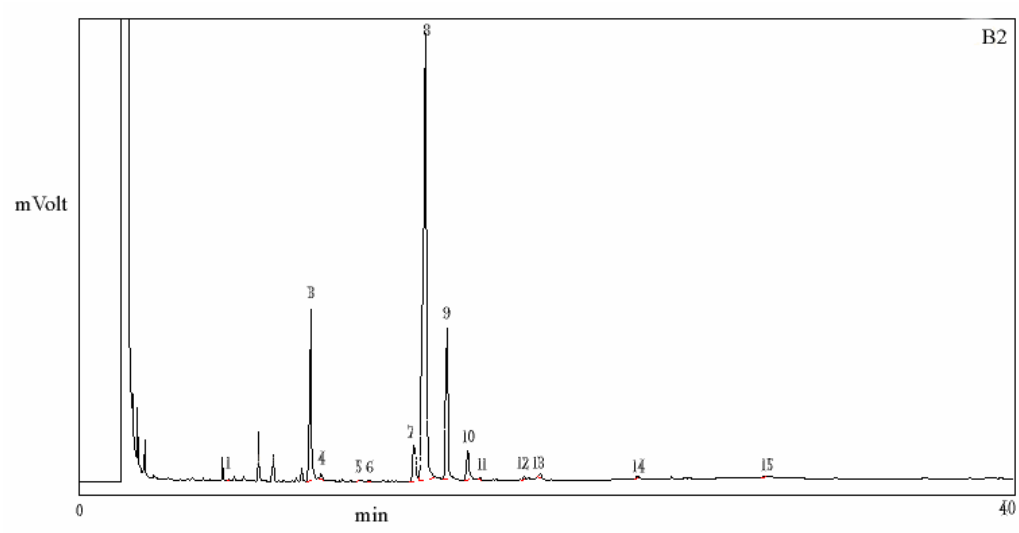


Figure V-2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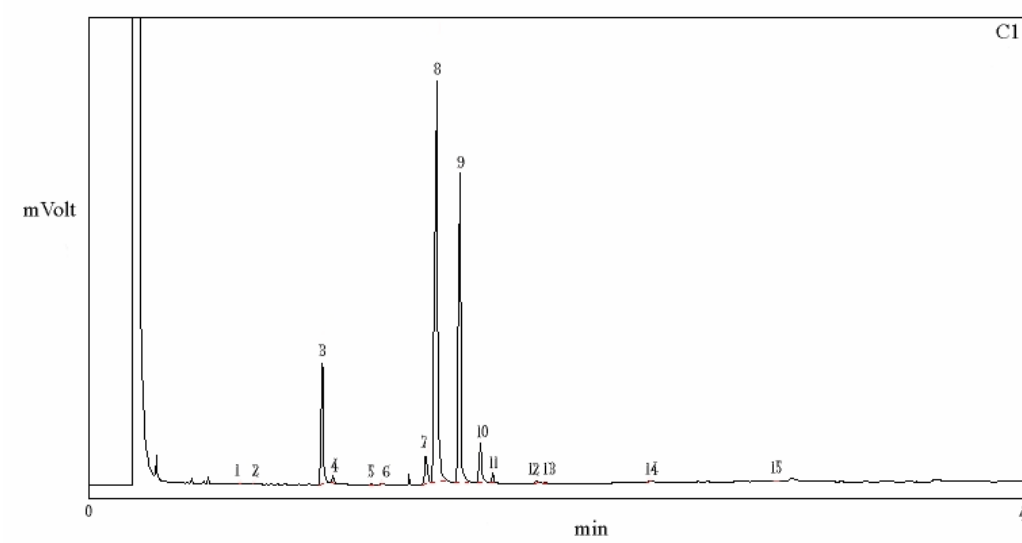
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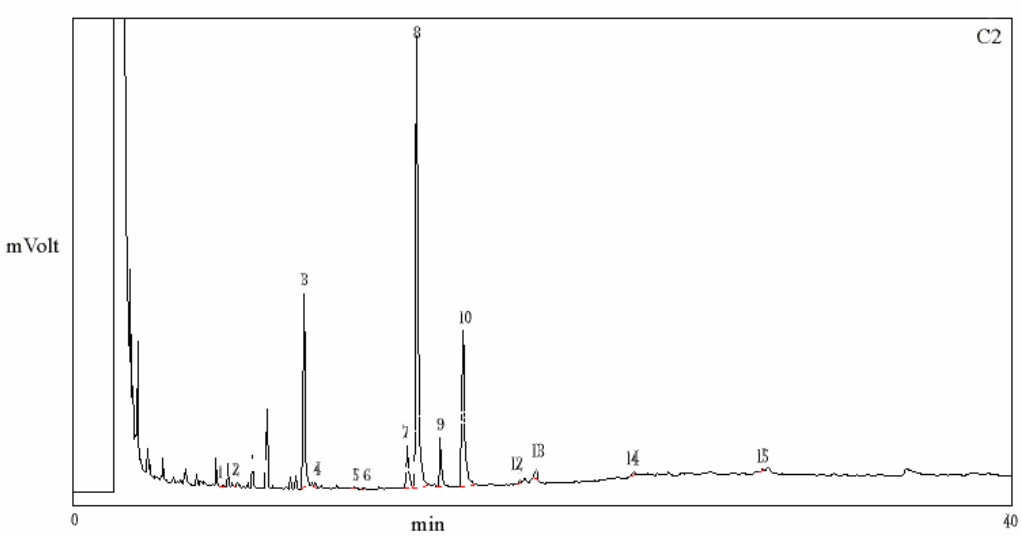


Figure V-2- GC-FID chromatograms of potato crisps FAMEs A- Month 0; B1- Month 1 darkness; B2- Month 1 lightness; C1- Month 3 darkness; C2- Month 3 lightness.

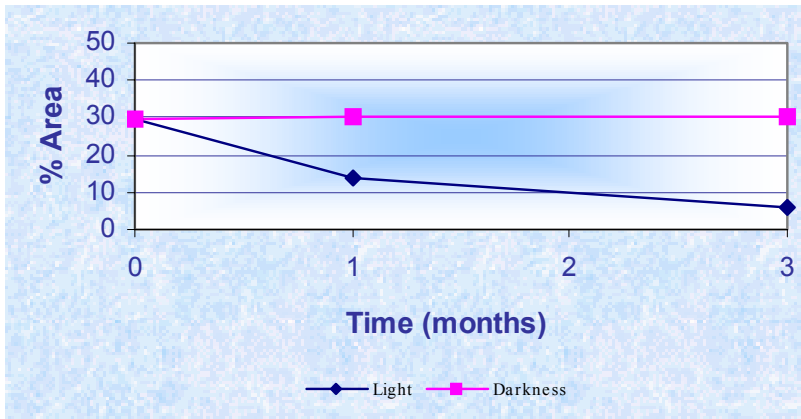


Figure V-3- Polyunsaturated FAMEs changes along a three month storage of potato crisps.

Among all fatty acid studied, linolenic acid was the most susceptible to oxidative deterioration due to the presence of the greatest number of double bonds [10]. Its % area has decreased from 0.83% (month 0) to 0.67%

and 0.05% (3 months later) respectively to potato crisps stored in the darkness and in the light (see table V-1).

Table V-1- FAMEs profile of potato crisps: A- Darkness; B- Light.

A Darkness	**Month 0**		**Month 1**		**Month 3**	
	Mean (% Área)	**DS**	**Mean (% Área)**	DS	**Mean (% Área)**	**DS**
$C_{14:0}$	0,074	0,016	0,053	0,007	0,058	0,001
$C_{14:1}$	0,002	0,001	0,001	0,000	0,002	0,000
$C_{16:0}$	11,243	0,377	10,595	0,461	11,234	0,496
$C_{16:1}$	0,525	0,018	0,469	0,010	0,477	0,004
$C_{17:0}$	0,065	0,006	0,028	0,014	0,060	0,007
$C_{17:1}$	0,068	0,003	0,057	0,003	0,058	0,003
$C_{18:0}$	3,485	0,052	3,061	0,081	3,268	0,048
$C_{18:1}$	53,733	0,403	54,800	0,430	54,331	0,317
$C_{18:2}$	29,037	0,182	29,775	0,257	29,278	0,335
$C_{18:3}$	0,826	0,089	0,622	0,109	0,666	0,053
$C_{20:0}$	0,352	0,038	0,195	0,004	0,211	0,015
$C_{20:1}$	0,173	0,011	0,116	0,004	0,125	0,007
$C_{22:0}$	0,287	0,031	0,127	0,002	0,129	0,008
$C_{24:0}$	0,129	0,018	0,100	0,026	0,104	0,018

B Light	**Month 0**		**Month 1**		**Month 3**	
	Mean (%)	**DS**	**Mean (%)**	DS	**Mean (%)**	**DS**
$C_{14:0}$	0,074	0,016	0,047	0,005	0,136	0,076
$C_{14:1}$	0,002	0,001	0,000	0,000	0,000	0,001
$C_{16:0}$	11,243	0,377	14,669	0,237	21,384	0,709
$C_{16:1}$	0,525	0,018	0,518	0,016	0,429	0,024
$C_{17:0}$	0,065	0,006	0,029	0,008	0,075	0,019
$C_{17:1}$	0,068	0,003	0,072	0,000	0,114	0,022
$C_{18:0}$	3,485	0,052	4,625	0,153	6,731	0,016
$C_{18:1}$	53,733	0,403	65,171	0,017	62,788	0,827
$C_{18:2}$	29,037	0,182	13,508	0,283	5,782	0,075
$C_{18:3}$	0,826	0,089	0,194	0,030	0,050	0,024
$C_{20:0}$	0,352	0,038	0,240	0,010	0,321	0,009
$C_{20:1}$	0,173	0,011	0,472	0,022	1,449	0,129
$C_{22:0}$	0,287	0,031	0,350	0,103	0,435	0,057
$C_{24:0}$	0,129	0,018	0,103	0,004	0,305	0,066

Nevertheless, % area data gives only limited information. It allows one to evaluate the effect of light, once the decrease of the polyunsaturated FAMEs fraction is more evident when potatoes are exposed to natural light during storage. However, it does not allow predicting the extension of this change in the FAMES contents (g/100g sample). This way, the quantification of fourteen FAMEs was carried out using the internal standard method. Nonadecanoic acid, which is not present in potato crisps according our preliminary tests, was selected as internal standard. The content of fourteen FAMEs of the potato crisps stored during 3 months it is presented in table V-2.

A considerable amount of papers has identified and quantified the fatty acids present in potato crisps [30-31]. Nevertheless this information is scarce with respect to the effect of light in the oxidation phenomenon during the storage period. Masson et al [32] have studied the progression of the deterioration of crisps stored at room temperature (daylight and darkness), and in darkness (at 40 ºC and –23 ºC). However, he studied other parameters aside from fatty acids.

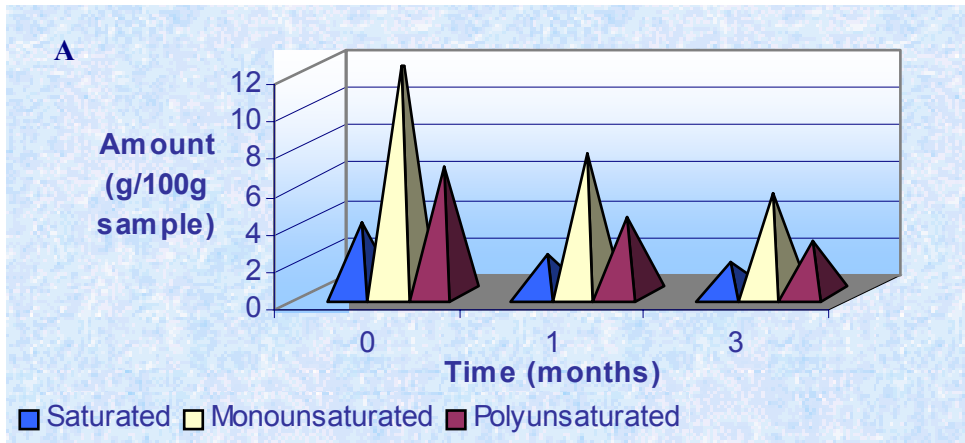
Figure V-4 shows that the FAMEs content decreased with storage time and this diminution was more significant when crisps were stored in presence of light.

After the first month, stored potato crisps under light present lower amounts of FAMEs that those stored in the darkness, however this difference is significative just for monoun- and polyunsaturated FAMEs (fig. V-5A). After 3 months (fig. V-5B) the difference between the two conditions, is more evident (about 22 times lower under light for polyunsaturated and 3.6 and 1.8 times lower for monounsaturated and saturated, respectively).

Table V-2- FAMEs content (g/100g sample) of potato crisps. **A-** Darkness; **B-** Lightness.

A- Darkness	*Month 0*		*Month 1*		*Month 3*	
	Mean	**DS**	**Mean**	**DS**	**Mean**	**DS**
$C_{14:0}$	0,016	0,000	0,006	0,001	0,004	0,001
$C_{14:1}$	0,001	0,000	0,000	0,000	0,000	0,000
$C_{16:0}$	2,353	0,007	1,279	0,151	0,959	0,177
$C_{16:1}$	0,086	0,001	0,047	0,005	0,034	0,008
$C_{17:0}$	0,011	0,000	0,003	0,002	0,005	0,001
$C_{17:1}$	0,016	0,000	0,008	0,000	0,006	0,001
$C_{18:0}$	0,829	0,002	0,426	0,049	0,323	0,067
$C_{18:1}$	12,054	0,029	7,329	0,580	5,183	1,156
$C_{18:2}$	6,533	0,019	3,989	0,309	2,801	0,638
$C_{18:3}$	0,155	0,001	0,067	0,015	0,050	0,008
$C_{20:0}$	0,099	0,000	0,030	0,003	0,023	0,004
$C_{20:1}$	0,058	0,000	0,022	0,003	0,017	0,003
$C_{22:0}$	0,092	0,001	0,022	0,002	0,016	0,003
$C_{24:0}$	0,040	0,004	0,019	0,005	0,013	0,001
Total	**22,671**		**13,581**		**9,765**	

B- Light	*Month 0*		*Month 1*		*Month 3*	
	Mean	**DS**	**Mean**	**DS**	**Mean**	**DS**
$C_{14:0}$	0,016	0,000	0,004	0,000	0,002	0,000
$C_{14:1}$	0,001	0,000	0,000	0,000	0,000	0,000
$C_{16:0}$	2,353	0,007	1,138	0,072	0,412	0,201
$C_{16:1}$	0,086	0,001	0,037	0,002	0,007	0,004
$C_{17:0}$	0,011	0,000	0,004	0,000	0,001	0,000
$C_{17:1}$	0,016	0,000	0,007	0,000	0,003	0,002
$C_{18:0}$	0,829	0,002	0,412	0,030	0,151	0,077
$C_{18:1}$	12,054	0,029	6,003	0,368	1,361	0,706
$C_{18:2}$	6,533	0,019	2,247	0,068	0,125	0,063
$C_{18:3}$	0,155	0,001	0,034	0,001	0,002	0,001
$C_{20:0}$	0,099	0,000	0,025	0,001	0,008	0,004
$C_{20:1}$	0,058	0,000	0,041	0,001	0,046	0,026
$C_{22:0}$	0,092	0,001	0,029	0,001	0,012	0,006
$C_{24:0}$	0,040	0,004	0,013	0,001	0,009	0,003
Total	**22,671**		**10,319**		**2,471**	



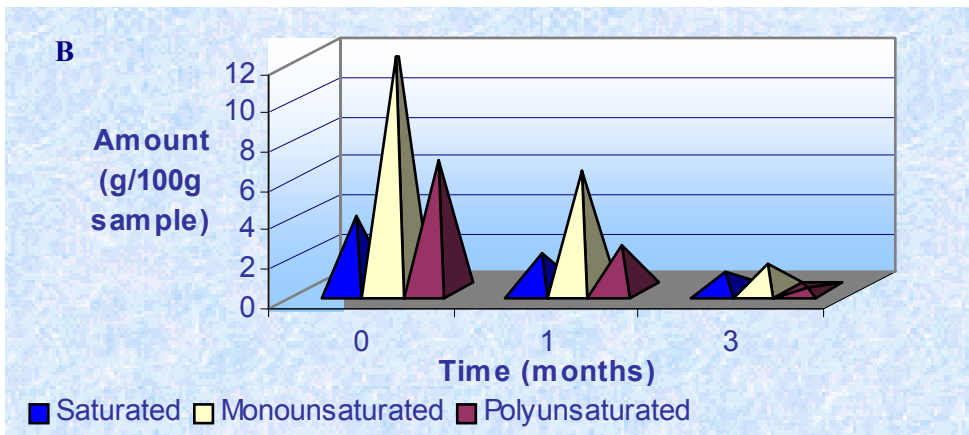


Figure V-4- Change of FAMES content during a three months storage: A- Light; B- Darkness.

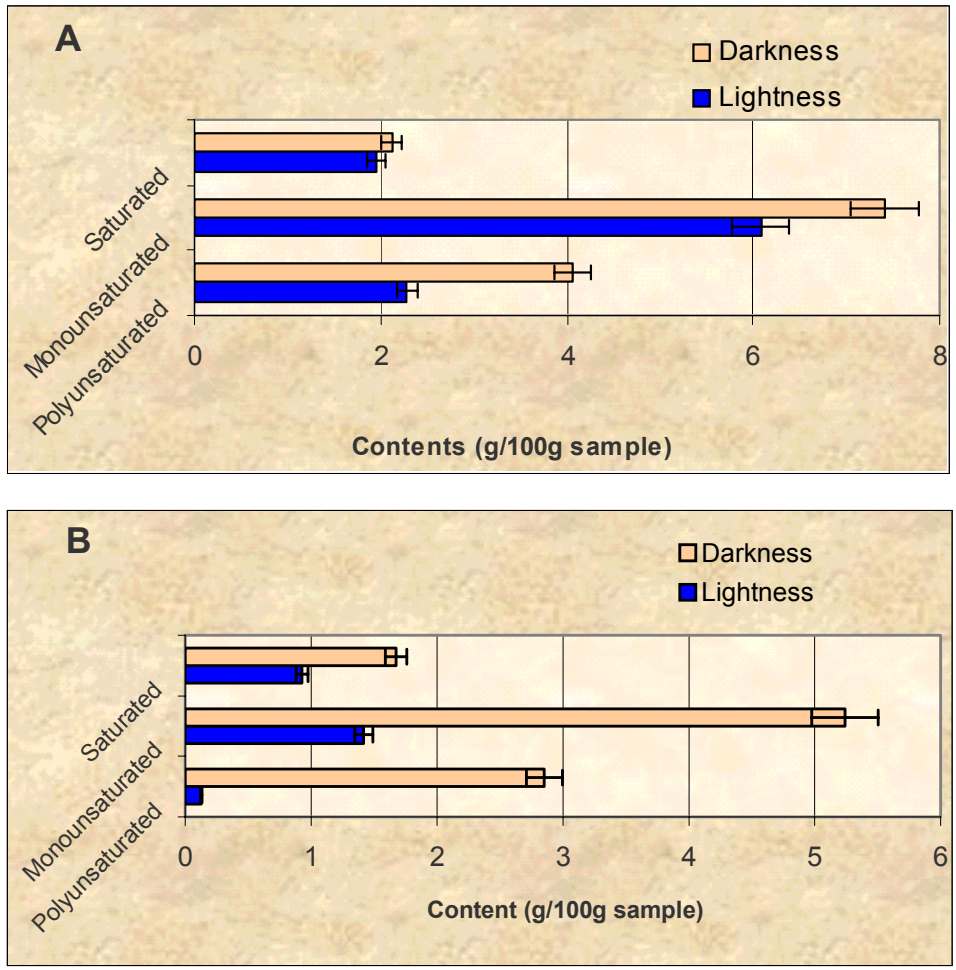


Figure V-5- Contents of saturated, monounsaturated and polyunsaturated fatty acids: A- First month; B- Third month.

Chromatograms of potato crisps stored for 1 and 3 months allow one to see the formation of new peaks. These peaks possibly correspond to new compounds generated in the oxidation process. With the aim of identifying these compounds, samples were injected in a gas chromatograph coupled with mass selective detector. Figure V-6 shows the chromatograms obtained. Table V-3 shows the identified compounds. Most of these compounds are fatty acids of shorter carbon chain and aldehydes.

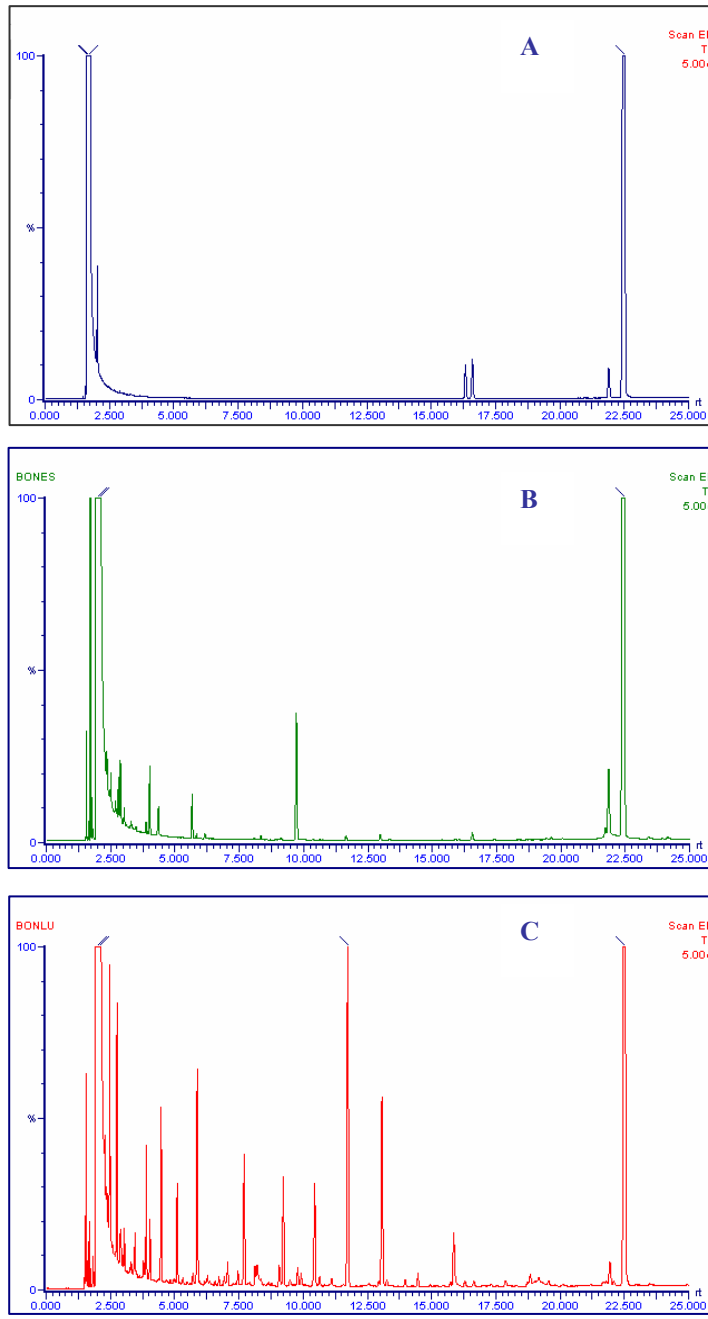


Figure V-6- GC-MS chromatograms. A- Standard; B- Darkness; C- Lightness.

In the presence of light during storage period, the number of new compounds formed was greater For instance benzaldehide and nonanal were only identified when storage was performed with natural light (see table V-3). Benzaldehide derived from linoleic acid while nonanal is derived from oleic acid [33]. Compounds yielded in both conditions presented a great content when crisps were stored under light.

Table V-3- Identification of compounds generated in the oxidative process.

Peak	Compounds	Wiley Number	t_r	Darkness		Light	
				Area	% Area	Area	% Area
1	Methane, chloro-	105	1,56	2640713	0,10	6504822	0,39
2	Acetic acid, methyl ester	118842	1,63	574677	0,02	1425787	0,09
3	Hexane	119434	1,70	70027024	2,72	1621421	0,10
4	Cyclopentane, methyl	119247	1,77	889464	0,03		
5	Benzene	119006	1,83			1203121	0,07
6	Benzene, methyl	119751	1,93	855560304	33,28	865039744	52,15
7	1,3,5-cycloheptatriene	119763	2,35	10593674	0,41	21362936	1,29
8	Hexanoic acid, methyl ester	123398	2,47			16093355	0,97
9	Benzene, ethyl	120878	2,51	9657625	0,38		
10	Hexanal, dimethyl acetal	12280	2,74	1839779	0,07	10224633	0,62
11	Benzaldehyde	120872	2,89			2429842	0,15
12	2-butenedioic acid (E)-dimethylester	125155	3,03	3767202	0,15		
13	Heptanoic acid, methyl ester	125196	3,04			1780932	0,11
14	benzenemethanol	121062	3,29	1594522	0,06	524608	0,03
15	Heptane, 1,1-dimethoxy	17742	3,45			2953484	0,18
16	Nonanal	125004	3,79			860296	0,05
17	Octanoic acid, methyl ester	127012	3,88	463714	0,02	6383934	0,38
18	Benzaldehyde dimethyl acetal	126250	4,02	3352612	0,13	2979109	0,18
19	1,1- dimethoxy-octane	23979	4,47			9184989	0,55

Table V-3 (continuation)

Peak	Compounds	Wiley Number	t_r	Darkness		Light	
				Area	% Area	Area	% Area
20	Nonanoic acid, methyl ester	128487	5,09			5414463	0,32
21	Hexanedioic acid, methyl ester	128628	5,32			434285	0,03
22	Dodecane, 1,1-dimethoxy	49390	5,84	340703	0,01	13268952	0,80
23	Decanoic acid, methyl ester	129692	6,72			581182	0,03
24	Heptanedioic acid, methyl ester	129780	7,06			1786384	0,11
25	Nonanoic acid, 9-oxo-methyl ester	129670	9,07			1888120	0,11
26	Octanedioic acid, dimethyl ester	36308	9,23			8459628	0,51
27	Trimethyl citrate	132833	9,72	10019181	0,39		
28	Decanoic acid, 9-oxo, methyl ester	35617	11,12			494297	0,03
29	Nonanedioic acid, dimethyl ester	42751	11,73			30312290	1,83
30	Decanedioic acid, methyl ester	132638	14,47			1182401	0,07
31	Tetradecanoic acid, methyl ester	133230	16,56	655930	0,02	541894	0,03
32	7-Hexadecenoic acid, methyl ester	64559	21,73	832084	0,03		
33	9-Hexadecenoic acid, methyl ester	64549	21,86	7289231	0,28	2354064	0,14
34	Hexadecanoic acid, methyl ester	134503	22,44	169065968	6,58	369390	0,02

V-4. Conclusions

The method applied in this paper is suitable for the analysis for fatty acids methyl esters in potato crisps. Data show that light greatly contributes for the development of the lipid oxidation process. Therefore, potato crisps should be stored in the dark, with an opaque film, in order to avoid or at least delay the oxidative process and this way improves product shelf life.

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APPENDIX I

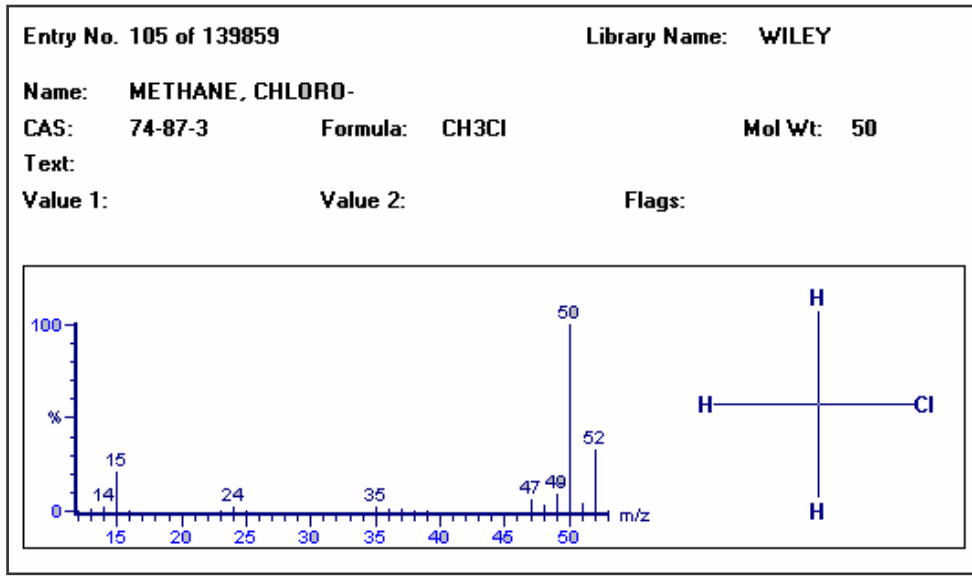

Figure V-7- Chloro-, methane GC-MS spectrum.

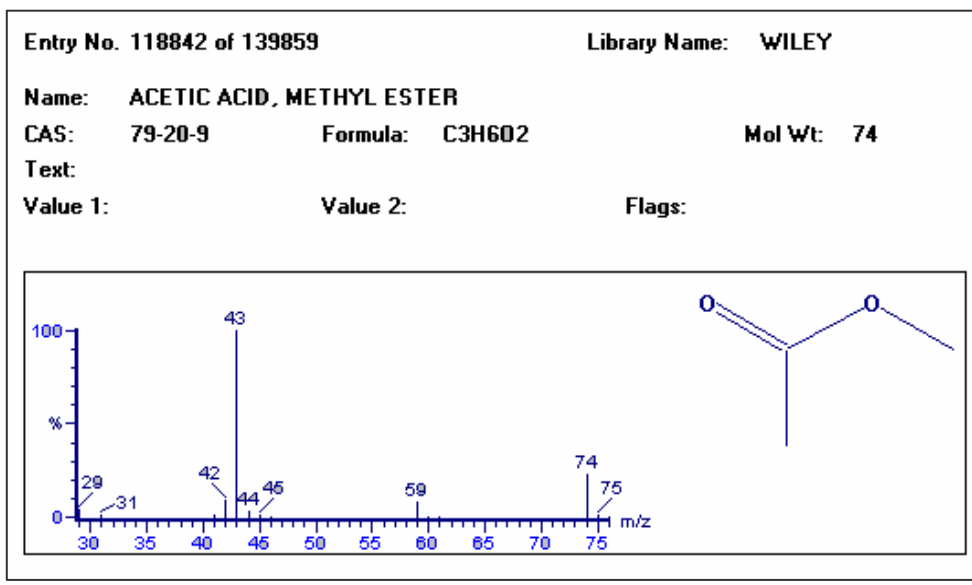

Figure V-8- Acetic acid methyl ester GC-MS spectrum.

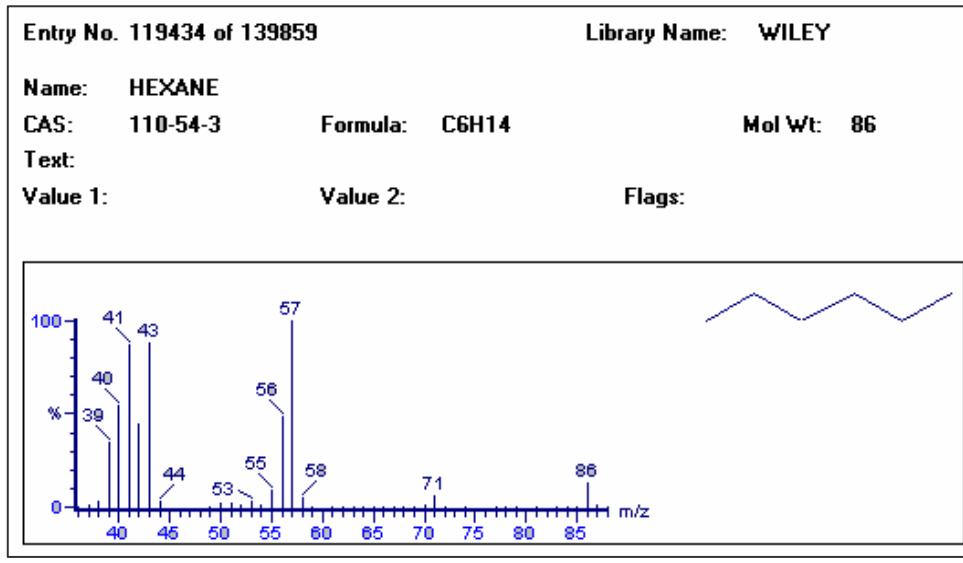

Figure V-9- Hexane GC-MS spectrum.

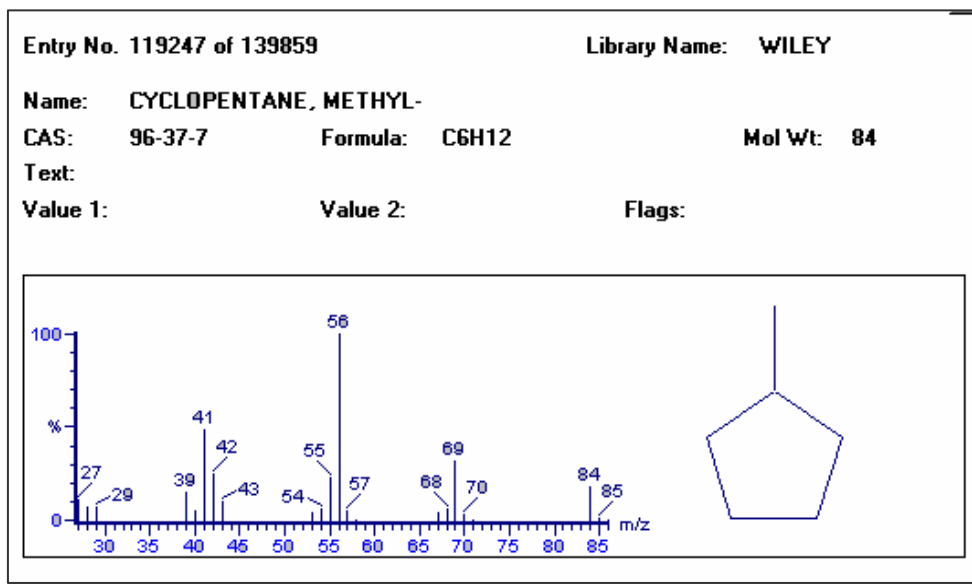

Figure V-10- Methyl-cyclopentane GC-MS spectrum.

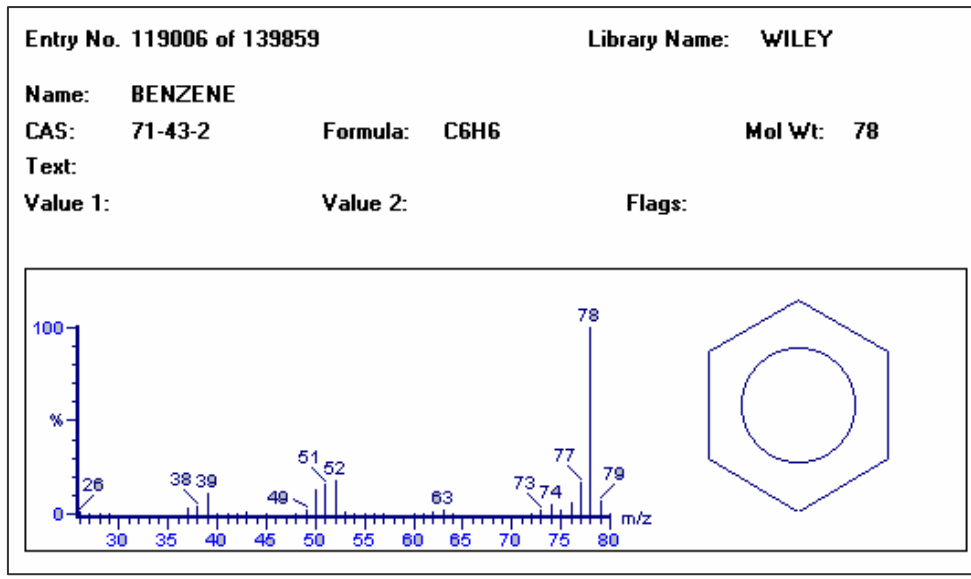

Figure V-11- Benzene GC-MS spectrum.

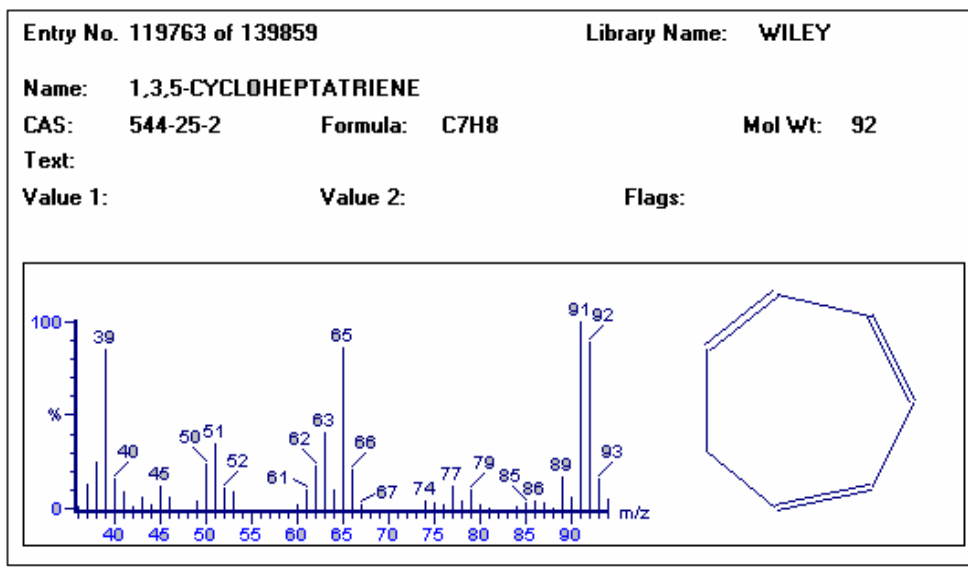

Figure V-12- 1,3,5-Cycloheptatriene GC-MS spectrum.

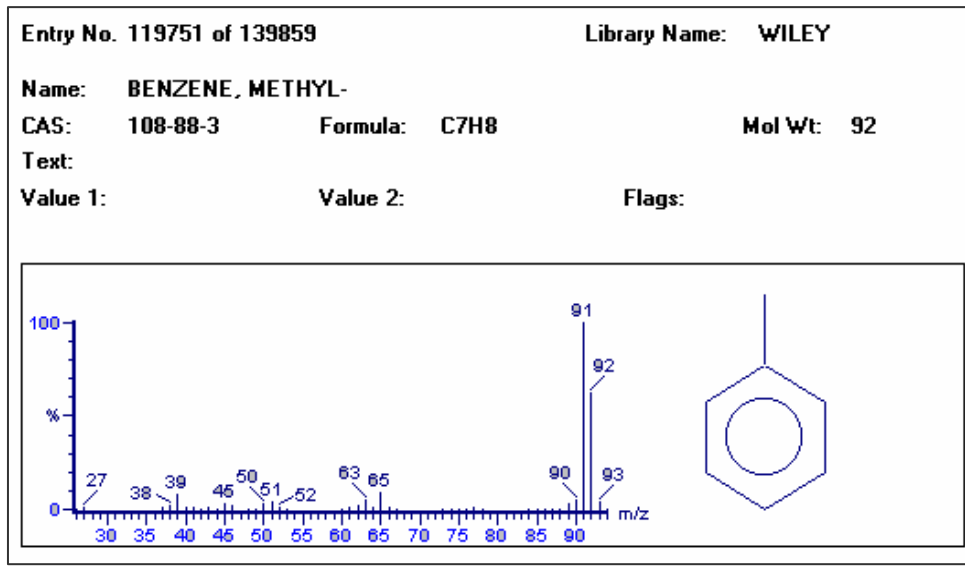

Figure V-13- Methyl-benzene GC-MS spectrum.

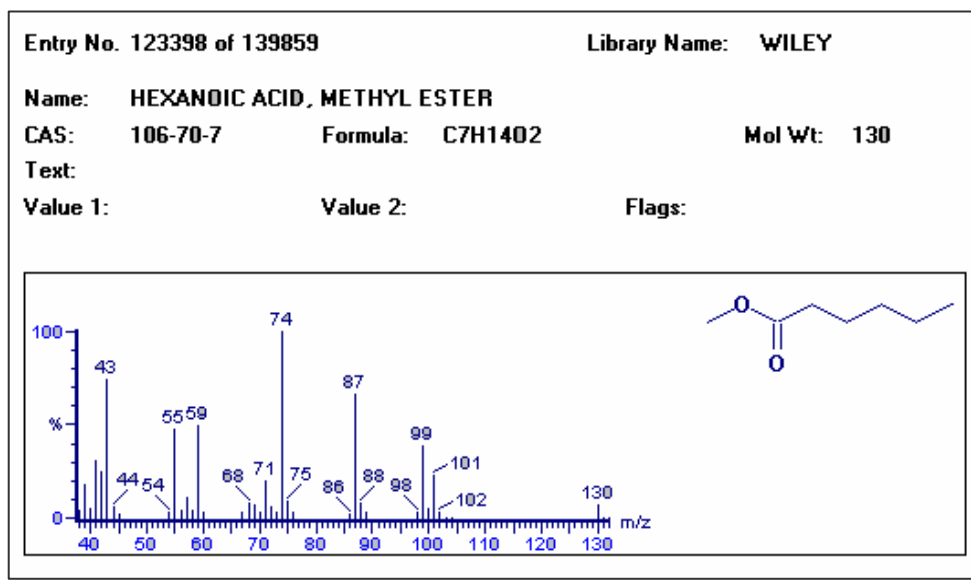

Figure V-14- Hexanoic acid methyl ester GC-MS spectrum.

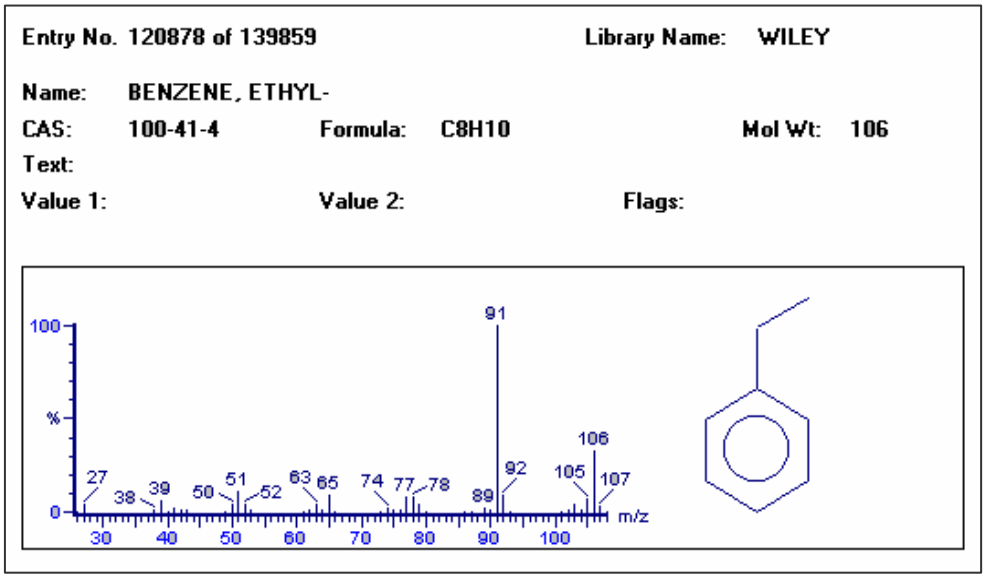

Figure V-15- Ethyl-benzene GC-MS spectrum.

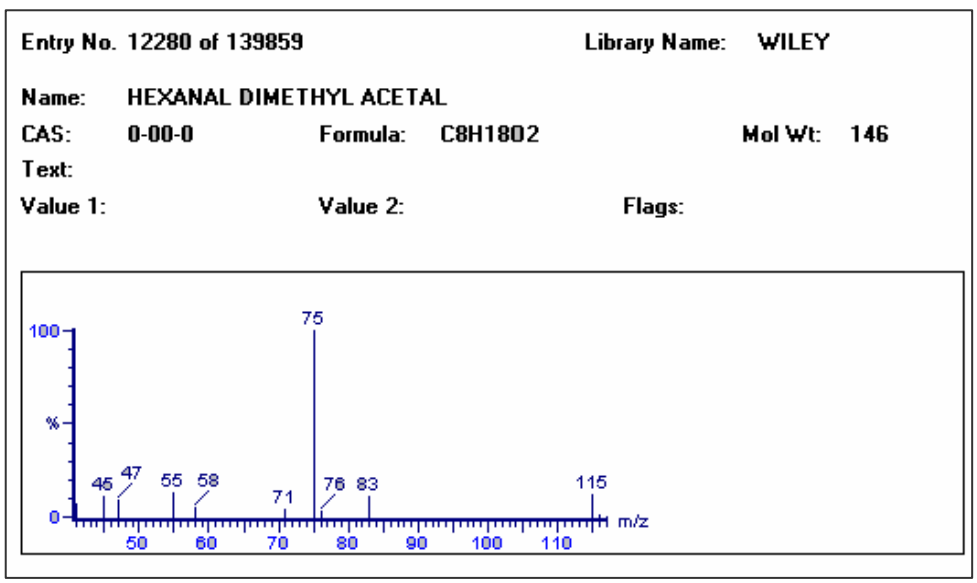

Figure V-16- Hexanal dimethyl acetal GC-MS spectrum.

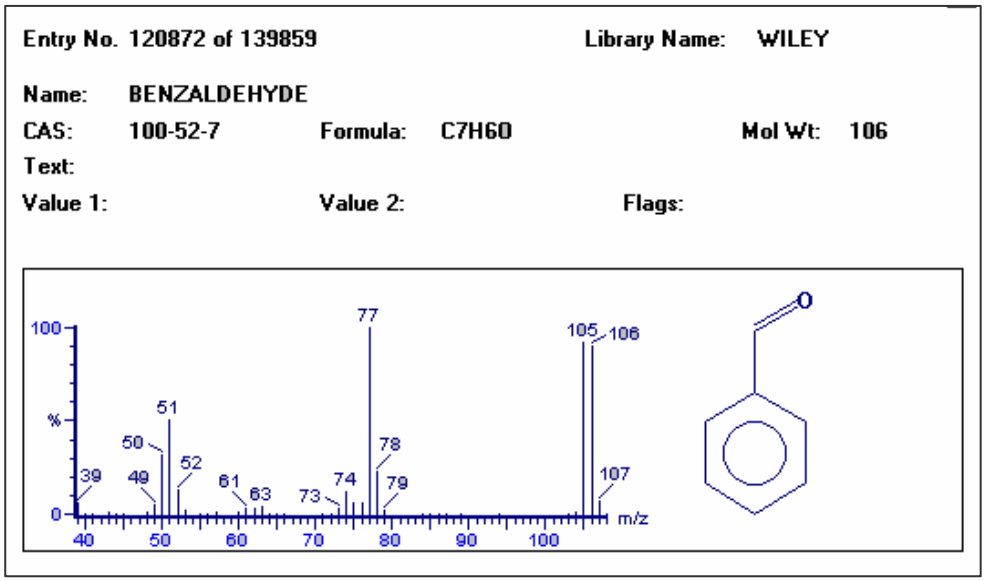

Figure V-17- Benzaldehyde GC-MS spectrum.

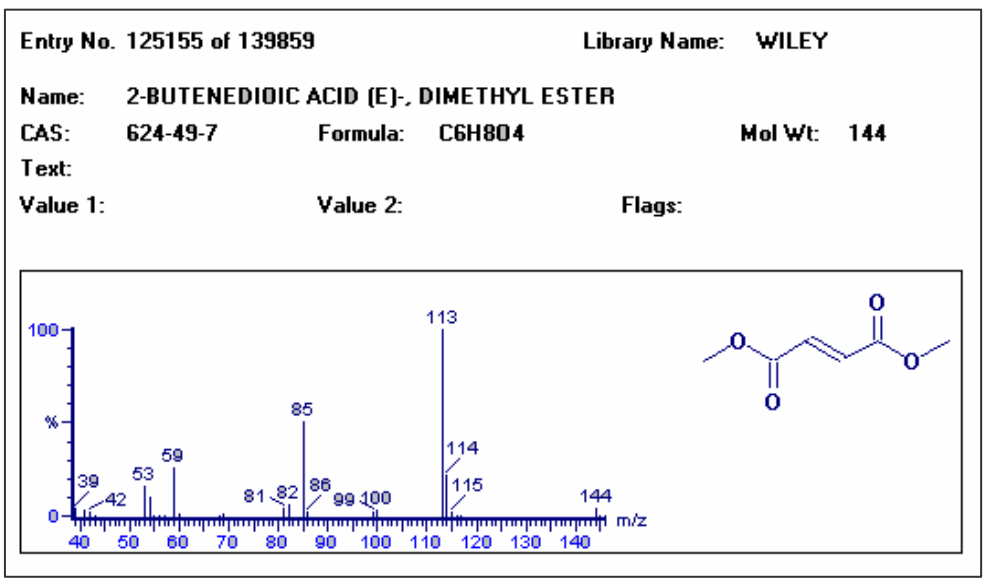

Figure V-18- 2-Butenedioic acid (E)-dimethylester GC-MS spectrum.

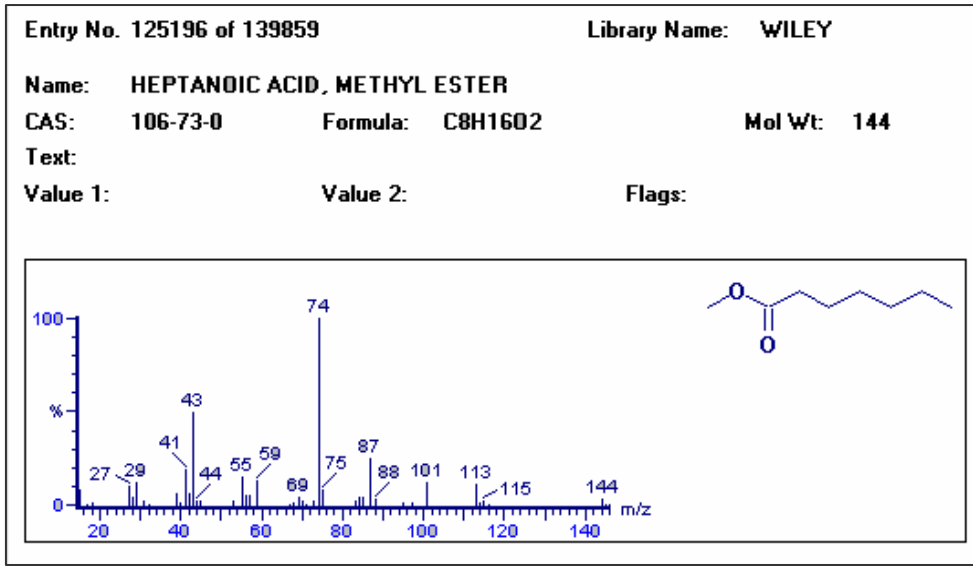

Figure V-19- Heptanoic acid methyl ester GC-MS spectrum.

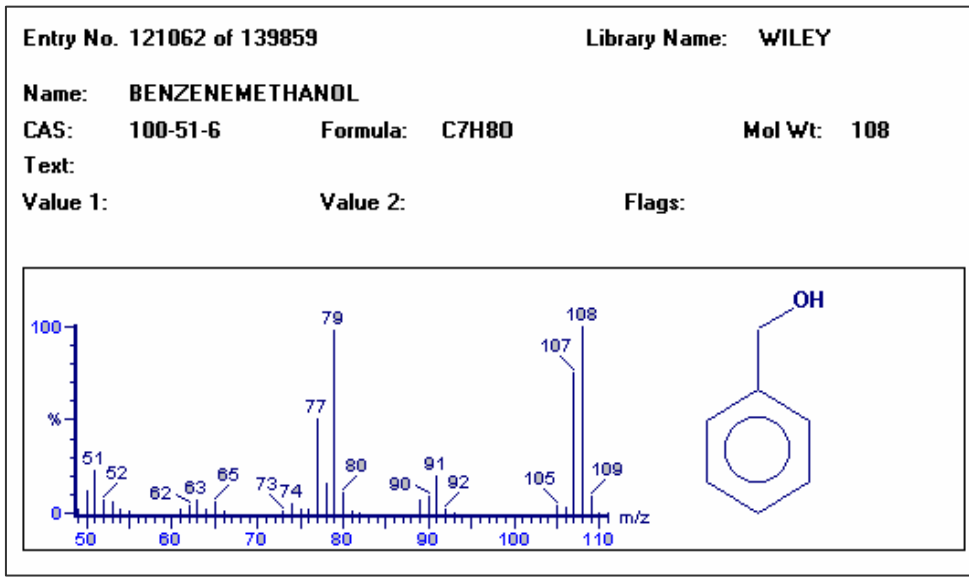

Figure V-20- Benzenemethanol GC-MS spectrum.

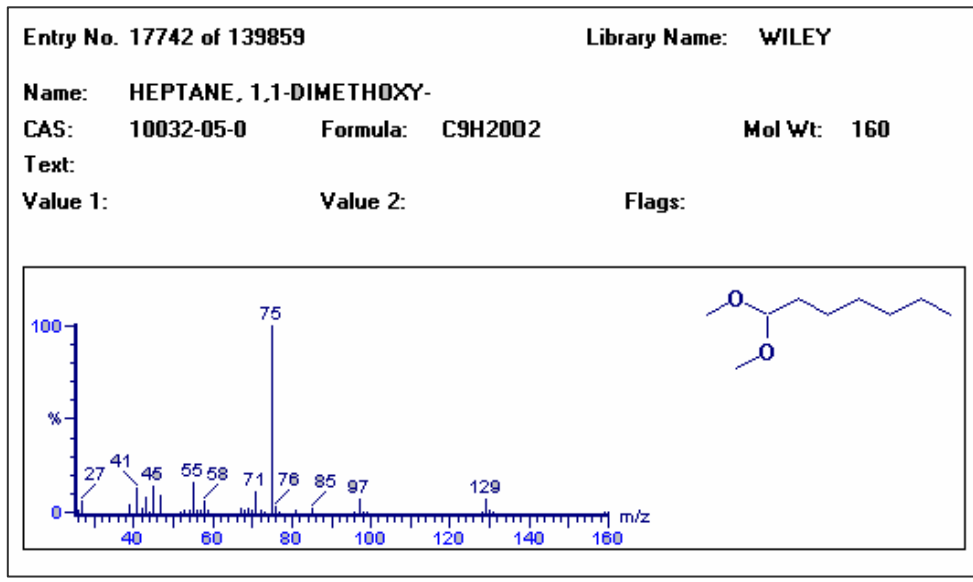

Figure V-21- Heptane, 1,1-dimethoxy GC-MS spectrum.

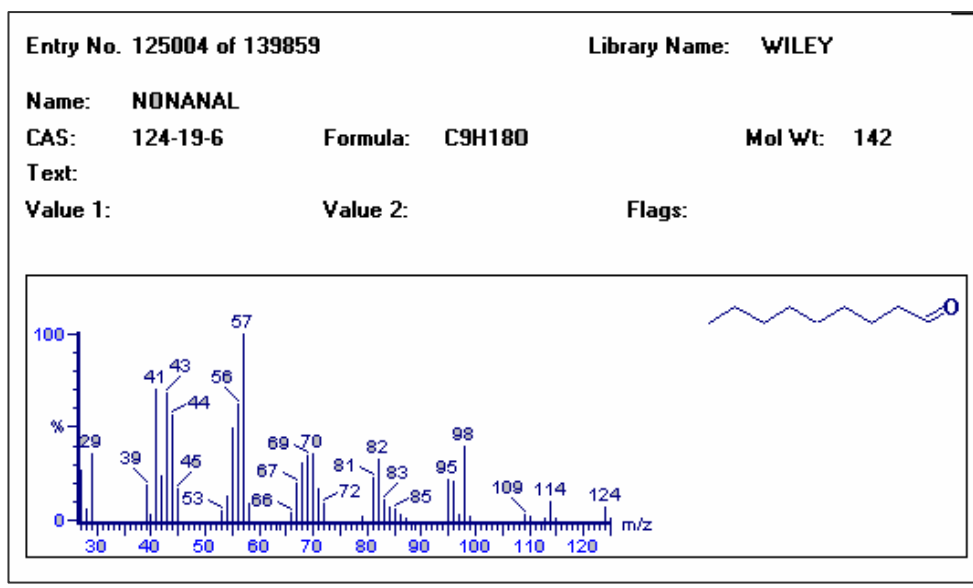

Figure V-22- Nonanal GC-MS spectrum.

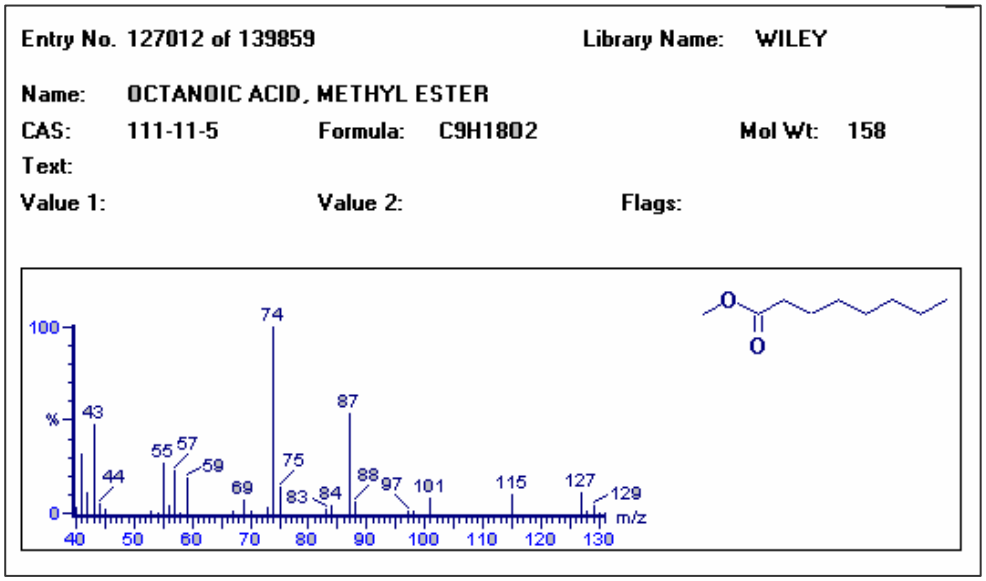

Figure V-23- Octanoic acid methyl ester GC-MS spectrum.

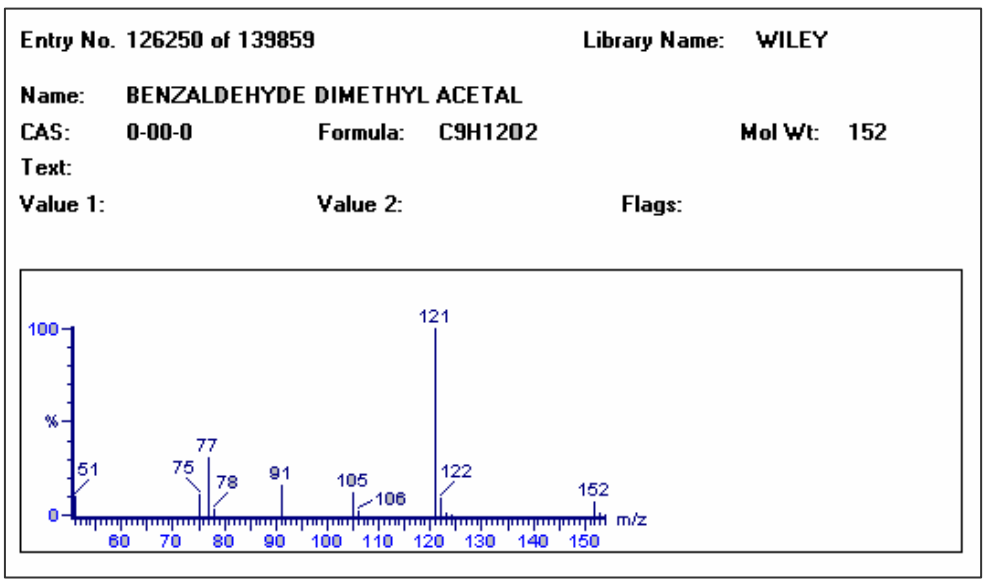

Figure V-24- Benzaldehyde dimethyl acetal GC-MS spectrum.

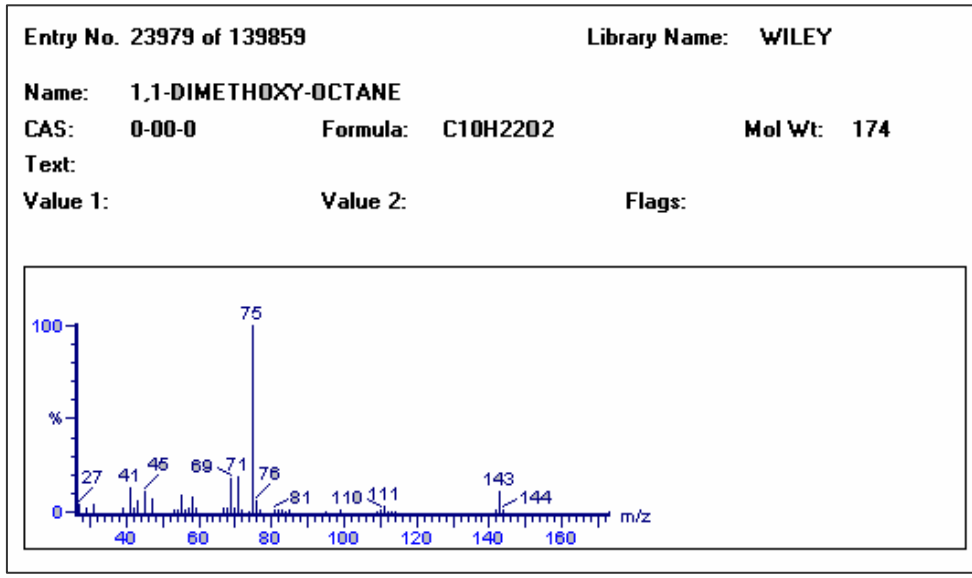

Figure V-25- 1,1- Dimethoxy-octane GC-MS spectrum.

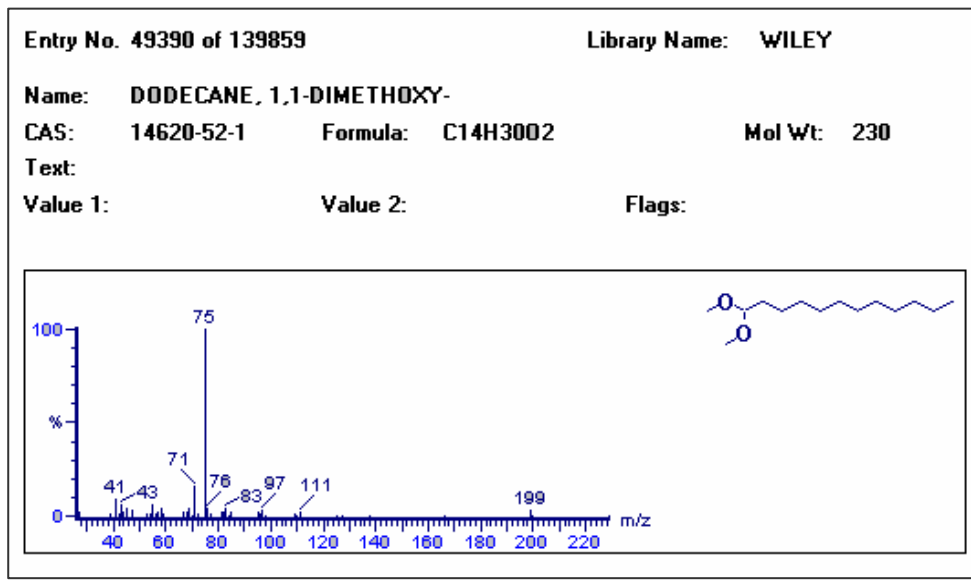

Figure V-26- 1,1-Dimethoxy dodecane GC-MS spectrum.

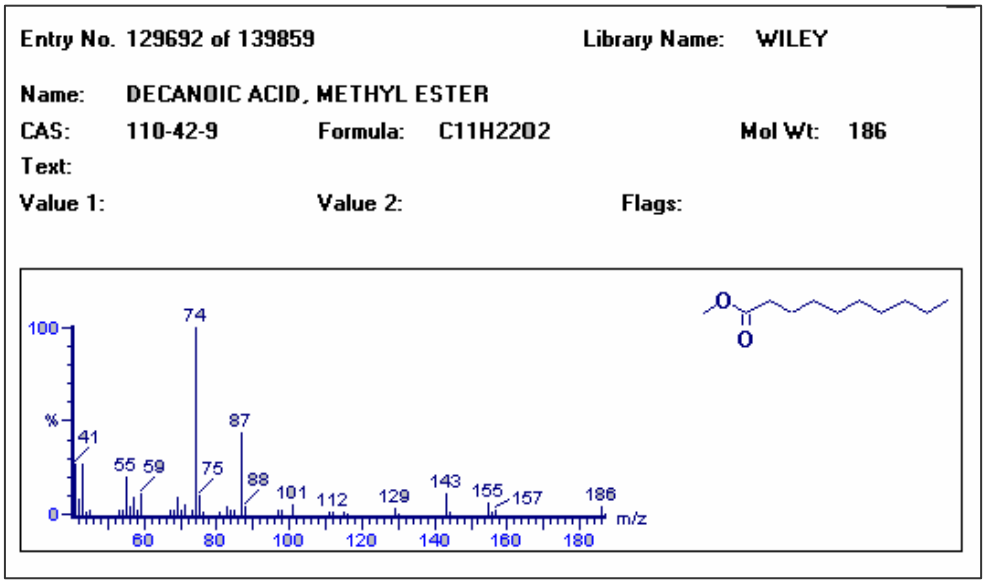

Figure V-27- Decanoic acid methyl ester GC-MS spectrum.

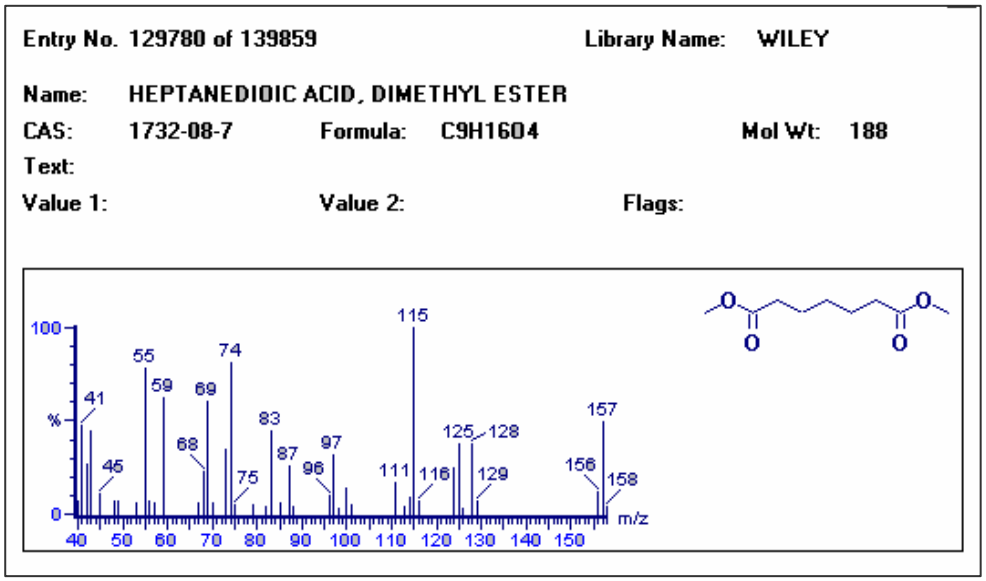

Figure V-28- Heptanedioic acid methyl ester GC-MS spectrum.

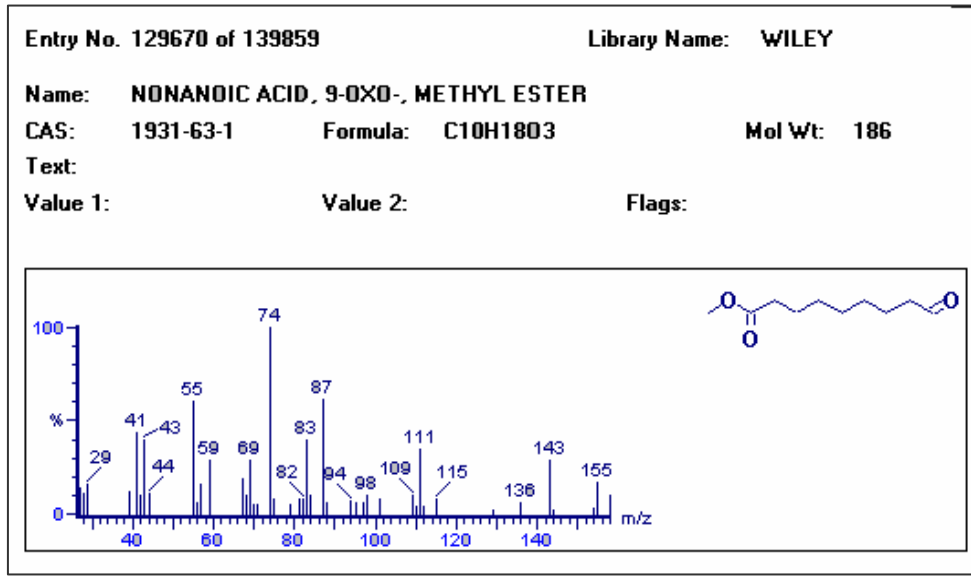

Figure V-29- Nonanoic acid, 9-oxo-methyl ester GC-MS spectrum.

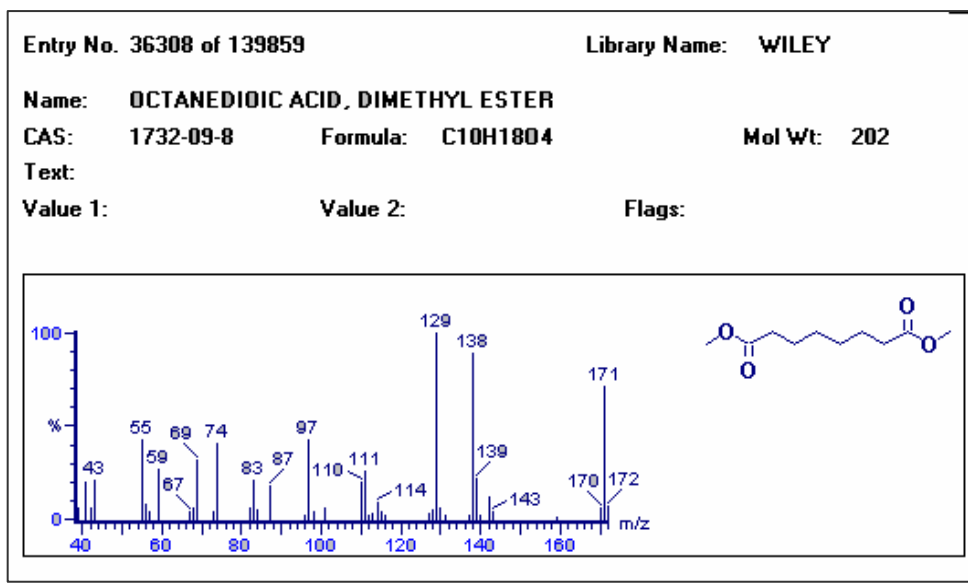

Figure V-30- Octanedioic acid, dimethyl ester GC-MS spectrum.

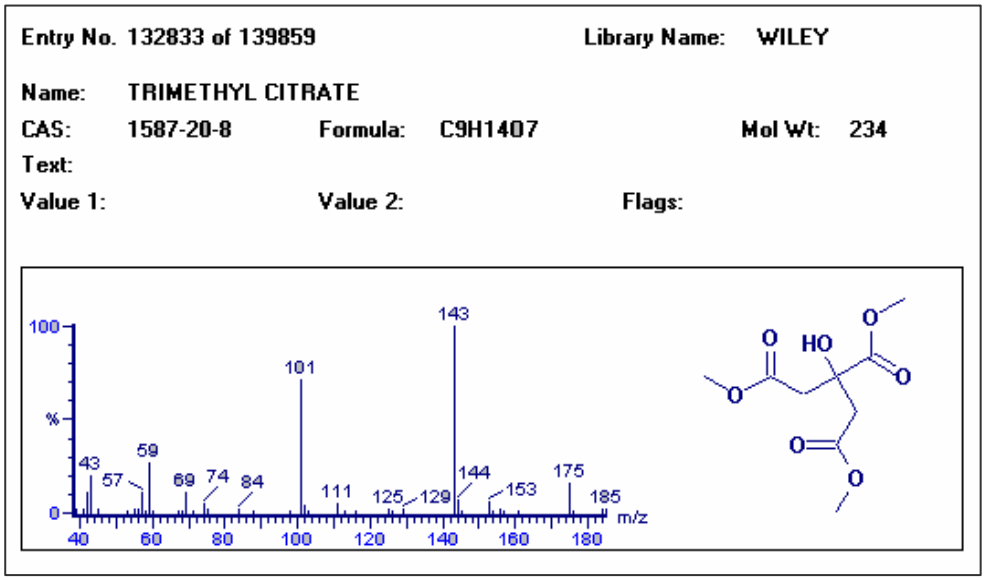

Figure V-31- Trimethyl citrate GC-MS spectrum.

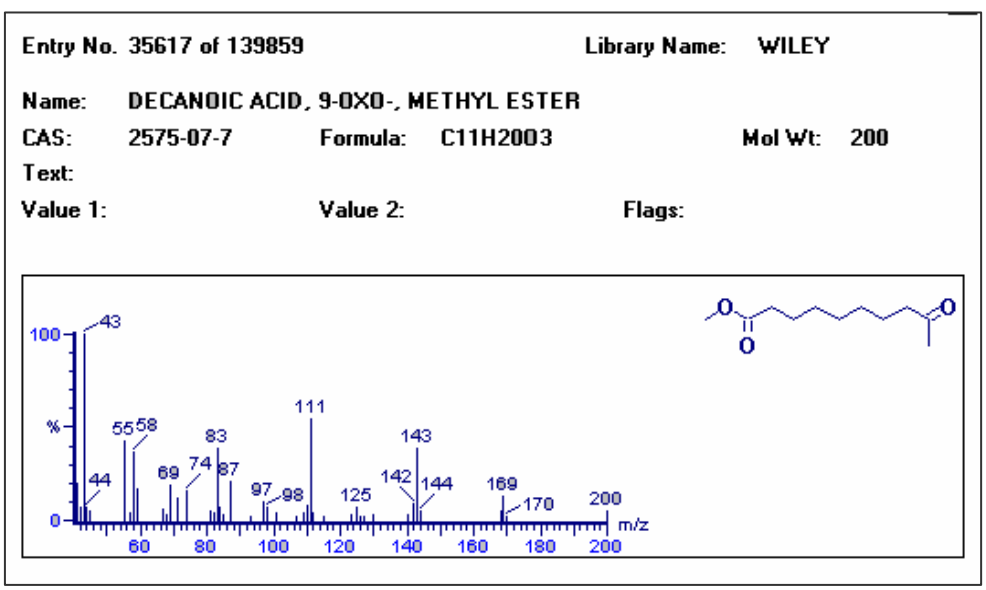

Figure V-32- Decanoic acid, 9-oxo, methyl ester GC-MS spectrum.

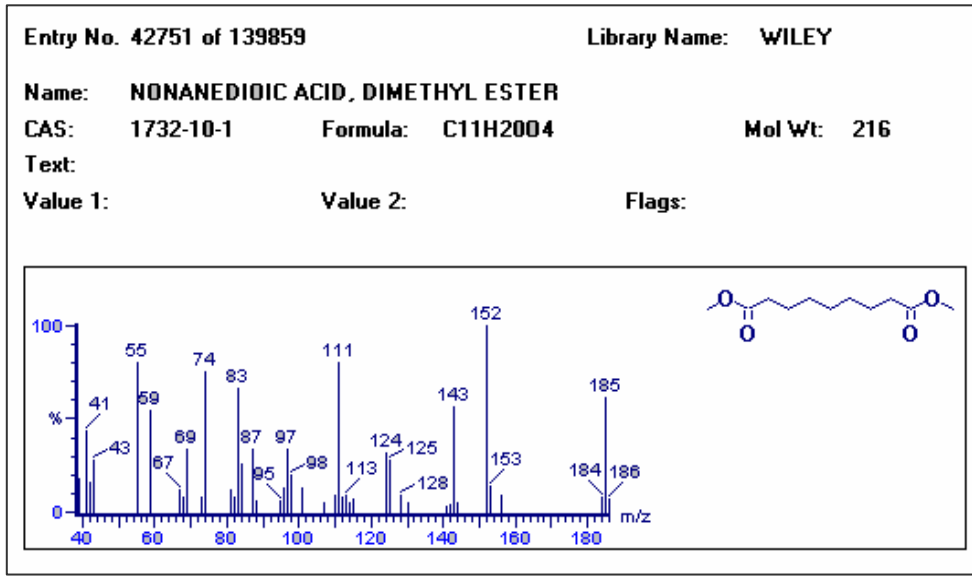

Figure V-33- Nonadioic acid dimethyl ester GC-MS spectrum.

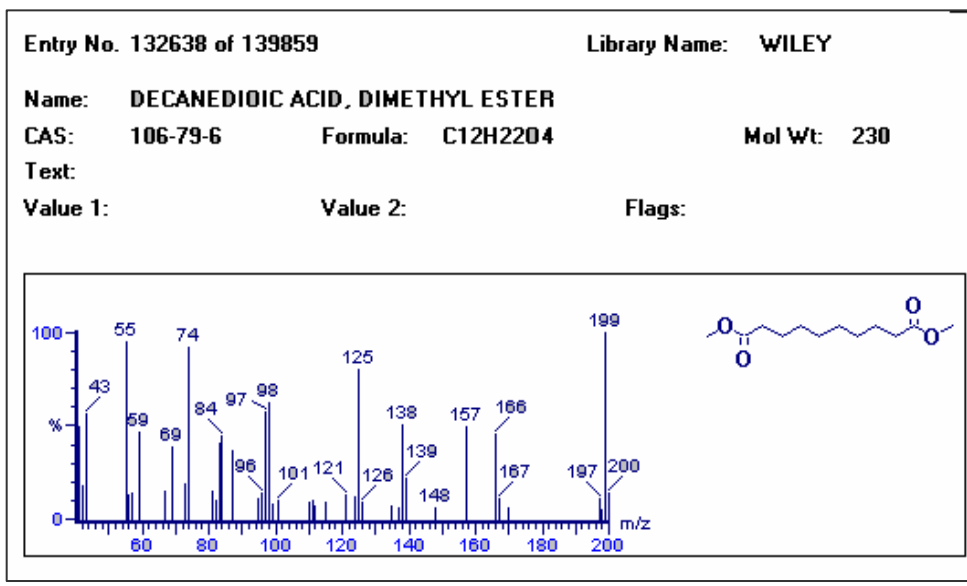

Figure V-34- Decanedioic acid methyl ester GC-MS spectrum.

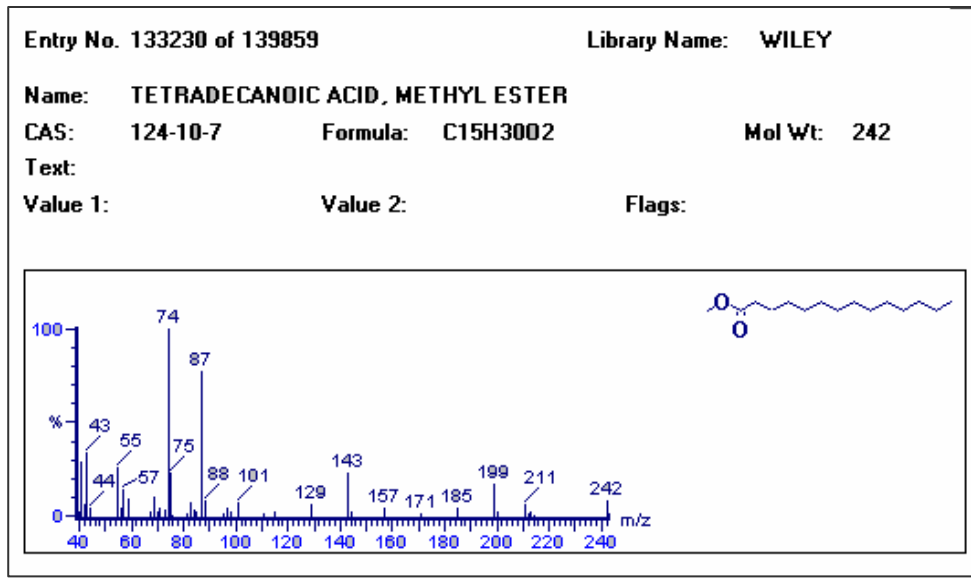

Figure V-35- Tetradecanoic acid methyl ester GC-MS spectrum.

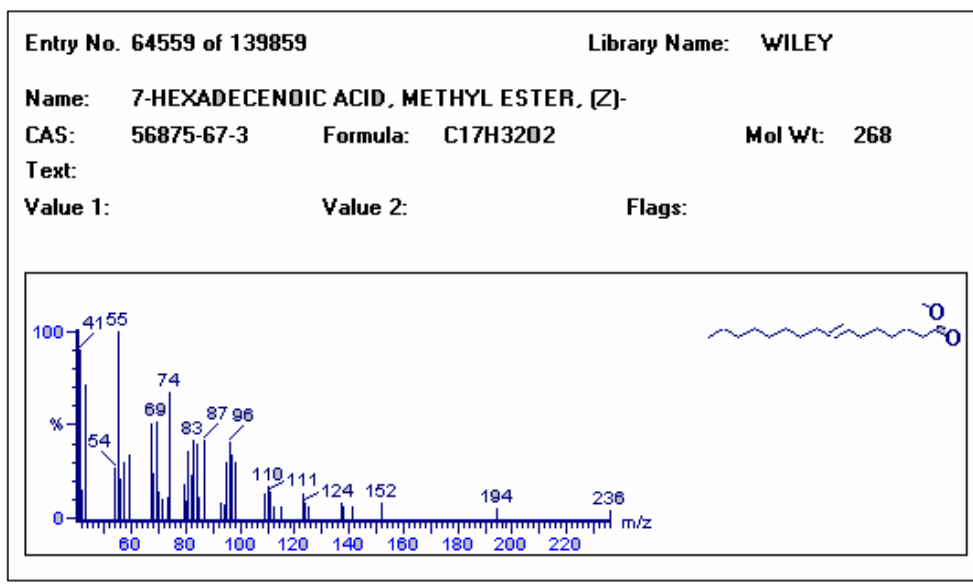

Figure V-36- 7-Hexadecenoic acid methyl ester GC-MS spectrum.

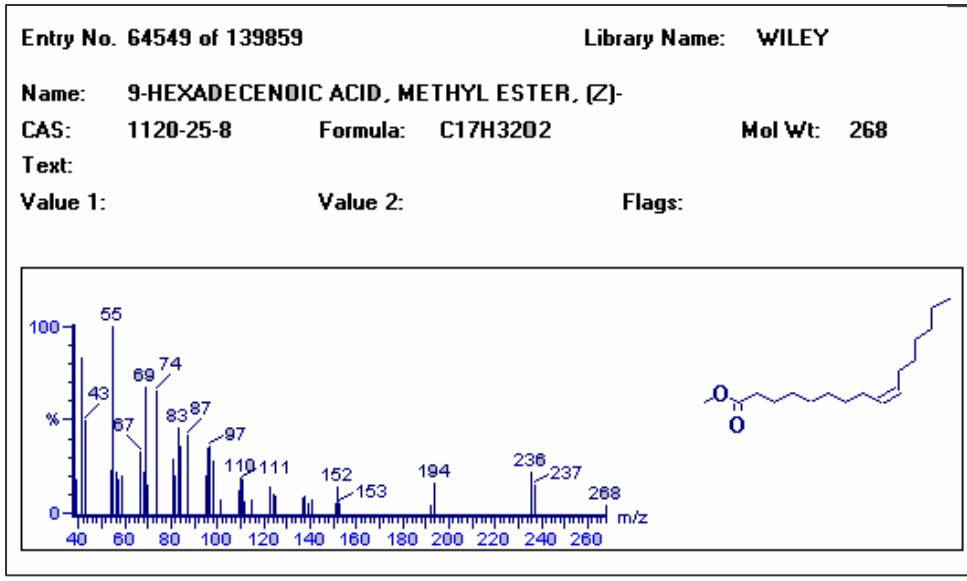

Figure V-37- 9-Hexadecenoic acid methyl ester GC-MS spectrum.

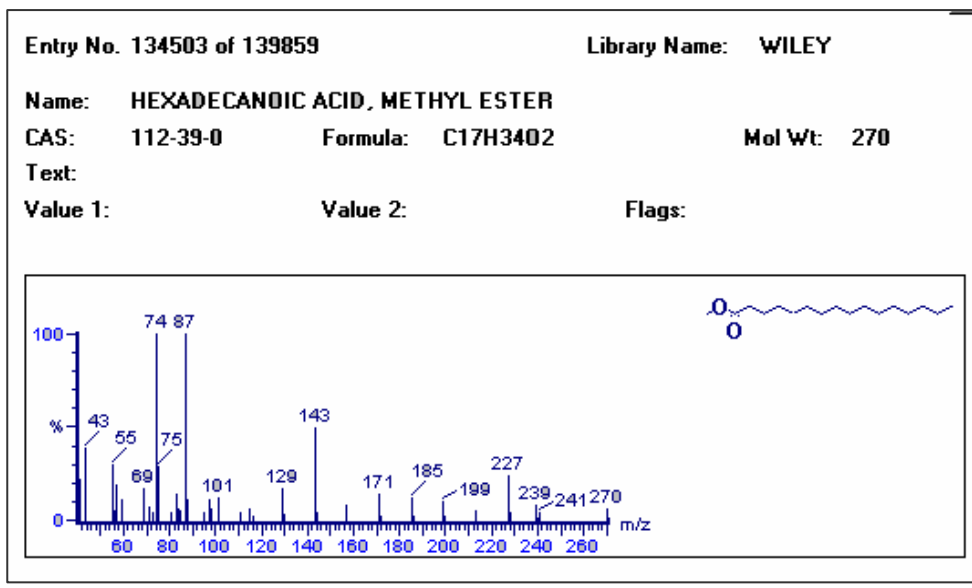

Figure V-38- Hexadecanoic acid methyl ester GC-MS spectrum.

VI- PROFILING FLAVOR COMPOUNDS OF POTATO CRISPS DURING STORAGE USING SOLID PHASE MICROEXTRACTION

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Abstract

Headspace solid phase microextraction (HS-SPME) was studied as a solvent free alternative method for the extraction and characterisation of volatiles in stored potato crisps by capillary gas chromatography coupled with mass detection.

Better results were obtained when extraction was carried out at 70ºC using a DVB/CAR/PDMS fiber. The fiber was exposed for 20 min (extraction time) to the sample headspace, immediately after an equilibrium time of 5 min (time needed to reach the equilibrium between sample and above headspace).

A total of 31 compounds were identified in oxidised potato crisps and resulted mainly from the degradation/rearrangement of lipids and carbohydrates.

Keywords: Solid Phase Microextraction; Volatile Compounds; Lipid Oxidation; Potato Crisps; Gas chromatography-Mass detection.

VI-1. Introduction

Consumers' acceptance of foodstuffs is closely related to its flavor. Therefore, it is not surprising the interest on developing newer methods for volatile compounds analysis.

Conventional methods for extraction and pre-concentration of volatiles include: liquid-liquid extraction [1], distillation [2-4], supercritical fluid extraction [5] and solid phase extraction [6]. These present several disadvantages: are time-consuming, use expensive and hazardous organic solvents, loss analytes during extraction, need sophisticated equipment and require large sample volume [7-9].

Static [10-11] and dynamic headspace [4, 12-13] as well as purge and trap [14] methods are fast, simple and solvent free, although presenting the risk of leaks and possible generation of artefacts [15].

In order of overcoming the main drawbacks of these approaches a method has been developed: solid phase microextraction (SPME) [8, 16]. This sample preparation technique, which enables the simultaneous extraction and pre-concentration steps, has been used, among in other applications, in the study of the volatile profile of foodstuffs [7]. It is very simple, fast, portable, inexpensive and can handle the sample matrix directly [17]. Therefore, reduce analysis time allowing to process a higher number of samples and avoiding loss of analytes. Furthermore, it presents an important advantage: does not use solvents, which contribute for environmental pollution, health hazard as well as purchase and disposal costs.

Due its advantages, SPME has already been applied to a wide range of matrices, like: meat [18-19]; fruits [15, 20-22]; truffles [23]; virgen olive oil [24]; cheese [7]; vinegar [9] and alcoholic beverages [25-27].

SPME bases on the adsorption of analytes directly from samples onto a coated fused silica fiber. The extraction of analytes can be performed with the fiber directly immersed in the sample (in the liquid state)- direct immersion SPME (DI-SPME) or otherwise, with the fiber exposed in the vapour phase above a gaseous, liquid or solid sample- headspace-SPME (HS-SPME). Using HS-SPME the fiber is not in contact with the sample, increasing, this way, the fiber lifetime. Moreover, HS-SPME is more recommended for highly volatile analytes once the low volatility of larger molecules may decrease the mass transfer from the sample to the headspace, originating a longer extraction time [1, 8, 17, 25].

Finally, the analytes are thermally desorbed in the GC injector or in a desorption chamber employing the mobile phase, when liquid chromatography is applied, and they are separated on the column. The result is a "fingerprint" chromatogram, where it is possible to determine compounds that are responsible for the off-flavors.

The high oil content of potato crisps, which come from the frying process, provides a great vulnerability to oxidative rancidity, reducing the crisps shelf-life [28].

The present paper reports the development of a SPME sampling method useful for the investigation of volatile compounds released during the storage of potato crisps.

Herein, crisps flavor was studied, by means of an accelerated storage test, which is commonly used to obtain information on product stability. The later was performed in order to identify the compounds formed during lipid oxidation process.

VI-2. Experimental

VI-2.1. Sampling

Potato crisps were purchased from a supermarket in Santiago de Compostela (Spain). They contained: 33.2% lipid; 7.2% protein; 14.3% sugar; and less than 1% sodium. After a GC-MS analysis, they were stored in presence of natural light. Potato crisps brand was selected regarding the type of oil used to fry and the packaging film employed. Selected crisps were fried in olive oil and were packed with a transparent film in order to evaluate the changes in the profile of volatiles substances under accelerated conditions of oxidation.

Immediately before analysis, potato crisps were ground using an electronic grinder (Taurus, G70, Lerida, Spain).

VI-2.2. Analytical standards

Hexanal (CAS 66-25-1), octanal (CAS 124-13-0), decanal (CAS 112-31-2), octanol (CAS 111-87-15) and hexanoic acid (142-62-1) were supplied from Sigma Aldrich (Madrid, Spain) and had a purity above 99%.

VI-2.3. SPME procedure

The SPME fibres and the manual holder were purchased from Supleco Co. (Bellefonte, PA). The following types of SPME fibres were used: poly(dimethylsiloxane) (PDMS)/divinylbenzene (DVB) with 65 μm thickness; carboxen (CAR)/PDMS with 75 μm thickness; DVB/CAR/PDMS with 50/30 μm thickness. Fibers were conditioned following the manufacturer's instructions previous to use: PDMS/DVB

was inserted 30 min at 260ºC; carboxen/PDMS was inserted 30 min at 280ºC while DVB/CAR/PDMS was inserted 4 h at 270ºC to condition.

Approximately 0.1g of ground sample was placed in a 20 ml clear glass vial (Sun International Trading, USA) and hermetically sealed with a Teflon-coated silicone cap. Vials were heated at 70ºC for 5 min to condition for the equilibrium time (time needed to reach the equilibrium between sample and above headspace).

After equilibrium time, the fiber was introduced into the vial and exposed to the sample headspace during 20 min (extraction time). Following sampling, the fiber was retracted and removed from the vial. Fibers were immediately thermal desorpted in the injection port during 3 min at 260ºC in order to prevent possible contamination. Each SPME sampling was conducted in triplicate.

VI-2.4. Gas chromatography/ Mass spectrometry

GC/MS analysis was performed in a mass selective detector MD 800 coupled to a gas chromatograph Fisons model 8000 (Manchester, UK).

The injector port was lined with a 0.8 mm I.D. narrow-bore glass liner (SGE, USA) and maintained at 260ºC. The fused silica capillary column DB-5 (30m x 0.25 mm i.d. x 1 μm film thickness) equivalent to a 5% phenyl, 95% methylsiloxane (DB J&W Scientific, CA; USA) was used.

The head pressure of the carrier gas helium (high purity) was 70kPa. The temperature program was set at an initial 40ºC for 1 min, followed by an increase of 20ºC/min to 120ºC, held for 8 min, then increased to 260ºC at a rate of 20ºC/min and held for 2 min. Purge and bottom valves were closed for 2 min.

All analyses were conducted under the same MS conditions. The MS detector was operated in the full scan mode with 70 eV electron ionisation, by scanning a mass range of m/z 35-300 in 0.45s.

The system was computer-controlled using the Masslab (version 1.4) software.

VI-2.5. Compounds identification

Components were identified by matching their mass spectra with the Wiley spectral library. Compounds were identified with a resemblance percentage above 85%.

Some of the most predominant flavor compounds were further identified by comparison of their retention times with those of pure standard compounds (hexanal, octanal, octanol, decanal and hexanoic acid).

VI-3. Results and Discussion

VI-3.1. Optimisation of extraction conditions

In the past few years the SPME technique has suffered important advances in order to achieved optimal results. Therefore, today are available several types of coating fibers for the compounds extraction which differ on the polarity (chemical nature) and thickness of the stationary phase [8].

The selection of the appropriate fiber should be made in accordance with the properties of the analytes. The amount of compounds extracted depends on the different affinities of the compounds for the fiber and on the competition phenomenon. This way, a non-polar fiber, like polydimethylsiloxane (PDMS), extracts, mainly, non-polar compounds

(volatile compounds) while a more polar fiber, such as polyacrylate (PA) presents a strong discrimination towards non-polar analytes, extracting from matrix more polar compounds (phenols and alcohols) [26].

Mixed phases such as CAR-PDMS, CW-DVB and DVB-CAR-PDMS present medium polarity, reducing the discrimination towards very nonpolar and polar volatile compounds. Therefore, they are preferred when a multicomponent analysis is carried out. Many authors have compared fiber coating to select the most appropriated [7, 11, 20, 26, 29]. Mixed fibers, which contain carboxen or/and divinybenzene absorbers presented better results when compared with "single fibers" (PDMS or PA fibers) [8, 11, 26, 30].

Therefore, a comparison of three mixed fibers: PDMS/DVB (partially crosslinked); CAR/PDMS (partially crosslinked); DVB/CAR/PDMS (highly crosslinked) was conducted to select the optimum fiber coating for the analysis of potato crisps volatiles. The inter-fiber comparison was performed, according F. Augusto et al [20], calculating the normalised extraction efficiency Nx. This parameter was defined as

$$N=100\ [\Sigma x/\Sigma(DVB/CAR/PDMS)]$$

where Σx is the sum of the areas of the detected peaks of stored potato crisps with the fiber x and Σ(DVB/CAR/PDMS) is the corresponding sum with DVB/CAR/PDMS fiber.

Fig. VI-1 shows the results obtained which reveal that the amounts extracted with DVB/CAR/PDMS are slight larger than the amounts extracted with the PDMS/DVB (R = 99.66%). PDMS/DVB has already been selected for the analysis of volatile compounds from infant milk powder oxidation [31] and for the analysis of volatile compounds of coffee beans [11] due to its good performance. CAR/PDMS showed the lowest extraction efficiency (N = 95.55%). In spite of the lower extraction

efficiencies of CAR/PDMS and DVB/PDMS, they have extracted a remarkable number of compounds. Results are an average of duplicate extractions and the variation between duplicates was greater for CAR/PDMS fiber (10.29%), 5.68% for PDMS/DVB fiber and smaller for DVB/CAR/PDMS (1.80%).

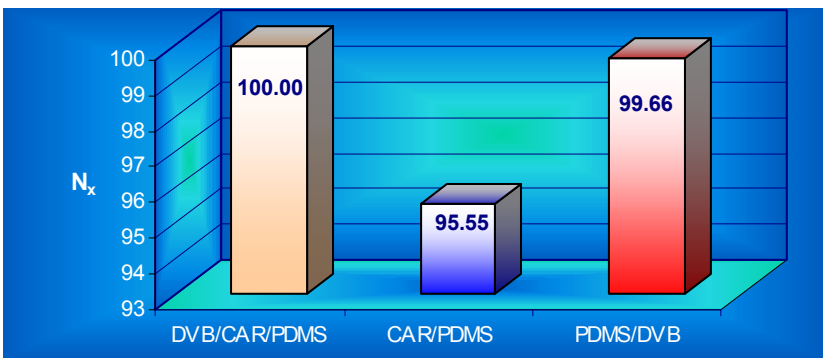

Figure VI-1- Normalized extraction efficiencies Nx measured for extractions with CAR-PDMS, PDMS-DVB, DVB-CAR-PDMS.

From these results, DVB/CAR/PDMS was selected for profiling volatiles of stored potato crisps. This fiber has already been chosen for the analysis of truffles aroma [23] and for determination of the fungicides in white wines [26] due to the presence of the adsorbents DVB and CAR.

With respect to the selection of the extraction mode, the HS-SPME mode was selected regarding all its inherent analytical advantages: the lowest detection limits, the shortest analysis time, the requirement of any sample preparation [17].

In SPME, the amount of compounds extracted depends also on the adsoption time, adsorption temperature and concentration of the analyte in the matrix [7-8].

The adsorption temperature and time were set at 20 min and 70ºC respectively for all analysis. Samples were heated to enhance the release

of analytes into the headspace. However, much attention has to be taken to avoid artifacts once high temperature increase the likelihood of sample alteration with consequent artifactual oxidation [29].

An equilibrium time (time needed to reach the equilibrium between sample and above headspace) of 5 min was set. Selected conditions were optimised in order to avoid fiber and/or detector saturation even for the more concentrated sample (more oxidised potato crisps).

Sample size is critical on the extraction once influences the amount of volatiles compounds extracted. Therefore, distinct quantities of sample were assayed (0.1-0.5g). A great sample amount produces fiber saturation while a small sample size may yield a considerable variation coefficient. Best results were obtained with 0.1 g sample, when using the 0.5 mm i.d. liner and 1 g sample with the 2 mm i.d. liner.

VI-3.2. Optimisation of desorption conditions

The injection port temperature as well as the desorption time were optimised to ensure that volatiles were totally desorbed from fiber. The optimal results were achieved with a temperature of 260ºC for 3 min. Memory effect of the fiber was excluded with the blank analysis performed after each run.

The fibers lifetime used to be approximately of 60 samples. Fiber ageing, which used to originate new peaks, was controlled with running blank samples. Moreover, fiber position in the GC was also studied, considering that the injector is not uniformly heated [17]. A desorption deep of 3 cm was chosen to perform all experiments. The normal inlet liner (2 mm i.d.) was changed by a narrow (0.8 mm i.d.) liner in order to improve GC resolution.

The internal diameter of the liner has a significant effect on the chromatographic peaks of extracted compounds. Peak broadening effect is remarkably minimized for analytes with low boiling points (b.p.) and almost eliminated for compounds with higher b.p.. Fig. VI-2 shows the chromatogram obtained from an oxidised crisps sample using a 2 mm liner. Table VI-1 compares the compounds identified in oxidised potato crisps after SPME extraction with two liners of different internal diameters. With the 2 mm i.d. liner, 27 compounds were identified, while using a 0.8 mm liner, 31 compounds were identified.

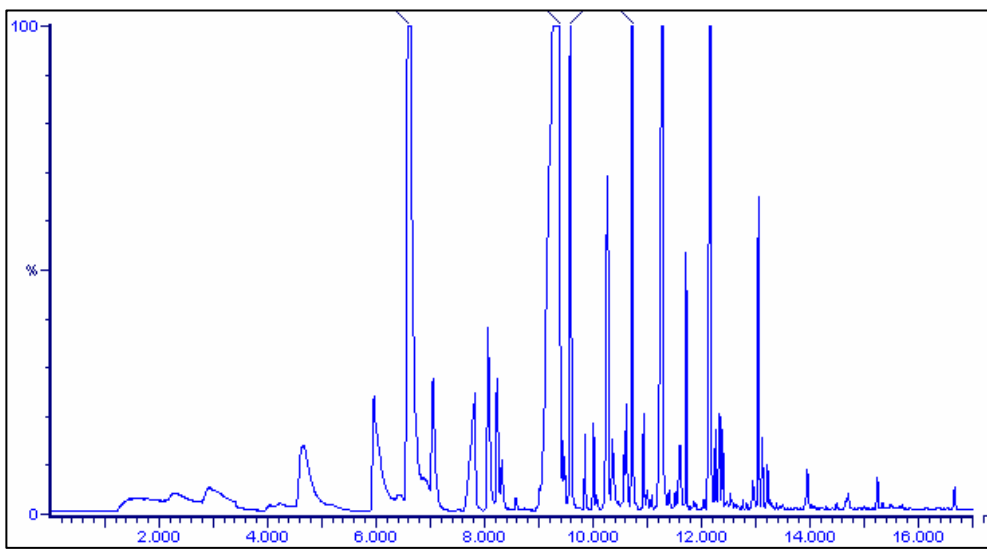

Figure VI-2- GC-MS chromatograms of potato crisps flavor after extraction with a 2 mm i.d. liner.

The repeatability of the method was determined for the two liners by using six replicate samples of potato crisps stored during the same period of time. Repeatability depends from compound to compound but, in general, using 1 g sample and 2 mm i.d. liner enables lower CV% (average 9.4%) than 0.1 g sample with a 0.8 mm liner (average 10.9%).

Table VI-1- Comparison of compounds identified using liners with different internal diameters.

Group	Compound	Liner	
		2 mm I.D.	**0.8 mm I.D.**
Alcohols	3-Methyl, 1-butanol	x	x
	Heptanol	x	x
	1-Octanol	x	x
	1,5-Heptadiene-3,4-diol		x
	1-Nonanol	x	x
Aldehydes	3-Methyl butanal	x	x
	Hexanal	x	x
	Heptanal	x	x
	Octanal	x	x
	Nonanal	x	x
	Decanal	x	x
	trans,trans-2,4-Nonadienal		x
	2-Undecenal		x
Carboxilic acids	Acetic acid	x	x
	Propanoic acid		x
	Hexanoic acid	x	x
	Heptanoic acid	x	x
	Octanoic acid	x	x
	2-Octenoic acid		x
	Nonanoic acid	x	x
	Decanoic acid	x	
Esters	Pentyl ester formic acid	x	
	Pentyl ester hexanoic acid	x	x
Furans	Tetrahydro-2-methyl furan		x
	2-Pentyl furan		x
Hydrocarbones	Pentyl oxirane	x	
	6-Methyl-1-Heptene		x
	Bycyclo-2,2,2-1-methyl-octane	x	
Ketones	2-Heptanone	x	x
	3(E), 3-octen-2-one	x	x
	2-Nonanone	x	x
	3-Nonen-2-one	x	x
	2-Decanone	x	x
Others	trans-tetrahydro-5,6-dimethyl-2H-2-pyranone		x
	5-Ethyl-didhydro-2(3)-furanone		x
	5-Pentyl-2(5)-furanone	x	
	5-Hexylhydro-2(3)-furanone	x	
		27	31

The main reason for this is the small sample size that, as we have already said, yields a considerable variation coefficient.

With the aim of obtaining a good chromatographic resolution from the headspace stored potato crisps, several ramp temperatures were studied. The chosen ramp (Experimental) allowed to separate better and therefore identify more volatile compounds released during the storage of potato crisps.

VI-3.3. Volatile compounds formed during crisps storage

A GC-MS analysis was carried out in order to identify the volatile compounds of stored potato crisps. Fig. VI-3 shows the total ion current (TIC) mass chromatogram of potato crisps after a 3-month storage period at room temperature in presence of natural light.

The identified and quantified (%) compounds in oxidised potato crisps are listed in table VI-2. The values correspond to the arithmetic mean ± standard deviations of 6 samples stored in the same conditions. These correspond to the following chemical families: Alcohols (1); Aldehydes (2); Carboxilic acids (3); Esters (4); Furans (5); Hydrocarbones (6); Ketones (7); Others (8). These compounds accounted for 87.8% of the total integrated peak areas.

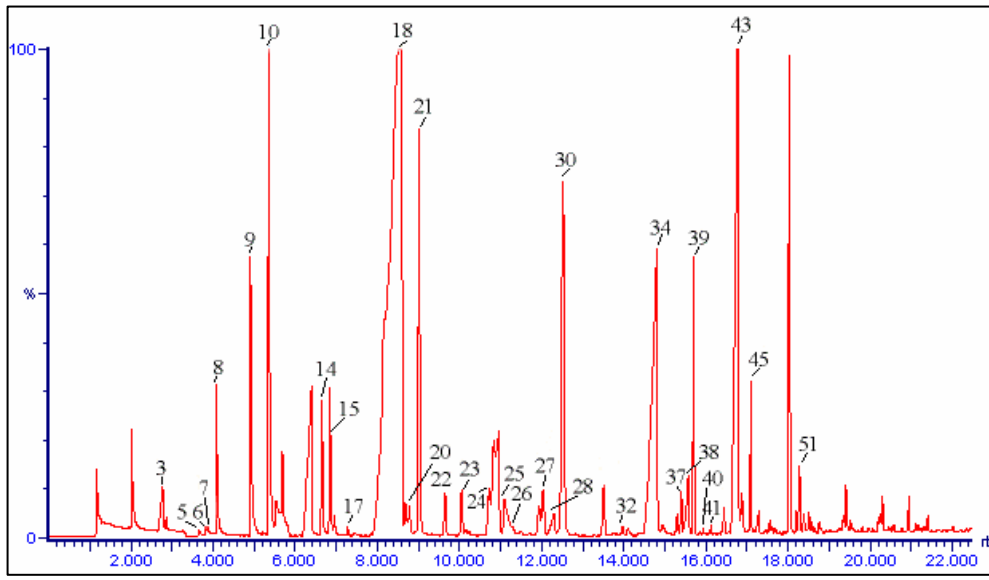

Figure VI-3- Total ion current (TIC) mass chromatograms of oxidized potato crisps.

A consideration that had to be taken in account was that no peaks appeared in the blank runs, thus indicating that no compounds due to the fiber or contamination were expected.

VI-3.3.1. Aldehydes

The aldehydes group constitute the second most important family of volatile compounds from a quantitative point of view in oxidised potato crisps (19%).

Most of the identified aldehydes presented straight chain such as hexanal, heptanal, octanal, nonanal, decanal, trans, trans-2,4-nonadienal and 2-undecenal.

Table VI-2- Compounds identified and quantified (%) in oxidized potato crisps after SPME extraction.

Peak No	Compound Name	RT (min)	Type	Oxidised potato crisps (% area ± SD)
3	Acetic acid	2,79	4	0,391 ± 0,084
5	Trans-tetrahydro-5,6-dimethyl-2H-2-pyranone	3,68	8	0,063 ± 0,010
6	Tetrahydro-2-methyl furan	3,83	5	0,055 ± 0,009
7	Propanoic acid	3,90	4	0,069 ± 0,015
8	3-Methyl butanal	4,11	1	0,966 ± 0,152
9	3-Methyl, 1-butanol	4,94	3	1,524 ± 0,126
10	Hexanal	5,39	1	6,220 ± 0,525
14	2-Heptanone	6,68	2	0,857 ± 0,082
15	Heptanal	6,88	1	0,960 ± 0,078
17	1-Heptene-6-methyl	7,31	6	0,060 ± 0,007
18	Hexanoic acid	8,77	4	38,365 ± 1,557
20	2-Pentyl furan	8,82	5	0,192 ± 0,046
21	Octanal	9,07	1	3,567 ± 0,137
22	Heptanol	9,70	3	0,400 ± 0,032
2	3(E), 3-Octen-2-one	10,11	2	0,436 ± 0,038
24	5-Ethyl didydro-2(3)-furanone	10,78	8	0,531 ± 0,107
25	Heptanoic acid	11,11	4	3,979 ± 0,660
26	1-Octanol	11,30	3	0,771 ± 0,084
27	2-Nonanone	12,01	2	0,369 ± 0,016
28	1,5-heptadiene-3,4-diol	12,10	3	0,531 ± 0,055
30	Nonanal	12,60	1	4,998 ± 0,147
32	3-Nonen-2-one	14,03	2	0,094 ± 0,008
34	Octanoic acid	14,90	4	8,848 ± 0,506
37	2-Decanone	15,44	2	0,268 ± 0,027
38	2-Octenoic acid	15,64	4	0,756 ± 0,069
39	Decanal	15,74	1	1,849 ± 0,062
40	trans,trans-Nona-2,4-dienal	15,97	1	0,032 ± 0,005
41	1-Nonanol	16,15	3	0,052 ± 0,005
43	Nonanoic acid	16,84	4	9,778 ± 0,864
45	Pentyl ester hexanoic acid	17,12	7	0,778 ± 0,041
51	2-Undecenal	18,23	1	0,113 ± 0,021

These compounds result from a degradative reaction of lipid oxidation of unsaturated fatty acids (oleic, linoleic and linolenic)- autoxidation- which is not enzimatically catalyzed once lipoxygenase and hydroperoxidases are destroyed during thermal processing [32-34].

Indeed, this compounds contribute to the aroma of oxidised crisps due to its low odour threshold [35].

Table VI-3 presents the aldehydes identified as well as the reaction responsible for their formation, compounds from which derive, type of aroma they present and flavor thresholds in oil and water.

Table VI-3- Aldehydes identified in oxidized potato crisps.

Aldehydes	**Reaction of formation**	**Substract**	**Aroma description**	**Odor threshold** (mg/kg)	
				Oil	**Water**
3-Methyl butanal	Strecker degradation	Leucine	-		3
Hexanal	Unsaturated fatty acids oxidation	Linoleic acid	Fatty-oily, green plant-like	320	12
Heptanal	Unsaturated fatty acids oxidation	Oleic/ linoleic acid	Fatty-oily, deep fried-like	3200	5
Octanal	Unsaturated fatty acids oxidation	Oleic/ linoleic acid	Fatty-oily, deep fried-like, soap-like	320	8
Nonanal	Unsaturated fatty acids oxidation	Oleic acid	Fatty-oily, fruity	13500	5
Decanal	Unsaturated fatty acids oxidation	Oleic acid	Orange like	6700	5
trans, trans,2,4-nonadienal	Unsaturated fatty acids oxidation	Linoleic acid	Oily, deep fried-like	2500	-
2-undecenal	Unsaturated fatty acids oxidation	Oleic acid	-	-	-

The amount of hexanal, heptanal, octanal and nonanal increase during storage. Thus, as would be expected, flavor quality decrease with storage time. Hexanal has been used an indicator of the state of lipid oxidation because its concentration increase during storage and it has a low perception threshold which means hexanal plays an important role in the off-flavors of rancid potato crisps. Hexanal come from linoleic acid via the 13-hydroperoxid. Nevertheless, can also appear later as an autoxidation product of the 2,4-decadienal [18, 32].

Besides linear aldehydes, a branched-chain aldehyde was also identified, 3-methyl butanal, which presents a bitter nut, green plant like

aroma [32, 36]. This compound is derived from the amino acid leucine [37]. Its major formation pathway seems to be the oxidative deamination-decarboxylation via Strecker degradation and it has already been identified in green beans [33] especially those pressure cooked once high temperature catalysis its formation reaction [32, 36].

VI-3.3.2. Ketones

Five ketones were identified (table VI-2), of which 3 were methylketones. Ketones as well as the others carbonyl compounds have their origin in the oxidative degradation of unsaturated fatty acids [4].

In many fruits and vegetables these compounds are only minor components but act as potential "feed chemical" for reactions which yield volatile flavor compounds. In potato crisps unsaturated fatty acids come, mainly, from the oil used to fry, in this case, olive oil [38]. Ketones are responsible for the flavor of many fruits; vegetables and dairy products like blue cheeses where they are by-products of the fermentation process [35].

VI-3.3.3. Alcohols

A total of five alcohols were identified. Higher-molecular-weight alcohols tend to exist in the liquid form due to the vapour pressure. Therefore, they have minor significance in the flavor once they present higher odours thresholds [36, 39]. Likewise other carbonyl compounds, they are yielded by chemical degradation of hydroperoxides of unsaturated fatty acids and some may result from the reduction of carbonyl compounds [40].

The alcohol, 3-methyl-1-butanol is generated via Strecker degradation. Leucine reacts with 2-ketoglutamic acid (the amino group acceptor) in a reaction catalysed by L-leucine amino transferase [33].

VI-3.3.4. Carboxylic acids

Carboxilic acids class were the most important compounds identified in potato crisps submitted to analysis after a three month storage. They constituted 65.35% of total peaks area. The most abundant compound in the volatile fraction was hexanoic acid (38.36%) (fig.VI-4).

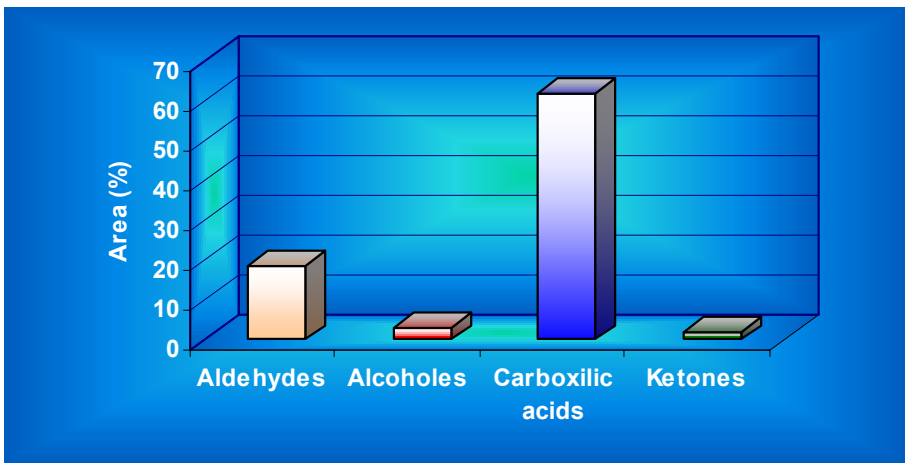

Figure VI-4- Profiles (% area) of the major volatile families of oxidized potato crisps.

Most of them are final products of lipid oxidation reactions, which result from the decomposition of products of fat or by deamination of amino acids [40], therefore they indicate that the analysed potato crisps presented an advanced lipid oxidation state. In general they present fruity, painty, acid and green-like aroma. E. C. Coleman and at [40] have reported the identification of acetic, propanoic, hexanoic and heptanoic acids in baked potato flavor.

VI-3.3.5. Esters

Esters result from the esterification of carboxylic acids and alcohols [35]. Esters, mainly the short chain acids, are responsible for fruity aromas [33]. Therefore, they have been found in olive oil [41]. In stored crisps just pentyl hexanoate was identified with the 0.8 mm liner.

VI-3.3.6. Furans

Two furans (2-methyl tetrahydrofuran and 2-pentyl furan) with a green and fruity aroma have been identified in oxidised potato crisps. 2-pentyl furan has already been identified in baked potatoes [40].

VI-3.3.7. Other compounds

Other compounds identified (1 hydrocarbon- 6-methyl-1-heptene- , 1 furanone and 1 pyranone) present minor importance to crisps flavor.

VI-4. Conclusions

The HS-SPME procedure is a non-evasive, solvent-free method presenting major advantages: simplicity, rapidity, high sensitivity and small sample volume.

The HS-SPME method is appropriated for the volatile profile study of potato crisps. It allowed the identification of 31 compounds, most of them resulted from degradation/ rearrangement of lipids and carbohydrates and have grassy notes.

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APPENDIX II

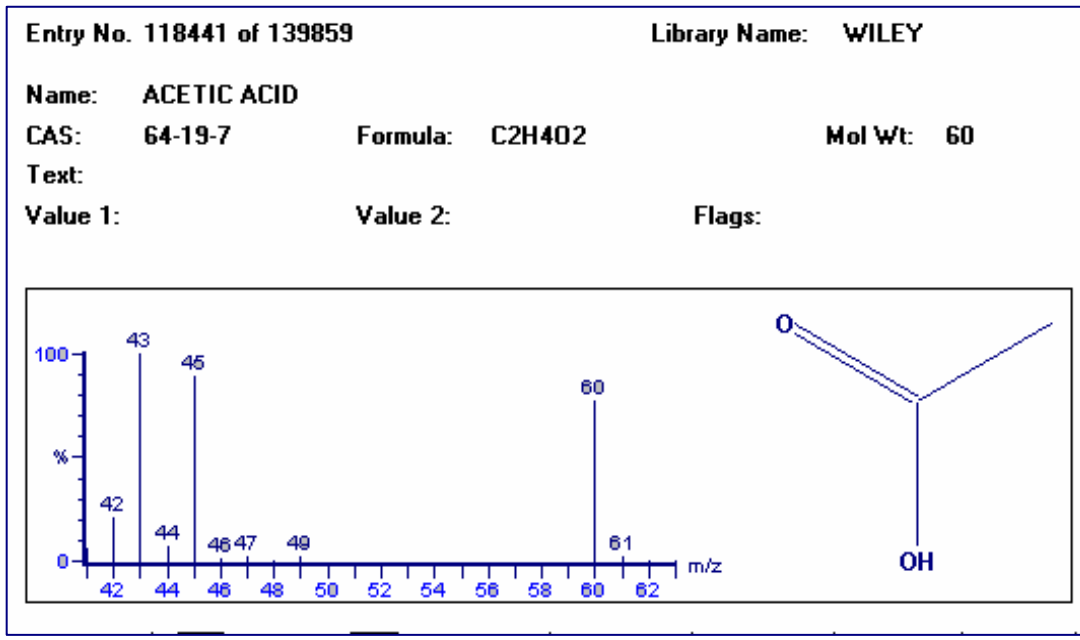

Figure VI-5- Acetic acid GC-MS spectrum.

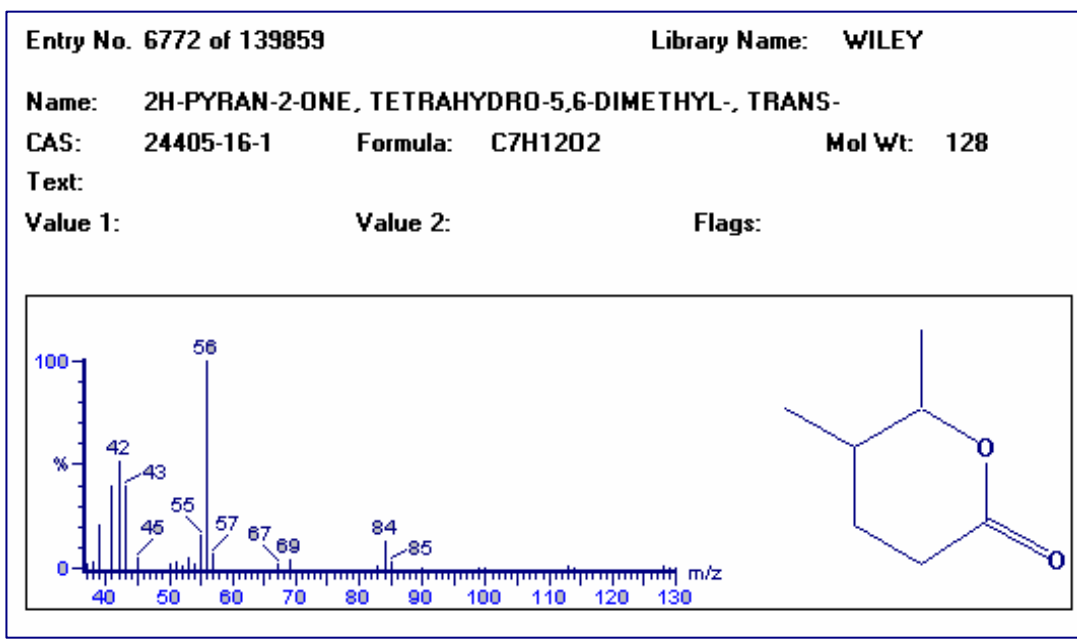

Figure VI-6- 2H-pyran-2-one, tetrahydro-5,6-dimethyl-, trans-, GC-MS spectrum.

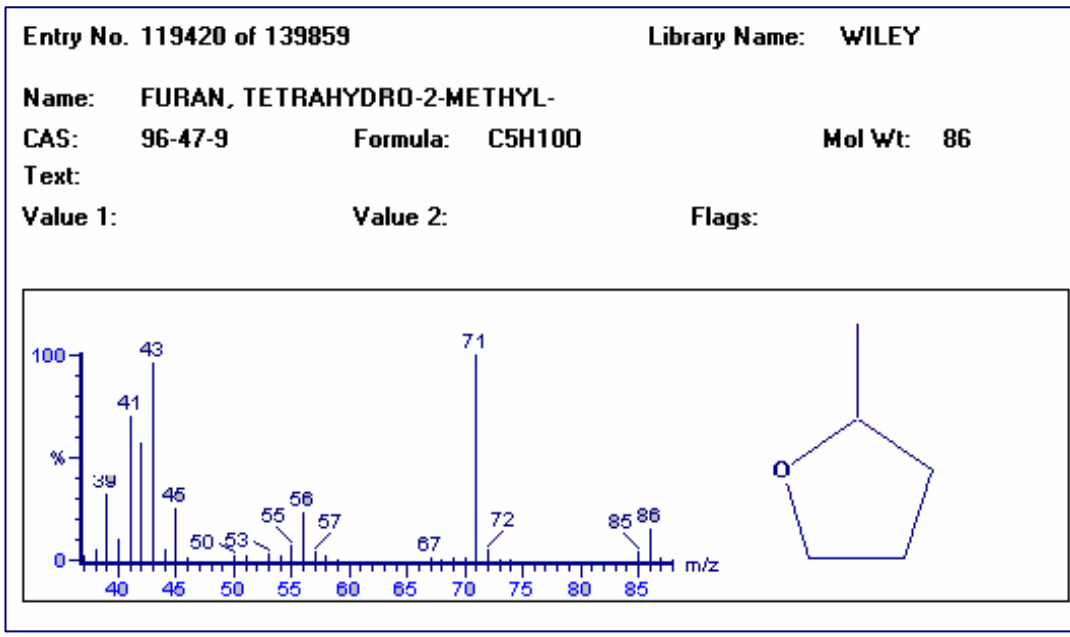

Figure VI-7- Tetrahydro-2-methyl-furan GC-MS spectrum.

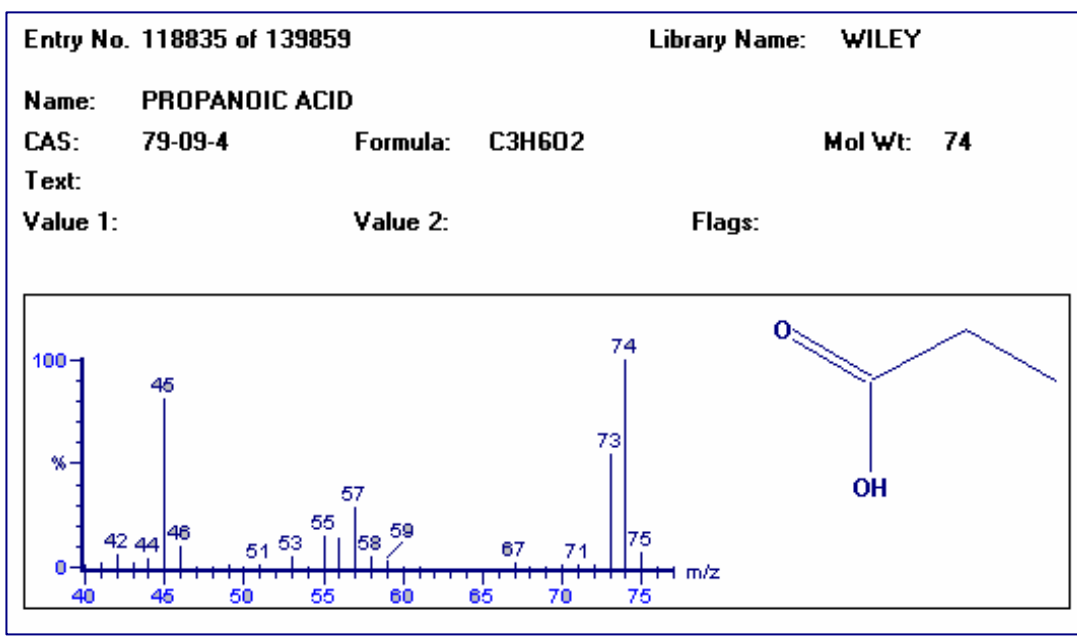

Figure VI-8- Propanoic acid GC-MS spectrum.

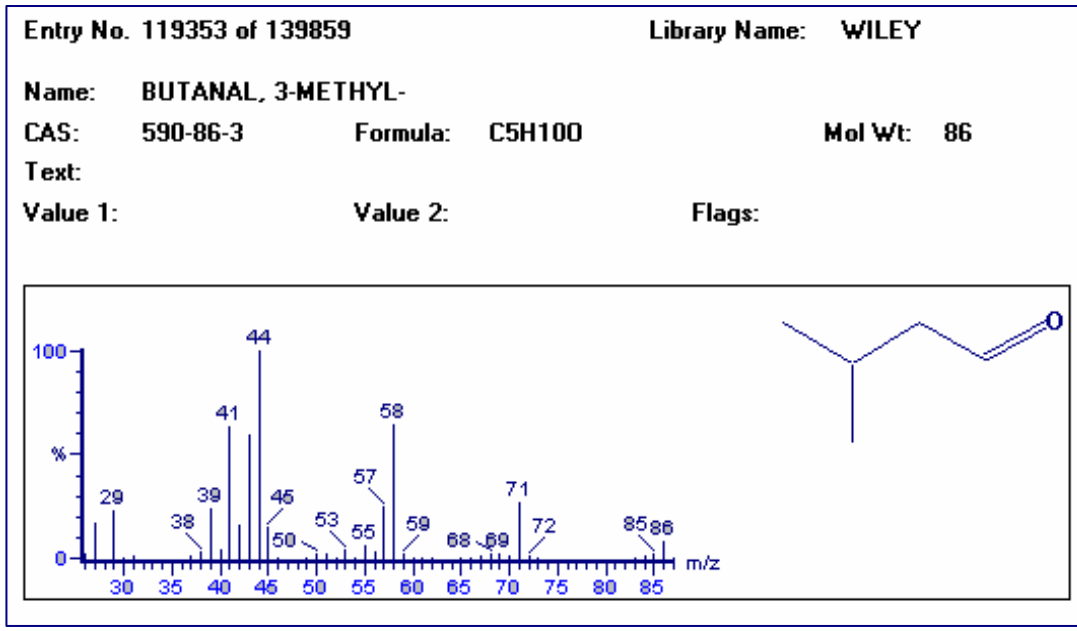

Figure VI-9- 3-Methyl butanal GC-MS spectrum.

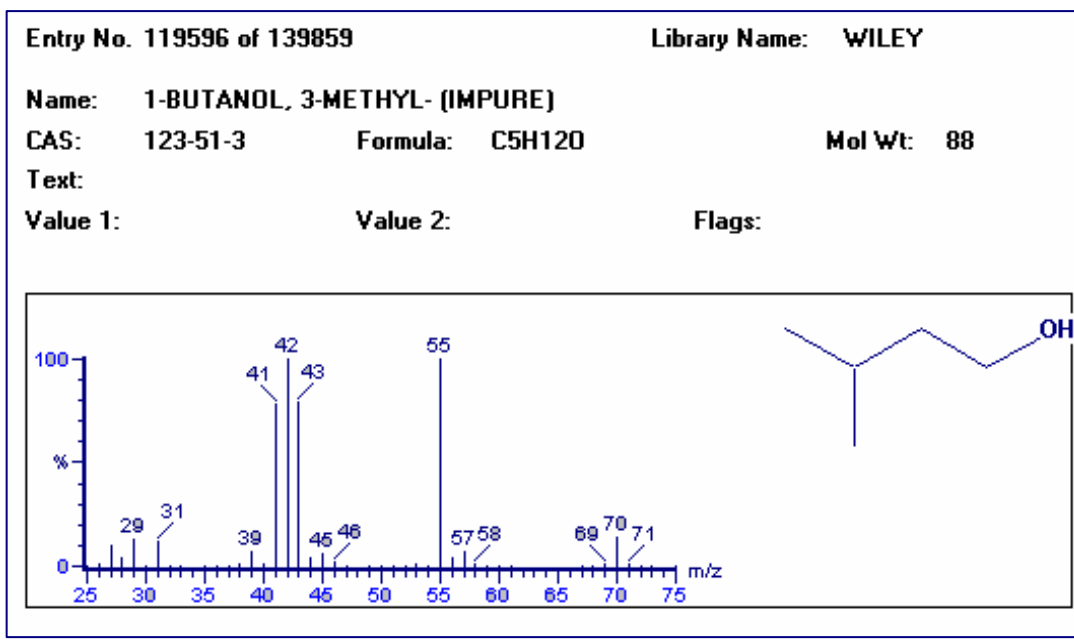

Figure VI-10- 1-butanol, 3-methyl (imp.) GC-MS spectrum.

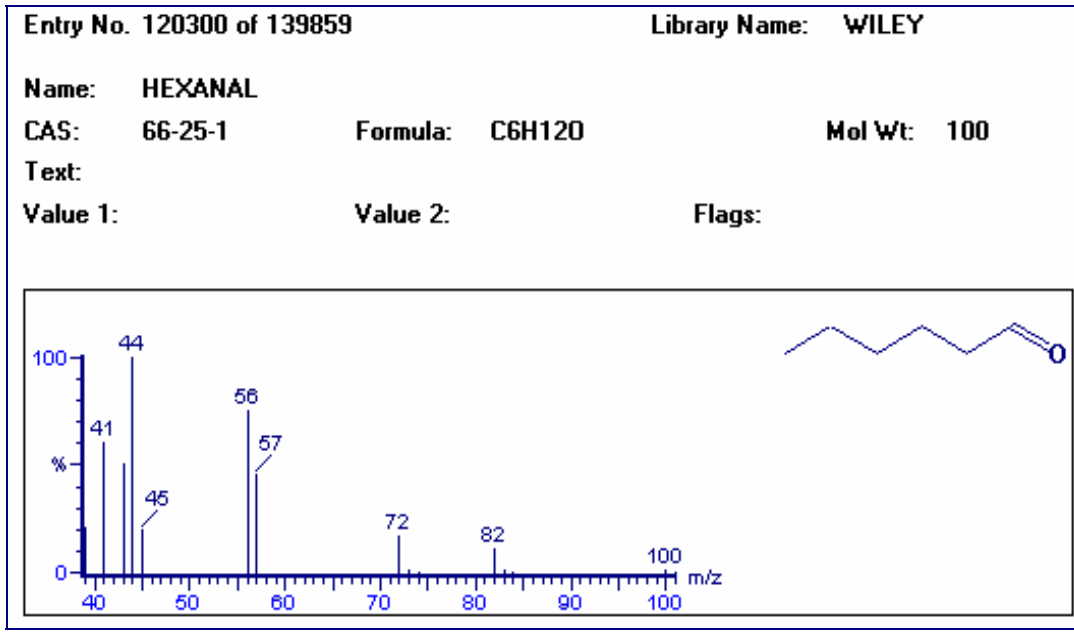

Figure VI-11- Hexanal GC-MS spectrum.

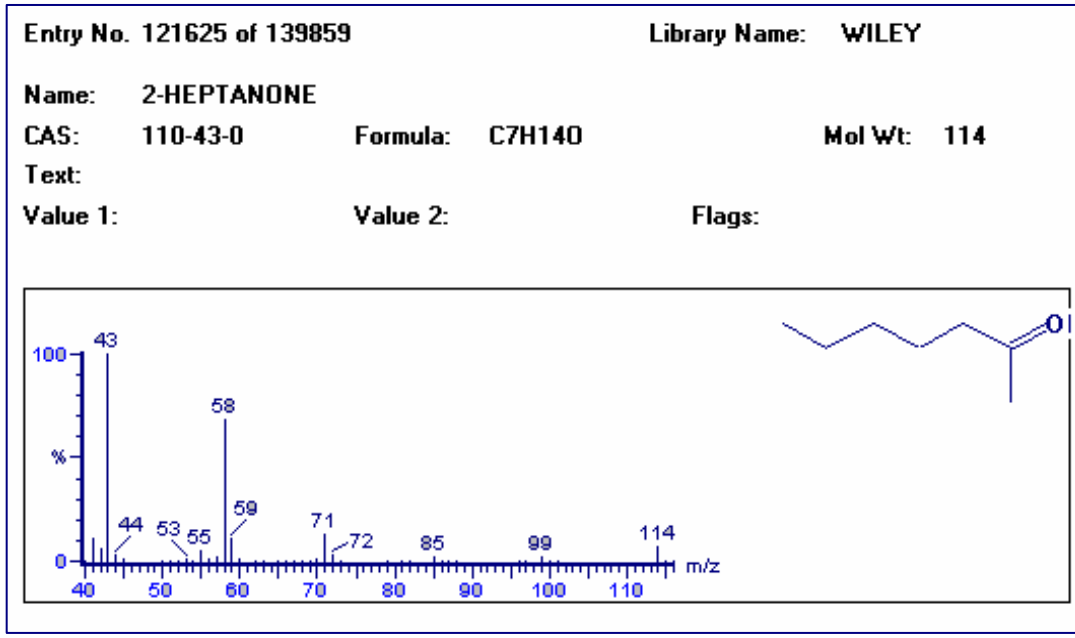

Figure VI-12- 2- Heptanone GC-MS spectrum.

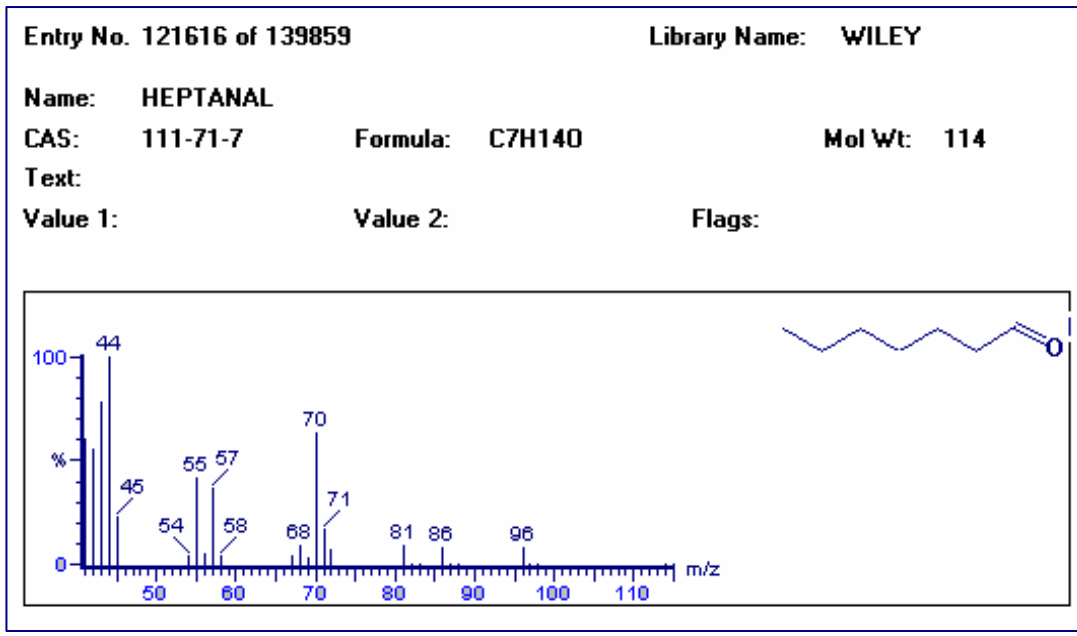

Figure VI-13- Heptanal GC-MS spectrum.

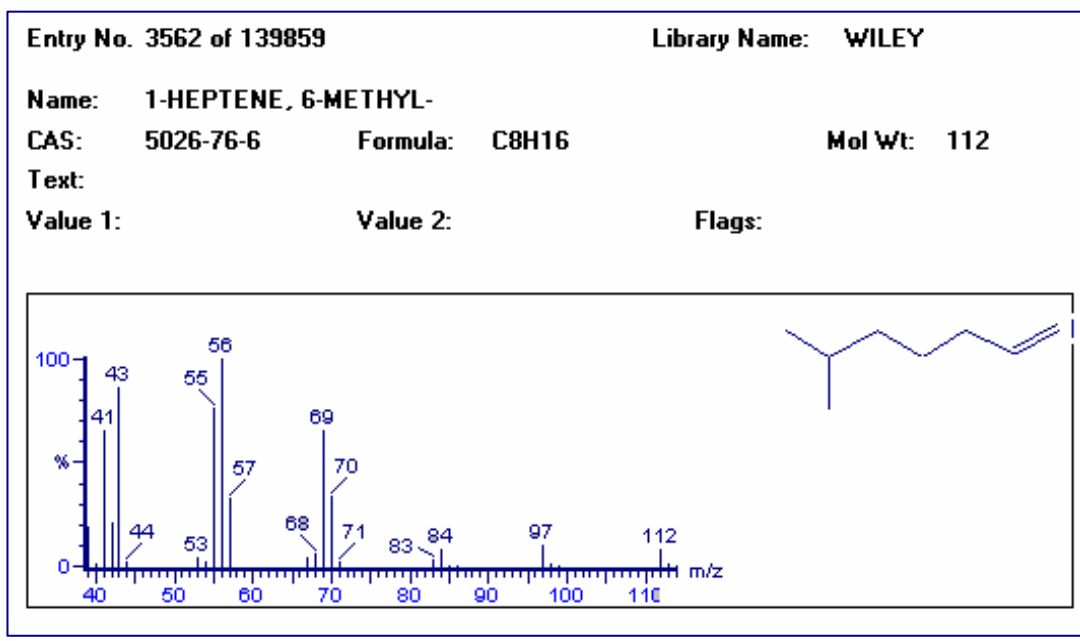

Figure VI-14- 1-Heptene- 6-methyl GC-MS spectrum.

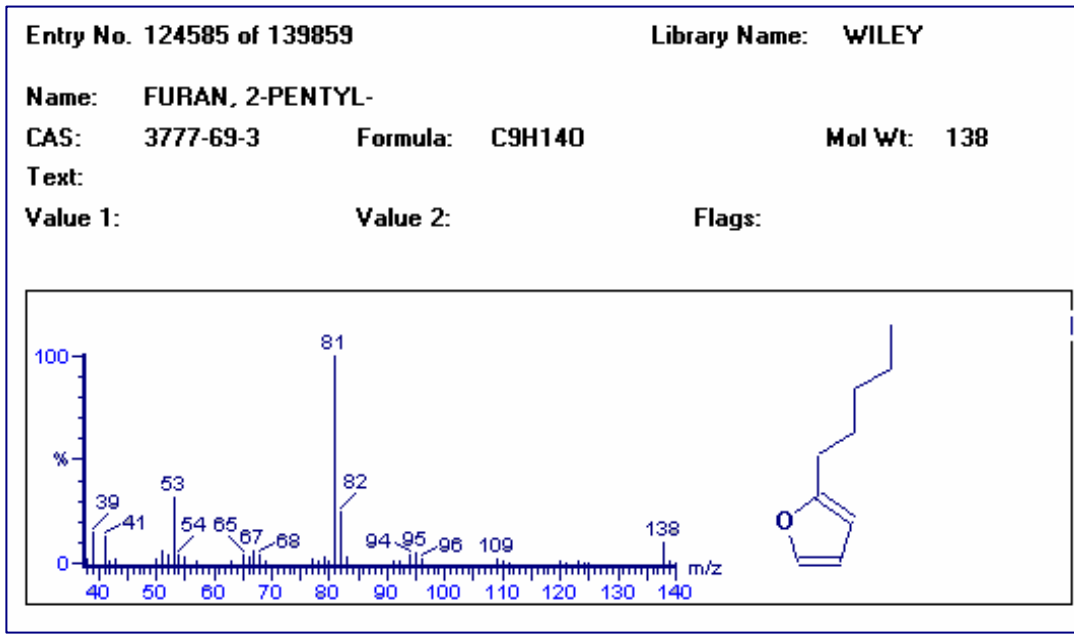

Figure VI-15- 2-Penthyl furan GC-MS spectrum.

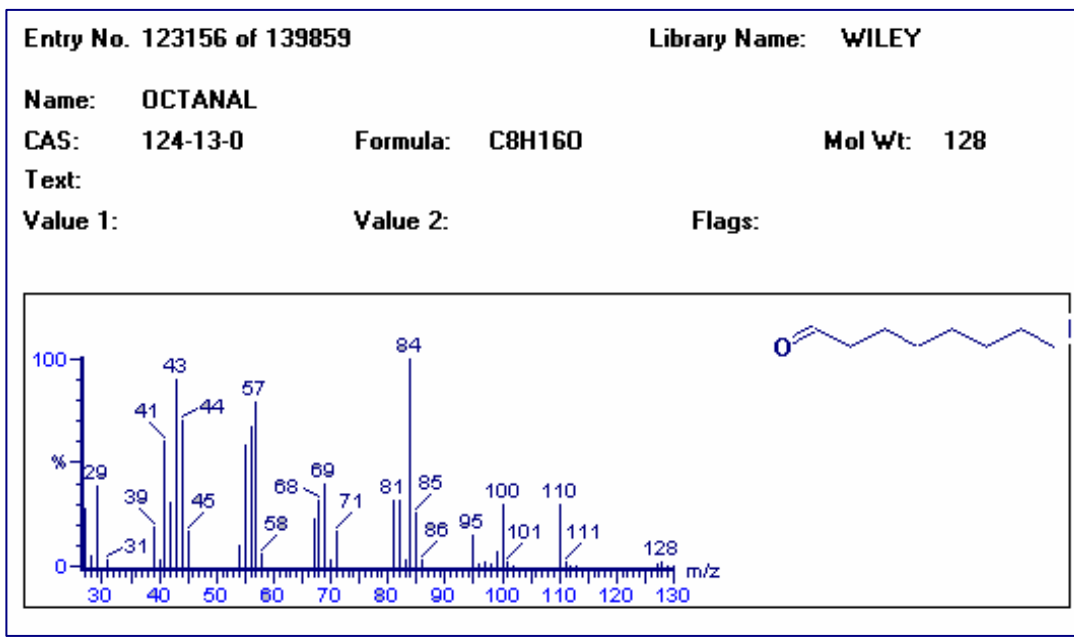

Figure VI-16- Octanal GC-MS spectrum.

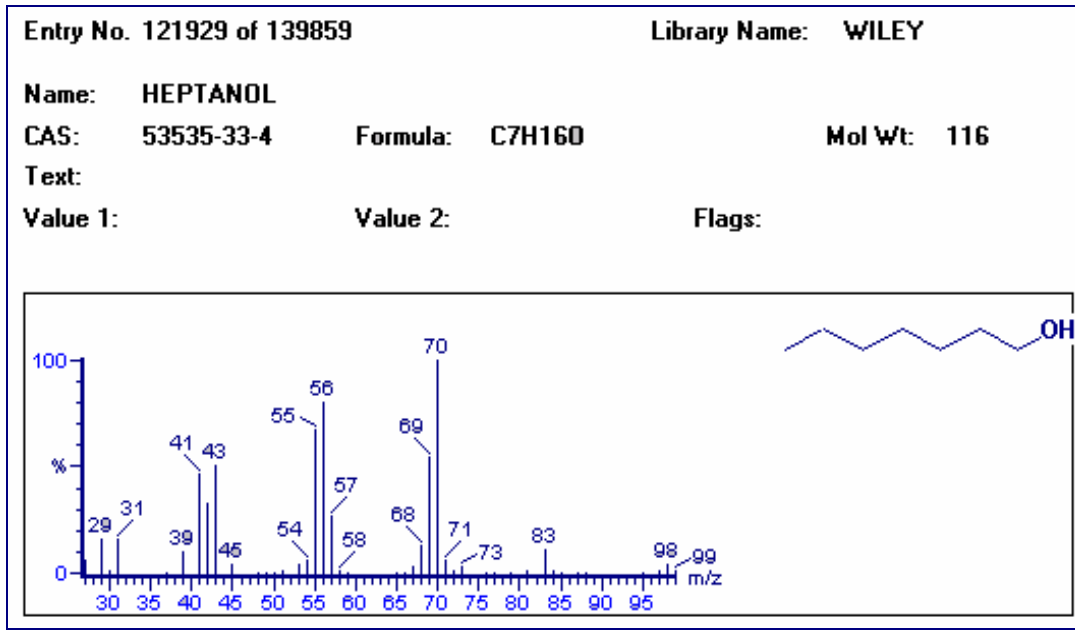

Figure VI-17- Heptanol GC-MS spectrum.

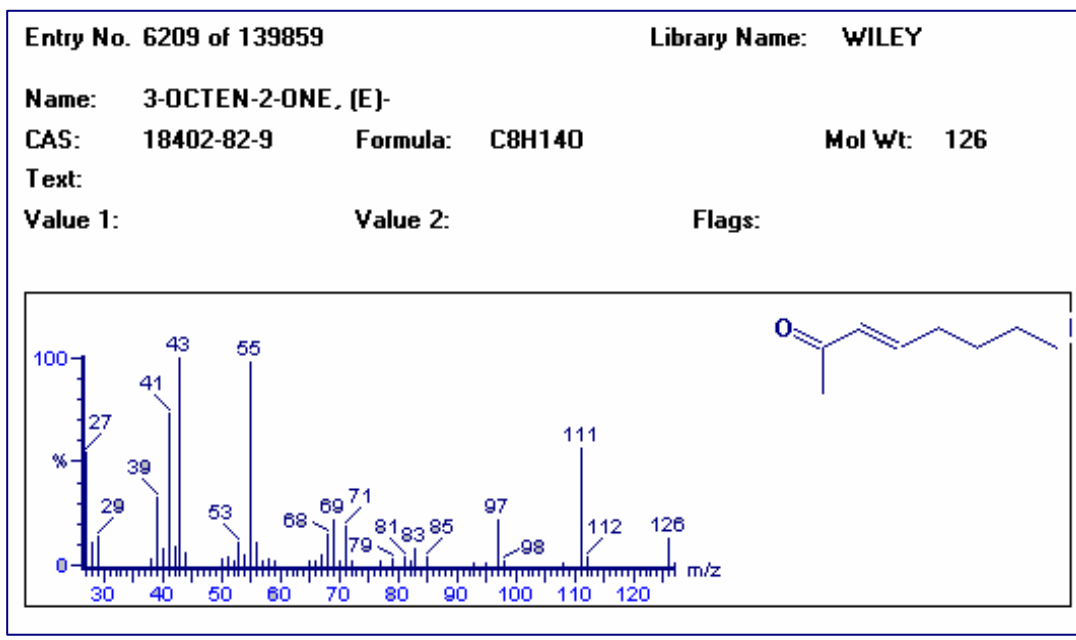

Figure VI-18- 3-Octen-2-one, (E) GC-MS spectrum.

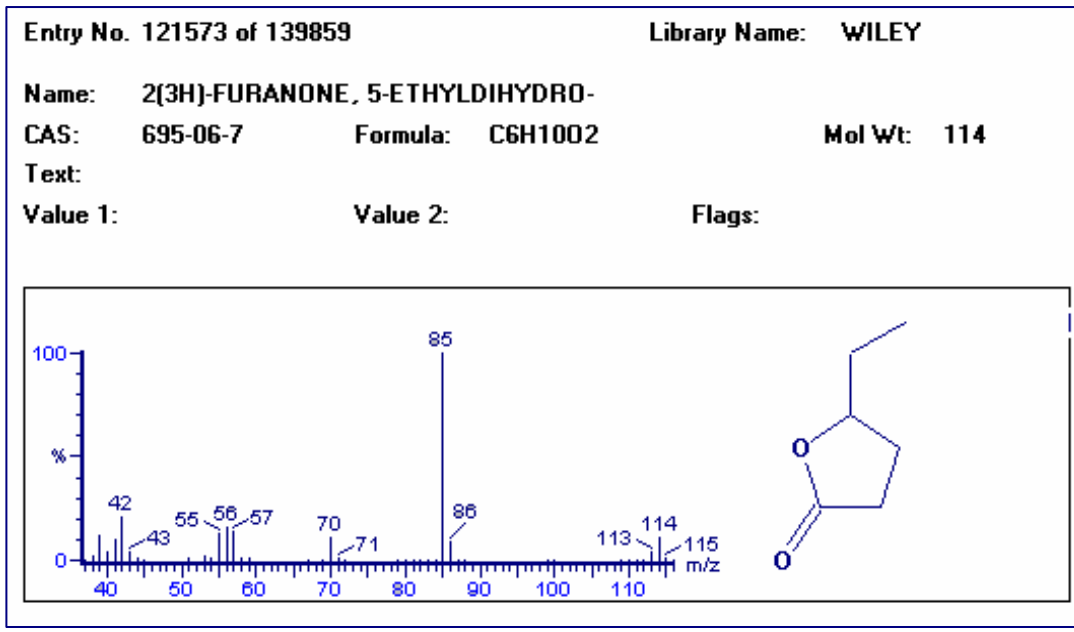

Figure VI-19- 5-ethyl didydro-2(3)-furanone GC-MS spectrum.

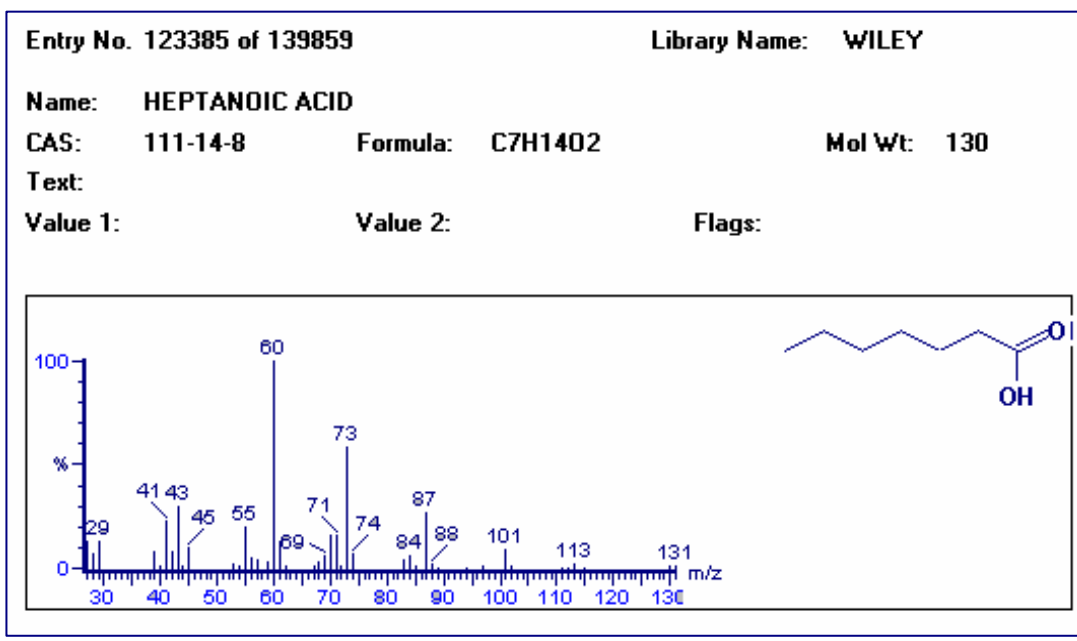

Figure VI-20- Heptanoic acid GC-MS spectrum.

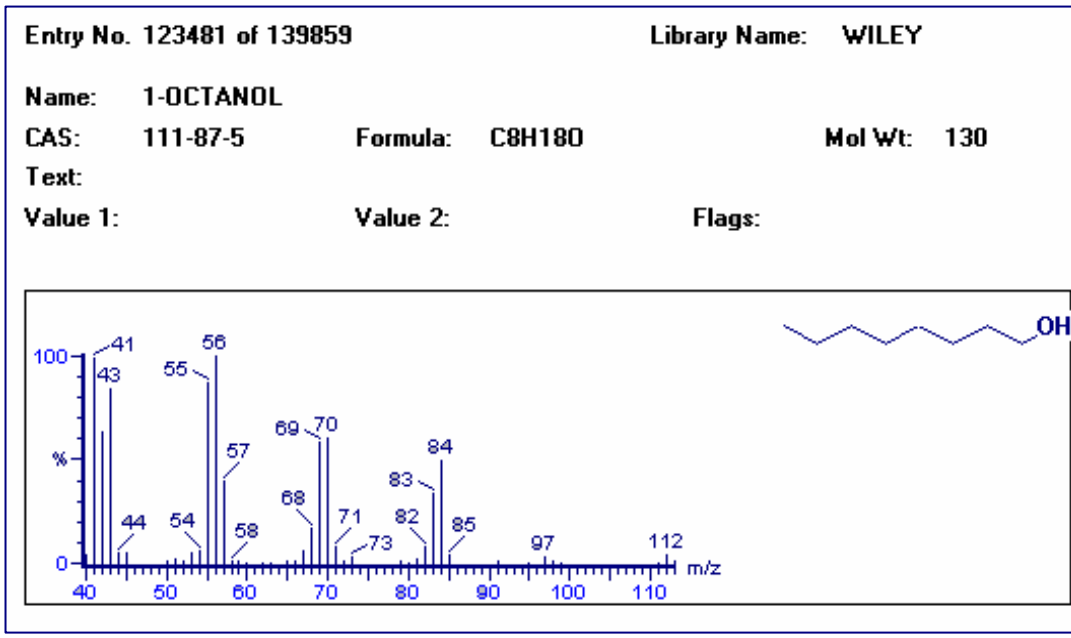

Figure VI-21- 1-Octanol GC-MS spectrum.

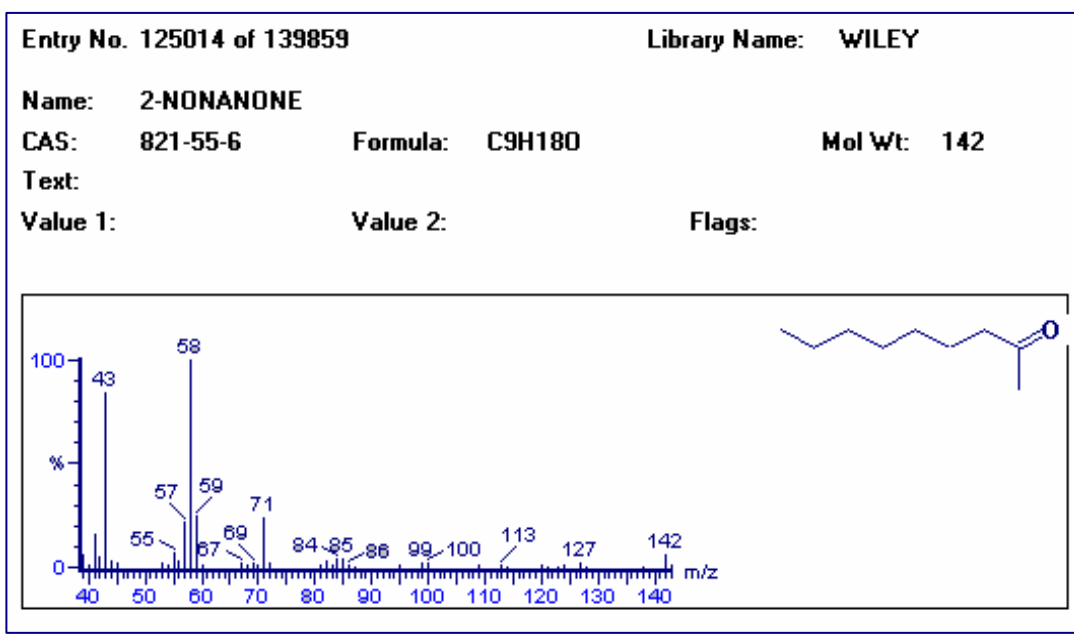

Figure VI-22- 2-Nonanone GC-MS spectrum.

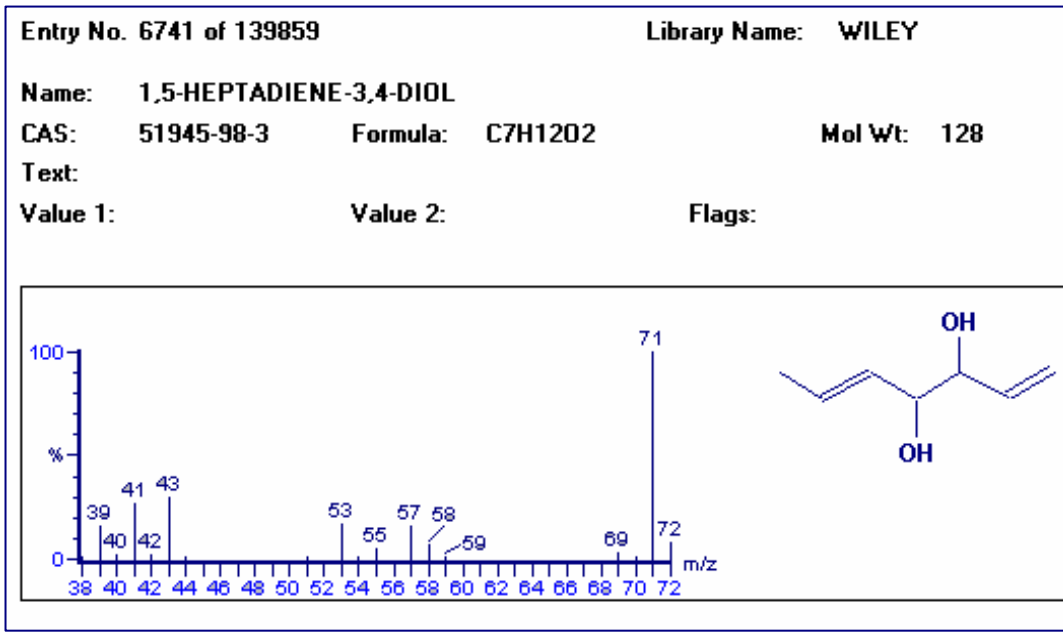

Figure VI-23- 1,5-heptadiene, 3, 4-diol GC-MS spectrum.

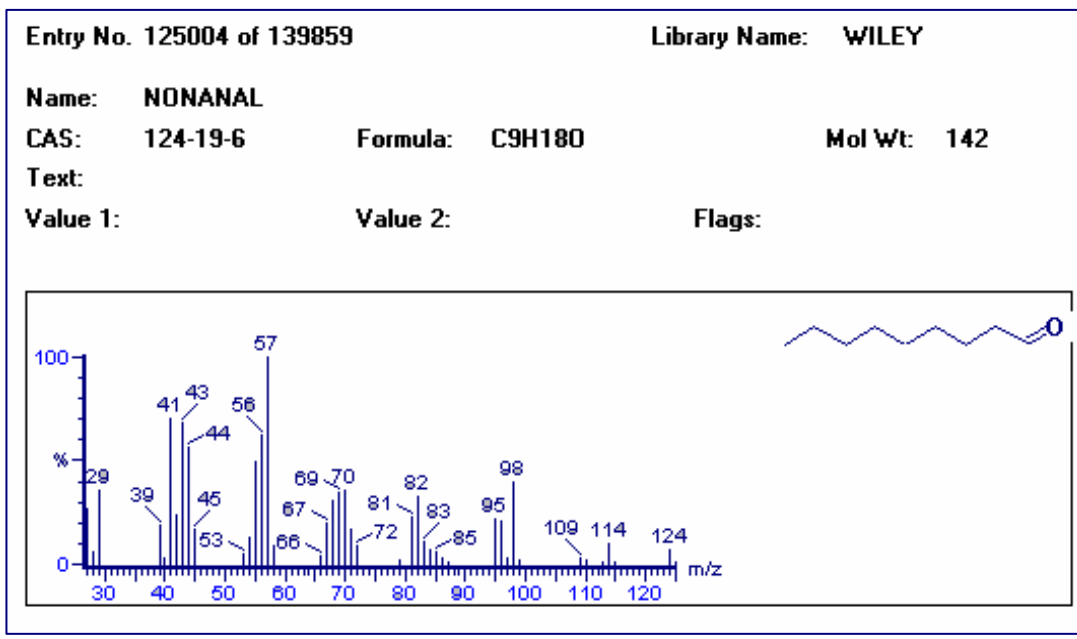

Figure VI-24- Nonanal GC-MS spectrum.

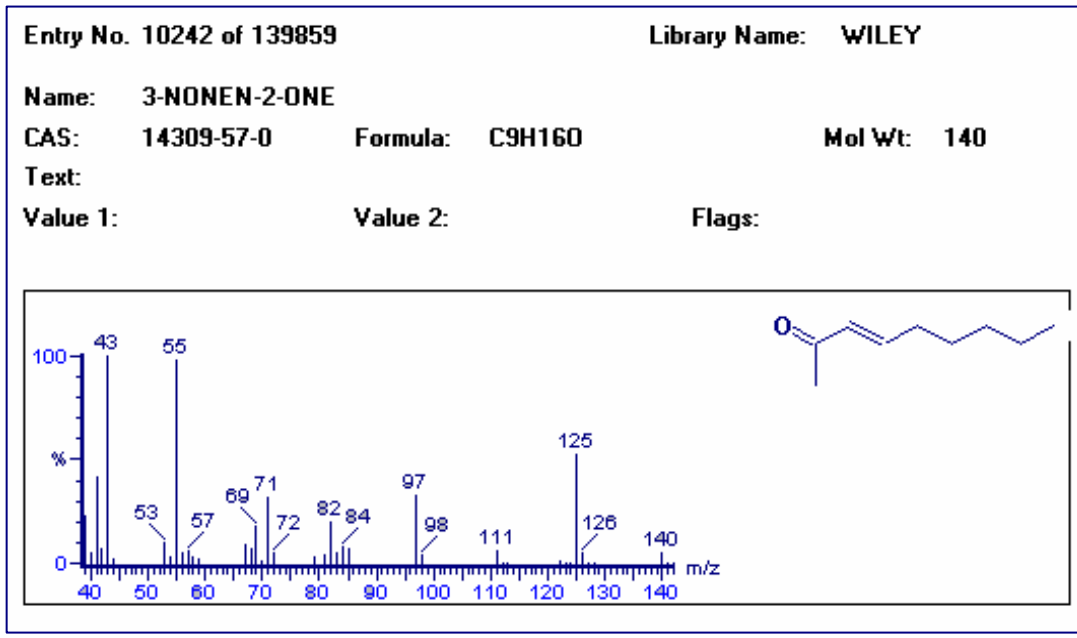

Figure VI-25- 3-Nonen-2-one GC-MS spectrum.

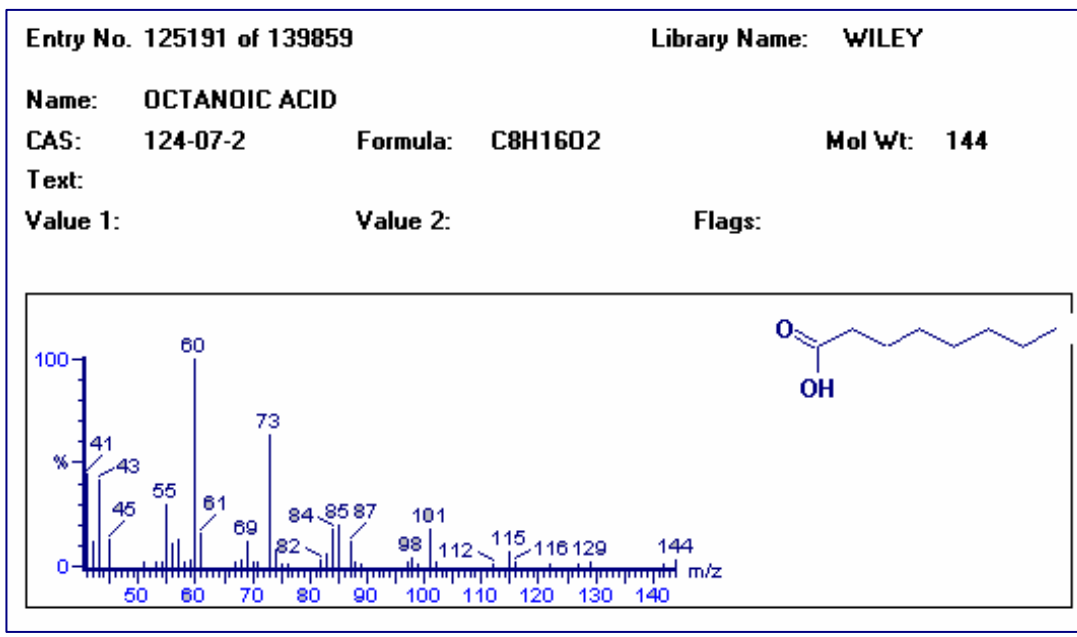

Figure VI-26- Octanoic acid GC-MS spectrum.

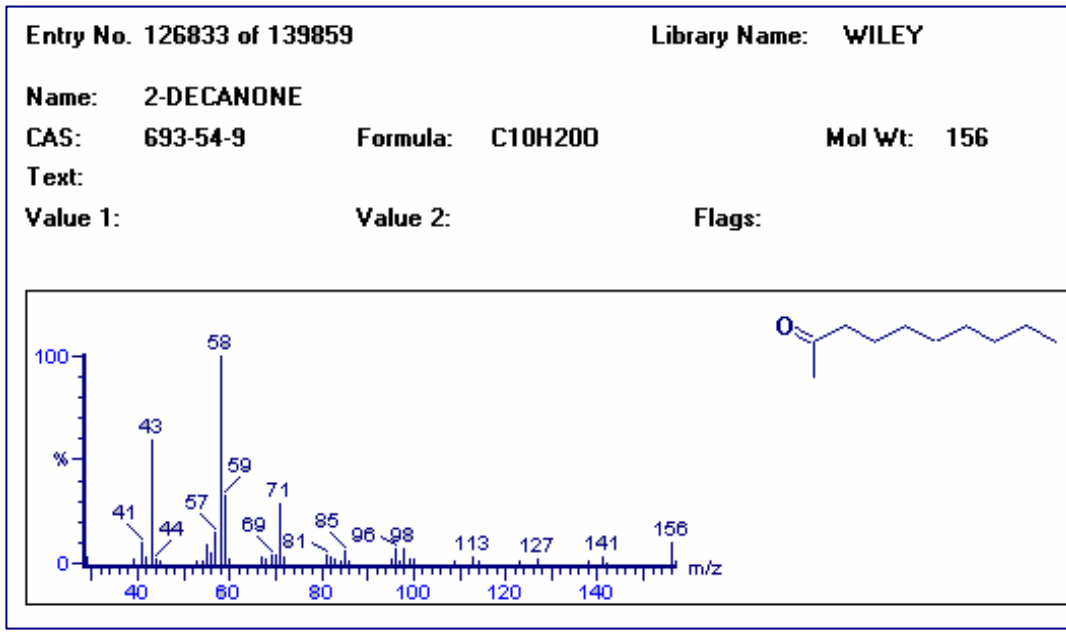

Figure VI-27- 2-Decanone GC-MS spectrum.

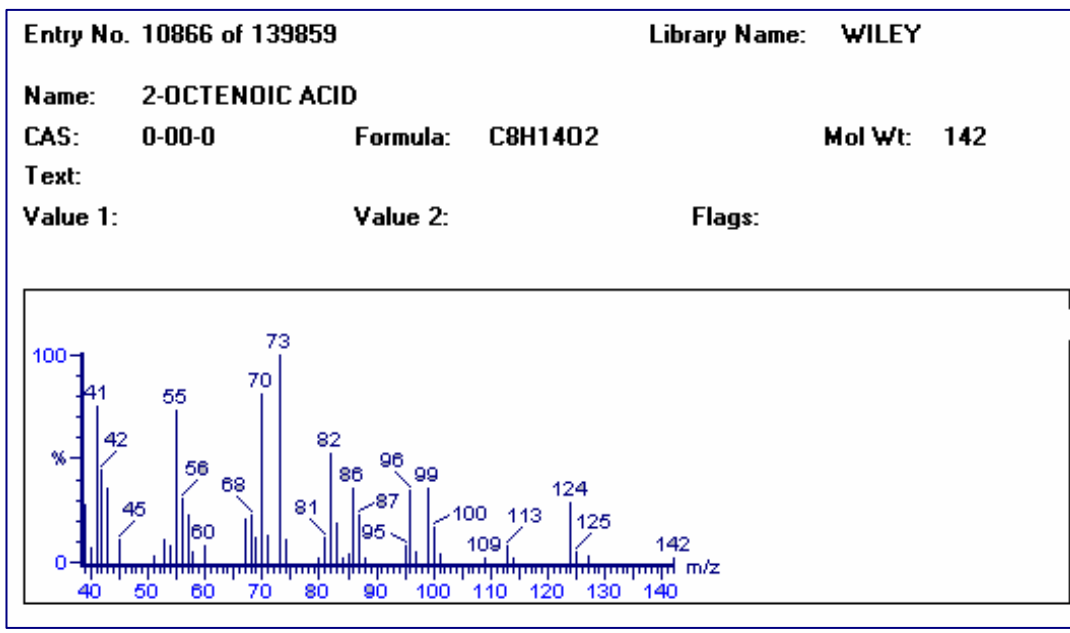

Figure VI-28- 2-Octanoic acid GC-MS spectrum.

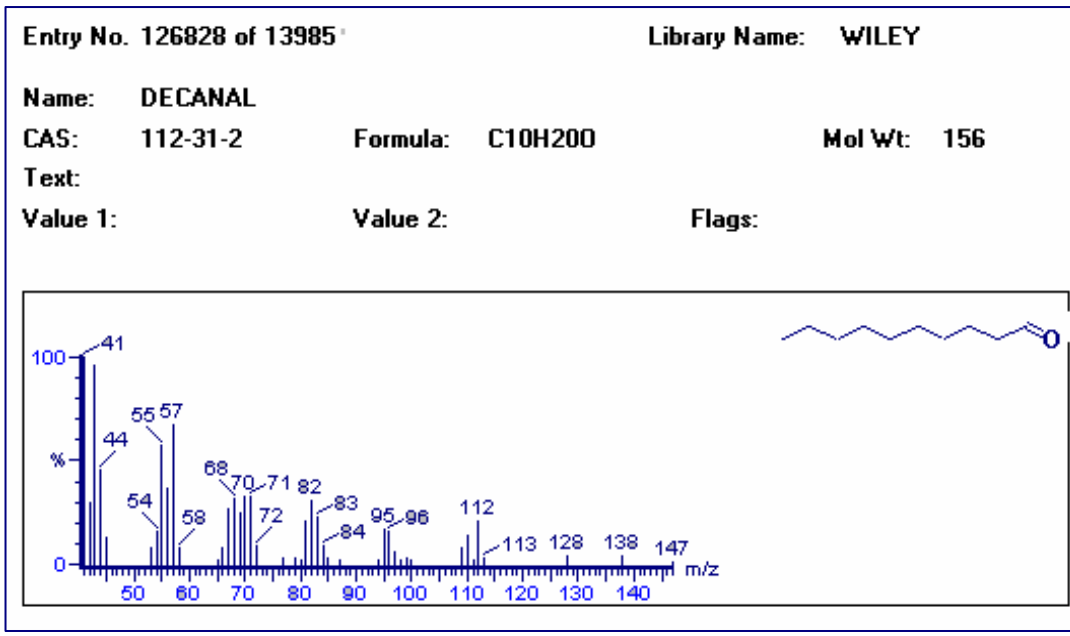

Figure VI-29- Decanal GC-MS spectrum.

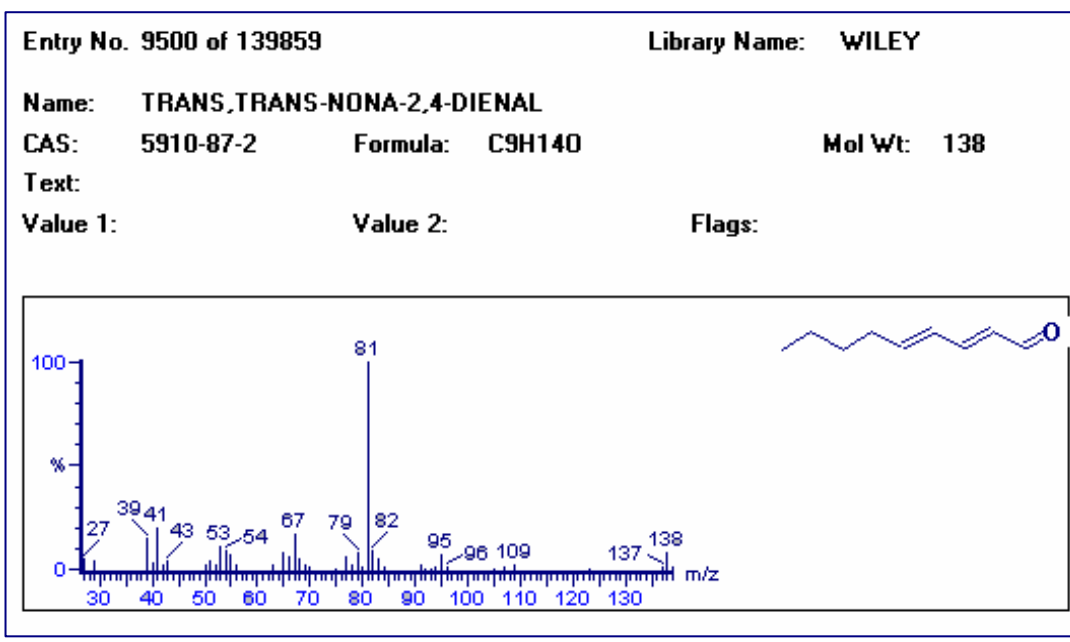

Figure VI-30- Trans, trans- nona-2,4-dienal GC-MS spectrum.

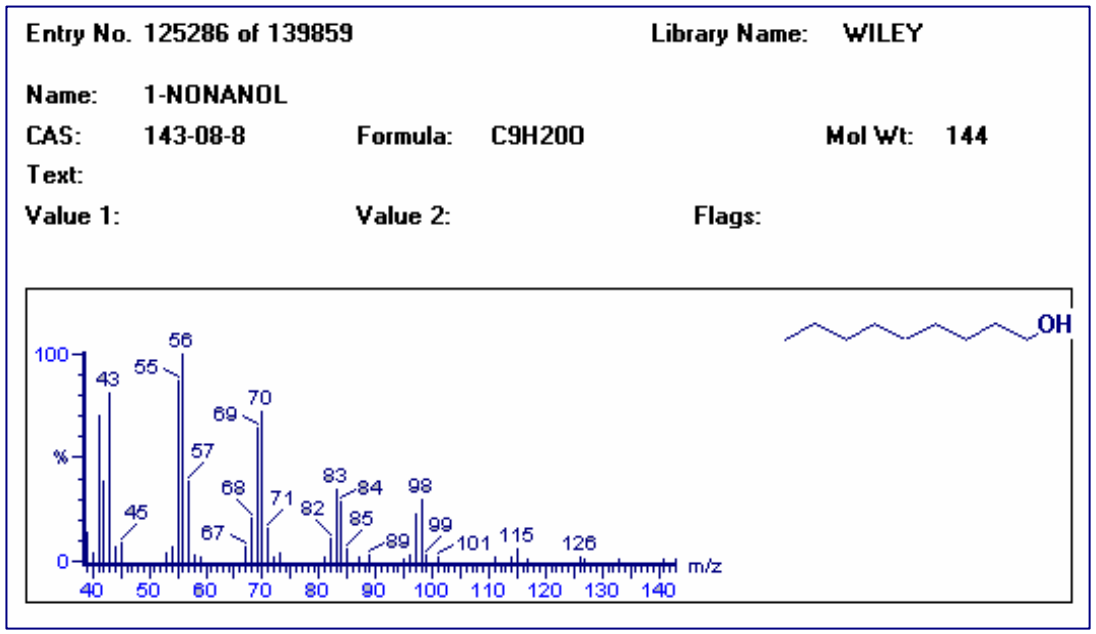

Figure VI-31- 1-Nonanol GC-MS spectrum.

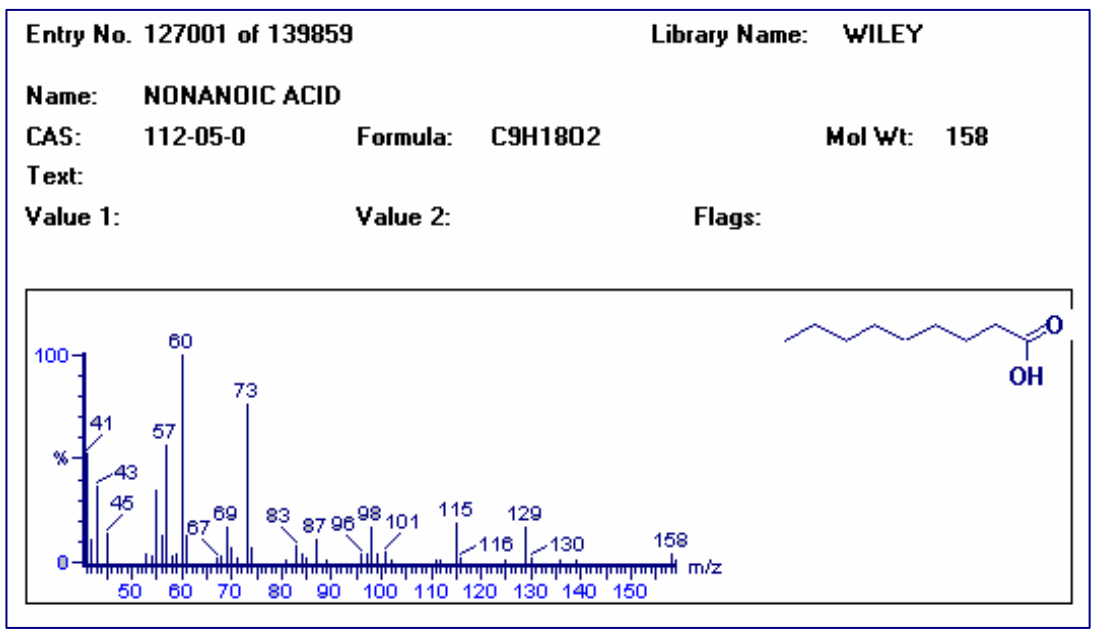

Figure VI-32- Nonanoic acid GC-MS spectrum.

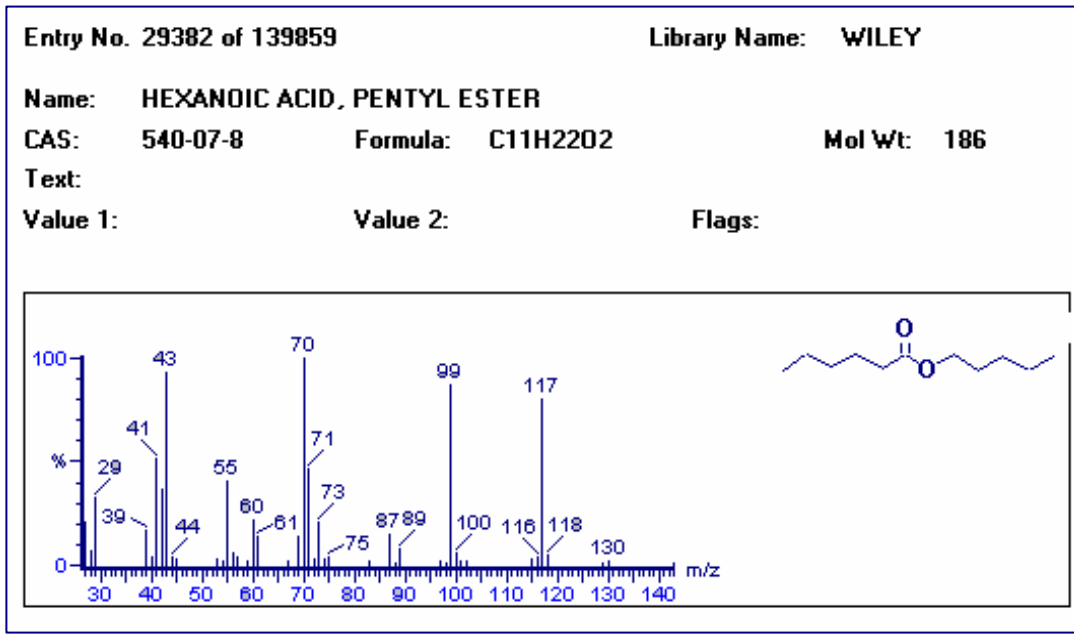

Figure VI-33- Penthyl ester, hexanoic acid GC-MS spectrum.

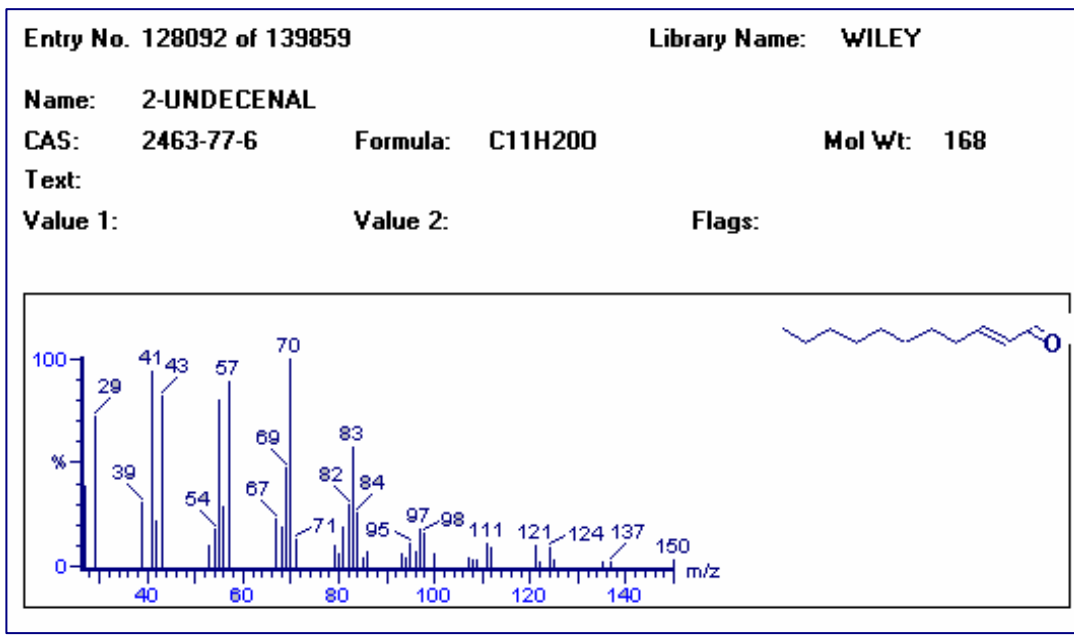

Figure VI-34- 2-Undecenal GC-MS spectrum.

VII- DETERMINATION OF HEXANAL AS INDICATOR OF THE LIPIDIC OXIDATION STATE IN POTATO CRISPS USING GAS CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Presented as a poster at: VII International Symposium on Analytical Methodology in the Environmental Field, XII Meeting of the Spanish Society of Analytical Chemistry (VIII ISAMEF/ SEQA 2003), A Coruña, Spain, 21-24 October, 2003.

Abstract

Hexanal (an oxidative state indicator) formed in the headspace of potato crisps during storage was evaluated using two different procedures. First, solid phase microextraction (SPME), an innovative sampling preparation methodology was used. It consisted on the absorption of analytes directly from samples and subsequent thermal desorption on the gas chromatograph (GC) injector. Then, a reverse-phase high-performance liquid chromatographic technique (RP-HPLC) was employed to quantify hexanal in the form of 2,4-dinitrophenylhydrazone derivative. Methods were evaluated in what concerns to validation parameters such as linearity, repeatibility and detection limit. GC (LOD= 1 ng/ml) method resulted in more sensitive method than HPLC (LOD= 9 ng/ml). The most suitable technique for hexanal measurement was selected.

Keywords: Food analysis, Oxidation; Hexanal; Lipids.

VII-1. Introduction

Deterioration of high fat content food generally occurs as result of lipid oxidation. This process is decisive on the reduction of food products shelf-life, once it is responsible for the production of undesirable odours, texture deterioration, decrease of nutritional value and formation of potentially toxic substances [1-2].

Food industry has great interest in preventing, as well as on the early detection of lipid oxidation due to the economical profits that can achieve directly from the increase of sells number and consumer acceptance because, this way, products will preserve all their qualities during a longer period and any change that might adversely affect the taste of the product is early detected [3]. Therefore, a substance (indicator) that could evaluate the state of lipid oxidation, advising or not the products' consumer is of great interest. Hexanal has become a known indicator thanks to be a major product of oxidation of fats and to increase with storage [4]. It is formed during the oxidation of linoleic acid via the 13-hydroperoxide and it has an odour described as "grassy" which contributes to off-flavours, and it is easily detected once have a low odour threshold (in water: 4,5 μg/Kg) [1-2, 5-6].

In the last few years hexanal has been determined in food matrixes, using many different sample preparation methods and detection techniques (e.g. gas chromatography, liquid chromatography, spectrophotometry) [7].

Hexanal has been evaluated in: cooked turkey [4], freeze-dried chicken myofibrils [2]; fish [8]; vegetable oils [9]; etc.

In this work, a study using two chromatographic techniques, HPLC and GC, is made, trying to find out which is the most suitable for the determination of hexanal in potato crisps.

In the first part of the paper, an innovative sample preparation technique, solid phase microextraction (SPME) followed by gas chromatography (GC), was used. SPME was introduced in the 90s by Arthur and Pawliszyn and has successfully been applied to the study of the volatile profile of foodstuffs [10].

SPME technique utilises a fiber (coated fused silica) which adsorb the analytes from samples. The fiber may be directly immersed in liquid samples (DI-SPME) or in the headspace of a gaseous, liquid or solid sample (HS-SPME). In spite of DI-SPME efficiency, it can not be performed in solid samples because the fiber is too weak to be pushed in solid material [3]. HS-SPME is more recommended because it allows shorter extraction times, a longer fiber lifetime (once the fiber it is not in contact with the sample) [11-12]. Finally, the fiber is inserted in the GC injection port for thermal desorption and subsequent gas chromatography.

In the second part of the work, an HPLC method is used. As previous authors have reported, HPLC presents advantages over GC because of the superior separation efficiency and due its major sensitivity. However, these methods require a pre- or post- column derivatization step. Numerous derivatization agents have been used [13]: dansylhydrazine [14], 4- dimethylaminobenzene-4'-sulphonylhydrazine and more frequently 2, 4- dinitrophenylhydrazine [15], which is well suited for the detection of traces [15]. Moreover, the use of HPLC with reversed-phase columns (RP-HPLC) results in a better resolution [15]. In the present paper hexanal is quantified as its 1, 4- dinitrophenylhydrazine derivative by RP-HPLC.

The paper's main purpose is to study the progress of hexanal during the storage of potato crisps, using two different methods GC and HPLC. The most suitable method for hexanal determination in crisps is selected regarding all outcomes and drawbacks.

VII-2. Experimental

VII-2.1. Sampling

Potato crisps were purchased from a local supermarket. According to the package information, they were fried in olive oil. Sample preparation procedures were performed at selected days. Meanwhile, they were stored in two different conditions. One group was stored in the darkness at room temperature, simulating the normal storage conditions. The other group was stored in presence of natural light at room temperature. This way, potato crisps stability was evaluated by means of an accelerated storage test in order to evaluate the suitability of hexanal as an early oxidation marker [1].

Potato crisps' brand used was selected regarding the type of package. A transparent packaging film was chosen in order to allow the study of the influence of light on the hexanal formation.

VII-2.2. Analytical Standards and Reagents

Hexanal [CAS No 66-25-1] was supplied from Sigma-Aldrich (Madrid, Spain) and had a purity above 99%. For SPME, an hexanal standard solution of 100 mg/l was prepared in water. Ultrapure water was obtained with a Milli Q filter system (Millipore, Bedford, MA).

All chemicals were of analytical grade. Acetonitrile, methanol, ethanol and n-hexane were from Merck (Darmstadt, Germany). Hexanal and 2,4-dinitrophenilhydrazine were purchased from Sigma (Steinheim, Germany). Sep-Pak DNPH-Silica cartridges were from Waters (Milford, MA).

VII-2.3. SPME Experimental Procedure

VII-2.3.1. Sample preparation

Potato crisps samples were ground with a commercial grinder, and homogenised. Approximately 0.1 g of sample was mixed with ultrapure water (1ml) in 20 ml vials (Sun International Trading, USA). The vials were hermetically capped with PTFE-faced silicone septum. Then, samples were mixed for 3 min in a vortex (Autovortex SA6, Stuart Scientific, Redhill, UK).

VII-2.3.2. Headspace-SPME (HS-SPME)

Following homogenisation, the vials were heated in a module (Stuart Scientific, UK) at 70ºC for 5 min to reach the equilibrium between the sample and above headspace [16].

The SPME holder (Supelco, Bellefonte, PA) was used to perform the experiments. A fused silica fiber coated with a 50/30 μm layer of divinylbenzene/ carboxen/ polydimethylsiloxane (DVD-CAR-PDMS, Supelco, Bellefonte, PA) was used to extract hexanal from potato crisps.

Prior to first use, fibers were conditioned, by inserting them for 4 h at 270ºC in the GC injection port. After equilibrium time, the holder needle was exposed to the headspace during 20 min (extraction time) at 70ºC. Then, the fiber was withdrawn and removed from the vial. Fibers were immediately thermally desorbed in the GC injector for 5 min at 250ºC to prevent contamination. A scheme of the SPME extraction procedure is presenting in figure VII-1.

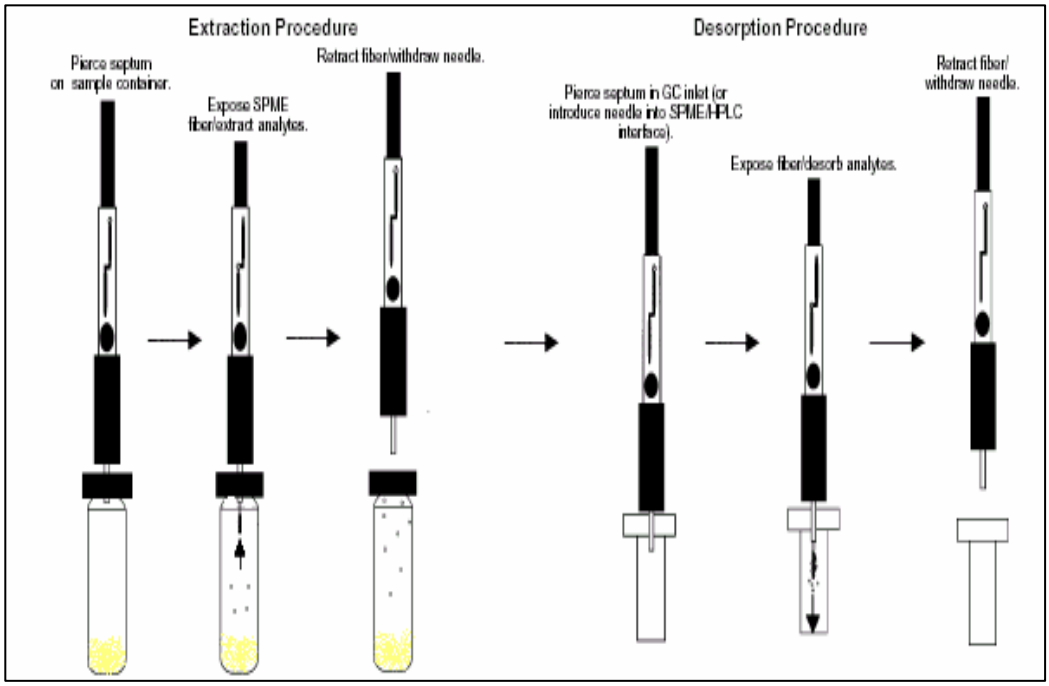

Figure VII-1- SPME-GC-MS procedure (webpage: http://www.sigmaaldrich.com/Graphics/Supelco/objects/8600/8564.pdf).

VII-2.3.3. Gas Chromatography-Mass Spectrometry system and conditions

A Fisons GC 8000 series Gas Chromatograph (Manchester, UK) equipped with a programmed split/splitless injector and a mass selective detector MD800 was used to perform all GC analysis.

A narrow-bore glass liner (SGE, USA) with 0.80 mm i.d. was used to substitute common liners in order to improve peaks resolution [11, 17]. A cross-link 5 % phenyl- 95% methyl siloxane (30 m x 0.25 mm i.d. x 1 µm film thickness) DB-5 column (DB J&W Scientific, CA, USA) was employed. Helium was used as carrier gas at a head pressure of 70 KPa.

The oven temperature was programmed as follows: 40ºC for 1 min, then to 120ºC at 20ºC/min, held for 8 min, then to 260ºC at 20ºC/min and

finally held for 2 min. The split valve was opened 2 min after injection. The injector temperature was 260ºC.

The mass spectras were obtained using a mass selective detector (MD 800, Manchester, UK) under electron ionization at a voltage of 70 eV and data acquisition was done at a scan rate of 0.45 s^{-1} over an m/z range of 35-300. The software used to process peak areas was Masslab (version 1.4). The confirmation of hexanal was performed by comparing the observed mass spectra with that recorded in Wiley Spectrometry Library and by identical retention time of a standard injected in the same conditions.

VII-2.4. LC Experimental Procedure

VII-2.4.1. Preparation of 2,4-DNPHi solution

The 2,4-DNPHi solution was prepared by mixing 90 ml of ethanol, 2 ml of hydrochloric acid, 50 mg of DNPH and 8 ml of water.

VII-2.4.2. 2,4-DNPH standard

DHPH derivative of hexanal was prepared by dissolving 1,8 mg 2,4-DNPH standard in 100 ml of ethanol in a volumetric flask (18 mg/l).

VII-2.4.3. Extraction

Hexanal was extracted from potato crisps, previously homogenized by magnetic stirring for 5 min, adding 5 ml of ethanol to 1g of sample. Then, 1 ml of the supernatant was removed and mixed with 1 ml of the 2,4-dinitrophenylhydrazine solution. The mixture was incubated at 40ºC for 45 min. (These mixtures were stable for 2 h stored at 4ºC). All samples

were filtered through a 0.5 µm Millipore filter (Bedford MA, USA) and afterwards analysed by HPLC-UV detection.

Several trials were carried out to optimise extraction of hexanal. Parameters studied were summarised in table VII-1. Two other extraction procedures were studied (table VII-1).

Table VII-1- Trials for extraction procedures optimisation of HPLC method.

EXTRACTION PROCEDURES		
Magnetic Stirring	Extraction Solvent Extraction Time Reaction Time Reaction Temperature	EtOH 5-10-15-30 min 30-45 min Room temp.,40ºC
Ultrasounds	Extraction Solvent Extraction Time Reaction Time Reaction Temperature	EtOH 15-30-60 min 30-45 min Room temp.,40ºC
DNPH-Silica Cartridges	Elution Solvent Elution Volume Sample Volume	EtOH-Hexane 2-3 ml 0.5-1 ml

At first, a method which homogenisation step consisted in ultrasounds stirring, then a method using DNPH-silica cartridges. The optimised procedure using DNPH-silica cartridges involve the solubilisation of 1g of sample to 5 ml of ethanol. Then, homogenization was performed for 5 min with constant magnetic stirring. The mixture was centrifuged at 5500 rpm for 15 min and then the supernatant was removed. An aliquot (1ml) of the supernatant was passed through the DNPH-silica cartridge, then it was dried for 10 min under vacuum and finally the hexanal was eluted with 2 ml of ethanol. This solution was filtered (0.22

µm Millipore) and then analysed by GC-MS. Schemes of the optimised extraction procedures with derivatization step are presented in table VII-2.

Table VII-2- Derivatization- HPLC-UV extraction procedures.

SPE-HPLC	**Magnetic/Ultrasounds stirring-HPLC**
1 g Sample + 5ml ethanol Magnetic stirring for 5 min ↓ Remove the supernatant Centrifuge (15 min at 5500 rpm) ↓ Pass 1ml through the DNPH-Silica cartridge ↓ Elute with 2 ml of ethanol, filtrate and inject into the HPLC	1 g Sample + 5ml ethanol Magnetic/ultrasounds stirring for 5 min ↓ Remove 1ml of the supernatant and mix with 1ml of 2,4-dinitrophenylhydrazine ↓ Incubate at 40ºC for 45 min ↓ Filtrate and inject into the HPLC

VII-2.4.4. Equipment and Chromatographic Conditions

LC-MS- just for hexanal identification

A SpectraPhysics series P200 liquid chromatograph equipped with a Rheodyne loop (50 µl) and a mass detector (Fisons VG Platform) (VG Biotech, Altrincham, UK) was used to identify hexanal. Full scan and single- ion recording (SIR) were obtained every second with a scan delay time of 0.1s. The column used was a Kromasil 100 C18 5 µm 15 x 0.4 cm and the mobile phase was acetonitrile/water (75:25) at a flow rate of 1ml/min performing in isocratic mode. Detector operated under the following conditions: negative atmospheric pressure chemical ionization

(APCI) mode; probe temperature 400ºC; source temperature 130ºC, cone voltage -20 V, electron multiplier voltage 700 V, drying gas nitrogen at 425 l/h, APCI sheet gas nitrogen at 175 l/h.

HPLC-UV- for hexanal quantification

The HPLC system (Hewlett Packard, CA USA) consisted of an HP1100 quaternary pump, an HP1100 degassing device, a 20µl injection loop (Rheodyne, Cotati, CA), an HP1100 UV detector, a column heater SP8792 (San José, CA, USA). The HPLC was controlled by a personal computer running HP Chem Station Software. Operating conditions were as follows: the mobile phase was methanol-water (75:25 v/v) at a flow rate of 1.5 ml/min, column Tracer Extrasil ODS2 (25 x 0.4 cm I.D., 5 µm particle size) set at 25ºC. The absorbance was monitored with an UV detector and set at 365 nm.

Figure VII-2- HPLC chromatograph (webpage: www.gmi-inc.com/ AnyLab/HP%201100b.jpg).

VII-3. Results and Discussion

VII-3.1. Methods Optimisation

-SPME/GC-MS

The formation of hexanal from potato crisps oxidation was evaluated using a DVB/CAR/PDMS fiber. Díaz et al had also selected this fiber of medium polarity to extract the volatile components from truffles because reduce discrimination toward very nonpolar and polar volatile compounds [18].

In the first approach, potato crisps samples were used in the dry state. Further experiments were performed with samples homogenised with water. Two factors contributed for the use of a slurry instead of a dry sample. In one hand, according to literature, the extraction of volatiles is much more efficient from the liquid state [1]. In the other hand, in order to achieve a reliable hexanal quantification, both standard solutions (used to construct the calibration curve as described in Experimental) and samples were in the liquid state and were subject to the same extraction procedure.

With the aim of avoiding artifacts, samples were extracted just after its preparation. For all experiments, the adsorption time and temperature, were set at 20 min and 70ºC, respectively. These conditions provided reproducible and sensitive results and avoided fiber saturation even for the more oxidised potato crisps. Regarding the desorption conditions, optimal temperature and time were 260ºC and 3 min, respectively. These were optimised in order to ensure total desorption from fibre and, this way, avoid carryover effects. Running blank samples were regularly made to check possible carryover or fiber ageing (which

originate new peaks). Figure VI-3 shows a GC-MS chromatogram of the sample after a 1-month storage stored in the lightness and in the darkness.

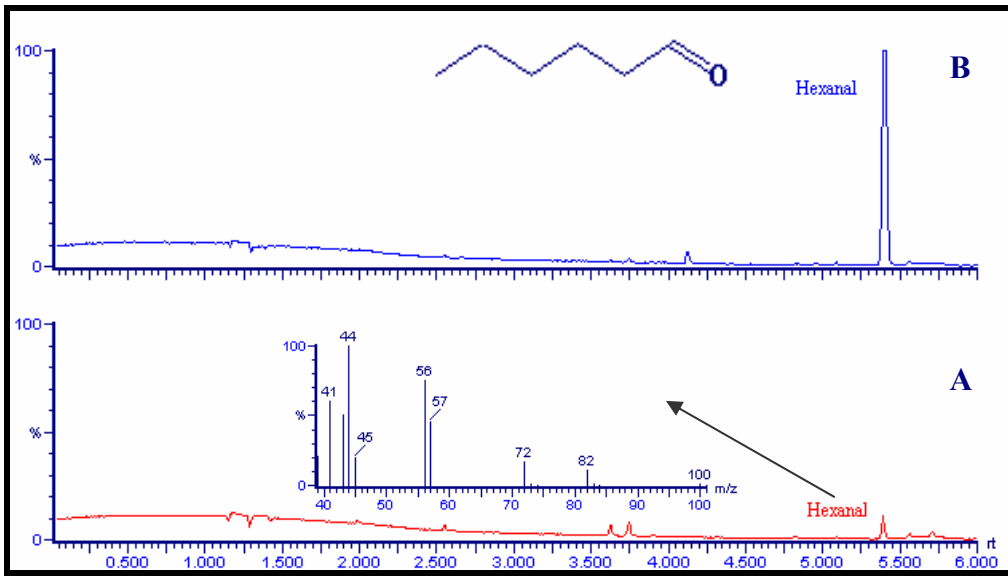

Figure VI-3- GC-MS chromatograms of potato crisps stored for one month: A- darkness and hexanal GC-MS spectra; B- lightness.

-Derivatization/RP-HPLC

Preliminary assays were performed in order to establish optimal extraction conditions (extraction volume, solvent extraction, reaction time, etc). Table VII-1 summarises the trials. Three extraction procedures were optimised: (1) with DNPH cartridges, (2) with magnetic stirring, (3) with ultrasounds stirring. The first and second extraction procedures differ only in the homogenisation step. Due to the importance of this step in the final result, assays were performed with magnetic stirring and ultrasounds. Magnetic stirring resulted in a more effective method.

Regarding the method which used cartridges, although its short analysis time, it resulted in a much more expensive procedure. For this reason, magnetic stirring method was selected. The derivatization reaction

is presented in figure VII-4. Figure VII-5 corresponds to HPLC-UV chromatograms of potato crisps samples.

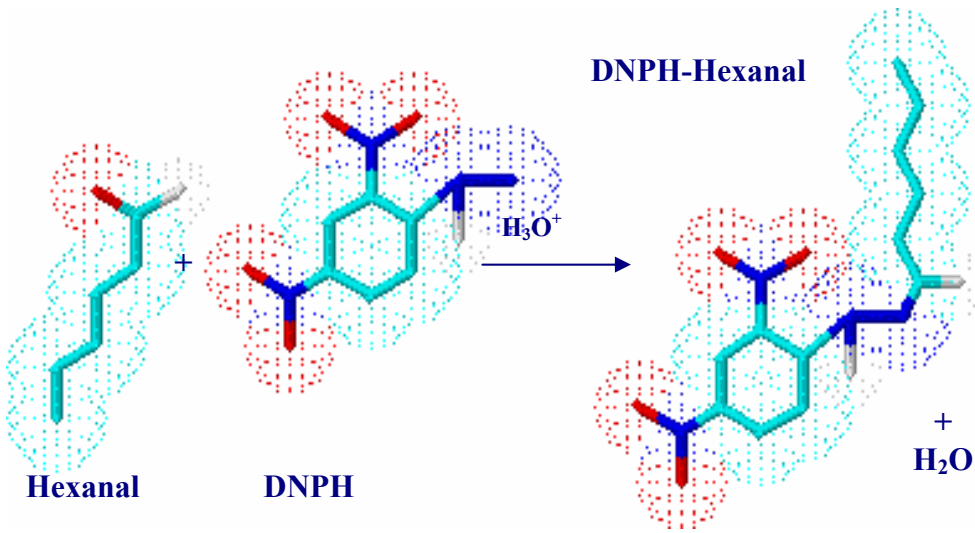

Figure VII-4 - Derivatization reaction of hexanal with dinitrophenylhydrazine (DNPH).

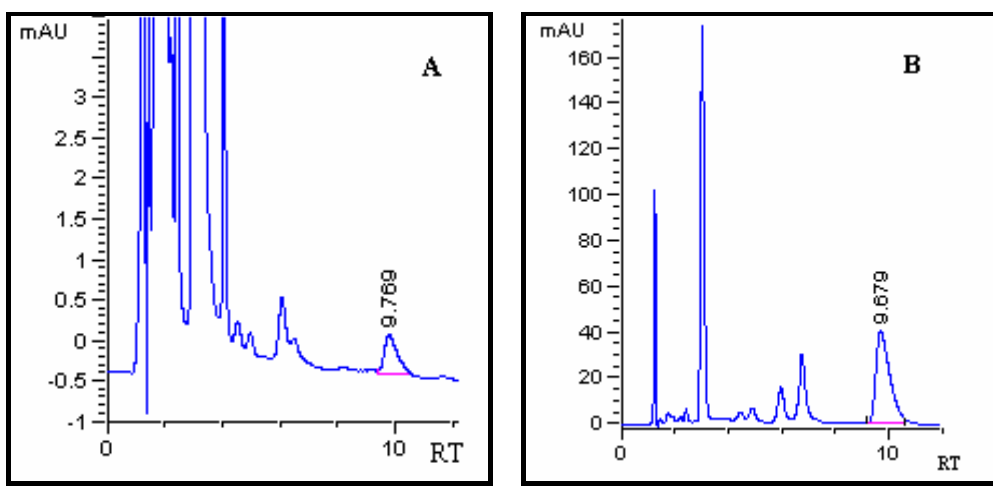

Figure VII-5- HPLC-UV chromatograms of potato crisps stored for 3-months: A- darkness; B- lightness.

Solutions stability was studied at regular intervals of 30 min at 4ºC for 5.5 h. Low temperatures allowed to decrease the reaction speed. Results showed that solutions were stable for 2 h at 4ºC, after this period they should not be injected once false results could be achieved.

With respect to hexanal identification, this was done by comparing of the retention time (tr) with the standard and confirmed by LC-MS

In order to achieve the best conditions in LC-MS, several assays were performed. SIR and full scan modes were used (figure VII-6). Several probe temperatures (350, 400 and 500ºC) and cone voltages (-20 and -30 V) were evaluated. Best response was achieved with 400ºC and -20V. Characteristic masses (m/z) were 182, 279. The first corresponded to the fragment $[M-(N=CH-(CH_2)_4-CH_3)]^-$ and the second to the ionization form $[M-H]^-$ of the molecule.

VII-3.2. Performance of analytical methods

Both methods were calibrated using a series of hexanal standards of known concentration. Calibration lines were constructed using five concentrations of standard, so that concentration in the sample was at the middle of the range. The equation obtained was y= 159.95 x- 18.658, the determination coefficient and the linearity range were 0.999 and 0.11-11.00 μg/ml respectively, for HPLC-UV method. For the GC-MS method, parameters of linearity were the follow: -3 x 10^6 (interception); 54609 (slope); 0.9894 (r^2) and 10-2000 μg/l (range).

The detection limit, estimated in accordance with ACS guidelines (defined as signal three times the height of the noise level) [19] was 9 ng/ml for HPLC-UV, whereas for GC-MS was 1 ng/ml. Yasuhara and Shibarnoto [20] have reported similar detection limits using a gas chromatographic method for quantitative of volatile aldehydes in fish flesh.

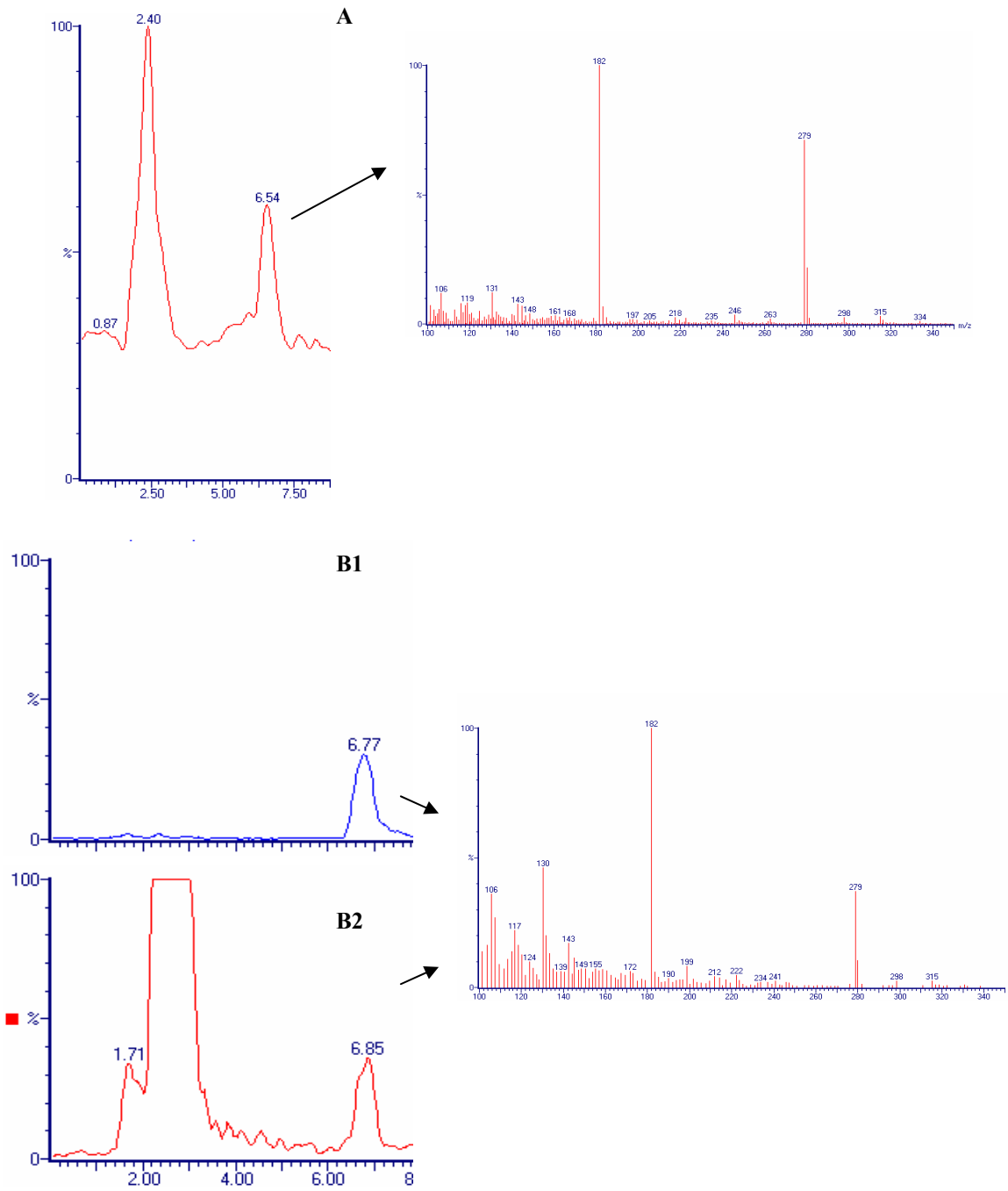

Figure VII-6- LC chromatograms with MS detection: A- Full scan mode for a hexanal standard solution and full scan mass spectra; B- SIR mode for the m/z 279 (1), 182 (2) and full scan mass spectra of correspondent potato crisps sample.

Repeatibility, was estimated as RSD% for the determination of six extracts (each prepared separately from the same homogenised sample). The result obtained for the HPLC method was 2.40% and for the GC was 7.56%.

Due to the best linearity and repeatibility of the HPLC method the assays of recovery were carried out just for the liquid chromatographic method. Recovery achieved after spiking samples, with about the same amount of hexanal present in oxidised samples, was 102 ± 10%.

VII-3.3. Hexanal profile and content during potato crisps storage

The GC-MS method was used to evaluate the suitability of hexanal as indicator of the lipid oxidation process. During a month (at 1, 4, 8, 15 and 30 days) analysis were performed in order to study the evolution of hexanal in both storage conditions.

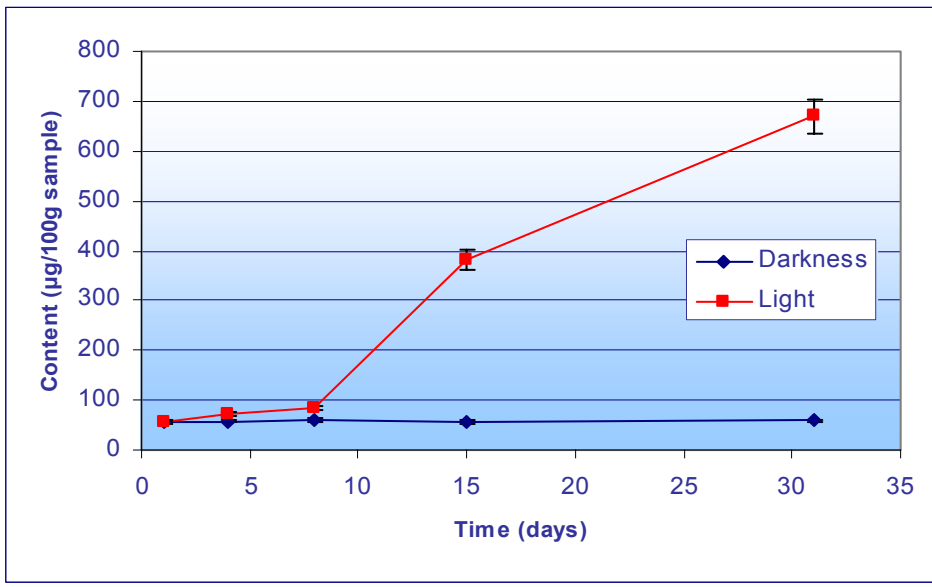

Figure VII-7– Hexanal content during an 1-month storage (SPME-GC).

Results (see figure VII-7) showed that in potato crisps stored in the darkness there was no hexanal change, disregarding minor fluctuations, while in potato crisps stored in presence of natural light there was a relevant increase, especially after the eighth day. Grosso et Resurrección [21] has also reported a similar hexanal evolution during a 110 days storage of peanuts.

On the other hand, an HPLC-UV method was developed in order to quantify hexanal, in samples stored at lightness and darkness. Regarding the results obtained with the GC method, which indicate there is no significative change when crisps are stored in the darkness, analyses were performed at day 1 and day 90 (just before validity expiration). The hexanal content has not changed significantly (Figure VII-8), although a slight increase has been observed (from 20.05 to 21.40 µg/100g sample).

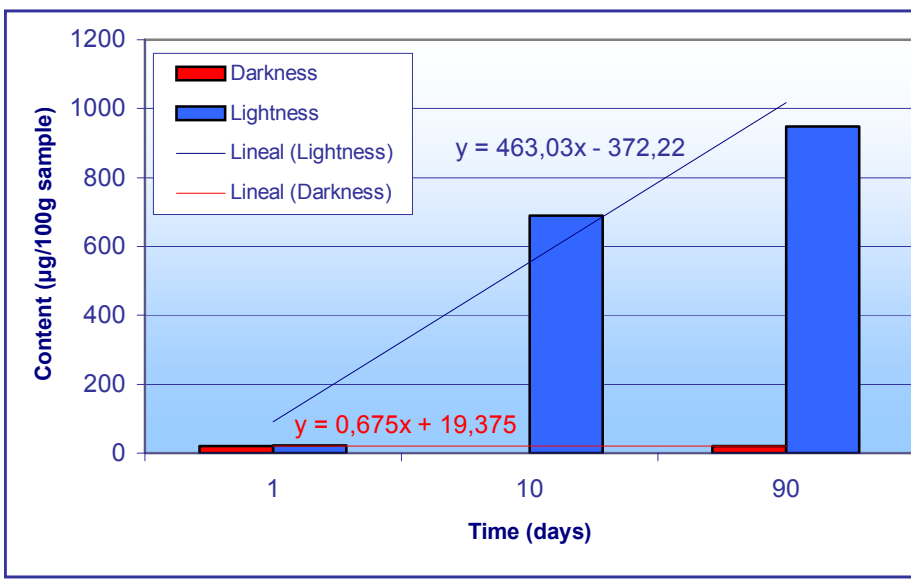

Figure VII-8- Hexanal content during a 3-month storage period (Derivatization- HPLC).

With respect to samples stored in presence of light, analysis were performed at days 1, 10, and 90, once it was expected an increase similar to the one found with the GC method. Indeed, with the HPLC method, it was also observed an amazing increased from 23.20 μg/100g sample at day 1 to 689.03 μg/100g sample at day 10 and 949.25 μg/100g at day 90. Therefore, although crisps were in the validity period, they were not acceptable for consumer because they presented a rancid odour and taste.

VII-4. Conclusions

The SPME method optimised in the present paper, is suitable for the identification and quantification of hexanal once presents a low detection limit. For the quantitative determination of hexanal in potato crisps, HPLC method represents a good alternative due to display an excellent repeatability and linearity.

In brief, our results indicated that hexanal is a good indicator of the lipid oxidation state of potato crisps because increases during storage and indicates deterioration of the sensorial proprieties.

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VIII- DETERMINATION OF TRICLOSAN IN FOODSTUFFS

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Abstract

A reverse-phase high-performance liquid chromatographic (RP-HPLC) method coupled with an ultraviolet detector was developed to determine triclosan in foodstuffs as result of migration from packaging materials. The method includes extraction with hexane, followed by evaporation to dryness and residue re-dissolution with ACN 90%.

Chromatographic separation was performed with a Kromasil 100 C18 (15 cm x 0,4 cm I.D., 5 μm particle size) at 30ºC and using ACN and water as mobile phases. Regarding recoveries, good results (higher than 83% and lower than 112%) were obtained for the four representative food matrixes selected (orange juice, chicken breast meat, gouda cheese and potato crisps).

Keywords: Triclosan, Biocides; Anti-microbial food packaging; Foodstuffs; High performance liquid chromatography.

VIII-1. Introduction

Triclosan (5-Cloro-2-(2,4-dichlorophenoxy) phenol) (Fig. VIII-1), Mw = 289.55, is a crystalline powder, soluble in many organic solvents, with very low solubility in water (10 mg/L), and with high lipophilicity (Log Kow (octanol-water) = 4.76) [1,2]. It has been first manufactured by Ciba-Geisy Co (Basel, Swizerland) under the trade name Irgasan DP 300 or Irgasan MP [3].

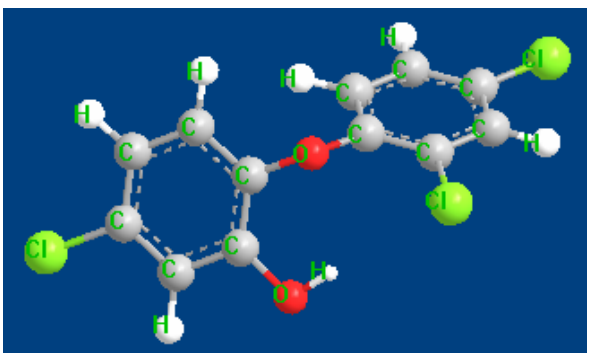


Figure VIII-1- Triclosan chemical structure.

During the last three decades, triclosan has been widely used as a broad spectrum antimicrobial and antifungal agent [3-6], and, it has been incorporated in various personal care products (soaps, cosmetics, deodorants, shower gels, mouthwashes and toothpastes) as well as in textiles, plastic consumer goods and toys [7-9]. As additive in plastics it prevents the growth of micro-organisms, avoiding the formation of stains and odours and, therefore, extends the polymer's shelf life. For this reason, its inclusion on food contact polymeric materials has been considered [7, 10].

Antimicrobial agents incorporated into plastic films (which encompass, for instance, organic acids and esters, enzymes and bacteriocins) must not undergo any change in food flavour or be

deactivated by food ingredients. They should have a controlled migration and a low minimum inhibitory concentration (MIC) [10]. At present, they have not been legislated in the European Union [11].

Lately, triclosan safety has been questioned in what accounts to environmental impact and human health risk. The United States Environmental Protection Agency (EPA) has registered triclosan as a pesticide [12]. Several studies were carried out in order to examine the occurrence and fate of triclosan in the aquatic environment (surface waters, wastewaters, treated waters, fish, sludges and sediments) [13-19].

Regarding the effect of triclosan in human health, toxicological studies have reported that triclosan is not a carcinogen or mutagen and it has rarely elicited irritation or sensitisation of the skin [3]. On the other hand, although, it was thought, for many years, that this general "biocide" did not have any specific target in the bacterial cell membrane, however recent studies reported it has. McMurry et al [5] have found that triclosan blocks the gene fabI or the enzyme that this gene encodes (enoyl reductase) in Escherichia Coli. Therefore, the biochemical pathway involved in lipid synthesis is specifically inhibited and bacteria are killed [9]. However, any mutation in fabI that may alter the enzyme or the overactivity of fabI inhibits antibacterial activity of triclosan which leads to the growth of strains which are triclosan-resistant.

These findings lead to the conclusion that triclosan can be considered an antibiotic, which can yield bacterial resistance. Therefore, it widespread use may not be advised.

In line with these facts, it is important to study the levels of triclosan migration into foodstuffs in contact with triclosan-containing plastics. In Foodmigrosure project [20], triclosan was chosen as a model migrant to study transport/partitioning processes into/within foods. The results obtained may be extrapolated to other substances of similar

characteristics regarding its use frequency of use in food contact materials and its analytical behavior.

In spite of the scarce bibliography available regarding the determination of triclosan in foodstuffs, several works have determined, in other matrices, this substance by high performance liquid chromatography with ultraviolet detection (HPLC-UV) [14-27] and by gas chromatography with mass detection (GC-MS) [16, 19].

This work describes an HPLC-UV method used for the identification and quantification of triclosan in food matrices. An analytical procedure was developed and optimised to determine triclosan in orange juice, chicken and cheese and potato crisps as representative samples of aqueous and acidic, proteic and fatty foods. Positive identification was carried out by HPLC-MS and GC-MS.

VIII-2. Experimental

VIII-2.1. Sampling

Food items (oranges, chicken breast, gouda cheese and potato crisps) were purchased from a local supermarket. Orange juice was prepared immediately before each analysis in order to prevent oxidation. Chicken breast was manually chopped up as much as possible, while cheese was homogenised with an ultra-turrax homogenizer and potato crisps with a commercial grinder.

VIII-2.2. Chemicals and standard solutions

All reagents were of analytical grade. Ethanol, acetonitrile (ACN) and hexane were from Merck (Darmstadt, Germany). Ultrapure water was

prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). Triclosan (CAS no 28064-14-4) was supplied from Fluka (cat. No 72779) and had a purity of 97%.

A primary stock solution of triclosan was prepared in ethanol (1,0 mg/ml). Intermediate standard solutions of triclosan were prepared in ACN and hexane (0.1-10.0 μg/ml). Solutions were stored in a refrigerator (4-10ºC) and remained stable for up to 6 months.

VIII-2.3. Sample preparation

For orange juice samples, 10 ± 0,01g were weighted in a 40 ml screw-cap centrifuge tube. A 10 ml volume of hexane was added and immediately hand-shaken for 10 min. Tubes were centrifuged at 1036 g for 10 min and the organic phase was removed. Extraction was repeated twice with 10 ml of hexane. Collected hexane phases were evaporated to dryness in a rotatory evaporator at 40ºC. Afterwards, the solid residue was re-dissolved with 10 ml ACN 90% (v/v) and homogenised by ultrasonics for 10 min. Finally the solution was filtered and an aliquot was transferred to an HPLC vial.

For chicken breast a preparation similar to orange juice was performed. 10 ± 0.01g was accurately weighted in a 40 ml screw-cap centrifuge tube. 2 ml of ACN and 10 ml of hexane were added and immediately hand-shaken for 10 min. Tubes were centrifuged at 1036 g for 10 min and the hexane phase was removed. Extraction was repeated twice with 10 ml hexane. Collected hexane phases were evaporated to dryness in a rotatory evaporator at 40ºC. Then the solid residue was re-dissolved with 10 ml ACN 90% (v/v) and homogenised by ultrasonics for 10 min. Finally the solution was filtered and an aliquot was transferred to an HPLC vial. Gouda cheese and potato crisps extraction was performed

as follows: 10 ± 0,01g of homogenised food matrix was extracted with 10 ml of hexane and hand-shaken for 10 min. Organic phases were separated by centrifugation (1036 g for 10 min). Extraction was repeated with 2 x 10 ml hexane and then, supernatants were pooled and evaporated in a rotatory evaporator. The fatty liquid residue obtained was extracted with 2 x 10 ml ACN. Collected phases were evaporated in the rotary evaporator and the residue was diluted to 10 ml with ACN 90% (v/v). Finally, the solution was homogenised by ultrasonics (10 min) and filtered. A 50 µl aliquot was injected in the HPLC.

VIII-2.4. Identification and quantification

Triclosan identification was carried out not only by comparison of its retention time and UV spectra of those of a pure standard injected on the HPLC-UV chromatograph, but also with respect to the mass spectra acquired by HPLC-MS and GC-MS.

Quantification was achieved by external standard, on the basis of a calibration line. The line consisted of a plot of peak area of triclosan against the concentration in seven calibration solutions.

VIII-2.5. Apparatus / Chromatographic conditions

HPLC-UV

The HPLC system (Hewlett-Packard, Waldbronn, Germany) was fitted with a HP1100 quaternary pump, a degassing device, an autosampler, a column thermostatting system and a diode array UV detector.

The HP ChemStation chromatographic software was used for data acquisition. Chromatographic separation was performed with a Kromasil

100 C18 (15 cm x 0,4 cm I.D., 5 μm particle size) (Teknokroma, Barcelona, Spain) at 30ºC.

A gradient elution method was employed. Within the first 2 min the mobile phase was 65% ACN/35% water, after which the ACN was raised to 100% within 15 min. The total run time of each analysis was 30 min to clean the column. The flow-rate was 1,0 ml/min.

HPLC-MS

A Navigator II liquid chromatograph (ThermQuest, Finnigan, Manchester, UK) equipped with an autosampler and a mass detector (Navigator II AQA, TermoQuest) was used to confirm the identity of triclosan.

The column and mobile phase were the same as in HPLC-UV analysis (flow rate 1 ml/min). The detector operating conditions were as follows: negative atmospheric pressure chemical ionisation (APCI) mode; probe temperature 250ºC; cone voltage -15 V, electron multiplier voltage 650V, full scan mode scanning a mass spectrum range of 100-400 amu (two scans for second). The system was computer-controlled using the Xcalibur (version 1.2) software.

GC-MS

The identity of triclosan was also confirmed by gas chromatography. A gas chromatograph Fisons (Manchester, UK) 8000 series instrument equipped with a mass selective detector MD 800 was used.

A fused silica capillary column DB-5 (30 m x 0.25 mm i.d. x 1 μm film thickness) (J&W Scientific, CA, USA) was employed. Helium was used as carrier gas and the head pressure was 70 kPa. The injector temperature was at 260ºC and the split ratio was 1:30. The column

temperature was held at 160ºC for 1 min, then increased at 15ºC to 260ºC which was held for 21 min. The MS detector was operated in the full scan mode with 70 eV electron impact, by scanning a mass range of m/z 50-300 in 0.45 s.

The software used to acquire data was Masslab (version 1.4.).

Other equipment

An ultra-turrax homogeniser (T25 basic, IKA® Labortechnik, Stanfen, Germany) was used to homogenise cheese samples. A commercial grinder (Taurus, G70, Lerida, Spain) was employed to grind potato crisps. An Eba 12 centrifuge (Hettich, Kirchlengern, Germany) was used to separate phases. A rotatory evaporator (RE 200, Bibby Sterilin, Stafforshire, UK) and an ultrasonics bath (5510 Bransonic, Danburry, CT, USA) were also used during sample preparation.

VIII-3. Results and Discussion

VIII-3.1. Preliminary assays and Method optimisation

Four representative food items were chosen trying to cover the analytical complexity of the food matrices: orange juice (aqueous and acidic liquid with a medium carbohydrates content); chicken breast meat (solid with a high protein content) and gouda cheese and potato crisps (solid with a high lipid content).

These food items were used to perform all analysis. Nevertheless other food items (apple sauce, milk UHT, tomato ketchup, cola, margarine, condensed milk, dark chocolate, toasted bread, wheat flour, rice and honey) were tested in order to evaluate possible interferences. No interference was found at triclosan retention time, except for wheat flour.

Several trials were carried out to optimise the sample preparation procedure and the chromatographic conditions. Depending on sample matrix composition, extraction procedure changed. High lipid content food, like gouda cheese and potato crisps, required an extended sample preparation method.

In order to establish an appropriated sample amount, distinct quantities (2, 5, 10 g) were assayed, good results were obtained with 10 g of sample. Besides sample amount, the sample homogenisation is of great importance once can greatly influence the extraction efficiency. Orange juice was extracted directly and it has not raised any analytical difficulty. However, solid samples, like chicken breast, cheese and potato crisps needed to be ground. Chicken was chopped up and due to its consistent, a slurry was obtained and considered suitable for an effective extraction procedure. Potato crisps were ground with a commercial grinder. Regarding cheese, in order to obtain a simple, rapid and reproducible method, several trials were tested such as the homogenisation in a mortar, with and without clean sea-sand. The most effective was the homogenisation with an ultra-turrax homogeniser.

Hexane was used as extraction solvent and in order to improve recoveries, the volume of solvent as well as the number of extractions were evaluated. The best results were achieved when extraction was repeated three times with 10 ml hexane and hand-shaken for 10 min. Vortex and ultrasonics were also tested, but better results were obtained with hand-shaken extraction.

Chicken extraction with hexane used to originate a compact mass, which did not desegregate, and foam, which interfered during phases dryness in the rotary evaporator. Therefore, in order to avoid both effects, 2 ml of ACN were added directly to the sample and homogenised by hand-shaking.

In what concerns to the extraction of triclosan from gouda cheese and potato crisps, two considerations need to be point out. First, the extraction with hexane (3 x 10 ml) does not provide a small solid residue after rotatory solvent evaporation just as in orange juice and chicken breast extractions. At the end, a fatty liquid residue (of approx. 4 ml) was obtained, which corresponds to the fat content of gouda cheese [28] and potato crisps. Secondly, to extract triclosan from fat, an immiscible solvent with fat, pure ACN, was used trying to avoid a clean up step with gel permeation chromatography (GPC). A double extraction, with 10 ml of solvent each, was performed with hand-shake for 10 min. Collected phases were evaporated to dryness. Then, extraction has progressed exactly as for juice and chicken sample preparation procedures.

During method development, chromatographic conditions were optimised in what accounts to the mobile phase, flow rate and column temperature. The optimal conditions for achieving a good chromatographic resolution were obtained starting the mobile phase gradient with 65% ACN/ 35% water, using a flow rate of 1ml/min and thermostatting the column at 30ºC.

The wavelengths used for triclosan determination were selected on basis of its highest absorbance peaks in UV scanning. The highest absorbance was found at 205 nm, other maximum were found at 235 and 280 nm. Figure VIII-2 shows the three-dimensional plot of the UV-visible spectra of a triclosan standard solution of 1 μg/ml. The HPLC chromatogram of the same solution is shown in figure VIII-3.

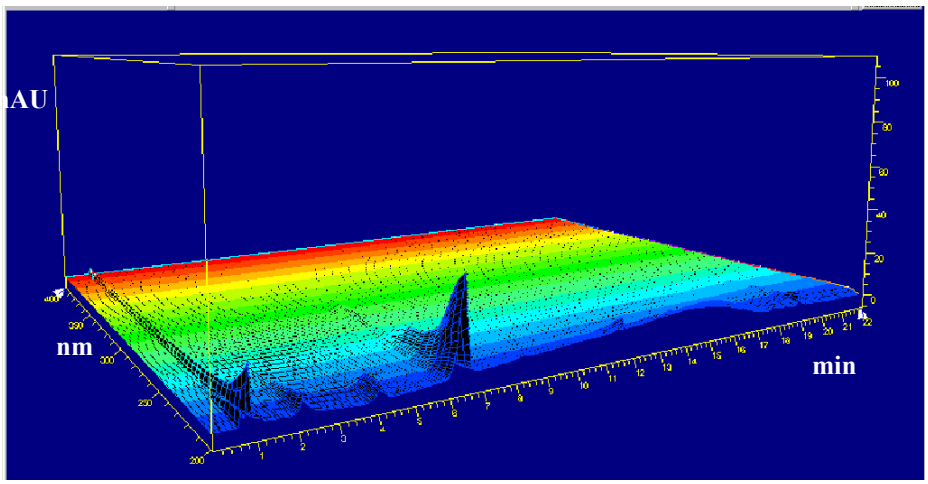


Figure VIII-2- Three-dimensional plot of the UV-visible spectra of a triclosan standard solution of 1 µg/ml.

Although 205 nm is not a selective wavelength it is the most sensitive. Sensitivity at this wavelength is 11.5 and 4.5 times higher than at 235 and 280 nm, respectively. These last wavelengths can be selected when sensitivity is not a limiting factor or when the signal at 205 nm is interfered.

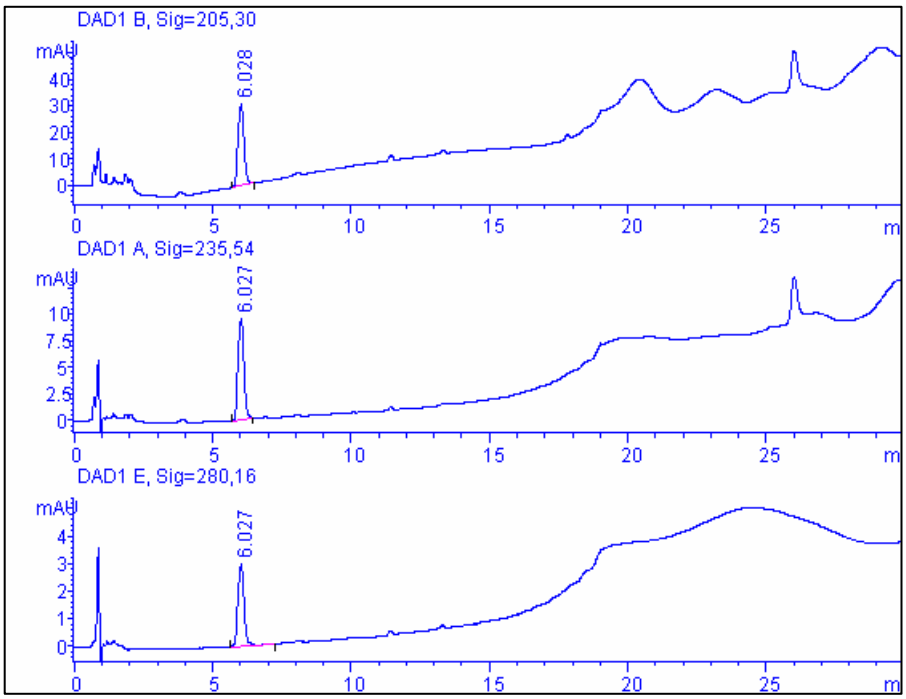


Figure VIII-3- Chromatograms of a triclosan standard solution of 1 µg/ml plotted at 205 nm, 235 nm and 280 nm.

Triclosan identity was confirmed by LC-MS (fig. VIII-4). Several assays were performed to achieve best conditions in LC-MS using a 50 mg/ml solution. SIM was selected towards full scan mode due to its sensitivity. Both positive and negative modes were tested. Negative mode was chosen to follow optimisation once provided better results.

Several probe temperatures (250-400ºC) and cone voltages (-10 to -40V) were evaluated. Optimal response was obtained with 250ºC and -15 V.

A chromatogram in SIM mode for the fragment m/z = 288 in an orange juice sample is showed in fig. VIII-4-A.

Fig. VIII-4-B shows the LC mass spectrum of triclosan. Characteristic mass 288 corresponds to the ionisation form [M-H$^-$] of triclosan molecule.

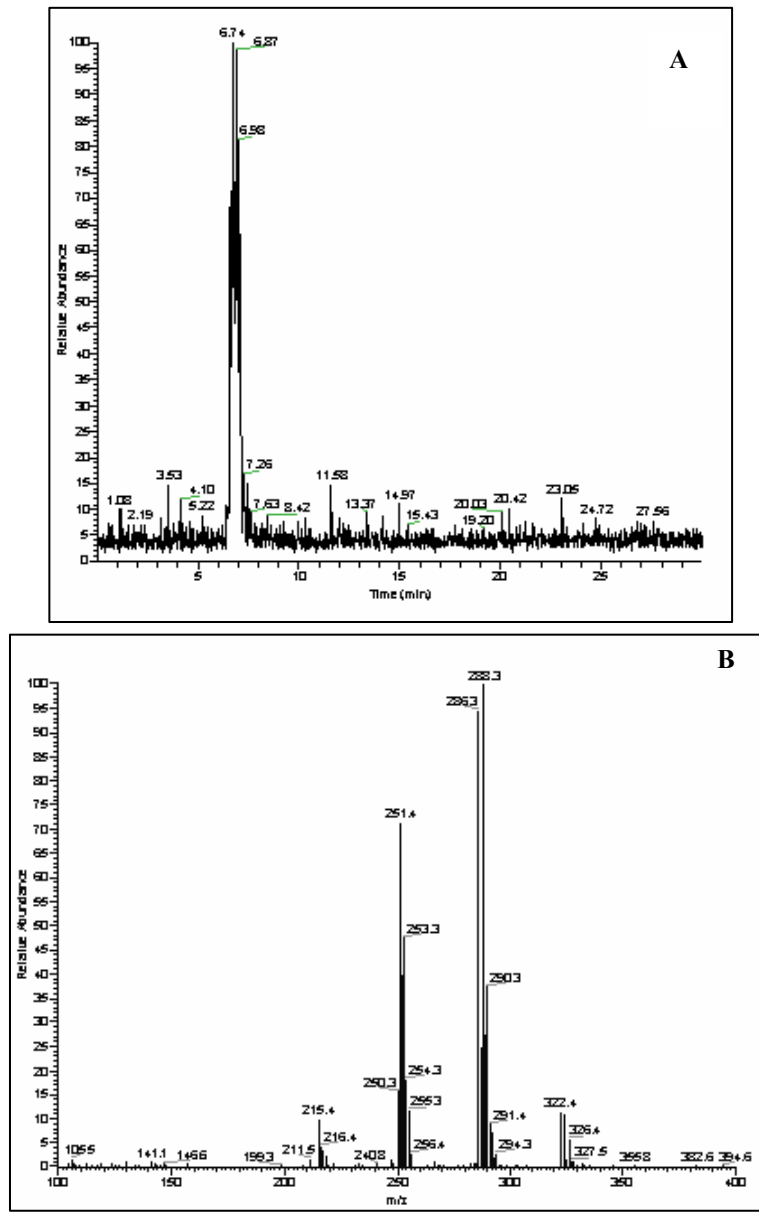


Figure VIII-4- HPLC chromatogram with MS detection (SIM mode) for the m/z 288 (A). Full scan mass spectra of triclosan (B).

Triclosan identity was also confirmed by GC-MS using a 50 mg/ml solution. A chromatogram in SIM mode for the mass 288 (exact mass 287.95) is showed in fig. VIII-5-A whereas fig. VIII-5-B shows the GC mass spectrum of triclosan. Triclosan was identified by comparison of its mass spectra with the one found in the Wiley spectral library.

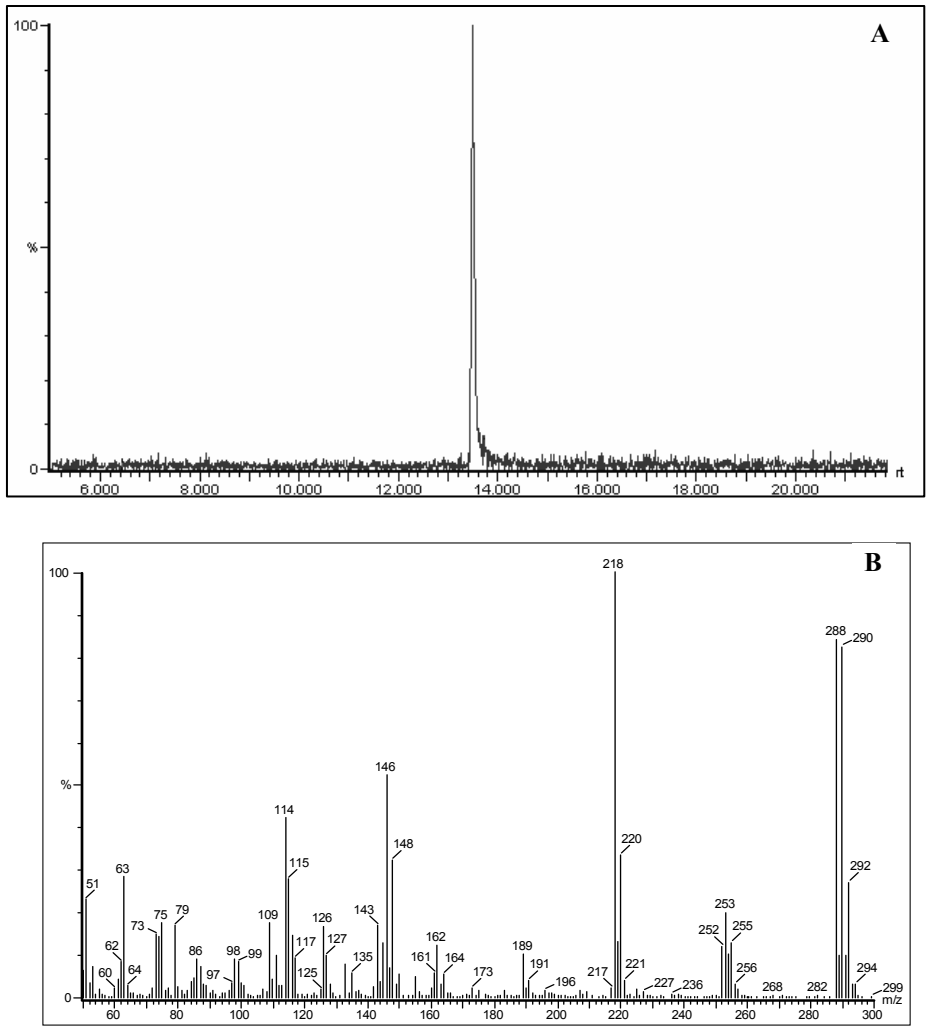


Figure VIII-5- GC chromatogram with MS detection (SIM mode) for the m/z 288 (A). Full scan mass spectra of triclosan (B).

VIII-3.2. Method validation

Calibration curve was linear over the concentration range of 0.1-10 μg/ml at 205 nm. Table VIII-1 presents calibration curves data which show an excellent correlation coefficient. These indicate suitability for triclosan quantification.

Detection limit (defined as signal three times the height of the noise level) was determined according the Guidelines of the American Chemical Society (ACS) [29]. The method was able to detect at least 25 ng/ml triclosan (table VIII-1).

The standard addition method was used to determine extraction recoveries. Samples were spiked at three concentration levels (5,0, 1,0 and 0,5 μg/ml) before extraction. Recoveries were estimated on basis of six determinations for each sample. Mean recoveries are listed in table VIII-2-4 and were always satisfactory, higher than 83% and lower than 112%. The HPLC chromatogram of orange juice, chicken breast and gouda cheese spiked samples with triclosan (0,5 μg/ml) is shown in figure VIII-6.

Table VIII-1- Parameters of calibration lines and detection limit for the determination of triclosan in foodstuffs.

Parameters of calibration curve	**λ = 205 nm**	**λ = 235 nm**	**λ = 280 nm**
Slope	465,38	142,6	48,62
Intercept	1,89	1,90	0,65
Correlation coefficient	1	0,9999	1
Range	0,1-10 μg/ml	0,1-10 μg/ml	0,1-10 μg/ml
Detection limit (LOD)	25 ng/ml	25 ng/ml	30 ng/ml

Table VIII-2- Results for recovery and repeatability at 205 nm in three food matrices (n=6).

Matrix	Level (µg/g)	Mean (µg/g)	Recovery (%)	Repeatability (RSDr, %)
Orange juice	5	4.86	97.2	2.5
	1	0.941	94.1	0.71
	0.5	0.519	103.8	8.8
Chicken breast	5	4.16	83.2	7.1
	1	0.838	83.8	4.7
	0.5	0.527	105.4	9.5
Gouda cheese	5	4.38	87.6	4.7
	1	0.887	88.7	12.2
	0.5	0.456	91.2	16
Potato crisps	5	4.91	98.2	5.62
	1	0.90	90.4	8.24
	0.5	0.47	93.4	13.3

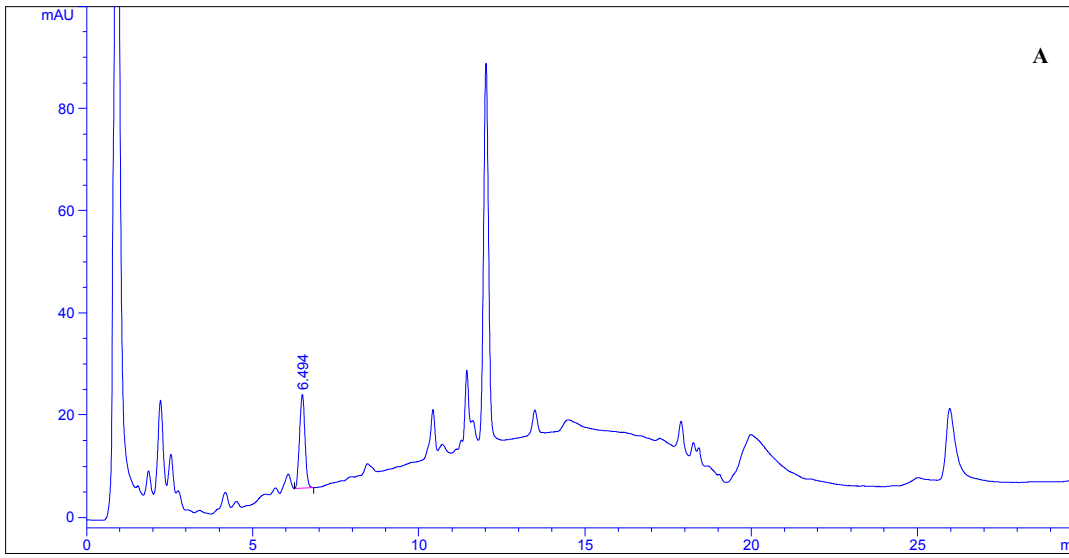
Table VIII-3- Results for recovery and repeatability at 234 nm in three food matrices (n=6).

Matrix	Level (µg/g)	Mean (µg/g)	Recovery (%)	Repeatability (RSDr, %)
Orange juice	5	4.84	96.8	0.47
	1	0.973	97.3	5.2
	0.5	0.483	96.5	3.2
Chicken breast	5	4.48	89.5	3.7
	1	0.899	89.9	3
	0.5	0.480	95.9	1.6
Gouda cheese	5	4.32	86.3	2.9
	1	1.03	102.9	15.8
	0.5	0.557	111.5	13
Potato crisps	5	4.19	83.7	9.46
	1	0.85	84.8	10.03
	0.5	0.47	93.4	11.29

Table VIII-4- Results for recovery and repeatability at 280 nm in four matrices (n=6).

Matrix	Level (µg/g)	Mean (µg/g)	Recovery (%)	Repeatability (RSDr, %)
Orange juice	5	4.97	99.4	0.98
	1	1	100.1	1.3
	0.5	0.519	103.9	6.4
Chicken breast	5	4.53	90.6	4.1
	1	0.961	96.1	3.0
	0.5	0.494	98.8	5.7
Gouda cheese	5	4.27	85.4	3.3
	1	0.837	83.7	8.9
	0.5	0.492	98.4	13.7
Potato crisps	5	4.65	92.9	11.30
	1	0.87	86.7	16.27
	0.5	0.44	87.1	8.93

Figure VIII-6



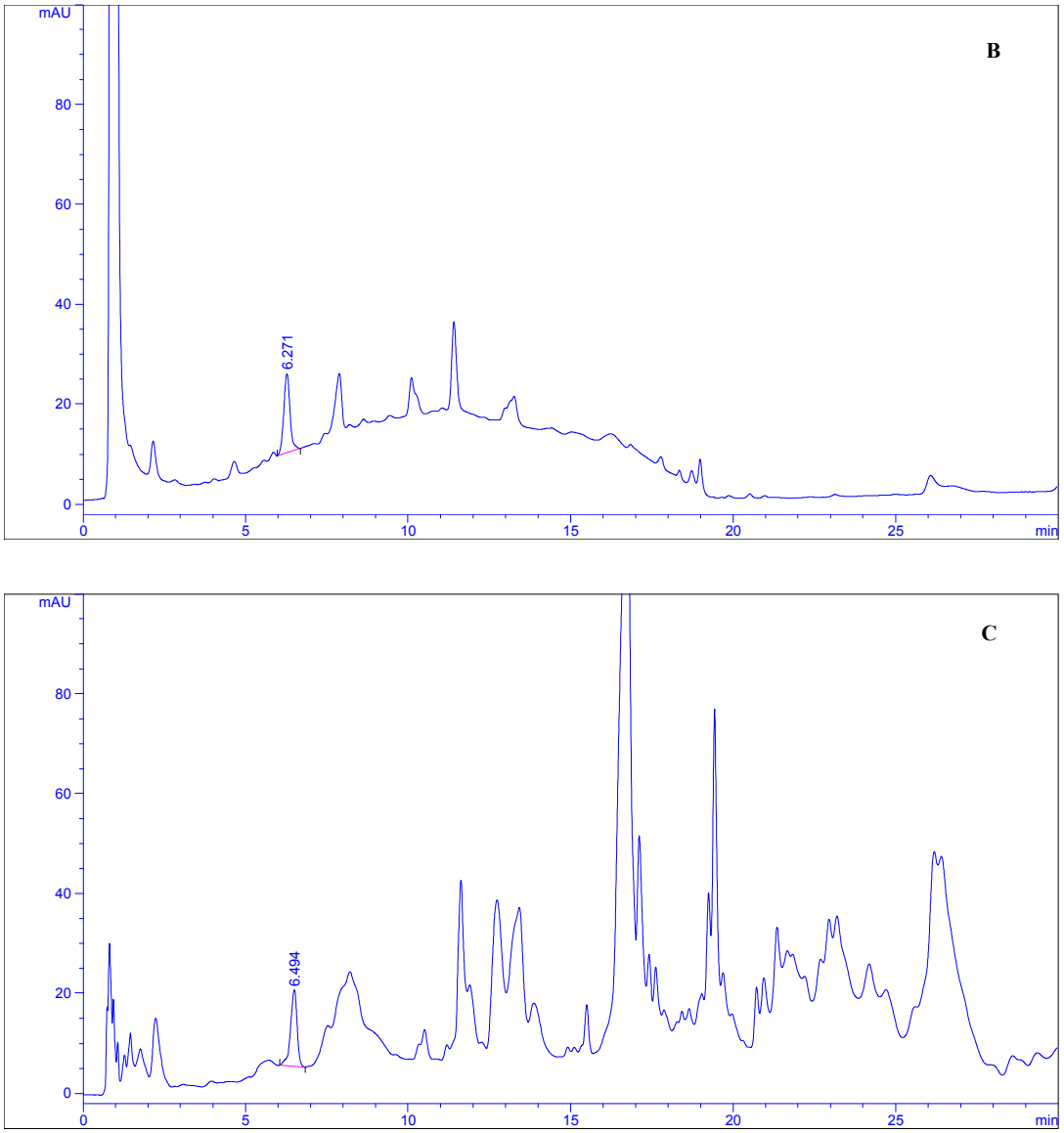


Figure VIII-6- HPLC chromatogram of an orange juice (A), chicken breast (B) and gouda cheese (C) samples spiked with triclosan (0,5 µg/ml).

VIII-4. Concluding remarks

The HPLC-UV method is suitable for the determination of triclosan in foodstuffs in the ranging from 0,1 to 10 mg/kg. HPLC-MS in APCI mode and GC-MS are appropriate for positive identification of triclosan.

Depending on the type of food matrix, the sample preparation method should be adapted. Future research is needed to evaluate the release kinetics of migration of triclosan from packaging material to food matrices.

Acknowledgments

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IX – CONCLUSIONES

PRIMERA: Se han comparado dos métodos cromatográficos (cromatografía líquida de alta resolución con detección ultravioleta y cromatografía gaseosa con detector de ionización de llama) para el análisis de los tres ácidos grasos insaturados más abundantes (ácido oleico, linoleico y linolénico) en patatas fritas en aceite de oliva envasadas comercialmente.

Con el método de cromatografía líquida de alta resolución se han determinado directamente los ácidos grasos mientras que con el de cromatografía gaseosa se han analizado los ésteres metílicos de los ácidos grasos. En ambos casos los datos referentes a la validación presentan elevados coeficientes de correlación (>0.9980) y buena precisión. El método de cromatografía líquida presenta una mejor precisión que el de cromatografía gaseosa, excepto para el ácido linolénico. Los datos de recuperación son siempre satisfactorios y superiores a 82.31%. El contenido en ácidos grasos calculado con el método de cromatografía líquida presenta una buena correlación cuando es comparado con los correspondientes valores del método de cromatografía gaseosa ($P>0.05$). El contenido en ácidos grasos disminuye significativamente ($P<0.05$) después de un periodo de tres meses de almacenamiento en presencia de luz. Para el análisis de estos tres ácidos grasos insaturados el método de HPLC sería el de elección, sin embargo, cuando se necesita analizar un mayor número de ácidos grasos, la cromatografía gaseosa es un método más conveniente y ventajoso.

SEGUNDA: El perfil de ácidos grasos de varias marcas de patatas fritas envasadas comercialmente ha sido evaluado utilizando el método de cromatografía de gases propuesto anteriormente. Los resultados indicaron que ninguna marca podría ser considerada representativa de las patatas

fritas en general. Sin embargo, las patatas presentan un perfil similar de ácidos grasos cuando son fritas en el mismo aceite. Así fue posible establecer tres grupos distintos de acuerdo con el ácido graso mayoritario: grupo 1- ácido oleico, grupo 2- ácido linoleico y grupo 3- ácido palmítico para a continuación identificar el tipo de aceite utilizado en el proceso de fritura de cada marca de patata frita.

Las patatas fritas envasadas comercialmente se almacenaron a temperatura ambiente y en la oscuridad, para evaluar los cambios en el perfil durante tres meses. A lo largo de este periodo de tiempo, los contenidos en ácido palmítico, ácido oleico y ácido linoleico no sufrieron cambios significativos. Con respecto al ácido linolénico, que en principio era previsible que fuese el primero en oxidarse, ya que tiene tres dobles enlaces, y que podría ser usado como indicador del estado inicial de oxidación, tampoco presentó ningún cambio significativo.

TERCERA: Para la evaluación de la influencia de la temperatura de almacenamiento y de la atmósfera del envase en el proceso de oxidación lipídica de las patatas fritas, han sido evaluados los cambios en el perfil de ácidos grasos con el método de cromatografía de gases propuesto inicialmente.

Los resultados del estudio permiten concluir que la atmósfera usada en el interior del envase influye en el progreso de los fenómenos oxidativos que tuvieron lugar en las patatas fritas estudiadas, a lo largo de los tres meses. Se estudiaron cuatro atmósferas distintas: aire, nitrógeno, vacío y bolsa con un absorbedor de oxígeno. Los mejores resultados se obtuvieron con el absorbedor de oxigeno, ya que ha redujo a niveles insignificantes la concentración residual de oxígeno, evitando los fenómenos oxidativos y aumentando la vida útil del alimento.

En relación al estudio de la influencia de la temperatura, se verificó que a temperatura ambiente no hubo cambios significativos ($P<0.05$) en el perfil de ácidos grasos en las cuatro marcas analizadas (fritas en distintos aceites), mientras que a 40ºC se produjo una disminución significativa de los ácidos poliinsaturados para todos los tipos de patatas fritas, a excepción de las que presentaban un menor contenido en grasa.

Por ello, para evitar o, por lo menos, retrasar el fenómeno de la oxidación lipídica se recomienda el control de estos dos parámetros durante el almacenamiento de las patatas fritas.

CUARTA: La influencia de la luz en el proceso de oxidación lipídica de las patatas fritas comerciales ha sido investigada en base a los cambios en el perfil (% área) y en el contenido (g/100g muestra) en ácidos grasos. La cuantificación se llevó a cabo usando el ácido nonadecanoico como estándar interno y el método de cromatografía gaseosa propuesto inicialmente. El perfil de ácidos grasos de las patatas fritas almacenadas en la oscuridad no sufrió cambios considerables, mientras que las almacenadas en presencia de luz natural presentaron cambios muy significativos, principalmente en los ácidos grasos poliinsaturados.

En relación a los contenidos en ésteres metílicos de ácidos grasos, se ha verificado una disminución en ambas condiciones de almacenamiento, aunque es más evidente para las muestras almacenadas en presencia de luz. Al cabo de los tres de meses de almacenamiento se detectó la aparición de nuevos compuestos que fueron identificados por cromatografía gaseosa acoplada a un detector de masas; estos fueron mayoritariamente ácidos grasos de cadena corta y aldehídos. Se concluye, por tanto, que la luz es un factor que contribuye de forma determinante en el proceso de oxidación lipídica.

QUINTA: Se ha propuesto un método de microextracción en fase sólida (SPME) seguido de cromatografía de gases con detector de masas, para la determinación de los compuestos volátiles generados en el proceso de oxidación lipídica de las patatas fritas.

Se han comparado varias fibras teniendo en cuenta la eficacia de extracción normalizada (Nx). Los mejores resultados se obtuvieron con la fibra DVB/CAR/PDMS, la cual presenta una polaridad media. Esta ha permitido identificar treinta y un compuestos, los cuales corresponden a las siguientes familias químicas: alcoholes, aldehídos, ácidos carboxílicos, ésteres, furanos, hidrocarburos, cetonas y otros. La mayoría de estos compuestos son el resultado de los procesos de degradación/reordenación de lípidos y carbohidratos.

SEXTA: Se han propuesto dos métodos para la evaluar la idoneidad del hexanal como indicador del proceso de oxidación lipídica. Primeramente, se ha optimizado un método de microextracción en fase sólida (SPME) seguido de cromatografía gaseosa con detección de masas. A continuación se ha puesto a punto un método de derivatización con 2,4-dinitrofenilhidrazina seguido de cromatografía líquida de alta resolución. Ambos métodos han demostrado ser adecuados para la identificación y cuantificación del hexanal. El método de cromatografía gaseosa presenta un límite de detección más bajo (1ng/ml) que el de cromatografía líquida (9ng/ml), sin embargo, se recomienda este último para la determinación del hexanal en patatas fritas dada su excelente repetibilidad y linearidad.

Los resultados indicaron que en las patatas fritas almacenadas en la oscuridad no hubo cambios en el contenido de hexanal, mientras que en las almacenadas a la luz se produjo un aumento significativo. Se verifica así, que el hexanal es un buen indicador del proceso de oxidación lipídica

ya que este aumenta durante el almacenamiento e indica la alteración de las propiedades sensoriales.

SEPTIMA: Para la determinación de triclosan, antimicrobiano incluido en plásticos para envasar alimentos, se propone un método de cromatografía líquida de alta resolución con detección ultravioleta a 205, 235 y 280 nm. El método ha sido validado en cuatro alimentos seleccionados como representativos del amplio rango de complejidad de las matrices alimentarias.

Los coeficientes de correlación de las rectas de calibración ($r>0,99$) muestran que el método es válido para la cuantificación de triclosan. Las recuperaciones del método, calculadas a tres niveles (5, 1 y 0.5 µg/ml) han sido satisfactorias (83.22-105.39%) en todos los casos.

La confirmación de la identidad del triclosan ha sido evaluada por cromatografía gaseosa y líquida, ambas acopladas a un detector de masas.

La fotografía de la portada fue tomada de la p□gina web:
www.vitro.com/envases/ ingles/home.htm.