Universidad de Santiago de Compostela

Facultad de Farmacia

Departamento de Química Analítica, Nutrición y Bromatología

Área de Nutrición y Bromatología

Determinación de la migración de sustancias químicas desde materiales poliméricos a los alimentos

Memoria para optar al grado de doctor

RAFAEL JAVIER PASEIRO CERRATO

Santiago de Compostela, Julio 2012

Dr. ANTONIO MOREDA PIÑEIRO, DIRECTOR DEL DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

INFORMA: Que Don Rafael Paseiro Cerrato presenta el trabajo titulado "Determinación de la migración de sustancias químicas desde materiales poliméricos a los alimentos" realizado bajo la dirección de la Dra. Ana Rodríguez Bernaldo de Quirós y del Dr. Perfecto Paseiro Losada, en los laboratorios de Bromatología de la Facultad de Farmacia de la Universidad de Santiago de Compostela.

Fdo. Dr. Antonio Moreda Piñeiro

Santiago de Compostela, Julio 2012

ANA RODRÍGUEZ BERNALDO DE QUIRÓS, PROFESORA CONTRATADA DOCTORA DEL ÁREA DE NUTRICIÓN Y BROMATOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA Y PERFECTO PASEIRO LOSADA, CATEDRÁTICO DEL ÁREA DE NUTRICIÓN Y BROMATOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

AUTORIZAN:

A Don Rafael Javier Paseiro Cerrato a presentar la Tesis titulada "Determinación de la migración de sustancias químicas desde materiales poliméricos a los alimentos" para optar al Grado de Doctor, la cual ha sido realizada bajo nuestra dirección en los 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 Julio de 2012.

Fdo. Dra. Ana Rodríguez Bernaldo de Quirós

Fdo. Dr. Perfecto Paseiro Losada

Fdo. Don Rafael Javier Paseiro Cerrato

AGRADECIMIENTOS

Quisiera expresar mi más sincero agradecimiento a,

Mis directores de tesis, la Doctora Ana Rodríguez Bernaldo de Quirós y el Doctor Perfecto Paseiro Losada, por su gran apoyo y orientación a lo largo de estos años para la realización de esta tesis. Sin ellos este trabajo no hubiera sido posible.

A los doctores Julia López Hernández, Raquel Sendón García, Asunción Lage Yusty y José Francisco Huidobro Canales por haberme guiado y por su valiosa orientación en muchos de los capítulos de esta tesis.

A los doctores Tim Begley y Gregory Noonan de la Food and Drug Administration de Estados Unidos y también al Doctor Roland Franz del Fraunhofer IVV Alemán, por haberme dado la oportunidad de colaborar con ellos durante tres meses en sus respectivos centros y contribuir activamente en mi formación.

Al subprograma de formación de personal investigador (ayudas FPI) por haberme otorgado la ayuda predoctoral de formación de personal investigador de referencia BES-2009-023016 asociada al proyecto de investigación AGL2008-04146, en el organismo Universidade de Santiago de Compostela, sin la cual todo esto no hubiera sido posible.

A mis compañeros del departamento Patricia, Letricia, Cristina, Maribel, Gonzalo, Pablo, Miguel y Joaquín, de los cuales tendré siempre un bonito recuerdo y una cuantas anécdotas que contar.

A todo el departamento de Química Analítica, Nutrición y Bromatología.

A toda mi familia. A mis padres, Carmen y Perfecto por su constate apoyo, cariño eterno y por haberme enseñado los valores que guían y guiarán los

senderos de mi vida A mis hermanos Perfecto, Carmen y Ana a los que quiero con todo mi corazón. A mis abuelos y abuelas, a los que querré siempre.

A mí querida novia Caroline por su constante apoyo y amor.

A mis amigos, por haber vivido y crecido conmigo, y por haberse caracterizado por estar siempre apoyándome y mostrándome el camino.

A todas las personas que no he mencionado por descuido y que han participado directa o indirectamente en la realización de esta tesis.

A todos, gracias.

A mis abuelas y abuelos, con todo mi cariño y amor.

INDICE GENERAL

INDICE DE TABLAS

VI-Characterization and analysis of two novel compounds yielded from the reaction among a common amine employed as monomers in food contact materials (MXDA) and fatty acids presents in fatty nature: instability of amines in fatty food

VII-Migration of photoinitiators by gas phase into dry foods

VIII-Study of the kinetic of migration of benzophenone, DPBD and uvitex ob: determination of diffusion and partition coefficients by direct contact and through the vapour phase

INDICE DE FIGURAS

ABREVIATURAS

APCI: Atmospheric-pressure chemical ionization

PAAs: Primary aromatic amines

DART: Direct analysis in real time

ESI: Electrospray ionization

KU: Kitchen utensil

MXDA: m-Xylenediamine

DPBD: Diphenylbutadiene

IR: Infrared spectroscopy

EI: Electronic impact

RMN: Resonancia magnetica nuclear

LDPE: low density polyethylene

HPLC: High performance liquid chromatography

ACN: Acetonitrile

MPPO: Modified polyphenylene oxide (tenax ®)

LMG: Límite de migración global

LME: Límite de migración específico

CM: Límite de cantidad residual en materia

FCM: Food contact materials

UPLC: Ultra performance liquid chromatography

TOF: Time of flight

LOD: Limit of detection

LOQ: Limit of quantification

DAD: Diode array detection

RASFF: Rapid alert system for food and feed

ITX: Isopropylthioxanthone

RESUMEN

El primer capítulo consiste en una introducción sobre los principales aspectos que se abordan en esta Tesis.

La cesión de sustancias químicas desde un material o artículo (envases, grandes contenedores, vajillas, cuberterías, útiles de cocina, etc.) al alimento con el que entra en contacto directo o indirecto se denomina migración.

En esta tesis se aborda el estudio de la migración a través de tres ejemplos de interés en el campo de la Seguridad Alimentaria: la problemática de las aminas polifuncionales, la problemática de los fotoiniciadores y por último, la de los de los aditivos alimentarios.

En el segundo capítulo, se lleva a cabo una amplia revisión bibliográfica relativa a los métodos cromatográficos utilizados para la determinación de aminas polifuncionales utilizadas como monómeros y aditivos en la fabricación de materiales destinados a entrar en contacto con alimentos incluidos en la lista positiva de la Unión Europea. Este estudio fue publicado como artículo en una revista internacional: *Comprehensive Reviews in Food Science and Food Safety*, en el volumen 9, Páginas 676-694 en Noviembre del 2010. También se presentó en *el 4th International Symposium on Recent Advances in Food Analysis*, Praga, República Checa. Del 4 al 6 de noviembre del 2009.

Las aminas polifuncionales son un grupo de sustancias muy reactivas y altamente utilizadas en la fabricación de materiales en contacto con alimentos. Algunos de estos compuestos pueden ser potencialmente peligrosos para la salud de los consumidores. Con el fin de verificar la migración de aminas desde el envase a los alimentos, es necesario el desarrollo de métodos para su determinación que sean lo suficientemente sensibles y fiables. Debido a la amplia variedad de estructuras químicas que tienen estos compuestos, no hay ningún método estándar para analizarlos de forma simultánea. La mayoría de los

procedimientos descritos en la literatura son métodos de un solo componente, sin embargo, los métodos multianalito para la determinación de aminas son muy escasos. De ahí que con fines de control resulte conveniente disponer de métodos multianalito para la determinación aminas polifuncionales que sean precisos y sensibles y permitan la detección de las mismas de una manera fácil, sencilla y poco costosa a los niveles exigidos por la legislación. El capítulo se divide en 2 secciones principales: monómeros y aditivos. Los monómeros se clasificaron en función de su estructura química: aminas alifáticas, alcohol aminas, aminas aromáticas, amidas, compuestos relacionados que contienen azufre, aminotriazinas, acrilamidas y otras aminas polifuncionales utilizados como monómeros. Los aditivos se clasificaron atendiendo a su efecto técnico en las siguientes categorías: lubricantes, estabilizantes UV, abrillantadores ópticos, aceleradores de la vulcanización, agentes tensosactivos, antioxidantes, antimicrobianos y otros aditivos. Hay algunas sustancias que pueden ser utilizadas como monómeros y aditivos, de modo que se ha dedicado un apartado especial a este tipo de sustancias. Esta clasificación se hizo en función a estos criterios para facilitar la comprensión. Además, en este capítulo también se describen las propiedades físico-químicas de las aminas objeto de estudio ya que se consideran imprescindibles a la hora de desarrollar métodos analíticos. Las propiedades físico-químicas presentadas fueron las siguientes: superficie topológica polar / Área de la superficie de VanderWaals, punto de ebullición, punto de fusión, presión de vapor, densidad y constante de la ley de Henry. Esta amplia revisión sirvió de base para el desarrollo de un método multianalito para la determinación de aminas polifuncionales empleadas como monómeros en la fabricación de materiales en contacto con alimentos.

En el capítulo tercero, se describe el desarrollo de un método analítico para la determinación simultánea de aminas polifuncionales utilizadas como monómeros en la fabricación de materiales destinados a entrar en contacto con alimentos. Este estudio se publicó como artículo en la revista internacional *Journal of Chromatography A*, Volumen 1218, Número 40, 7 de octubre de 2011, páginas 7105-7109. Parte de este trabajo también se presentó como poster en el congreso internacional *IFT Annual Meeting and Food Expo*®, en Chicago IL, EE.UU. Del 17 al 20 julio 2010 y en el *7th Aegean Analytical Chemistry Days* (AACD 2010). Del 29 septiembre al de 3 octubre del 2010. Las aminas se analizaron por cromatografía líquida de alta resolución con detección ultravioleta (HPLC-UV) después de una derivatización con cloruro de dansilo. Las aminas empleadas en este estudio fueron: hexametilentetramina, etilendiamina, 1,4 - diaminobutano, m-xililendiamina, hexametilendiamina, isoforonodiamina y etanolamina. La derivatización se realizó mediante la adición de 1 ml de la solución de cloruro de dansilo (5 mg / ml preparada en acetona) a 1 ml de la solución estándar que contenía las aminas. El pH de la solución se ajustó a 9,7 con $NaHCO_3$. La solución se mezcló y se incubó 60 min a 80 °C. La separación cromatográfica se realizó en una columna Kromasil ODS (C18) (150 x 3,20 mm, 5 μm) termostatizada a 25 °C. Las fases móviles empleadas para el análisis fueron metanol y agua. La elución se llevó a cabo en gradiente. Las aminas se detectaron a 254 nm o a 246 nm, dependiendo de donde estuviera el máximo de absorción en sus espectros UV. Estos compuestos se identificaron mediante la comparación de sus tiempos de retención y de sus espectros UV con las de los estándares de las mismas. El método fue validado en términos de linealidad, límite de detección, repetibilidad y recuperación. LC-MS/MS, utilizando una fuente APCI en el modo de ion positivo, fue utilizada como técnica de confirmación. El análisis de confirmación identificó la formación de los

derivados disustituidos del cloruro de dansilo con la mayoría de las aminas empleadas para este estudio excepto para la etanolamina, donde se identificó el producto monodansilado del mismo. La estabilidad de las aminas se testó en los siguiente simulantes de alimentos: Agua destilada, ácido acético al 3%, etanol al 10%, etanol al 50% y en aceite de oliva. Las condiciones empleadas para determinar la estabilidad de las aminas en los simulantes fueron 10 días a 40 °C. Los resultados mostraron que las aminas tenían una estabilidad aceptable en la mayoría de los simulantes de alimentos, sin embargo, en el aceite de oliva se observó una pérdida de 100% para todos los analitos. El método propuesto es preciso y sensible y podría ser utilizado como una herramienta analítica excelente para la determinación de las aminas en los laboratorios de control.

El capítulo cuatro de la tesis trata sobre el análisis de la histamina como indicador de vida útil en productos del mar. Este estudio ha sido publicado en un número especial del *Italian Journal of Food Science*, páginas 51-54 en el año 2011. También fue presentado como póster en el *Shelf Life International Meeting*. Zaragoza, España. Del 23 al 25 junio 2010. El trabajo recibió el "SLIM Poster award". La histamina es una amina biogénica formada por la descarboxilación enzimática del aminoácido histidina. La intoxicación por histamina puede causar varios efectos adversos tales como: dolor de cabeza, erupción cutánea, náuseas, vómitos, urticaria, palpitaciones cardíacas, etc. Además, se considera como indicador de deterioro de los alimentos y se utiliza como un marcador para el control de calidad de los alimentos. Muestras de surimi fueron seleccionadas como alimento representativo de origen marino para la determinación de la histamina. La histamina se determinó previa derivatización con cloruro de dansilo por medio de cromatografía líquida de alta resolución con detección ultravioleta. La extracción de la histamina se llevó a

cabo por la adición de 25 ml de HCl 0,1 N a 5 g de muestra. La mezcla se homogeneizó durante 10 minutos. El sobrenadante se eliminó y el residuo se volvió a extraer con 25 ml de HCl 0,1 N. Los sobrenadantes se juntaron y llevaron a 50 ml; la solución se almacenó posteriormente a 4 ºC durante la noche para favorecer la precipitación de los lípidos y las proteínas. Transcurrido este periodo de tiempo, una alícuota de la solución se filtró y se derivatizó. La derivatización de la histamina se realizó mediante la adición de 1 ml de solución de cloruro de dansilo (5 mg / ml) a 1 ml de muestra y 300 µL de solución saturada de $NaHCO_3$; a continuación la mezcla se incubó a 80 °C 60 min. La separación cromatográfica se logró utilizando una columna Kromasil ODS (C18) (150 x 3,20 mm, 5 µm) termostatizada a 25 °C. Un programa de elución en gradiente fue utilizado empleando metanol y agua como fases móviles. El detector de diodos se fijó a 254 nm. El contenido de histamina en surimi estaba comprendido en un rango de 1.0 a 2.5 mg / kg, por lo tanto, las muestras de surimi analizadas cumplen con la normativa europea (CE) n º 1441/2007.

En el capítulo cinco se trata la problemática de las aminas aromáticas primarias (PAAs en inglés). Las PAAs son en general un grupo de sustancias que provocan efectos perjudiciales para la salud humana. Estas pueden ser utilizadas o formarse durante la producción de polímeros, tintas y/o adhesivos. Numerosos métodos analíticos emplean la cromatografía líquida de alta resolución con detección UV o la espectrometría de masas para la determinación de PAAs en simulantes de alimentos. El análisis mediante una nueva e innovadora técnica denominada DART (en inglés, Direct Analysis in Real Time, lo que podría traducirse como análisis directo en tiempo real) empleando espectrometría de masas, no requiere preparación de la muestra y ha sido aplicada con éxito en la determinación de varios compuestos en materiales destinados a entrar en

contacto con alimentos. El objetivo de este estudio es determinar si el DART-MS podría ser utilizado como método de screening y sustitutivo en la determinación de PAAs en utensilios de cocina en lugar de realizar la migración en simulantes de alimentos y posterior análisis por las técnicas comúnmente utilizadas. Para ello se realizaron análisis de utensilios de cocina empleando DART-MS y también realizando los análisis de migración correspondiente en el simulante alimenticio acido acético al 3% y su posterior análisis por cromatografía líquida de alta resolución con altas presiones acoplado a un detector MS/MS. Varias aminas aromáticas primarias se han utilizado en este trabajo incluyendo anilina ,4-cloro-2 ,5-dimethoxyaniline, 4,4 '-oxidianilina, o-toluidina, o-tolidina, 2-naftilamina, 2-metil-m-fenilendiamina, 2,6-dimetilanilina, bencidina, m-phenilendiamina, 4-aminobifenilo, 2,4 diaminotolueno y 4,4'-diaminodifenilmetano. El método cromatográfico ha sido validado en términos de linealidad, recuperación, repetibilidad, límites de cuantificación y detección. La cuantificación de muestras en ácido acético al 3% se ha llevado a cabo utilizando patrones internos con estructuras químicas similares a las aminas. Una columna Phenomex Synergi (C18) Hidro-RP 80 A, (150 x 2 mm de diámetro y 4µm de tamaño de las partícula) se utilizó como fase estacionaria. La temperatura se fijó a 30 ºC. Un gradiente de metanol con acetato de amonio 5 mM y H_2O: metanol (95:5 v/v) con 5 mM de acetato amónico se utilizó como fase móvil. La detección se realizó a través de ionización por electrospray (ESI) en el modo positivo. Por otra parte, los parámetros de DART se optimizaron antes del análisis y las condiciones óptimas se obtuvieron aplicando un voltaje de 15 V en el orificio 1 y poniendo la temperatura del gas a 500 °C. Los datos muestran que DART es una valiosa herramienta que puede emplearse como método de screening para identificar PAAs in utensilios de cocina.

El capítulo seis de esta tesis doctoral se dedica al estudio de los compuestos formados por la reacción entre las aminas y componentes de los alimentos de naturaleza grasa. Se describe por primera vez la síntesis y caracterización de los productos obtenidos como resultado de la reacción de m-xililendiamina (MXDA), una amina polyfuntional ampliamente utilizada como monómero en la fabricación de materiales en contacto con alimentos, con el ácido oleico y ácido palmítico, dos compuestos seleccionados como ácidos grasos modelo. Primeramente se sintetizaron los compuestos por dos rutas distintas. Una vez aislados se llevó a cabo una completa caracterización e identificación de ambos productos empleando varias técnicas como la espectroscopia de infrarrojos (IR) impacto electrónico (EI) espectrometría de masas, RMN de protón y carbono 13, espectrofotometría UV y LC-MS/MS. Los compuestos resultantes fueron dos moléculas que pertenecen a la familia de las amidas de los ácidos grasos, estas dos moléculas resultantes fueron denominadas con el nombre de dioleamida y dipalmitida. Con el fin de estimar la toxicidad de los nuevos compuestos sintetizados, se aplicaron las conocidas reglas Cramer, las cuales estiman cuál puede ser la toxicidad de las moléculas en función de su estructura química. Los resultados muestran que dichas sustancias son susceptibles de tener una alta toxicidad. Con el fin de demostrar y verificar la formación de estos compuestos en muestras reales, se le añadió una concentración conocida de MXDA a muestras de aceite de oliva. Consecuentemente, se procedió al desarrollo de un método rápido y simple para extraer las amidas de los ácidos grasos de la matriz de aceite. Posteriormente, las sustancias extraídas se identificaron por LC-MS/MS. El método de extracción se logró después de realizar una saponificación en frío del aceite empleando KOH 0,5 N a 40 ºC durante 24 h. A continuación, 2 ml de agua Milli-Q se añadieron a 2 ml de la muestra. Después, se añadieron 2 ml de una solución saturada de NaCl seguido por 6 ml de cloroformo. La

solución se mezcló cuidadosamente y después se centrifugó (5000 rpm, 10 min, 4 ºC). La capa inferior de cloroformo se separó y se evaporó a sequedad. A continuación, se añadió 1 ml de acetonitrilo al residuo insaponificable sobrante de la evaporación y se llevó al ultrasonidos durante 1 hora a 40 ºC. La solución de acetonitrilo se inyectó en el cromatógrafo de líquidos acoplado al detector de masas. Para realizar los análisis se empleó una fuente APCI. La separación cromatográfica se logró utilizando una Ace 3 C18 HL (30 mm x 3,0 mm, 3 μm de tamaño de partícula, Advanced Chromatography Technologies). La temperatura se fijó a 30 ºC. Los análisis se llevaron a cabo en modo isocrático con acetonitrilo como fase móvil. Ambos compuestos fueron identificados con éxito en las muestras reales de aceite de oliva. Estos productos pueden representar un peligro para la salud, por lo tanto, se deben realizar más estudios sobre la posible toxicidad de estas nuevas sustancias y sus posibles efectos sobre la salud humana.

En el capítulo siete se estudia la migración de siete fotoiniciadores a través de la fase de vapor. Este estudio ha sido publicado en el *Journal of Agriculture and Food Chemistry*, en el año 2009, Volumen 57, páginas 10211-10215. También fue presentado como póster *en 4th International Symposium on Recent Advances in Food Analysis*. Praga, República Checa. Del 4 al 6 de noviembre, 2009. La Benzofenona y sus derivados se emplean como fotoiniciadores para la formación de tintas utilizadas en la impresión de envases de alimentos. Varios estudios han demostrado la migración de los fotoiniciadores desde el papel y el cartón a los alimentos a través de contacto directo. Sin embargo, los datos relativos a la migración a través de la fase de vapor son muy escasos. En este estudio, la migración de la benzofenona y algunos de sus derivados a través de la fase gaseosa se ha evaluado. Para llevar a cabo el ensayo de migración, una cera de

polietileno enriquecida con los fotoiniciadores se empleó como fuente de liberación de fotoiniciadores. Para realizar el estudio de migración de los fotoiniciadores se emplearon cinco alimentos (bizcocho, pan, cereales, pasta y arroz). En el capítulo también se presentan datos importantes relativos a las estructuras químicas y propiedades fisicoquímicas de los fotoiniciadores. Los análisis se han realizado utilizando un método de HPLC-DAD. Se utilizó una columna Kromasil 100 C18 (25 x 0,4 cm ID, 5 μm). La fase móvil utilizada fue un gradiente de acetonitrilo y agua. Las longitudes de onda seleccionadas fueron 254 nm para la mayoría de fotoiniciadores a excepción del 4-benzoylbifenil donde la longitud de onda seleccionada fue 290 nm. Los estudios de migración se realizaron bajo condiciones aceleradas (70 ºC, 48 h), que simula el peor caso de migración. La porosidad de los alimentos también se estudió como parámetro de importancia que influye activamente en la migración. La porosidad se midió empleando un picnómetro. La cinética de migración para cada compuesto también ha sido ha evaluada. El equilibrio se alcanzó después de 150 horas para la mayoría de los fotoiniciadores. Los resultados confirman que la mayoría de los compuestos migran al bizcocho, el cual tiene un alto contenido en grasa y es un alimento con una elevada porosidad. De los fotoiniciadores estudiados se observó una mayor migración de la 2-hidroxi benzofenona, benzofenona y 4-metil benzofenona, estos compuestos presentan unas presiones de vapor elevadas, lo que explicaría la elevada migración de los mismos. En resumen, se puede concluir que los alimentos con alto contenido de grasa y altos valores de porosidad están expuestos a niveles altos de migración de fotoiniciadores.

En el Capítulo ocho se estudia la cinética de migración por contacto directo y a través de la fase de vapor de tres migrantes modelo, la benzofenona, el Difenilbutadieno (DPBD) y el Uvitex OB. Por atra parte, también se

determinaron los coeficientes de difusión y partición. Además, los coeficientes de difusión determinados de forma experimental se van a utilizar para estimar coeficientes de difusión a otras temperaturas empleando la ecuación de Arrhenius. Este estudio ha sido presentado en *el IFT Annual Meeting and Food Expo*®. Chicago IL, EE.UU. Del 17 al 20 julio de 2010. En este capítulo, la migración de los tres aditivos seleccionados (benzofenona, DPBD y Uvitex OB) por contacto directo y a través de la fase de gas son comparados a través de los coeficientes de partición y difusión. Para evaluar la migración de los aditivos a través de la fase gaseosa, el bizcocho (alimento selecionado para este estudio) y un film de polietileno contaminado con los aditivos fueron colocados, sin contacto directo entre ellos, en un recipiente de vidrio que se cerró herméticamente. Para los ensayos de migración por contacto directo, el film de polietileno de baja densidad (LDPE) se ponía en contacto por ambos lados con el bizcocho, posteriormente las muestras se envolvieron en papel de aluminio y se llevaron a una estufa para realizar el ensayo de migración. Las muestras se almacenaron a distintas condiciones de exposición de tiempo-temperatura. Los migrantes se extrajeron de los films de polietileno con etanol (70 ºC, 24 h) y a continuación se analizaron por cromatografía líquida de alta resolución con detección ultravioleta (HPLC-UV). Una columna Kromasil C18 (25 x 0.32 cm, 5 µm de tamaño de partícula) se utilizó como fase estacionaria. La fase móvil estaba compuesta por agua y otro eluyente compuesto por 30% de tetrahidrofurano y 70% metanol. La detección de los analitos fue la siguiente: 256 nm para benzofenona, 330 nm para DPBD y 372 nm para Uvitex OB. La temperatura se fijó en 30 ºC durante todo el programa. Los resultados indicaron que en ambos casos la migración de benzofenona se produce en gran medida. Sin embargo, para DPBD y Uvitex OB la migración sólo se produce por contacto directo a todas las temperaturas estudiadas.

En el último capítulo de este trabajo, se desarrolla un método para la determinación de natamicina en alimentos. Este trabajo ha sido presentado en el *Shelf Life International Meeting*. Zaragoza, España. Desde el 23 hasta el 25 junio de 2010. La superficie de los alimentos es susceptible de ser contaminada por microorganismos. Esta contaminación puede provocar el deterioro de los alimentos y los hace inadecuados para el consumo. Uno de los sistemas más comúnmente utilizados para evitar la contaminación superficial es la aplicación directa de agentes antimicrobianos en la superficie de los alimentos. La natamicina es un antibiótico que pertenece al grupo de los macrólidos poliénicos producidos por el actinomicetes *Streptomyces natalensis*; este aditivo alimentario (E235) se emplea generalmente como agente antifúngico para aumentar la vida útil de algunos productos alimenticios. La máxima concentración de natamicina en el producto final que permite la legislación europea es 1 mg/dm^2 y no debe ser detectable a una profundidad de 5 mm. El empleo de natamicina se permite en algunos tipos de quesos y embutidos. Con fines de control para cumplir con la legislación alimentaria europea se necesitan métodos rápidos, precisos y sensibles. El objetivo del presente estudio es desarrollar un método cromatográfico rápido y sencillo con detección DAD para determinar natamicina en muestras de alimentos. Los alimentos empleados comprenden varios tipos de quesos, entre ellos el fresco y blando, semicurado, curado, Camembert y Roquefort. Siete tipos de embutidos diferentes, incluyendo tipos de jamón y chorizo. También se incluyeron en este estudio salchichas y doce vinos comerciales blancos y tintos. LC-MS/MS con ionización por electrospray (ESI) en modo positivo se utilizó como técnica de confirmación. La natamicina se extrajo de las muestras de alimentos utilizando 0,5 g de cada muestra y posteriormente se añadieron 10 ml de metanol acidificado con ácido acético. La

solución resultante se agitó durante 5 minutos. Después, 1 ml de la solución se retiró y se diluyó con 2 mL con agua Milli-Q. Las muestras se almacenaron a -22 ºC durante la noche. Finalmente, las muestras se filtraron y se inyectaron en el sistema de HPLC. Las muestras de vinos se filtraron y se inyectaron directamente en el HPLC. La separación se realizó en una Kromasil ODS C18 (150 x 3,20 mm de diámetro interno, 5 µm de tamaño de partícula) termostatizada a 25 º C. Un gradiente de acetonitrilo y agua Milli-Q se empleó como fase móvil. Se seleccionaron tres longitudes de onda correspondientes a los tres máximos del espectro de absorción característico de la natamicina, éstas fueron 291,4, 304,4 y 319,4 nm. El método fue validado en lo que se refiere a linealidad, límites de detección y cuantificación, recuperación y repetibilidad. Los datos mostraron que dos muestras excedieron el límite establecido por la legislación europea. La natamicina también fue detectada en muestras en las que su uso no está permitido.

ABSTRACT

In the first chapter of this thesis, a summary is presented. The principal topics of this work such as the importance of the food contact materials, migration of the compounds from the materials into food or food simulants, the safety and analytical topics of the polyfunctional amines as well as the photoinitiators and food additives are reported.

In the second chapter, an extensively review concerning the chromatographic methods used for the determination of polyfunctional amines used as monomers and additives in the manufacture of food contact materials included in the positive list of the European Union is presented. This study was published as paper in an international journal: *Comprehensive Reviews in Food Science and Food Safety*. Volume 9, pages 676–694, November 2010. It was also presented as poster in the international congress *4th International Symposium on Recent Advances in Food Analysis*. Prague, Czech Republic. November 4–6, 2009. Polyfunctional amines are a group of substances very reactive and frequently employed in the manufacture of food contact materials. Some of these compounds can be potentially dangerous for the consumers' health. In order to verify the migration of these compounds from the packaging into the food, sensitive and reliable methods are required. Because of the wide variety of chemical structures there are no standard methods to analyze polyfunctional amines. Most of the procedures reported in the literature are single-component methods; however multi-analyte methods to determine several amines are very scarce. Thereby, the development of accurate and sensitive methods for the simultaneous determination of amines that allow the determination of the analytes at the regulatory levels are required. The review is divided into 2 main sections: monomers and additives. Monomers are classified according to their chemical structure as it follows: aliphatic amines, alcohol amines, aromatic

amines, amides, related compounds containing sulphur, aminotriazines, acrylamides and other polyfunctional amines used as monomers. Additives are classified according their use into the following categories: lubricants, UV stabilizers, optical brighteners, accelerators for vulcanization, surfactants, antioxidants, antimicrobial agents and other additives. There are some substances that may be used even as monomers as well as additives, so that, a special sections of this review is targeted to the additive-monomers. This order should make for easy understanding. Since the knowledge of many physicochemical properties of the substances is crucial in the selection of a suitable analytical method, physicochemical experimental and estimated properties of the substances as: Topological polar surface area / vanderWaalsSurface Area, boiling point, melting point, vapour pressure, density, and Henry's law constant are considered in this review.

In third chapter, the development of an analytical method for the simultaneous determination of polyfunctional amines used as monomers in the manufacture of food packaging materials is presented. This study was published as paper in an international journal: *Journal of Chromatography A,* Volume 1218, Issue 40, 7 October 2011, Pages 7105–7109. Part of this work was also presented as poster in the international congress *IFT Annual Meeting and Food Expo*®. Chicago IL, USA. July 17-20, 2010 and in the *7th Aegean Analytical Chemistry Days* (AACD2010) September 29 - October 3, 2010. Amines were analyzed by high-performance-liquid-chromatography with diode-array detection (HPLC-DAD) after derivatization with dansyl chloride. The amines employed in this study were hexamethylenetetramine, ethylenediamine, 1,4-diaminobutane, *m*-xylylenediamine, hexamethylenediamine, isophoronediamine and ethanolamine. The derivatization was performed by adding to 1 mL of the standard solution

containing the amines, 1 mL of the dansyl chloride solution (5 mg/mL prepared in acetone), the pH of the solution was adjusted to 9.7 with $NaHCO_3$. The solution was mixed and incubated 60 min at 80 ºC. Chromatographic separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm, 5 μm) thermostatted at 25 ºC. The mobile phases employed for the analysis were methanol and water. Amines were detected at 254 nm or at 246 nm, depending on the maximal absortion of their UV spectra. Amines were identified by comparing their retention times and UV spectra with those of pure standards. The method was validated in terms of linearity, limit of detection, repeatability and recovery. LC-MS/MS using atmospheric pressure ionisation (APCI) in the positive ion mode was used as confirmatory technique. The confirmatory analysis identified the disubstituted dansyl derivatives for all amines except for ethanolamine were the the monodansylated product has been identified. The stability of the amines was tested in distilled water, 3% acetic acid, 10% ethanol, 50% ethanol and olive oil under the most common testing conditions (10 days at 40 ºC). Results showed that amines had an acceptable stability in most of simulants, however, in the olive oil a loss of 100% was observed for all analytes. The proposed method is precise and sensitive and could be used as an excellent analytical tool for determination of amines in control laboratories.

Chapter four of the thesis is entitled “Analysis of histamine as indicator of shelf life in seafood”. This study has been published in the *Italian Journal of Food Science*, Special issue, 51-54, 2011. It was also presented as a poster in *Shelf Life International Meeting. Zaragoza*, Spain. June 23-25, 2010. The work received the “SLIM Poster award”. Histamine is a biogenic amine formed by enzymatic decarboxylation of the amino acid histidine. Histamine intoxication can cause several and serious undesirable effects including headache, rash, nausea,

vomiting, urticarias, cardiac palpitation and so on. Furthermore, it is considered as indicator of deterioration in foods and used as a marker for food quality control. Surimi samples have been selected as representative seafood to determine histamine. The amine has been detected after derivatization with dansyl chloride employing HPLC-UV detection. It was extracted adding 25 mL of 0.1 N HCl to 5 g of sample, the mixture was homogenized for 10 min. The supernatant was removed and the residue was re-extracted with 25 mL of 0.1 N HCL. The supernatants were combined and made up to 50 mL. The solution was stored at 4 ºC overnight in order to precipitate lipids and proteins. Then, an aliquot of the solution was filtered and derivatized. The dansylated derivative of histamine was formed by adding to 1 mL of sample 1 mL of dansyl chloride solution (5 mg/mL) and 300 μL of saturated $NaHCO_3$ solution; then the mixture was incubated at 80 ºC 60 min. The chromatographic separation was achieved using a Kromasil ODS (C18) (150 x 3.20 mm, 5μm) column thermostated at 25ºC. A gradient elution program was used employing methanol and water as mobile phases. The diode array detector was set at 254 nm. Histamine contents ranged from 1.0 to 2.5 mg/kg, thus, surimi samples are in good agreement with the actual European regulation (CE) No 1441/2007 which establishes a caution level of 100-200 mg/kg in seafood.

In chapter five the safety and analytical issues of primary aromatic amines (PAAs) are treated. PAAs are a group of substances, with undesirable health effects, that can be formed or used during the production of polymers, inks and adhesives. Numerous analytical methods, using liquid chromatography with UV and/or mass spectrometry detection, for the determination of PAAs in food simulants, have been developed. Direct analysis in real time mass spectrometry (DART-MS) requires very little sample preparation and has been successfully

applied as a screening method to determine compounds in food contact materials. The aim of this study was to determine if DART-MS could be utilized, in place of migration testing, as a screening method for PAAs in kitchen utensils. Kitchen utensils migration has been performed in 3% (w/v) acetic acid, which represents the worst case for the migration, results have been analyzed by UPLC-MS/MS. DART analysis has been compared with UPLC-MS/MS method. Several primary aromatic amines have been used in this study including Aniline, 4-chloro-2,5-dimethoxyanile, 4,4´-oxydianiline, o-Toluidine, o-Tolidine, 2-Naphthylamine, 2-methyl-m-phenylendiamine, 2,6-dimethylaniline, Benzidine, m-phenylendiemine, 4-aminobiphenyl, 2,4-Diaminotoluene and 4,4´-Diaminodiphenylmethane. The chromatographic method has been validated in terms of linearity, recovery, repeatability, LOQ and LOD. Quantification in 3% acetic acid samples has been carried out using internal standards with similar chemical structures to the amines. A Phenomex Synergi (C18) Hidro-RP 80 A, (150 x 2 mm i.d 4 µm particle size) was used as stationary phase. Temperature was set at 30 ºC. A gradient of methanol with 5mM ammonium acetate and H_2O: methanol (95:5 v/v) with 5 mM ammonium acetate was used as mobile phase. Mass detection was performed using electrospray ionization (ESI) in the positive mode. On the other hand, DART parameters have been optimized before analysis and the optimal conditions were voltage of 15 V in orifice 1 and gas temperature at 500 ºC. Of the employed samples included in this study, four samples of polyamide which previously contains PAAs where analyzed Furthermore, three polypropylene samples were used as control. DART detect PAAs in all polyamide analyzed samples. So that, DART can be used as a powerful screening tool to identify PAAs in kitchen utensils samples.

Chapter six is devoted to study the compounds formed as a result of the reaction between amines and fatty components of food. In this study for the first time is described the synthesis and characterization of the products obtained as a result of the reaction of *m*-xylylenediamine (MXDA), a polyfuntional amine widely used as monomer in the manufacture of food contact materials, with oleic acid and palmitic acid, two compounds selected as model fatty acids. To carry out the experiment, firstly the two compounds were synthesized by two different routes. Afterwards, a complete characterization and identification of both products were carried out employing several techniques as Infrared spectroscopy (IR), Electron Impact (EI) mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy, UV spectrophotometry and LC/MS/MS. The resulting compounds were two molecules belonging to the family of fatty acid amides, dioleamide and dipalmitide. Cramer rules have been applied in order to estimate toxicity of the compounds showing that dioleamide and dipalmitide are susceptible to have high toxicity. The formation of these compounds in olive oil samples was tested spiking a known concentration of MXDA in the olive oil. A rapid and simple method was developed to extract the fatty acid amides from the fatty matrix and consequently identified by LC-MS/MS. Extraction method was achieved after cold saponofication with 0.5 N KOH at 40 ºC during 24 h. Then, 2 ml of milli-Q water were added to 2 ml of the sample. Afterwards, 2 ml of a saturated solution of NaCl were incorporated followed by 6 ml of cloroform. The solution was mixed and then centrifugated. The lower chloroform layer was removed and evaporated to dryness. Afterwards, 1 ml of acetonitrile was incorporated and sonicated at 40 ºC for 1h. The acetonitrile solution was injected into a LC/MS/MS system. MS data were acquired in the positive ion mode employing atmospheric pressure chemical ionization (APCI). Chromatographic separation was achieved using an Ace 3 C18 HL (30 mm x 3.0 mm, i.d. 3 μm particle size,

Advanced Chromatography Technologies). Temperature was set at 30 ºC. Analyses were carried out in isocratic mode using acetonitrile as mobile phase.
Both compounds have been successfully identified. These products could represent a hazard on the human heath, thereby, more studies concerning to the toxicity of these substances should be performed.

In the chapter seven, the migration of seven photoinitiators through the vapour phase was investigated. This study has been published in the *Journal of Agriculture and Food Chemistry*, 2009, 57, 10211-10215. It was presented as poster at *4th International Symposium on Recent Advances in Food Analysis*. Prague, Czech Republic. November 4–6, 2009.
Benzophenone and other related derivatives are components of UV inks widely used as photoinitiators in the printing of food packaging. These types of printing inks are environmentally friendly since no organic solvents are included in their formulation. Several studies have demonstrated the migration of the photoinitiators from the paper and board to the food via direct contact. However, data concerning the migration through the vapor phase is very scarce. The development and validation of accurate and sensitive methods to analyze potential migrants is essential in order to guarantee the food safety.
In this study, the migration of seven benzophenone-based photoinitiators through the gas phase was evaluated. To carry out migration test an additive enriched polyethylene wax was used as a source to release photoinitiators. Migration of the photoinitiators into five selected dry foods including cake, bread, cereals, pasta and rice has been tested. Additionally, important data concerning about chemical structures and physicochemical properties of the photoinitiators is also presented. Analyses have been performed using an HPLC-DAD method. A Kromasil 100 C18 (25 x 0.4 cm i.d., 5 μm) was used as column. The mobile

phase was a gradient consisted of acetonitrile and water. The selected wavelengths were 254 nm for most of photoinitiators except for 4-benzoylbiphenyl where the selected wavelengths was 290 nm. The migration studies were performed under accelerated conditions (70 ^{0}C, 48 h) which simulate the worst case migration. Porosity of the food has been tested employing a pycnometer. Cinetic of migration for the compound has been also evaluated; equilibrium was reached after approximately 150 h. Results confirm that highest contents of the migrated photoinitiators were found in cake, which contains higher fat contents and has more porosity. Of these seven photoinitiators studied, the higher migration was observed for 2-hydroxy benzophenone, benzophenone and 4-methyl benzophenone, this could be explained because of the higher vapor pressures of these compounds. In summary, it is concluded that foods with high fat content and high porosity values are exposed to high migration levels of photoinitiators.

Chapter eight treats about the study of the kinetic of migration by direct contact and through the vapour phase of three model migrants, benzophenone, Diphenylbutadiene (DPBD) and Uvitex OB and it was also reported the determination of diffusion and partition coefficients. Additionally, Arrhenius equation was applied in order to estimate diffusion coefficients at any temperature. This study was presented as poster in *IFT Annual Meeting and Food Expo*®.Chicago IL, USA. July 17-20, 2010. In this chapter, migration of three selected additives (benzophenone, DPBD and Uvitex OB) by direct contact and through the gas phase, are compared. Additionally, partition and diffusion coefficients are calculated. To evaluate the migration of the additives through the gas phase, the cake and the fortified low density polyethylene (LDPE) film were placed with no direct contact between them in a glass container that was

hermetically closed. Besides, to achieve direct migration, a Low density polyethylene film was place in contact by both sides with the cake; the samples were then overwrapped in aluminium foil and stored in an oven. The samples were stored for different temperature-time exposure conditions (20 ºC, 40 ºC and 60 ºC). Migrants were extracted from the plastic films with ethanol (70 ºC, 24 h) and then analyzed by high-performance liquid chromatography with diode array detection. A Kromasil C18 (25 x 0.32 cm I.D., 5 μm particle size) column was used as stationary phase and a binary mobile phase composed by water and a mixture composed by 30% tetrahydrofurane and 70% methanol were used. The wavelengths used were 256 nm for benzophenone, 330 nm for DPBD and 372 nm for Uvitex OB. Temperature was set at 30 ºC in the entire program. The results indicated that in both cases benzophenone migration occurs in large extent. However for DPBD and Uvitex OB migration occurs only by direct contact.

In the last chapter of this work, a method to determine natamycin is presented. This work has been presented in the congress *Shelf Life International Meeting*. Zaragoza, Spain. June 23-25, 2010 and it will be submitted to an international Journal.

The surface of some foods is subject to contamination by microorganisms. This contamination can causes food deterioration and makes them unsuitable for consumption. One of the systems most commonly used is the direct application of antimicrobial agents on the food surface. Natamycin is an antibiotic that acts by binding to sterols, especially ergosterol, in the fungal cell membrane and belongs to the group of poliene macrolides produced by actinomycetes *Streptomyces natalensis*. This food additive (E235) is usually employed as antifungical agent to increase shelf life of some foodstuffs. The maximum

concentration of natamycin in the final product allowed by the European Legislation is 1 mg/dm^2 and should not be detectable at 5 mm depth. The natamycin employements is allow in cheeses and dry sausages. Rapid, sensitive and accurate methods for compliance with European food legislation are required. Regarding the toxicity of the antifungal agent the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) of the European Food Safety Authority (EFSA) reported that the levels of natamycin allowed for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages were not of safety concern.

The aim of the present study was to develop a simple and rapid high performance liquid chromatographic method with diode-array detection to determine natamycin in food samples (comprising several types of cheese; fresh soft, semi-cured, cured, Camembert and Roquefort; seven different sausages including salami, ham, "chorizo", and sausages and twelve commercial white and red wines) that can be used for quality control. LC-MS/MS using electrospray ionization (ESI) in positive mode was used as confirmatory technique. Natamycin was extracted from food samples employing 0.5 g of each sample and adding 10 ml of methanol acidified with 0.001% acetic acid then shaked for 5 minutes. Afterwards, 1 ml of the solution was removed and diluted to 2 ml with Milli-Q water. Samples were stored at -22 ºC overnight. Finally, the samples were filtered and injected in the HPLC system. Wine samples were filtrered and injected directly in the HPLC. Separation was achieved on a Kromasil ODS (C18) (150 x 3.20 mm i.d., 5 μm particle size) column thermostatted at 25 ºC. A gradient of acetonitrile and milli-Q water was used as mobile phase. Three selected wavelengths were set in DAD detector, 291.4, 304.4 and 319.4 nm, corresponding to the three absorption peaks of the characteristic natamycin spectrum. The method was validated with respect to

linearity, limits of detection and quantification, recovery and repeatability. Data showed that two samples exceeded the limit established by the European Legislation. Natamycin was also detected in samples in which its use is not allowed.

I-INTRODUCCIÓN

I.1. Materiales en contacto con alimentos

Los materiales destinados a entrar en contacto con alimentos son objetos destinados a entrar en contacto con los mismos sea de forma directa o de forma indirecta, como pueden ser, envases, utensilios de cocina, vajillas, máquinas y un largo etcétera. De entre ellos cabe destacar la importancia de los envases. Los envases alimenticios están destinados a proteger el alimento de factores externos como la luz, oxígeno, microorganismos, entre otros. Al interponerse entre el entorno y el alimento reduce mucho su deterioro y a su vez garantiza la calidad de los alimentos. Además los envases permiten transportar y conservar los alimentos para que estos lleguen en buen estado al consumidor. Todos los materiales en contacto con alimentos (Acrónimo en inglés FCM Food Contact Materials) se encuentran regulados bajo la legislación europea, que si bien no está totalmente desarrollada, intenta la armonización de las distintas leyes de los estados miembros eliminando disparidades entre ellas. Todos los FCM están sujetos al reglamento marco (CE) 1935/2004 y deben de estar fabricados conforme a las buenas prácticas de fabricación, de tal forma que se minimizan los efectos nocivos que éstos puedan representar para la salud humana (Reglamento (CE) 2023/2006). Los FCM pueden estar fabricados con distintos materiales, tales como siliconas, plásticos, cartón, etc. Para los distintos materiales y objetos se pueden establecer medidas especificas tales como listas de sustancias autorizadas empleadas para la fabricación de los mismos (Conocidas como listas positivas, de las cuales hablaremos más adelante) y restricciones entre los que se encuentran límites de migración específico y global de las sustancias empleadas para su fabricación, especificaciones de pureza y condiciones de uso de dichas sustancias. De entre la lista de materiales y objetos entre los que se pueden aplicar medidas específicas solo cuatro están regulados a

nivel de la UE, éstos son: Materiales y objetos activos e inteligentes, cerámica, plásticos y celulosa regenerada. Los demás están regulados bajo las legislaciones nacionales de los estados miembros si es que las hay.

I.2. Materiales plásticos

Los polímeros son macromoléculas formadas por moléculas de más bajo peso molecular unidas por un proceso llamado polimerización. Los polímeros son empleados en la fabricación de los materiales plásticos. Los plásticos estan formados por polímeros de muy alto peso molecular, como es el caso de los termoplásticos (poliamidas, polietileno, etc.) y de hasta peso molecular esencialmente infinito, como es el caso de los polímeros termoestables (epoxy-amina, aminoplastos UF, etc.). Los polímeros empleados para la fabricación de plásticos que posteriormente estarán en contacto con los alimentos pueden sintetizarse empleando monómeros (con los cuales se sintetiza la cadena polimérica) y los aditivos (los cuales están destinados a modificar o proteger las propiedades de los plásticos). Estas sustancias están catalogadas y se recogen en el reglamento 10/2011 y se basan en el principio de las listas positivas. Solo las sustancias recogidas en esta lista podrán utilizarse en la fabricación de materiales poliméricos. La inclusión en esta lista se establece en base a criterios toxicológicos para proteger la salud del consumidor. Los polímeros en sí no son un problema desde el punto de vista de la salud ya que aunque éstos migraran, su peso molecular es tan elevado que no sería absorbido por el organismo, ya que se reconoce que las sustancias de un peso molecular mayor a los 1000 Daltons no son biodisponibles por vía oral. Sin embargo, las sustancias de partida que se emplean para la fabricación sí que tienen un peso molecular inferior a los 1000 Daltons.

Estas sustancias no tienen por qué reaccionar completamente en el proceso de polimerización, en el caso de los monómeros, ó, en el caso de los aditivos un pequeño porcentaje puede migrar desde el polímero a los alimentos. Aquí estriba uno de los principales problemas de la migración. Por otro lado, estas sustancias de partida pueden producir reacciones colaterales con otros compuestos, degradarse y formar otros productos de reacción poco conocidos. Estas sustancias que no están añadidas de forma intencionada se conocen como NIAS (En inglés, No Intentionally Added Substances) y también son susceptibles de migrar y podrían generar un problema para la salud del consumidor.

Ref. No	CAS No	Name	Restrictions and/or specifications
(1)	(2)	(3)	(4)
58720	000111-14-8	Heptanoic acid	
58960	000057-09-0	Hexadecyltrimethylammonium bromide	SML = 6 mg/kg
59120	023128-74-7	1,6-Hexamethylene-bis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionamide)	SML = 45 mg/kg
59200	035074-77-2	1,6-Hexamethylene-bis(3-(3,5-di-tert-butyl~4-hydroxyphenyl)propionate)	SML = 6 mg/kg
59280	000100-97-0	Hexamethylenetetramine	SML(T) = 15 mg/kg (22) (35)(expressed as Formaldehyde)
59360	000142-62-1	Hexanoic acid	
59760	019569-21-2	Huntite	
59990	007647-01-0	Hydrochloric acid	
60025	---	Hydrogenated homopolymers and/or copolymers made of 1-decene and/or 1-dodecene and/or 1-octene	In compliance with the specifications laid down in Annex V. Not to be used for articles in contact with fatty foods.

Figura 1. Vista parcial de las listas positivas.

I.3. Fenómeno de la migración

El empleo de FCM han contribuido a reducir al mínimo los factores externos que deterioraban los alimentos, asimismo, han contribuido a garantizar la integridad y calidad de los productos tanto como para conservar sus propiedades organolépticas. Sin embargo, otro tipo de problemas surgieron del empleo de estas nuevas tecnologías, como es el fenómeno de transferencia de masa de estos materiales al alimento, este fenómeno se conoce con el nombre de migración y las sustancias que migran se les conocen con el nombre de migrantes. La migración es un proceso inevitable y predecible y que es necesario tener en cuenta para garantizar la salud humana. La migración de las sustancias está sujeta a restricciones, las principales restricciones son:

Límite de migración global (LMG)- "Los materiales y objetos plásticos no cederán sus constituyentes a los simulantes alimentarios en cantidades que superen en total los 10 miligramos de constituyentes liberados por decímetro cuadrado de superficie de contacto (mg/dm^2)".
No obstante para los lactantes y niños de corta edad establece que los materiales no cederán sus constituyentes a los simulantes alimentarios en cantidades que superen en total los 60 miligramos de constituyentes liberados por kilogramo de simulante alimentario.

Límite de migración específico (LME)- Se aplica a sustancias individuales o a un grupo de ellas y fija la cantidad máxima que puede ser cedida al producto alimenticio, se expresa en mg de sustancia/kg de alimento.

Con respecto a las sustancias para las que no se establezca límite de migración específica ni otras restricciones, se aplicará un límite genérico de migración específica de 60 mg/kg.

Cantidad máxima permitida en el material u objeto (CM o CMA)- Se aplica a sustancias individuales o a un grupo de ellas y fija la cantidad máxima que puede contener el material en peso o superficie. Se establece para aquellas sustancias que es más apropiado realizar su control en el propio material de contacto.

La migración de sustancias se realiza en conformidad con las Directivas 82/711/CEE y modificaciones (Directivas 93/8/CEE y 97/48/CE y 85/572/CEE). Ya que el análisis de las sustancias en alimentos es complicada se suelen utilizar simulantes de alimentos que facilitan los análisis evitando así emplear matrices complejas. Los simulantes empleados son el agua destilada para alimentos acuosos que tengan un pH > 4,5; el ácido acético al 3% (p/v) para alimentos ácidos y alimentos acuosos que tienen un pH < 4,5; el etanol al 10% (v/v) para alimentos alcohólicos (la concentración de etanol se debe ajustar a la graduación alcohólica real del alimento si es superior al 10%(v/v)) y el aceite de oliva refinado para alimentos grasos. Existen también ensayos sustitutivos del aceite de oliva como el isoctano, etanol 95% (v/v) y óxido de polifenileno modificado (MPPO) que se pueden emplear ya que el aceite es una matriz compleja para el análisis.

Según el nuevo Reglamento (UE) no 10/2011 de la Comisión de 14 de enero de 2011, los simulantes empleados son el etanol 10% (v/v) Simulante A; el ácido acético al 3% (p/v) Simulante B; el etanol al 20% (v/v) Simulante C; etanol 50% (v/v) Simulante D1; aceite vegetal Simulante D2 y poli(óxido de 2,6-difenil-p-fenileno), tamaño de partícula 60-80 malla, tamaño de poro 200 nm Simulante E.

Los simulantes alimentarios A, B y C se asignan a alimentos que tengan carácter hidrofílico y sean capaces de extraer sustancias hidrofílicas. El simulante B se usará para alimentos que tengan un pH inferior a 4,5. El simulante alimentario C debe usarse para alimentos alcohólicos con un contenido de alcohol de hasta un 20 %, y para alimentos que contengan una cantidad importante de ingredientes orgánicos que lo hagan ser más lipofílico.

Los simulantes D1 y D2 se asignan a alimentos que tengan carácter lipofílico y sean capaces de extraer sustancias lipofílicas. El simulante alimentario D1 se usará para alimentos alcohólicos con un grado alcohólico superior al 20 % y para aceite en emulsiones acuosas. El simulante D2 se usará para alimentos que contengan grasas libres en la superficie.

El simulante alimentario E se destina a ensayar la migración específica en alimentos secos.

Según este nuevo reglamento para cribar la migración específica, los simulantes alimentarios pueden sustituirse por sucedáneos si, con arreglo a datos científicos, los sucedáneos de simulantes sobrestiman la migración en comparación con los simulantes alimentarios regulados.

Por lo tanto, para conocer mejor el fenómeno de la migración de estos productos a los alimentos, es necesario desarrollar métodos analíticos de referencia, fiables y seguros para la identificación y cuantificación de los migrantes y así garantizar la salud del consumidor.

I.4. Aminas polifuncionales

Las aminas son sustancias por lo general muy reactivas y algunas de ellas pueden ser tóxicas. Estos compuestos pueden ser empleados como monómeros o aditivos

en la elaboración de FCM. Las aminas permitidas en la elaboración de materiales poliméricos destinadas a entrar en contacto están incluidas en las listas positivas de la UE, habiendo sido éstas permitidas debido a que han pasado las correspondientes evaluaciones toxicológicas.

Las aminas cuando migran pueden permanecer inalteradas o formar nuevos compuestos con otras sustancias, ya que algunas de ellas son altamente inestables y reactivas. Estas nuevas sustancias formadas son completamente desconocidas y no están evaluadas toxicológicamente, pudiendo representar un riesgo para la salud de los consumidores, con lo que deberían ser identificadas y cuantificadas.

En la literatura revisada se detecta la falta de métodos multianalito para la determinación de las aminas. La mayoría de los métodos descritos son procesos largos, tediosos y caros que analizan las aminas individualmente. Con lo que se necesitan métodos para la determinación simultánea de aminas que faciliten el análisis en los laboratorios de control

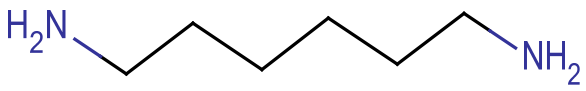


Figura 2. Ejemplo de amina polifuncional. Hexamethylene diamine.

I.4.1. Aminas biógenas

Algunas aminas se forman de manera natural debido a la descomposición alimentos como pescados, carnes, vegetales y vinos entre otros. Estas aminas se denominan aminas biógenas, entre las que se encuentran la histamina, putresceína y cadeverina entre otras, pudiendo ser estas utilizadas como marcadores biológicos para comprobar la calidad de los alimentos o comprobar si su estado de conservación en los envases ha sido la apropiada. Además algunas de ellas están incluídas en las listas positivas de la UE, para su uso como monómeros o aditivos en la fabricación de materiales destinados a entrar en contacto con alimentos.

El desarrollo de metodología analítica en este campo también es necesario para garantizar la salud del consumidor.

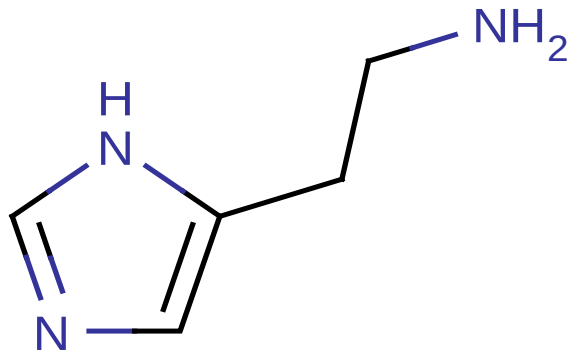


Figura 3. Ejemplo de amina biogénica. Histamine.

I.4.2. Aminas aromáticas primarias (PAAs)

Las PAAs son sustancias muy reactivas y con efectos adversos sobre la salud humana. Precisamente debido a su alta reactividad y relativo bajo coste, éstas son empleadas en la fabricación de múltiples productos tales como, textiles, tintas, poliuretanos, productos farmacéuticos, etc. Los poliuretanos se utilizan en algunos casos como adhesivos. Si en el proceso de curado no reaccionan de forma completa, en contacto con agua pueden liberar PAAs siendo un peligro para la salud humana. Los límites establecidos por las autoridades europeas son extramendamente bajos 10 μg/L debido a los efectos adversos que tienen sobre la salud humana, incluso en algunos países como USA no se permite su detección en los alimentos o simulantes de alimentos. Solo la m-phenilendiamina está incluida en la lista positivas teniendo un límite de migración específico de 0.02 mg/Kg. Varios estudios han demostrado que la migración de PAAs desde utensilios de cocina a simulantes de alimentos (especialmente el acido acético al 3% (p/v)), exceden los límites de migración específicos establecidos por la UE. Además en los últimos años el número de alertas publicadas en el Sistema de Alerta Rápido para Alimentos y Piensos (RASFF) notificando la presencia de estas sustancias en utensilios de cocina han aumentado considerablemente. Es por tanto necesario el desarrollo de métodos analíticos fiables, precisos y sensibles para la identificación y cuantificación de estas sustancias.

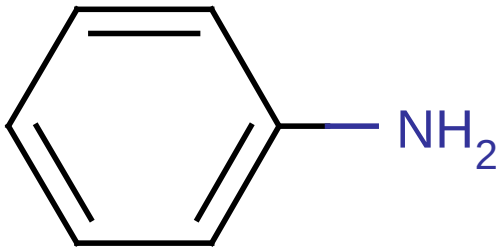


Figura 4. Ejemplo de amina aromática primaria. Aniline.

I.5. Fotoiniciadores

Los fotoiniciadores son sustancias que se emplean en la producción de tintas que están destinadas a ser utilizadas en distintos tipos de envases que van a estar en contacto con los alimentos. Estos reaccionan de forma general con monómeros y oligómeros de acrilatos bajo el efecto de la luz ultravioleta emitida por lámparas de luz ultravioleta produciendo un proceso denominado foto polimerización, en el cual se produce el secado (también denominado el "curado") de de dichas tintas. Unas de las sustancias más empleadas para este proceso son la benzofenona y sus derivados. Cabe destacar que este tipo de sustancias son ampliamente utilizadas debido a su bajo coste y la baja contaminación que producen en el ambiente. A la hora de formar la red polimérica, hay sustancias que pueden quedar sin reaccionar y pueden ser susceptibles de pasar a los propios alimentos. Uno de los primeros escándalos que dieron a conocer la problemática de la migración de fotoiniciadores, se dió debido a la presencia de ITX en alimentos infantiles y posteriormente por la benzophenona y derivados

en productos derivados de cereales. La migración de estas sustancias a los alimentos pueden darse por tres vías posibles: A través del sustrato sobre el que han sido aplicados; otra ruta sería mediante el enrollado o apilamiento del material para su almacenamiento y posterior uso (un proceso conocido como set-off); o por último a través de la fase de vapor, una vía poco explorada y desconocida.

I.6. Aditivos alimentarios

En la mayoría de las veces el empleo de envases para la conservación de los alimentos no es suficiente para garantizar la calidad de los mismos. Los aditivos alimentarios son sustancias cuya adición intencionada tienen un propósito de mejora tecnológica a los alimentos. Hay varios tipos de aditivos tales como estabilizadores, correctores de la acidez, antioxidantes y conservadores entre otros, que garantizan que los alimentos conserven sus propiedades y lleguen en buenas condiciones a los consumidores.

La natamicina o pimaricina, se utiliza como conservante para los alimentos. En la anterior legislación europea en el anexo III de la Directiva 95/2/EC, se permitía el uso de la natamicina (conservante E-235) a nivel superficial en queso curado o madurado: duro, semiduro y semiblando o también en embutidos crudos-curados permitiendo una dosis máxima de 1 mg/dm^2 de superficie (no presente 5 mm de profundidad). El anexo II del Reglamento (CE) nº 1333/2008 recientemente publicado establece las mismas condiciones de uso para la aplicación del aditivo. Debido a que se encontraron niveles de natamicina en vinos provenientes de Argentina en los cuales su uso no está permitido, el análisis de esta sustancia despertó interés en el campo de la seguridad alimentaria. Con el objetivo de garantizar la salud de los consumidores y que se

usen conforme establece la legislación es necesario desarrollar métodos analíticos seguros y sensibles para la determinación de aditivos alimentarios.

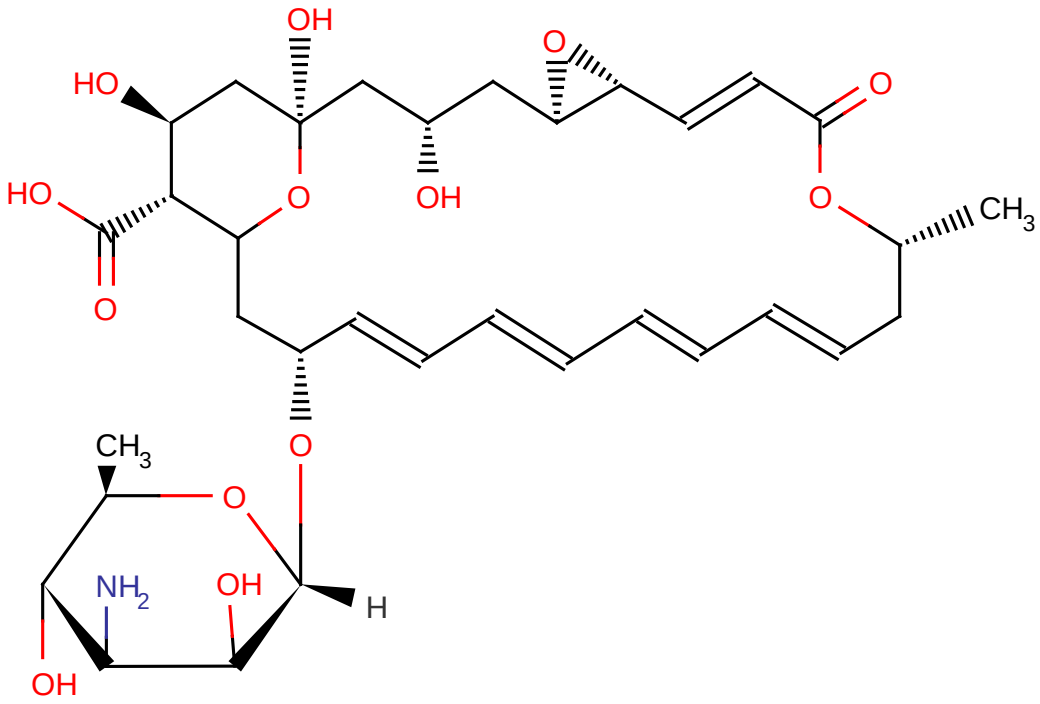


Figura 5. Ejemplo de aditivo alimentario. Natamycin.

I.7. Objetivos de la tesis doctoral

Realizar una profunda revisión bibliográfica de los métodos analíticos empleados para la determinación de aminas polufuncionales usadas como monómeros y aditivos en la fabricación de FCM.

Desarrollo de nuevos métodos multianalito para la determinación de aminas en alimentos y simulantes de alimentos. También es objeto de esta tesis la detección e identificación de los compuestos que se generan por la reacción de las aminas y los componentes grasos de los alimentos.

Estudio de la cinética de migración de fotoiniciadores y otros migrantes modelo por contacto directo y a través de la fase de vapor y desarrollo de métodos analíticos para su determinación.

Puesta a punto de una metodología analítica para la determinación de aditivos alimentarios, sustancias de especial importancia para la conservación de los alimentos. En concreto nos centraremos en la determinación de natamicina en distintos alimentos.

Todos estos estudios serán objeto de publicación en revistas internacionales y comunicaciones a congresos internacionales.

II-CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF POLYFUNCTIONAL AMINES AND RELATED COMPOUNDS USED AS MONOMERS AND ADDITIVES IN FOOD PACKAGING MATERIALS: A STATE-OF- THE-ART REVIEW

Published at: Comprehensive Reviews in Food Science and Food Safety. Volume pages 676–694, November 2010.

Presented as a poster at: 4th International Symposium on recent advances in food analysis. Prague, Czech Republic. November 4–6, 2009.

Abstract

Polyfunctional amines are a group of substances commonly used as additives or monomers in food-contact materials. These substances can migrate into foodstuffs and, consequently, may be potentially dangerous for human health. Due to their different chemical structures and physicochemical properties there does not exist a standard method to analyze polyfunctional amines. This review aims to provide an update on the chromatographic methods used for the determination of polyfunctional amines that are commonly used in the manufacture of food packaging materials. Detailed information regarding chromatographic conditions, (mobile phases, chromatographic columns, detection systems, and so on) is provided. Moreover, chemical structures and physicochemical properties of the substances studied are also presented.

Keywords: Polyfunctional amines, monomers, additives, food packaging, chromatographic analysis.

II.1. Introduction

Polyfunctional amines are a group of substances that are very reactive and widely employed as monomers and additives in the manufacture of food contact materials. These compounds can migrate from the material into food and may be potentially dangerous for the consumers' health.

The European Union in its Directive 2002/72/EC has established the authorized substances that can be used in the plastic materials intended to be in contact with food as well as their overall and specific migration limits. These substances are evaluated by the Scientific Committee for Food (SCF) before their inclusion on the positive list. The evaluations are based on toxicological studies (Hamdani and others 2002).

Some of the typical food packaging applications of polyfunctional amines are cooking utensils made from melamine-formaldehyde plastics (Lund and Petersen 2006) or, for example, wraps used for sausages and cooked meat made with nylon-6 and nylon-12 which are synthesized from caprolactam and laurolactam, respectively (Stoffers and others 2003; Bradley and others 2004).

Regarding the additives, amines are used as antioxidants (for example, Irganox 1098); UV stabilizers (for example, hydroxybenzotriazoles); optical brighteners (for example, bis-benzoxazoles); lubricants (for example, oleamide, erucamide), and surfactants (for example, EDTA) among others.

In order to confirm the migration of these compounds from the packaging to the food sensitive and reliable methods are required. Because of the wide variety of chemical structures there no standard method to analyze polyfunctional amines. The analytical techniques commonly used include liquid and gas chromatography. Most of these methods require a chemical derivatization in order to achieve a suitable sensitivity. The majority of the procedures reported in

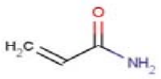
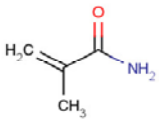
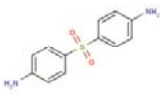
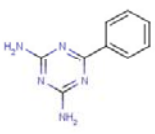
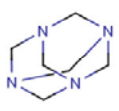
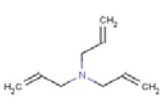
the literature are single-component methods, but methods for the simultaneous analysis of several amines are very scarce. Therefore, development is necessary of multi-analyte methods that are sensitive, accurate, and that allow to verify if a material used in the manufacture of food packaging is in compliance with the European Directive.

In this article we present an exhaustive review of the chromatographic methods for the determination of polyfunctional amines commonly used in food packaging materials. The review is divided into 2 main sections (a) monomers and (b) additives. Monomers are classified according to their chemical structure and additives are classified into the following categories: lubricants, UV stabilizers, optical brighteners, accelerators for vulcanization, surfactants, antioxidants, antimicrobial agents, and other additives. This order should make for easy understanding. Furthermore, physicochemical properties of the substances considered in this review are also reported.

II.2. Physicochemical properties

Seeing that the knowledge of many physicochemical properties of the substances is crucial in the selection of a suitable analytical method, such detailed data are provided in Table 1 and Table 2. Information was obtained from different sources (Sci Finder, 2007; ChemIDplus Advanced; Instant JChem).

Table 1- Chemical structure, physicochemical properties, and specific migration limits of some monomers included in the positive list of the European Union.

Structure	MW	Formula	CAS No	Name	T/V	Bp	MP	pKa	Log P	Vp	He Law	D	SML (mg/kg)
	71.08	C3H5NO	000079-06-1	Acrylamide	0.42[b]	192.6[a]	84.5[a]	15.35[b]	-0.67[a]	0.007[a]	0.000000001[b]	1.13[a]	0.01
	85.10	C4H7NO	000079-39-0	Methacrylamide	0.32[b]	211.5[b]	110[a]	15.46[b]	-0.228[b]	0.182[b]	N.A.[b]	0.942[b]	0.02
	248.30	C12H12N2O2S	000080-08-0	4,4'-Diaminodiphenyl sulfone	0.28[b]	N.A.[b]	175.5[a]	2.41[a]	0.97[a]	2.68E-08[b]	3.11E-14[b]	1.392[a]	5
	187.20	C9H9N5	000091-76-9	Benzoguanamine	0.36[b]	495.8[b]	226.5[a]	N.A.[b]	1.36[a]	0.000000119[b]	4.11E-11[b]	1.4[a]	
	140.19	C6H12N4	000100-97-0	Hexamethylenetetramine	0.06[b]	N.A.[b]	263[a]	5.28[b]	-4.15[b]	0.004[a]	1.64E-09[b]	1.3[a]	15
	137.22	C9H15N	000102-70-5	Triallylamine	0.01[b]	155.5[a]	94[a]	8.31[a]	2.59[a]	3.64[a]	0.0000827[b]	0.809[a]	

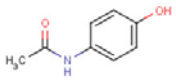
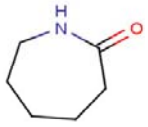
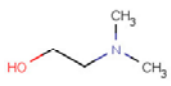
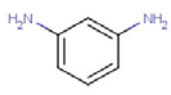
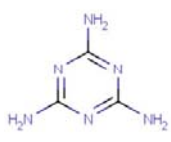
Structure	MW	Formula	CAS No	Name	T/V	Bp	MP	pKa	Log P	Vp	He Law	D	SML (mg/kg)
	151.16	C8H9NO2	000103-90-2	N-(4-hydroxyphenyl)acetamide	0.22[b]	500[a]	170[a]	9.38[a]	0.46[a]	0.000007[b]	6.42E-13[b]	1.4[a]	0.05
	113.16	C6H11NO	000105-60-2	Caprolactam	0.15[b]	270[a]	69.3[a]	16.61[b]	0.66[b]	0.00607[b]	2.53E-08[b]	1.02[a]	15
	60.10	C2H8N2	000107-15-3	Ethane-1,2-diamine	0.43[b]	117[a]	11.1[a]	9.922[a]	-2.04[a]	12[a]	1.73E-09[a]	0.9[a]	12
	89.14	C4H11NO	000108-01-0	Dimethylaminoethanol	0.12[b]	134[a]	-59[a]	9.31[a]	-0.94[b]	3.18[a]	0.000000373[b]	0.89[a]	18
	108.14	C6H8N2	000108-45-2	1,3-Phenylenediamine	0.31[b]	285[a]	63.5[a]	4.98[a]	-0.33[a]	0.00321[b]	1.25E-09[b]	1.14[a]	0.02
	126.12	C3H6N6	000108-78-1	Melamine	0.73[b]	557.5[b]	345[a]	5[a]	-1.37[a]	1.82E-12[b]	1.84E-14[b]	1.6[a]	30

Table 1 - (Continued)

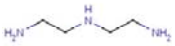
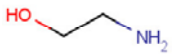
Structure	MW	Formula	CAS No	Name	T/V	Bp	MP	pKa	Log P	Vp	He Law	D	SML (mg/kg)
	88.15	C4H12N2	000110-60-1	1,4-Diaminobutane	0.29[b]	158.5[a]	27.5[a]	10.8[a]	-0.7[a]	4120[b]	1.82E-09[b]	0.864[b]	
	103.17	C4H13N3	000111-40-0	1,4-Diaminobutane	0.32[b]	207[a]	-39[a]	10.45[a]	-2.13[b]	0.232[a]	0.000000315[b]	0.96[a]	5
	116.20	C6H16N2	000124-09-4	1,6-Diaminohexane	0.21[b]	205[a]	41.5a	11.02[a]	0.35[b]	0.256[b]	3.21E-09[b]	0.93[a]	2.4
	61.08	C2H7NO	000141-43-5	2-Aminoethan-1-ol	0.40[b]	171[a]	10.5[a]	9.499[a]	-1.31[a]	0.404[a]	3.25E-08[b]	1.02[a]	0.05
	43.07	C2H5N	000151-56-4	Aziridine	0.13[b]	56[a]	-77.9[a]	8.04[a]	-0.28[b]	213[a]	0.0000121[b]	0.8[a]	0.01
	215.33	C12H25NO2	000693-57-2	12-Aminododecanoic acid	0.15[b]	349.7[b]	183.25[a]	4.78[b]	3.077[b]	0.00000795[b]	N.A.[b]	0.957[b]	0.05

Table 1 - (Continued)

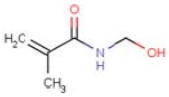
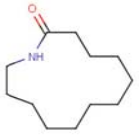
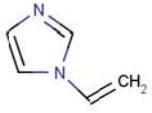
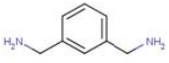
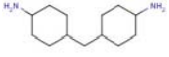
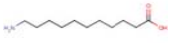
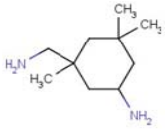
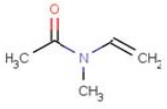
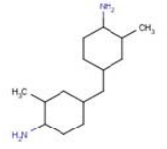
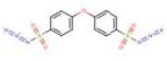
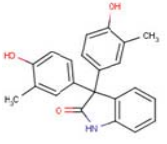
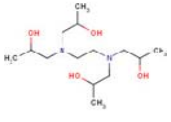
Structure	MW	Formula	CAS No	Name	T/V	Bp	MP	pKa	Log P	Vp	He Law	D	SML (mg/kg)
	115.13	C5H9NO2	000923-02-4	N-Methylolmethacrylamide	0.28[b]	306.3[b]	N.A.[b]	13.08[b]	-0.927[b]	0.0000728[b]	N.A.[b]	1.052[b]	0.05
	197.32	C12H23NO	000947-04-6	Laurolactam	0.08[b]	348[a]	152[a]	16.91[b]	2.92[a]	0.00000731[b]	0.000000139[b]	0.884[b]	5
	94.11	C5H6N2	001072-63-5	1-Vinylimidazole	0.13[b]	191.8[b]	N.A.[b]	6.07[b]	0.39[b]	0.506[b]	N.A.[b]	1.0414[a]	
	136.19	C8H12N2	001477-55-0	m-Xylylenediamine	0.23[b]	247[a]	N.A.[b]	N.A.[b]	0.15[b]	N.A.[b]	N.A.[b]	N.A.[b]	0.05
	210.36	C13H26N2	001761-71-3	bis(4-Aminocyclohexyl)methane	0.13[b]	320[a]	15[a]	10.87[b]	3.26[b]	0.000327[b]	N.A.[b]	0.96[a]	0.05
	201.31	C11H23NO2	002432-99-7	11-Aminoundecanoic acid	0.16[b]	334.9[b]	191[a]	10.67[b]	-0.16[b]	0.0000236[b]	N.A.[b]	0.965[b]	5

Table 1 - (Continued)

Table 1 - (Continued)

Structure	MW	Formula	CAS No	Name	T/V	Bp	MP	pKa	Log P	Vp	He Law	D	SML (mg/kg)
	170.30	C10H22N2	002855-13-2	Isophoronediamine	0.15[b]	247[a]	10[a]	10.74[b]	1.9[b]	0.015[a]	4.4E-09[b]	0.92[a]	6
	99.13	C5H9NO	003195-78-6	N-Vinyl-N-methylacetamide	0.12[b]	164.5[b]	N.A.[b]	-0.35[b]	-1.367[b]	1.96[b]	N.A.[b]	0.896[b]	
	238.41	C15H30N2	006864-37-5	3,3'-Dimethyl-4,4'-diaminodicyclohexylmethane	0.12[b]	342[a]	-7[a]	11.02[b]	3.472b	0.00218[b]	N.A.[b]	0.95[a]	0.05
	380.36	C12H8N6O5S2	007456-68-0	4,4'-Oxybis(benzenesulfonyl azide)	0.34[b]	N.A.[b]	N.A.[b]	N.A.[b]	N.A.[b]	N.A.[b]	N.A.[b]	N.A.[b]	
	345.39	C22H19NO3	047465-97-4	3,3-bis(4-Hydroxy-3-methylphenyl)-2,3-dihydro-1H-indol-2-one	0.14[b]	560.4[b]	N.A.[b]	9.81[b]	4.026[b]	3.66E-13[b]	N.A.[b]	1.292[b]	1.8
	292.41	C14H32N2O4	000102-60-3	N,N,N,'N'-Tetrakis(2-hydroxypropyl)ethylenediamine	0.16[b]	369.1[b]	25[a]	14.23[b]	-2.08[b]	0.000000599[b]	N.A.[b]	1.1[b]	

a = Experimental; b = Estimated; T/V = Topological polar surface area/vanderWaalsSurfaceArea; Bp = Boiling point; Mp = Melt point; Vp = Vapor pressure; D = Density; SML = Specific migration limit; He Law = Henry's law constant (atm-m3/mole) 25 ºC.

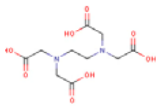
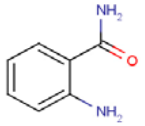
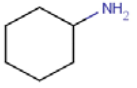
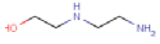
Structure	MW	Formula	CAS No	Name	T/V	Bp	Mp	pKa	Log P	VP	He Law	D	SML (mg/kg)
	292.24	C10H16N2O8	000060-00-4	Ethylenediaminetetraacetic acid	0.39[b]	N.A.[b]	245[a]	0.26[a]	-3.86[b]	4.98E-13[b]	1.17E-23[b]	0.086[a]	
	136.15	C7H8N2O	000088-68-6	2-Aminobenzamide	0.36[b]	300[a]	110.5[a]	15.77b	0.35[a]	0.0000263[b]	7.83E-13[b]	1.233[b]	0.05
	99.17	C6H13N	000108-91-8	Cyclohexanamine	0.13[b]	134[a]	-17.7[a]	10.63a	1.49[a]	10.1[a]	0.00000416[a]	0.86[a]	
	593.02	C38H76N2O2	000110-30-5	N,N'-Ethylenebisstearamide	0.05[b]	720.3b	145[a]	15.53b	14.995[b]	1.33E-20[b]	N.A.[b]	0.97[a]	
	104.15	C4H12N2O	000111-41-1	N-(2-Aminoethyl)ethanolamine	0.29[b]	239[a]	-38[a]	7.21a	-2.13[b]	0.00819[a]	1.1E-13[b]	1.0254[a]	0.05
	337.58	C22H43NO	000112-84-5	Erucamide	0.06[b]	474.2[b]	77.5[a]	16.61b	5.3[b]	5.82E-09[b]	0.00000284[b]	0.874[b]	

Table 2: Chemical structure, physic chemical properties, and specific migration limits of some additives included in the positive list of the European Union.

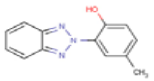
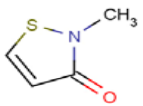
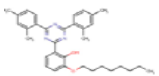
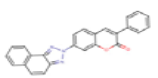
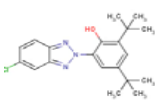
Structure	MW	Formula	CAS No	Name	T/V	Bp	Mp	pKa	Log P	VP	He Law	D	SML (mg/kg)
	225.25	C13H11N3O	002440-22-4	2-(2'-Hydroxy-5'methylphenyl) benzotriazole **(Tinuvin P)**	0.16[b]	N.A.[b]	130[a]	0.74[b]	4.31[a]	7.95E-08[b]	6.12E-14[b]	1.3[b]	30
	115.15	C4H5NOS	002682-20-4	2-Methyl-2,3-dihydro-1,2-thiazol-3-one	0.33[b]	182.8[b]	50[a]	-2.03[b]	0.053[b]	0.797[b]	N.A.[b]	1.293[b]	0.02
	509.68	C33H39N3O2	002725-22-6	2,4-bis(2,4-Dimethylphenyl)-6-(2-hydroxy-4-n-octyloxyphenyl)-1,3,5-triazine	0.08[b]	695.2[b]	89.5[a]	8.45[b]	11.655[b]	5.67E-20[b]	N.A.[b]	1.088[b]	0.05
	339.60	C22H45NO	003061-75-4	Behenamide	0.06[b]	471.1[b]	N.A.[b]	16.61[b]	9.393[b]	4.78E-09[b]	N.A.[b]	0.865[b]	
	389.41	C25H15N3O2	003333-62-8	3-Phenyl-7-{3,4,5-triazatricyclo[7,4,0,0^{2,6}]trideca-1(9),2,5,7,10,12-hexaen-4-yl}-2H-chromen-2-one	0.12[b]	661.1[b]	N.A.[b]	0.4[b]	7.124[b]	2.39E-17[b]	N.A.[b]	1.35[b]	
	357.88	C20H24ClN3O	003864-99-1	2-(2'-Hydroxy-3,5'-di-tert-butylphenyl)-5-chlorobenzotriazole (Tinuvin 327)	0.09[b]	469.2[b]	154.75[a]	9.41[b]	7.812[b]	2.0E-9[b]	N.A.[b]	1.18[b]	30

Table 2 - (Continued)

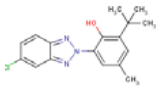
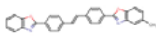
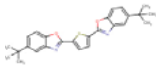
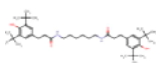
Structure	MW	Formula	CAS No	Name	T/V	Bp	Mp	pKa	Log P	VP	He Law	D	SML (mg/kg)
	315.80	C17H18ClN3O	003896-11-5	2-(2'-Hydroxy-3'-tert-butyl-5'-methylphenyl)-5-chlorobenzotriazole **(Tinuvin 326)**	0.11[b]	460.4[b]	139[a]	9.49[b]	6.584[b]	4.24E-09[b]	N.A.[b]	1.26[b]	30
	428.48	C29H20N2O2	005232-99-5	2-Cyano-3,3-diphenylacrylic acid, ethyl ester	0.09[b]	578.9[b]	N.A.[b]	0.87[b]	8.88[b]	8.56E-13[b]	N.A.[b]	1.261[b]	0.05
	430.56	C26H26N2O2S	007128-64-5	2,5-Bis(5-tert-butyl-2-benzoxazolyl)thiophene **(Uvitex OB)**	0.13[b]	531.2[b]	N.A.[b]	1.24[b]	8.985[b]	7.83E-11[b]	N.A.[b]	1.185[b]	0.6
	590.06	C40H79NO	010094-45-8	Octadecylerucamide	0.02[b]	671.6[b]	N.A.[b]	16.43[b]	18.092[b]	6.68E-18[b]	N.A.[b]	0.857[b]	5
	505.90	C34H67NO	016260-09-6	Oleylpalmitamide	0.03[b]	618.6[b]	N.A.[b]	16.43[b]	14.904[b]	3.14E-15[b]	N.A.[b]	0.858[b]	5
	636.95	C40H64N2O4	023128-74-7	l,6-Hexamethylene-bis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionamide) (Irganox 1098)	0.08[b]	740.1[b]	156[a]	12.08[b]	8.796[b]	1.19E-22[b]	N.A.[b]	1.021[b]	45

Table 2 - (Continued)

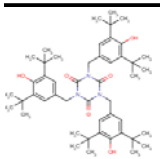
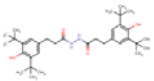
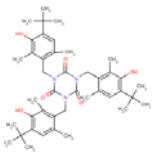
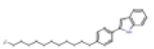
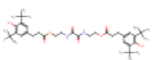
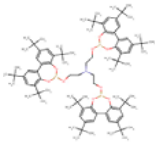
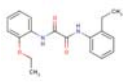
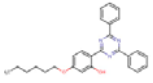
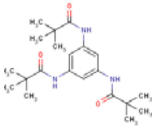
Structure	MW	Formula	CAS No	Name	T/V	Bp	Mp	pKa	Log P	VP	He Law	D	SML (mg/kg)
	4.08	C48H69N3O6	027676-62-6	1,3,5-Tris(3,5-di-tert-butyl-4-hydroxybenzyl)-1,3,5-triazine-2,4,6(1H,3H,5H)-trione (Irganox 3114)	0.09[b]	757.9[b]	N.A.[b]	11.45[b]	10.336[b]	8.89E-24[b]	N.A.[b]	1.12[b]	5
	552.79	C34H52N2O4	032687-78-8	N,N'-Bis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionyl)hydrazide (Irganox 1024)	0.10[b]	652.6[b]	N.A.[b]	11.1[b]	8.373[b]	1.24E-17[b]	N.A.[b]	1.054[b]	15
	699.92	C42H57N3O6	040601-76-1	1,3,5-Tris(4-tert-butyl-3-hydroxy-2,6-dimethylbenzyl)-1,3,5-triazine-2,4,6(1H,3H,5H)-trione	0.11[b]	793.8[b]	N.A.[b]	11.36[b]	8.031[b]	3.55E-26[b]	N.A.[b]	1.172[b]	6
	361.56	C26H35N	052047-59-3	2-(4-dodecylphenyl)-1H-indole	0.02[b]	521[b]	N.A.[b]	16.86[b]	10.981[b]	1.95E-10[b]	N.A.[b]	0.99[b]	0.06
	696.91	C40H60N2O8	070331-94-1	2,2'-Oxamidobis[ethyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate]	0.13[b]	N.A.[b]	N.A.[b]	11.48[b]	8.688[b]	N.A.[b]	N.A.[b]	1.105[b]	

Table 2 - (Continued)

Table 2 - (Continued)

Structure	MW	Formula	CAS No	Name	T/V	Bp	Mp	pKa	Log P	VP	He Law	D	SML (mg/kg)
	1464.93	C90H132NO9P3	080410-33-9	2,2',2''-Nitrilo[triethyl tris(3,3',5,5'-tetra-tert-butyl-1,1'-biphenyl-2,2'-diyl)phosphite]	0.04[b]	N.A.[b]	N.A.[b]	5.35[b]	33.6[b]	N.A.[b]	N.A.[b]	N.A.[b]	5
	312.36	C18H20N2O3	023949-66-8	2-Ethoxy-2'-ethyloxanilide **(Tinuvin 312)**	0.14[b]	N.A.[b]	N.A.[b]	10.81[b]	3.632[b]	N.A.[b]	N.A.[b]	1.216[a]	30
	425.52	C27H27N3O2	147315-50-2	2-(4,6-Diphenyl-1,3,5-triazin-2-yl)-5-(hexyloxy)phenol	0.11[b]	645.6[b]	N.A.[b]	8.48[b]	8.752[b]	2.89E-17[b]	N.A.[b]	1.15[b]	0.05
	375.51	C21H33N3O3	745070-61-5	N-[3,5-bis(2,2-Dimethylpropanamido)phenyl]-2,2-dimethylpropanamide	0.13[b]	601.8[b]	N.A.[b]	13.88[b]	4.156[b]	1.95E-14[b]	N.A.[b]	1.112[b]	0.05

a = Experimental; b = Estimated; T/V = Topological polar surface area/vanderWaalsSurfaceArea; Bp = Boiling point; Mp = Melt point; Vp = Vapor pressure; D = Density; SML = Specific migration limit; He Law = Henry's law constant (atm-m3/mole) 25 ºC.

II.3. Monomers

An updated review of the available analytical techniques for the determination of amines commonly used as monomers in the manufacture of food contact materials is presented.

II.3.1. Aliphatic amines

Aliphatic amines such as 1,4-diaminobutane and 1,6-diaminohexane are monomers commonly used in the plastics industry to fabricate materials to be in contact with food. Since these compounds do not present appropriate fluorescence characteristics, nor show suitable absorption in the UV-Vis region, their determination frequently involves a chemical derivatization to enhance analytical sensitivity. Among derivatizing agents, dansyl chloride is one of the most widely used. Many methods reported in the literature include the separation of the dansyl derivatives of the amines on a conventional reversed-phase C18 column and with and an elution system consisting of acetonitrile and water. The UV detection is performed at 254 nm (Dugo and others 2006; Chiacchierini and others 2006; Innocente and others 2007; Saaid and others 2009).

Saarinen (2002) employed another mobile phase that consisted of 0.2 M ammonium acetate at pH 5, water, and acetonitrile. The flow rate used was 0.65 mL/min. The separation was performed on a Spherisorb ODS-2 (250 mm x 3 mm, i.d. 5 μm particle size). The derivatized amines were identified using a fluorescence detector (λ_{ex} 250 nm, λ_{em} 540nm). The method showed a good sensitivity with limits of detection of 0.5 mg/kg.

A gradient of 2 eluents, (A) 0.1 mol/L ammonium acetate and (B) acetonitrile, was used by Mao and others (2009) to analyze the compounds. A Kromasil C_{18} (150 mm x 4.6 mm, i.d. 5 μm particle size) was used as stationary phase.

Molins-Legua and others (1999) performed the derivatization on a C18 solid-phase support. The amines were separated on a C18 Lichrospher (125 mm x 4 mm, i.d. 5 μm particle size) using acetonitrile-imidazol solution (1 mM, pH 7.0) (70:30 v/v) as the mobile phase in gradient elution mode. λ_{ex} 252 nm and λ_{em} 500 nm were selected as the optimum wavelengths. A limit of detection of 10 ng/mL was obtained for all polyamines studied.

Moret and others (2005) evaluated 2 derivatizing agents, dansyl chloride (DCl) and *o*-phthaldialdehyde (OPA). The separation was performed on C18-Kromasil (250 mm x 4.6 mm, i.d. 5 μm particle size). The mobile phases used were: a gradient of acetonitrile and water for dansyl chloride derivatives and a gradient elution system composed of water, 0.07 M phosphate buffer at pH 7.0, and acetonitrile for *o*-phthaldialdehyde derivatives. UV-Vis (λ 254 nm) detection was used for DCl derivatives and fluorescence (λ_{ex} 330 nm and λ_{em} 440 nm) for OPA derivatives.

A method that involves a pre-column derivatization with CEOC (2-(9-carbazole) ethyl chloroformate) was reported by You and Zhang (2002). The analyses were performed on a Hypersil BDS C18 column (200 mm x 4.6 mm, i.d. 5 μm particle size) and using a gradient system of 2 eluents: (A) 30 % acetonitrile in 0.02 mol L^{-1} acetate buffer (pH 4.0) and (B) acetonitrile-water (95:5 v/v) at a flow rate of 1 mL/min. The fluorescence derivatives were determined at λ_{ex} 293 nm and λ_{em} 360 nm.

An isocratic reversed-phase liquid chromatographic method has been developed by Zhang and others (2008a). In their work, the authors synthesized a new fluorescent derivatizing agent, 3-(4-chlorobenzoyl) quinoline-2-carboxaldehyde. A C18-Kromasil column (250 mm x 4.6 mm, i.d. 5 μm particle size) was used as the stationary phase and methanol-tetrahydrofuran-8 mM pH 6.0 phosphate

buffer solution (78:2.5:19.5 v/v/v) as the mobile phase. Detection was performed at λ_{ex} 480 nm and λ_{em} 545 nm.

Recently, Deng and others (2009) developed a method to determine amines based on micellar electrokinetic capillary chromatography with laser-induced fluorescence detection. N-hydroxysuccinimidyl fluorescein-*O*-acetate was used as the derivatizing agent. The separation was performed on an uncoated fused-silica capillary column (600.2 mm, 500 mm to the detector, 75 μm, i.d.) and using 25 mM pH 9.6 boric acid electrolyte containing 60 mM sodium dodecyl sulfate (SDS). Detection was done at λ_{ex} 488 nm and λ_{em} 520 nm.

Bomke and others (2009) used liquid chromatography coupled to electrospray ionization mass spectrometry operated in a positive mode to analyze amines. The amines were separated on a BioWidePore C18 Supelco (150 mm x 4.6 mm, i.d. 5 μm particle size) column after derivatization with succinimidylferrocenyl propionate and using a gradient system of 2 eluents: (A) 100 mmol ammonium formate and 200 μL formic acid in 1 L deionized water (pH 4) and (B) acetonitrile at a flow rate of 0.45 mL/min.

Casella and others (2008) determined the amines without previous derivatization by using cation-exchange chromatography with suppressed conductivity detection. The underivatized amines were separated on an Ion Pac CS17 (250 mm x 4 mm) cation-exchange column (macroporous 7 μm 55% cross-linked poly(ethylvinylbenzene-divinylbenzene) in gradient elution mode and using 50 mM H_2SO_4 and water as the mobile phase.

Another aliphatic polyamine commonly used as monomer in food packaging materials is diethylene triamine. Saito and others (1992) reported a liquid chromatographic method with fluorescence detection that involves the derivatization and the separation of the amine in the same column, a reversed-phase C_{18} Asahipak ODP-50 (150 mm x 4.6 mm, i.d.) thermostated at 40 ºC. The

mobile phase consisted of a mixture of 50 mM sodium borate buffer (pH 9.9)-acetonitrile (77:23 v/v) containing 2 mM o-phthaldehyde and N-acetyl-L-cysteine as derivatizing agents. The fluorescence derivatives were detected at λ_{ex} 330 nm and λ_{em} 430 nm.

Cycloaliphatic polyamines, such as 4,4'-methylenebiscyclohexylamine and isophorone diamine, and araliphatic amines such as *m*-xylylenediamine are usually employed as curing agents for epoxy resins (Ellis 1993). In food packaging, epoxy resins have been extensively used as inner coatings for cans. The use of epoxyresins in food contact materials has been reviewed by Simal-Gándara and others (1998).

Paseiro-Losada and others (1999) developed a high-performance liquid chromatographic method with fluorescence detection to determine *m*-xylylenediamine (MXDA) previously derivatized with fluorescamine in the food simulant olive oil. The column used was a Spherisorb ODS2 Sugelabor (150 mm x 4.6 mm, i.d. 5μm particle size) and the mobile phase was a mixture consisting of borate buffer-water-methanol (18:37:45 v/v/v) at a flow rate of 1 mL/min. The fluorescence detector was set at λ_{ex} 394 nm and λ_{em} 480 nm. The method exhibited an excellent sensitivity with a limit of detection of 9.2 μg/L.

In a previous study Paseiro-Losada and others (1998) determined MXDA in the official EU aqueous food simulants; distilled water, 3% (w/v) acetic acid and 15% (vol. /vol.) ethanol. The detection limits in the food simulants were, 3.9, 9.6 and 9.3 μg/L, respectively. In another work Paseiro-Losada and others (1992) identified the fluorescamine derivatized MXDA by thermospray mass spectrometry. A C_{18} (100 mm x 4.6 mm, i.d. 5 μm particle size) and a mixture of acetonitrile-0.1 M ammonium acetate buffer (30:70 v/v) were used as a column and mobile phase, respectively.

Paseiro-Losada and others (1991) developed a reversed-HPLC method with fluorescence detection for the simultaneous determination of bisphenol A diglycidyl ether (BADGE) and MXDA in epoxyresins. BADGE was analyzed directly while MXDA was previously derivatized with fluorescamine. Analytes were extracted from the epoxyresins by using a mixture of chloroform-methanol (25:75 v/v). In a later work the specific migration limit of MXDA from an epoxy-amine formulation into water-based food simulants was studied by Simal-Gándara and others (1993).

More recently, liquid chromatography coupled to mass spectrometry appears to be a suitable analytical tool to analyze these compounds. Applications of this technique for the determination of potential migrants from food packaging materials have been reported in a previous review (Simal-Gándara and others 2002).

Marand and others (2004) developed a LC-MS/MS (ESI) method to determine diamines (for example, isophorone diamine) previously derivatized with perfluorofatty acid anhydride. The separation was performed on Xterra® C_{18} (50 mm x 1.0 mm, i.d. 2.5 μm particle size) and using a gradient of 2 eluents: (A) acetonitrile-water (5:95 v/v) and (B) acetonitrile-water (95:5 v/v) at a flow rate of 70 μL/min. The analyses were performed in a negative ionization mode. In the same study, the stability of the standard solution of amines prepared in 1 M H_2SO_4 was evaluated, and no degradation was observed for one week when stored at 4 ºC.

A LC-MS (ESI) method to quantify dicyclohexylmethane-4, 4'-diamine (DMDA) and dicyclohexylmethane-4,4-diisocyanate (DMDI) in pharmaceutical polymers has been reported by Zhang and others (2008). Polymer samples were extracted by using 0.1% formic acid. The analyses were carried out on an ACE C18 column (100 mm x 4.6 mm, i.d. 5 μm particle size) HiChrom, Reading,

UK). Methanol and 0.1% (v/v) formic acid was used as the mobile phase in gradient elution mode at a flow rate of 0.7 mL/min. Amines were quantified in a positive ionization mode.

Paik and others (2006) determined 1,6-diaminohexane as N-ethoxycarbonyl-N-pentafluoropropionyl derivatives by GC-FID and GC-MS. A DB-5 MS column (SE-S4 bonded phase, 30 m x 0.25 mm, i.d. x 0.25 μm film thickness) was used for the GC-FID system. The oven temperature was programmed from 60 ºC to 290 ºC and helium was used as the carrier gas at the flow of 1 mL/min. In the GC-MS system the analytes were separated on an Ultra-2 (SE-S4 bonded phase, 25 m x 0.20 mm, i.d. 0.11 μm film thickness) and using a program temperature from 100 ºC to 300 ºC. The flow rate of the carrier gas was 0.5 mL/min. Under these analytical conditions, the limit of detection was 1.2 ng.

The standard CEN methods (European Commission Report EUR 17610EN) to determine ethylenediamine and 1,6-diaminohexane in food contact materials involves the derivatization of the amines with ethyl chloroformate followed by analysis with GC-FID. The analysis was performed on a DB-1 (30 m x 0.32 mm, i.d. x 0.25 μm film thickness) and with a program from 100 ºC to 270 ºC.

GC-FID is the technique also selected by Demertzis and others (1995) to determine hexamethylenediamine in the four official EC food simulants.

Watson and others (1999) developed a gas chromatography-negative chemical ionization mass spectrometry method to analyze 4,4'-methylenebiscyclohexylamine (DMDA) and 4,4'-methylenecyclohexylisocyanate (DMDI). The analytes were extracted from the polymer with buffer (0.1 M potassium phosphate pH 3.1) and determined by previous derivatization with heptafluorobutyric anhydride on a HP-1 (12 m x 0.2 mm, i.d. x 0.33 μm film thickness) column. Helium was used as carrier gas and methane as the reagent gas. The temperature program was from 100 to 300 ºC.

Since DMDI could not be determined directly, its quantification was performed after decomposition to DMDA.

Methods of analysis of 3,3'-dimethyl-4,4'diaminodicyclohexylmethane and triallylamine are described in the Community Reference Laboratory for Food Contact Materials (CRL FCM).

The method to determine 3,3'-dimethyl-4,4'diaminodicyclohexylmethane, known as Laromin C260, involves analysis by GC-FID using a chromosorb W with 15% PPG-4000 (1 m x 3.175 mm, i.d.) column.

Head space gas chromatography is the technique described to analyze triallylamine.

A gas chromatographic method with selective nitrogen detection to determine ethyleneimine in food simulants is described in a European Commission Report (EUR 17610EN). The separation is performed on a DB-1 column (30 m x 0.32 mm, i.d. x 0.25 μm film thickness) and using a temperature program from 100 ºC to 200 ºC. Hydrogen is the carrier gas.

II.3.2. Alcohol amines

Alcohol amines such as ethanolamine and dimethylamino ethanol are included in EU directives on plastic materials in contact with food. These compounds are used as starting substances in the production of food packaging materials. A chemical derivatization followed by liquid chromatography analysis is the common procedure to analyze alcohol amines.

Zhang and others (2008b) determined ethanolamine by high-performance liquid chromatography with FLD after derivatization with 3-(4-fluorinebenzoyl)-2-quinoline carboxaldehyde. The analyses were performed on a Zorbax Eclipse XDB-C_8 (150 mm x 4.6 mm, i.d. 5 μm particle size) column and using a gradient

solvent system consisting of (A) water and (B) methanol. The fluorescence detector was set at λ_{ex} 480 nm and λ_{em} 546 nm. With the developed method a limit of detection of 2 nM was obtained.

A liquid chromatographic method coupled to electrospray mass spectrometry in positive ionization mode was used by Fournier and others (2008) to analyze aliphatic, aromatic, and alcohol amines (for example, ethanolamine). The analytes were separated on a Phenomenex Luna C18 column (150 mm x 2.0 mm, i.d. 3 μm particle size) after previous derivatization with dansyl chloride and using a gradient system of 2 eluents: acetonitrile and water containing 1% formic acid at a flow rate of 0.2 mL/min. The mass spectrometer was operated under the following conditions: nebulization gas, 20 psi; drying gas (N_2), 8 L/min; capillary temperature, 350 ºC; and capillary voltage, 3500 V.

The method approved by the European Committee for Standardization (CEN) (European Commission Report EUR 17610EN) to determine dimethylaminoethanol comprises GC-FID analysis using a DB-1 (25 m x 0.32 mm, i.d. x 0.5 μm film thickness of dimethylpolysiloxane) column and an oven program from 45 ºC to 250 ºC. DB-1 (15 m x 0.25 mm, i.d. x 0.25 μm film thickness of dimethylpolysiloxane) is also a suitable stationary phase to perform the analyses). Gas chromatography coupled to mass spectrometry is used as a confirmatory technique.

II.3.3. Aromatic Amines

Aromatic amines are used as starting materials in the production of articles intended to be in contact with food. Due to the high toxicity of these amines, the specific migration limits (SML) are very low. Therefore, sensitive methods are necessary to guaranty the efficiency in the control of these substances.

Mortensen and others (2005) developed a LC-ESI-MS/MS to determine several aromatic amines (for example, m-phenylenediamine) in aqueous food simulants. The separation was performed on a Zorbax SB-C3 column (150 mm x 2.1 mm, i.d. 5 μm particle size) and using a binary elution system consisting of (A) 4.7 mM pentafluoropropionic acid in methanol and (B) 4.7 mM pentafluoropropionic acid in Milli-Q water. The mass spectrometer operated in positive ionization mode under the following conditions: capillary voltage 1.0 kV; cone voltage 20 V; desolvation temperature 400 ºC, and the nebulizing gas was nitrogen.

Baranowska and others (2002) proposed an isocratic and reversed-phase liquid chromatographic method with DAD to determine m-phenylenediamine. The analytical column used was a RP 18 Lichrosorb column (250 mm x 4 mm, i.d. 7 μm particle size) and a mixture of methanol-water (55:45 v/v) at a flow rate of 1 mL/min was used as the mobile phase. The authors proposed derivative spectrometry as a complementary analytical tool to LC.

A GC method with a nitrogen-selective detector to determine 1,3-phenylenediamine is described in European Commission Report EUR 17610EN. The column used was a (12 m x 0.32 mm, i.d. x 0.25 μm film thickness) phenyl-equivalent modified siloxane. The oven program was set from 90 ºC to 280 ºC. Helium was used as the carrier gas.

II.3.4. Amides

Caprolactam and laurolactam are monomers of polyamides Nylon-6 and Nylon 12, widely used in food packaging materials (Stoffers and others 2003; Bradley and others 2004). After manufacture, monomers can still remain without reaction and like low- molecular-weight oligomers in the material will be in contact with

foodstuffs. Due to the elevated temperature to which they are exposed and due to their low molecular weight they may be able to migrate (Sanches-Silva and others 2006).

Migration of caprolactam into foods packaged in Nylon-6 was determined by LC-MS (Bradley and others 2004). The extraction of caprolactam from foodstuff was performed with ethanol, water, and heptane. Caprylactam was used as internal standard. The separation was carried out on a reversed-phase Genesis C18 120 (250 mm x 3 mm, i.d. 4 μm particle size) column with a gradient of acetonitrile and water; the selected ions monitored were m/z 69, 79, and 114 for caprolactam and 142 for the internal standard.

Stoffers and others (2003) proposed a HPLC-DAD method to analyze laurolactam. A Hypersil ODS5 column (125 mm x 4 mm, i.d. 5 μm particle size) thermostated at 40 ºC and a gradient of methanol and water were used; and 207 nm was selected as the optimal wavelength. Mass spectrometry MS (APCI) was used as a confirmatory technique; mass spectra acquisition was performed in full scan mode (m/z =100-1200). The limits of detection obtained with the HPLC-DAD and HPLC-MS systems were 1.5 and 0.05 μg/mL, respectively.

To analyze 12-aminododecanoic acid in olive oil a HPLC-FLD method has been described in the CRL-FCM database. The analysis was performed after a derivatization step with fluorescamine. To carry out the analysis a Zorvax C8 (200 mm x 4.6 mm, i.d.) column was used, the detector was set at λ_{ex}390 nm λ_{em} 480 nm. To extract the amine from the olive oil sample, pentane was used as extraction solvent. In order to enhance the extraction, a mixture of ethanol-water and buffer solution of pH 9.5 was added. Similar chromatographic conditions have been reported in the CRL-FCM database to analyze 11-aminoundecanoic acid.

A high-performance liquid chromatographic method with ultraviolet detection at 250 nm to analyze 4-hydroxyphenylacetamide is reported in the CRL FCM database. A binary gradient elution system consisting of (A) acetonitrile and (B) 0.01N H_3PO_4 and a Bondapak C18 (300 mm x 3.9 mm, i.d. 10 μm particle size) column were used to carry out the analyses.

Gas chromatography coupled to mass spectrometry was employed for the determination of caprolactam as degradation product resulting from irradiation of multilayer PA-6 films. The extraction of the compound was performed by using solid-phase microextraction. The best results were achieved with PDMS (polydimethylsiloxane) fiber. The optimal chromatographic conditions were the following: a HP-5MS (30 m x 0.25 mm, i.d. x 0.25 μm film thickness) was used as stationary phase, helium was employed as carrier gas, and the oven temperature was from 80 ºC to 320 ºC. In the second part of the work the migration of the compounds to food simulants was evaluated. It was observed that caprolactam migrated into water and 95% ethanol food simulants. The method was validated in the food simulants, the results showed an excellent sensitivity with limits of detection of 0.61 and 0.05 μg/g in water and in 95% ethanol, respectively. The recovery values obtained were 132.3% and 83.7% for each simulant (Felix and others 2008).

GC-FID has been employed for laurolactam determination in Nylon 12 films. Ethanol (95%) and water were used as extraction solvents. A DB624 (30 m x 0.32 mm, i.d. x 1.8 μm film thickness) was utilized as stationary phase, hydrogen was used as carrier gas, and the temperature was increased at a rate of 10 ºC/min from 180 ºC to 240 ºC. Caprylactam was used as internal standard. Under these analytical conditions the limits of detection and quantification were 0.04 μg/mL and 0.15 μg/mL, respectively (Stoffers and others 2003).

In a recent study, Araújo and others (2008) extracted caprolactam from irradiated multilayer PA-6 films for food packaging by using methanol as the extraction solvent. The analysis was performed by GC-FID and confirmed by GC/MS/MS. A DB-1701 (30 m x 0.25 mm, i.d. x 0.25 μm film thickness) and a factor four VF5-MS capillary column (30 m x 0.25 mm, i.d. x 0.25 μm film thickness) were used for GC-FID and GC-MS/MS, respectively. The oven program was from 110 ºC to 200 ºC and the detector temperature was set at 250 ºC. Identical conditions were used for both systems. The method provided a good recovery, with values higher than 90% and a limit of detection of 0.2 ng.

II.3.5. Related compounds containing sulfur

Molecules containing sulfur are frequently employed in polymeric materials. Some thermoplastic materials contain sulfur atoms in the form of sulfide (S) and sulfone (SO2) (Domininghaus 1988). Some monomers containing sulfur in their structure such as dapsone and 4,4' oxydibenzenesulfonyl azide are listed in EU directives on plastics in contact with food. Because they can still remain without reacting and consequently migrate into food they are potential contaminants. Analytical methods for the determination of these compounds are of interest to control laboratories.

HPLC coupled to electrochemical detection (ECD) or MS are the most frequently used techniques for the analysis of dapsone (Kwadijk and Toraño 2002). Several sulfonamides, including dapsone, have been determined by HPLC-MS/MS, employing a gradient of 2 solvents: Solvent A (water containing 0.5% formic acid and 1 mM nonylfluoropentanoic acid) and solvent B (methanol-acetonitrile (50:50 v/v) containing 0.5% formic acid), the separation

was performed on a Zorvax SB C18 column (50 mm x 2.1 mm, i.d. 1.8 µm particle size) (Mohamed and others 2007).

Parkin and Boddy (1998) suggested a HPLC-UV method to determine dapsone. An octadecyl silica (300 mm x 3.9 mm, i.d. 10 µm particle size) column and methanol-water (20:80 v/v) containing ammonium as the mobile phase were used. Detection was set at 290 nm. The same wavelength was employed by Kwadijk and Toraño (2002); they proposed an isocratic elution system consisting of water-acetonitrile-glacial acetic acid and triethylamine (80:20:1.0:0.05, v/v/v/v) and a Discovery C18 (150 mm x 4.6 mm, i.d. 5 µm particle size) column to analyze the amine.

Toluene and methanol have been employed for residual 4,4' oxydibenzenesulfonyl azide extraction in propylene resins. Two stationary phases were evaluated. With the normal phase, isooctane and methylene chloride were used as mobile phase, whereas with the reversed phase a gradient of water containing 0.1% H_3PO_4 and acetonitrile-methanol (50:50 v/v) containing 0.1% H_3PO_4 were used as the elution system. The UV detector was set at 254 nm (CRL-FCM database).

II.3.6. Aminotriazines

Melamine is an amino resin employed as monomer in several plastic materials. It can also be used as an additive-like cross-linking agent.

For the analysis of melamine, several authors have used HPLC-UV. Lund and Petersen (2006) have tested the migration of melamine using the CEN method. To carry out the experiment a Lichrosorb NH2 (200 mm x 4.6 mm, i.d. 5 µm particle size) column and an isocratic mobile phase composed of acetonitrile-5 mM phosphate buffer (75:25 v/v) were used. Chromatograms were registered at

230 nm. Similar conditions have been employed by Muñiz-Valencia and others (2008), but they used a Luna CN column (250 mm x 4.6 mm, i.d. 5 µm particle size) and a Synergi fusion-RP (SF) column (250 mm x 4.6 mm, i.d. 4 µm particle size). The best results were achieved with the Luna CN column.

Other authors have proposed a system consisting of 2 columns of Prodigy ODS (250 mm x 4.6 mm, i.d. 5 µm particle size) connected in series (Ehling and others 2007).

Hamilton and O'Neal (2003) extracted melamine from Nylon 6/6.6 with a solution composed by octanesulfonic acid and isopropanol. The separation was carried out on a µBondpack C18 (300 mm x 3.9 mm, i.d.) column and an isocratic elution system composed of 8% 0.2 mol/L sodium phosphate monobasic, 42% 0.2 mol/L sodium phosphate dibasic heptahydrate, and 50% water at a flow rate of 1 mL/min. The UV detector was set at 230 nm. A limit of detection of 2.8 ppm for free melamine in Nylon polymer was obtained with this method.

Another way to determine melamine is by capillary zone electrophoresis. Yan and others (2009) developed a capillary zone electrophoresis method with diode array detection to analyze the contaminant in dairy products. The optimum wavelength was λ 206 nm.

A rapid HPLC method to analyze benzoguanamine was described in European Commission Report EUR 17610EN. UV detection was done at λ 230 nm employing acetonitrile-phosphate buffer pH 6.5 (75:25 v/v) as the mobile phase.

LC/MS/MS was employed to determine melamine. A Synergi Polar-RP column (150 mm x 4.6 mm, i.d. 4 µm particle size) was used. The mobile phase was a gradient consisting of A (10 mM ammonium acetate in water), B (acetonitrile), and C (0.1% formic acid in water) at a flow rate of 500 µL/min (Filigenzi and others 2008).

Melamine was analyzed in aluminum adhesives by GC using 2 types of detectors: MS (mass spectrometer) and AED (atomic emission detector) with a temperature- programmable pyrolyzer. In order to carry out the experiment, an Ultra Alloy-5 metal capillary column (30 m x 0.25 mm, i.d. x 0.25 μm film thickness) was employed. The temperature of the column was established at 35 ºC for 3 min and increased to 320 ºC at a rate of 10 ºC/min. Helium was used as the carrier gas (Nakamura and others 2000).

Benzoguanamine is a monomer used as cross-linking agent for thermosetting coatings. A GC/MS method to analyze the analyte is reported in the CRL-FCM database. The column used was a RSC 300 (15 m x 0.18 mm, i.d. x 0.25 μm film thickness) and the oven program was set from 120 ºC to 280 ºC. The injector temperature was set at 280 ºC.

II.3.7. Acrylamides

Acrylamide is another important amino resin commonly used in plastic materials, paper, cosmetics, and more. (Alpmann and Morlock 2008). Due to the high toxicity of these components, in the past few years many analytical methods have been developed.

High-performance liquid chromatography with previous derivatization with 2-mercaptobenzoic acid has been widely used for the determination of acrylamide (Bagdonaite and others 2008; Shi and others 2009).

Paleologos and Kontominas (2005) proposed a normal-phase high-performance liquid chromatographic method to determine acrylamide and methacrylamide. An Aminex HPX-87H column (300 mm x 7.8 mm) was used as stationary phase and a mixture of water and acetonitrile containing 0.01 M sulfuric acid as the mobile phase. The UV detector was set at λ200 nm.

Another method to determine acrylamide as reported in European Commission Report EUR 17610EN involves the use of Dionex Ion pac ICE-ASI (250 x 7.6 mm) column and a mixture composed by water, acetonitrile, and sulfuric acid as the elution system. Detection was done at 202 nm.

Lee and others (2007) determined acrylamide in food employing solid-phase microextraction (SPME) coupled to a GC-PCI-MS-MS. The stationary phase was a DB-WAX (30 m x 0.25 mm, i.d. x 0.25μm film thickness) fused silica capillary column; helium was the carrier gas with a flow rate of 1 mL/min and the injector temperature was 210 ºC; the oven initial temperature was 80 ºC, then was increased at a rate of 15 ºC/min to 220 ºC. With the proposed method a limit of detection of 0.1 μg/L was obtained.

Biederman and Grob (2008) observed that 3-hydroxy propionitrile may cause an interference with acrylamide in some foods using GC-MS owing to the fact that they are detected by the same molecular mass using GC-MS with chemical ionization (CI).

Another monomer used for the manufacture of certain plastic materials is N-(hydroxymethyl)methacrylic amide. The method reported in the CRL-FCM involves analysis by HPLC-UV. The separation was performed on a Purospher RP 18sec column (250 mm x 3 mm, i.d. 5 μm particle size) and using a gradient composed of A (water and phosphoric acid) and B (acetonitrile and phosphoric acid) as mobile phase. The UV-detector was set at 205 nm.

II.3.8. Other polyfunctional amines used as monomers

Another polyfunctional amine also included in the European Union positive list and widely used is 3,3-bis (3-methyl-4-hydroxyphenyl)-2-indolinone.

The method approved by CEN (European Committee for Standardization) (European Commission Report EUR 17610EN) for its determination which involves reversed-phase liquid chromatographic analysis with UV detection (λ 235 nm). A binary gradient elution system consisting of (A) orthophosphoric acid-water-acetonitrile (0.7:620:380 v/v/v) and (B) acetonitrile and a Spherisorb ODS2, C18 column (250 mm x 4.6 mm, i.d. 5 μm particle size) are used as mobile phase and stationary phase, respectively.

N-vinyl N-methylacetamide and 1-vinylimidazole act as co-polymers in the manufacture of plastic materials. Two gas chromatographic methods are described in the Community Reference Laboratory for Food Contact Materials (CRL-FCM) database.

N-vinyl N-methylacetamide was determined by GC-FID using a CP Wax 52 CB column (10 m x 0.53 mm, i.d. x 2.0 μm film thickness) as stationary phase; the injection is performed in a split mode (1:10). For 1-vinylimidazole a GC method with N specific detector has been reported, the stationary phase employed was a DB wax column (30 m x 0.25 mm, i.d. x 0.5 μm film thickness).

II.4. Additives-monomers

Some amines included in the positive list of the European Union can be used as monomers or additives in the manufacture of food contact materials.

A HPLC-FLD method using previous derivatization with OPA (*o*-phthaldehyde) and ME (2-mercaptoethanol) to determine ethylenediamine has been reported by Paz-Pino and others (2003). Their analyses were performed on a ODS 2 Waters Spherisorb (150 mm x 4.6 mm, i.d. 5 μm particle size) column using isocratic conditions with methanol-water (65:35 v/v) as the mobile phase. The analyses were carried out in less than 10 min and the fluorescent derivatives were detected

at 330 nm (excitation) and 450 nm (emission). The limits of detection and quantification obtained with the reported method were 0.09 and 0.25 mg/L, respectively.

Crea and others (2005) determined dietylenetriamine and ethylenediamine (EDA) using high-performance ion exchange chromatography. A Dionex IonPac CS14 cation-exchange column (4 x 250 mm i.d.) was used as analytical column protected by an IonPac CG14 (4 x 50 mm i.d.) guard column. The mobile phase was a mixture of $HClO_4$-$NaClO_4$-acetonitrile; and the amines were detected amperometrically.

The method to determine hexamethylenetetramine (HTM) in food simulants reported in the European Commission Report EUR 17610EN includes the determination of HTM as formaldehyde by spectrophotometry (λ 574 nm).

Hexamethylenetetramine is used as accelerator in rubber vulcanization and as a stabilizer of lubricating oils among other applications (Koga and others 1985).

A GC method has been developed for the determination of HTM in water. Extraction is started by CH2Cl2 forming a charge-transfer complex with iodine which is then decomposed with methanolic KOH. To carry out the determination a FTD (flame thermionic detector) is used. The column is a glass column (2 m x 3 mm i.d.) packed with 5% PEG-20 M on Uniport HPS. The oven temperature is maintained at 220 ºC and the flow rate of N_2 is 50 mL/min (Koga and others 1985).

II.5. Additives

Additives are incorporated into polymers to modify or enhance their properties as well as to increase their shelf-life. Antioxidants, antimicrobial agents, UV stabilizers, lubricants, optical brighteners, and surfactants are some of the

additives frequently used in food-contact materials. An overview of the chromatographic methods applied to the plastic additive analyses is presented below. Detailed information regarding stationary phase, mobile phase composition, temperature program, and detection system is reported.

II.5.1. Lubricants

Lubricants primarily act by controlling (reducing) friction. Among the lubricant additives, slip agents are used to reduce the adhesion between the polymer and the metallic walls of processing equipment (Salamone 1996). Fatty acid amides are widely used as slip additives in food-packaging materials. Several methods have been reported in the literature to analyze these compounds including liquid and gas chromatography.

Brengartner (1986) determined 12 fatty acid primary amides (for example, oleamide, erucamide, behenamide) in different commercial materials by using gas chromatography with flame ionization detection (GC-FID). The amides were extracted from plastics with 2-propanol. The separation was performed on a fused silica 30 m SP-2330 (90% bis-cyanopropyl/10% phenylcyanopropyl polysiloxane) (30 m x 0.32 mm, i.d. x 0.2 μm film thickness) column. Helium was used as carrier gas and the oven temperature was programmed from 200 to 260 ºC.

A GC-FID method was also used by Cooper and Tice (1995) to analyze oleamide, stearamide, and erucamide. Two different columns and 2 oven programs were employed. A CP-Sil 5CB (Chrompack) (25 m x 0.32 mm, i.d. x 1.2 μm film thickness) with a ramp of temperature from 150 to 320 ºC; and BP1 (SGE) column (25 m x 0.32 mm, i.d. x 1.2 μm film thickness) with a temperature program from 80 to 325 ºC.

Ding and others (2008) reported on the use of a GC-MS system to identify 17 fatty acid amides from coal samples. The analytical column used was a HP-5MS (cross-link 5% PH ME siloxane, 30 m x 0.25 mm, i.d. x 0.25 μm film thickness).

Farajzadeh and others (2006) proposed 2 chromatographic techniques: GC-FID and HPLC-UV to analyze oleamide, stearamide, and erucamide in polyethylene samples. With the gas chromatographic method the analytes were separated on a Carbowax 20M (20 m x 0.25 mm, i.d. x 0.5 μm film thickness). The oven program was from 150 to 220 ºC and nitrogen was used as carrier gas. The chromatographic conditions employed for the HPLC analyses were as follows: a C_{18} Nova Pak column (150 mm x 3.9 mm, i.d. 5 μm particle size) was used as the stationary phase and acetonitrile: methanol (60:40, v/v) as the mobile phase. The flow rate and column temperature were 0.5 mL/min and 25± 3 ºC, respectively. The detection was done at 202 nm. Total analysis time does not exceed 8 min. However, when real samples were analyzed by HPLC, the peak corresponding to oleamide was not suitably separated because of interferences in the first part of the chromatogram; this drawback was avoided with the GC method. Regarding performance characteristics both methods showed a good linearity with correlation coefficients (R^2 ≥0.997); the GC method presented better sensitivity with lower detection limits (oleamide 20 mg/L; stearamide 15 mg/L and erucamide 30 mg/L).

In a recent study, Gaudin and others (2007) developed a novel method to determine N-N'-ethylenebisstearamide in polymer samples by using HPLC with evaporative light scattering detection (ELS). The additive was extracted from the polymer by a dissolution/precipitation procedure, using $CHCl_3$ and methanol as extraction solvents.

The analysis was performed on a normal-phase Diol column (250 mm x 4.6 mm, i.d. 5 μm particle) thermostated at 50 ºC, and trichloromethane was used as

mobile phase at a flow rate of 1 mL/min. The ELS operated with a tube temperature of 75 ºC and a flow rate of nebulizing air of 2.0 L/min. The method exhibited an excellent sensitivity with a limit of detection of 0.8 μg/mL.

II.5.2. UV Stabilizers

UV stabilizers are often used in food-contact materials, such as polyethyleneterephthalate (PET). These additives are added to polymers to protect them from UV radiation and, consequently, prevent the degradation not only of the polymer but also the food (Salamone 1996; Monteiro and others 1998).

Chemically, a wide variety of UV absorbers are available. Derivatives of hydroxybenzotriazoles and hydroxybenzophenones are extensively used.

Regarding the analytical techniques used to determine these additives, various methods including reversed- and normal-phase high-performance liquid chromatography (NP-HPLC, RP-HPLC), gas chromatography (GC), and size-exclusion chromatography- high-performance liquid chromatography (SEC-HPLC) have been described in the literature.

A RP- HPLC-UV method to determine 3 derivatives of benzotriazoles is reported by Gennaro and others (1999). The separation was performed on a Chrompack C_{18} column (150 mm x 4.6 mm, i.d. 5 μm paricle size) protected by a guard column (5 mm x 3 mm, i.d. 5 μm paricle size) with identical packaging. A gradient system composed by acetonitrile and water at a flow rate of 1 mL/min was used as the mobile phase. The detector was set at 343 nm.

A mobile phase consisting of a mixture of acetonitrile-water was also used by Spyropoulos (1998) to analyze 2-ethoxy-2-ethyloxyanilide, known as Tinuvin 312, in aqueous and fatty food simulants. The column used was a Nucleosil 120,

C18 (250 mm x 4.6 mm, i.d. 5 μm particle size). The UV detector was set at 296 nm.

Hodgeman (1981) employed a normal stationary phase to analyze hydroxybenzophenones and hydroxybenzotriazole derivatives. After acetylation, the UV stabilizers were separated on a Spherisorb silica gel column (150 mm x 4.6 mm, i.d. 5 μm particle size) and with an isocratic mobile phase consisting of 0.1% isopropanol in methylene chloride at a flow rate of 1.0 mL/min. Chromatograms were monitored at 254, 280, and 313 nm.

A SEC-HPLC method was developed by Monteiro and others (1996) to determine antioxidants and UV stabilizers such as 2-(2'-hydroxy-5-methylphenyl) benzotriazole (Tinuvin P) in polyethyleneterephthalate (PET) bottles. The antioxidant was extracted from the samples by using dichloromethane. The analyses were carried out on a column of PL-GEL 50 Å (300 mm x 7.5 mm, i.d.). The column temperature was set at 30 ºC. *n*-Hexane:dichloromethane (75:25 v/v) at a flow-rate of 1.0 mL/min was used as the mobile phase. Detection was at 280 nm. With the proposed method a limit of detection of 0.1 μg/g was obtained.

SEC has also been reported as a technique used as a clean-up procedure to separate interfering substances before the analysis of additives. In a study conducted by Nerín and others (1995) a system consisting of two HPLC columns separated by an automatic switching valve was designed to determine antioxidants and UV stabilizers including 2(3'-*tert*-butyl-2'-hydroxy-5'methylphenyl)-2H-5-chlorobenzotriazole (Tinuvin 326) and 2(2'-hydroxy-3',5'-di-*tert*-butylphenyl)-2H-5-chlorobenzotriazole (Tinuvin 327). In the first column size-exclusion chromatography was performed on PL-Gel 50Å (300 mm x 7.5 mm i.d.). In the second one, a normal-phase separation was carried out on a Nucleosil 100-7 OH column (250 mm x 4.6 mm, i.d. 7 μm paricle size). Both

columns were thermostated at 30ºC. The mobile phase used was a mixture of n-hexane: dichloromethane (73:27, v/v) and the flow rate was 0.9 mL/min. Two wavelengths were selected, 254 and 280 nm.

Gas chromatography coupled to mass spectrometry was used by Monteiro and others (1998) to determine hydroxybenzophenone and hydroxybenzotriazole derivatives in PET bottles. The analytes were separated on a DB1701 capillary column (60 m x 0.25 mm, i.d. x 0.25 μm film thickness). The oven temperature was programmed from 100 to 280 ºC. Helium was used as carrier gas. Only Tinuvin P was detected in the samples analyzed.

Michelsen and others (2007) also employed a GC-MS method to analyze Tinuvin P. The stationary phase used was a CP-SIL 8CB wall-coated open tubular (WCOT) low-bleed fused-silica MS column (30 m x 0.25 mm, i.d. x 0.25 μm film thickness). The temperature column was set from 50 to 280 ºC; the carrier gas used was helium.

II.5.3. Optical Brighteners

Optical brighteners enhance the appearance of plastic materials by protecting their color from adverse environmental conditions. Thus, they can prevent yellowing and discoloration that polymers can undergo under unfavorable conditions. From a chemical point of view, bis-benzoxazoles (bis-benzoxazolyl-stilbene and bis-benzoxazolyl- thiophene) are widely employed in thermoplastics (Jervis 2003).

Direct spectroscopic and chromatographic methods have been applied to determine these additives.

Quinto-Fernández and others (2003) proposed a spectrofluorimetric method to determine 2,5-bis (5-tert-butyl-2-benzoxazolyl) thiophene, known as UVITEX

OB, in an olive oil simulant. The measures were performed directly without sample treatment. The fluorescence wavelengths selected were λ_{em} 433 nm λ_{ex} 394 nm.

A reversed-phase chromatographic method to determine UVITEX OB has been described by Paseiro and others (2006). A Phenomenex Luna C18 (250 mm x 4.6 mm, i.d. 5 μm particle size) column and a gradient system of 3 eluents: (A) acetonitrile containing 1% v/v acetic acid, (B) water, and (C) tetrahydrofuran at a flow rate of 1.2 mL/min was used. The detection wavelength was 330 nm.

Regarding the analysis of 4,4'bis (benzoxazolyl) stilbene, a UV/Vis spectrophotometry and a reversed-phase liquid chromatographic method with fluorescence detection are described in the CRL FCM database. The chromatographic analysis involves the separation on a Zorbax StableBond (SR)^{TM} C_{18} (150 mm x 4.6 mm, i.d. 5 μm particle size) column and using a mixture of 0.14% H_3PO_4 (pH 2)-acetonitrile-tetrahydrofuran as the mobile phase. The optical brightener is then detected at λ_{ex} 365 nm and λ_{em} 430 nm.

II.5.4. Accelerators for vulcanization

Primary amines, like cyclohexylamine, are used as accelerators for vulcanization (Ash and Ash 1999). Liquid chromatography is the technique most extensively used to analyze these compounds. Since aliphatic amines do not exhibit suitable absorptivity a derivatization step is essential to improve the sensitivity.

An isocratic and reversed-phase liquid chromatographic method with UV detection to determine cyclohexylamine has been reported by Casals and others (1996). Trinitrobenzenesulfonic acid (TNBS) was used as the derivatizing agent. The analysis was performed on a Spherisorb ODS2 column (150 mm x 4.6 mm, i.d. 5 μm particle size) using acetonitrile-0.01 M monoammonium phosphate

(60:40 v/v) (pH 3.5 adjusted with 4 M phosphoric acid) as the mobile phase at a flow rate of 1 mL/min in isocratic elution mode. The wavelength was set at 335 nm. With the proposed method recoveries higher than 99% were achieved.

m-Toluoyl chloride was selected by Simon and Lemacon (1987) as derivatizing agent. In this study two normal-phases, a silica column and Nucleosil CN-bonded silica column (250 mm x 4.6 mm, i.d. 5 μm particle size) and two reversed-phase Nucleosil C_{18} and Spherosil C_{18} columns (150 mm x 4.5 mm, i.d. 5 μm particle size) were evaluated. Acetonitrile-water and a mixture of isooctane 90% methylene chloride 9% and 2-propanol 1% were used as mobile phases for reversed and normal phase separations, respectively. The flow rate was 1 mL/min and the wavelength was set at 240 nm.

Rampfl and others (2008) analyzed the amines by high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC/ESI-MS) operated in a positive mode. The determination was performed on a Discovery HSF5 (150 mm x 2.1 mm, i.d. 3 μm particle size) column thermostated at 40 ºC and using a gradient elution system consisting of acetonitrile and water with 0.02% formic acid at a flow rate of 400 μL/min.

Jegorov and others (1990) used thin-layer chromatography as the separation technique, and previous derivatization of amines with 1-fluoro-2,4-dinitrobenzene. Reversed phase HPTLC RP 18 (100 mm x 100 mm) plates with acetonitrile as eluent were used. The wavelength selected was 369 nm.

II.5.5. Antioxidants

Polymers are constantly in contact with oxygen in each stage of their life cycle. Contact with oxygen results in degradation, as well as surface cracks, discoloration, loss of gloss or transparency, chalking, and loss of mechanical

properties such as strength, elongation, toughness, and so on. In order to avoid oxidation and, consequently, degradation of polymeric materials, antioxidants are added to the polymer (Wang 2000). The antioxidants are dispersed in the polymer matrix without significantly affecting the molecular structure. We can divide the antioxidants into 2 classes: Primary antioxidants are radical scavengers or hydrogen donors, as well as chain reaction breakers. These are hindered phenols and secondary aryl amines. And on the other hand, secondary antioxidants are peroxide decomposers. These are thioesters and organophosphites. Frequently, both kinds of antioxidants are used together due to their synergistic effect (Salamone 1996; Carraher 2007). The use of additives such as antioxidants avoids adverse effects and gives stability to the polymers.

Several methods have been reported in the literature for the determination of antioxidants. The analysis of these compounds is not easy because of their low concentration in the polymer (between 100 and 1000 ppm of each primary and secondary antioxidant depending on the application and the processing conditions) and due to interferences from the polymer matrix (Wang 2000). Generally, the first step to determine antioxidants includes their separation from the polymer. Lesellier and Tchapla (1993) pointed out that when the Soxhlet system was used an incomplete extraction of high-molecular-weight antioxidants was achieved, and supercritical fluid extraction was proposed as an excellent alternative.

Murteanu and others (1987) analyzed several antioxidants (for example, Irganox 1024 and Irganox 3114) and light stabilizers (such as Tinuvin P and Tinuvin 327) in polyolefins by HPLC. The separation was performed on a LiChrosorb RP-18 (250 mm x 4 mm, i.d. 5 μm particle size) column; 3 isocratic mobile phases were tested: 100% acetonitrile; acetonitrile-water (90:10 v/v), and acetonitrile-water (80:20 v/v). Chromatograms were detected at 254 nm and 280

nm. In this work the IR detector was also used. The authors suggested using the UV254/UV280 ratio to have qualitative information about the structure of the molecule and for the detection of overlapping peaks.

Kim and others (2007) determined antioxidants by RP-HTLC. Three kinds of elevated- temperature columns were tested. The analytical conditions were: Zichrom PBD (100 mm x 4.6 mm, i.d. 3 μm particle size) column, thermal gradient (40-150 ºC) and UV wavelength 225 nm. The study showed that the use of Zichrom PBD columns with a thermal gradient improve the separation efficiency and resolution of the peaks while reducing elution time.

In a study reported by Lesselier and Tchapla (1993) different antioxidants (for example, Irganox 1098) and light stabilizers (Tinuvin 327) were analyzed under isocratic conditions. With the proposed method they did not achieve a proper separation within a reasonable analysis time. In order to improve the separation, the quaternary gradient system consisted of methanol, water, tetrahydrofuran and acetonitrile was used. Three columns were evaluated: LiChrospher 100 RP 18 (250 mm x 4 mm, i.d. 5 μm particle size), Brownlee Spheri 5-ODS (250 mm x 4.6 mm, i.d. 5 μm particle size) and Ultrabase UB 225 (250 mm x 4.6 mm, i.d. 5 μm particle size). The analytes were determined with light-scattering coupled to an UV-detector.

Block and others (2006) conmpiled a little library of MS spectra of several additives including antioxidants (Irganox 565 and 3114), lubricants (erucamide and oleamide), and UV absorbers (Tinuvin 326 and 327) by LC-MS. The APCI mass spectra were recorded in positive and negative modes. A Waters Nova-Pak C18 (150 mm x 3.9 mm, i.d. 4 μm particle size) column protected by a guard column with the same packaging was employed to perform the separation.

Carrott and others (1998) proposed the use of supercritical fluid chromatography with APCI-MS detection for the determination of different additives such as

oleamide, erucamide, tinuvin 327, and irganox 3114. The column employed was a reversed-phase C18 (250 mm x 4.6 mm, i.d.) and CO_2 with methanol as modifier gradient was used as the mobile phase.

A liquid chromatographic method for the determination of additives including Irganox MD-1024 and kenamide was reported by Duderstadt and Fisher (2008). Mass spectrometry (APPI and APCI) and UV were used as detection systems. Two reversed phases, Zorbax Eclipse XDB-C8 (100 mm x 3 mm, i.d. 3.5 μm particle size) and Zorbax Eclipse XDB-C18 (150 mm x 3 mm, i.d. 3.5 μm particle size) and 2 mobile phases, methanol-water (50:50, v/v) and acetonitrile-water (50:50, v/v), were employed. For the UV detector, 210 nm was selected as the optimal wavelength.

Another different method to analyze antioxidants (Irganox MD 1024) has been described by Wang (2000). The method consisted of a pyrolysis-gas chromatography-MS. For the Py-GC method a DB-5 (10 m x 0.10 mm, i.d. x 0.4 μm film thickness) fused silica capillary column was used. The temperature program was from 50 ºC to 320 ºC, then the pyrolysis products were separated in a DB-5 (30 m x 0.25 mm, i.d. x 1.0 μm film thickness) fused-silica capillary column using a linear gradient of temperature from 40 ºC to 320 ºC.

II.5.6. Antimicrobial agents

Most of the synthetic polymers are not attacked by microorganism, as fungi and bacteria, but they often allow surface growth. Polymer materials such as celluloses, vegetable oil coatings, and so on, are often subject to microbiological deterioration. And on the other hand, some synthetics containing linkages can be recognized by microorganism and may be susceptible to attack. Thus,

microorganism inhibitors are currently employed, so it is important to consider their toxicity and analyze them.

For the analysis of microorganism inhibitors Rafoth and others (2007) proposed a GC-MS method. 2-Methyl-3-isothiazolinone was analyzed by using a DB 5-MS (30 m x 0.25 mm, i.d. x 1 μm film thickness) fused-silica capillary column, helium as carrier gas, and with a column oven temperature program started at 50 ºC up to 280 ºC. The proposed method showed an excellent sensitivity (LOQ 0.28 μg/L and LOD 0.085 μg/L) and a good linearity in the range 100-1000 ng/L (R^2 0.992).

Wu and others (2008) developed a method to determine preservatives in cosmetics (2-methyl-3-isothiazolinone) employing ultra-performance liquid chromatography with PDA (photoelectric diode array). Methanol was used as extraction solvent and a complete recovery (92.9%) was obtained. Chromatograms were registered in UV scan mode (254-280 nm). The column and mobile phase employed were UPLC BEH C18 (50 mm x 2.1 mm, i.d. 1.7 μm particle size) and a gradient of formic acid and methanol. With the reported method a limit of detection of 0.175 μg/mL was obtained.

II.5.7. Surfactants

Surface-active substances (surfactants) are amphiphilic compounds. This amphiphilic property is responsible of the capacity for concentrate and aggregate solutions in supramolecular structures (Cheng and others 2007). EDTA is a kind of surfactant widely used owing to the considerable versatility in industrial, pharmaceutical, agricultural, and household uses (Laine and Matilainen 2005; Maleki and others 2009.) Furthermore, they are present in cleaning agents and detergents based on perborates as stabilizers. Besides these applications EDTA is

used as chelating agent for heavy metals and as stabilizer for colors and flavors (Cagnasso and others 2007).

Laine and Matilainen (2005) determined the chelating agent EDTA by HPLC–UV in less than 5 min using a mobile phase containing sodium acetate, tetrabutylammonium bromide in water, and methanol at pH 3.5. A Luna C18 (250 mm x 4.6 mm, i.d. 5 μm particle size) column was employed as stationary phase; detection was done at 254 nm. The detection limit was 0.27μmol/L.

Cagnasso and others (2007) have developed a method using HPLC with a DAD detector set at 257 nm for the determination of EDTA in nonalcoholic beverages. The method was carried out employing a RP C18 column with a mobile phase consisting of a mixture of 0.01 M ammonium phosphate monobasic-acetonitrile-40% tetrabutylamonium hydrochloride (90:10:0.2, v/v/v), pH 2.42. A limit of detection of 0.6 mg/L was achieved.

Maleki and others (2009) used a dispersive liquid-liquid micro-extraction in conjunction with HPLC-DAD to determine EDTA in water at low concentration (LOD 1.7 μg/L). A Shimpack VP-ODS (250 mm x 4.6 mm, i.d. 5μm particle size) was used as a stationary phase. Elution was carried out under isocratic conditions; using a mobile phase composed of a mixture of 0.01 M acetate buffer and 2 mM tetrabutylamonium hydroxide at pH=4.0 with at flow rate of 1 mL/min. The wavelength selected was 300 nm. The linearity of the method was studied in the range 3-50 μg/L and a correlation coefficient of 0.9982 was obtained.

Triisopropanolamine was used with PET for the solvolytic degradation of waste PET; it was also employed for polyurethanes and epoxy resin reactivation. When that product (PET/TEA) is formed, it can be used as a cross-linking agent with new beneficial properties such as low viscosity and flexibility. In a study carried out by Spychaj and others (2004) SEC-HPLC was used to analyze PET/TEA.

N,N'-diethylaminoethanol and lysosomotropic amino alcohol were determined by potentiometric detection and compared with cation-exchange HPLC-UV. HPLC separations were performed using a universal cation-exchange HPLC column (100 mm x 4.6mm, i.d. 7 μm particle size) coupled to a pre-column (7.5 mm x 4.6 mm, i.d. 7 μm particle size); detection was done at 230 nm. The mobile phases used were: acetonitrile-40 mM phosphoric acid (15:85, v/v) or acetonitrile-1.95 M acetic acid (15:85, v/v) (Bazylak and Nagels 2002).

II.5.8. Other polyfunctional amines used as additives

A Nucleatin/clarifying agent (N-[3,5-bis-(2,2-dimethyl-propionylamino)-phenyl]-2,2-dimethyl-propionamide) was analyzed using HPLC-UV. A gradient of water and acetonitrile (+0.01 %TFA) was used as mobile phase. The detector was set at 260 nm and the stationary phase was a Symmetryshield C18 column (150 mm x 3.9 mm, i.d. 5 μm particle size) (CRL-FCM database).

Ethyl 2-cyano-3,3-diphenylacrylate is an additive employed in the production of certain plastic materials. A HPLC-UV method for its determination is reported in the CRL-FCM database. To perform the analysis a Fluorix® 120E (250 mm x 4.6 mm, i.d.) column was used, the mobile phase was a mixture composed of water-acetonitrile-phosphoric acid (350:650:1 v/v/v). The detector was set at 300 nm.

GC/MS was used as the analytical technique for the determination of N-methylpyrrolidone (NMP). To carry out the analysis a CAM (30 m x 0.25 mm, i.d. x 0.25 μm film thickness) column was employed, the specific masses were 99.10 m/z and 98.05 m/z (CRL-FCM databse).

Anthranilamide, a scavenger for acetaldehyde, is an additive used in the manufacture of PET. A LC/MS/MS method using a reversed-phase Luna C18 (2)

column (150 mm x 2 mm, i.d. 3 μm particle size) and a mobile phase consisting of methanol-aqueous acetic acid 0.1% (60:40, v/v) as the isocratic elution system is reported in CRL-FCM database.

Acknowledgements

The study was financially supported by the Ministerio de Ciencia e Innovación, ref. Nº AGL/2008-04146 "MIGRAMIN". The authors are grateful to "Ministerio de Ciencia e Innovación" for a Predoctoral Fellowship FPI (ref. BES-2009-023016) awarded to Rafael Paseiro-Cerrato.

References

Alpmann A, Morlock G. 2008. Rapid and sensitive determination of acrylamide in drinking water by planar chromatography and fluorescence detection after derivatization with dansulfinic acid. J Sep Sci 31:71-77.

Araújo HP, Félix JS, Manzoli JE, Padula M, Monteiro M. 2008. Effects of g-irradiation on caprolactam level from multilayer PA-6 films for food packaging: Development and validation of a gas chromatographic method. Radiat Phys Chem 77:913-917.

Ash M, Ash I. 1999. Food packaging. Chemicals and materials electronic handbook. New York: Synapse Information Resources, Inc.

Bagdonaite K, Derler K, Murkovic M. 2008. Determination of acrylamide during Roasting of coffee. J Agric Food Chem 56:6081-6086.

Baranowska I, Pieszko C, Raróg D, Pielesz A. 2002. Analysis of some aromatic amines by means of derivative spectrophotometry. J Environ Sci Heal A 37:1841-1848.

Bazylak G, Nagels LJ. 2002. Potentiometric detection of N,N'-diethylaminoethanol and lysosomotropic amino alcohols in cation exchange high-performance liquid chromatography systems. Anal Chim Acta 472:11-26.

Biedermann M, Grob K. 2008. In GC-MS, acrylamide from heated foods may be coeluted with 3-hydroxy propionitrile. Eur Food Res Technol 227:945-948.

Block C, Wynants L, Kelchtermans M, De Boer R, Compernolle F. 2006. Identification of polymer additives by liquid chromatography-mass spectrometry. Polym Degrad Stabil 91:3163-3173.

Bomke S, Seiwert B, Dudek L, Effkemann S, Karst U. 2009. Determination of biogenic amines in food samples using derivatization followed by liquid chromatography/mass spectrometry. Anal Bioanal Chem 393:247-256.

Bradley EL, Speck DR, Read WA, Castle L. 2004. Method of test and survey of caprolactam migration into foods packaged in nylon-6. Food Addit Contam 21:1179–1185.

Brengartner DA. 1986. The determination of fatty acid primary amides by capillary gas chromatography. J Am Oil Chem Soc 63:1340-1341.

Cagnasso CE, López LB, Rodríguez VG, Valencia ME. 2007. Development and validation of a method for the determination of EDTA in non-alcoholic drinks by HPLC. J Food Compos Anal 20:248-251.

Carraher ChE. 2007. Introduction to polymer chemistry. Boca Ratón (FL): Taylor & Francis Group.

Carrott MJ, Jones DC, Davidson G. 1998. Identification and analysis of polymer additives using packed-column supercritical fluid chromatography with APCI mass spectrometric detection. Analyst 123:1827-1833.

Casals I, Reixach M, Amat J, Fuentes M, Serra-Majem L. 1996. Quantification of cyclamate and cyclohexylamine in urine samples using high-performance liquid chromatography with trinitrobenzenesulfonic acid pre-column derivatization. J Chromatogr A 750:397-402.

Casella IG, Palladino GA, Contursi M. 2008. Determination of aliphatic amines by cation-exchange chromatography with suppressed conductivity detection after solid phase extraction. J Sep Sci 31:3718-3726.

ChemIDplus Advanced United States National Library of Medicine National Institutes of Health (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp)

Cheng L, Mewes D, Luke A. 2007. Boiling phenomena with surfactans and polymeric additive: A state-of-the-art review. Int J Heat Mass Transf 50:2744-2771.

Chiacchierini E, Restuccia D, Vinci G. 2006. Evaluation of two different extraction methods for chromatographic determination of bioactive amines in tomato products. Talanta 69:548-555.

Community Reference Laboratory for Food Contact Materials (CRL-FCM). Available from: http://crl-fcm.jrc.it/resource_centre/smm.php

Cooper I, Tice PA. 1995. Migration studies on fatty acid amide slip additives from plastics into food simulants. Food Addit Contam 12:235-244.

Crea F, De Robertis A, Sammartano S. 2005. Chromatographic behaviour of open-chain polyamines NH2-$(CH2)_2$-[NH-$(CH2)_2$]n-NH2 and their quantitative determination in sea water by high-performance ion-exchange chromatography. J Chromatogr Sci 43:342-347.

Demertzis PG, Simal-Gándara J, Franz R. 1995. A convenient group method for the gas chromatographic determination of aliphatic diamines in the four official EC food simulants. Dtsch Lebensm Rundsch 91:35-38.

Deng YH, Wang H, Zhong L, Zhang HS. 2009. Trace determination of short-chain aliphatic amines in biological samples by micellar electrokinetic capillary chromatography with laser-induced fluorescence detection. Talanta 77:1337-1342.

Ding MJ, Zong ZM, Zong Y, Ou-Yang XD, Huang YG, Zhou L, Wang F, Cao JP, Wei XY. 2008. Isolation and identification of fatty acid amides from shengli coal. Energ Fuel 22:2419-2421.

Domininghaus H. 1988. Plastics for engineers: materials, properties, applications. Düsseldorf, Germany: Hanser Publishers.

Duderstadt RE, Fischer SM. 2008. Effect of organic mobile phase composition on signal responses for selected polyalkene additive compounds by liquid chromatography-mass spectrometry. J Chromatogr A 1193:70-78.

Dugo Gmo, Vilasi F, La Torre GL, Pellicanò TM. 2006. Reverse-phase HPLC/DAD determination of biogenic amines as dansyl derivatives in experimental red wines. Food Chem 95:672-676.

Ehling S, Tefera S, Ho IP. 2007. High-performance liquid chromatographic method for the simultaneous detection of the adulteration of cereal flours with melamine and related triazine by-products ammeline,ammelide, and cyanuric acid. Food Addit Contam 24:1319-1325.

Ellis BE. 1993. Chemistry and technology of epoxy resins. Glasgow: Blackie Academic & Professional.

EU Commission Directive 2002/72/EC relating to plastic materials and articles intended to come into contact with foodstuffs. Official Journal of the European Union, L220 of 15.08.2002;18-58.

European Commission Report. (EUR 17610EN) (1997). Development of methods of analysis for monomers and other starting substances with SML and/or QM limits in directives 90/128/EEC and 92/39/ECC.

Farajzadeh MA, Ebrahimi M, Ranji A, Feyz E, Bejani V, Martin AA. 2006. HPLC and GC methods for determination of lubricants and their evaluation in analysis of real samples of polyethylene. Microchim Acta 153:73-78.

Félix JS, Monteiro M, Manzoli JE, Padula M, Pezo D, Romero J, Nerín C. 2008. Identification and migration of degradation compounds from irradiation of multilayer polyamide 6 films for meat foodstuffs and cheese. Anal Bioanal Chem 391:847-857.

Filigenzi MS, Puschner B, Aston LS, Poppenga RH. 2008. Diagnostic determination of melamine and related compounds in kidney tissue by liquid chromatography/tandem mass spectrometry. J Agric Food Chem 56:7593-7599.

Fournier M, Lesage J, Ostiguy C, Van Tra H. 2008. Sampling and analytical methodology development for the determination of primary and secondary low-molecular-weight amines in ambient air. J Environ Monitor 10:379-386.

Gaudin K, Ho-Sung H, Bleton J, Joseph-Charles J, Dallet P, Puig P, Dubost JP. 2007. Determination of N,N'-ethylenebisstearamide additive in polymer by normal phase liquid chromatography with evaporative light scattering detection. J Chromatogr A 1167:27-34.

Gennaro MC, Gianotti V, Alberi F, Angelino S, Scagliotti M. 1999. Chromatographic determination of UV absorbers in car paints. J Liq Chromatogr Relat Technol 22:2689-2700.

Hamdani M, Thil L, Gans G, Feigenbaum AE. 2002. Safety assessment of polymeric additives for food packaging: hydrolysis of polymeric plasticizers by digestive fluids. J Appl Polym Sci 83:956–966.

Hamilton DW, O'Neal PA. 2003. Analytical methods for the quantification of free melamine and cyanuric in nylon 6/6,6 copolymer. J Sep Sci 26:510-514

Hodgeman DKC. 1981. Analysis of 2-hydroxybenzophenone and 2'-hydroxyphenylbenzotriazole UV stabilizers by high-performance liquid chromatography. J Chromatogr 214:237-242.

Innocente N, Biasutti M, Padovese M, Moret S. 2007. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. Food Chem 101:1285-1289.

Instant JChem was used for structure database management, search and prediction, Instant JChem 4.2.1, 2008, ChemAxon (http://www.chemaxon.com).

Jegorov A, Třiska J, Zahradníčková H. 1990. Determination of higher aliphatic amines and cyclohexylamine by TLC after derivatization with 1-fluoro-2,4-dinitrobenzene. Fresenius J Anal Chem 338:302-303.

Jervis DA. 2003. Optical brighteners: improving the colour of plastics. Plastics Additives & Compounding 5:42-46.

Kim B, Yang D, Ok JH. 2007. Analysis of polymer additives in high-temperature liquid chromatography. J Chromatogr Sci 45:16-21.

Koga M, Shinohara R, Akiyama T. 1985. Gas chromatographic determination of trace amounts of hexametylenetetramine in water using an iodine charge-transfer complex. Anal Sci 1:381-384.

Kwadijk S, Toraño JS. 2002. High-performance liquid chromatographic method with ultraviolet detection for the determination of dapsone and its hydroxylated metabolite in human plasma. Biomed Chromatogr 16:203-208.

Laine P, Matilainen R. 2005. Simultaneous determination of DTPA, EDTA and NTA by UV-visible spectrometry and HPLC. Anal Bioanal Chem 382:1601-1609.

Lee M, Chang L, Dou J. 2007. Determination of acrylamide in food by solid-phase microextraction coupled to gas chromatography-positive chemical ionization tandem mass spectrometry. Anal Chim Acta 582:19-23.

Lesselier E, Tchapla A. 1993. Sequential optimization of the separation of a complex mixture of plastics additives by HPLC with a quaternary gradient and a dual detection system. Chromatographia 36:135-143.

Lund KH, Petersen JH. 2006. Migration of formaldehyde and melamine monomers from kitchen- and tableware made of melamine plastic. Food Addit Contam 23:948-955.

Maleki R, Nezhad NM, Samadi N, Farhadi K. 2009. Trace determination of EDTA from water samples using dispersive liquid-liquid microextraction coupled with HPLC-DAD. Microchim Acta165:97-101.

Mao H, Chen B, Qian X, Liu Z. 2009. Simultaneous determination of twelve biogenic amines in serum by high-performance liquid chromatography. Microchem J 91:176-180.

Marand Å, Karlsson D, Dalene M, Skarping G. 2004. Determination of amines as pentafluoropropionic acid anhydride derivatives in biological samples using liquid chromatography and tandem mass spectrometry. Analyst 129:522-528.

Michelsen VB, Moe G, Skålevil R, Jensen E, Lygre H. 2007. Quantification of organic eluates from polymerized resin-based dental restorative materials by use of GC/MS. J Chromatogr B 850:83-91.

Mohamed R, Hammel YA, LeBreton MH, Tabet JC, Jullien L, Guy PA. 2007. Evaluation of atmospheric pressure ionization interfaces for quantitative measurement of sulfonamides in honey using isotope dilution liquid chromatography coupled with tandem mass spectrometry techniques. J Chromatogr A 1160:194-205.

Molins-Legua C, Camping-Falcó P, Sevillano-Cabeza A, Pedrón-Pons M. 1999. Urine polyamines determination using dansyl chloride derivatization in solid-phase extraction cartridges and HPLC. Analyst 124:477-482.

Monteiro M, Nerín C, Reyes FGR. 1996. Determination of UV stabilizers in PET bottles by high-performance size-exclusion chromatography. Food Addit Contam 13:575-586.

Monteiro M, Nerín C, Rubio C, Reyes FGR. 1998. A GC/MS method for determining UV stabilizers in polyethyleneterephthalate bottles. J High Resol Chromatogr 21:317-320.

Moret S, Smela D, Populin T, Conte LS. 2005. A survey on free biogenic amine content of fresh and preserved vegetables. Food Chem 89:355-361.

Mortensen SK, Trier XT, Foverskov A, Petersen JH. 2005. Specific determination of 20 primary aromatic amines in aqueous food simulants by

liquid chromatography-electrospray ionization-tandem mass spectrometry. J Chromatogr A 1091:40-50.

Muñiz-Valencia R, Ceballos-Magaña SG, Rosales-Martinez D, Gonzalo-Lumbreras R, Santos-Montes A, Cubedo-Fernández-Trapiella A, Izquierdo-Hornillos RC. 2008. Method development and validation for melamine and its derivatives in rice concentrates by liquid chromatography. Application to animal feed samples. Anal Bioanal Chem 392:523-531.

Murteanu D, Isfan A, Isfan C, Tincul I. 1987. High-performance liquid chromatographic separation of polyolefin antioxidants and light-stabilisers. Chromatographia 23:7-14.

Nakamura S, Takino M, Daishima S. 2000. Analysis of pressure sensitive adhesives by GC/MS and GC/AED with temperature programmable pyrolyzer. Anal Sci 16:627-631.

Nerín C, Salafranca J, Cacho J, Rubio C. 1995. Separation of polymer and on-line determination of several antioxidants and UV stabilizers by coupling size-exclusion and normal-phase high-performance liquid chromatography columns. J Chromatogr A 690:230-236.

Paik MJ, Choi Y, Kim KR. 2006. Simultaneous profiling analysis of alkylphenols and amines by gas chromatogrphy-mass spectrometry. Anal Chim Acta 560:218-226.

Paleólogos EK, Kontominas MG. 2005. Determination of acrylamide and methacrylamide by normal-phase high-performance liquid chromatography and UV detection. J Chromatogr A 1077:128-135.

Parkin JE, Boddy MR. 1998. Development of a chromatographic method of analysis for glucosyl-amines formed from dapsone. J Liq Chromatogr Relat Technol 21:2131-2142.

Paseiro-Losada P, Paz-Abuín S, Vázquez-Odériz L, Simal-Lozano J, Simal-Gándara J. 1991. Quality control of cured epoxy resins: determination of residual free monomers (m- xylylenediamine and bisphenol A diglycidyl ether) in finished product. J Chromatogr A 585:75-81.

Paseiro-Losada P, Simal-Gándara J, López-Mahia, P. 1992. Identification of fluorescamine-derivatized m-xylylenediamine RP-HPLC peaks by thermospray mass spectrometry. J Chromatogr Sci 30:267-270.

Paseiro-Losada P, Simal-Gándara J, Sanmartin-Fenollera P, Pérez-Lamela C, López-Fabal F. 1998. m-Xylylenediamine determination in the oficial EU aqueous food simulants. J Chromatogr Sci 36:554-560.

Paseiro-Losada P, Simal-Gándara J, Sanmartín-Fenollera P, Pérez-Lamela C. 1999. Determination of *m*-xylylenediamine in the official EU food simulant olive oil. Chromatographia 50:453-456.

Paseiro P, Simoneau C, Franz R (Editors). 2006. Compilation of analytical methods for model migrants in foodstuffs: Collection of methods descriptions. EUR22232 EN. European Communities.

Paz-Pino B, Pérez-Lamela C, Cancho-Grande B, Simal-Gándara J. 2003. HPLC method for determining ethylenediamine migration from epoxy-amine food packaging coatings into EU food simulants. Food Addit Contam 20:308-312.

Quinto-Fernández EJ, Pérez-Lamela C, Simal-Gándara J. 2003. Analytical methods for food-contact materials additives in olive oil simulant at sub-mg kg-1 level. Food Addit Contam 20:678-683.

Rafoth A, Gabriel S, Sacher F, Brauch H. 2007. Analysis of isothiazolinones in environmental waters by gas chromatography-mass spectrometry. J Chromatogr A 1164:74-81.

Rampfl M, Mair S, Mayer F, Sedlbauer K, Breuer K, Niessner R. 2008. Determination of primary, secondary and tertiary amines in air by direct or

diffusion sampling followed by determination with liquid chromatography and tandem mass spectrometry. Environ Sci Technol 42:5217-5222.

Saaid M, Saad B, Hashim NH, Ali ASM, Saleh MI. 2009. Determination of biogenic amines in selected Malaysian food. Food Chem 113:1356-1362.

Saarinen MT. 2002. Determination of biogenic amines as dansyl derivatives in intestinal digesta and feces by reversed-phase HPLC. Chromatographia 55: 297-300.

Saito K, Horie M, Nose N, Nakagomi K, Nakazawa H. 1992. Determination of polyamines in foods by liquid chromatography with on-column fluorescence derivatization. Anal Sci 8:675-680.

Salamone JC. 1996. Polymeric materials encyclopedia. In Ohkatsu Y, Stevens MP, Maiti SN. Additives (Agents sustaining properties; property and processing modifiers; types and applications) vol. 1 Boca Ratón (Florida): CCR Press, Inc. pp.108-130.

Sanches-Silva A, Sendón-García R, Cooper I, Franz R, Paseiro-Losada P. 2006. Compilation of analytical methods and guidelines for the determination of selected model migrants from plastic packaging. Trends Food Sci Technol 17:535-546.

SciFinder. 2007. American Chemical Society, SciFinder Scholar.

Shi Z, Zhang H, Zhao X. 2009. Ultrasonic-assisted precolumn derivatization-HPLC determination of acrylamide formed in Radix asparagi during heating process. J Pharm Biomed Anal 49:1045-1047.

Simal-Gándara J, López-Mahia P, Paseiro-Losada P, Simal-Lozano J, Paz-Abuín S. 1993. Overall migration and specific migration of bisphenol A diglycidyl ether monomer and m-xylylenediamine hardener from an optimized epoxy-amine formulation into water-based food simulants. Food Addit Contam 10:555-565.

Simal-Gándara J, Paz-Abuín S, Ahrné L. 1998. A critical review of the quality and safety of BADGE-based epoxy coatings for cans: implications for legislation on epoxy coatings for food contact. Crit Rev Food Sci Nutr 38:675-688.

Simal-Gándara J, Damant AP, Castle L. 2002. The use of LC-MS in studies of migration from food contact materials: a review of present applications and future possibilities. Crit RevAnal Chem 32:47-78.

Simon P, Lemacon C. 1987. Determination of aliphatic primary and secondary amines and polyamines in air by high-performance liquid chromatography. Anal Chem 59:480-484.

Spychaj T, Pilawka R, Spychaj S, Bartkowiak A. 2004. Tertiary alkanolamines as solvolytic agents for poly(ethylene terephthalate). Evaluation of the products as epoxy resin hardeners. Ind Eng Chem Res 43:862-874.

Spyropoulos DV. 1998. Stability testing of the plastics additives 2,4-dihydroxy-benzophenone, 2,2'-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-n-octyloxybenzophenone, 2-ethoxy-2-ethyloxanilide (tinuvin 312) and 2,2'-methylenebis(4-methyl-t-tert-butyl phenol) in aqueous and fatty food simulants. Food Addit Contam 15:362-369.

Stoffers NH, Brandl F, Linssen JPH, Franz R. 2003. Development and validation of analytical methods for monomeric and oligomeric migrants from nylon 12 packaging materials. Food Addit Contam 20:410–416.

Wang FC. 2000. Polymer additive analysis by pyrolysis-gas chromatography IV. Antioxidants. J Chromatogr A 891:325-336.

Watson DG, Xin LL, Midgley JM, Carr D. 1999. Analysis of a residual diamine in a pharmaceutical polymer using solid-phase extraction with analysis by gas chromatography-mass spectrometry. J Pharm Biomed Anal 19:917-921.

Wu T, Wang C, Wang X, Ma Q. 2008. Simultaneous determination of 21 preservatives in cosmetics by ultra-performance liquid chromatography. Int J Cosmet Sci 30:367-372.

Yan N, Zhou L, Zhu Z, Chen X. 2009. Determination of melamine in dairy products, fish feed, and fish by capillary zone electrophoresis with diode array detection. J Agric Food Chem 57:807-811.

You J, Zhang Y. 2002. Determination of biogenic amines by RPHPLC with fluorescent detection after derivatization with 2-(9-carbazole)ethylchloroformate (CEOC). Chromatographia 56:43-50.

Zhang N, Wang H, Guo XF, Zhang HS. 2008a. Determination of primary biogenic amines in various food products using isocratic HPLC with 3-(4-chlorobenzoyl)-quinoline-2-carboxaldehyde as a new precolumn fluorogenic reagent. J Sep Sci 31:2804-2812.

Zhang N, Zhao YZ, Zhang HS, Wang H. 2008b. Sensitive determination of aliphatic amines by high-performance liquid chromatography with a new fluorogenic probe 3-(4-fluorinebenzoyl)-2-quinoline carboxaldehyde. J Sep Sci 31:38-46.

Zhang T, Watson DG, Lu D, Carr D, Trager L. 2008. Determination of cross-linking residues in a pharmaceutical polymer by liquid chromatography-high resolution full- scan mass spectrometry. Talanta 76:509-512.

.

III-ANALYTICAL METHOD FOR THE SIMULTANEOUS DETERMINATION OF POLYFUNCTIONAL AMINES USED AS MONOMERS IN THE MANUFACTURE OF FOOD PACKAGING MATERIALS

Published at: Journal of Chromatography A Volume 1218, Issue 40, Pages 7105–71097, October 2011,

Presented as a poster at:

IFT annual meeting and food expo®.Chicago IL, USA. July 17-20, 2010

7th Aegean Analytical Chemistry Days (AACD2010) September 29-October 3, 2010.

Abstract

This paper describes the development of a multi-analyte method for the determination of polyfunctional amines commonly used as monomers in the manufacture of food contact materials. Amines were analyzed by high-performance-liquid chromatography with diode-array detection (HPLC-DAD) after derivatization with dansyl chloride. The chromatographic analysis and the derivatization conditions were optimized. The proposed method was validated in terms of linearity, limits of detection and repeatabilities. The method showed an excellent sensitivity (LOD ≤ 0.05μg/mL) and appropriate repeatabilites (R.S.D. (n=7) ≤ 5%)). LC-MS/MS was used as a confirmatory technique.

The stability of the amines in five food simulants (distilled water, 3% acetic acid, 10% ethanol, 50% ethanol and olive oil) under the most common testing conditions (10 days at 40 ºC) was also studied. Results showed that amines had an acceptable stability in aqueous simulants but in the olive oil a loss of 100% was observed for all analytes.

Keywords: polyfunctional amines, multi-analyte method, food packaging materials, food simulants, stability.

III.1. Introduction

The Regulation EU No 10/2011 [1] establishes the monomers, starting substances and additives that are authorized in the manufacture of materials intended to come into contact with foodstuffs as well as the restrictions which they are subject such as global and specific migration limits (SML). Among the substances included in the positive list, polyfunctional amines are a great group of substances widely employed in the production of food packaging materials either as monomers or additives. Several reviews about analytical methods to determine these compounds have been reported in the literature [2-5]. Low molecular substances such as additives, residual monomers and oligomers can migrate from the material into the food and constitute a risk for the health of the consumers. To verify the migration as well as to evaluate the stability of these compounds in the foodstuffs accurate and sensitive methods are necessary. Due to the complexity of food samples the legislation (EU Council Directive 85/572/EEC) [6] allows to carry out the migration tests using food simulants instead of real samples with the aim to simplify the analysis.

Since the amines are potential migrants the toxicity of these compounds should be taken into account. The SML are established based on toxicological studies, so that if the migration of the target analytes occurs to a large extent, at levels higher than the SML, could be a potential danger for the consumers' health [7].

From the chemical point of view the analysis of aliphatic amines is not a simple task since these compounds do not absorb in the ultraviolet region nor they have suitable fluorescence properties. They did not determine directly by gas chromatography. The derivatization is the common approach in order to overcome this drawback. Several derivatizing agents have been employed;

among them *o*-phthaldehyde (OPA) [8] dansyl chloride (Dns-Cl) [9-13] and fluorescein isothiocyanate (FITC) [14] are some of the most commonly used.

Compared with OPA, the use of Dns-cl presents some advantages, this reagent allows derivatize not only primary amines but also secondary amines, in addition the dansyl derivatives could be detected either by UV-Vis or fluorescence. Besides, as it has been previously reported dansyl derivatives are more stable than those obtained with OPA [9, 15, 16].

The analytical techniques commonly used to determine polyfunctional amines used in the fabrication of food contact materials include liquid and gas chromatography; currently, the majority of the methods are single-component being very scarce the procedures that analyse simultaneously several amines [17, 18]. In addition, due to the different chemical structures and physicochemical properties there is not a standard method to analyze these compounds.

This paper describes the first attempt to develop a multi-analyte method to determine polyfunctional amines used as monomers in the manufacture of food packaging materials. For this purpose amines used commonly as curing agents, alcohol amines and aliphatic amines were selected. A suitable separation was achieved on a reversed ODS (C18) (150 x 3.20 mm, 5μm) stationary phase after a derivatization with dansyl chloride. The linearity, sensitivity and repeatability of the proposed method were also studied. In addition, the behaviour of the amines in food simulants under the most common testing conditions (10 days at 40ºC) was investigated by means of the test of stability.

LC–MS/MS using atmospheric pressure ionisation (APCI) in the positive ion mode was applied to confirm the results.

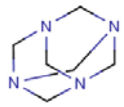
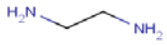
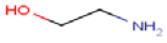
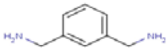
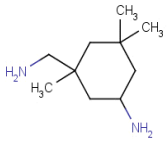
III.2. Materials and methods

III.2.1. Chemicals and analytical standards

Amine standards were supplied as follows: hexamethylenetetramine (99%) (HTM), ethylenediamine (≥99%) (EDA), 1,4- diaminobutane (99%) (1,4-DAB), *m*-xylylenediamine (99%) (MXDA), hexamethylenediamine (98%) (HMDA), and isophoronediamine (≥ 99%) (IPDA) by Aldrich (Steinheim, Germany) and ethanolamine (98%) (EA) by Merck (Schuchardt). The chemical structures and SML of the amines are summarized in Table 1.

Stock standard solutions of polyfunctional amines at a concentration of 1000 mg/L were prepared in 0.1 M HCl and stored at 4 ºC in the dark. Working standard solutions were prepared by dilution of stock solutions.

All chemicals were of analytical grade. Methanol, acetone, hydrochloric acid tetrahydrofuran and n-hepatane were from Merck (Darmstadt, Germany); sodium bicarbonate from Vorquímica (Vigo, Spain) and dansyl chloride (Dns-cl) from Fluka (Steinheim, Germany). Water used for all solutions was obtained from Milli-Q water purification system (Millipore) (Bedford, MA, USA). The olive oil used was purchased in a local supermarket.

Structure	Amines	CAS No	SML (mg/kg)
	Hexamethylenetetramine (HTM)	000100-97-0	15
	Ethylenediamine (EDA)	000107-15-3	12
	1,4-Diaminobutane (1,4-DAB)	000110-60-1	60
	Hexamethylenediamine (HMDA)	000124-09-4	2,4
	Ethanolamine (EA)	000141-43-5	0,05
	*m-X*ylylenediamine (MXDA)	001477-55-0	0,05
	Isophoronediamine (IPDA)	002855-13-2	6

SML: Specific Migration Limit

Table 1.- Chemical structures and specific migration limits of the selected amines.

III.2.2. Chromatography

III.2.2.1. HPLC-UV analysis

The HPLC HP1100 system (Hewlett-Packard, Waldbronn, Germany) consisted of a quaternary pump, a degassing device, an autosampler, a column thermostatting system, a diode-array detector (DAD), a fluorescence detector and Agilent Chem-Station for LC and LC/MS systems software. Operating conditions were as follows: the chromatographic separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm, 5 μm) thermostatted at 25 ºC. The mobile phases consisted of (A) methanol and (B) water. The injection volume was 20 μL. EDA; 1,4-DAB; MXDA; HMDA and IPDA were detected at 254 nm and HTM and EA were detected at 246 nm. Amines were identified by comparing their retention times and UV spectra with those of pure standards.

III.2.2.2. HPLC-MS analysis

The HPLC–MS/MS system comprised an Agilent 1200 liquid chromatograph fitted with a degasser, a quaternary pump, an autosampler and a column oven controlled by Chemstation (version B.01.03, Agilent Technologies, Palo Alto, CA, USA), all coupled to an Agilent 6330 Ion Trap mass detector controlled by Ion Trap LC/MS (version 6.1, Bruker Daltonic GmbH) software.

MS data were acquired in the positive ion atmospheric pressure chemical ionization (APCI) mode. Mass spectra were monitored in the mass range *m/z* 50–650 and 100-1100. Nitrogen was used as nebulizer and dry gas; the collision gas for ion fragmentation in MS/MS was helium. Optimized MS/MS detector settings were: dry temperature 350 ºC, vaporizer temperature 250 ºC, nebulizer

gas 20 psi, dry gas at 5.0 L/min, capillary voltage was -2000 V, corona 4 μA, end plate offset -500 V, skimmer 40 V, capillary exit 136 V, octopole 1 DC 12 V, octopole 2 DC 1.7 V, octopole RF 200 Vpp, lens 1 -5 V, lens 2 -60 V, trap drive 59.9.4

The column used was a Luna C18 (2) HST (Phenomenex 50 mm x 3 mm, i.d. 2.5 μm particle size from Phenomenex) and the mobile phases were the same as in the HPLC–UV system, adjusting the gradient to 22 minutes at a flow rate of 0.3 mL/min. The injection volume was 2 μL.

III.2.3. Derivatization procedure

The derivatization was conducted by adding to 1 mL of the standard solution containing the amines, 1 mL of the dansyl-chloride solution (5 mg/mL prepared in acetone), the pH of the solution was adjusted to 9.7 with $NaHCO_3$. The solution was mixed in a vortex and incubated 60 min at 80 ºC. Prior HPLC analysis the solution was filtered through 0.50 μm PTFE membrane filter (Advanted, Toyo Roshi Kaisha, Ltd. Japan).

III.2.4. Quantification

Quantification was performed on the basis of linear calibration plots of peak area against concentration. Calibration lines were constructed based on five concentration levels of standard solutions. The concentration levels used to construct the calibration curves were 0.5; 1; 5; 10; 20 mg/L for HTM and 1,4-DAB; 0,5; 1; 2,5; 5; 10 mg/L for EDA, HMDA and IPDA and 0,05; 0,5; 1; 5; 10 mg/L for EA and MXDA. Each point of the calibration curve is the average of three peak-area measurements.

III.2.5. Stability test

The stability of the amines was evaluated in five food simulants: distilled water, 3% (w/v) acetic acid, 10% (v/v) ethanol; 50% (v/v) ethanol and olive oil as fatty food simulant. The test conditions selected were 10 days at 40 ºC. To perform the stability study 20 mL headspace vials containing the food simulant were spiked with known amounts of amines (5 mg/L) and then were subject to the testing conditions. For the aqueous simulants a standard solution of amines prepared in 0.1 M HCl was used to spike the simulants. In the case of the olive oil, 1 mL of the standard solution was evaporated under a nitrogen stream and the residue was re-dissolved in a known volume of tetrahytdrofuran. This solution was used to spike the simulant. After the exposure time, the aqueous simulants were analyzed by HPLC directly after derivatization. In the fatty food simulant the amines were extracted from the olive oil by using a mixture of 0.1 M HCl-*n*-heptane (2:1 v/v) prior to derivatization. Three replicates were performed for each simulant.

III.3. Results and Discussion

III.3.1. Optimization of derivatization conditions

As it has been pointed out earlier the lack of a suitable chromophore or fluorophore group makes difficult the direct analysis of aliphatic amines. The derivatization is the common procedure to improve the sensitivity. This process is affected by many factors, such as time, temperature and pH among others. The derivatization conditions were optimized with regard to reaction time and temperature, concentration of the derivatization agent and pH. The assays were

carried out by using standard solutions at a concentration level of 5 and 10 mg/L. The effect of the temperature on the derivatization reaction was evaluated in the range of 40-80 ºC. It was observed that higher temperatures led to largest peak-areas. In the same assay the reaction time was also investigated; 30, 60, 90 and 120 min were tried. After 60 min of reaction time highest peak-areas were obtained for all amines except for HTM and EA. For these two last amines 90 min were needed to complete the derivatization reaction. Since the differences observed for HTM and EA were not significant and the rest of amines showed the highest response after 60 min of reaction, 80 ºC and 60 min was selected as the best option for the simultaneous determination of the amines. Two different concentrations of dns-cl were tested (5 and 20 mg/mL). No significant differences were observed; therefore 5 mg/mL was chosen as suitable concentration to produce quantitative results. Some authors have suggested that it is important to remove the excess of the derivatization reagent to avoid interferences [9, 11]. The addition of ammonia to neutralize the excess of Dns-cl was also studied. Contrary to expected an interfering peak appeared in the middle of the chromatogram when ammonia was added, and consequently it was not used in successive analyses.

It is well known that pH plays a key role in the efficiency of the derivatization reaction [19-22]. To guarantee a complete derivatization the reaction should be conducted under basic conditions. The influence of this factor was investigated in the pH range 8-10. All amines exhibited the maximum response at pH 9.7 except EDA which showed the highest intensity at pH 8.6. A pH of 9.7 was selected as the best compromise for the analysis of all amines in a single-run. Under the optimized conditions the derivatization reaction yield was above 95%.

III.3.2. Optimization of the chromatographic conditions and performance characteristics

In developing the chromatographic method, several mobile phases initial proportions (A, methanol; B, water) were assayed: (A-B 30:70 v/v); (A-B 60:40 v/v) and (A-B 50:50 v/v). A suitable separation was achieved starting the mobile phase gradient with methanol-water (50:50 v/v).

On basis of UV maximal absorption of the dansyl derivatives the detection at 254 nm was selected for EDA; 1,4-DAB; MXDA; HMDA and IPDA and at 246 nm for HTM and EA.

Under the optimized conditions a suitable separation of the amines was achieved. A chromatogram of a standard mixture of amines (10 mg/L) is presented in Figure 1. As can be observed the IPDA occurs as cis/trans isomers with a cis/trans isomer ratio of 75/25.

The linearity of the method was tested by using a series of amine standards solutions of known concentration. The calibration curves were constructed using five concentration levels and they were fitted to a linear equation. Parameters of linearity are presented in Table 2. All amines studied showed a good linearity, determination coefficients were in all cases greater than 0.985. The limits of detection, (defined as signal three times the height of the noise level) were calculated according American Chemical Society ACS guidelines [23] (Table 2). The proposed method exhibited an excellent sensitivity with limits of detection lower than or equal to 0.05 μg/mL. Dugo et al. [10] have reported similar detection limit for 1,4-DAB whereas Latorre-Moratalla et al. [24] have reported values slightly higher.

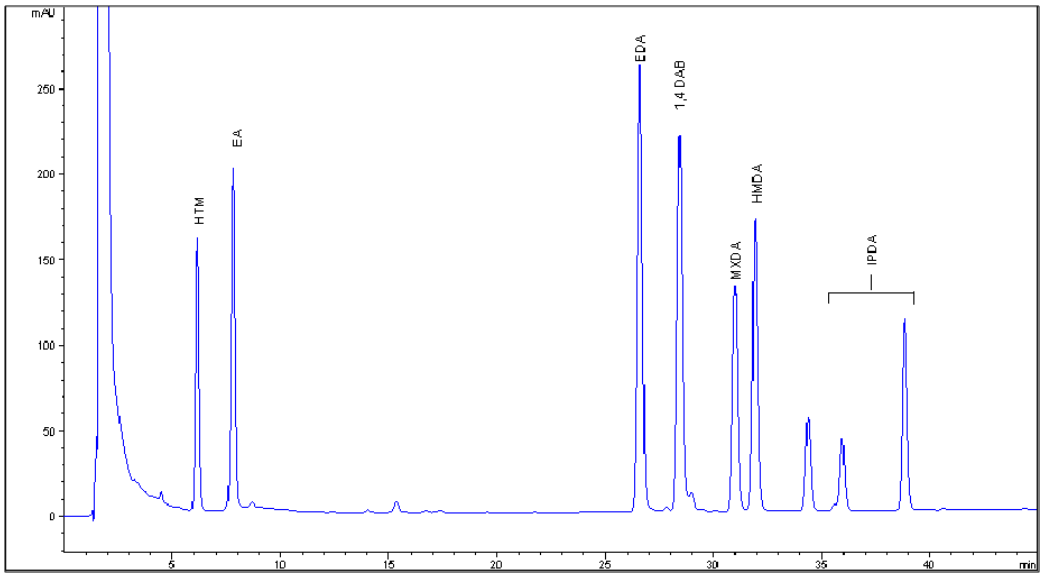


Figure 1.- HPLC-Chromatogram of polyfunctional amines acquired under the following analytical conditions: The separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm, 5 µm). A gradient consisting of methanol (A) and water (B) at a flow rate of 0.5 mL/min was used. The HPLC elution program started with 50% of each mobile phase and gradually increased to 90% methanol and 10% water within 45 min.

This method allows detection of the amines at levels below the specific migration limits (Directive 2002/72/EC) and could be used for the routine analysis of amines in control laboratories.

Repeatabilities, expressed by means of the percentage of R.S.D. (R.S.D. % (n=7)), was determined by analysing seven replicates of a standard solution of amines at a concentration level of 5 mg/L (Table 2). For the amines studied the repeatabilities were lower than 5% except for HTM.

Amines	Range of linearity (mg/L)	Equation	R^2	LOD (mg/L)	% Repeatability (R.S.D. (n= 7))
HMTA	0.5-20	y = 264.7x + 45.11	0.9850	0.025	5.18
EA	0.05-10	y = 177.1x + 1.436	0.9994	0.025	3.55
EDA	0.5-10	y = 337.6x - 85.35	0.9996	0.025	2.86
1,4-DAB	0.5-20	y = 318.6x - 28.02	0.9988	0.025	2.78
MXDA	0.05-10	y = 108.6x + 2.037	0.9870	0.025	2.38
HMDA	0.5-10	y = 173.7x + 9.522	0.9991	0.025	2.56
IPDA	0.5-10	y = 115.8x + 1.623	0.9985	0.050	2.90

LOD: Limit of detection

Table 2.- Parameters of linearity and repeatabilities of the selected amines.

III.3.3. Study of the stability of amines in food simulants

The stability study was conducted under the most common testing conditions, 10 days at 40 ºC. The percentage of recovery of each amine in the five simulants considered in this study is presented in Table 3. In aqueous food simulants the amines showed an acceptable stability with recovery values ranging from 69.6% to 131.0% except for HTM in 3% acetic acid that presented a recovery around 57.3%. Regarding the fatty food simulant, the amines were unstable in olive oil after 10 days at 40ºC. Under these conditions a loss of 100% was observed for all amines. These results suggest that amines present a great reactivity in mediums with a fatty nature.

Food Simulant	HTM	EA	EDA	1,4DAB	MXDA	HMDA	IPDA
Distilled water	107.7±4.45	96.6±1.82	104.5±4.30	89.9±3.96	91.8±3.70	94.0±2.94	96.9±1.95
3% Acetic acid	57.3±6.13	80.3±7.05	117.7±12.70	116.8±4.09	131.0±10.83	127.6±6.21	125.5±11.99
10% EtOH	97.9±2.85	96.1±3.82	98.3±3.88	88.1±1.56	90.2±0.75	95.0±1.97	97.8±0.51
50% EtOH	85.8±2.99	86.6±1.67	70.1±0.25	75.1±2.25	69.6±1.49	84.8±2.33	77.3±1.89
Olive oil	0	0	0	0	0	0	0

Recoveries (mean (%) ± S.D. (n=3)) were estimated on the basis of determination after spiking the food simulants with known amounts of amines.

Table 3. - Stability test: Recovery values of the amines in the food simulants.

Future work will be addressed to identify the reaction products which can migrate into food and may be a potential hazard for the consumers' health.

III.3.4. HPLC-MS/MS analysis

Peaks detected by UV were identified by LC MS (APCI+) in scan mode as corresponding to the disubstituted derivatives for EDA, 1,4-DAB, HMDA, MXDA, IPDA and the monosubstituted derivative for EA. In the case of HTM the main ion detected was m/z 251, which corresponds to the Delépine reaction product of HTM with Dns-cl (dansylamide). Several authors have observed that under acidic conditions HTM breaks down to ammonia and formaldehyde [25, 26]. This explains the formation of the dansylated derivative of ammonia. Once the precursor ions were selected, MS2 mode was used to identify the main product ions (Table 4). At least two product ions could be detected for each amine, which could be used for quantification and confirmation purposes. For HTM, the main product ion (251>234) corresponded to the lost of ammonia; EA lost the ethanol group (295>252); MXDA (bidansylated) yielded the $[M+H-C_{12}H_{14}N_2O_2S]^+$ ion (603>353), resulting from the loss of a dansylamide group; EDA main transition (527 >464) is the result of the loss of a dansyl group; 1,4-DAB breaks down releasing a dansylamide group (555>304); HMDA main transition was 583>519, but also others could be detected corresponding to the loss of a dansyl group (583>349) and a dansyl amide group (583>332). For IPDA, the $[M+H- C_{12}H_{14}N_2O_2S]^+$ ion (637>387) was dominant and it was due to the loss of a dansyl amide group.

Amine	Retention time (min)	SCAN m/z (APCI+)	Dansylated reaction product	MS(2) m/z
hexamethylenetetramine	4.3	251	dansylamide	251>234 251>170
ethylenediamine	18.1	527	bidansylated	527 >463 527 >234 527>276 527>293
1,4-diaminebutane	19	555	bidansylated	555>304. 555>321
m-xylylenediamine	20.2	603	bidansylated (+++)	603>353 603>523 603>168
	11.2	369	monodansylated	369>170 369>234 369>288
hexamethylenediamine	20.6	583	bidansylated	583>519 583>332 583>320 583>349
isophoronediamine	22.6, 23.8	637	bidansylated	637>387 637>339 637>554
aminoethanol	6	295	monodansylated	295>252 295>280

Table 4.- LC MS (APCI+) experimental data.

III.4. Conclusion

Briefly, a multi-analyte method for the determination of polyfunctional amines used as monomers in the manufacture of food packaging materials is described for the first time. The proposed method is sensitive and precise and could be

used as an excellent analytical tool for the routine determination of amines in control laboratories.

Under the testing conditions (10 days, 40ºC) amines showed an acceptable stability in aqueous food simulants, however in olive oil a loss of 100% was observed for all analytes. This indicates that amines react with components present in fatty mediums. The migration of the reaction products formed into food may constitute a risk to consumer health. Future work will be devoted to the identification, determination and toxicity of these products.

Acknowledgements

The study was financially supported by the Ministerio de Ciencia e Innovación, ref. Nº AGL/2008-04146 "MIGRAMIN". Authors are grateful to "Ministerio de Ciencia e Innovación" for the Predoctoral fellowship FPI (ref. BES-2009-023016) awarded to Rafael Paseiro-Cerrato. The authors are also grateful to C. Casal, P. Blanco and G. Hermelo for their excellent technical assistance.

References

[1] Official Journal of the European Communities, L12 1-89, Commission Regulation (EU) No 10/2011 of 14 January 2011.

[2] R. Paseiro-Cerrato, A. Rodríguez-Bernaldo de Quirós, R. Sendón, J. Bustos, M.I. Santillana, J.M. Cruz, P. Paseiro-Losada, Compr. Rev. Food. Sci. Food Saf. 9 (2010) 676.

[3] A. Önal, Food Chem. 103 (2007) 1475.

[4] M. Careri, F. Bianchi, C. Corradini, J. Chromatogr. A 970 (2002) 3.

[5] J. Simal-Gándara, A.P. Damant, L. Castle, Crit. Rev. Anal. Chem. 32 (2002) 47.

[6] Official Journal of the European Communities, L372, 14-21, Council Directive (85/572/EEC) of 19 December 1985.

[7] EFSA Note for guidance for petitioners presenting an application for the safety assessment of a substance to be used in food contact materials prior to its authorisation. Updated on 30/07/2008.

[8] K. Saito, M. Horie, N. Nose, K. Nakagomi, H. Nakazawa, Anal. Sci. 8 (1992) 675.

[9] N. Innocente, M. Biasutti, M. Padovese, S. Moret, Food Chem. 101 (2007) 1285.

[10] G. Dugo, F. Vilasi, G.L. La Torre, T.M. Pellicanò, Food Chem. 95 (2006) 672.

[11] M.T. Saarinen, Chromatographia 55 (2002) 297.

[12] E.H. Soufleros, E. Bouloumpasi, A. Zotou, Z. Loukou, Food Chem. 101 (2007) 704.

[13] Y.M. Lu, X.H. Chen, M. Jiang, X. Lv, N. Rahman, M.S. Dong, Y. Gujun, Food Control 20 (2009) 593.

[14] L.M.L. Nollet, Food Analysis by HPLC, Marcel Dekker, Inc., Basel, 2nd ed., 2000.

[15] M. Saaid, B. Saad, N.H. Hashim, A. Salhin, M. Ali, M.I. Saleh, Food Chem. 113 (2009) 1356.

[16] S. Bomke, B. Seiwert, L. Dudek, S. Effkemann, U. Karst, Anal. Bioanal. Chem. 393 (2009) 247.

[17] P. Paseiro-Losada, J. Simal-Gándara, P. Sanmartín-Fenollera, C. Pérez-Lamela, Chromatographia 50 (1999) 453.

[18] B. Paz-Pino, C. Pérez-Lamela, B. Cancho-Grande, J. Simal-Gándara, Food Addit Contam. 20 (2003) 308.

[19] H. Mao, B. Chen, X. Qian, Z. Liu, Microchem. J. 91(2009) 176.

[20] Y.M. Lu, X. Lu, X.H. Chen, M. Jiang, C. Li, M.S. Dong, Food Chem.100 (2007) 1424.

[21] C. Molins-Legua, P. Camping-Falcó, A. Sevillano-Cabeza, M. Pedrón-Pons, Analyst 124 (1999) 477.

[22] S. Moret, L.S. Conte, J. Chromatogr. A 729 (1996) 363.

[23] American Chemical Society (ACS), Subcommittee of Environmental Analytical Chemistry, Anal. Chem. 52 (1980) 2242.

[24] M.L. Latorre-Moratalla, J. Bosch-Fusté, T. Lavizzari, S. Bover-Cid, M.T. Veciana-Nogués, M.C. Vidal-Carou, J. Chromatogr. A 1216 (2009) 7715.

[25] I. Bodik, J. Derco, M. Drtil, Environ. Biotechnol. 42 (1991) 71.

[26] K.O. Colquhoun, Water Sci. Technol. 30 (1994) 95.

IV-ANALYSIS OF HISTAMINE AS INDICATOR OF SHELF LIFE IN SEAFOOD

Publised in: Italian Journal of food science, Special issue, 51-54, 2011

Presented as a poster at: Shelf Life International Meeting. Zaragoza, Spain. June 23-25, 2010

Abstract

In the present study, a reversed high performance liquid chromatographic method with diode array detection was developed to analyze histamine in sea food samples. The separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm, 5μm) column thermostated at 25ºC and using a gradient of Milli-Q water and methanol as mobile phase. The biogenic amine was extracted from seafood samples by using 0.1 N HCl and derivatized with dansyl chloride. Histamine contents ranged from 1.0 to 2.5 mg/kg.

Keywords: Chromatographic analysis, derivatization, food deterioration, histamine, shelf-life.

IV.1. Introduction

Histamine is a biogenic amine resulting of the enzymatic decarboxylation of the amino acid histidine. It is considered an indicator of deterioration in foods and frequently used as a biomarker for food quality control. The European regulation (CE) No 1441/2007 establishes a caution level of 100-200 mg/kg in seafood.

Several undesirable effects on the human health such as, hypotension, nausea, headache, rash, cardiac palpitation, emesis and even intracerebral anaphylactic shock, haemorrhage and death in very severe cases caused by high levels of histamine have been reported in the literature (Chiacchierini et al. 2006; Innocente et al. 2007).

Numerous analytical procedures have been developed for the determination of histamine including, enzyme-linked-immunoassays (ELISA), colorimetric methods and chromatographic methods. Among them, high performance liquid chromatography appears as the most suitable technique to analyze the biogenic amine. Due to these compounds do not have a suitable fluorophore or chromophore group a derivatization is essential in order to enhance the sensitivity. Several derivatizing agents have been employed; the most commonly used are *o*-phthaldialdehyde, dansyl chloride and fluorescamine (Dugo et al 2006; Saito et al. 1992).

The aim of this paper is to determine the histamine content in sea food samples by high-performance liquid chromatographic with diode array detection after derivatization with dansyl chloride.

IV.2. Materials and Methods

IV.2.1. Reagents and standard solutions

All reagents were of analytical grade. Methanol, acetone and hydrochloric acid were from Merck (Darmstadt, Germany); sodium bicarbonate from Vorquímica (Vigo, Spain) and dansyl chloride (Dns-cl) from Fluka (Steinheim, Germany). Water used for all solutions was obtained from a Milli-Q water purification system (Millipore; Bedford, MA). Standard of histamine was purchased from Aldrich. Stock standard solution of histamine was prepared in 0.1 N HCl and stored at 4 ºC in the darkness. Working solutions were prepared by dilution.

IV.2.2. Instrumentation

HPLC-analysis were performed on a HP1100 system (Hewlett–Packard Waldbronn, Germany) equipped with quaternary pump, a degassing device, an autosampler, a column thermostatting system, a diode-array detector (DAD), a fluorescence detector and Agilent Chem-Station for LC and LC/MS systems software.

IV.2.3. Samples and extraction procedure

Surimi was selected as representative seafood and was purchased in a local supermarket. Histamine was extracted as follow; 25 mL of 0.1 N HCl were added to 5 g of sample, the mixture was homogenized by magnetic stirring for 10 min. The supernatant was removed and the residue was re-extracted with 25 mL of 0.1 N HCl. The supernatants were combined and made up to 50 mL. The

solution was stored at 4 ºC overnight in order to precipitate lipids and proteins. Then, an aliquot of the solution was filtered and derivatized. The dansylated derivative of histamine was formed by adding to 1 mL of sample 1 mL of dansyl chloride solution (5 mg/mL) and 300 μL of saturated $NaHCO_3$ solution; then the mixture was incubated at 80ºC 60 min. Detailed information regarding derivatization procedure is reported in a paper submitted for publication.

IV.2.4. Chromatographic conditions

The chromatographic separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm, 5μm) column thermostated at 25ºC.The mobile phases consisted of A (Milli-Q water) and B (Methanol). The gradient elution program was as follows: 0 min (50% A /50% B); 45 min (10% A / 90% B) 50 min (50% A / 50% B). The flow rate was 0.8 mL/ min and the injection volume 20 μL. The diode array detector was set at 254 nm.

IV.3. Results and Conclusions

Since histamine has neither suitable absorption in the UV-Vis region nor fluorescence characteristics a derivatization step is essential in order to improve the sensitivity. According to the literature dansyl chloride is one of the most suitable derivatizing agents for biogenic amines. A method that involves the derivatization of amine with dansyl chloride followed by a reversed phase high performance liquid chromatography with detection at 254 nm was used to analyze the histamine content in surimi samples.

A typical chromatogram of the dansylated derivative of the amine is shown in Figure 1.

Identification of histamine was made by comparison of the retention time and UV spectra with that of pure standard. A spectrum of the dansylated derivative is presented in Figure 2.

Quantification was carried out with the external standard method. Calibration line was constructed based on four concentration levels of standard solutions within 1–23 mg/L range and it was fitted to a linear equation $y = 132.67\ x - 45.102$ (R^2 0.9995).

The recovery of the method was estimated on the basis of determination after spiking the samples with known amounts of histamine (10 mg/L). Satisfactory recoveries were achieved (102.8 ± 2.8%). Once the analytical conditions were established the method was applied to determine histamine in surimi samples. The concentrations found in the samples analyzed ranged from 1.0 to 2.5 mg/kg.

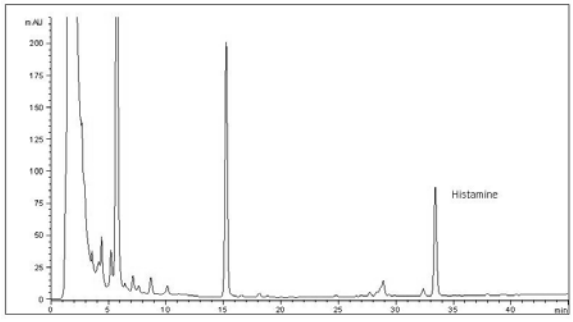


Fig 1. HPLC chromatogram of a surimi sample.

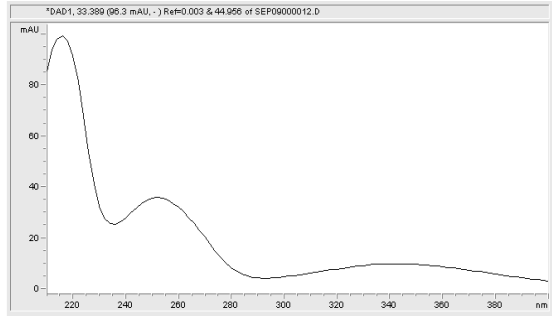


Fig 2. UV spectra of the dansylated derivative.

Acknowledgements

The study was financially supported by the Ministerio de Ciencia e Innovación, ref. Nº AGL/2008-04146 "MIGRAMIN". Authors are grateful to "Ministerio de Ciencia e Innovación" for the Predoctoral fellowship FPI (ref. BES-2009-023016) awarded to Rafael Paseiro-Cerrato.

References

Chiacchierini, E., Restuccia, D. and Vinci, G. 2006. Evaluation of two different extraction methods for chromatographic determination of bioactive amines in tomato products. Talanta 69: 548.

Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

Dugo. G., Vilasi F., La Torre G.L. and Pellicanò T.M. 2006. Reverse phase HPLC/DAD determination of biogenic amines as dansyl derivatives in experimental red wines. Food Chem. 95:672.

Innocente N., Biasutti M., Padovese M. and Moret S. 2007. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. Food Chem. 101:1285.

Saito, K., Horie, M., Nose, N., Nakagomi, K. and Nakazawa, H. 1992. Determination of polyamines in foods by liquid chromatography with on-column fluorescence derivatization. Anal Sci. 8:675.

V-DEVELOPMENT OF A RAPID SCREENING METHOD TO DETERMINE PRIMARY AROMATIC AMINES (PAAS) IN KITCHEN UTENSILS USING DIRECT AN
ALYSIS IN REAL TIME.

Abstract

Primary Aromatic Amines (PAAs) are a group of substances with undesirable health effects. Several studies have been recently published showing that migration of PAAs from some kitchen utensils into acetic acid 3% (w/v) exceeded the specific migration limit (SML) established by the European Union regulations. Numerous analytical procedures have been developed for their determination; nevertheless, all needs previously samples treatment. Direct Analysis in Real Time (DART), is a new technique that has been successfully applied as screening method to determine substances in food contact materials. The aim of this work is to determine if DART-MS could be utilized as a screening method for PAAs determination in kitchen utensils. DART analysis results have been compared with UPLC/MS/MS method which has been developed and validated in terms of linearity, repeatability, recovery, LOD and LOQ.

Keywords: Primary aromatic amines, Direct Analysis in Real Time (DART), Screening method UPLC/MS/MS, Kitchen utensils.

V.1. Introduction

Primary aromatic amines (PAAs) are a group of substances very reactive that may be susceptible of produce cancer, allergy, genotoxic and toxically effects even at low concentration. Due to the high reactivity of PAAs, they are commonly used as starting substances in the production of pesticides, rubbers, textiles, pharmaceutical, explosives, cosmetics, polyurethanes and azo-dyes among others (Mortensen et al. 2005, McCall et al. 2012). Polyurethanes are frequently used in multilayer's films as adhesives. In some cases, if the curing process is not complete, isocyanates can not totally react and consequently yield PAAs in contact with water, being and hazard for the human health. On the other hand, because of their low cost and their broad colour spectrum, PAAs are widely used in the production of azo-dyes which are used in several materials, such as toys, medical devices, and clothing among others.

In 2004 Brede et al. identified aniline (ANL) migration as contaminant in polyamide cooking utensils. Afterwards, in 2005, Mortensen et al. published a paper where PAAs migration from black nylon kitchen utensils (KU), especially ANL and 4,4'-Methylenedianiline (MDA), exceed the permitted limit laid down by the UE legislation. After this, several alerts have been issued on the Rapid Alert system for Food and Feed (RASFF) notifying high levels of the PAAs which migrated from Food Contact Materials (FCM), especially those came from China. The UE issued in the repealed Commission Directive (EU) No 2002/72/EC that plastic materials or articles should not release PAAs into food in detectable levels, being 10 µg/Kg the maximum amount of PAAs that can migrate into food or food simulant. Following the same legislation frame, articles intended for repeated uses, should be in compliance with the legislation in the third migration test. However, in the new Commission Regulation (EU) No

10/2011 of 14 January 2011, is set that PAAs yielded from polyamide kitchenware should be compliant in the first migration test, this regulation will be apply from the 01/01/2013. Furthermore, due to most of samples came from China, in the Commission Regulation (EU) No 284/2011 of 22 March 2011 has been establish that articles came from China should be compliant with UE legislation and a declaration confirming that polyamide and melamine plastic kitchenware comply with UE PAAs requirements should be presented.

The techniques commonly used in the PAAs determination are liquid and gas chromatography coupled to mass detectors. Many papers have been published in the literature to analyse PAAs. In order to determine aniline in Polyamide 66, three different techniques have been applied by Brede et al. (2003) including HPLC-UV, GC-MS and spectrophotometric methods. For GC-MS PAAs determination solid phase analytical derivatization with trifluoroacetic anhydride was used. GC/MS was also used by Kawakami et al. (2010) to determine PAAs in textile products. PAAs may be also determined by capillary electrophoresis coupled with transient isotachophoretic stacking (Wang et al. 2009). Zhao et al. (2008) quantified PAAs using a precolumn fluorescent derivatization by HPLC-FDL. The system was coupled to a MS detector for online identification Garrigos et al. (1998) employed supercritical fluid extraction and gas chromatography with flame ion ionization to determine 4-Cl-o-toluidine, 4-aminobiphenyl (4-AB) and 2-naphtylamine (Naph) in finger-paints for children. In order to determine banned aromatic amines including ANL, benzidine (BZ), 4,4'oxydianiline (OXN) among others, García-Lavandeira et al. (2010), used LC coupled to a tandem mass spectrometry(MS). On the other hand, Schubert et al.(2011) employed LC-ESI/MS to analyze PAAs in mainstream water pipe smoke. In a recent article, McCall et al. (2012) have successfully applied UPLC-ESI to determine PAAs black nylon kitchen utensils.

All the methods presented above, required a previous sample treatment, causing long and expensive methods. Direct Analysis in Real Time (DART) is a new technique developed in 2003 and published in 2005 for Cody et al. (2005) and commercialized by JEOL. This technique consists of metastable gas plasma that makes contact with the liquids, gas or solid samples and desorbs the compound of interest from the samples. The substances suffer a chemical ionization and consequently are able to be analyzed by a mass detector. So this method allows direct detection of compounds without sample preparation. In the last years, different samples including, pharmaceuticals, textiles, abuse drugs and FCM among others, have been successfully analyzed. This technique allows to develop rapid, not time-consuming, cheap and environmentally friendly methods to analyze substances directly in the samples. In the best of our knowledge, DART has not yet been used for PAAs determination in FCM. To carry out DART analysis, only little sample is required. DART can be coupled to different detectors including, orbitrapMS (Vaclavik et al. 2010, Self et al. 2012) and TOFMS (Cajka et al. 2008, and Vaclavik et al. 2009) among others. Furthermore, DART has been successfully applied as screening method (Ackerman et al. 2009, Edison et al. 2011 and Pfaff et al. 2011).

This paper describes the first attempt to use DART coupled to an accuTOFMS as a rapid screening method to detect PAAs in FCM. Four black nylon kitchen utensyls which contains PAAs were used in this study.DART results were compared with an UPLC/MS/MS method which has been validated in terms of linearity, repeatability, recovery, LOD and LOQ.

V.2. Materials and methods

V.2.1. Standards and internal standards

Aniline (CAS Nº62-53-3, purity ≥99,5%) ,4-chloro-2,5-dimethoxyanile (4-Cl-2,5-DMA) (CAS Nº6358-64-1, purity 97%), 4,4´-oxydianiline (CAS Nº101-80-4, purity 97%),o-Toluidine (O-Tl)(CAS Nº95-53-4, purity 97%), o-Tolidine (3,3'-DMB) (CAS Nº119-93-7, purity ≥ 97%) ,2-Naphthylamine (CAS Nº91-59-8), 2-methyl-m-phenylendiamine(2-m-PDA) (CAS Nº823-40-5, purity 97%), 2,6-dimethylaniline (2,6-DMA) (CAS Nº87-62-7, purity 97%), Benzidine (CAS Nº92-87-5, purity ≥ 95%), m-phenylendiemine (m-PDA) (CAS Nº108-45-2, purity ≥99%),4-aminobiphenyl (CAS Nº92-67-1), 2,4-Diaminotoluene (2,4-DAT) (CAS Nº95-80-7, purity 98%),4,4´-Diaminodiphenylmethane (MDA) (CAS Nº101-77-9, purity ≥ 97%) were purchased from Sigma-Aldrich. 2,4-Diaminotoluene-α,α,α-d3 99.2 atom %D) (CAS Nº 71111-08-5) (DAT-d3), 4-aminobiphenyl-d9 99.5 atom %D (4-AB-d8) (CAS Nº344298-96-0), 4,4-methylenedianiline-2,2´,6,6´, N, N, N´, N´, -d8 98.5 atom% D (MDA-d8) (CAS Nº 1219795-26-2) were from CDN isotopes. Pointe-Claire, Quebec, Canada. Aniline-d5 (ANL-d5) 100mg, from Neat Supelco analytical, Bellefonte, PA USA). Polyethylene glycol 600 (PEG 600) was supplied by Chem. Service.

V.2.2. Solvents

H2O Optima® for LC/MS, Methanol (MeOH) Optima® for LC/MS, Acetonitrile Optima® for LC/MS was obtained from Fisher Scientific, Fair Lawn, New Jersey. Ethyl Alcohol (EtOH) absolute from ACROS, New Jersey, USA. Acetic acid, Glacial and Ammonium Hydroxide, 20% from ULTRE® II. Ultrapure Reagent, J.T. Baker®.Canada. Formic acid from Fluka metals basis by Sigma-Aldrich.

V.2.3. FTIR

All samples were analyzed using a FTIR Nicolel 6700 Thermo scientific Smart performer, controlled by OMNIC 7.3 software Thermo Electron Corporation. The FT-IR spectrometer was operating in the range from 4000 to 400 cm-1.

V.2.4. UPLC-MS/MS

Waters Acquity ultra performance liquid chromatography with a sample manager, binary Manager AB SCIEW QTRAP 5500 AB Applied Biosystens MDS Analytical Technologies was used. The mobile phase was MeOH with 5mM ammonium Acetate (A) and H2O: MeOH (95:5) with 5 mM Ammonium Acetate (B). HPLC elution programme is presented in table 1. Separation was performed on a Phenomex Synergi (C18) Hidro-RP 80 A, (150 x 2 mm i.d 4µm particle size). Temperature was set at 30ºC. The injection volume was 20 µL and the flow rate was 0.3 mL/min. MRM conditions were as follows: Curtain gas 45, collision gas was set in High mode, ion spray voltage 5500 V, temperature

550ºC ion source gas 1 = 50 , ion source gas 2 = 60 , the interface heater was in mode On, declustering potential 70 and entrance potential 16.

Time(min)	A%	B%	Flow (mL/min)
0	0	100	0.3
8	95	5	0.3
11	95	5	0.3
11.10	0[a]	100[a]	0.3
19	0[b]	100[b]	0.3

(A) MEOH with 5mM ammonium Acetate

(B) H2O: MeOH (95:5) (5 mM Ammonium Acetate)

[a] Return to initial conditions

[b] Re-equilibration

Table 1- HPLC elution profile program.

V.2.5. DART-accu TOFMS

A DART ionSense® real-time science solutions (Direct Analysis in Real Time) couples to an JEOL accuTOF JMS-T100LC controlled by MassCenter Main Version 1.3.0.1000 and MassCenter System Version 1.3.10b have been employed. DART conditions were as follows: Needle voltage= 0 V, Ring lens Voltage = 5, Orifice 1 voltage = 15 V, Orifice 2 voltage= 5 V, Temperature

orifice 1= 105 ºC, Detector voltage ramping 2400 V, Peaks voltage 300 V, Bias voltage 30 m Pushe vias voltage= -20 V, focus lens voltage 10.0 V, quadrupole lens voltage= -20 V, right/left -1 V, top/botton 6.5, reflectrom voltage 800 V, heater temperature 500ºC positive mode(+) 0.5 mm/s (right). Helium and nitrogen were used as gases. Nitrogen was used to rise up and drop down the temperature and helium was used as the ionization gas. Mass spectra data was revised using the software *Search From List*, Version 4.1, using a threshold of zero and one and mass tolerance of 5 mmu. (mDa).

V.2.6. Standard solutions preparation

Concentrated EtOH stock standard solutions were prepared separately using an accurate quantity of each PAA and of each internal standard (IS) in a volumetric flask. As 2-m-PDA has limitation of solubility, a makeup solution of 100 µg/mL was prepared then sonicated for 30 min to achieve a completely dissolution. A mixed PAAs solution in acetic acid 3% (w/v) of 5 µg/mL for PAAs and of 1 µg/mL for IS were prepared separately from the concentrated EtOH solutions. All solutions were stored in the refrigerator at 6 ºC for up to 1 month for the acetic acid 3% (w/v) solutions and for 3 months for the EtOH solutions. To prepare the sample solution, 20 µL of the IS solution was added to 1 mL of the PAAs solution, afterwards, in order to neutralize the solution, 200 µL of Ammonium Hydroxide, 20% was added and mixed thoroughly.

V.2.7. Quantification and stability test

Quantification was achieved using a calibration line basis on the different calibration levels within 0.1-50 µg/L range and the response area. The response area was calculated dividing the peak area ratio of each PAAs from the peak area ratio of the corresponding IS. The PAA and the IS were identified by the retention time and the relation area among the quantification and the confirmation ions. The selection of the IS to each amine was based in chemical structure such as ANL – d9 was used as IS for Naph, ANL, 2,6-DMA, O-tl and 4-Cl-2,5-DMA. DAT-d3 was used for 2,4-DAT, m-PDA and 2-m-PDA. 4-AB-d9 was employed for BZ, 4-AB and 3,3'-DMB. Finally, MDA-d8 was used as IS for MDA and OXN. On the other hand, to perform the stability test, 100 mL of the food simulant were spiked with 10 µg/L of the PAAs and placed in a glass container cover with aluminium foil. Test condition selected were 100 ºC for 2h. LOD, LOQ and RSD were also calculating for each PAA. A briefly resume of this paragraph is presented in table 1.

V.2.8. Articles selection

Seven KU samples were used for in this study. Four were black nylon kitchen utensyls which previously contain PAAs. Three samples were of polypropylene (PP) and were used as reference samples. In order to calculate the surface area of samples, aluminium foil was cut out in small pieces of 1 dm^2 each one and accurately weighted, the SD (n=8) was 0.0047, and the average was used to estimate the surface of the kitchenware articles. Kitchenware articles were wrapped with aluminium foil and then the aluminium foil was accurately weighted to estimate the surface area.

PAAs	Rt(min)	LOD(µg/L)	LOQ(µg/L)	RSD(n=7)	Recoveries(mean (%)±S.D(n=5)	R^2	Range of linearity(µg/L)	Equation
ANL	5.05	0.5	1	3.10	89.55±0.5	0.9999	1-50	y=20.828x-0.0696
2,6-DMA	7.07	0.5	1	5.61	88.61±0.58	0.9999	1-50	y=11.62x-0.988
o-Tl	6.14	0.5	1	13.23	95.05±0.72	0.9999	1-25	y=10.551x-0.2232
2,4-DAT	3.98	0.1	0.5	2.41	87.18±0.51	0.9998	0.5-25	y=33.013x-0.0446
BZ	5.58	0.5	1	4.8	109.73±0.95	0.9999	1-50	y=0.24x-0.1326
Naph	7.15	0.5	1	4.98	88.12±0.38	0.9988	1-50	y=5.4752x-1.3404
4-AB	7.9	1	5	5.10	95.81±0.56	0.9989	5-50	y=2.1872x-3.1991
m-PDA	2.97	0.1	0.5	3.28	105.88±0.48	0.9995	0.5-25	y=222.17-0.0416
4-Cl-2,5-DMA	6.78	0.1	0.5	3.94	72.40±0.71	0.9986	0.5-25	y=2.4215x-0.3873
MDA	6.45	?0.1	0.1	2.82	100.04±0.38	0.9994	0.1-50	y=21.149x-0.244
OXN	5.65	0.1	0.5	4.68	112.11±0.90	0.9998	0.5-50	y=24.006x-0.1045
3,3′-DMB	6.73	0.5	1	4.88	60.45±1.19	0.9997	1-50	y=0.7303x-0.2161
2-m-PDA	3.35	0.5	1	10.45	91.57±0.46	0.9986	1-50	y=87.673x-0.1617

LOD, limit of detection. LOQ, limit of quantization.RSD, Relative standard deviation .Rt, Retention time.

Table 1. Parameters of repeatabilities, recovery and linearity of the PAAs in acetic acid 3 % (w/v)

V.2.9. Migration Test

In order to carry out migration tests, each sample was placed in a beaker and then filled out with a known amount of acetic acid 3% w/v, then cover with aluminium foil and placed in an oven during 2h. Migration was carried out at 100ºC in the case of polyamide samples and at 70 ºC for PP samples. Technical guidelines on testing the migration of PAAs from polyamide kitchenware and of formaldehyde from melamine kitchenware 1st edition 2011 (Simoneau et al. 2011) was used as reference. In order to developed a method that not required high amount of solvent, glass marbles were placed into the glassware to increase the volume and use little simulant. After migration, 20 µL of IS solution was spiked into a vial that contains 1 mL of the migration solution, afterwards, the solution was neutralized and mixed with 200 µL of Ammonium Hydroxide 20%, then analyzed by UPLC-MS/MS. The migration test were carried out three times, however, as the second migration is not relevant in this study, in order to earn time and solvent, KU were place together in the 2nd migration in a big glass container with enough food simulant and this solution was not analyzed.

V.2.10. DART analysis

To carry out DART/MS analysis, three small pieces of each KU were undertaken with a cutter from three different parts intended to be in contact with food. Afterwards, pieces were analyzed separately by DART coupled to an accuTOF. Small pieces of samples have been taken with tweezers and placed in the source manually, trying to repeat the same position for each sample. Each sample was hand-placed close to the exit grid electrode just slightly above the centre of the exit gas, during approximately 5 seconds to be analyzed. Before start DART

analysis and, in order to perform a mass calibration, a tip slightly soaked with a PEG 600 solution was employed as mass calibrate.

All PAAs in an EtOH solution were determined separately by DART in the positive ion mode at a concentration level of 10 µg/mL, except for ANL, where a concentration of 5 µg/mL was used. All PAAs except ANL were satisfactory identified with a mass tolerance of 1 mDa. Moreover, a test with mixed PAAs was carried out three times at different concentration levels including 0.1, 0.5, 1, 5 and 10 µg/mL. All PAAs were detected in the range of 1 to 10 µg/mL except for ANL and 4-Cl-2,5-DMA, were the LOD should be above those levels.

V.3. Results and discussion

V.3.1. Migration condition

Samples were analyzed by DART and UPLC-MS/MS. IR spectrum was obtained in order to identify polymers and to compare with KU specification.

In order to carry out the migration test, KU were placed in different glassware. To not use lot of simulant, marbles were placed into an appropriate glassware to increase volume, resulting the use of little simulant and consequently enhancing the sensitivity in the PAAs detection by UPLC-MS/MS. Acetic acid 3% (w/v) has been selected as simulant because it represents the worst case for the PAAs migration. The set of glassware-marbles-simulant-KU was accurately weighted and then cover with aluminum foil in order to prevent simulant evaporation. Migration was achieved placing the set in a stove at 100ºC for 2h in the case of polyamide samples and at 70ºC for PP samples. After migration, the set was cooled down to room temperature and was accurately weighted again and simulant were added in order to obtain the initial weight. 20 µL of IS solution

was spiked into a vial which contains 1 mL of the migration solution, afterwards, the solution was neutralized and mixed thoroughly with 200 µL of Ammonium Hydroxide 20%, then analyzed by UPLC-MS/MS.

UE Regulation 10/2011 states that results should be expressed in µg/kg and the results normalized to a ratio surface/volume of 6 dm^2 per kilogram of food or food simulant, thus, taking in account that density of acetic acid 3% is approximately one, obtained results were converted to µg/kg. Hence, migration in first and third test has been expressed in µg/kg.

V.3.2. UPLC-MS/MS method

The UPLC-MS/MS method was validated in terms of linearity, recovery, RSD, LOQ and LOD. PAAs calibration curve were constructed at different concentrations level (0.1-50 µg/L) and were fitted to lineal plot. Linearity show excellent values of R^2 (> 0.9986) and slopes values between 0.24 and 87.67. Regarding to the stability of PAAs, all present acceptable values, except for 4-Cl-2,5-DMA and 3,3'-DMB were stability was 72.40 and 60.45 respectively. RSD (n=7), was achieved analyzing 7 PAAs standard solution of 10 µg/L in acetic acid 3% (w/v). Except for O-Tl and 2-m-PDA, repeatability was lower of 6%. In order to determine LOD, Guideline for data acquisition and data quality evaluation in environmental chemistry was used as reference frame. The presented method shows and excellent sensitivity (LOD between 0.1 µg/L and 1 µg/L) and all LOQ were below the SML of 10 µg/L. In the method developed by FERA, IS is employed to determine PAAs in acetic acid 3% (w/v) by LC-MS/MS. Following this method as guideline, four IS were used and assign to PAAs taken in account the similarity in their chemical structure. So that, DAT-d3 was used for 2,4-DAT, m-PDA and 2-m-PDA. 4-AB-d9 for 4-AB, BZ and

3,3'-DMB. MDA-d8 was employed with MDA and OXN, and ANL-d9 was assigned for, ANL, Naph, O-tl, 2,6-DMA, and 4-Cl-2,5-DMA.

In UPLC analysis single and doubled blanks (with IS) were analyzed before samples. Since the sensitivity is 0.1 μg/L, is possible to detect little traces of the PAA. However, 4-AB, BZ, MDA, OXN and 3,3'-DMB present detectable level in the single and double blank. As these PAAs are commonly used in the manufacture of dyes and adhesives, the presence of them at a low concentration could be as consequence of the employment in the manufacture of some of the plastics which are in contact with the solvent along the method. Hence, in order to discard false positives, the blanks with the higher response ratio PAAs/IS area was subtracted from the PAAs/IS area ratio of the samples.

The following transition ions were used as quantification and confirmation ions respectively: ANL (94.1/77.0, 94.1/51.2).2,6-DMA (122.0/105.2, 122.0/103.1). O-Tl (108.1/91.1, 108.1/65.1). 2,4-DAT (123.0/106.2, 123.0/108.2 and 123.0/79.0). BZ (185.0/167.2, 185.0/139.1). Naph (144.0/127.1, 144.0/77.1). 4-AB (170.0/152.2, 170.0/153.3 and 170.0/128.0). m-PDA (109.0/109.0/92.0).4-Cl-2,5-DMA (188.23/173.2, 188.3/158.2 and 188.3/130.2). 3,3'-DMB (213.1/198.2, 213.1/181.1). MDA (199.3/106.3, 199.3/79.3). OXN (201.1/108.3, 201.1/80.2). 2-m-PDA (123.0/106.1, 123.0/79.2 and 123.0/108.1). On the other hand, the transition ions used for IS were as follows: ANL-d5 (99.2/82.2), 4-AB-d9 (179.4/134.4), DAT-d3 (126.3/180.2), MDA-d8 (203.0/108.1).

V.3.3. DART method

In order to improve DART parameters, individual high concentration of PAAs in EtOH solution of approximately 1000 µg/L were used. Hajslova et al. (2011), explain in an extensive review the influence in DART analysis of the gas temperature, the sample position, the employment of dopants, the matrix effects and the automation, among others. The authors describe that, usually, compounds have more response at 300 ºC; however, in this case we found that at 500ºC PAAs have more response. The voltage of the orifice 1 is another parameter that was improved. It has been studded the influence of the voltage of the orifice 1 on the PAAs sensitivity. It was found that 15 V were the best condition on PAAs determination. At a 40 V some PAAs such as 2,6-DMA and 4-Cl-DMA start to yield products and at 70 V TOFMS detect successfully all PAAs expected products. Since the aim of this study is to develop a screening method, the detection of the quantification and confirmatory ions at 70 V were not taken in account and data will be used for further studies. So that, a voltage of 15 V in the orifice 1 has been selected as parameter to this study. The entire detected ions were the protonated molecular ions.

In order to calibrate the mass spectrum, in each real time chromatogram, PEG 600 was used as mass calibrate at the beginning and at the end of the analysis. Afterwards, an EtOH blank was used in order to reject any presence of PAAs, then, samples were analyzed by triplicate in groups of four or five. With the improved conditions, individual solutions of PAAs were used at concentrations of 10 µg/L, except for ANL where a 5 µg/L solution was employed. All PAAs except ANL were successfully determined with mass difference of 1 mDa. Some test were carried out in acetic acid 3% (w/v) and a loss of sensitivity was observed, hence, in order to determine LOD, mixed PAAs standard solutions

were make up in EtOH in the range of 0.1, 0.5, 1, 5 and 10 mg/L and analyzed by triplicate. At concentrations below of 1 mg/L no PAAs were detected. m-PDA, 2,6 DAT, 2-m-PDA and BZ were detected at 1 mg/L. 4-AB and Naph were identified at 5 mg/L. In the analyzed PAAs 10 mg/L solution, 3,3'-DMB, OXN, MDA, O-Tl and 2,6-DMA were detected. In a mixed solution of 9 PAAs ANL was detected at levels of 1 mg/L. The unique PAA which was not identified was 4-Cl-2,5-DMA. In summary, data show that PAAs do not have the same sensitivity.

Seven KU analyzed by DART. In order to improve the treatment of the samples in DART analysis, samples were analyzed in three different ways: rubbing the sample with a glass tip, immersing little pieces of samples in the acetic solution and finally, analyzing directly the raw material. Samples rubbed with a glass tip and the samples that were immersed in the acetic simulant have no satisfactory results; nevertheless, in the analyzed raw material PAAs were identified, therefore, all DART analysis were achieve analyzing small pieces of sample of the raw material. The small pieces were taken with tweezers and placed in the source manually, trying to repeat the same position for each sample in order to obtain a better repeatability. On the other hand, the three PP samples were used as reference samples without PAAs.

V.3.4. Migration results and DART analysis

V.3.4.1. UPLC-MS/MS results

As it was expected, all polyamide samples analyzed by UPLC were positive in PAAs.Three samples exceed the EU migration limit in the first migration and 2 of them in third migration tests. Black nylon kitchen utensils which exceed

migration limit of 10 µg /Kg in the first migration test, have specially high concentrations in MDA and ANL, and some were also positive in o-Tl, Naph, 3,3'-DMB, 2-m-PDA and 2,6-DAT, but at a low concentrations (usually below of 10 µg /Kg). From the samples which contain levels of ANL and MDA above 100 µg/kg in the 1st test, were non compliant in the third migration test.

V.3.4.2. DART results

To have an extensive view of the DART detection capacity, mass spectra interpretation has been carried out without a threshold, so ion masses with little relative abundance have been taken in account. All ions were in the range of ± 5mDa of the expected mass and were the protonated molecular ions. Within the result of the overall mass spectrum chromatogram, DART detect PAAs in all polyamide samples, and only in one PP sample a PAA was identified but at a low relative abundance. Samples with high concentration of PAAs in polyamide polymer have usually high relative abundance in DART analysis. Nevertheless, some samples were false positives and negatives in PAAs, so that, more studies in order to enhance DART sensitibilty should be performed in order to evoid these drawbacks. Briefly, DART is in good agreement with UPLC-MS/MS analysis and it could be applied as a good sreeaning method to determine PAAs in KU, especially in those samples which contain high PAAs levels in polymer.

V.4. Conclusion

Seven samples have been used in this study. UPLC-MS/MS and DART/accuTOF have been compared in order to determine if DART may be used as screening method to identified PAAs in KU. UPLC-MS/MS method has

been developed and validated in terms of linearity, repeatability, recovery, LOD and LOQ. DART has show that is a powerful tool to identified samples with PAAs in KU, especially those with high concentration in polymer, being useful in order to carry out rapid screening methods. However, false positives and negatives in the polimeric material can occur. Further studies are required in order to enhance DART sensitivity such employing dopands, using an autosampler to improve repeability and enhancing number of analysis in order to be compliant with legislation.

Acknowledgements

Authors are grateful to "Ministerio de Ciencia e Innovación" for the Predoctoral fellowship FPI (ref. BES-2009-023016) awarded to R. Paseiro-Cerrato.

References

Ackerman, L.C., Noonan, G.O., & Begley, T.H. (2009). Assessing direct analysis in real-time-mass spectrometry (DART-MS) for the rapid identification of additives in food packaging. *Food additives and Contaminants*, 26, No. 12, 1611-1618.

ACS Committee on environmental improvement. Guideline for data acquisition and data quality evaluation in environmental chemistry. (1980). *Analytical Chemistry*, 52, 2242-2249.

Brede, C., Skjevrak, I. & Herikstad, H. (2003). Determination of primary aromatic amines in water food simulant using solid-phase analytical derivatization followed by gas chromatography coupled with mass spectrometry. Journal of Chromatography A, 983, 35-42.

Brede, C., & Skjevrak, I. (2004). Migration of aniline from polyamide cooking utensils into food simulants. *Food additives and Contaminants*, 21, No. 11, 1115-1124.

Cajka, T., Vaclavik, L., Riddellova, K. & Hajslova, J. (2008) GC–TOF-MS and DART–TOF-MS: Challenges in the Analysis of soft drinks. *LC GC Europe*, 21, issue 5, 250-256.

Commision Directive 2002/72/EC of 6 August relating to plastic materials and articles intended to come into contact with foodstuff, Official Journal of the European Communities, L220, 2002, p. 18.

Commision Regulation No 10/2011 of 14 January 2011 on to plastic materials and articles intended to come into contact with food, Official Journal of the European Union, L12, 2011, p. 1.

Commision Regulation No 284/2011 of 22 March 2011 laying down specific conditions and detailed procedures for the import of polyamide and melamine plastic kitchenware originating in or consigned from the People's Republic of

China and Hong Kong Special Administrative Region, China, Official Journal of the European Union, L77, 2011, p. 25.

Cody, R. B., Laramée, J. A. & Durst, H. D. (2005). Versatile new ion source for the analysis of materials in open air under ambient conditions. *Analytical Chemistry*, 77, 2297-2302.

Edison, S.E., Lin, L.A.. & Parrales, L. (2011) Practical considerations for the rapid screening for pesticides using pressure desorption ionization with high-resolution mass spectrometry *Food additives and Contaminants*, 28, No. 10, 1393-1404.

European commission, Joint research center, Institute for health and consumer protection. Simoneau, C, ed (2011) Technical guidelines on testing the migration of PAAs from polyamide kitchenware and of formaldehyde from melamine kitchenware 1st edition. EUR 24815 EN.

García-Lavandeira, J., Salgado-Petinal, C., Blanco, E. & Cela, R. (2010). A sensitive and efficient procedure for the high throughput determination of banned aromatic amines in textiles and leather products aided by advanced sample composition. *Analytical and Bioanalytical Chemistry*, 397, 751-763.

Garrigós, M.C., Reche, F., Pernías, K., Sánchez, A. & Jiménez, A. (1998). Determination of some aromatic amines in finger-paints for children's use by supercritical fluid extraction combined with gas chromatography. *Journal of Chromatography A*, 819, 259-266.

Hajslova, J., Cajka, T., &Vaclavic, L. (2011). Challenging applications offered by direct analysis in real time (DART) in food-quality and safety analysis. *Trends in Analytical Chemistry*,30,No 2,204-218.

Kawakami, T., Isama, K., Nakashima, H.,Tsuchiya T , & Matsuoka, A. (2010). Analysis of primary aromatic amines originated from azo dyes in commercial

textile products in Japan.. *Journal of Environmental Science and Health Part A*, 45, 1281-1295.

McCall, E., Keegan, J., & Foley,B. (2012). Primary aromatic amine migration from polyamide kitchen utensils: method development and product testing. *Food Additives and Contaminants*, 29, No. 1, 149-160.

Mortensen, S.K., Trier, X.T., Foverskov, A., & Petersen, J.H. (2005). Specific determination of 20 primary aromatic amines in aqueous food simulants by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Journal of Chromatography A*, 1091, 40-50.

Pfaff A.M. & Steiner, R.R. (2011) Development and validation of AccuTOF-DART™ as a screening method for analysis of bank security device and pepper spray components. *Forensic Science International*, 206, 62-70.

Schubert, J., Kappenstein, O., Luch, A. & Schulz, G. (2011). Analysis of primary aromatic amines in the mainstream waterpipe smoke using liquid chromatography-electrospay ionization tandem mass spectrometry. *Journal of Chromatography A*, 1218, 5628-5637.

Self, R. L. & Wu, W. (2012) Rapid quantitative analysis of phthalates added to food and nutraceutical products by direct analysis in real time/ orbitrap mass spectrometry. *Food Control*, 25, 13-16.

The Food and Environment Research Agency (FERA) (2009). Standard operating procedure for the determination of primary aromatic amines in 3% (w/v) aqueous acetic acid using LC-MS/MS. Sand Hutton, York, YO41 1LZ (UK).

Vaclavik, L., Cajka, T., Hrbek, V. & Hajslova, J. (2009) Ambient mass spectrometry employing direct analysis in real time (DART) ion source for olive oil quality and authenticity assessment. *Analytica Chimica Acta* 645 56-63.

Vaclavik, L., Zachariasova, M., Hrbek, V. & Hajslova, J. (2010) Analysis of multiple mycotoxins in cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to high resolution mass spectrometry. *Talanta*, 82, 1950-1957.

Wang, X. & Chen, Y. (2009). Determination of aromatic amines in food products and composite food packaging bags by capillary electrophoresis coupled with transient isotachophoretic stacking. *Journal of Chromatography A*, 1216, 7324-7328.

Zhao, X & Suo, Y. (2008). Analysis of primary aromatic amines using precolumn derivatization by HPLC fluorescence detection and online MS identification. *Journal of Separation Science,* 31, 646-658.

VI-CHARACTERIZATION AND ANALYSIS OF TWO NOVEL COMPOUNDS YIELDED FROM THE REACTION BETWEEN A COMMON AMINE EMPLOYED AS MONOMERS IN FOOD CONTACT MATERIALS (MXDA) AND FATTY ACIDS PRESENTS IN FATTY NATURE: INSTABILITY OF AMINES IN FATTY FOOD

Abstract

It is a well known fact that amines are not stable in food of fatty nature. In this study for the first time is described the synthesis and characterization of the product obtained as a result of the reaction of amines in a fatty medium. Based on the well known reactions among amines and acid and esters groups, two novel compounds have been synthesized using m-xylylenediamine (MXDA) (a secondary amine widely used as monomer in the manufacture of food contact materials) and two fatty acids, which occurs in most of fats oleic and palmitic acid. The resulting compounds were two molecules belonging to the family of fatty acid amides, dioleamide and dipalmitide. A complete characterization of both products were carried out employing several techniques as Infrared spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, Electron Impact (EI) mass spectrometry, LC-MS/MS and UV spectrophotometry. The results obtained by the different techniques were well correlated. In the second part of the work, the formation of these compounds in real samples was evaluated. For this purpose certain volume of olive oil was spiked with a known amount of MXDA. Olive oil was selected as a fatty medium since it is a widely-consumed food and additionally is used as a fatty food simulant in migration studies. A simple and rapid method was developed to extract the fatty acid amides from the fatty matrix and was identified by LC-MS/MS.

A Chemical software were used to predict the toxicity of the synthesized compounds.

Keywords: amines, olive oil, stability, MXDA, oleic acid, triplamitin, palmitic acid.

VI.1. Introduction

Polyfunctional amines are very reactive substances extensively used either as monomers or additives in the manufacture of materials intended to be in contact with food. The regulation (EU) No 10/2011 includes the positive list of monomers, starting substances and additives authorized in food contact materials and the restrictions which they are subject, the overall migration limit (OML) and the specific migration limit (SML) defined as "the maximum permitted amount of non-volatile substances released from a material or article into food simulants" and "the maximum permitted amount of a given substance released from a material or article into food or food simulants", respectively.

Plastic materials can transfer these potential migrants to foods and may be a hazard for health. It is generally recognized, that substances with low molecular weight (< 1000 Da) can be absorbed in the gastrointestinal tract, and consequently involve a risk to consummers' health (EFSA, 2008).

In order to verify the compliance of the food contact materials with the legislation and to assure its safety, migration assays under controlled conditions (time, temperature and food simulant) must be performed. Nevertheless, some of the allowed substances are unstable under the migration assay conditions; they can react with other compounds or undergo a degradation process. The resulting substances -degradation products, reaction products- are migrants potentially toxic, but however, not restrictions are considered for these compounds in the regulations, nevertheless, their safety is responsibility of manufactures.

It is a well-known fact that amines are not stable in simulants of fatty nature. Under the common migration testing conditions (10 days, 40 ºC) amines disappeared (Paseiro-Cerrato et al., 2011).They react with components present in the fatty medium, to form the corresponding amides. In fats and oils the fatty

acids occur linked to glycerine yielding triglycerides by an ester group or as free fatty acids.

Reactions of amines through acid and ester groups to yield amides are well known in the field of organic chemistry (Streitwieser and Heathcock, 1987, Thornton and Neilson, 1985). In the early fifties, several authors synthesized fatty acid amides from amines and fatty acids (Swern et al., 1949, Roe et al., 1952 and Roe et al., 1952). Many years after, Cravatt et al. (1995, 1996) verified that similar compounds belonging to the class of fatty acid amides had pharmacological effects.

On the other hand, in past years several authors (Posada de la Paz et al, 1996, Schurz et al, 1996, Calaf et al 2001) related aniline derivatives with the toxic oil syndrome.

Taking into account these findings and observations our investigation group decided study in depth the behaviour of amines that could be present in food packaging materials, such as residual monomers or additives, when come in contact with fatty foods.

In the present paper for the first time is reported the synthesis and characterization of the product obtained as a result of the reaction of the amines in a fatty medium (Olive oil).

m-Xylylenediamine (MXDA) was selected as a model amine, since it is commonly employed as curing agent for epoxy resins (Ellis, 1993) because of its high reactivity. In food packaging these resins are usually employed as inner coatings for cans (Simal-Gándara, et al. 1998, Paseiro-Cerrato et al. 2010).The two major fatty acids of olive oil, oleic and palmitic acid were selected to simulate the fatty medium.

For a complete characterization several techniques including, Infrared spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, Electron Impact (EI) mass

spectrometry, LC/MS/MS using an atmospheric pressure ionisation (APCI) source and UV spectrophotometry were applied.

NMR spectroscopy has demonstrated to be especially interesting for structural elucidation. The two-dimensional NMR experiments are particularly useful for this purpose. For establishing correlations within the molecule HH COSY (Correlation Spectroscopy) and TOCSY (Total Correlation Spectroscopy) were performed.

The study was completed by the estimation of the toxicity of the synthesized compound based on the Cramer rules (Cramer et al. 1978).

Experimental results were supported by data obtained with the prediction softwares.

The fatty amide formation reaction was evaluated under real conditions. For that, an olive oil sample was spiked with a known concentration of MXDA. For extracting the fatty amide formed from the olive oil a simple and rapid method was developed. The identification was performed by LC-MS/MS.

VI.2. Experimental section

VI.2.1. Materials and Reagents

All chemicals were of analytical grade. Chloroform (Cl3CH), tetrahydrofuran (THF), hydrochloric acid, n-heptane and acetonitrile (ACN) (grade HPLC) were from Merck (Darmstadt, Germany). Acetonitrile LC-MS CHROMASOLV® was from Fluka (Steinheim, Germany). M-xylylenediamine (MXDA) (CAS Nº 1477-55-0 from Aldrich (Steinheim, Germany). Glyceryl triplamitate, (Tripalmitine) minimum 85% (CAS Nº 555-44-4) was from Sigma. KBr (potassium bromide) for IR spectroscopy and Potassium hydroxide pellets were from Merck

(Darmstadt, Germany). Chloroform – d, 99.8 atom % D (CDCl3) (CAS Nº 865-49-6) was supplied by Aldrich (Steinheim, Germany). Sodium chloride (NaCl) was from Vorquímica (Vigo, Spain). Milli-Q water was obtained by a purification system (Millipore) (Bedford, MA, USA). Olive oil (0.4º) was obtained in a local supermarket.

VI.2.2. Preparation of the model compounds

Dipalmitide was prepared by mixing tripalmitin (1g) and the amine (0.16g) (MXDA) into a headspace vial. Immediately after, the mixture was heated in an oven at 70 ºC for 24 h. The components were added in the 1:1 stoichiometric ratio. After the 24 h, the compound was cooled down to a room temperature.
In order to purify the sample and remove fatty remains, 1 mL of n-heptane was added to the product and then centrifugated during 10 min. This process was repeated two times.
The compound obtained as a result of the reaction between MXDA and oleic acid was synthetized in National Center for Food, Spanish Agency for Food Safety and Nutrition and kindly supplied for the present study.

VI.2.3. Infrared spectroscopy (IR)

KBr pellets were prepared by mixing thoroughly approximately 90 mg of KBr and 1 mg of the sample; then the mixture was pressed during 1 minute. IR spectra of the samples were acquired by using a Genesis II FTIR ^{TM}, Mattson spectrometer. The measurements were performed in the transmittance mode in the range of 400- 4000 cm^{-1}.

VI.2.4. ^{13}C and ^{1}H Solid state NMR spectroscopy

Once the product was obtained and after ensuring it was completely dried, it was pulverized. About 35 mg were used to perform the ^{13}C NMR analysis. In order to establish correlations within the molecule two- dimensional 2D ^{1}H experiments including COSY and TOCSY were carry out. To perform 1H NMR, COSY and TOCSY analysis a makeup solution of 1mM in CDCl3 were prepared.
The ^{1}H and ^{13}C spectra were recorder under standard conditions, employing a Bruker DX-500 avance spectrometer (500 MHz) at 27 ºC. Acquisition time and relaxation delay were set at 1 and 12s, respectively. Data points were 5482 Hz and 32 K respectively. Data analysis was performed using MestReC, NMR Data Processing Made Easy 4.9.9.9 software.

VI.2.5. Electron Impact (EI) mass spectrometry

Direct mass spectrometric analysis in solid state was performed using a Micromass Autospec. The electron impact (EI) ionization source was operated in positive mode. Spectra were recorder under standard conditions at 70eV. Data were processed using Masslynx version 4.1 software.

VI.2.6. UV–visible spectrophotometer

In order to investigate the maximum absorbance of the synthesized product, the compounds were appropriately dissolved in acetonitrile (ACN) 0.1 mg/100 mL for dipalmitide and 1mg/10mL for dioleamide. However, in the case of dipalmitide due to it has not enough solubility in ACN, ultrasounds at 40 ºC during 60 min was employed in order to achieve a completely dissolution. The

solutions were then analyzed using a Kontron Uvikon 922 Uv-vis double beam Spectrophotometer. Spectra were recorded from 190 to 400 nm.

VI.2.7. LC-MS/MS analysis

An HPLC/MS system comprising an Accela 1250 pump fitted with a degasser, a quaternary pump coupled to a triple quadrupole mass spectrometer TSQ Quantum Access max controlled by Xcalibur (Thermo Fisher Scientific, San José, CA, USA) was used.

In order to obtain parent masses and products of the compounds, LC/MS/MS parameters have been optimized. MS data were acquired in the positive ion mode employing atmospheric pressure (APCI) by direct infusion. An isocratic elution of ACN was used as mobile phase with a flow rate of 500 µL/min. A Hamilton syringe pump with a syringe size volume of 500 µL was employed for direct injection with flow rate syringe pump set at 10 µL/min. Mass spectra were monitored in the mass range 100-800 m/z. MS detector settings were as follows: Discharge current 4 V, capillary temperature 331 ºC and vaporizer temperature for dipalmitide was 400 ºC and 366 ºC for dioleamide. Collision pressure and collision energy was set at zero. Nitrogen was used as auxiliary gas (pressure 16 arbitrary units) and sheath gas (pressure 30 psi). The same parameters were after employed to performed SRM analysis.

In order to achieve chromatographic separation, an Ace 3 C18 HL (30 mm x 3.0 mm, i.d. 3 µm particle size, Advanced Chromatography Technologies) was used. Analyses were carried out in isocratic mode using ACN as mobile phase. Total run was 20 minutes. Flow rate was 500 µl/min and injection volume was 10 µl.

VI.2.8. Toxicity estimation

In order to estimate toxicity of molecules, Toxtree (Estimation of Toxic Hazard - A Decision Tree Approach) version 2.5.0, based in Cramer rules was used.

VI.2.9. Preparation of the spiked olive oil samples

The spiked samples were prepared as follows: 10 g of olive oil were weighed in a headspace vial. One mL of a solution of 85 µg/mL of MXDA in HCl 0.1 N was evaporated under nitrogen steam and re-dissolved in a known volume of THF, afterwards; it was spiked in the oil. The head space vial was hermetically closed, mixed thoroughly and stored in an oven at 40ºC for 10 days. Two replicate experiments were made.

VI.2.10. Extraction of the fatty acid amides from olive

2.5 g of the spiked samples were removed and saponificated using 25 mL of a KOH 0.5 N solution prepared according to Casares et al., (1967). Saponification was performed at 40 ºC during 24 h. To perform extraction from the saponificated oil, 2 mL of milli-Q water were incorporated to 2 mL of the sample. Afterwards, 2 mL of a saturated solution of NaCl were added followed by 6 mL of Cl_3CH. The resulting solution was slightly mixed in order to prevent emulsion formation. The solution was then centrifugated at 4 ºC during 10 min at 5000 rpm. Two phases were separated; one mL of the lower chloroform layer was removed and evaporated to dryness to obtain the unsaponifiable residue. After that, 1 mL of ACN was incorporated to the unsaponifiable residue and later

sonicated at 40 ºC for 1h in order to achieve completely dissolution of the substance in ACN. The ACN solution was injected into a LC-MS/MS system.

VI.3. Results and discussion

VI.3.1. Dioleamide and dipalmitide syntheses

VI.3.1.1. Dipalmitide syntheses

The reactions among amines and ester groups have been widely described in literature (Streitwieser and Heathcock, 1987, Thornton and Neilson, 1985); on the other hand and as mentioned above, several authors have reported the instability of certain types of amines in olive oil simulant (Paseiro-Cerrato et al. 2010; Brede et al. 2004). Taking as a starting point these facts, the reaction of m-Xylylenediamine (MXDA) in a fatty medium was investigated.

MXDA was selected as a model amine because, as it has been commented on earlier, is typically employed in food packaging applications. Its specific migration limit (SML) established in the Regulation EU No 10/2011 is 0.05 mg/kg.

Tripalmitin is a triacylglycerol that contains three molecules of palmitic acid which is a saturated fatty acid present in most fats and oils, so that, it was selected to simulate common component fats.

In order to obtain the compound free of impurities, a purification step was performed by whashing the product with n-heptane.

The experiments revealed that amines react with tripalmitin to form the corresponding amide, which using UIPAC name would correspond with N-{[3-

(hexadecanamidomethyl)phenyl]methyl} hexadecanamide, but, in order to abbreviate the name, it will be called dipalmitide.

VI.3.1.2. Dioleamide syntheses

Oleic acid is a monounsaturated acid present in most fats and oils, indeed, occurs in large extend in olive oil.The resulting product is the (9E)-N-({3-[(9E)-octadec-9-enamidomethyl]phenyl}methyl)octadec-9-enamide using IUPAC name. It will be named dioleamide.

The resulting products were characterized through FTIR, ^{1}H NMR, ^{13}C NMR, UV spectrum, EI and LC/MS. Furthermore, Chem Draw and Instant JChem softwares have been used in order to draw both molecules, to estimate molecular properties and theoretical ^{1}H NMR and ^{13}C NMR spectrums. Molecular structures of both compounds with useful data and chemical properties are presented in figures (1, 2) and table 1.

VI.3.2. Infrared spectroscopy (IR)

Since IR spectroscopy is one of the main techniques used to study the molecular structures, IR analysis was conducted to characterize the product. Figure 3 shows representative FTIR spectra of the KBr pellet containing the dipalmitide formed as a result of the reaction between MXDA and tripalmitin, before and after washing with n-heptane.

The bands at 1643 and 1552 cm^{-1} correspond to amide and aromatic group, respectively. Two other bands at 2811 and 3481 cm^{-1} assigned to alcane and amine group are also present. When both spectra are compared the band at 1742 cm^{-1} that corresponds to ester group disappears after the treatment with n-

heptane. The spectra data confirm the formation of the amide between the triplamitin and the amine.

The same bands can be clearly appreciated in the dioleamide spectrum. It can be observed that the band which corresponds to 1643 and 1552 cm^{-1} has a higher intensity, that maybe due to the presence of the double bond (insaturation) in the aliphatic chain. (Figure 4)

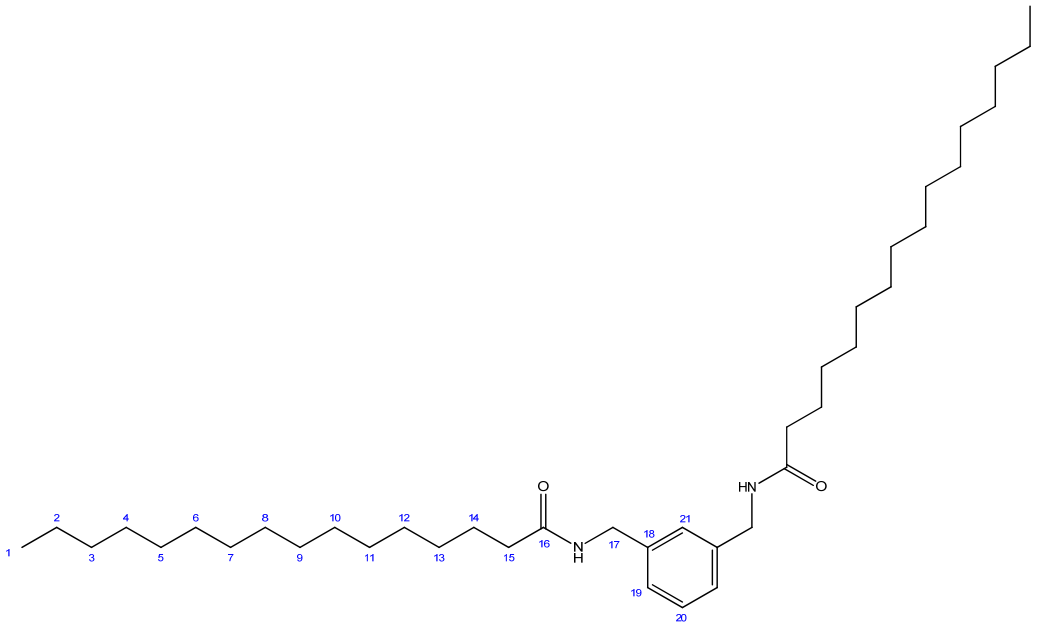


Figure 1. Numbered dipalmitide structure

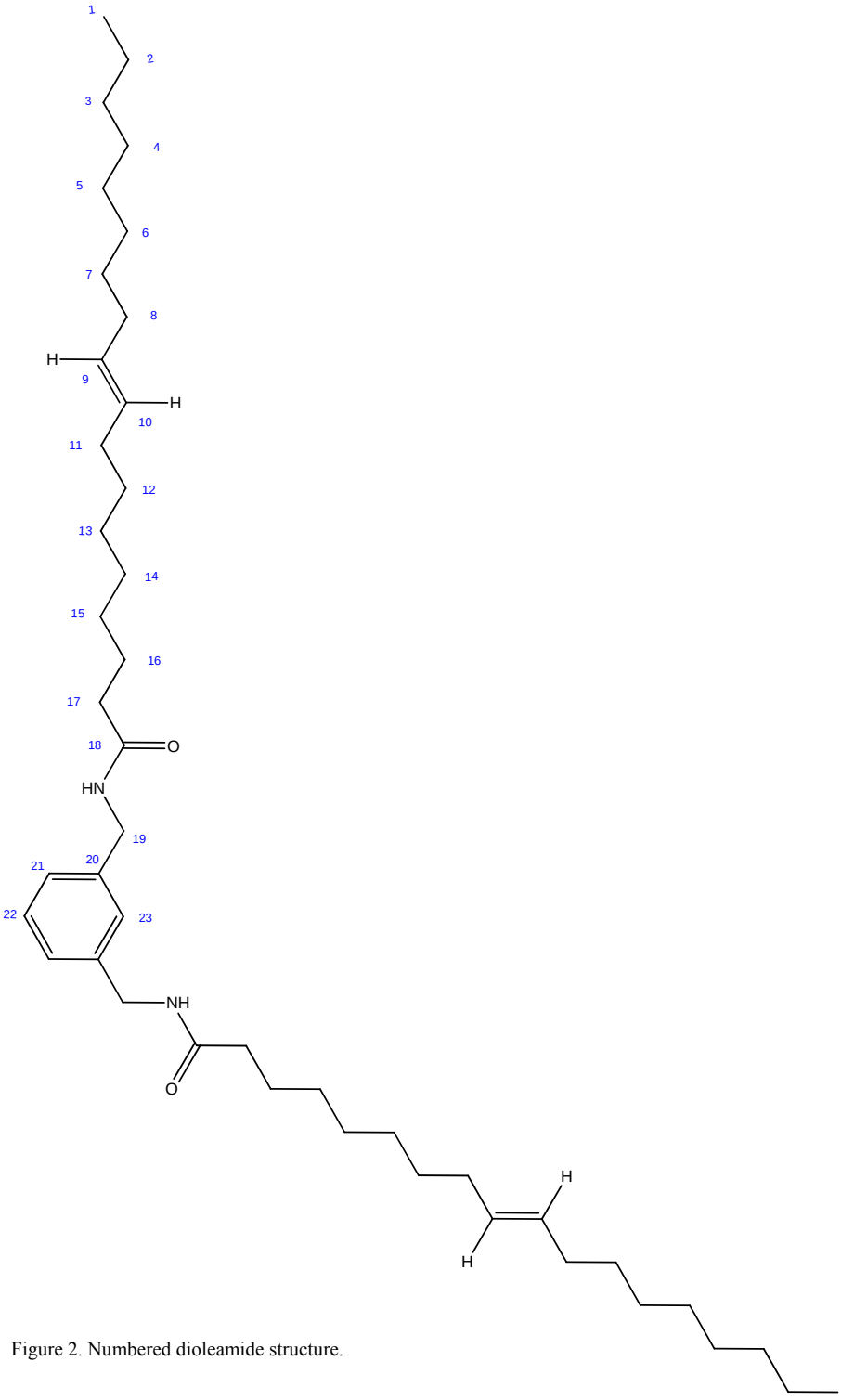


Figure 2. Numbered dioleamide structure.

Name	UIPAC name	SMILES	MW	Log P	Ac. pka	Bs. pka	TPSA	VWSA	TV
Dipalmitide	N-{[3-(hexadecanamidomethyl)phenyl]methyl}hexadecanamide	CCCCCCCCCCCCCCCC(=O)NCc1cccc(CNC(=O)CCCCCCCCCCCCCCC)c1	613.01	12.82	15.48	-0.22	58.20	1214	0.05
Dioleamide	(9E)-N-({3-[(9E)-octadec-9-enamidomethyl]phenyl}methyl)octadec-9-enamide	CCCCCCCC\C=C\CCCCCCCC(=O)NCc1cccc(CNC(=O)CCCCCCC\C=C\CCCCCCCC)c1	665.09	13.88	15.49	-0.22	58.20	1280	0.05

Table 1. Estimated physicochemical properties of dioleamide and dipalmitide. MW= Molecular weight; Ac.pKa=Strongest acidic pKa; Bs.pKa=Strongest basis pKa;TPSA=Topological polar surface area;VWSA=VanderWaalsSuyrfaceArea;TV= Topological polar surface area/ VanderWaalsSuyrfaceArea

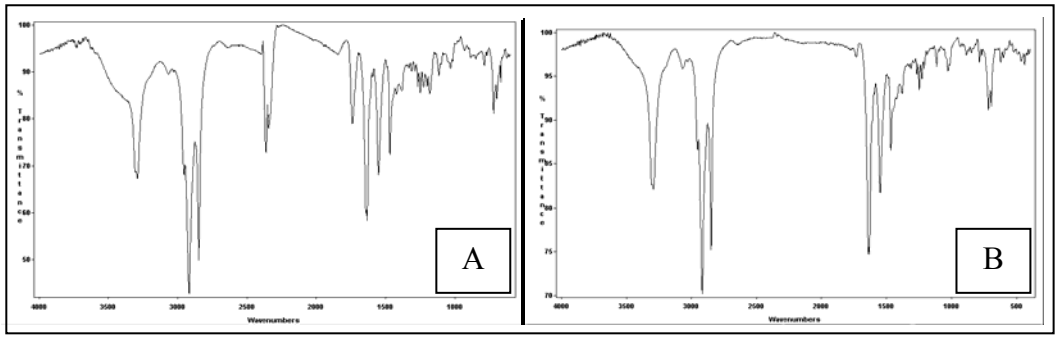


Figure 3. IR spectrum of dipalmitide before (A) and after (B) washing with n-heptane

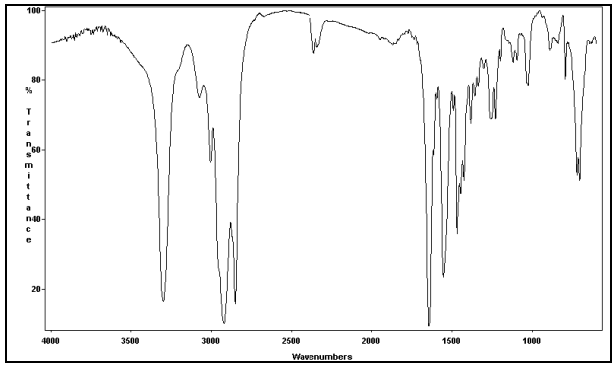


Figure 4. IR spectrum of Dioleamide

VI.3.3. ^{13}C and ^{1}H NMR spectroscopy

NMR provides valuable information about chemical structure. In addition, does not require sample preparation.

^{1}H NMR, COSY and TOCSY analysis were carried out employing a 1mM solution in CDCL3 of the compounds. The ^{13}C NMR analyses were performed in solid state due to the lack of sensitivity obtained when the product was dissolved in deuterated chloroform. The NMR ^{13}C spectrum of the figure 5, correspond to the dipalmitide product and showed signals at 173.23; 69.34; 62.35; 23.69 and 13.54 δ ppm attributed to C=O(C 16); B-CH2-CO-NH-R(C 15); R-CO-NH-CH2- (C 17) ; CH3-CH2- (C 2) and terminal CH3-(C 1), respectively.

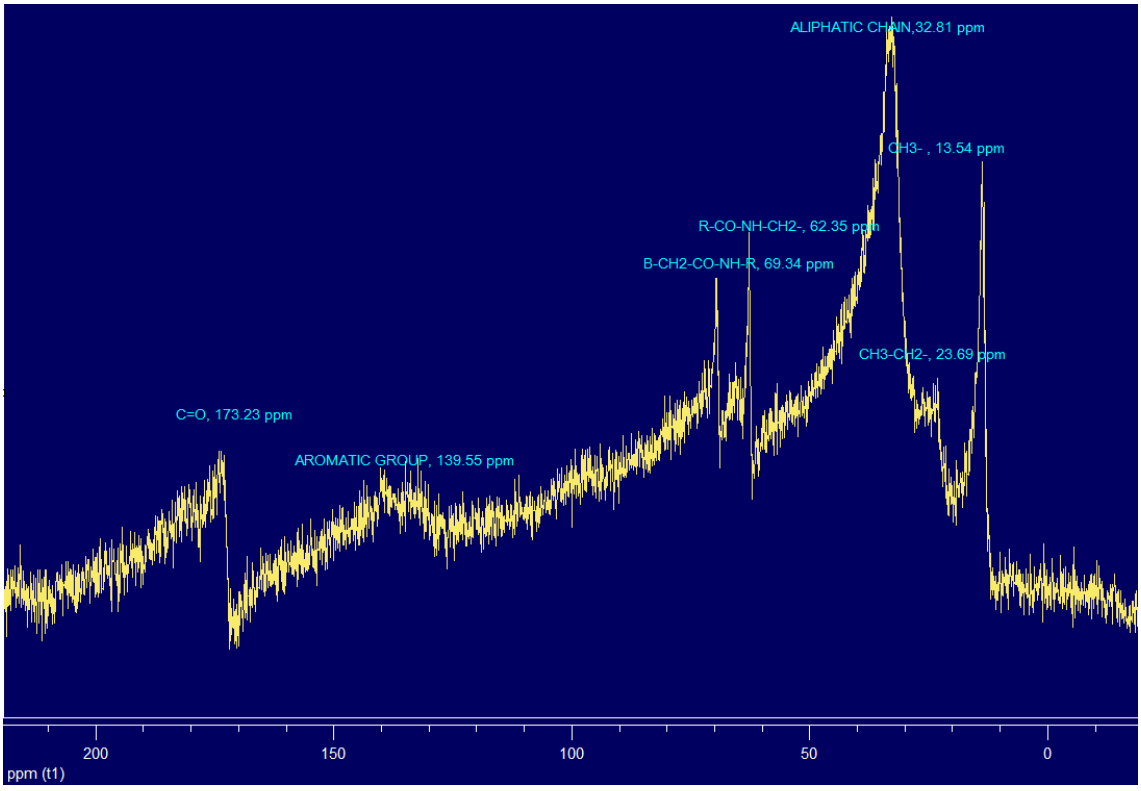


Figure 5. ^{13}C NMR of Dipalmitide.

Additionally, exhibited a weak signal at 139.55 ppm and a broad signal at 32.81 ppm corresponding to aromatic group (C 18-21) and aliphatic chain (C 3-14), respectively.

In the NMR ^{13}C spectrum of the dioleamide (Figure 6) appears a new signal at 127.64 δ ppm which corresponds to (C=C) double bond characteristic of the oleic acid (C 9-10).

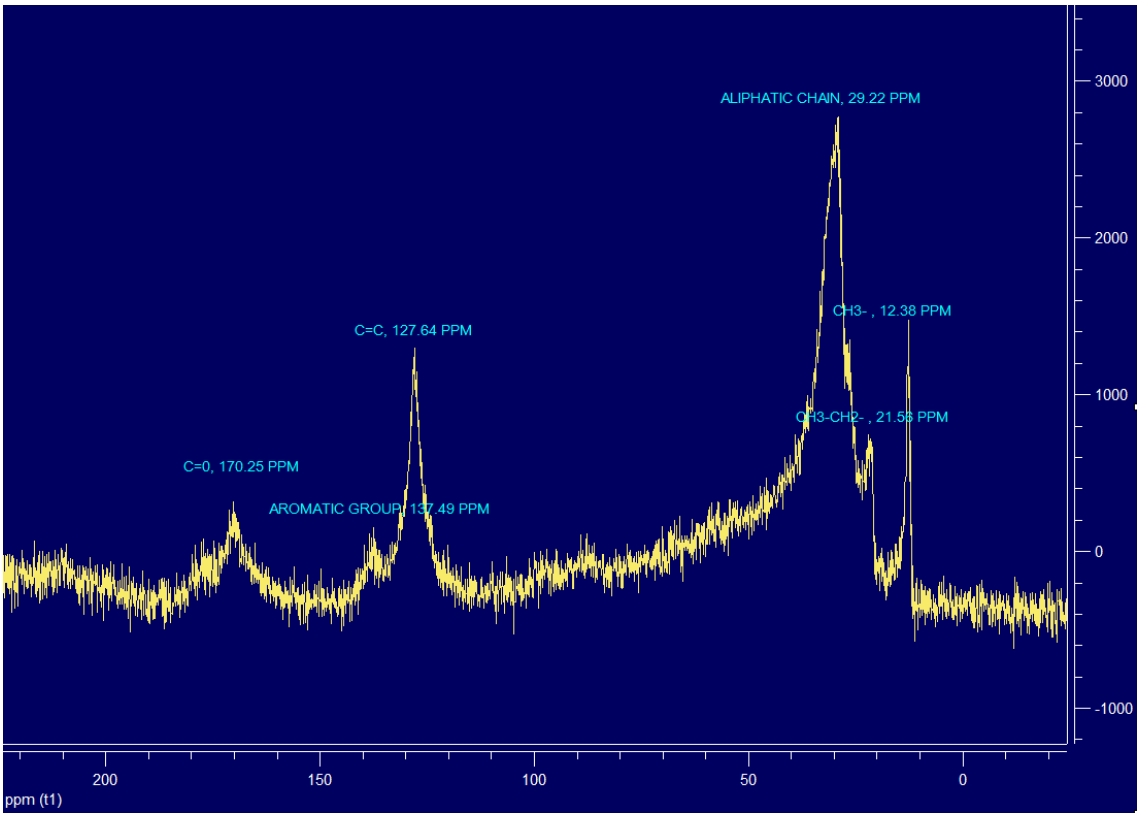


Figure 6. ^{13}C NMR of Dioleamide

The results obtained are in good agreement with the predicted spectra obtained using Chem Draw software, where similarities among the expected and obtained spectra have been observed. Predicted signals were 172.9;36.5;43.9;22.7 and

14.1 δ ppm assigned to C=O (C 16); B-CH_2-CO-NH-R (C 15) ; R-CO-NH-CH_2- (C 17); CH_3-CH_2- (C 2) and terminal CH3- (C 1) groups respectively in dipalmitide. On the other hand, for dioleamide compound, 130.6 δ ppm was the shift signal assigned to the double bond (C 9-10).

A representative ^{1}H NMR spectrum of the dipalmitide is illustrated in Figure nº 7. The signals shifts at 4.42 δ ppm and 5.73 δ ppm are attributed to the protons of –NH- and -NH-CH_2 (C 17) respectively. A well-defined peak which corresponds to the protons of -CO-CH_2- (C 15) is observed at 2.20 δ ppm and CO-CH2-CH2- (C 14) at 1.67 δ ppm. Protons of the aromatic ring (C 18-21) appear at 7.21 δ ppm as a multiplet. Additional peaks corresponding to the protons of the aliphatic chain (C 2- 13) and terminal -CH3 (C 1) group are observed at 1.28 and 0.91 δ ppm, respectively.

The ^{1}H NMR spectrum of dioleamide (Figure 8) showed the similar shifts than dipalmitide spectrum except for the signals corresponding to the influence of the double bond, where 4.05 δ ppm belongs to the double bonds hydrogen signals (C 9-10). Furthermore, 1.79 and 1.91 δ ppm signals belongs to CH2-C=C-(C8 and C11) and CH2-CH2-C=C- (C 7 and C 12), respectively.

Prediction for dipalmitide in ^{1}H NMR is quite similar to experimental data. Number of peak and shifts are agree with experimental data, with the exception that signal assigned to -NH- is set at 8.03 ppm whilst experimental data establish the peak at 5.73 ppm as well as in dioleamide and in dipalmitide spectrum.

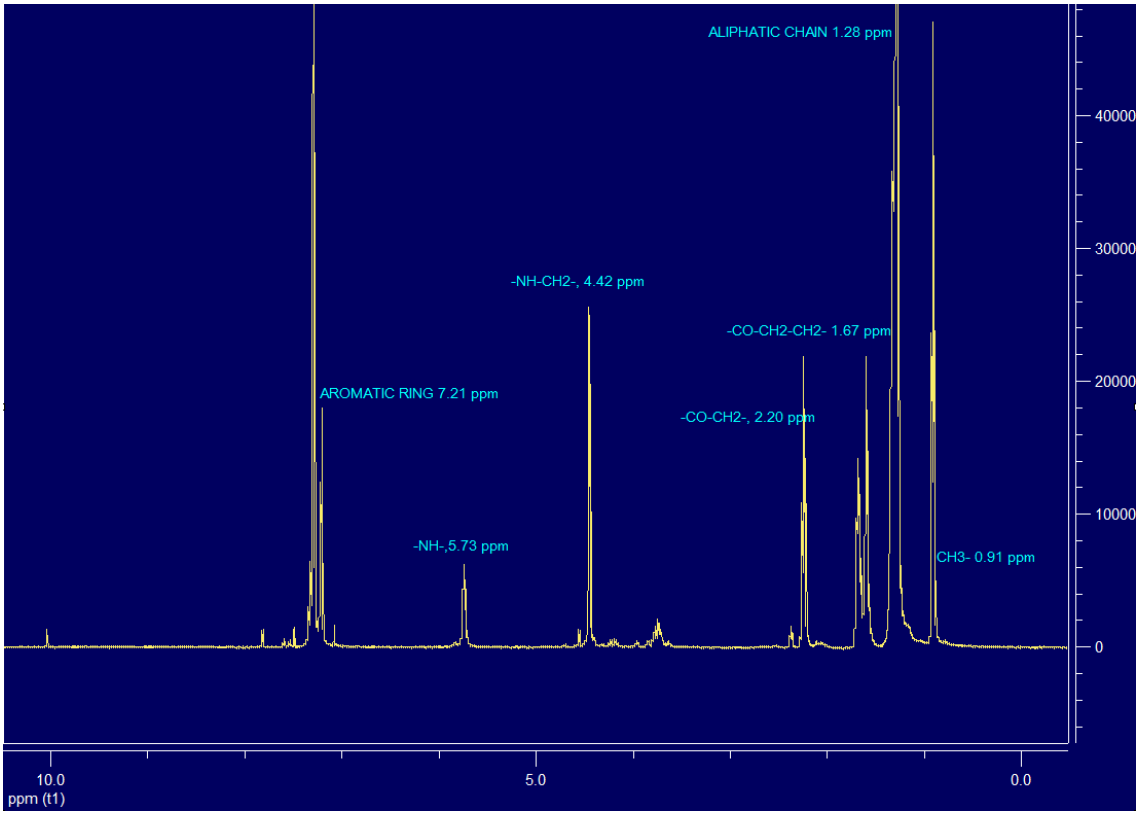


Figure 7. ^{1}H NMR of Dipalmitide

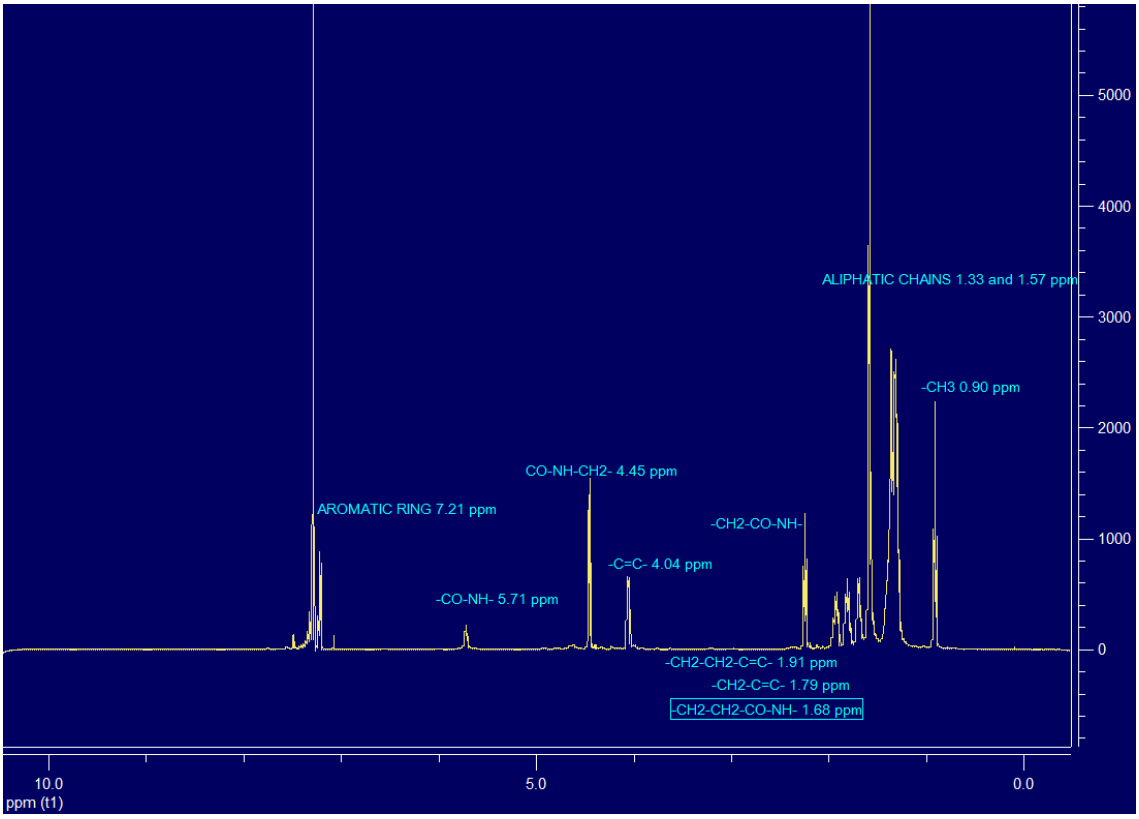


Figure 8. ^{1}H NMR of dioleamide

VI.3.4. COSY and TOCSY spectrums

Dipalmitide COSY spectra shows that the aliphatic chain (C 2- 13) (1.28 δ ppm) is set to the protons of the signals at 0.91 and 1.67 δ ppm. Furthermore, the signals 2.20 and 4.4 δ ppm are coupled with signals 1.67 and 5.7 δ ppm respectively (Fig 9 A). A briefly resume of dipalmitide ^{1}H NMR characteristic spectrum is presented in table 2 including semi quantitative integration valour and spin-spin coupling. Regarding to the diolemide COSY spectrum, the

connectivity of the signal associated to the double bond at 4.05 δ ppm with signals correspond to the CH2-C=C-(C8 and C11) and CH2-CH2-C=C- (C 7 and C 12) at 1.79 and 1.91 δ ppm respectively, is the main difference between both COSY spectrum as is remarked in the figure 9B. It can be observed the absence of the signal corresponding to the double bond and related signals in the COSY dipalmitide spectrum

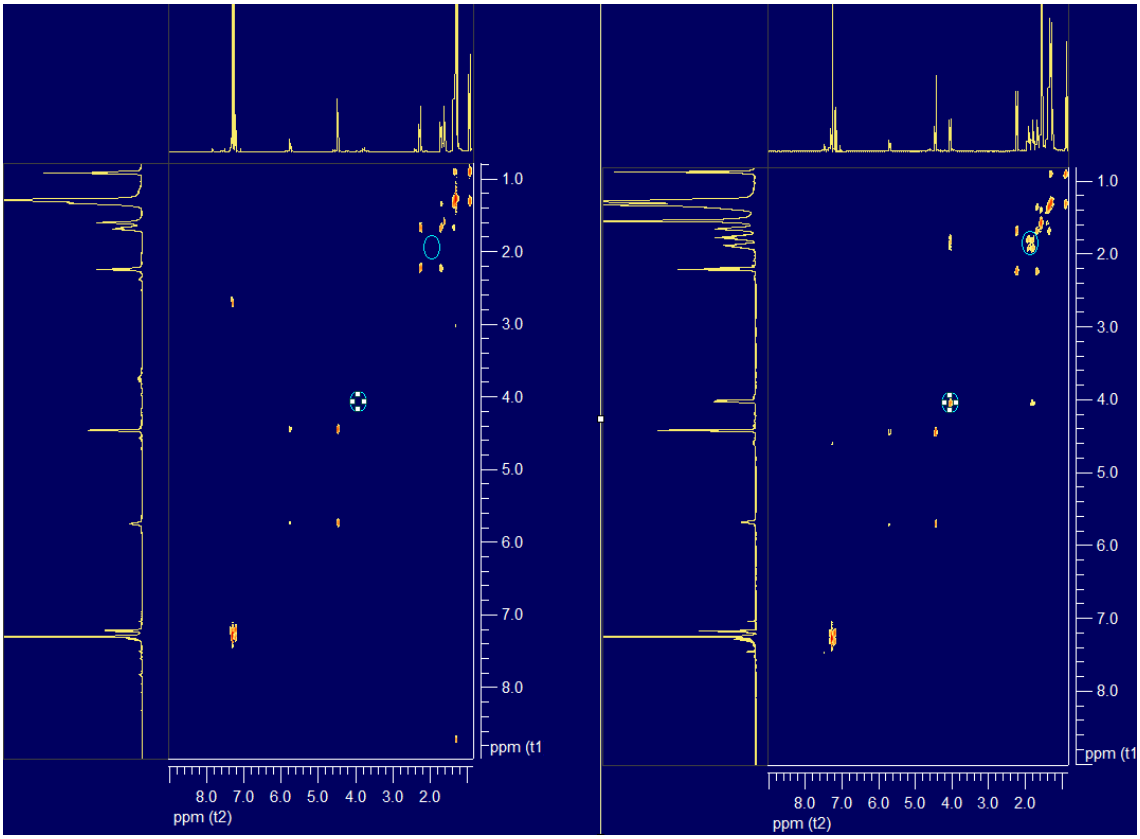


Figure 9: Comparison among dipalmitide (A) and dioleamide (B) COSY spectrum. Observe that signal corresponding to the double bonds are not present in the dipalmitide spectrum.

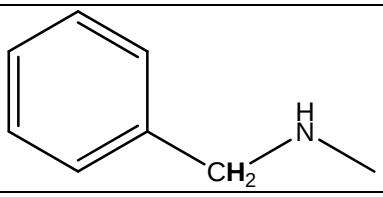
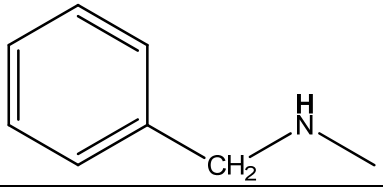
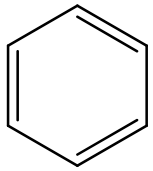
Structure	Ppm	Integration valor	Spin-spin coupling	COSY coupling
$C\mathbf{H}_3$-CH_2-	0.91	3	Triplet	With 1.28 ppm
-($C\mathbf{H}_2$)$_{24}$-	1.28	24.64	Singlet	With 0.91 and 1.67 ppm
-CO-CH_2-$C\mathbf{H}_2$-(CH_2)$_{24}$-CH_3	1.67	2.03	Pentet	With 1.28 and 2.20 ppm
-CO-$C\mathbf{H}_2$-CH_2-(CH_2)$_{24}$-CH_3	2.20	1.77	Triplet	With 1.65
	4.42	1.83	Doublet	With 5.73 ppm
	5.73	0.83	Singlet	With 4.42
	7.21	-	Multiplet	Not coupled

Table 2. Dipalmitide ^{1}H NMR characteristic spectrum.

Likewise, in both COSY spectrums, signals which belong to the protons of –NH- and -NH-CH_2 (C 17) are not linked with the rest of the spectrum because it belongs to a different spin system as it was expected.

Regarding to the TOCSY spectrum, only dioleamide TOCSY spectrum has been obtained. TOCSY signal of diolemide show the connectivity of the diolemide structure and the different spin system from C1 to C17 and for -NH- and C19 protons (Figure 10). Due to the sensitivity limitations of the TOCSY experiment when dipalmitide was analyzed data is not available.

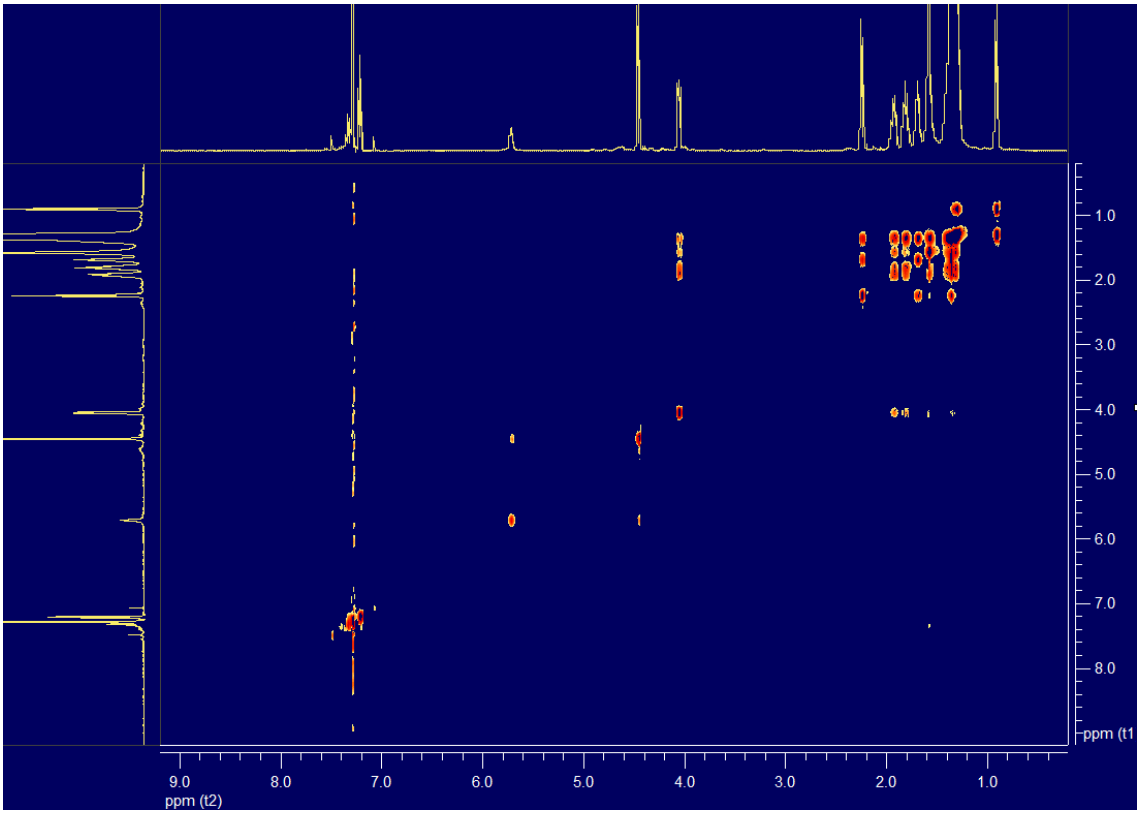


Figure 10. TOCSY of dioleamide.

VI.3.5. Mass spectrometry characterization and UV spectrum.

Mass spectra of dioleamide and dipalmitide obtained by Electron Impact Mass Spectrometry supplied data about their molecular weight. The spectra display the molecular ion peak at *m/z* 664.2 and *m/z* 612.2, corresponding to dioleamide and dipalmitide, respectively.

These results are in good agreement with the prediction of exact mass of the chemical structure which is 664.6 *m/z* and 612.6 *m/z*.

The UV properties of the products have been also tested. Solutions of dioleamide and dipalmitide in ACN have been employed for the UV spectrum determination. Dioleamide and dipalmitide have their maximal absorption at 196 and 205 nm, respectively.

VI.3.6. LC-MS/MS

LC-MS analysis by direct infusion described the two expected quasi molecular ions at *m/z* 613.6 and 665.6 employing APCI source. Results are in good concordance with the expected masses of the compounds obtained in EI and with software prediction of the molecular mass.

Electrospray ionization was firstly employed; however, no satisfactory results were obtained.

To carry out the analysis, solutions of 0.1 µg/mL for diolemide and for dipalmitide were employed to perform the chromatographic separation in SRM mode. Diolemide and dipalmitide peaks were detected at 6.30 and 6.66 min respectively under isocratic conditions with ACN as mobile phase. Several fragments have been obtained for both compounds which are summarized in table 3. Diolemide main transition is 665.6>384.2, resulting from the loss of one

monounsaturated aliphatic chain including the amide group. Fragment 401.3 *m/z*. corresponds to the loss of the aliphatic chain. On the other hand, dipalmitide has as main ion product 358.2 *m/z* which corresponds to the loss of the saturated aliphatic chain including the amide group. Besides, fragment 375.2 *m/z* corresponds to the loss of the aliphatic chain.

Furthermore, both compounds have a common break down releasing the aliphatic chains including the amide group yielding the fragment 105.1 *m/z*.

Compounds	Transition	Collision energy (V)
Diolemide	665.6>401.3	17
	665.6>384.2	23
	665.6>105.1	39
Dipalmitide	613.6>375.2	14
	613.6>358.2	21
	613.6>105.1	36

Table 3. Single reaction monitoring (SRM): Dioleamide and dipalmitide products.

VI.3.7. Toxicity estimation

To estimate toxic hazards of our compounds, Cramer rules have been applied. These rules allow classifying molecules based on their molecular structure in a fast and simple way. Cramer rules classified molecules in three classes of toxicity: Class I, II and III. Class I expects low toxicity and class III suggests substantial toxicity. Class II would be an intermediate level. To achieve compounds classification, software based on these rules has been used. The obtained results revealed that both compounds belong to class III, therefore, the

toxicity of the compounds should be investigated in order to establish possible hazards in human health.

VI.3.8. Identification of the products in olive oil real sample

The dioleamide and dipalmitide formation reaction was evaluated under real conditions using olive oil to simulate the fatty medium.

After spiking a known amount of MXDA in the oil, the samples were subject to the common migration testing conditions (40 ºC during ten days). After this time, a cold saponification was performed at 40 ºC during 24 h in order to avoid chemical bond breakage of amide group. After that, 2 mL of milli-Q water were incorporate to 2 mL of the sample, then 2 mL of a saturated solution of NaCl were added. The saturated NaCl solution play an important role in the extraction, due to it enhances phase's separation. Cl_3CH was employed, as a organic solvent, because the compound has a good solubility on it. The resulting solution was slightly mixed in order to prevent emulsion formation. Since the fatty amides formed were not very soluble in ACN, the unsaponifiable residue was sonicated at 40 ºC for 1 h in an ultrasonic bath in order to improve the solubility. Besides, standard solutions of diolemide and dipalmite at a concentration of 0.1 µg/mL were spiked in Cl_3CH to validate the extraction of amide after saponification. The ACN solution was analyzed by LC-MS/MS. The identification of the products in the olive oil samples was confirmed by LC-MS/MS Identification of dipalmitide and dioleamide was achieved using the confirmatory ions and retention time (Figure 10). The presence of the compounds demonstrate that amines react with the acid or/and ester group of fatty acid forming the corresponding amide; this fact could explain the instability of amines in fats.

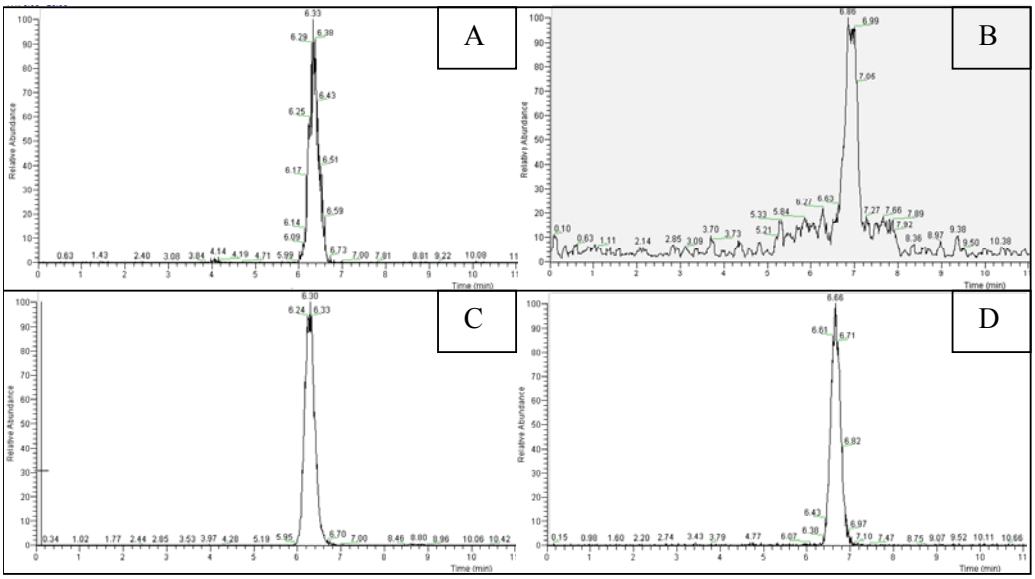


Figure 11.LC/MS/MS chromatogram of dioleamide and dipalmitide in spiked olive oil samples (A and B respectively).LC/MS/MS chromatogram of dioleamide and dipalmitide spiked stock standard solution of 0.1 µg/mL in $CHCl_3$ after saponification in olive oil samples (C and D respectively).

VI.4. Conclusion

In the present work we report for the first time a simple approach for the preparation of fatty acid amides as a result of the reaction of amines in a fatty medium. A complete characterization of the resulting compound by using several analytical techniques including ^{1}H and ^{13}C NMR spectroscopy, Infrared spectroscopy, UV, spectrophotometry, Electron Impact (EI) mass spectrometry, LC-MS/MS is also presented. Results obtained demonstrate good concordance among all techniques.

Once the compound was identified and isolated the fatty amide formation reaction was tested under real conditons using olive oil as fatty medium. A simple and rapid method to extract the fatty amides from olive oil was developed. Diolemide and dipalmitide were successfully identified by LC-MS/MS. Furthermore, toxicity of the compounds is predicted by Cramer rules. These products could represent and hazard on the human heath, thus, more studies concerning to the toxicity of these substances should be performed. The compounds could be dangerous itself or can yield the amines in the organism due to biological process. Thereby, investigations about real behavior of those compounds in the human body are necessary. On the other hand, futures work concerning migration from polymers made of amines present in food contact materials will be tested and evaluated in order to guarantee consumers heath.

Acknowledgements

The study was financially supported by the Ministerio de Ciencia e Innovación, ref. Nº AGL/2008-04146 "MIGRAMIN". Authors are grateful to "Ministerio de Ciencia e Innovación" for the Predoctoral fellowship FPI (ref. BES-2009-023016) awarded to Rafael Paseiro-Cerrato. The authors are also grateful to C. Casal, P. Blanco and G. Hermelo for their excellent technical assistance.

References

Calaf, R.E.; Peña, J.; Paytubi, S.; Blount, B.C.; Posada de la Paz. M.; Gelpi, E.; Abian,J. 2001, 73, 3828-3837.

Casares R. Tratado de análisis químico.Tomo III; Editorial Casares: Madrid, 1967. Brede, C.; Sjevrak. Food Addit Contam; 2004, 21, 11, 1115-1124.

Commission Regulation (EU) No 10/2011, Off. J. Eur. Commun. L12 (January) (2011) 1.

Cramer, G. M.; Ford, R. A.; Hall, R. L. Food Cosmet. Toxicol. 1978, 716, 255-276.

Cravatt, B. F.; Prospero-Garcia, O.; Siuezdak, G.; Gilula, N. B.; Henriksen, S.J.; Boger, D. L.; Lerner, R.A. Science, 1995, 268, 1506-1509.

Cravatt, B. F.; Giang, D. K.; Mayfield, S. M.; Boger, D. L.; Lerner, R.A.; Gilula, N. B. Nature, 1996, 384, 83-87.

EFSA (2008). Note for guidance for petitioners presenting an application for the safety assessment of a substance to be used in food contact materials prior to its authorisation. http://www.efsa.europa.eu/en/efsajournal/doc/21r.pdf (Updated 30/07/2008).

Ellis B. E. Chemistry and technology of epoxy resins; Blackie Academic & Professional: Glasgow, 1993.

Paseiro-Cerrato, R.; Rodríguez-Bernaldo de Quirós, A.; Sendón, R.; Bustos, J.; Santillana, M. I.; Cruz, J. M.; Paseiro-Losada, P. Compr. Rev. Food Sci. Food Saf. 2010, 9, 676-694.

Paseiro-Cerrato, R.; Rodríguez-Bernaldo de Quirós, A.; Sendón, R.; Bustos, J.; Ruíz, E.; Cruz, J. M.; Paseiro-Losadaa, P. J. Chromatogr; A 2011, 1218, 7105–7109.

Paseiro-Losada P.; Simal-Gándara J.; Sanmartín-Fenollera P.; Pérez-Lamela, C. Chromatographia; 1999, 50, 453–456.

Philo, M. R.; Jickells, S. M.; Damant, A. P.; Castle, L. J. Agric. Food Chem. 1994, 42, 1497-1501.

Posada de la Paz. M.; Philen, R. M.; Borda, I.A.; Socias, J.M.S.; Gómez de la Cámara, A.; Kilbourne, E.M. Fd Chem. Toxic. 1996, 34, 3, 251-257.

Roe. E. T.;Stutzman, J. M.; Scanlan, J. T.; Swern, D. J .Am. Oil Chem. Soc. 1952, 29, 1, 18-22.

Roe. E. T.; Miles, T. D.; Swern, D. J. Am. Chem. Soc. 1952, 74 (13), 3442-3443

Schurz, H.H.; Hill, R.H.; Posada de la Paz. M.; Philen, R. M.; Borda, I.A.; Bailey, S.L.; Needham, L. L. 1996, 9, 1001-1006.

Simal-Gándara J.; Paz-Abuín S.; Ahrné, L. Crit Rev Food Sci Nutr. 1998, 38, 675–688.

Streitwieser, A Jr.; Heathcock C.H. Química orgánica; Interamericana: Madrid, 1987.

Swern, D.; Stutzman, J. M.; Roe. E. T . J. Am. Chem. Soc. 1949, 71 (9), 3017-3019.

Thornton, R.; Neilson, R. Química orgánica; Fondo educativo interamericano: Mexico D.F, 1985.

Winton, A. L.; Winton, K. B. Structure and composition of foods. Vol 1. Cereals, nuts, oil seeds. Jonh Wiley and Sons, 1946.

VII-MIGRATION OF PHOTOINITIATORS BY GAS PHASE INTO DRY FOODS

Published at: The Journal of Agriculture and Food Chemistry, 2009, 57, 10211-10215.

Presented as a poster at: 4th International Symposium on recent advances in food analysis. Prague, Czech Republic. November 4–6, 2009.

Abstract

Photoinitiators are components widely used in UV-cured inks for printing food packaging. In the present study, the migration of seven photoinitiators through the vapor phase was investigated. To perform the migration test, an additive enriched polyethylene wax was used as a source to release photoinitiators. The method was applied to evaluate the migration of the photoinitiators into five selected dry foods (cake, bread, cereals, rice and pasta). The highest level of migration was found in the cake. Parameters affecting to the migration process were evaluated and high migration level was found to correlate with both the porosity as well as the fat content.

In addition, the kinetics of migration of the photoinitiators from the additive enriched wax into the cake were studied under accelerated conditions.

Keywords: Photoinitiators, migration, vapor phase, dry foods.

VII.1. Introduction

Last February a notification from the German authorities concerning the migration of 4-methylbenzophenone from cardboard to certain breakfast cereals was issued on the Rapid Alert System for Food and Feed (RASFF). As a result of the notification the European Commission asked European Food Safety Authority (EFSA) to evaluate if the presence of 4-methylbenzophenone found in cereals constituted a risk for the health and also to evaluate if the existing Tolerable Daily Intake (TDI) for benzophenone and hydroxybenzophenone could be applied to 4-methylbenzophenone. The Scientific Panel on food contact materials, enzymes, flavourings and processing aids (CEF) concluded that short term consumption of cereals contaminated with 4-methylbenzophenone at the levels reported did not constitute a risk for the health; but also indicated that if the use of 4-methylbenzophenone is to be continued, more data are required to carry out a full risk assessment. On the other hand, the Panel concluded that the TDI of benzophenone could not be applied to 4-methylbenzophenone and in addition hydroxybenzophenone should not be included in the TDI of benzophenone because of the lack of supporting data *(1)*.

Benzophenone-based derivatives are widely used as photoinitiators for UV-cured inks. These types of printing inks are environmentally friendly since no organic solvents are included in their formulation *(2, 3)*. In the food packaging field, UV-cured inks, like the one including benzophenone as photoinitiator, are widely used for printing the external face of the packaging. If there is not an effective barrier, ink components, like photoinitiators with low molecular weights, can permeate through the material and migrate to the food *(4, 5)*.

Several studies have demonstrated the migration of benzophenone from paper and board to food and food simulants via direct contact *(5-8)* however information about the migration through the vapor phase is very scarce *(9-12)*.

Johns et al. *(5)* studied the migration of ink components from cartonboard to food during frozen storage; and they observed that under low temperature conditions (-20 ºC) the migration of benzophenone occurs even when there is no direct contact between the packaging and the food.

The development and validation of accurate and sensitive methods to analyze potential migrants is essential in order to guarantee the food safety. The techniques commonly used to determine photoinitiators are liquid chromatography with UV detection and gas chromatography with flame ionization detector *(7, 13, 14)*. Chromatographic techniques coupled to mass spectrometry have also been successfully applied in the analysis of these contaminants *(4, 15)*.

Pastorelli et al. *(13)* reported a rapid and specific reverse-phase liquid chromatographic method with diode-array detector to quantify benzophenone in packaging materials and cake samples.

Sagratini et al. *(15)* developed a multi-component method to analyze five ink photoinitiators in packaged beverages. Analyses were performed by gas chromatography-mass spectrometry. The presence of benzophenone in the samples was confirmed by liquid chromatography atmospheric-pressure photoionization mass spectrometry (APPI)/MS/MS).

In the present paper the migration of seven benzophenone-based photoinitiators including 4-hydroxybenzophenone, methyl-2-benzoylbenzoate, benzophenone, 2-hydroxybenzophenone, 4-methylbenzophenone, 4-benzoylbiphenyl and 4,4'-bis(diethylamino) benzophenone through the vapor phase was studied. To perform the migration test an additive enriched polyethylene wax was used as

the releasing source for photoinitiators. The method was applied to evaluate the migration of the photoinitiators into five selected dry foods. Parameters that influence migration process were evaluated. Migration kinetics were studied under accelerated conditions.

VII.2. Materials and methods

VII.2.1. Reagents and standard solutions

Standards of 4-hydroxybenzophenone (purity 98%), methyl-2-benzoylbenzoate (purity 97%), 2-hydroxybenzophenone (purity 99%), 4-methylbenzophenone (purity 99%), 4-benzoylbiphenyl (purity 99%) and 4,4'-bis(diethylamino) benzophenone (purity 99%) were supplied by Aldrich and benzophenone (purity 99%) was provided by Fluka.

Chemical structures and physico-chemical properties of the photoinitiators studied are shown in Table 1. The data was collected from different databases (SciFinder 2007, ChemIDplus Advanced).

Polyethylene wax Licowax PE 520 (non-polar and low-molecular 105 polyolefin waxes; drop point: 120 ºC; density: 0.93g/cm^3) was obtained from Clariant Ibérica, S. A. (Barcelona, Spain).

All solvents were HPLC-grade. Acetonitrile was provided by Merck (KgaA, Darmstadt, Germany) and ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Stock standard solutions (300 mg/L) of photoinitiators were prepared in acetonitrile and stored at 4ºC in the darkness.

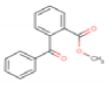
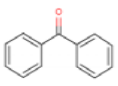
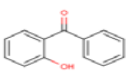
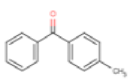
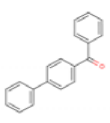
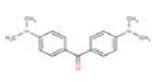
Structure	Formula	Name	CAS No	Molecular weight	Melt Point (ºC)	Boil Point (ºC)	log P (octanol-water)	Vap Press (mm Hg) 25ºC	Henry's Law Constant (atm-m3/mole) 25ºC
	$HOC_6H_4COC_6H_5$	4-hydroxybenzophenone	1137-42-4	198.22	132-135	150-160	3.07	1.00E-05*	2.02E-10*
	C15-H12-O3	methyl-2-benzoylbenzoate	606-28-0	240.254	52	351	2.7	1.53E-05*	1.25E-08*
	C13H10O	benzophenone	119-61-9	182.22	47.8	305.4	3.18	8.2E-04*	0,00000194*
	C13-H10-O2	2-hydroxybenzophenone	117-99-7	198.22	40	127-133	3.52	4.39E-04*	2.54E-08*
	C14-H12-O	4-methyl benzophenone	134-84-9	196.2	59.5	328.1*	3.690*	1.94E-4*	_
	C19-H14-O	4-benzoylbiphenyl	2128-93-0	258.3	100	420	4.827*	3.11E-7*	_
	C21 H28 N2 O	4,4'-bis(diethylamino)benzophenone	90-93-7	324.4	95.5-96.5	475.7±30.0*	5.994±0.394*	3.25E-9*	_

Table 1.- Chemical structures and physicochemical properties of the photoinitiators. (*) Estimated; (-) Not found.

VII.2.2. Food samples

For migration studies five dry foods were selected: cake (naturally leavened baked cake), toasted bread, breakfast cereals (corn flakes), pasta (durum wheat semolina pasta) and rice (white parboiled).
All foodstuffs were bought in a local supermarket.

VII.2.3. Migration studies

An additive enriched polyethylene wax was used as a photoinitiators release system. It was prepared according to the procedure reported by Sanches-Silva et al. *(16)* with a slight modification. Briefly, a solution containing about 2 mg of each photoinitiator was carefully added to 3 g of wax. Once the solvent was evaporated, the mixture was heated at 140 ºC for 2 h. The enriched wax was then cooled at room temperature. To verify the concentrations of the photonitiators, a small piece of the wax was extracted with the procedure mentioned below and analyzed by HPLC-DAD.
Food samples were placed on a metallic net and were separated from each other with a glass ring (∅ = 3.5 cm and the height = 0.9 cm). The additive enriched wax (area = 46.6 cm^2) was placed on the bottom of a glass container (V = 720 mL) without direct contact to food to ensure that the migration of the photoinitiators occurred through the vapor phase. The migration studies were performed under accelerated conditions (70 ^{0}C, 48 h).
For the studies of migration kinetics, the cake was selected as the representative of dry food. The cake samples (m = 3 g) were placed in a 45 mL glass jars without direct contact with the wax (area = 2.9 cm^2) (Figure 1). The glass jars

were then hermetically closed and stored at 70 ºC for 5, 10, 24, 34, 48, 144 and 360 h.

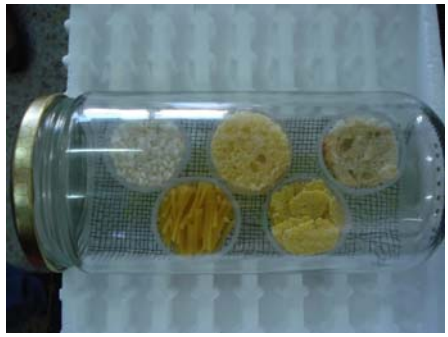


Figure 1.-System employed to perform the migration test

VII.2.4. Photoinitiators extraction

Food samples were extracted using the method described previously in literature *(13)*. Acetonitrile was used as extraction solvent.

VII.2.5. Chromatography

HPLC-analyses were performed on a HP1100 system (Hewlett- Packard, Waldbronn, Germany) equipped with a HP1100 quaternary pump, a degassing device, an autosampler, a column thermostatting system, a diode-array detector (DAD) and Agilent ChemStation for LC and LC/MS systems software.

The separation was performed on a Kromasil 100 C18 (25 x 0.4 cm i.d., 5 μm) column, using binary mobile phase consisted of acetonitrile and water; the gradient elution program is shown in Table 2. The flow rate was 1mL/min and

the injection volume was 20 μL. The selected wavelengths were 254 nm for 4-hydroxybenzophenone, methyl-2-benzoylbenzoate, benzophenone, 2-hydroxybenzophenone, 4-methylbenzophenone, and 4,4'-bis(diethylamino) and 290 nm for 4-benzoylbiphenyl. Data concerning the validation of the chromatographic method are described in a paper submitted for publication.

Time(min)	A%	B%
0	50%	50%
10	0%	100%
15	0%	100%
16	50%	50%[a]
20	50%	50%[b]

(A)Water; (B) Acetonitrile
[a] Return to initial conditions
[b] Re-equilibration

Table 2.- HPLC elution profile program

VII.3. Results and discussion

The migration of benzophenone and derivatives into dry foodstuffs through the vapor phase was investigated. The assays were carried out under accelerated conditions (70 ºC, 48 h) (Figure 2). The foodstuffs used for the study were analyzed prior experiments (blank sample) and no detectable quantities of the migrants under interest were found from these blank food samples. All photoinitiators studied here were found to migrate into the food, except 4,4'-bis (diethylamino) benzophenone, probably due to its low vapor pressure (3.25 x 10^{-9} mmHg). The conditions used in the migration test here, simulate the worst case.

The highest contents of migrated photoinitiators were found in cake. This is not surprising since the compounds are lipophilic. The fat content was approximately 10.7 times higher in cake than in pasta and about 3.6 times higher than in cereals (Table 3).

Food	Fat (%)	Proteins (%)	Carbohydrate (%)
Cake (*)	11.6	4.4	78.1
Bread	7	13	70.5
Cereal	3.2	9	72.2
Pasta	1.08	12.84	67
Rice	0.5	7.84	77.2

(*) Data obtained from the USDA database *(17)*

Table 3.- Chemical composition of the foods studied according the nutritional label

These results are in good agreement with those reported by Anderson and Castle *(4)* and Triantafyllou et al. *(7)* for benzophenone. Anderson and Castle *(4)* analyzed 71 food samples selected randomly from a total of 143 samples packaged in printed carton board, in which benzophenone had been detected. The highest value corresponded to a high fat chocolate packaged in direct contact with cartonboard.

In the study conducted by Triantafyllou et al. *(7)*, the migration of different contaminants from recycled paper and board into foods with different fat contents was evaluated. They observed that the highest migration levels were found in the foodstuff with the highest fat content.

Of these seven photoinitiators studied, the higher migration was observed for 2-hydroxy benzophenone, benzophenone and 4-methyl benzophenone. This could be explained with the higher vapor pressures of these compounds.

Another factor that seems to affect the migration process is the porosity. In order to evaluate the influence of this parameter, the true density of the selected food was measured using a helium pycnometer (Mycropicnometer, Quanta-Chrome, MPY-2). From the data of density, the porosity (ε) was calculated by means of this equation

$$\varepsilon = (1 - d_{apparent}/d_{true}) \times 100$$

where $d_{apparent}$ is the apparent density (mg/mL of the sample including air) and d_{true} is the true density (mg/mL of the sample excluding air). The porosity values obtained for cake, bread, cereal, pasta and rice were 30.6%, 35.9%, 8.8%, 3.7% and 4.8%, respectively. It was observed that the foodstuffs with a porous structure, such as cake and bread, presented the highest migration values.

These results suggest that the porosity strongly influences the migration process; high porosity values contribute to high migration levels.

In the cake, the concentrations of the photoinitiators increased over time until the equilibrium was reached after approximately 150 h (Figure 3). However, for 2-hydroxybenzophenone not even that time was enough to reach the equilibrium. On the other hand, in the case of 4-benzoylbiphenyl, it was only after 48 h when the migration was on detectable level. This could be due to the low vapor pressure compared to the rest of photoinitiators.

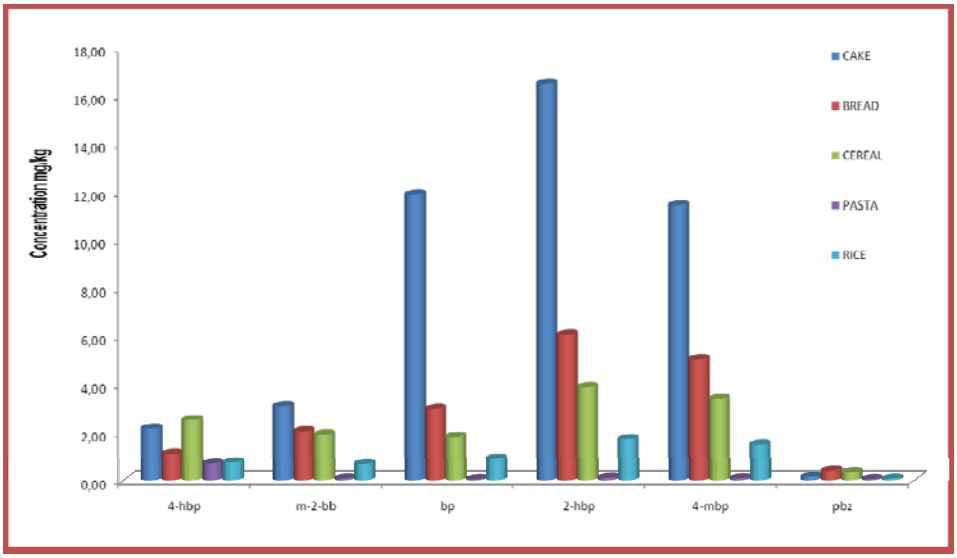


Figure 2.- Migration of benzophenone-based derivatives into dry foodstuffs, at 70ºC for 48 h.

In summary, the results obtained in this study show that the migration of benzophenone-based photoinitiators through the vapor phase occurs to a large extent. And furthermore, both the porosity and the fat content of the food have strong influence on the migration process. Foods with high fat content and high porosity values are exposed to high migration levels.

Acknowledgement

The study was financially supported by the Xunta de Galicia (Proj. n INCITE08PXIB203096PR). The authors are grateful to Ms Lidia Pereiro Expósito for the excellent technical assistance.

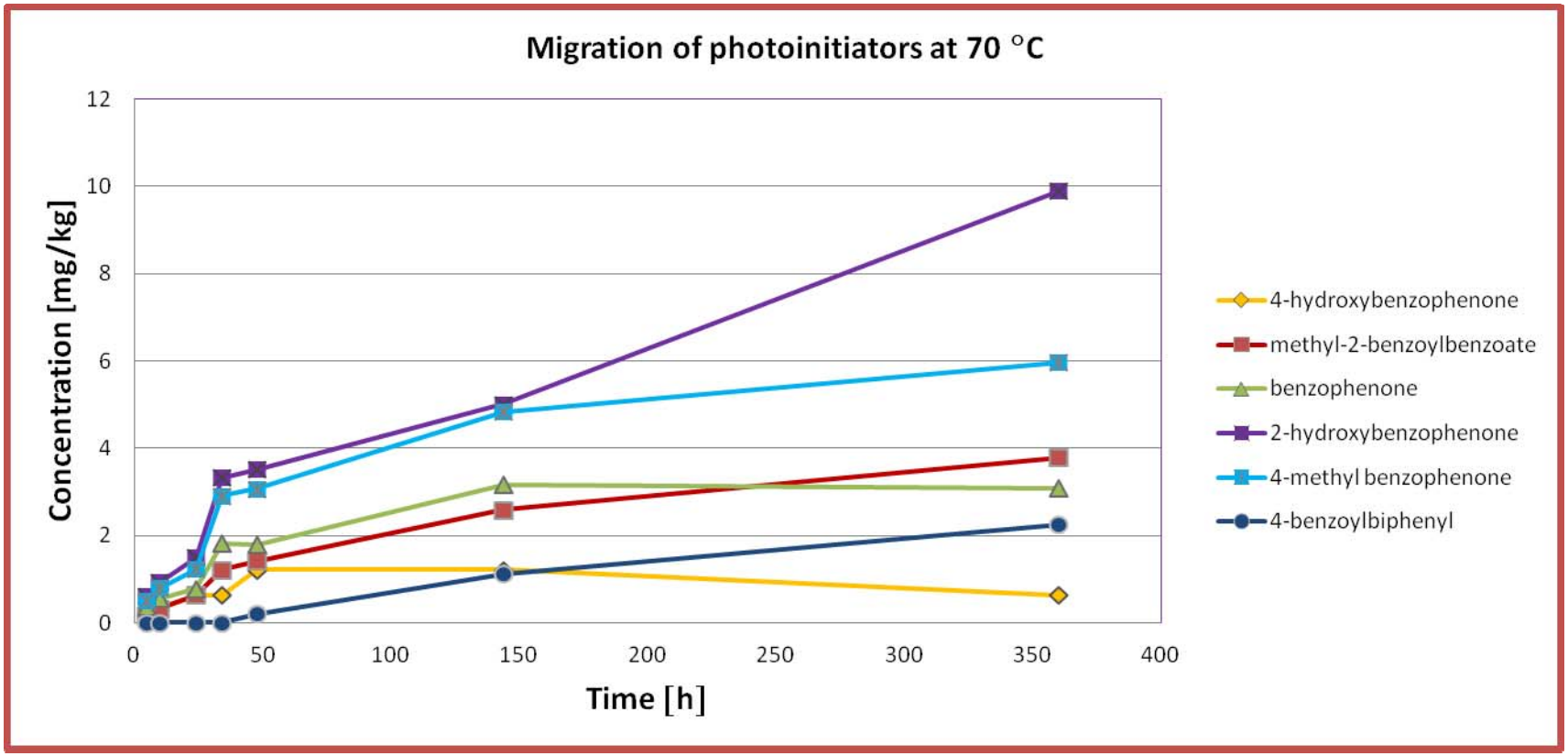


Figure 3.- Kinetic of migration of photoinitiators into cake at 70 ºC.

LITERATURE CITED

(1) Scientific Opinion of EFSA prepared by the Panel on food contact materials, enzymes, flavourings and processing aids (CEF) on Toxicological evaluation of benzophenone. *EFSA J.* **2009,** *1104*, 1-30.

(2) Wang, Z. W.; Huang, X. L.; Hu, C. Y. A systematic study on the stability of UV ink photoinitiators in food simulants using GC. *Packag. Technol. Sci*. **2009**, *22*, 151-159.

(3) Papilloud, S.; Baudaz, D. Analysis of food packaging UV inks for chemicals with potential to migrate into food simulants. *Food Addit. Contam*. **2002**, *19*, 168-175.

(4) Anderson, W.; Castle, L. Benzophenone in cartonboard packaging materials and the factors that influence its migration into food. *Food Addit. Contam*. **2003**, *20*, 607-618.

(5) Johns, S. M.; Jickells, S. M.; Read, W. A.; Castle, L. Studies on functional barriers to migration. 3. Migration of benzophenone and model ink components from cartonboard to food during frozen storage and microwave heating. *Packag. Technol. Sci.* **2000**, *13*, 99-104.

(6) Triantafyllou, V. I.; Akrida-Demertzi, K.; Demertzis, P. G. Migration studies from recycled paper packaging materials: development of an analytical method for rapid testing. *Anal. Chim. Acta* **2002**, *467*, 253–260.

(7) Triantafyllou, V. I.; Akrida-Demertzi, K.; Demertzis, P. G. A study on the migration of organic pollutants from recycled paperboard packaging materials to solid food matrices. *Food Chem*. **2007**, *101*, 1759-1768.

(8) Nerín, C.; Asensio, E. Migration of organic compounds from a multilayer plastic-paper material intended for food packaging. Anal. Bioanal. Chem. **2008**, *389*, 589-596.

(9) Jickells, S. M.; Poulin, J.; Mountfort, K. A.; Fernández-Ocaña, M. Migration of contaminants by gas phase transfer from carton board and corrugated board box secondary packaging into foods. *Food Addit. Contam*. **2005**, *22*, 768-782.

(10) Nerín, C.; Cacho, J.; Gancedo, P. Plasticizers from printing inks in a selection of food packagings and their migration to food. *Food Addit. Contam.* **1993**, *10*, 453-460.

(11) Nerín, C.; Batlle, R.; Cacho, J. Design of a test for migration studies in the vapour phase. *Food Addit. Contam.* **1998**, *15*, 84-92.

(12) López, P.; Batlle, R.; Salafranca, J.; Nerín, C. Efficiency of Whole and Skimmed Powdered Milk for Trapping Volatile Compounds Released from Plastic Containers in High-Temperature Applications. *J. Food Prot.* **2008**, *71,* 1889-1897.

(13) Pastorelli, S.; Sanches-Silva, A.; Cruz, J. M.; Simoneau, C.; Paseiro-Losada, P. Study of the migration of benzophenone from printed paperboard packages to cakes through different plastic films. *Eur. Food Res. Technol.* **2008**, *227*, 1585-1590.

(14) Sanches-Silva, A.; Pastorelli, S.; Cruz, J. M.; Simoneau, C.; Castanheira, I.; Paseiro-Losada, P. Development of a method to study the migration of six photoinitiators into powdered milk. *J. Agric. Food Chem*. **2008**, *56*, 2722-2726.

(15) Sagratini, G.; Caprioli, G.; Cristalli, G.; Giardiná, D.; Ricciutelli, M.; Volpini, R.; Zuo, Y.; Vittori, S. Determination of ink photoinitiators in packaged beverages by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. *J. Chromatogr., A* **2008**, *1194*, 213-220.

(16) Sanches Silva, A.; Cruz, J. M.; Sendón García, R.; Franz, R.; Paseiro Losada, P. Development of an in-house method for the incorporation of model migrants in polyethylene films and determination of diffusion constants in food. *Eur. Food Res. Technol*. **2008**, *226*, 1357–1363.

(17) US Department of Agriculture, Agricultural Research Service, 2009, USDA National Nutrient Data. http://www.nal.usda.gov/fnic/foodcomp/

VIII-STUDY OF THE KINETIC OF MIGRATION OF BENZOPHENONE, DPBD AND UVITEX OB: DETERMINATION OF DIFFUSION AND PARTITION COEFFICIENTS BY DIRECT CONTACT AND THROUGH THE VAPOUR PHASE.

Presented as a poster at:

IFT annual meeting and food expo®.Chicago IL, USA. July 17-20, 2010

Abstract

Benzophenone is a photoinitiator for UV-cured inks widely used for printing the external face of food packaging. Uvitex OB and DPBD are optical brighteners used as additives to protect plastics from photo-degradation and enhance life time of polymers. Numerous studies have revealed the migration of these additives into food via direct contact, however there is less information about the migration through the gas where there is indirect contact between the packaging and the food.

The kinetic of migration of benzophenone, Uvitex OB and DPBD from fortified polyethylene films into cake by direct contact and through the gas phase was studied. The cake was selected as a representative dry food with a high fat content.

To perform the migration tests fortified plastic containing additives were place in contact by both sides with the cake; the samples were then overwrapped in aluminium foil and stored in an oven at 20 ºC, 40 ºC and 60 ºC.

To evaluate the migration of benzophenone, DPBD and Uvitex OB through the gas phase, the cake and the fortified polyethylene film were placed with no direct contact using glass ring in a glass container that was hermetically closed. The samples were stored for different temperature-time exposure conditions.

The compounds have been extracted from the plastic films with ethanol (70 ºC, 24 h) and were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD). Migration into the cake was calculated by mass difference, as the amount lost from the film. In order to verify that the migration process was effective, the migrants' concentration in cake samples was checked. Relevant parameters as partition and diffusion coefficients between food and

plastic film have been calculated. Arrhenius equation was applied in order to estimate the diffusion coefficient at any temperature.
The results indicated that the migration of benzophenone by direct contact and through the gas phase happens to a significant extent and for DPBD the migration only took place at 60ºC through the vapor phase. As it was expected, not migration for Uvitex OB through gas phase has occurred.

Keywords: Benzophenone, Uvitex OB, Diphenylbutadiene, migration, vapor phase, kinetic, diffusion coefficients, partition coeffients.

VIII.1.Introduction

From the point of view of Food Safety food packaging has been a matter of concern for European authorities. As a result of the interaction of food and the packaging mass transference phenomena can occur. As a consequence low molecular weight, components can pass from the material to the foodstuffs and constitute a risk for the human health. The European legislation through the Regulation (EU) No 10/2011 establishes the authorized substances that can be used as monomers or additives in the manufacture of food contact materials (FCM). It is based on the called "Positive list". The regulation also establishes the restrictions which they are subject such as specific migration limit (SML) and overall migration limit (OML), those are based on toxicological studies (1)

To verify the compliance of FCM with the legislation migration test conditions (time, temperature and food simulant) are specified. Since these experiments are usually time-consuming, and involve the use of high quantities of solvents and reagents, the legislation allows the use of mathematical diffusion models to estimate the migration whenever there is scientific evidence that real migration is overestimated. These models are based on the Fick's second law.

Within the European project " Flavourings, additives and food contact material exposure task" (FACET) several model migrants with suitable physico-chemical properties (polarity, molecular weight, solubility and so on) were selected to study the kinetic of migration, including benzophenone, diphenylbutadiene (DPBD) and Uvitex OB among others.

Benzophenone is a substance that can be used as a UV stabilizer or as a photoinitiator for UV-cured inks for printing the external face of food packaging. Although numerous studies have revealed the migration of this photoinitiator from paper into food via direct contact (2-4). Recently, it has been reported a

study through the gas phase, where there is not contact with the packaging (5). The authors concluded that the fatty content of food and the porosity as well as the vapor pressure of the migrants play an import role on the process.

DPBD is a model migrant used for fatty foods. Uvitex OB is subtances used as optical brightener, it mainly acts protecting the colour of the polymer against the adverse environmental conditions avoiding the discoloration. It has been reported the migration of both analytes by direct contact into fatty foods (6-8). However, to the best of our knowledge the information about the migration through the vapor phase is very scarce.

Migration from food contact materials into food is a process inevitable and predictable if relevant parameters as partition coefficient, diffusion coefficient, molecular weight and so on are well known. Migration is a process inevitable and predictable that obeys to diffusion Fick's laws which depends on the type of material, the kind of food, the nature of the migrant and the time-temperature conditions of the contact.

The scope of this study is to determine kinetic of migration of benzophenone, DPBD and uvitex from LDPE additivated films into cake by direct contact and through the vapour phase. The cake was selected as a representative dry food with a high fat content and with considerable porosity. Kinetic migration test were performed at three different temperatures. Additionally, partition and diffusion coefficients between film and food samples were determined. On the other hand Arrhenius equation was employed in order to estimate diffusion coefficient at any temperature.

VIII.2.Materials and methods

VIII.2.1. Chemicals and Reagents

Standard of Trans, trans-1,4-diphenyl-1,3-butadiene (DPBD) (CAS number 538-81-8; purity ≥98) and Benzophenone (CAS number 119-61-9) were supplied by Sigma-Aldrich. 2,5-bis (5-tert-butyl-2-benzoxazolyl) thiophene or Uvitex OB purity 99 % (CAS number 7128-64-5) was provided by Fluka. Chemical structure and important chemical properties are presented in table 1.

All chemicals were of HPLC-grade. Ethanol absolute and acetonitrile were purchased from Merck (Darmstadt, Germany). Tetrahidrofurane was provided by Scharlau (Barcelona, made in the European Union). Ultrapure water was obtained employing a Milli-Q filter system (Millipore Bedford, MA).

Stock standard solutions were prepared in Ethanol absolute.

VIII.2.2. Stock standard solutions and calibration curve

Stock standard solutions were prepared in Ethanol absolute. To obtain stock standard solutions, an appropriate quantity of each standard was accurately weighted in a volumetric flask and then filled up with ethanol. The calibration curve was constructed using the standard solution in the range from 0.05-10$\mu g.ml^{-1}$. Each measurement was made by triplicate. The solution were injected and analyzed by High-Performance-Liquid-Chromatography (HPLC). The calibration line for each model substance was obtained by plotting the peak area (average of the three areas) of the substances against the concentration in the calibration solutions in $\mu g.ml^{-1}$. Regression parameters and correlation coefficients have been calculated.

VIII.2.3. Cake samples

Cake samples were selected as representative dry food to conduct migration through vapour phase and by direct contact. Cake was obtained in a local supermarket.

The cake was sliced with a low thickness in pieces of a dimension of 1 x 10 cm2 and then were accurately weighted.

VIII.2.4. Preparation of LDPE additivated films

The thickness of the LDPE films used in the study was measured at different points along the film with a hand-held micrometrer making the average of ten measurements. The initial concentration of the component in LDPE additivated film was 589.2 ± 18.9 µg/g, 379.6±4.3 µg/g and 845.27±10.326 µg/g to benzophenone, DPBD and Uvitex OB respectively.

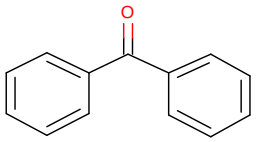
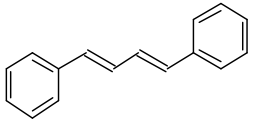
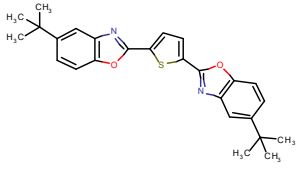
	Structure	MW	MP	BP	Vp	Henry's law	Log P
Benzophenone		182	47.8	305.4	8.2E-05*	1.94E-06	3.18
DPBD		206.287	152	350	3.54E-04*	1.56E-04*	4.76*
Uvitex		430.569	198-199	531.2	7.83E-11 Torr*	N.A	8.61*

Table 1.- Chemical structures and physicochemical properties of the employed additives. (*) Estimated; MW- Molecular weight; MP - Melt point (ºC); BP-Boiling point (ºC); Vp-Vapor pressure (mm Hg ºC); Henry's law- Henry's law constant (atm-m3/mole 25ºC); Log P- Log P octanol-water.

VIII.2.5. Migration test

To perform the migration tests by direct contact the additivated LDPE film containing benzophenone, DPBD and Uvitex was put in contact by both sides with two cake samples; the cake was sliced as described above, the LDPE additivated film was cut in pieces (1 x 10 cm^2). of the same dimension that the food sample. Films and food were accurately weighted. Then the set was wrapped in aluminium foil. All samples were stored in a plastic bag at different selected temperatures (20, 40 and 60 ºC) during different selected times (table 1). Samples were prepared by duplicate.

To evaluate the migration of benzophenone, DPBD and Uvitex through the gas phase, the two cake samples and the fortified polyethylene film were placed with no direct contact between them in a glass container that was hermetically closed and stored in an oven. For that purpose, a glass ring (ø = 3.5 cm and the height = 0.9 cm) was used to separate the two cake samples from the releasing source. The set was placed in a glass container (volume =0.5 L) hermetically closed (Figure 1). The time-temperature storage conditions are presented in Table 1.

Temperature (ºC)	Time (h)
20	2; 4; 8; 12; 24; 48; 96; 168; 240; 360; 552*
40	1; 2; 4; 8; 12; 24; 48; 96; 168; 240; 384*
60	0,5; 1; 2; 4; 8; 12; 24; 48; 96; 168; 240*

(*) Additional-time for migration test through the vapour phase

Table2. Time-temperature migration for direct contact and though vapor phase

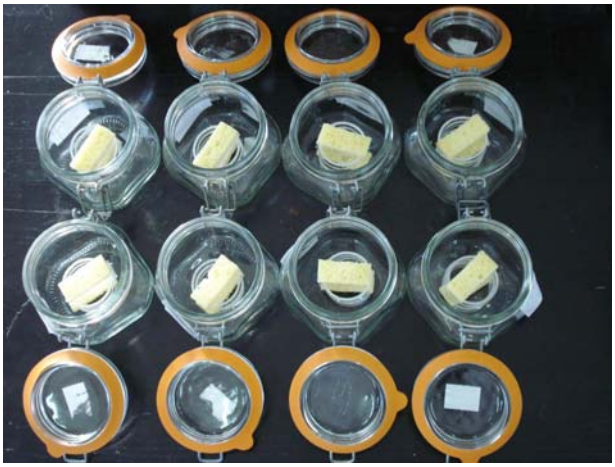


Figure 1. Glass container used to perform the assay by gas phase.

VIII.2.6. Extraction from additivated LDPE films

The extraction of the migrants from LDPE films were performed as follows: films samples were placed in a 60 ml flask with 50 ml of ethanol for 6 hours at 70 ºC. After extraction, it was verify that no loss of solvent occurs during the incubation period. The plastic film was separated from the liquid phase. An aliquot of the liquid phase was filtered by a 0.50 µm filter and analyzed it by HPLC/DAD.

VIII.2.7. Extraction from cake samples

Although the migration into the cake was calculated by mass difference, as the amount lost from the film, in order to verify that the migration process was effective some cake samples were analyzed 6 hours at 70 ºC using ethanol as a extraction solvent. An aliquot of the extraction solution was filtered with a 0.50 µm filter. The analyses were carried out by high-performance-liquid

chromatography with diode-array detector (HPLC-DAD) and the quantification was performed by the external standard method and analyzed it by HPLC

VIII.2.8. HPLC conditions

HPLC analyses were performed on a HP1100 system (Hewlett- Packard, Waldbronn, Germany) equipped with a HP1100 quaternary pump, a degassing device, an autosampler, a column thermostatting system and a diode-array detector (DAD). The stationary phase was a Kromasil C18(25 x 0.32cm I.D., 5 μm particle size) column. A binary mobile phase composed by an eluent A: Water and an eluent B: 30% THF and 70% methanol (v/v). The gradient elution program started at 30% A in the first four minutes. Then, eluent B was gradually increased to 100% to minute 10 afterwards maintained to minute 17. A post-time of 3 minutes was employed to ensure a total cleaning of the column. Total run was 20 minutes Temperature was set at 30ºC in the entire program. The flow rate was 0.5 $ml.min^{-1}$ and the injection volume was 20 μl. The selected wavelengths employed for the compounds detection were 256 nm for benzophenone, 330 nm for DPBD and 372 nm for Uvitex OB. Identification was carried out by comparison of the retention time and UV spectra of the pure standard of each component

VIII.3 Results and discussion

VIII.3.1.Kinetic of migration

The kinetic of migration through the vapor phase and by direct contact of three model migrants (benzophenone, DPBD, Uvitex OB) was studied. The assays

were performed under the time-temperature conditions described above. Since the migration through the vapor phase is slower than for direct contact additional times were analyzed.

To check the homogeneity of film, each 10 samples two were analyzed to determine initial concentration of the migrants in the film. It was confirmed that after 6 hours at 70 ºC a complete extraction was achieved.

The quantification of the analytes was made by the external standard method: calibration curves based on six concentration levels were constructed. The following equations and determination coefficients were obtained: y = 216,28x - 5,6753 (R^2 0.9999) for benzophenone; y = 559,73x - 22,856 (R^2, 0.9998) for DPBD and y = 276,97x + 4,2375 ($R^2$0.9999) for Uvitex OB.

Figure 2 shows the kinetic of the three analytes at the temperatures studied. As can be seen by direct contact the migration of benzophenone and DPBD is higher than the migration of Uvitex OB at any temperature. In the case of the migration through the vapor phase as it was expected benzophenone migrated at 20, 40 and 60 ºC. However, for DPBD only at 60 ºC, it can be appreciated a slightly diffusion and no migration was observed for Uvitex OB.

VIII.3.2.Calculation of partition coefficient LDPE/Food ($K_{P/F}$)

In order to calculate partition coefficient between the LDPE film and the food for each model substance, the following equation was used:

$$K_{P/F} = \frac{C_{P\,eq} \times W_F}{(C_{P\,t} - C_{P\,eq}) \times W_P}$$

Where:

$K_{P/F}$ is the partition coefficient between the LDPE and the food.

$C_{P\,i}$ is the initial concentration of substance in the PE, in $\mu g.g^{-1}$.

$C_{P\,eq}$ is the concentration of substance in the LDPE at equilibrium, in $\mu g.g^{-1}$.

W_F is the food weight, in g.

W_P is the polymer weight, in g.

The partition coefficients obtained experimentally were in good agreement with those estimated for benzophenone and DPBD when the assay was performed by direct contact. However in the case of Uvitex OB seems that the equilibrium was not reached hence the partition coefficients determined experimentally and estimated were not in concordance. Results are presented in table 2

The partition coefficients determined for benzophenone through the vapor phase were similar to those obtained by direct contact which means that the analyte migrated through both routes in the same extent (Figure 3).

For DPBD and Uvitex OB no migration through the vapor phase was observed, except for DPBD at 60 ºC where the equilibrium was reached and $K_{p/f}$ obtained was similar to that calculated by direct contact.

VIII.3.3. Calculation of diffusion coefficient

In order to estimate diffusion coefficient, migration models are based on the 2nd Fick law:

$$\frac{\partial c}{\partial t} = D_P \frac{\partial^2 c}{\partial x^2}$$

Where C is the concentration of the migrant in the plastic film at time t and distance from the origin. D_p is the diffusion coefficient of the migrant in the plastic material. Migration estimation can be performed using equation presented above. The analytical solutions of the Fick equation were developed by Crank. Crank equation is commonly used to calculate parameters as partition and diffusion coefficients:

$$\frac{m_{F,t}}{A} = 0.1 c_{P,0} \rho_P d_P \left(\frac{\alpha}{1+\alpha}\right)\left[1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left(-D_P t \frac{q_n^2}{d_P^2}\right)\right]$$

Where:

$$\alpha = \frac{1}{K_{P,F}} \frac{V_F}{V_P}$$

Where:

mF,t = migrant amount in food at time t (µg)

α= is the mass ratio of migrant in food to that in packaging film at equilibrium;

Dp = is the diffusion coefficient (cm^2/s)

Dp is the thickness of packaging film (cm)

Qn= is the positive roots of the equation tan qn = -α. qn;

t is the migration time (s).

A = Area of the plastic in contact with food.

Cp,o= Initial concentration of the migrant in the plastic (mg/kg).

ρP = Plastic density

VP = Plastic volumen (cm^3)

VF = Food volumen (cm^3)

Diffusion coefficients were only calculated in the experiments performed by direct contact for all the compounds studied, diffusion coefficients increase with temperature (Table 3).

On the other hand, once the diffusion coefficients at three different temperatures have been calculated, it is possible to predict diffusion coefficients at any temperature (so that, migration) employing Arrhenius equation:

$$D = D_0 e^{\frac{E_A}{RT}} \qquad \ln D = \frac{-E_A}{R} \cdot \frac{1}{T} + \ln D_0$$

Where,

E_A is the activation energy in kJ/mol

R is the gas constant kJ/□mol/K

T is the temperature (K)

D is the diffusion coefficient (cm^2/ s).

D_0 is pre-exponential factor (cm^2/ s).

Using this equation it is possible to calculate diffusion coefficients for the selected compound at any temperature (See figure 4).

VIII.4. Conclusions

Briefly, kinetic migration of benzophenone, DPBD and Uvitex OB from an additivated LDPE film to cake samples through vapor phase and direct contact

was studied. Diffusion and partition coefficients have been also determined obtaining satisfactory results. The results indicated that in both cases benzophenone migration occurs in large extent. DPBD reach migration by direct contact at 40 and 60 ºC, meanwhile, only at 60 ºC migration occurs through vapor phase. Migration by gas phase does not occur for Uvitex OB. Only by direct contact migration occurs above 20 ºC In most of cases estimated valor for partition coefficients of the three selected compounds are in good agreement with experimental data. Arrhenius equation has been successfully applied in order to obtain data concerning diffusion coefficients at any temperature.

Acknowledgments

This work was funded by the European Union under Grant Agreement 211686 (Project FACET - Flavours, Additives and food Contact material Exposure Task) The authors are grateful to "Ministerio de Ciencia e Innovación" for a Predoctoral Fellowship FPI (ref. BES-2009-023016) awarded to Rafael Paseiro-Cerrato. The authors are also grateful to C. Casal, P. Blanco and G. Hermelo for their excellent technical assistance.

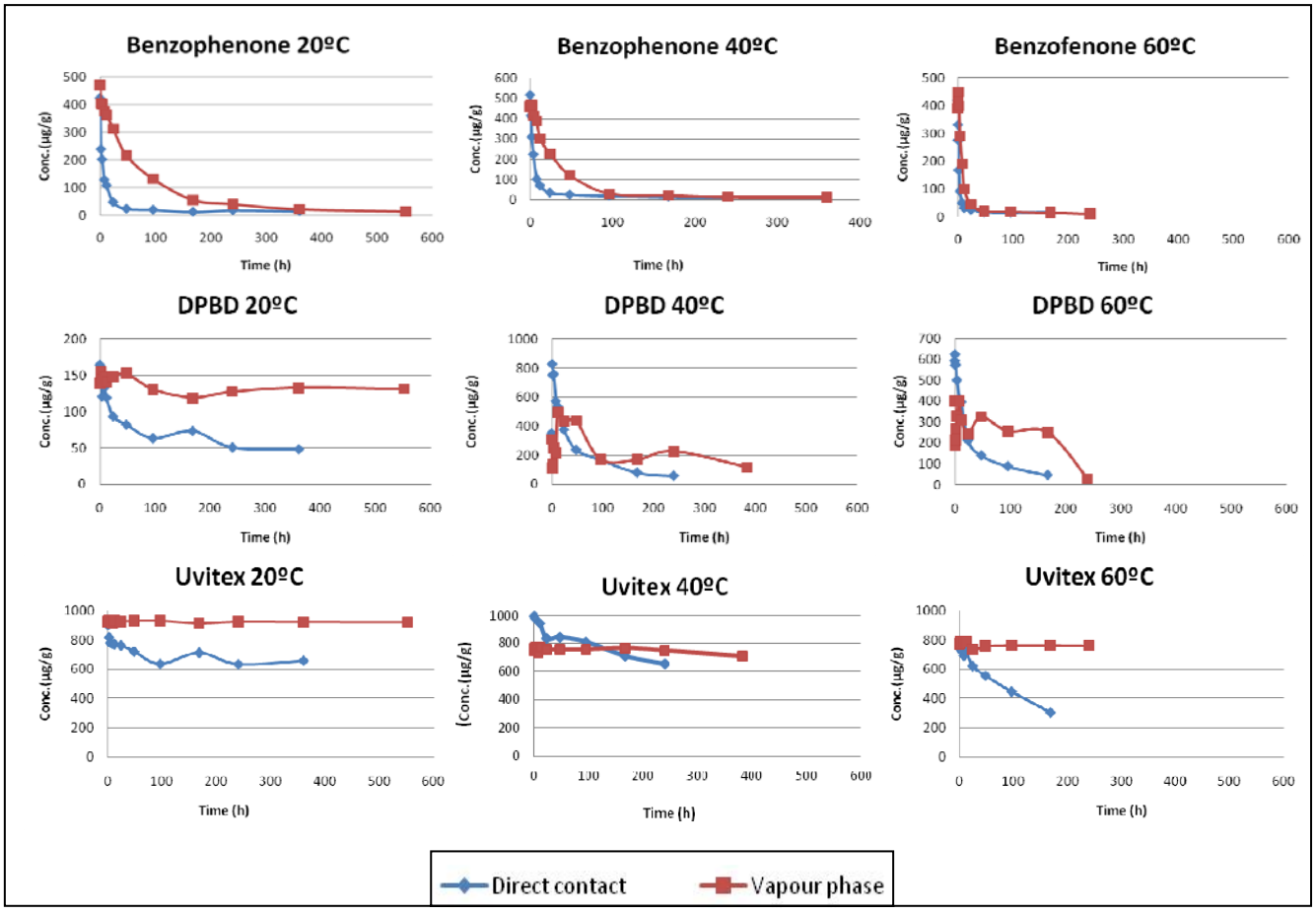


Figure 2 . Migration from LDPE additivated films to cake by direct contact and through vapor phase at different times-temperature

	Benzophenone			DPBD			Uvitex OB		
Temperature	$K_{p/f\ est}$	$K_{p/f\ exp}$	$K_{p/f\ exp\ vp}$	$K_{p/f}$	$K_{p/f\ exp}$	$K_{p/f\ exp\ vp}$	$K_{p/f}$	$K_{p/f\ exp}$	$K_{p/f\ exp\ vp}$
20ºC	1.5	1.1	0.95	15	12.14	536.95	80	80.1	27119.4
40ºC	0.5	0.357	0.34	0.8	0.817	7.47	0.01	66.26	181.09
60ºC	0.6	0.602	0.58	0.7	1.14	0.998	0.01	8.63	79.03

Table 3. K p/f est is the estimated partition coefficient via direct contact; K p/f exp is the experimental partition coefficient via direct contact and K p/f exp vp is the experimental partition coefficient though gas phase.

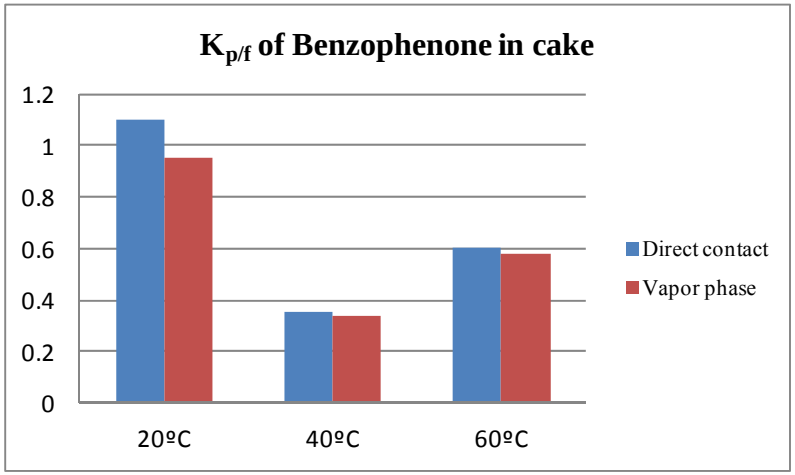


Figure 3. $K_{p/f}$ of benzophenone at three different selected temperatures in cake though vapor phase and by direct contact.

Temperature		Benzophenone		DPBD		Uvitex OB	
T (ºC)	1/T (K^{-1})	D ($cm^2 \cdot s^{-1}$)	ln D	D ($cm^2 \cdot s^{-1}$)	ln D	D ($cm^2 \cdot s^{-1}$)	ln D
20	3.41E-03	1.47E-09	-20.34	1.96E-10	-22.35	9.36E-11	-23.09
40	3.19E-03	9.22E-09	-18.50	1.18E-09	-20.56	5.13E-11	-23.69
60	3.00E-03	1.77E-08	-17.85	1.36E-09	-20.42	2.13E-10	-22.27

Table 4. Employed parameters applied to Arrhenius equation to estimate diffusion coefficients at any temperature.

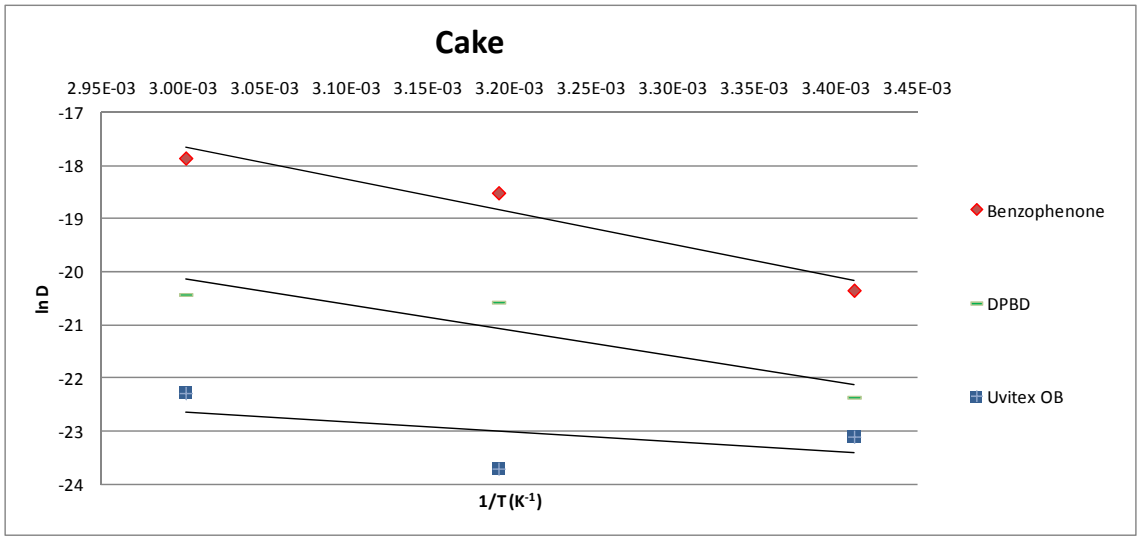


Figure 4. Equation plot for benzophenone, DPBD and Uvitex OB in cake samples predicted by Arrhenius equation.

REFERENCES

(1) Commission Regulation (EU) No 10/2011, Off. J. Eur. Commun. L12 (January)
2011, 1-89.

(2) Triantafyllou, V. I.; Akrida-Demertzi, K.; Demertzis, P. G. Migration studies from recycled paper packaging materials: development of an analytical method for rapid testing. Anal. Chim. Acta 2002, 467, 253–260.

(3) Triantafyllou, V. I.; Akrida-Demertzi, K.; Demertzis, P. G. A study on the migration of organic pollutants from recycled paperboard packaging materials to solid food matrices. Food Chem. 2007, 101, 1759–1768.

(4) Nerín, C.; Asensio, E. Migration of organic compounds from a multilayer plastic-paper material intended for food packaging. Anal. Bioanal. Chem. 2008, 389, 589–596.

(5) Rodríguez-Bernaldo de Quirós, A.; Paseiro-Cerrato, R.; Pastorelli, S.; Koivikko, R.; Simoneau, C.; Paseiro-Losada, P. Migration of Photoinitiators by Gas Phase into Dry Foods. J. Agric. Food Chem. 2009, 57, 10211–10215.

(6) Sanches-Silva, A.; Cruz, J.M.; Sendón-García, R.; Franz, R.; Paseiro-Losada, P. Kinetic migration studiesfrom packaging films into meat products. Meat Sci. 2007, 77, 238-245.

(7) Sanches-Silva, A.; Cruz, J.M.; Sendón-García, R.; Franz, R.; Paseiro-Losada, P. Time-temperature study of the kinetics of migration of DPBD from plastics into chocolate, chocolate spread and margarine. Food Res. Int. 2007, 40,679-686.

(8) Quinto-Fernández E.J.; Pérez-Lamela, C.; Simal-Gándara, J. Analytical methods for food-contact materials additives in olive oil simulant at sub-mg kg-1 level. Food Addit Contam. 2003, 20, 678–683.

IX-RAPID METHOD TO DETERMINE NATAMYCIN BY HPLC-DAD IN FOOD SAMPLES FOR COMPLIANCE WITH EU FOOD LEGISLATION.

Presented as a poster at: Shelf Life International Meeting. Zaragoza, Spain. June 23-25, 2010

Abstract

Natamycin is an antibiotic belonging to the group of polyene macrolides extensively employed as additive to prevent the microbial deterioration in food. The Annex III of the Directive 95/2/EC establishes the maximum levels of the antifungal allowed in the final product.

Reliable and sensitive methods for the analysis of natamycin are required to guarantee compliance with food legislation as well as to improve consumer protection.

In the present paper, a simple and rapid high performance liquid chromatographic method with diode-array detection (HPLC-DAD) to determine natamycin in food samples was developed. Natamycin was extracted from food samples by using methanol acidified with acetic acid. The chromatographic separation was performed on a reversed-phase Kromasil ODS (C18) (150 x 3.20 mm i.d., 5 μm particle size) and the analysis was completed within 6 min. The method was validated in terms of linearity, limits of detection and quantification, repeatability and recovery and satisfactory results were achieved.

With the proposed method natamycin levels were determined in different food items. Data showed that two samples exceeded the limit established by the Annex III of the Directive 95/2/EC. Natamycin was also detected in samples in which its use is not allowed.

LC-MS/MS using electrospray ionisation (ESI) in positive mode was employed to confirm the results obtained by HPLC-DAD.

Keywords: Natamycin, antifungal agent, HPLC-DAD, food samples, HPLC-MS.

IX.1. Introduction

The surface of some foods, such as cheese or sausage, is subject to contamination by microorganisms. This contamination causes deterioration in food, which diminishes food quality and consumers acceptance.

Polymeric films additivated with antimicrobial agents or methods that involve a direct application on the food surface including dipping, spraying or brushing have been extensively used as effective systems to inhibit or retard the growth of the microorganisms. These systems can enhance the food safety and extend the shelf-life of the food products[1-6].

Among the antimicrobial substances employed by the food industry, natamycin has been proven effective in controlling microbial growth. Natamicyn or pimaricin is an antibiotic belonging to the group of polyene macrolides produced by actinomycetes *Streptomyces natalensis*[3,7]; these antibiotics act by binding to sterols, especially ergosterol, in the fungal cell membrane[8]. As a natural preservative has been widely used for the surface treatment of cheeses and dry sausages[7,9,10].

According to the Annex III of the Directive 95/2/EC, the maximum levels of natamycin allowed in the final product should not exceed 1 mg/dm^2 and should not be present at a depth greater than 5 mm[11].

The European Food Safety Authority (EFSA) by request of the European Commission published a scientific opinion on the use of natamycin as food additive. The Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded that the levels of natamycin allowed for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages were not of safety concern[8].

Recently, it was published that German authorities had detected natamycin in wines from Argentina. Its use in wines is not allowed in the EU. The sale of the wines was prohibited and withdrawn from the market. Due to the antifungal is used as a cleaning product in wine cellars, natamycin could be present in the wine as a result of an accidental contamination.

Regarding the analytical methods to determine natamycin in food samples, the process generally involves a solid-liquid extraction by using organic solvents followed by a spectrophotometric[12] or chromatographic analysis with UV detection[4,8].

Dos Santos Pires et al.[12] determine natamycin spectrophotometrically in sliced mozzarella cheese samples after extraction with a mixture of acetonitrile-phosphoric acid, (4:1 v/v).

Koontz et al.[4] quantify the antifungal by reversed-phase high-performance liquid chromatography (RP-HPLC). The separation was performed on a (4.6 x 150 mm, 5 μm) Waters Spherisorb C8 column and using a gradient system composed by (A) methanol-water-acetic acid, (60:40:5, v/v/v), (B) 100% water; and (C) 100% methanol. Detection was carried out at 304 nm.

Recently, liquid chromatography coupled to mass spectrometry have appeared as a powerful analytical tool to determine the additive at very low concentrations in wine samples[13,14].

This study aims to develop a simple and rapid method to determine natamycin in food samples that can be used for quality control. cheeses and dry sausages LC-MS/MS using electrospray ionization (ESI) in positive mode was used to confirm the results.

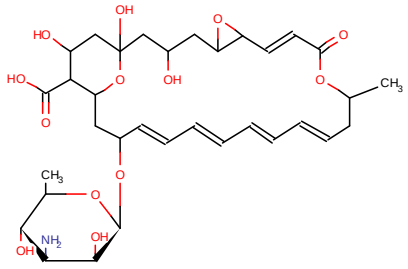
IX.2. Materials and methods

IX.2.1. Reagents

Standard of pimaricin or natamycin (CAS no 7681-93-8), from Streptomyces chattanoogensis, minimum 95% (HPLC) was supplied by Sigma (Steinheim, Germany). The chemical structure and physicochemical properties of natamycin are presented in Table 1. The information was obtained from ChemIDplus Advanced[15]. All reagents were of analytical quality. Acetonitrile hypergrade for LC-MS, methanol and acetonitrile HPLC grade and acetic acid were from Merck (Darmstadt, Germany); formic acid solution puriss. p.a. for HPLC, 50% in water was from Fluka (Steinheim, Germany); water used for all solutions was obtained from Milli-Q water purification system (Millipore) (Bedford, MA, USA).

IX.2.2. Standard solution preparation

Stock standard solutions were prepared by dissolving an accurate quantity of natamycin in methanol acidified with 0.001% acetic acid in a volumetric flask. The flask was shaken until a homogenous solution was formed. The stock standard solution had a concentration of 100 µg/mL. Finally, the solution was diluted with methanol acidified with 0.001% acetic acid until the desired concentration was achieved.

Chemical structure	Physico chemical characteristics	
	Formula	$C_{33}H_{47}NO$
	CAS No	7681-93-8
	MW	665.7
	Melting Point	290 ºC[a]
	log P (octanol-water)	-3.670[b]
	Water solubility	4100 mg/L (21ºC)

a: Experimental

b: Estimated

Table 1. – Chemical structure and physicochemical characteristics of natamycin

IX.2.3. Apparatus

IX.2.3.1. HPLC-UV analysis

An HPLC HP1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with a quaternary pump, a degassing device, an autosampler, a column thermostatting system, a diode-array detector (DAD), and Agilent Chem-Station for LC and LC/MS systems software was used. Separation was performed on a Kromasil ODS (C18) (150 x 3.20 mm i.d., 5 µm particle size) column thermostatted at 25 ºC. Acetonitrile (A) and Milli-Q water (B) were used as mobile phase. Samples were eluted in gradient mode. The gradient elution programme is shown in Table 2. Three selected wavelengths were set in DAD detector, 291.4, 304.4 and 319.4 nm, corresponding to the three absorption peaks of the characteristic natamycin spectrum (Figure 1 A). The injection volume was 20µL.

Time(min)	A%	B%	Flow (mL/min)
0	30	70	0.5
10	60	40	0.5
15	60	40	0.5

(A) Acetonitrile (B) Water

Table 2. - HPLC elution profile program.

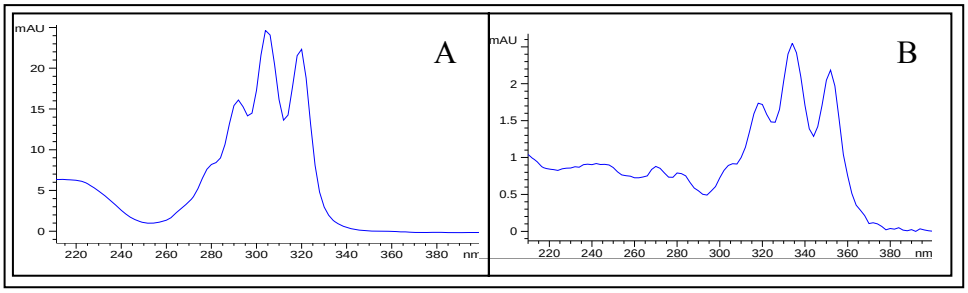


Figure 1. UV spectrum of natamycin (A), UV spectrum of unknown substance (B).

IX.2.3.2. LC-ESI/MS/MS analysis

An HPLC/MS/MS system comprising an Accela autosampler and Accela 1250 pump fitted with a degasser, a quaternary pump and a column oven coupled to a

triple quadrupole mass spectrometer TSQ Quantum Access max controlled by Xcalibur was used (Thermo Fisher Scientific, San José, CA, USA).

MS data were acquired in the positive ion mode employing electrospray ionization (ESI). Mass spectra were monitored in the mass range *m/z* 100-800. Optimized MS/MS detector settings were: Spray voltage 3000 V, vaporizer temperature 350 ºC, ion transfer tube temperature 350 ºC. Nitrogen was used as sheath gas (pressure 20 psi) and as auxiliary gas (pressure 5 arbitrary units), Argon was used as the collision gas (1.5 mTorr) and tube lens voltage was 69 V. The stationary phase was an Ace 3 C18 HL (150 mm x 3.0 mm, i.d. 3 μm particle size, Advanced Chromatography Technologies) and the gradient elution was the same as in the HPLC-DAD system and the mobile phase was composed by acetonitrile 0.1 % formic acid (A) and Milli-Q water 0.1 % formic acid (B).

IX.2.4. Food samples

Twenty six samples were analyzed comprising several types of cheese; fresh soft, cured, semi-cured, Roquefort and Camembert; seven different sausages including ham, "chorizo", salami and sausages and twelve commercial white and red wines from different regions of Spain and from Argentina. All samples were purchased in local supermarkets.

IX.2.5. Sample preparation

Prior to analysis, solid samples were carefully cut in slices from the outside to the centre of the sample with approximately 2 mm of thickness. Three representative parts of the sample were analyzed; the rind, the layer just below

the rind and the layer at a depth of 5 mm from the outside. All samples presented a surface of 2.25 cm^2. Analyses were carried out in duplicate.

Natamycin was extracted from the samples as follows: approx. 0.5 g was extracted with 10 mL methanol acidified with 0.001% acetic acid and was hand-shaken for 5 minutes. Afterwards, 1 mL of the solution was removed and diluted to 2 mL with Milli-Q water. Samples were stored at -22 ºC overnight. Finally, the samples were filtered through 0.50 μm PTFE membrane filter and injected into the chromatographic system.

Wine samples were filtered through a 0.50 μm PTFE membrane filter and directly injected into the chromatograph without previous treatment.

IX.2.6. Identification and quantification

Identification of natamycin was made by comparison of the retention time and UV-Vis spectrum of the sample with that of a pure standard solution. Quantification was carried out with the external standard method. Calibration line was constructed based on five concentrations levels of standard solution within the 0.1-5 μg/mL range. Each point is the average of two peak-area measurements.

IX.3. Results and discussion

IX.3.1. Analytical method

The use of natamycin as food additive is regulated by the Annex III of the Directive 95/2/EC[11]. Rapid, sensitive and accurate methods for compliance with European food legislation are required. To the best of our knowledge there are

few methods to determine the additive in food samples. The analysis generally comprises a solid-liquid extraction with organic solvents such as methanol or acetonitrile followed by a chromatographic analysis.

In the present work we intended to develop a simple, rapid and sensitive method for the routine determination of the antifungal. Appropriate extraction was achieved with methanol acidified with 0.001% acetic acid and water. The extracts were stored at -22 ºC overnight in order to precipitate lipids and proteins. In addition, the extraction process described here is an environmentally friendly procedure and consumes a small volume of solvent, 10 mL per sample.

A suitable chromatographic resolution of the peak of natamycin was achieved with a reversed-phase (C-18) and a gradient elution system consisting of acetonitrile and water. Under these conditions the analysis was completed within 6 min. Typical RP-HPLC chromatogram of a standard solution is shown in Figure 2.

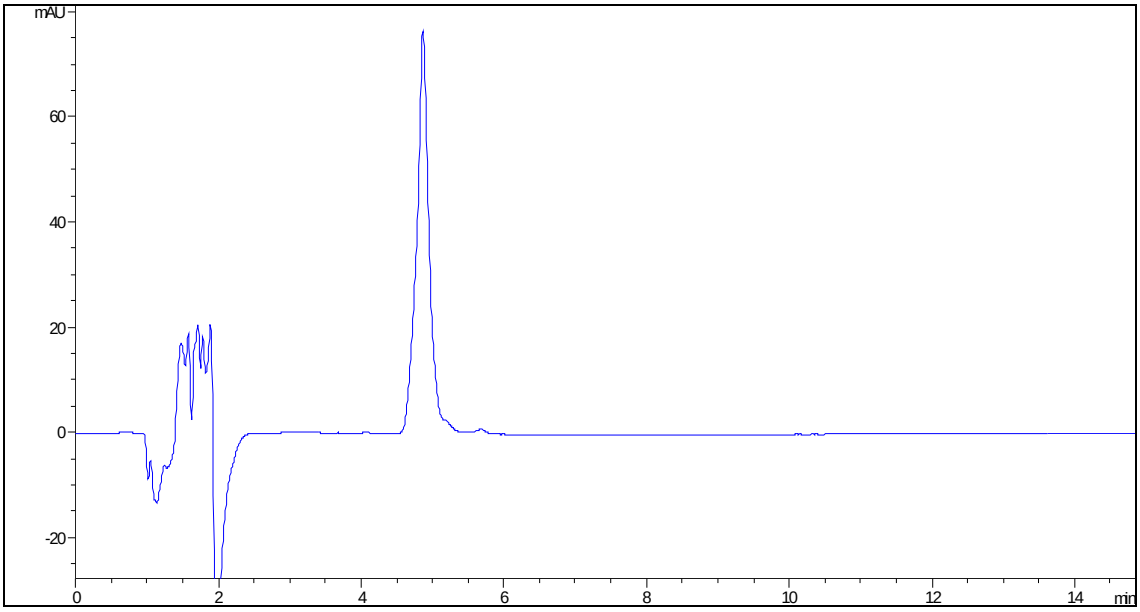


Figure 2. Chromatogram of a standard solution of natamycin recorded at 319 nm.

IX.3.1.1. In house method validation

The proposed method to determine natamycin in food samples was validated in terms of linearity, limits of detection and quantification, repeatability and recovery.

The method was calibrated using a series of natamycin standard solutions of known concentrations. The calibration curve was constructed using five concentration levels and it was fitted to a linear equation within the 0.1-5 μg/mL range. The equation obtained was y = 162.01x + 8.5899 and the determination coefficient 0.9991.

UV spectrum of natamycin shows three major absorption peaks in the range 290-320 nm. Although 304 nm is the wavelength commonly used to quantify the antifungal,[4,10] due to spectral interferences from other compounds, 319 nm was selected as the best option for the analysis of complex matrices as are food samples.

The limits of detection and quantification (defined as a signal three and ten times, respectively the high of noise level) determined in accordance with the Analytical Chemical Subcommittee guidelines[16] were 0.01 μg/mL and 0.05 μg/mL, respectively, which correspond approximately to 9 $\mu g/dm^2$ and 45 $\mu g/dm^2$ employing this extraction procedure. According the legislation the maximum level of natamycin allowed in the final product should not exceed 1 mg/dm^2, thus the proposed method has sufficient sensitivity to detect the additive at the regulatory level.

The repeatability was determined by analyzing ten replicates of standards at a concentration level of 1 mg/L expressed as the percentage of R.S.D. (% R.S.D.(n= 10)) resulted to be 1.36%± 2.36.

Recoveries (mean (%) ± S.D. (n=3)) were estimated on the basis of determination after spiking the samples (cheese sample nº 1 and sausage sample nº 3) with known amounts of natamycin at a concentration level of 2 μg/mL. Appropriate recoveries were achieved for both types of samples; 90.3% ±11.4 and 92.4%±6.5 for cheese and sausage samples, respectively.

IX.3.2. Food samples analysis

The Annex III, part C of the Directive 95/2/EC[11] establishes the use of natamycin as additive for the surface treatment of hard, semi-hard and semi-soft cheese and dry, cured sausages at a maximum level of 1 mg/dm^2 and should not be present at a depth of 5 mm.
A total of twenty six samples including cheeses, sausages and wines bought in local supermarkets were analyzed to evaluate the natamycin content. Each sample of cheese and sausage was divided into three parts, as has been described above, and subsequently analyzed. Foods where addition of natamycin is not allowed were also included in the study.

Results corresponding to cheese and sausage samples expressed as mg/dm^2 are listed in tables 3 and 4, respectively. Concentrations ranged from non-detectable to 5.9 mg/dm^2. Samples 1 and 7 corresponding to semi-cured and cured cheese exceeded the maximum allowable limit in the rind. This result could be attributed to an unsuitable incorporation of the antifungal onto cheese surface. However, no detectable quantities of natamycin were found in the internal zone. Samples 3 and 4 (soft cheeses), for which natamycin use is not allowed, showed values of 5.9 and 0.41 mg/dm^2 in the rind. As it was expected no natamycin was detected in samples 2 and 6. On the other hand, one unknown substance with a

retention time of 9.1 min, which had a similar spectrum to the natamycin, was found in the rind of sample number 5. This spectrum also presents three peaks of maximal absorption, but at different wavelengths with respect to natamycin: 318.4, 334.4 and 352.4 nm (Figure 1 B). Similarities among this two spectrums maybe because of those two substances belongs to the same class of polyene macrolides, as it was found for other substances as amphotericin B and tetrafungin among others, where a similar spectra with different wavelengths have been observed[17-19].

Regarding the sausage samples, none of the products analyzed exceeded the limit of 1 mg/dm^2 established by the European Directive. Only the sample 3 presented natamycin on the casing.

With respect to wine samples no natamycin was found, at the limit of detection level, in any of the samples tested.

Samples		Representative zones		
Number	Type	Rind Concentration (mg/dm^2)	Under the rind (< 5 mm depth) Concentration (mg/dm^2)	Internal (≥ 5 mm depth) Concentration (mg/dm^2)
1	Semi-cured cheese	4.9±1.1	0.16±0.18	N.D.
2	Roquefort	N.D.	N.D.	N.D.
3	Soft and fatty cheese	5.9±0.49	< LOQ	N.D.
4	Soft cheese	0.42±0.05	0.35±0.05	N.D.
5	Camembert	N.D.	N.D.	N.D.
6	Fresh cheese	N.D.	N.D.	N.D.
7	Cured cheese	1.4±0.38	< LOQ	N.D.

N.D.: Not detected
LOQ: Limit of quantification
n= 2

Table 3.- Natamycin in cheese samples.

Samples		Representative zones		
Number	Type	Casing Concentration (mg/dm^2)	Under the casing (< 5 mm depth) Concentration (mg/dm^2)	Internal (≥ 5 mm depth) Concentration (mg/dm^2)
1	Ham	N.D.	N.D.	N.D.
2	Cured ham	N.D.	N.D.	N.D.
3	"Chorizo" Brand 1	0.45±0.04	< LOQ	< LOQ
4	Salami	N.D.	N.D.	N.D.
5	"Chorizo" Brand 2	< LOQ	< LOQ	< LOQ
6	Sausage Brand 1	N.D.	N.D.	N.D.
7	Sausage Brand 2	N.D.	N.D.	N.D.

N.D.: Not detected
LOQ: Limit of quantification
n= 2

Table 4.- Natamycin in sausages samples.

IX.3.3. HPLC-MS analysis

A LC-ESI-triple quadrupole analyzer system that operated in the positive-ion mode was used as a confirmatory technique. Under the analytical conditions described for the HPLC-MS system, natamycin exhibited a fragment at *m/z* 666.6 which corresponds to the protonated natamycin ion $[M + H]^+$. With the same LC/MS conditions, sample number 5 has been also analyzed and two molecular ions have been identified at 511.3 and 533.4 *m/z.* However, due to the lack of a standard, the identification of this substance has been not possible. Further studies are required in order to identify this compound.

Natamycin MS/MS analysis was also carried out by isolating and fragmenting the protonated natamycin ion. Identification of the main products is presented in the table 5.

Briefly, the proposed method is simple, rapid, environmentally friendly and has sufficient sensitivity to detect the antifungal at the regulatory level, so the method could be applicable as screening tool for compliance with European food legislation.

HPLC/ESI-MS/MS provide a powerful tool to confirm the results obtained by HPLC–DAD analysis.

Acknowledgments

Authors are grateful to “Ministerio de Ciencia e Innovación” for the Predoctoral fellowship FPI (ref. BES-2009-023016) awarded to R. Paseiro-Cerrato. R. Sendon is grateful to the “Parga Pondal” Program financed by “Consellería de Innovación e Industria, Xunta de Galicia” for her postdoctoral contract. The authors are also grateful to C. Casal, P. Blanco and G. Hermelo for their excellent technical assistance.

LITERATURE CITED

(1) Pintado, C. M. B. S.; Ferreira, M. A. S. S.; Sousa, I. Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin. *Food Control* **2010**, *21,* 240-246.

(2) Türe, H.; Eroğlu, E.; Soyer, F.; Özen, B. Antifungal activity of biopolymers containing natamycin and rosemary extract against Aspergillus niger and Penicillium roquefortii. *Int. J. Food Sci. Technol.* **2008**, *43,* 2026–2032.

(3) Koontz, J. L.; Marcy, J. E. Formation of natamycin: cyclodextrin inclusion complexes and their characterization. *J. Agric. Food Chem.* **2003a**, *51,* 7106-7110.

(4) Koontz, J. L.; Marcy, J. E.; Barbeau, W. E.; Duncan, S. E. Stability of natamycin and its cyclodextrin inclusion complexes in aqueous solution. *J. Agric. Food Chem.* **2003b**, *51,* 7111-7114.

(5) Fajardo, P.; Martins, J. T.; Fuciños, C.; Pastrana, L.; Teixeira, J. A.; Vicente, A. A. Evaluation of a chitosan-based edible film as carrier of natamycin to improve the storability of Saloio cheese. *J. Food Eng.* **2010**, *101,* 349–356.

(6) Hanušová, K.; Dobiáš, J.; Klaudisová, K. Effect of packaging films releasing antimicrobial agents on stability of food products. *Czech. J. Food Sci*. **2009**, *27,* S347-S349.

(7) Moreira de Oliveira, T.; Ferreira Soares, N.; Magela Pereira, R.; de Freitas Fraga, K. Development and evaluation of antimicrobial natamycin-incorporated film in Gorgonzola cheese conservation. *Packag. Technol. Sci.* **2007**, *20,* 147–153.

(8) Scientific Opinion of EFSA prepared by the Panel on Food Additives and Nutrient Sources added to Food (ANS) on the use of natamycin (E 235) as a food additive. *EFSA J.* **2009**, *7*, 1412-1437.

(9) Var, I.; Erginkaya, Z.; Güven, M.; Kabak, B. Effects of antifungal agent and packaging material on microflora of Kashar cheese during storage period. *Food Control* **2006**, *17,* 132–136.

(10) Hanušová, K.; Šťastná, M.; Votavová, L.; Klaudisová, K.; Dobiáš, J.; Voldřich, M.; Marek, M. Polymer films releasing nisin and/or natamycin from polyvinyldichloride lacquer coating: Nisin and natamycin migration, efficiency in cheese packaging. *J. Food Eng.* **2010,** *99,* 491–496.

(11) European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. *Off. J.* **1995**, *L061*, 1.

(12) dos Santos Pires, A. C.; Ferreira Soares, N.; de Andrade, N. J.; Mendes da Silva, L.; Peruch Camilloto, H. G.; Campos Bernardes, P. Development and evaluation of active packaging for sliced mozzarella preservation. *Packag. Technol. Sci.* **2008**, *21,* 375–383.

(13) Mariño Repizo, L.; Dante Martínez, L.; Olsina, R. A.; Cerruti, S.; Raba, J. A novel and rapid method for determination of natamycin in wines based on ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry: validation according to the 2002/657/EC European decision. *Anal. Bioanal. Chem*. **2012**, *402,* 965-973.

(14) Roberts, D. P. T.; Scotter, M. J.; Godula, M.; Dickinson, M.; Charlton, A. J. Development and validation of a rapid method for the determination of natamycin in wine by high-performance liquid chromatography coupled to high resolution mass spectrometry. *Anal. Methods* **2011**, *3,* 937-943.

(15) ChemIDplus Advanced United States National Library of Medicine National Institutes of Health. Available from: http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp. Accessed April 2011.

(16) American Chemical Society (ACS). Subcommittee of environmental analytical chemistry. *Analytical Chemistry* **1980**, *52,* 2242–2280.

(17) Slisz, M.; Cybulska, B.; Mazershi, J.; Grzybowska, J.; Borowski, E. Studies of the effects of antifungal cationic derivatives of amphotericin B on human erythrocytes. *J. Antibiot.* **2004**, *57*, 669-678.
(18) Veiga, M.; Fabrega, J. Tetrafungin, a new polyene macrolide antibiotic 1. Fermentation, isolation, characterization, and biological properties. *J. Antibiot.* **1983**, *36,* 770-775.
(19) Molzahn, S. W.; Woods, R. A. Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae. J. Gen. Microbiol.* **1972,** *72*, 339-348.

CONCLUSIONES

1. Se ha realizado una extensa revisión sobre los métodos empleados para la determinación de aminas polifuncionales. Se ha observado que los métodos para la determinación de las aminas de forma simultánea son muy escasos. Además, se incluyen propiedades fisicoquímicas de dichas aminas experimentales o estimadas y que son de utilidad para el desarrollo de métodos analíticos.

2. Se ha desarrollado un método multianalito para la determinación de aminas polifuncionales utilizadas como monómeros en la fabricación de materiales de envasado de alimentos. El método descrito es sensible, preciso y puede ser utilizado como una buena herramienta analítica para la determinación de las aminas en los laboratorios de control. Bajo las condiciones de migración más comunes (40 ºC durante diez días) las aminas muestran una estabilidad aceptable en simulantes de alimentos, a excepción del aceite de oliva, donde se observó una pérdida de 100% de las aminas. Esto indica que las aminas pueden reaccionar con los componentes presentes en el medio graso. La migración de los productos de reacción formados en los alimentos puede constituir un riesgo para la salud de los consumidores. Como resultado de este estudio un posterior trabajo ha sido desarrollado con el fin de identificar qué tipo de compuestos se forman a través de la posible reacción entre las aminas y los componentes de naturaleza grasa de los alimentos.

3. Se ha desarrollado un método para determinar histamina que implica un proceso de derivatización con cloruro de dansilo. El método fur optimizado y aplicado con éxito para controlar el contenido de la amina biógena en muestras de alimentos de origen marino. Los niveles de histamina encontrados en las muestras utilizadas, se encuentran por debajo de los límites establecidos por la legislación europea.

4. Se ha realizado una comparación de UPLC-MS/MS con DART para la determinación de aminas aromáticas primarias con el fin de determinar si DART

se puede utilizar como método de cribado para la identificación de las mismas en utensilios de cocina. El método cromatográfico se ha validado en términos de linealidad, la repetibilidad, recuperación, límite de detección y cuantificación. DART demuestra que es una herramienta útil y poderosa para identificar muestras de aminas aromáticas primarias en los polímeros de una forma rápida y sencilla.

5. Por primera vez, los compuestos resultantes de la reacción entre aminas (MXDA) y dos ácidos grasos presentes en la mayoría de las grasas han sido caracterizados y extraídos de muestras reales. Estos compuestos pertenecen a la familia de las amidas de ácidos grasos. El aislamiento e identificación de estos productos de reacción podría explicar la inestabilidad de las aminas en los alimentos grasos. La caracterización completa de los compuestos se llevó a cabo mediante el uso de varias técnicas, incluyendo NMR protón y carbono 13, espectroscopia infrarroja, espectrofotometría ultravioleta, impacto electrónico y por medio de espectrometría de masas. Los compuestos identificados y aislados fueron la diolemida y la dipalmitida. La reacción ha sido probada en condiciones reales utilizando aceite de oliva como medio graso. Se ha desarrollado un método sencillo y rápido para extraer las amidas a partir de aceite de oliva y se ha aplicado satisfactoriamente. La diolemida y la dipalmitida se identificaron con éxito por LC-MS/MS. También se emplearon las reglas de Cramer para hacer una estimación de su posible toxicidad. Estos productos pueden representar un peligro para la salud humana, por lo tanto, de deben realizar más estudios sobre la toxicidad de estas sustancias.

6. Se ha estudiado la migración de benzofenona y algunos de sus derivados empleados como fotoiniciadores a través de la fase de vapor. Los resultados muestran que la migración de benzofenona y algunos derivados relacionados se producen en gran medida a través de la fase gaseosa. Además, la porosidad y el

contenido de grasa en el alimento tienen una gran influencia en el proceso de migración por esta vía. La presión de vapor de estos compuestos puede tener un papel esencial en la migración.

7. Se ha estudiado la cinética de migración, a través de la fase de vapor y por contacto directo, de la benzofenona, del difenilbutadieno y del Uvitex OB desde un film de polietileno de baja densidad aditivado a muestras de bizcocho. Los coeficientes de difusión y partición han sido determinados. Los resultados indican que la benzofenona migra en su totalidad por contacto directo y a través de la fase de vapor. Sin embargo, la migración del difenilbutadieno y del Uvitex OB se produce básicamente por contacto directo a las temperaturas estudiadas.

8. Se ha desarrollado un método de análisis para la determinación de natamicina en alimentos. El método propuesto es simple, rápido y respetuoso con el medio ambiente. Además, tiene sensibilidad suficiente para detectar natamicina en los niveles que estipula la legislación, por lo que el método podría ser utilizado como método de rutina para el cumplimiento con la legislación alimentaria europea. Sólo dos de las muestras utilizadas en este estudio tenían niveles superiores al límite establecido por la legislación europea de aditivos. Los resultados de este estudio se han confirmados por espectrometría de masas.

CONCLUSIONS

1. An extensive review concerning methods employed to determine polyfuncional amines have been reported. Methods to determine several amines simultaneously are very scarce and should be developed. On the other hand, physicochemical properties of the substances determined experimentally or estimated have been included since they are important in order to develop analytical methods.

2. A multi-analyte method for the determination of polyfunctional amines used as monomers in the manufacture of food packaging materials has been carried out. The described method is sensitive and precise and could be used as a good analytical tool for the routine determination of amines in control laboratories.

Under the most common testing conditions (40 ºC for ten days) amines showed an acceptable stability in food simulants, except in olive oil, where a loss of 100% was observed for all amines. This indicates that amines could react with components present in fatty medium. The migration of the reaction products formed into food could constitute a risk for consumers' health. As a result of this study a later work has been developed in order to identify what types of compounds are formed with the possible reaction among amines and components of the fatty nature.

3. A method which involves a derivatization process with dansyl chloride to determine histamine in seafood has been presented. The optimized method was successfully applied to monitor the histamine content in sea food samples. On the other hand histamine levels found in surimi seafood are below the limits established by the European legislation.

4. An ultra performance liquid chromatograph coupled a tandem spectrometrer (UPLC-MS/MS) and direct analysis in real time (DART) coupled to an accuTOF (time of flight) have been compared in order to determine if DART may be used as screening method to identified primary aromatic amines (PAAs) in kitchen

utensils. An UPLC/MS/MS method has been developed and validated in terms of linearity, repeatability, recovery, LOD and LOQ. DART has shown that is a powerful tool to identified samples with a high concentration of PAAs in polymers, being useful in order to carry out rapid screening methods.

5. For the first time, the yielded compounds that occur by the reaction between amines (MXDA) and two fatty acids present in most of fats have been study. The resulting molecules belong to the family of fatty acid amides and may explain instability of amines in fatty food. A complete characterization of the resulting compounds by using several analytical techniques including ^{1}H and ^{13}C NMR spectroscopy, Infrared spectroscopy, UV spectrophotometry, Electron Impact (EI) mass spectrometry, LC-MS/MS have been used successfully. The compounds were identified and isolated. The reaction was tested under real conditions using olive oil as fatty medium. A simple and rapid method to extract the amides from olive oil was developed. Diolemide and dipalmitide were successfully identified by LC-MS/MS. On the other hand, toxicity of the compounds is predicted by Cramer rules. These products could represent a hazard for the human heath; consequently, more studies concerning the toxicity of these substances should be performed.

6. A study of the migration of benzophenone-based photoinitiators through the vapor phase has been carried out. Results show that migration of benzophenone and some related derivatives occurs in to a large extent. Furthermore, porosity and the fat content of the food have strong influence on the migration process. Vapor pressure of the benzophenone related derivatives may have a special role in migration though vapor phase.

7. Kinetic migration, through vapour phase and direct contact, of benzophenone, diphenyl butadiene and Uvitex optical brightener from an additivated low density polyethylene films into cake samples was studied. Diffusion and partition

coefficients have been also determined. The results indicated that in both cases benzophenone migration occurs in large extent.

8. A method to determine natamycin in food has been developed. The proposed method is simple, rapid and environmentally friendly. The method has sufficient sensitivity to detect the antifungal at the regulatory level, so the method could be applicable as screening tool for compliance with European food legislation. Only two samples analyzed in this experiment exceeded the limit established by the European Legislation. Results have been confirmed by HPLC tandem mass spectrometry.