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ESTUDIO DE LA MIGRACIÓN DE DISTINTOS COMPONENTES DE LOS MATERIALES PLÁSTICOS A LOS ALIMENTOS

Memoria presentada para optar al Grado de Doctor

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INFORMA: Que Dña. Raquel Sendón García presenta el trabajo titulado "ESTUDIO DE LA MIGRACIÓN DE DISTINTOS COMPONENTES DE LOS MATERIALES PLÁSTICOS A LOS ALIMENTOS" realizado bajo la dirección del Dr. Perfecto Paseiro Losada y de la Dra. Mª Concepción Pérez Lamela, en los laboratorios de Bromatología de la Facultad de Farmacia de la Universidad de Santiago de Compostela.

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AUTORIZAN a Dña. Raquel Sendón García a presentar la Tesis titulada "ESTUDIO DE LA MIGRACIÓN DE DISTINTOS COMPONENTES DE LOS MATERIALES PLÁSTICOS 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 Febrero de 2005.

Fdo. Dr. Perfecto Paseiro Losada Fdo. Dra. Mª Concepción Pérez Lamela.

Fdo. Dña. Raquel Sendón García.

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ABREVIATURAS

ABS	Acrilonitrilo/Butadieno/Estireno
ADI	Aceptable Daily Intake (Ingesta Diaria Admisible)
AESA	Agencia Española de Seguridad Alimentaria
APCI	Atmospherical Presure Chemical Ionization (Ionización Química a Presión Atmosférica)
ATBC	Tri-n-butil citrato
BADGE	Bisfenol A diglicidil éter
BFDGE	Bisfenol F diglicidil éter
BHT	Hidroxitolueno Butilado
Bp	Boiling Point (Punto de Ebullición)
BSE	Bovine Spongifrom Encephalopathy (Encefalopatía Espongiforme Bovina)
CAS	Chemical Abstracts Service
Da	Daltons
DEHA	Bis 2-diethylehxyl adipate
DIPN	Diisopropilnaftaleno
DPBD	Dipheynilbutadiene
DMF	Dimetilformamida
E/VA	Etileno/Acetato de Vinilo
EFSA	European Food Safety Authority (Agencia Europea de Seguridad Alimentaria)
Em	Emisión
ESI	Electrospray Ionization
EUFIC	European Food International Council (Consejo Europeo sobre la Alimentación)
EVOH	Etileno/Alcohol Vinílico
Ex	Excitación
FAO	Food And Agriculture Organization of the United Nations (Organización de las Naciones Unidas para la Agricultura y la Alimentación)

FDA	U. S. Food and Drug Administration (Administración de Drogas y Alimentos de los EE.UU)
FCM	Food Contact Materials (Materiales en Contacto con Alimentos)
FID	Flame Ionization Detector (Detector de ionización de llama)
Fl	Fluorescencia
Fp	Flash Point (Punto de Inflamación)
GC	Gas Chromatography (Cromatografía en fase gaseosa)
GPC	Gel Permeation Chromatography (Cromatografía de Gel Permeación)
GTA	Glicerol Triacetato
HDPE	High Desnity Poliethylene (Polietileno de Alta Densidad)
HPLC	High Performance Liquid Chromatography (Cromatografia de Líquidos de Alta Resolución)
i.d.	Internal diameter (Diámetro interno)
ILSI	International Life Sciences Institute
IUPAC	International Union of Pure and Applied Chemistry (Unión Internacional de Química Pura y Aplicada)
LC-MS	Liquid chromatography- Mass Spectrometry (Cromatografia de gases-Espectrometria de Masas)
LDPE	Low Density Polyethylene (Polietileno de Baja Densidad)
LOD **DL**	Limit of Detection (Límite de detección)
Log Kow	Coeficiente de Partición Octanol-Agua
M **MW**	Peso Molecular
MAE	Microwave-assisted extraction (Extracción por microondas)
mp	Melting Point (Punto de Fusión)
MERCOSUR	Mercado Común del Sur
MEK	Methyl Ethyl Ketone (Metiletilcetona)
MPPO	Modified polyphenylene oxide (óxido de polifenileno modificado) Tenax ®

m/z	Mass to charge ratio (Relación masa carga)
NOGE	Novolac glicidil éteres
NPLC	Normal Phase Liquid Chromatography (Cromatografía Líquida en Fase Normal)
PA	Poliamidas Alifáticas Semicristalinas
PAN	Poliacrilonitrilo
PB	Polibuteno
PC	Policarbonato
PE	Polietileno
PET	Poli (tereftalato de etileno)
PMP	Polimetilpenteno
PP	Polipropileno
PS	Poliestireno
PTFE	Polytetrafluorethylene (Politetrafluoretileno o Teflón)
PVC	Poli (cloruro de vinilo)
PVDC	Poli (cloruro de vinilideno)
r^2	Coeficiente de determinación
Ref. Nº	Substance Reference Number given by the Commission services
RSD	Relative Standard Deviation (Desviación estándar relativa)
rpm	Revoluciones por minuto
SAN	Estireno/Acrilonitrilo
SB	Estireno/Butadieno
SEC	Size Exclusion Chromatography (Cromatografía de Exclusión por Tamaño)
SFC	Supercritical Fluid Chromatography (Cromatografía de Fluidos Supercríticos)
SIR	Single Ion Recording
SML	Specific Migration Limit (Límite de Migración Específico)
SPE	Solid Phase Extraction (Extracción en Fase Sólida)
SPME	Solid Phase Microextraction (Microextración en fase sólida)
TDI	Tolerable Daily Intake (Ingesta Diaria Tolerable)
THF	Tetrahidrofurano

TSP	Thermospray Ionization
UV	Ultravioleta
v/v	Volumen/volumen
w/v	Weight/volume (peso/volumen)

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

RESUMEN

Los materiales en contacto con alimentos son todos aquellos materiales y artículos destinados a entrar en contacto directo con los alimentos, incluidos los materiales de envasado pero también cubiertos, platos, máquinas de procesado, recipientes, etc. Este término incluye así mismo, los materiales y artículos que están en contacto con agua destinada para consumo humano, pero no incluye las redes de suministro de aguas públicas o privadas.

Así bien, aunque esta definición no sólo incluye a los envases alimentarios, son estos los que tienen una mayor relevancia debido a su uso tan extendido.

Entre las funciones del envase se encuentran la de contener el producto (función esencial) y mantener la calidad del mismo. Sin embargo, hay una característica muy importante y que se debe exigir a todos los envases, y es que no se produzcan interacciones con su contenido. Las modernas técnicas de envasado, con la utilización de nuevos materiales, han solucionado muchos problemas de higiene, pero plantean otros nuevos. Hay que asegurarse que los materiales utilizados no sean tóxicos o susceptibles de interaccionar con los alimentos que van a contener.

Los recubrimientos son materiales plásticos que proporcionan una protección necesaria y suplementaria al material base y al producto. Estos materiales se aplican sobre una superficie (generalmente metálica) formando una fina película continua, con el fin de aumentar la inercia envase-alimento.

Los principales recubrimientos usados son bisfenol A epoxi resinas y/o epoxi novolacs; ambas son mezclas complejas de especies químicas de naturaleza fenólica de diferentes pesos moleculares. Las resinas epoxi bisfenol A son principalmente productos de condensación de bisfenol A y epiclorhidrina y las epoxi novolacs son productos de condensación del formaldehído con fenol seguido de una epoxidación. Todas son sustancias de partida empleadas en la elaboración de recubrimientos plásticos usados en la industria de los envases alimentarios.

Habitualmente, las industrias alimentarias compran sus materiales de envasado ya recubiertos con este tipo de resinas y no conocen exactamente su composición. Por esto es conveniente desarrollar métodos que permitan evaluar la migración de estos compuestos y así garantizar el cumplimiento de la legislación

europea sobre recubrimientos basados en resinas epoxi y materiales en contacto con alimentos.

En el **capítulo II** (***Determination of compounds from epoxy resins in food simulants by HPLC-fluorescence***) de esta Tesis Doctoral se describe el desarrollo de un método analítico de cromatografía líquida de alta resolución en fase reversa con detector de fluorescencia que permite la separación y cuantificación de bisfenol A diglicidil éter (BADGE), bisfenol F diglicidil éter (BFDGE) y sus derivados clorados e hidroxilados, así como de los oligómeros del BADGE y de los novolac glicidil éteres (NOGE).

El método desarrollado ha sido aplicado al análisis de una resina de tipo epoxi destinada a su uso como recubrimiento para envases alimentarios. A esta resina se le aplicaron dos métodos de curado diferentes, uno a temperatura ambiente durante 24 horas y otro a 100ºC durante una hora.

Los ensayos de migración se han realizado en tres simulantes alimentarios, establecidos por la legislación europea y que son: ácido acético al 3 % (*p/v*), (simulante para alimentos ácidos acuosos) etanol al 10 % (*v/v*) (simulante para alimentos con un contenido en etanol menor al 10 %) y aceite de oliva rectificado (simulante para los alimentos grasos). Los simulantes acuosos han sido analizados directamente, mientras que para llevar a cabo el análisis de los extractos de aceite de oliva, se aplicó un método de extracción rápido y sencillo. También se realizaron ensayos de extracción con acetonitrilo, comparando estos resultados con los obtenidos para los simulantes.

La cuantificación de los distintos compuestos se ha llevado a cabo utilizando la calibración externa, y los parámetros de caracterización del método fueron satisfactorios.

Debido a que no existen patrones comerciales de los oligómeros del BADGE y NOGE, se han construido dos rectas de calibración usando BADGE y BFDGE, que han sido usadas para cuantificar sus oligómeros. Con estas rectas de calibración hechas con los patrones comerciales puros (de BADGE y BFDGE) se obtuvieron en todos los casos coeficientes de determinación mayores a 0,996 y límites de detección de adecuados. La concentración del BFDGE ha sido calculada como la suma de sus tres isómeros. Para la cuantificación de los derivados del

BADGE y BFDGE el procedimiento usado es similar. De esta manera todos los resultados están expresados como BADGE y BFDGE asumiendo que los factores de respuesta de estos derivados son similares a los de los patrones puros.

La preparación de los derivados clorados e hidroxilados del BADGE y BFDGE se ha realizado aplicando dos procesos diferentes para cada uno, para así poder identificar los derivados obtenidos. La preparación de los derivados hidroxilados se ha llevado a cabo mediante una disolución de los patrones en agua y su posterior mantenimiento a una temperatura elevada. Los derivados clorados se obtienen mediante la adición de ácido clorhídrico a una disolución concentrada de los patrones. En el caso del BFDGE la existencia de tres isómeros dificulta mucho esta identificación. Experimentalmente se comprobó que 2 horas son suficientes para la obtención de los derivados hidroxilados (BADGE.H_2O, BADGE.$2H_2O$, BFDGE.H_2O, BFDGE.$2H_2O$) y 20 minutos son suficientes para la obtención de los derivados clorados (BADGE.H_2O.HCl, BADGE.2HCl, BFDGE.H_2O.HCl, BFDGE.2HCl).

Para la confirmación positiva de la identidad de los derivados y oligómeros se ha empleado la cromatografía liquida en fase reversa con detector de espectrometría de masas.

Se comprobó que el recubrimiento analizado era del tipo BADGE, no se encontró ningún derivado tipo NOGE o BFDGE y los únicos derivados cuantificados después de los ensayos de migración fueron derivados hidroxilados y oligómeros del BADGE. A pesar de que estaban presentes oligómeros con M>1000 Da, estos no fueron cuantificados porque no eran objeto de este estudio.

El comportamiento de la migración ha sido el esperado ya que en los simulantes acuosos, el BADGE se hidroliza después de haber migrado a estos simulantes. No se han detectado oligómeros del BADGE en estos simulantes, debido a su baja solubilidad. En cambio, los extractos de aceite de oliva contenían oligómeros del BADGE y en ellos no se detectaron cantidades significativas de los derivados hidroxilados. Los extractos de acetonitrilo contenían los mismos compuestos epoxi que el aceite de oliva, lo cual confirmó los datos obtenidos en el ensayo de migración con este último.

Los datos obtenidos muestran que el recubrimiento epoxídico analizado cumple la legislación europea. Pero cuando se usa el ensayo alternativo con el acetonitrilo y el recubrimiento no ha sido sometido a un tratamiento posterior a alta

temperatura, la cantidad total de sustancias migrantes (incluidos los oligómeros del BADGE) es más alta que en el ensayo de migración para el aceite de oliva. Se ha observado también que para todas las muestras sometidas a un proceso posterior a alta temperatura, se obtienen valores de migración menores.

El objetivo del **capítulo III** (***Determination of BADGE and its hydrolysis and chlorohydroxy derivatives by LC-MS***) ha consistido en desarrollar un método que permita tanto la cuantificación como la identificación positiva del BADGE y de todos sus derivados usando un método de cromatografía líquida de alta resolución en fase reversa acoplada a un espectrómetro de masas vía una sonda de ionización química a presión atmosférica (APCI).

La legislación europea establece que la suma del BADGE, sus derivados hidroxilados (BADGE.H_2O y BADGE.$2H_2O$) y clorados (BADGE.HCl, BADGE.2HCl y BADGE.H_2O.HCl) no debe exceder el límite de migración específica de 1 mg/kg de alimento o simulante.

La cuantificación de estas sustancias se ha llevado a cabo usando el modo SIR ("single ion recording") después de haber seleccionado los fragmentos más característicos en los espectros de masa obtenidos. Así para el BADGE y BADGE.H_2O se optó por el modo APCI (+) y los fragmentos seleccionados se corresponden con los "clusters" formados por el propio analito y una molécula de acetonitrilo $[M+CH_3CNH]^+$. Para el BADGE.$2H_2O$ se seleccionó el modo APCI (-) y el fragmento correspondiente al ión cuasi molecular ha sido considerado el más selectivo.

Para el BADGE.HCl el fragmento más abundante corresponde al $[M+CH_3CNH]^+$. El caso del BADGE.2HCl es más difícil de explicar porque el fragmento más abundante y simultáneamente más específico podría corresponder a un cluster similar al que forma el BADGE.

El BADGE.H_2O.HCl se ha determinado usando APCI (-) en vez de (+) debido a la imposibilidad de separarlo cromatográficamente del BADGE.H_2O. De esta forma se seleccionó un fragmento muy selectivo correspondiente a $[M-H]^-$.

El método ha sido evaluado en acetonitrilo al 90% (v/v) y en agua. En ambos casos se obtienen para todas las sustancias coeficientes de determinación

mayores a 0,99 y límites de detección satisfactorios, comparándolos con los límites de migración específica establecidos en la legislación.

Una vez optimizado el método, se han realizado análisis aleatorios con latas vacías, extrayéndolas con acetonitrilo. No se ha encontrado ninguna de las sustancias citadas, lo cual demuestra la garantía de inocuidad de las latas evaluadas. Estos ensayos de extracción son actualmente utilizados por varios autores ya que el acetonitrilo es un buen disolvente de extracción y se consigue así, tener una idea de la seguridad de estos materiales antes de ser usados como envases para alimentos.

En el **capítulo IV** (***Determination BFDGE and its related compounds by HPLC-MS***), al igual que en el anterior, se describe la puesta a punto de un método analítico basado en la cromatografía líquida de alta resolución en fase reversa acoplada a un espectrómetro de masas y usando la ionización química a presión atmosférica (APCI) para la separación, cuantificación e identificación del bisfenol F diglicidil éter y sus derivados hidroxilados y clorados.

La legislación europea relacionada con el uso de determinados compuestos epoxídicos como materiales en contacto con alimentos, establece que la suma de los niveles de migración del BFDGE y sus derivados no debe exceder el valor de 1 mg/kg de alimento o simulante (o 1 mg/6dm^2 en el material evaluado). Para el NOGE se establece (desde marzo de 2003), que la cantidad de estos componentes con más de dos anillos aromáticos, y con al menos un grupo epoxi, y sus derivados con moléculas de cloro, y un peso molecular menor de 1000 Da, deben ser no detectables a niveles inferiores a 0,2 mg/6dm^2.

Así, una vez optimizadas todas las condiciones analíticas y para establecer los parámetros de cuantificación, se han realizado ensayos iniciales con disoluciones concentradas de estos compuestos para obtener la máxima información posible de sus espectros de masas.

En la producción de BFDGE, la condensación entre el fenol y el formaldehído puede ocurrir en las posiciones *orto* y *para* del anillo fenólico. Esta es la razón por la que este compuesto está formado por tres isómeros y puede estar acompañado por compuestos de tres o más anillos. Esto hace que su separación cromatográfica tenga una mayor dificultad si la comparamos con la del BADGE.

Además, también es importante destacar que sólo hay disponibles en el mercado tres patrones comerciales (BFDGE, BFDGE.2H_2O y BFDGE.2HCl); así para los que no existen estos patrones es necesario preparar mezclas que contengan los demás derivados (BFDGE.H_2O, BFDGE.HCl y BFDGE.H_2O.HCl) tal y como se describe en el capítulo II, pero la separación cromatografica y la identificación individual es por tanto, realmente difícil.

La ionización de estos compuestos tiene un perfil similar a la observada en el capítulo III para el BADGE. Una vez seleccionados los iones más selectivos se han construido las rectas de calibrado (sólo para aquellos compuestos disponibles en el mercado como patrones puros). Se han obtenido en todos los casos coeficientes de determinación mayores de 0,99 y límites de detección adecuados, teniendo en cuenta el límite de migración específica.

En cuanto al NOGE, únicamente es posible la cuantificación de los derivados de hasta cuatro anillos aromáticos, pero la única restricción de este método es el límite de detección, inconveniente que puede solucionarse usando un paso previo de concentración. La principal ventaja de este método es que al utilizar el modo SIR ("selecting ion recording") se consigue diferenciar mezclas complejas.

Durante los últimos años, el sector de la industria conservera ha demostrado una evidente preocupación por la posible contaminación de sus productos por los compuestos anteriormente citados. Esto ha hecho posible el estudio descrito en el **capítulo V** (***Migration survey of BADGE and BFDDGE from canned fish and seafood, in Spanish market during last years***). Se han evaluado los niveles de BADGE y BFDGE en 389 muestras de conservas de pescado suministradas directamente por industrias nacionales durante los últimos seis años.

Para dicha evaluación se ha utilizado un sencillo método de extracción de las muestras, y el método de análisis cromatográfico descrito en el capítulo II.

En todas las muestras se ha realizado un análisis cuantitativo del BADGE y BFDGE, y también se han identificado otros derivados.

Los resultados encontrados muestran una evolución en la concentración de estos compuestos epoxídicos. Así, en el año 1999, se detectaron niveles de BADGE en casi un 50 % de las muestras, pero siempre en niveles inferiores a los límites establecidos en la legislación. En cuanto al BFDGE se encontraron valores

que excedían los límites legales en un 4 % de las muestras. Desde este año (1999) y hasta finales del 2001, sólo un 1 % de las muestras superó el límite de 1 mg/kg, y en los siguientes tres años (2002-2004) ninguna muestra alcanzó este valor. Comparando estos datos con los obtenidos previamente en nuestro laboratorio, se puede concluir que estos compuestos se encuentran en concentraciones inferiores a las que se obtenían a finales de los años 90.

En la Unión Europea existe legislación de obligado cumplimiento, que define los límites máximos de migración global y migración específica, para envases plásticos en contacto con alimentos. Las investigaciones científicas relacionadas con la migración potencial y con el comportamiento de los materiales de envasado han demostrado que la difusión y la migración pueden ser fenómenos previsibles y en principio descritos matemáticamente. Sin embargo, la escasa información relativa al comportamiento de muchas sustancias potencialmente migrantes y la creciente importancia que tienen los temas de seguridad alimentaria, ha suscitado el interés de esta por los materiales plásticos en contacto con alimentos. Entre las acciones que la Unión Europea ha decidido iniciar está el financiamiento de un proyecto de investigación llamado “FOODMIGROSURE”, que actualmente siendo desarrollado en varios centros europeos de investigación, entre los incluido está incluido nuestro laboratorio. El principal objetivo de este proyecto es proporcionar una herramienta novedosa y económica para la estimación de la exposición del consumidor a sustancias migrantes que provengan de los materiales en contacto con alimentos. Esta herramienta está basada en un modelo de migración físico-química que describe matemáticamente los procesos de migración desde los plásticos a alimentos reales, bajo cualquier condición de contacto previsible.

Dentro de este proyecto europeo existen tres secciones principales.

- La primera incluye la definición y selección de los materiales plásticos objeto de estudio, sustancias modelo y alimentos representativos de cada grupo.
- La segunda hace referencia al trabajo de investigación experimental que enfocado a obtener los datos físico-químicos de los migrantes modelo seleccionados.
- La tercera, pretende hacer una evaluación multidisciplinar e interactiva, así como llevar a cabo la ejecución del trabajo.

Incluidos en la segunda sección del plan de trabajo del proyecto existen dos módulos diferenciados. En el primer módulo se distinguen tres partes: el desarrollo de métodos analíticos y el diseño experimental para las dos siguientes partes; los estudios de cinética de migración desde filmes plásticos a alimentos y los estudios de procesos de transporte y partición de los migrantes modelo en los alimentos.

Los **capítulos VI, VII y VIII** recogen el trabajo integrado en el primer módulo en la sección correspondiente al desarrollo de los métodos analíticos y diseño experimental.

Así, una vez que el proyecto ha seleccionado los materiales plásticos de referencia, se han elegido también migrantes modelo de diferentes propiedades físicas y químicas que han sido estudiadas en los capítulos VI y VII.

En el **capítulo VI** (***Compilation of analytical methods to determine migration of additives and monomers from plastics to foodstuffs***), las sustancias evaluadas fueron: Irganox 1076®, difenilbutadieno, Chimassorb 81®, Uvitex OB®, caprolactama, benzofenona, difenil ftalato y bis (2-dietilhexil) adipato (DEHA).

En primer lugar se ha realizado una extensa revisión bibliográfica de las propiedades físicas y químicas de los compuestos seleccionados. Debido a que muchos de estos datos estaban incompletos, algunas de estas propiedades se evaluaron en nuestro laboratorio.

Para cada sustancia se recopilaron los diferentes datos y las propiedades disponibles como el número CAS, la fórmula y el nombre químico, peso molecular, punto de fusión y ebullición, solubilidad en agua y en varios solventes, estructura química, uso, coeficiente de reparto y propiedades espectrales, todas ellas consideradas importantes para el posterior desarrollo de métodos analíticos.

También se ha llevado a cabo una revisión bibliográfica de todos los métodos analíticos disponibles para cada compuesto en polímeros, simulantes y alimentos. La bibliografía relacionada con este tema, es escasa y da más énfasis al contenido total del migrante en polímeros y a la migración en simulantes, que a la migración en alimentos. Además, los métodos analíticos disponibles están aún en fase de desarrollo, no habiendo por tanto métodos de aprobación general. Por

último, se ha verificado que algunos de los protocolos encontrados en la bibliografía no están descritos con suficiente detalle de forma que permitan su repetición en otros laboratorios. Esta es una situación común cuando una metodología analítica se encuentra en su fase inicial de desarrollo. En conclusión, actualmente, no están disponibles, protocolos analíticos para la determinación de gran mayoría de los migrantes potenciales.

Para completar esta revisión se realizaron estudios preliminares para conocer experimentalmente las características de estas sustancias. Estos consistieron en el estudio de las propiedades de ultravioleta y de fluorescencia, y la posibilidad del uso de la cromatografía líquida con detector de espectroscopía de masas y de la cromatografía gaseosa.

Los compuestos evaluados en el **capítulo VII** (***State of the art of analytical strategies to evaluate different migrants from food packaging materials***) han sido: estireno, bisfenol A, 1-octeno, limoneno, di-isopropilnaftaleno (DIPN), laurolactama, triacetin, tri-n-butilacetil citrato (ATBC), hidroxitolueno butilado (BHT) y triclosan.

Al igual que en el capítulo anterior se ha revisado la bibliografía disponible sobre los métodos analíticos en polímeros, simulantes de alimentos y alimentos, y aunque escasa, se ha encontrado más información basada en el análisis de simulantes y polímeros que en alimentos.

Se evaluaron también en el laboratorio diferentes propiedades espectroscópicas, ya que en la mayoría de los casos, estas no estaban recogidas en la bibliografía y realmente se consideran el punto de partida en el desarrollo de los métodos analíticos.

En ambos capítulos se sugieren y discuten unas guías generales para la determinación de estos compuestos en alimentos teniendo en cuenta las propiedades de cada molécula.

Varios métodos analíticos han sido desarrollados en el marco del proyecto "FOODMIGROSURE". En el **capítulo VIII** (***Determination of DPBD by LC-UV-Fluorescence in foodstuffs***) se describe uno de ellos, concretamente el desarrollado para el análisis del difenilbutadieno (DPBD).

Este compuesto es un absorbedor de la luz ultravioleta incorporado en un amplio rango de materiales poliméricos. Su función es, una vez que ha sido añadido al material, absorber los rayos ultravioletas de la luz y cederlos como luz azul. Esta luz azul interactúa con el color amarillo y le da al material la apariencia de ser más blanco.

Para poder determinar los niveles de migración en alimentos, se ha desarrollado un método analítico para determinar el DPBD en tres alimentos con propiedades físico-químicas muy diversas: zumo de naranja (alimento acuoso, ácido y con un contenido medio de hidratos de carbono), pechuga de pollo (alimento sólido con un alto contenido en proteínas) y queso gouda (alimento sólido con un alto contenido en grasa).

El método analítico está basado en la cromatografía líquida de alta resolución en fase reversa con detectores de ultravioleta y fluorescencia, obteniéndose una buena linearidad. La determinación de este compuesto en los alimentos se ha llevado a cabo mediante la extracción con hexano y posterior cambio de solvente compatible con la técnica analítica empleada. Para la identificación positiva de esta sustancia se ha empleado la cromatografía gaseosa con detector de espectrometría de masas.

Para evaluar la precisión del método se han analizado muestras de los tres alimentos conteniendo tres niveles diferentes de concentración de DPBD. Las recuperaciones obtenidas han sido satisfactorias.

Se han realizado estudios con otros alimentos para evaluar la posibilidad de aplicar este mismo método. Los alimentos analizados son: compota de manzana, leche UHT, ketchup, cola, margarina, leche condensada, chocolate, pan tostado, harina, arroz y miel. No se han encontrado en ningún caso interferencias al tiempo de retención del DPBD.

CAPÍTULO I: INTRODUCCIÓN

I.1.- LA SEGURIDAD ALIMENTARIA

Entre las preocupaciones relacionadas actualmente con nuestro entorno, una de las más importantes es la calidad y la seguridad de los alimentos que comemos. Aspectos relacionados con los cultivos modificados genéticamente, microorganismos peligrosos, la incidencia y la transmisión de la encefalopatía espongiforme bovina (BSE), son sólo algunas de las pocas áreas problemáticas que reciben una atención frecuente y amplia (Hester, 2001).

El diccionario de la Real Academia Española define seguridad como "*cualidad de seguro*", y este a su vez define seguro como "*libre y exento de todo peligro, daño o riesgo*". Dicha definición aplicada al campo de la alimentación toma una importancia muy relevante.

Para la FAO (Organización de las Naciones Unidas para la Agricultura y la Alimentación), "e*xiste seguridad alimentaria cuando todas las personas tienen en todo momento acceso físico y económico a suficientes alimentos inocuos y nutritivos, para satisfacer sus necesidades alimentarias y sus preferencias en cuanto a los alimentos, a fin de llevar una vida activa y sana*". Existe seguridad si se dan cuatro condiciones: una oferta y disponibilidad de alimentos adecuadas; la estabilidad de la oferta sin fluctuaciones ni escasez en función de la estación o del año; el acceso a los alimentos o la capacidad para adquirirlos; la buena calidad e inocuidad de los mismos.

En el entorno de los países de la Unión Europea la consecución de las tres primeras condiciones está generalizada, por lo que podríamos decir que existe seguridad alimentaria cuando los alimentos están en buenas condiciones higiénicas y son inocuos para la salud.

La seguridad y calidad alimentarias son campos muy amplios. Para abarcarlos totalmente sería necesario un análisis completo de la cadena alimentaria, desde la semilla o genotipo del ganado, pasando por la agricultura, el procesado, la formulación, envasado, distribución, venta, almacenamiento doméstico y finalmente el consumo.

Las posibilidades de que un alimento se contamine comienzan desde el momento de su cosecha y continúan hasta el momento en que es consumido. En

general, los riesgos relativos a seguridad alimentaria se pueden clasificar en dos amplias categorías:

La *contaminación biótica* (p. ej.: bacterias, hongos, virus o parásitos). Esta categoría puede provocar toxiinfecciones alimentarias, es decir, síntomas más o menos graves en la mayoría de los casos en un tiempo corto.

Los *contaminantes abióticos,* que comprenden sustancias químicas de origen natural (micotoxinas), residuos de medicamentos de uso veterinario (antibióticos, hormonas), metales pesados (mercurio) u otros residuos (pesticidas) introducidos de forma involuntaria o accidental en los alimentos durante su cultivo o cría, su elaboración, su transporte o su envasado.

El que un contaminante pueda suponer un riesgo para la salud o no, depende de muchos factores, entre ellos la absorción y la toxicidad de la sustancia, el nivel presente en el alimento, la cantidad de alimentos contaminados que se consumen y el tiempo de exposición a ellos. Además, las personas tienen diferentes sensibilidades a estos compuestos químicos, y hay otros factores de la dieta que pueden influir en sus consecuencias tóxicas. Un factor aún más complicado, con respecto a los contaminantes químicos, es que muchos de los estudios sobre la toxicidad de los contaminantes, se extrapolan, por necesidad, de estudios realizados en animales, y no siempre se sabe con absoluta seguridad si las sustancias tienen los mismos efectos en los humanos (EUFIC, 2004).

I.1.1 Instituciones que vigilan la seguridad alimentaria

Después de una serie de sobresaltos en los años 90 (BSE, dioxinas...) los cuales mermaron la confianza del consumidor en la cadena alimentaria, la Unión Europea concluyó que era necesario crear un nuevo cuerpo científico encargado de proporcionar consejo independiente y objetivo en problemas de seguridad alimentaria. Su primer objetivo, tal y como se afirma en el Libro Blanco de la Seguridad Alimentaria *"...contribuirá a lograr un elevado nivel de protección de la salud de los consumidores y, por consiguiente, a recuperar y conservar la confianza de éstos."*. El resultado ha sido la creación de la Agencia Europea de Seguridad Alimentaria (European Food Safety Authority, EFSA).

La EFSA proporciona asesoramiento científico en todos los problemas relacionados con la seguridad de alimentos y piensos (incluyendo la salud y el

bienestar animal y protección de las plantas) y aporta asesoramiento científico en nutrición en relación con la legislación comunitaria (EFSA, 2004).

En el ámbito estatal, en julio de 2001, se creó la Agencia Española de Seguridad Alimentaria (AESA) como organismo autónomo adscrito al Ministerio de Sanidad y Consumo. Tal y como se cita en la ley 11/2001 del 5 de julio de 2001, en su artículo 2 :"*La Agencia se crea con el objetivo general de promover la seguridad alimentaria, como aspecto fundamental de la salud pública, y de ofrecer garantías e información objetiva a los consumidores y agentes económicos del sector agroalimentario español, desde el ámbito de actuación de las competencias de la Administración General del Estado y con la cooperación de las demás Administraciones públicas y sectores interesados."* (AESA, 2004).

El sistema de seguridad de alimentos de los Estados Unidos está basado en leyes federales y estatales fuertes, flexibles, en conocimientos científicos y en la responsabilidad legal de la industria de producir alimentos seguros. Este sistema se guía por los principios siguientes: sólo se pueden comercializar alimentos seguros y sanos; las directrices en seguridad alimentaria tienen un soporte científico; el gobierno tiene la responsabilidad sobre la entrada en vigor; se espera que los fabricantes, los distribuidores, los importadores y otros, cumplan las directrices, siendo responsables si no lo hacen; y el proceso regulador es transparente y accesible al público. Distintas administraciones están implicadas en este sistema.

La Food and Drug Administration (FDA) es el organismo responsable de legislar en Estados Unidos, entre otros aspectos, sobre las normas de etiquetado y envasado, así como también sobre los estándares de calidad de alimentos, que se comercializan en ese país.

Dentro de esta legislación se encuentra una sección específica para ingredientes alimentarios y envasado alimentario. En esta sección no sólo se incluye cualquier sustancia usada en la producción, tratamiento, envasado, transporte o almacenamiento de alimentos, sino que también, se establece la necesidad de que cualquiera de estas sustancias pase por un proceso de acreditación de inocuidad previo a su comercialización.

En cuanto a la situación en Suramérica, en 1991 se creó el Mercado Común del Sur, (MERCOSUR) que está constituido por Argentina, Brasil, Paraguay y Uruguay que constituyen los cuatro Estados-Parte junto, con Bolivia y Chile como estados asociados. El órgano ejecutivo del MERCOSUR es el Grupo Mercado

Común (GMC) que entre sus funciones está la de coordinar y orientar las tareas de los diferentes Subgrupos de trabajo. Dentro del Subgrupo de Trabajo nº 3 se creó la Comisión de Alimentos. Los parámetros fundamentales con que trabaja esta Comisión son: conceptos de salubridad, a partir de la verificación de la inocuidad y seguridad alimentaria; transparencia y equivalencia, para dotar al consumidor de toda la información necesaria de los productos; y prácticas no engañosas al consumidor en cuanto a asegurar la calidad del producto elaborado. Todo esto determina trabajar de manera coordinada en dos ámbitos, uno horizontal para asegurar la inocuidad de los alimentos, y otro vertical a partir de la elaboración de los reglamentos verticales.

En todos los casos para la armonización de las legislaciones preexistentes en estos Estados Partes se considera la normativa internacional vigente, constituyendo el Codex Alimentarius el patrón principal de orientación, complementado, principalmente, con la legislación de la Unión Europea y de la Food and Drugs Administration (FDA), según corresponda (MERCOSUR, 2004).

I.2.- MATERIALES EN CONTACTO CON ALIMENTOS

Los materiales en contacto con alimentos son todos aquellos materiales y artículos destinados a entrar en contacto directo con los alimentos, incluidos los materiales de envasado pero también máquinas de procesado, cubiertos, platos, recipientes, etc. Este término incluye así mismo, los materiales y artículos que están en contacto con agua destinada para consumo humano, pero no incluye las redes de suministro de aguas públicas o privadas.

Así bien, aunque esta definición no sólo incluye a los envases alimentarios, son estos los que tienen una mayor relevancia debido a su uso tan extendido.

En general los alimentos son perecederos, por lo que necesitan ciertas condiciones de conservación y manipulación. La conservación de alimentos como medio para prevenir la escasez ha sido una de las preocupaciones de la humanidad. Actualmente los alimentos se presentan en varios envases, fabricados a partir de diferentes materias primas. Así, el envasado alimentario es un área muy extensa de

estudio que engloba varias disciplinas, como pueden ser la química, la microbiología, la ciencia y teología de los alimentos y la ingeniería.

En la sociedad actual, el envasado es fundamental y esencial. Envuelve, realza y protege los artículos que compramos, desde el procesado y manufactura pasando por el manipulado y almacenamiento, hasta el consumidor final. Sin el envasado los modernos canales de venta serían casi imposibles.

A pesar de la importancia y del papel crucial que juega el envasado, es a menudo considerado como un mal necesario o como un coste innecesario. Además a la vista de muchos consumidores el envase es, en el mejor de los casos como algo superfluo, y en el peor como un serio derroche de recursos y una amenaza medioambiental. Este punto de vista surge debido a que las funciones que el envase realiza son desconocidas o no totalmente tenidas en cuenta. Cuando la mayoría de los consumidores entra en contacto con un envase, su función en la mayoría de los casos ha finalizado, y es quizás comprensible, que por esto no sea valorado. (Robertson 1993).

Entre las funciones del envase se encuentran la de contener el producto (función esencial) y mantener la calidad del mismo. Sin embargo, hay una característica muy importante, y que se debe exigir a todos los envases, y es que no se produzcan interacciones con el contenido del mismo. Las modernas técnicas de envasado, con la utilización de nuevos materiales, han solucionado muchos problemas de higiene pero plantean otros nuevos. Hay que asegurarse que los materiales utilizados no sean tóxicos o susceptibles de interaccionar con los alimentos que van a contener.

I.2.1 Papel y Cartón

El papel y el cartón son algunos de los más antiguos y versátiles materiales de envasado disponibles en el mercado hoy en día. Es un material paradójico, pues puede ser permanente o efímero, frágil o resistente, barato o caro, en abundancia o escaso.

Estos dos materiales, solos o en combinación con otros, ha sido usados en el envasado alimentario o en contacto con alimentos durante muchos años. Ambos están hechos a partir de fibras de celulosa son obtenidas de árboles. Químicamente, la celulosa pura consiste en largas cadenas de β-glucosa de peso molecular

variable. La celulosa tiene una estructura lineal o fibrosa, en la que se establecen múltiples puentes de hidrógeno entre los grupos hidroxilo de distintas cadenas yuxtapuestas de glucosa, haciéndolas impenetrables al agua, y originando fibras compactas que constituyen la pared de las células vegetales. Estas fibras se agrupan entre ellas en haces, y estos dan lugar a la fibra de papel. Al papel se le llama por lo general cartón cuando su densidad es mayor a los 224 g/m^2 (ILSI 2004).

Papel y cartón pueden ser usados en contacto con alimentos de maneras muy diversas, tanto directamente como indirectamente, y también solo o laminado con otros materiales como plásticos o papel de aluminio. Entre los varios tipos que se usan hoy en día en el envasado alimentario tenemos:

- Envase de papel: natural o blanqueado, recubierto o asociado con otros materiales, puede encontrarse en forma de bolsa.
- Envase de cartón: Se puede encontrar como cajas para productos de pastelería o como recipiente. Es el envase más utilizado en productos dirigidos directamente al consumidor, como comida congelada o como recipiente para alimentos líquidos.
- Cartón ondulado: Marrón o blanco, resistente a la humedad o a la compresión, estos estuches constituyen el mayor volumen de papel y cartón usado para aplicaciones en contacto con alimentos.

Ha habido un significante aumento del uso de estos materiales en los últimos 50 años, debido a varias razones: es sólido y adaptable, es práctico, se recicla fácilmente y está hecho de materia renovable (ILSI 2004).

I.2.2 Materiales plásticos

Los materiales plásticos son compuestos macromoleculares orgánicos obtenidos por polimerización, policondensación, poliadición o algún otro proceso similar, a partir de moléculas de peso molecular inferior o por modificación química de macromoléculas naturales. En su formulación intervienen los monómeros de partida, así como otros compuestos habitualmente de bajo peso molecular que se adicionan intencionadamente como coadyudantes del proceso de fabricación, o

modificadores de las características del producto final; "aditivos" destinados a conseguir determinados efectos técnicos (plastificantes, antioxidantes, colorantes...).

Según sus características termodinámicas, los plásticos pueden clasificarse en (Sarria Vidal 1999):

- ✓ Termoplásticos:
  - Poliolefinas: como el polietileno (PE), el etileno/acetato de vinilo (E/VA), el etileno/alcohol vinílico (E/AL o VEO), el polipropileno (PP), el polibuteno (PB) y el polimetilpenteno (PMP).
  - Plásticos de Poliestireno: como el poliestireno (PS), el estireno/acrilonitrilo (SAN), estireno/butadieno (SB) o PS de alto impacto (HIPS) y el acrilonitrilo/butadieno/estireno (ABS).
  - Plásticos clorados: como el policloruro de vinilo rígido (PVC) y el policloruro de vinilideno (PVDC).
  - Plásticos fluorados: como el polifluoretileno o teflón (PTFE).
  - Plásticos tipos éster: como el politereftalato de etileno (PET) y el policarbonato (PC).
  - Plásticos con grupos de nitrógeno (poliamidas): Como las poliamidas alifáticas semicristalinas (PA), las poliamidas semiaromáticas cíclicas o el poliacrilonitrilo (PAN).
- ✓ Plásticos termoestables:
  - Fenoplastos o plásticos fenólicos (PF).
  - Aminoplastos.
  - Resinas Poliéster.
  - Resinas epoxi.
- ✓ Elastómeros
- ✓ Plasticos de altas prestaciones

Los recubrimientos son materiales plásticos que proporcionan una protección necesaria y suplementaria al material base y al producto. Estos materiales

de recubrimiento se aplican sobre una superficie formando una fina película continua, con el fin de aumentar la inercia envase-alimento. Estos recubrimientos ya se utilizaban en la prehistoria, estando compuestos en aquel momento por sustancias como grasa de animales, tierras coloreadas y carbón. Aunque esto no se corresponde con nuestra noción de un recubrimiento moderno, están basados en el mismo principio.

En la mayoría de los casos, el recubrimiento se aplica directamente sobre una hojalata u otro material base antes de la formación del envase. El recubrimiento interior de las latas es muy importante porque previene la reacción de los metales con los ingredientes del alimento enlatado. El exterior de la lata es pintado para prevenir la corrosión y por razones decorativas.

I.2.3 Otros

El vidrio es un material duro, frágil y transparente. A pesar de comportarse como sólido, es un líquido sobreenfriado y amorfo (sin estructura cristalina).

Los recipientes de vidrio se conocen desde la más remota antigüedad, y alcanzaron su gran difusión en la época del imperio romano, al difundirse las técnicas de soplado con caña, pero el uso de estos recipientes como envases, garantizando la conservación de alimentos y bebidas durante largo tiempo, y facilitando su transporte, es mucho más reciente.

Los envases de primera fabricación (los que se obtienen por conformación del vidrio fundido, cuya composición básica es silicato de sodio y calcio) son los más comúnmente usados para alimentos y bebidas.

El uso de cerámicas esmaltadas en envases para conservar alimentos y bebidas es infrecuente, aunque crece día a día; es muy común en cambio, su uso en vajilla para cocinar y servir, en recipientes para consumo rápido y equipamientos del más diverso tipo. A diferencia de los envases de vidrio, la composición química de los esmaltes utilizados es muy variable y se emplean variadas tecnologías para su fabricación (Catalá y Gavara, 2002).

I.3.- ASPECTOS LEGISLATIVOS DE LOS MATERIALES EN CONTACTO CON ALIMENTOS

El contacto existente entre el envase y el alimento introduce la posibilidad de transferir constituyentes procedentes del envase a éstos. Con el fin de asegurar que este fenómeno no produzca ningún daño en la salud del consumidor, los materiales destinados a entrar en contacto con alimentos están sometidos a investigación y a legislaciones específicas.

I.3.1 La legislación Europea

La legislación de la Unión Europea (UE) tiene como objetivo final, la total armonización en todos los estados miembros, partiendo de las legislaciones nacionales o generando nuevas disposiciones a partir de los conocimientos científicos existentes. Las normativas son de obligado cumplimiento en todos los estados pertenecientes a la Unión Europea.

La primera directiva marco sobre materiales y artículos en contacto con alimentos (89/109/CEE, actualmente derogada por el Reglamento nº 1935/2004) establecía el principio de "naturaleza inerte" de los materiales y la "pureza" de las sustancias alimenticias, diciendo que: *Los materiales y artículos en contacto con alimentos no deben transferir al alimento componente alguno en cantidades tales que pudiera "perjudicar la salud humana" y/o "llevar algún cambio inaceptable en la composición del alimento o bien un deterioro en las propiedades organolépticas características".*

Desde la adopción de esta directiva han surgido una serie de nuevas cuestiones que debían ser tenidas en cuenta en la legislación, así, se han realizado importantes avances tecnológicos en el ámbito del envasado de alimentos, y se ha de garantizar mejor la trazabilidad y el etiquetado de los materiales y objetos destinados a entrar en contacto con alimentos.

El principal objetivo del envasado de alimentos es protegerlo contra los riesgos físicos, biológicos y químicos. Tradicionalmente se han desarrollado materiales de envasado para evitar interacciones con los alimentos y en particular, para reducir al máximo la liberación de sus componentes ("migración") en los

mismos. En consecuencia, la legislación comunitaria en vigor hasta la incorporación del nuevo reglamento marco, pedía que se aumentase al máximo la inercia de los materiales en contacto con alimentos y que se redujese al máximo la contaminación de los mismos. Los materiales de envasado no podían ocasionar modificaciones inaceptables en la composición de los alimentos o en las características organolépticas de éstos. Este sigue siendo el principio básico del actual reglamento, pero en él se han incorporado los "materiales y objetos activos en contacto con alimentos", que a diferencia de los materiales y objetos tradicionales en contacto con alimentos, no son inertes por su diseño, y por lo tanto pueden modificar la composición o las propiedades organolépticas de los alimentos, pero únicamente si estas modificaciones cumplen la legislación vigente relativa a alimentos, como por ejemplo, la legislación de aditivos alimentarios.

En la anterior directiva marco (89/109/CEE) también se establecía el principio de las listas positivas de sustancias autorizadas y de los grupos de materiales y objetos que deberían ser regulados mediante medidas específicas. También se establecían los procedimientos y los criterios que debían seguirse en la elaboración y la adopción de medidas de aplicación, incluida la evaluación de sustancias por el comité científico de la alimentación humana. El nuevo reglamento establece procedimientos más detallados para la evaluación de la seguridad y la autorización de las sustancias que deberán utilizarse en la fabricación de los materiales en contacto con alimentos.

Al elaborar una lista positiva, el procedimiento propuesto en el reglamento puede resumirse de la siguiente forma:

Los interesados en comercializar una nueva sustancia para un material destinado a entrar en contacto con alimentos deben solicitarlo a la autoridad competente. Esta autoridad informa a la AESA y la esta es la encargada de informar a todos los estados miembros así como a la Comisión. En un plazo definido la AESA emite un dictamen y lo hace público.

En la siguiente tabla se resume la legislación al respecto, incluyendo tanto las Directivas Comunitarias derogadas como la legislación actualmente en vigor.

DIRECTIVAS	CONTENIDO
REGLAMENTO MARCO	
1935/2004	Sobre los materiales y objetos destinados a entrar en contacto con alimentos y por el que se derogan las Directivas 80/590/CEE y 89/109/CEE.
DIRECTIVAS MARCO DEROGADAS	
80/590/CEE	Determinación del símbolo que puede acompañar a los materiales y objetos destinados a entrar en contacto con productos alimenticios.
89/109/CEE	Aproximación de las legislaciones de los Estados Miembros sobre los materiales y objetos destinados a entrar en contacto con productos alimenticios.
DIRECTIVAS ESPECÍFICAS	
82/711/CEE	Establece las normas de base necesarias para la verificación de la migración de los constituyentes de los materiales y objetos de materia plástica destinados a entrar en contacto con los productos alimenticios.
84/500/CEE	Aproximación de las legislaciones de los Estados Miembros sobre objetos de cerámica destinados a entrar en contacto con productos alimenticios.
85/572/CEE	Determina la lista de los simulantes que se deben utilizar para controlar la migración de los componentes de los materiales y objetos de plástico destinados a entrar en contacto con los productos alimenticios.
93/8/CEE	Se modifica la Directiva 82/711/CEE que establece las normas de base necesarias para la verificación de la migración de los componentes de los materiales y objetos de materia plástica destinados a entrar en contacto con productos alimenticios.
93/10/CEE	Relativa a los materiales y objetos de película de celulosa regenerada destinados a entrar en contacto con productos alimenticios.
93/111/CE	Se modifica la Directiva 93/10/CEE relativa a los materiales y objetos de película de celulosa regenerada destinados a entrar en contacto con productos alimenticios.
97/48/CE	Se modifica por segunda vez la Directiva 82/711/CEE del Consejo que establece las normas de base necesarias para la verificación de la migración de los componentes de los materiales y objetos de materia plástica destinados a entrar en contacto con productos alimenticios.
2002/72/CE	Relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios.
2004/14/CE	Se modifica la Directiva 93/10/CEE relativa a los materiales y objetos de película de celulosa regenerada destinados a entrar en contacto con productos alimenticios.
2004/1/CE	Se modifica la Directiva 2002/72/CE en lo relativo a la suspensión de la utilización de la azodicarbonamida como agente expansor.
2004/19/CE	Se modifica la Directiva 2002/72/CE relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios.

Tabla I.1: Resumen de la legislación aplicable a materiales destinados a entrar en contacto con alimentos.

DIRECTIVAS	CONTENIDO
DIRECTIVAS DEROGADAS POR LA 2002/72/CE	
90/128/CEE	Relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios.
92/39/CEE	Se modifica la Directiva 90/128/CEE.
93/9/CEE	Se modifica la Directiva 90/128/CEE.
95/3/CEE	Se modifica la Directiva 90/128/CEE.
96/11/CEE	Se modifica la Directiva 90/128/CEE.
99/91/CE	Se modifica la Directiva 90/128/CEE.
2001/62/CE	Se modifica la Directiva 90/128/CEE.
2002/17/CE	Se modifica la Directiva 90/128/CEE.
SUSTANCIAS ESPECÍFICAS	
78/142/CEE	Aproximación de las legislaciones de los Estados Miembros sobre materiales y objetos que contengan cloruro de vinilo monómero, destinados a entrar en contacto con productos alimenticios.
80/766/CEE	Relativa a la determinación de cloruro de vinilo en el producto acabado.
81/432/CEE	Establece el método comunitario de análisis para el control oficial del cloruro de vinilo cedido por los materiales y objetos destinados a entrar en contacto con productos alimenticios.
93/11/CEE	Relativa a la cesión de N-nitrosaminas y de sustancias N-nitrosables por las tetinas y chupetes de elastómeros o caucho.
2002/16/CE	Relativa a la utilización de derivados epoxídicos en materiales y objetos destinados a entrar en contacto con productos alimenticios.
2004/13/CE	Por la que se modifica la Directiva 2002/16/CE relativa a la utilización de determinados derivados epoxídicos en materiales y objetos destinados a entrar en contacto con productos alimenticios.

Tabla I.1 (continuación): Resumen de la legislación aplicable a materiales destinados a entrar en contacto con alimentos.

I.3.2 La legislación estadounidense

En los Estados Unidos, los materiales para el envasado de alimentos se clasifican generalmente como aditivos alimentarios (al igual que muchas de las sustancias añadidas intencionadamente a los alimentos), ya que pueden pasar a ser componentes de los alimentos por medio de procesos normales de difusión.

Los aditivos alimentarios están sujetos a una evaluación con respecto a su inocuidad previa su comercialización.

Existen procesos de petición y notificación (que se aplican estrictamente a los aditivos que están en contacto con los alimentos, incluyendo los envases) estipulados por las leyes para servir como medios para la evaluación de inocuidad por parte de la FDA.

La "Política de Umbral de Regulación" permite la exención de la necesidad de desarrollar una reglamentación para una sustancia para ser usada en contacto con los alimentos, cuando la exposición diaria estimada en la dieta del consumidor, con respecto al uso previsto de esta sustancia, esté por debajo del valor umbral.

La "Política de Constituyentes" se aplica a las impurezas cancerígenas que estén en los aditivos alimentarios no considerados como cancerígenos en sí mismos. Aunque las leyes de los EE.UU. prohíben que cualquier sustancia considerada cancerígena cuando se consuma por humanos o animales se apruebe como aditivo alimentario, las impurezas cancerígenas se pueden valorar mediante procedimientos de evaluación cuantitativa del riesgo, siempre que el aditivo no sea cancerígeno.

I.3.3 La legislación en el MERCOSUR

Atendiendo al concepto de aptitud sanitaria, para la fabricación de envases y equipamientos alimentarios, sólo se permite el uso de sustancias que estén enumeradas en las llamadas listas positivas. Estas sustancias son tanto materiales básicos, como aditivos y otros componentes menores. En estas listas positivas pueden especificarse restricciones en el uso de ciertos componentes de los materiales de envasado. Estas restricciones pueden adoptar la forma de límites de composición, límites de migración específica, prohibiciones de usar determinadas sustancias para contacto con un tipo de alimentos, o autorizaciones para usar determinadas sustancias sólo en ciertos materiales de envasado. Otro factor a tener en cuenta es el límite de migración total o global, para lo cual se realizan ensayos de cesión en los que se cuantifica la cantidad total de componentes que se han transferido al simulante del alimento.

Finalmente se establece que los materiales de envasado no deben modificar los caracteres sensoriales de los productos que contienen.

I.4.- LA MIGRACIÓN DE COMPONENTES DESDE EL ENVASE AL ALIMENTO

De las diferentes interacciones que tienen lugar en el sistema envase/alimento/entorno, la migración de distintos componentes del material de envase alcanza una particular importancia, por su incidencia en la calidad y seguridad de los alimentos envasados.

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

Por este motivo en la Unión Europea, existe legislación de obligado cumplimiento, que define los límites máximos de migración global y migración específica, para envases plásticos en contacto con alimentos. Las investigaciones científicas relacionadas con la migración potencial y con el comportamiento de los materiales de envasado han demostrado que la difusión y la migración pueden ser fenómenos previsibles y en principio descritos matemáticamente.

Así, si se establece un modelo de migración físico-química que pueda describir matemáticamente los procesos de migración desde los plásticos a los alimentos reales, sería de inestimable utilidad:

- Como una única herramienta para estimar la exposición del consumidor, en cuanto a la migración dentro del marco convencional de condiciones del sistema de regulación alimentaria de la Unión Europea, aplicando el peor caso posible de exposición.
- Junto con los datos estadísticos obtenidos del consumo alimentario y de los estudios de envases plásticos alimentarios para estimar una real, o peor caso posible, exposición del consumidor en cualquier situación. Se lograría a través de la flexibilidad del cálculo de la proporción de la migración en cualquier alimento, desde cualquier plástico, en contacto en cualquier condición.

I.5.- OBJETIVOS

1.- Desarrollar un método multirresiduo basado en HPLC-FL, para la separación y cuantificación de BADGE, BFDGE, sus derivados y oligómeros del BADGE y NOGE en simulantes de alimentos que permita evaluar la conformidad del recubrimiento con la legislación europea.

2.-Desarrollo de un método analítico basado en HPLC-MS, para identificar y cuantificar los derivados del BADGE procedentes de recubrimientos epoxi empleados en envases alimentarios.

3.-Aplicar y optimizar el método cromatográfico anterior para identificar y cuantificar BFDGE, sus derivados y NOGEs de peso molecular mayor.

4.-Cuantificar la migración de BADGE y BFDGE en muestras reales e identificar otros derivados, mediante la aplicación de los métodos cromatográficos anteriormente optimizados.

5.- Realizar una extensa revisión bibliográfica de las propiedades físicas y químicas de 18 compuestos seleccionados por el proyecto FOODMIGROSURE. Llevar a cabo además, una revisión bibliográfica de todos los métodos analíticos disponibles para cada compuesto en polímeros, simulantes y alimentos.

6.- Desarrollar un método analítico basado en cromatografía líquida de alta resolución en fase reversa con detectores de ultravioleta y fluorescencia para determinación de los niveles de migración del difenilbutadieno en tres alimentos con propiedades físico-químicas muy diversas: zumo de naranja, pechuga de pollo y queso gouda.

I.6.- ARTÍCULOS EN REVISTAS

Sendón García R., Paseiro Losada P., Pérez Lamela C., 2003. Determination of Compounds from Epoxy Resins in Food Simulants by HPLC-Fluorescence. Chromatographia, 58 (5/6) 337-342.

Sendón García R., Paseiro Losada P., 2004. Determination of bisphenol A diglycidyl ether and its hydrolysis and chlorohydroxy derivatives by liquid chromatography-mass spectrometry. Journal of Chromatography A, 1032 (1/2) 37-43.

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Compilation of Analytical Methods to Determine Migration Kinetics of Selected Additives/Monomers in Plastics to Foodstuffs. ILSI Europe 3rd International Symposium on Food Packaging - Ensuring the Safety, Quality and Traceability of Foods. Barcelona, España, 17-19 de noviembre, 2004.

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DIRECTIVA DEL CONSEJO 80/590/CEE, de 9 de junio de 1980 relativa a la determinación del símbolo que puede acompañar a los materiales y objetos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 151/21 de 19.06.80).

DIRECTIVA DEL CONSEJO 80/766/CEE, de 8 de julio de 1980 relativa a la determinación del método comunitario de análisis para el control oficial del contenido de cloruro de vinilo monómero en los materiales y objetos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 213/42 de 16.08.80).

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DIRECTIVA DEL CONSEJO 82/711/CEE, de 18 de octubre de 1982 que establece las normas de base necesarias para la verificación de la migración de los constituyentes de los materiales y objetos de materia plástica destinados a entrar en contacto con productos alimenticios. (DOCE N° L 297/26 de 23.10.82).

DIRECTIVA DEL CONSEJO 84/500/CEE, de 15 de octubre de 1984, relativa a la aproximación de las legislaciones de los Estados Miembros sobre objetos de cerámica destinados a entrar en contacto con productos alimenticios. (DOCE N° L 277/12 de 20.10.1984).

DIRECTIVA DEL CONSEJO 85/572/CEE, de 19 de diciembre de 1985 por la que se determina la lista de los simulantes que se deben utilizar para controlar la migración de los componentes de los materiales y objetos de material plástico destinados a entrar en contacto con los productos alimenticios. (DOCE N° L 372/14 de 31.12.85).

DIRECTIVA DEL CONSEJO 89/109/CEE, de 21 de diciembre de 1988 relativa a la aproximación de las legislaciones de los Estados miembros sobre los materiales y objetos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 40/38 de 11.02.89).

DIRECTIVA DEL CONSEJO 90/128/CEE, de 23 de febrero de 1990 relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 75 de 21.03.90).

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DIRECTIVA DE LA COMISIÓN 93/10/CEE, de 15 de marzo de 1993 relativa a los materiales y objetos de película de celulosa regenerada destinados a entrar en contacto con productos alimenticios. (DOCE Nº L 93/27 de 17.4.1993).

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DIRECTIVA DE LA COMISIÓN 93/111/CE, de 10 de diciembre de 1993 por la que se modifica la Directiva 93/10/CEE relativa a los materiales y objetos de

película de celulosa regenerada destinados a entrar en contacto con productos alimenticios. (DOCE N° L 310/41 de 14.12.1993).

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DIRECTIVA DE LA COMISIÓN 96/11/CE, de 5 de marzo de 1992 por la que se modifica la Directiva 90/128/CEE relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 61/26 de 12.03.96).

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DIRECTIVA DE LA COMISIÓN 99/91/CE, de 23 de noviembre de 1999 por la que se modifica la Directiva 90/128/CEE relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios. (DOCE N° L 310/41 de 4.12.99).

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CAPÍTULO II: DETERMINATION OF COMPOUNDS FROM EPOXY RESINS IN FOOD SIMULANTS BY HPLC-FLUORESCENCE

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Presentado como póster en: First International Symposium in Separation and characterization of Natural and Synthetic Macromolecules, Ámsterdam, Holanda, 5-7 de febrero, 2003.

ABSTRACT

A fluorimetric-detection RP-HPLC method is developed for the separation and quantification of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE), its hydrolysis and chlorohydroxy derivatives, and NOGE and BADGE oligomers. This method was applied to test an epoxy resin used as a coating for food contact packaging materials with two different heat treatments in the curing process. Migration of these compounds was evaluated in three food simulants: 3% (*w/v*) acetic acid, 10% (*v/v*) ethanol, rectified olive oil and also in acetonitrile. A simple extraction procedure was also applied to obtain olive oil extracts. Quantification was performed by using external calibration, and correlation coefficients were greater than 0,996 and the limit of detection (LOD) was 0.02 mg L^{-1} for all substances. HPLC-MS method was applied to confirm derivatives and oligomers identity.

KEYWORDS

HPLC-Fluorescence, bisphenol A diglycidyl ether (BADGE) and derivatives, bisphenol F diglycidyl ether (BFDGE) and derivatives, NOGE and BADGE oligomers, epoxy resins.

II.1.- INTRODUCTION

Epoxy resins are used as internal surface coating for food cans (sea products, vegetables, beer, soft drinks, powder milk), big storage vessels (wine, water) and for various types of food containers. The success of these epoxy formulations as coatings is due to its desirable flavour-retaining characteristics, its excellent chemical resistance, and its outstanding mechanical properties.

Main resins used are Bisphenol A epoxy resins and/or Epoxy Novolacs resins; both are a complex mixture of chemical species and the chemical expression that better defines these oligomeric series are shown in Figure II.1. This figure also show the eight compounds of a molecular weight less than 1000 Da. Bisphenol A epoxy resins are mainly condensation products of Bisphenol A with epichlorhydrin and Epoxy Novolacs are made by condensing formaldehyde with phenol, followed by epoxidation [1]. They are starting substances used to manufacture surface coatings. If reaction process or cure conditions were not applied in a proper way, and/or finished coating is not correctly crosslinked, components of Bisphenol A epoxy resins or Epoxy Novolacs resins and its reaction products could migrate to food [2].

Recently, many articles have reported the safety of the use of BADGE (Bisphenol A diglycidyl ether), BFDGE (Bisphenol F diglycidyl ether), NOGE (Novolac glycidyl ethers) and its derivatives in materials in contact with foodstuffs. This interest have also been noted in European Legislation; for this reason Directive 2002/16/CE [3] establishes that the sum of the migration levels of BADGE, BADGE.H_2O, BADGE.HCl, BADGE.2HCl, BADGE.H_2O.HCl and BADGE.$2H_2O$ shall not exceed the limit of 1 mg Kg^{-1} in foodstuffs or in food simulants (or 1 mg 6 dm^{-2} in the material tested [4]). The same specific migration limit is established for BFDGE and its respective derivatives (Figure II.2). Concerning to the use of NOGE (Figure II.1), the quantity of its components shall not be detectable in food contact materials at the detection limit of 0.2 mg 6 dm^{-2}, since March 2003 [3].

Furthermore of migration of above mentioned compounds, it is also possible the migration into food of unknown and potentially toxic substances. This problem has been reported by some researchers who show chromatograms with unidentified components after migration assays in food simulants [5, 6] or in food products [7, 8]. Analytical problems increase when these compounds react with food components [9], due to its identification becomes more difficult still.

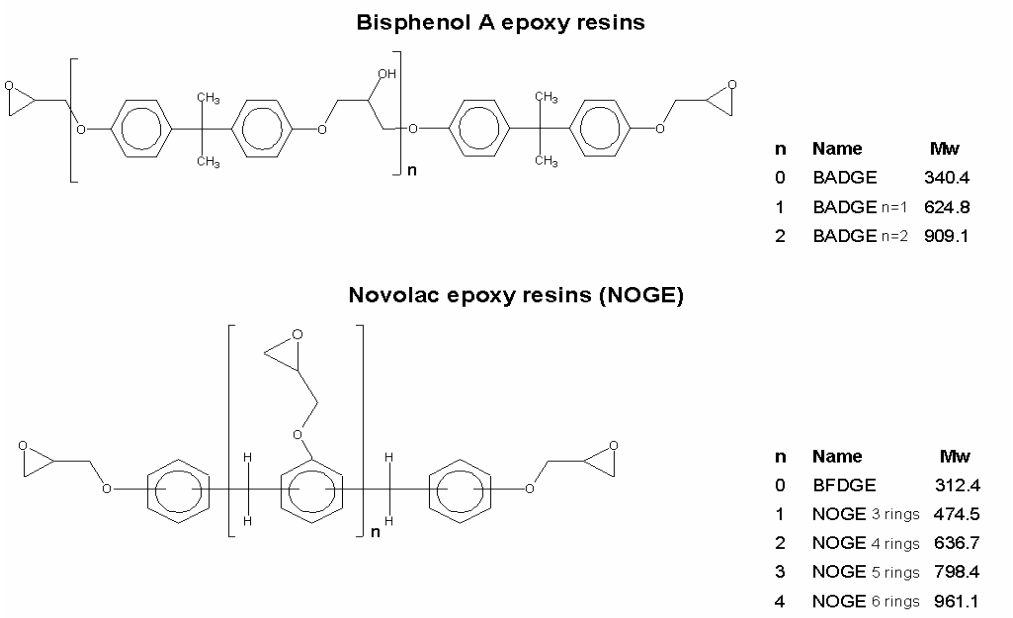

Figure II.1: Compounds of low Molecular Weight in BADGE / NOGE series.

Additionally, quantification of NOGE is not easy because of the high number of components [10]. Apart from that, migration processes depend on many factors and some authors have studied kinetics migration of BADGE resins in aqueous food simulants [11, 12] and olive oil [13].

Usually, food industries purchase the packaging material already coated with these resins and they do not know exactly the epoxy coatings composition. So, it is convenient to develop methods to analyse migrant compounds and therefore to guarantee the compliance of epoxy resins based coatings with food packaging European Legislation.

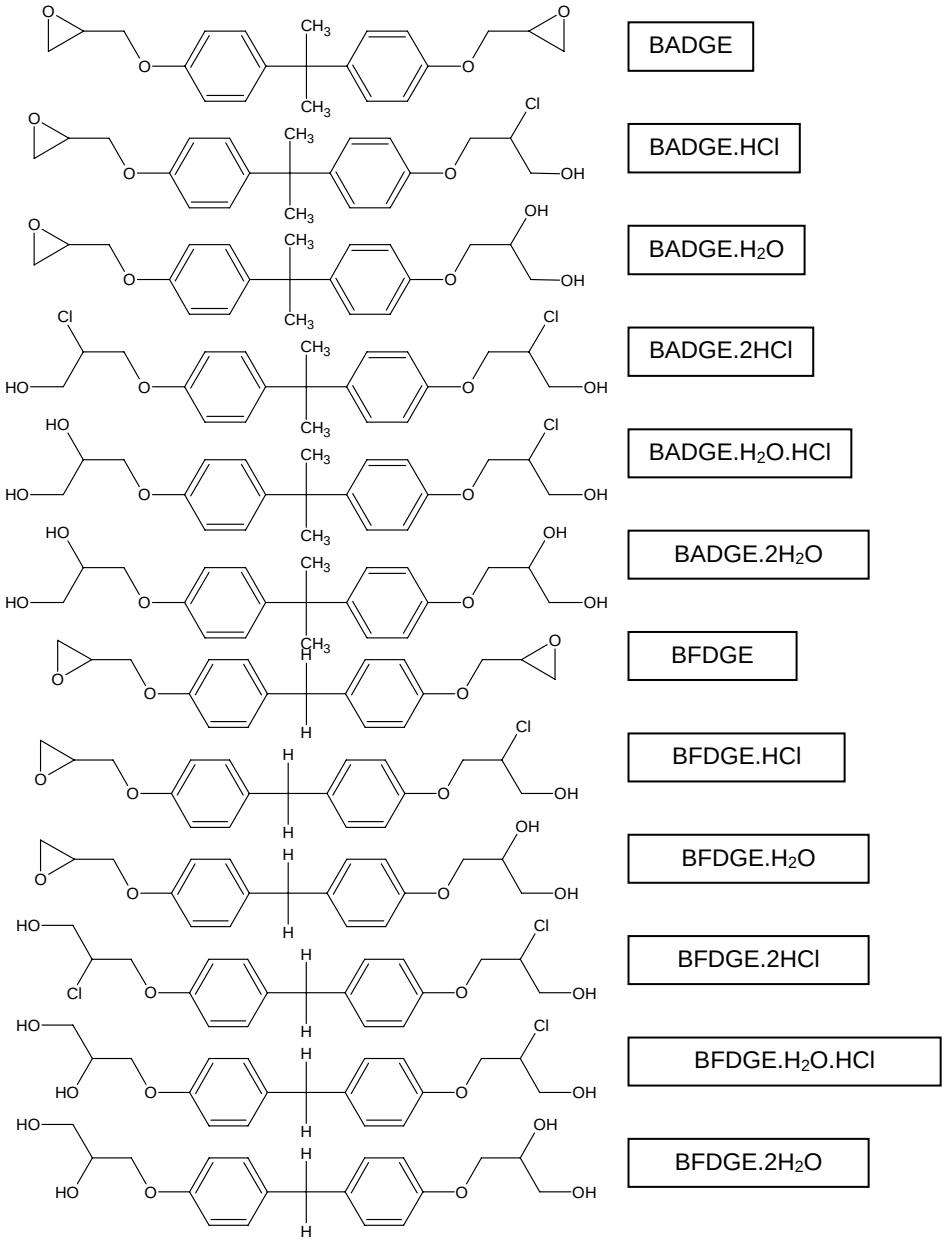

Figure II.2: Chemical structures of BADGE, *p,p*-BFDGE and its derivatives.

II.2.- EXPERIMENTAL

II.2.1 Reagents

Acetonitrile was HPLC grade and the other solvents used (ethanol, acetic acid and tetrahydrofuran) were analytical grade. All were supplied by Merck (Darmstadt, Germany) and purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Rectified olive oil was used as a fatty food simulant. Standards of Bisphenol A diglycidyl ether (BADGE, CAS: 1675-54-3, (≥97%)) and Bisphenol F diglycidyl ether (BFDGE, CAS: 2095-03-6, (total of three isomers (≥97%): *ortho-ortho, para-para, ortho-para*) were purchased from Fluka Chemie AG (Buchts, Switzerland). Poly (phenyl glycidyl ether)-*co*-formaldehyde (NOGE, Average M ca. 605) and Poly (Bisphenol A -*co*- epichlorohydrin) glycidyl end-capped (BADGE oligomers, Average M ca. 1075) were from Sigma-Aldrich (Steinheim, Germany).

II.2.2 Standards

Two different stock solutions of BADGE and BFDGE containing 1000 mg L^{-1} were prepared in tetrahydrofuran. In the same way, NOGE and BADGE oligomers were dissolved in tetrahydrofuran at a concentration of 1000 mg L^{-1} respectively. Intermediate standards solutions were prepared by dissolving appropriate amounts of all compounds in 90% (*v/v*) acetonitrile to yield a concentration of 1 mg L^{-1}.

Hydrolysis products, BADGE.H_2O and BADGE.$2H_2O$ were obtained by dissolution of 1 mL of BADGE stock solution in 50 mL of water and storing this flask for 2 hours at 60ºC.

Chlorohydrins of BADGE were obtained by adding to 5 mL of the stock solution 0.25 mL of HCl 0.05N. After 20 min, an intermediate solution was prepared by filling to the mark with 250 mL of 90% acetonitrile. This intermediate solution contains a mixture of BADGE, BADGE.HCl and BADGE.2HCl.

The hydrolysis product of BADGE chlorohydrin (BADGE.H_2O.HCl) was obtained by dissolving 5 mL of chlorohydrins stock solutions in 250 mL of water and storing this solution for 2 hours at 60ºC. Hence, all BADGE derivatives were prepared.

The same procedure was followed to obtain all BFDGE derivatives (BFDGE.H_2O, BFDGE.HCl, BFDGE.$2H_2O$, BADGE.H_2O.HCl and BFDGE.2HCl).

Hydrolysis and chlorohydrins compounds obtained from BADGE and *p,p*-BFDGE are shown in Figure II.1.

II.2.3 Apparatus and conditions

Chromatographic measurements were performed with a Hewlett Packard (Waldbronn, Germany) system comprised of a HP1100 liquid chromatograph fitted with a quaternary pump, an autosampler, a column oven, a fluorescence array detector and HP Chemstation data analysis software (version A.06.01). A Kromasil 100 C18 column (5µm i.d., 150mm x 40mm) from Teknokroma (Barcelona, Spain) was used for the separation.

Chromatographic conditions were developed in a previous article [5]. The mobile phase consisted of acetonitrile/water 30:70 (*v/v*) in an isocratic mode for two minutes, followed by a gradient to 80% acetonitrile for eighteen min, another gradient to 100% acetonitrile for three min and finally an isocratic elution during seven min. The flow rate was 1 mL min^{-1}. The injection volume was 50 µL. The column oven temperature was kept at room temperature. Fluorescence detection was performed with excitation and emission wavelengths of 225 nm and 305 nm, respectively.

Identification of selected compounds was carried out using a mass spectrometer (VG Platform II, Fisons) in positive mode by atmospheric pressure chemical ionisation (APCI). The following instrument parameters were applied: probe temperature: 500 ºC, ionisation source temperature: 130 ºC, cone voltage: 30 V, electron multiplier voltage: 700 V, sheath gas nitrogen at 200 L h^{-1} and drying gas nitrogen at 250 L h^{-1}.

II.2.4 Test samples

The polymer coating tested was an epoxy paint intended to use as a coating into foodstuffs. Glass discs, each with an area of 1.22 dm^2, were painted with the polymer coating on two sides. All of them were curing for 24 h at room temperature. Several of them were also subjected to a post curing treatment for 1 h at 100ºC.

II.2.5 Analysis of samples

All discs painted were immersed in 100 mL of each aqueous simulant (3% (*w/v*) acetic acid and 10% (*v/v*) ethanol) and in 100 g of fatty simulant (rectified olive oil) into hermetic glass cells. All of them were sterilized for 30 min at 121ºC following migration assays conditions described in European Legislation [14]. Alternative analysis were carried out by immersing painted discs in 100 mL of 90% acetonitrile in hermetic glass cells maintained at 40ºC for 24 h. All migration tests were performed by duplicate.

Aqueous simulants (3% (*w/v*) acetic acid and 10% (*v/v*) ethanol) and 90% acetonitrile extracts were filtered through a PTFE 0.45 µm 13 mm syringe filter, and injected into the chromatograph. Olive oil simulant was extracted according to the procedure described in a previous work [15]: 5 mL of heptane were added to 5 g of olive oil and mixed. Then, 10 mL of 90% acetonitrile were added and the mixture was shaken vigorously for 1 min and subsequently was centrifuged for 5 min at 1500 rpm. Acetonitrile phase was taken up, filtered through a PTFE 0.45 µm 13 mm syringe filter and injected.

II.3.- RESULTS AND DISCUSSION

Chromatographic protocol [5] has been prolonged in 5 min in order to improve the separation of substances with a higher molecular weight (e.g. NOGE and BADGE oligomers). This method has been successfully applied for the separation of Bisphenol A epoxy resins, NOGE and its derivatives as is shown in Figure II.3.

As individual standards of NOGE and BADGE oligomers ($n \geq 1$) are not commercially available, two different calibration lines of BFDGE and BADGE were used to quantify NOGE and BADGE oligomers, respectively. These calibration lines were constructed using BADGE and BFDGE (pure standards) and had correlation coefficients (r) greater than 0.996. The concentration of BFDGE was calculated as the sum of its three isomers. To quantify all BADGE and BFDGE derivatives a similar procedure was followed. Hence, results were expressed in BADGE for all BADGE

derivatives and in BFDGE for all BFDGE derivatives, assuming that derivatives response factors are equal as BADGE and BFDGE, respectively.

Test samples with the polymer coating (with and without post curing treatment) were analysed by duplicate for each simulant and acetonitrile as has been described previously. Results are summarized in Table II.1.

	Cured	**Post cured**
3% acetic acid	0.112±0.002	0.078±0.007
10% ethanol	0.126±0.004	0.091±0.008
Olive oil	0.031±0.002	0.007±0.001
90% acetonitrile	0.616±0.0173	0.151±0.004

Table II.1: Summary migration results for all BADGE related compounds expressed as mg BADGE dm^{-2}.

The preparation of hydrolysis products and chlorohydrins of BADGE and BFDGE was performed by applying two different procedures for each one in order to ascertain derivatives obtained. In the case of BFDGE, the existence of three isomers (*para-para, ortho-para* and *ortho-ortho*) makes the chromatographic separation and the identification more difficult. Experimentally, it has been proved that 2 hours are needed to yield all hydrolysis derivatives (BADGE.H_2O, BADGE.$2H_2O$, BFDGE.H_2O, BFDGE.$2H_2O$) and 20 minutes are enough time to obtain chlorohydrins derivatives (BADGE.H_2O.HCl, BADGE.2HCl, BFDGE.H_2O.HCl, BFDGE.2HCl) (Figure II.3).

Although BADGE derivatives were commercially available as standards products very recently, they also can be obtained in the laboratory [16, 17].

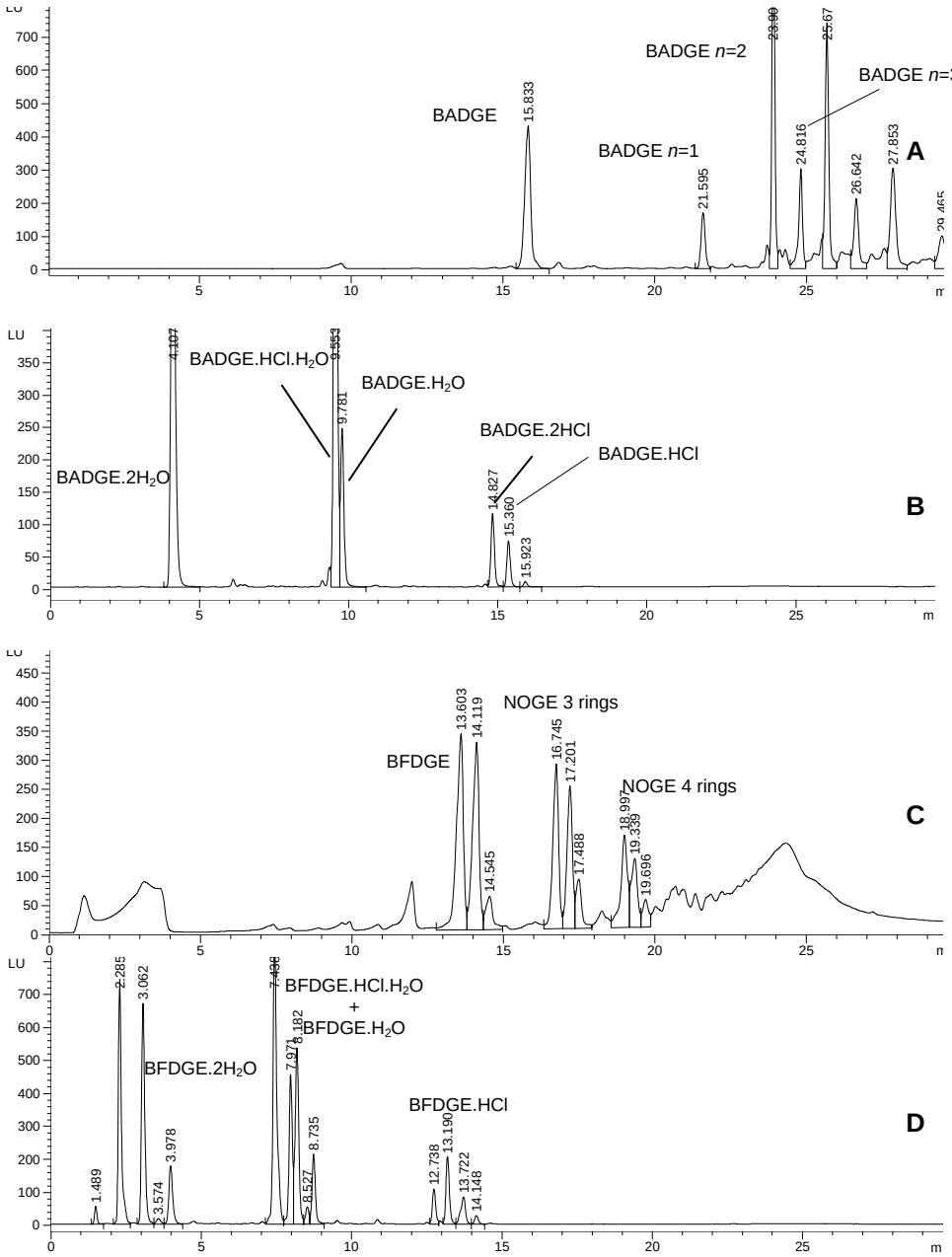

Figure II.3: Chromatograms of Bisphenol A epoxy resin (**A**), NOGE resin (**C**) and all derivatives obtained from BADGE and BFDGE standards (**B** and **D**).

The HPLC-MS confirmation method was applied to verify the identity of the compounds, derivatives and oligomers, from the epoxy paint in the extracts obtained after the migration assays. BADGE and its hydrolysis derivatives were previously identified [17]. BADGE oligomers were identified by its mass spectrum. Masses corresponding to the [M + acetonitrile]$^+$ adduct of the different oligomers were present in the total ion chromatogram. So, for BADGE *n*=0 was detected the mass 382 [M(340)+42]$^+$, for BADGE *n*=1, mass 666 [M(624)+42]$^+$ and for BADGE *n*=2 mass 951 [M(909)+42]$^+$ (Figure II.4). BADGE oligomers with higher molecular weight than 1000 Da do not have such interest because it is generally accepted that compounds with M>1000 Da are non-bioavaliable by oral way, so they could not pass through physiological membranes.

The procedure described in this work is simpler than the one used for other authors [18, 19] and allows the identification of a larger number of compounds.

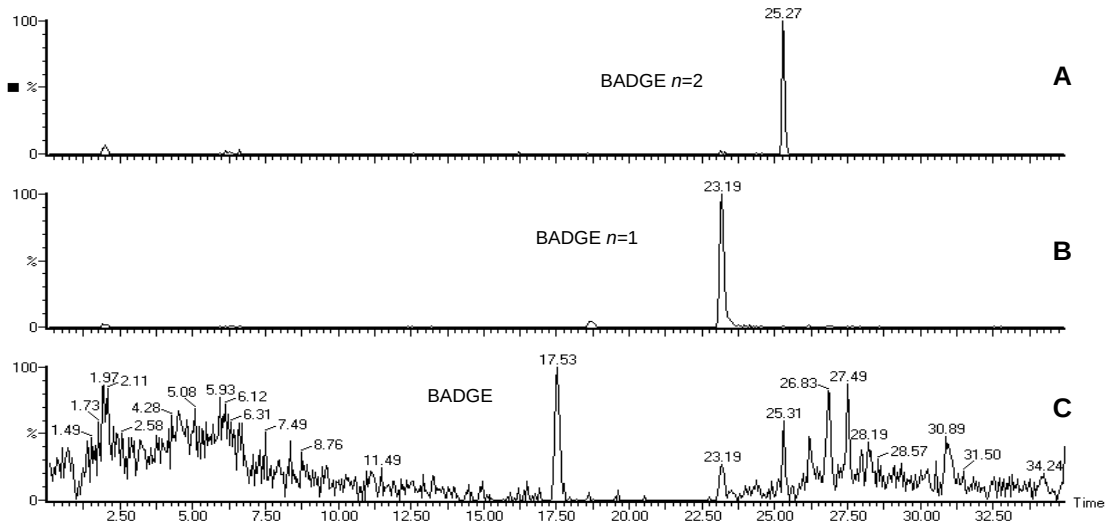

Figure II.4: HPLC-MS chromatogram of a sample without post cured treatment extracted with 90% acetonitrile acquired in single ion recording mode: mass 951(**A**), mass 666 (**B**) and mass 382 (**C**).

Epoxy coating tested was a BADGE resin-type because none NOGE neither BFDGE derivatives were found in the sample chromatograms (Figure II.5); and the only derivatives identified after the migration assays were hydrolysis products and oligomers provided by a BADGE coating, as shown in Table II.2. Although BADGE oligomers with M>1000 Da were present, they were not quantified because it was not the aim of this work.

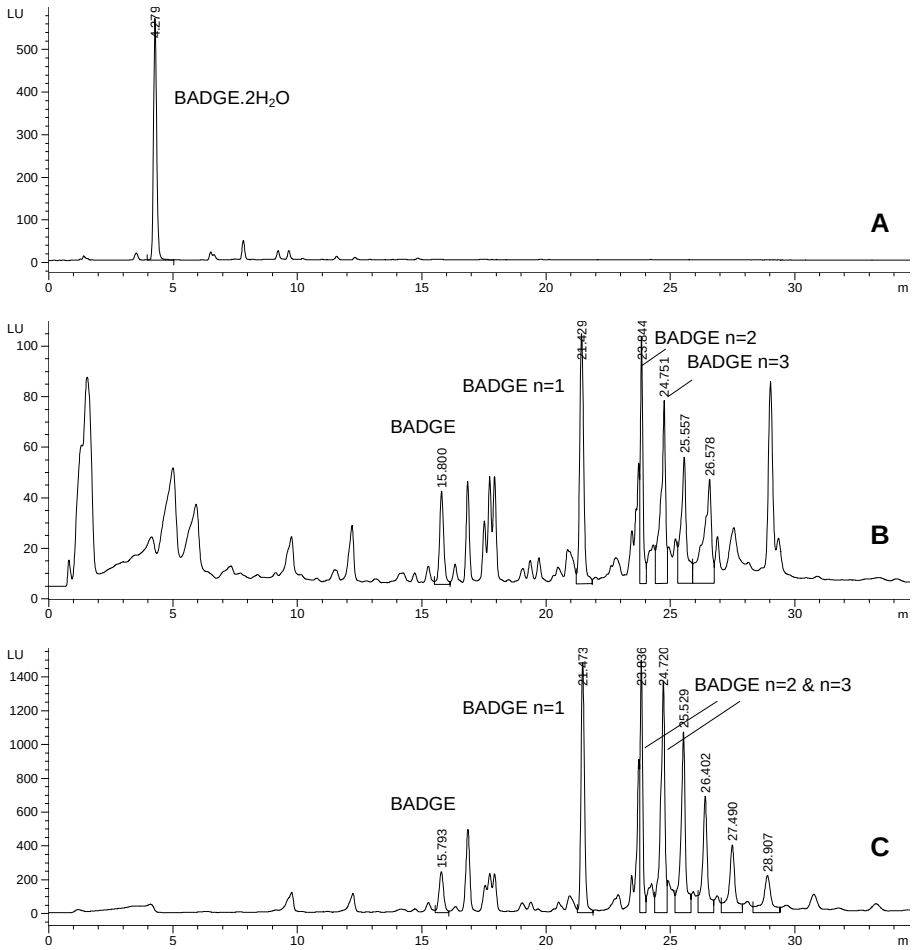

Figure II.5: Chromatograms of samples without post cured treatment: 3% acetic acid (**A**), olive oil (**B**) and 90% acetonitrile (**C**).

This migration behaviour is expected in aqueous food simulants due to the BADGE (*n*=0) is hydrolysed after its migration to the simulant [10, 11]. BADGE oligomers are not detectable in aqueous simulants because of its very low solubility (Figure II.5 A). Meanwhile olive oil extracts contain BADGE oligomers (Table II.2) as found by other researchers in oily canned foods [9] but do not contain significant hydrolysed derivatives (Figure II.5 B).

Acetonitrile extracts contain the same epoxy substances as olive oil extracts (Figure II.5 C), which confirm the obtained data in the migration assay with olive oil.

Analysis of oil samples is always troublesome and some researchers have applied different procedures to extract epoxy compounds from fatty canned foods [8, 9]. The extraction process applied in this work is simple and could be used to carry out the analysis in real samples with surrounding oil.

As it is shown in Table II.1, the epoxy coating complies EU legislation [3]. But when, the alternative assay with acetonitrile is used and coatings have not been post cured, the total amount of migrates identified (including BADGE oligomers) is higher than the amounts obtained with the migration test in olive oil for not post cured coatings; it is also observed that all samples subjected to the post curing treatment show lower migration values.

	3% acetic acid		10% ethanol		Olive oil		90% acetonitrile	
	Y	N	Y	N	Y	N	Y	N
BADGE.2H$_2$O	0.078±0.007	0.112±0.002	0.087±0.009	0.125±0.005	n.d	n.d	n.d	n.d
BADGE.H$_2$O.HCl	n.d.	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d
BADGE.H$_2$O	n.d.	n.d.	0.004±0.001	0.0010±0.000	n.d	n.d	n.d	0.034±0.003
BADGE.2HCl	n.d.	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d
BADGE.HCl	n.d.	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d
BADGE *n*=0	n.d.	n.d.	n.d.	n.d.	n.d	0.007±0.001	0.006±0.000	0.052±0.003
BADGE *n*=1	n.d.	n.d.	n.d.	n.d.	0.007±0.001	0.015±0.001	0.058±0.002	0.241±0.005
BADGE *n*=2	n.d.	n.d.	n.d.	n.d.	n.d	0.009±0.001	0.087±0.002	0.289±0.005

Y = with post cured treatment
N = without post cured treatment
n.d.=not detectable

Table II.2: Results for BADGE derivatives and oligomers migration expressed as mg BADGE dm^{-2}.

Current legislation [3] establishes migration levels of BADGE, BFDGE and its derivatives as well as NOGE but does not make any reference with respect to the BADGE oligomers of low molecular weigh (M<1000), which also should be included in regulations in order to protect consumer safety, due to BADGE oligomers concentration can be higher than BADGE itself.

Acetonitrile helps to test this type of materials and seems to be a good extractor as it is maintained by other authors [20].

ACKNOWLEDGMENTS

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CAPÍTULO III: DETERMINATION OF BISPHENOL A DIGLYCIDYL ETHER AND ITS HYDROLYSIS AND CHLOROHYDROXY DERIVATIVES BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

European Legislation establishes that the sum of the migration levels of bisphenol A diglycidyl ether (BADGE), its hydrolysis (BADGE.H_2O and BADGE.$2H_2O$) and chlorohydroxy (BADGE.HCl, BADGE.2HCl and BADGE.H_2O.HCl) derivatives shall not exceed the limit of 1 mg/Kg in foodstuffs or food simulants. A reversed phase high performance liquid chromatographic method combined with mass spectrometry detection (HPLC-MS) using the atmospheric pressure chemical ionisation (APCI) is developed for the separation, quantification and identification of the interesting compounds. Quantification of the analytes was carried out in the single ion recording mode (SIR), once their characteristic masses were selected from their full spectra, by using an external calibration. The optimised method was suitable for the migration evaluation of these compounds in different samples.

KEYWORDS

HPLC-MS; Bisphenol A diglycidyl ether (BADGE); Hydrolysis products; Chlorohydroxy derivatives.

III.1.- INTRODUCTION

Epoxy resins are used to make internal surface coatings for food cans (sea products, vegetables, beer, soft drinks, powder milk), big storage vessels (wine, water) and various types of food containers. Bisphenol A epoxy resins are mainly condensation products of Bisphenol A with epichlorhydrin [1]. If the reaction process or cure conditions were not applied in a proper way, and finished coating is not correctly crosslinked, components of Bisphenol A epoxy resins and reaction products formed could migrate to food [2], and in some cases react with food components giving new compounds.

Recent studies about the toxicity of these compounds [3-5], have demonstrated that the genotoxic effect of bisphenol A diglycidyl ether (BADGE) was stronger than the genotoxic effect of BADGE.H_2O and BADGE.$2H_2O$. Regarding BADGE.HCl, its genotoxic effect was comparable to those obtained to BADGE.H_2O. These studies support the hypothesis that the degree of toxicity of epoxy compounds depends mainly of the concentration of unreacted epoxy groups, although the case of BADGE.2HCl is different because it does not present any epoxy group, but its genotoxicity is probably due to the presence of Cl groups.

BADGE can also be used in organosols in order to remove hydrochloric acid, which results in formation of BADGE.HCl, BADGE.2HCl and BADGE.H_2O.HCl [6]. BADGE may easily hydrolyse in contact with aqueous and acidic food forming BADGE.H_2O and BADGE.$2H_2O$ [7] (Figure III.1).

European Legislation establishes that the sum of the migration levels of BADGE, its hydrolysis (BADGE.H_2O and BADGE.$2H_2O$) and chlorohydroxy (BADGE.HCl, BADGE.2HCl and BADGE.H_2O.HCl) derivatives shall not exceed the limit of 1 mg/Kg in foodstuffs or in food simulants or 1 mg/6dm^2 in the material tested (conventional conversion factor that expresses the relationship between the surface area of materials and the quantity of foodstuffs in contact therewith [8,9]).

The most widely used techniques for the analysis of BADGE are normal and reverse phase high performance liquid chromatography (NPLC and RP-HPLC) with fluorescence detection [10-18], as well as its hydrolysis and chlorohydroxy derivatives [12,16,19-21]. Several researches have applied gas chromatography (GC) coupled with mass spectrometry detection to determine these compounds [22-

25]. Most of the times this technique was used just to confirm positively the presence of these compounds after HPLC quantification with fluorescence detection [11,14,21,26].

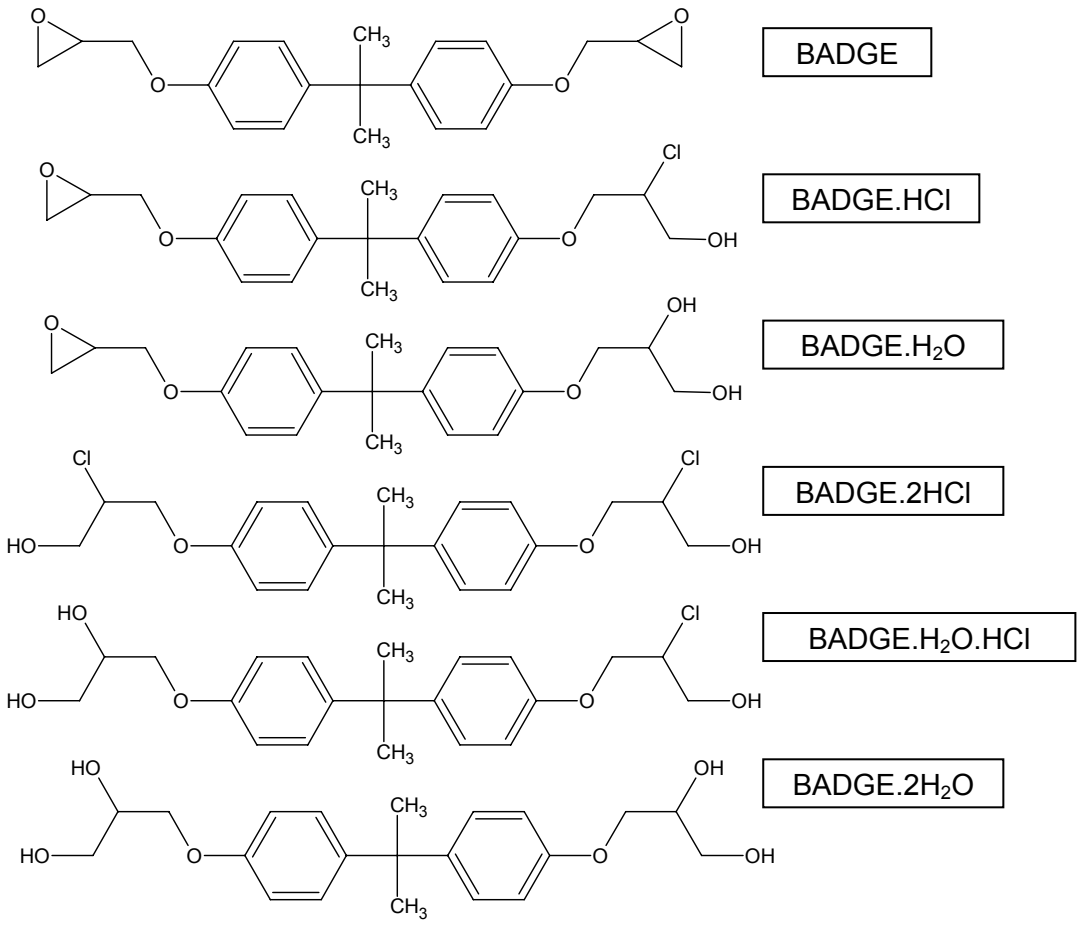


Figure III.1: Chemical structures of BADGE and its derivatives.

Some works are focused on the identification of these substances and other oligomers of higher molecular weight using HPLC with detection by atmospheric pressure chemical ionisation (APCI) [27-30], thermospray mass

spectrometry (TSP) [31] and electrospray ionisation (ESI) [29,32]. The characterization of BADGE hydrolysis products has also been described by other authors [33]. Very few works are focused on the quantification using an HPLC-MS technique [34], but this last case is limited to BADGE and BADGE.2H_2O.

The objective of this work is to develop a method that allows both, the quantification and positive identification of BADGE and its derivatives using an RP-HPLC-MS technique (APCI), after its majority characteristic masses were selected. This will facilitate the migration studies in complex food or in food simulants, once this way possible interferences are minimized.

III.2.- EXPERIMENTAL

III.2.1. Chemicals and reagents

Acetonitrile was HPLC grade, supplied by Merck (Darmstadt, Germany) and ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). Standards of Bisphenol A diglycidyl ether (BADGE, CAS No. [1675-54-3], (≥97%)), Bisphenol A (2,3-dihydroxypropyl) glycidyl ether (BADGE.H_2O, CAS No. [76002-91-0], (≥97%)), Bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE.2H_2O, CAS No. [5581-32-8], (≥97%)), Bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether (BADGE.HCl, CAS No. [13836-48-1], (≈95%)), Bisphenol A bis(3-chloro-2-hydroxypropyl) ether (BADGE.2HCl, CAS No. [4809-35-2], (≥99%)) and Bisphenol A (3-chloro-2-hydroxypropyl) (2,3-dihydroxypropyl) ether (BADGE.H_2O.HCl, CAS No. [227947-06-0], (≥98%)) were all purchased from Fluka Chemie AG (Buchts, Switzerland).

Individual stock solutions of all compounds containing 1000 mg/l, were prepared in acetonitrile and were kept at -20ºC. Intermediate standards solutions in 90% (v/v) acetonitrile and water were prepared by dissolving appropriate amounts of all compounds to yield a concentration of 10 mg/l. Calibration solutions in water and 90% (v/v) acetonitrile were prepared from these intermediate solutions. Intermediate solutions in 90% acetonitrile were kept at 4ºC and intermediate solutions in water were kept at -20ºC.

III.2.2. Equipment

The HPLC–MS system comprised a Spectra-Physics Series P200 liquid chromatograph equipped with a 50μl injection loop Rheodyne, and a Fisons VG Platform mass detector (VG Biotech, Altrincham, UK), controlled by VG Mass Lynx software (all from SP Thermo Separation Products, Altrincham, UK). The column was a Kromasil 100 C 18 (15 × 0.4 cm I.D., 5μm particle size) (Teknokroma, Barcelona, Spain).

III.2.3. Chromatographic conditions

Elution conditions were developed in a previous article [16]. The mobile phase was acetonitrile-water 30:70 (v/v) in an isocratic mode for two min, followed by a gradient to 80% acetonitrile for eighteen min, another gradient to 100% acetonitrile for three min and finally an isocratic elution during seven min. The flow rate was 1.0 ml/min. Detector settings: positive or negative atmospheric pressure chemical ionisation (APCI) mode, probe temperature 500ºC, ionisation source temperature 130ºC, cone voltage +30 or -30 V, electron multiplier voltage 700 V, drying gas nitrogen at 425 l/h, APCI sheet gas nitrogen at 175 l/h, full-scan mode detection range m/z 100–500 and selected ion recording (SIR) (Table III.1).

Analyte	APCI (+)		APCI (-)	
	Selected Ion (+ *m/z*)	Other ions	Selected Ion (- *m/z*)	Other ions
BADGE	382[a]	191		
BADGE.HCl	418[a]	382		
BADGE.2HCl	382[*]	191		
BADGE.H_2O	400[a]	209		
BADGE.H_2O.HCl			393[b]	283,227
BADGE.$2H_2O$			375[b]	301,227

[a] Fragment corresponding to $[M+CH_3CNH]^+$

[b] Fragment corresponding to $[M-H]^-$

[*] Unknown fragment

Table III.1: Selected ions for quantification.

III.2.4. Samples

Conventional empty 3-piece type cans (with easy-open lids) were provided by the industry. Cans and lids were extracted separately and both were extracted only for the surface intended to be in contact with foodstuffs. Cans were completely filled with acetonitrile and stored for 4 hours at 40ºC. They were covered to prevent evaporation. In this way, an extract of all surfaces was obtained. For the extraction of the lids, special glass cells were used: the lids were located inside the cap cell which is recovered with teflon and turning over the cells once they were closed. Therefore only the internal surface of the lid was in contact with the acetonitrile. These cells were kept for 4 hours at 40ºC. The exposure conditions were the same for all cans tested.

For the HPLC analysis, 0.1 ml of water was added to 0.9 ml of the extract obtained and it was filtered through a PTFE 0.45 µm 13 mm syringe filter, and injected into the chromatograph. In any case no step of cleaning up was applied.

III.3.- RESULTS AND DISCUSSION

III.3.1. Mass Spectrometry

The first part of this work was focused in stablishing the optimums detector conditions. Analysis by this technique is greatly influenced by the cone voltage, which determines the degree of fragmentation of analyte ions. Cone voltages of 10, 30 and 50 V (in (+) and (-) mode) were evaluated for each substance in order to set a cone voltage that allowed to obtain selective ions for each compound. Probes temperatures of 250 and 500ºC were tested and although it did not greatly affect the degree of fragmentation, it was finally set at 500ºC due to the low volatility of all compounds.

Although full scan mode gives more information, it was chosen the SIR mode for the quantification, since this way the sensitivity was improved (about fifty times).

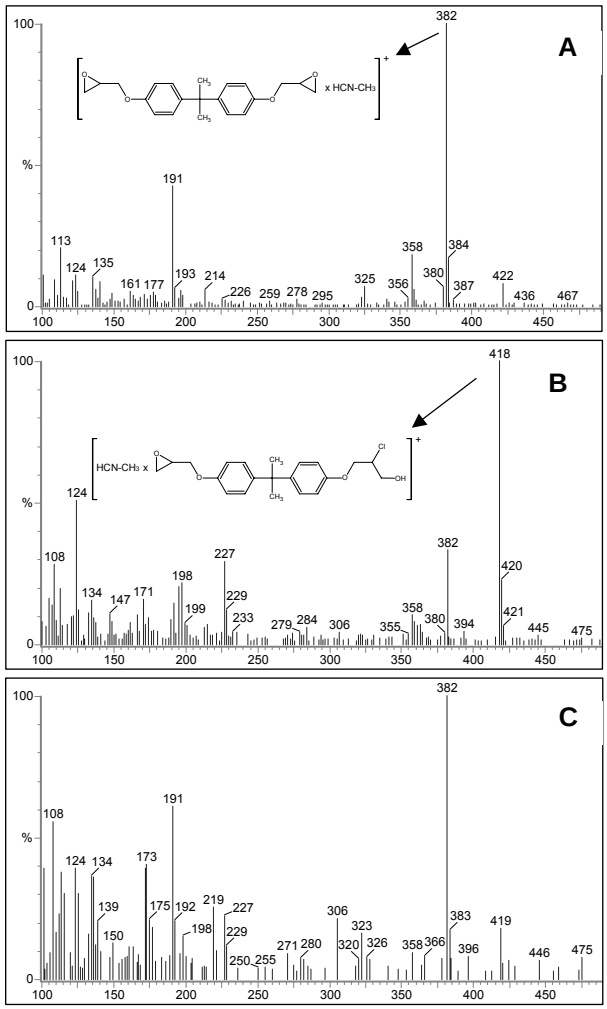


Figure III.2.1: MS spectra of BADGE (**A**), BADGE.HCl (**B**) and BADGE.2HCl (**C**).

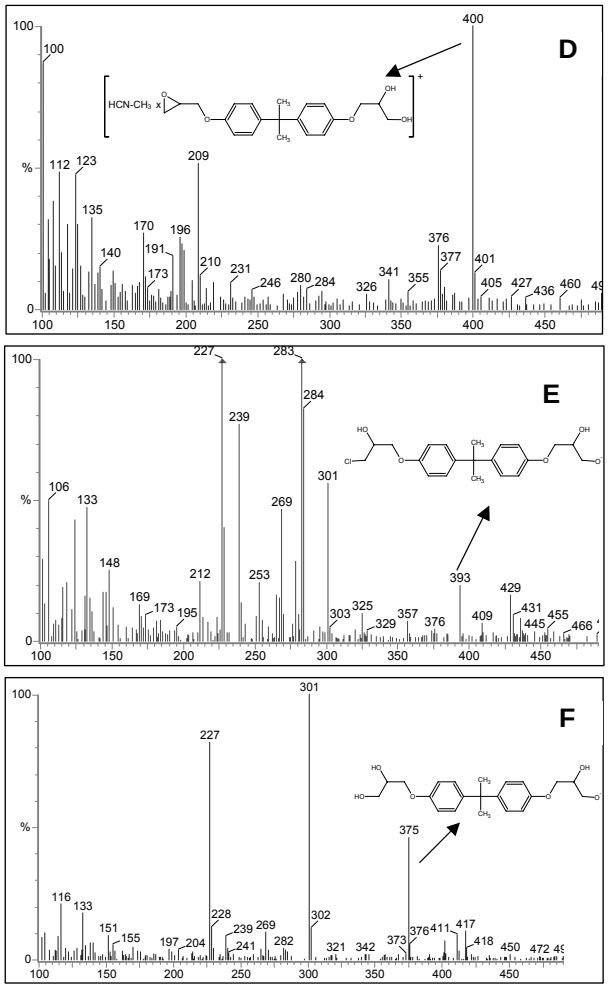


Figure III.2.2: MS spectra of BADGE.H_2O (**D**), BADGE.H_2O.HCl (**E**) and BADGE.$2H_2O$ (**F**).

Once detector conditions were optimised and to establish the SIR parameters for the quantification, initial tests on concentrate standard solutions of 10 mg/l were carried out using the full scan mode to obtain the maximum information from their mass spectra (Figure III.2.1 and III.2.2). BADGE, BADGE.H_2O and BADGE.$2H_2O$ were characterized by other authors [33] using the APCI technique. Thus, for BADGE and BADGE.H_2O, APCI (+) was used, and fragments corresponding to the clusters comprising these analytes and a molecule of acetonitrile were observed to be the most abundant ($[M+CH_3CNH]^+$) (Figure III.2.1.A and III.2.2.D). For BADGE.$2H_2O$, APCI (-) was used and the fragment corresponding to the $[M-H]^-$ was selected to quantify due to its selectivity (Figure III.2.2.F).

For BADGE.HCl the major fragment corresponds to the $[M+CH_3CNH]^+$ (Figure III.2.1.B) which coincides with other authors [27]. The case of BADGE.2HCl is more difficult to explicate. It could be explained if the molecule loses its Cl atoms and hydroxyl groups of the chlorohydrin act as nucleophilic reagents, giving rise to a conjugated acid from which the epoxide arises by the elimination of a proton [35]. So the formed molecule would act as BADGE, forming a cluster with a molecule of acetonitrile.

BADGE.H_2O.HCl was determined using the APCI (-) mode instead of (+) mode due to the impossibility of undergoing a chromatographic separation from BADGE.H_2O. Hence, a very selective fragment of BADGE.H_2O.HCl corresponding to its $[M-H]^-$ (Figure III.2.2.E) ion was selected to determine it.

Furthermore for the substances that were determined using the APCI (-) mode, other fragments (Table III.1) were present in a more relative abundance, they were not selected because they were common to more species. Table III.2 shows possible structures of these fragments.

III.3.2. Chromatography

Chromatographic protocol has been optimised previously in order to improve the separation of these substances. Not all BADGE derivatives have response in the same ionisation mode, so two injections of each sample were always necessary in negative and positive mode. Figure III.3 and III.4 show chromatograms acquired in both positive and negative mode.

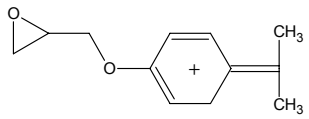
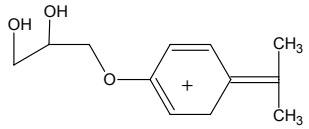
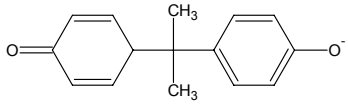
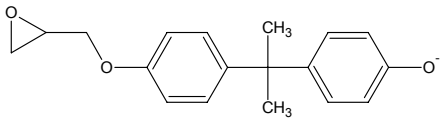
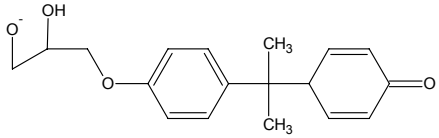
Ion	Structure	APCI
191		(+)
209		(+)
227		(-)
283		(-)
301		(-)

Table III.2: Possible structures of other fragments.

III.3.3. Method validation

The method was calibrated using series of standards (mixtures of all substances) in 90% acetonitrile of known concentrations. The relationship between known concentrations and measured areas was assessed by linear regression (five calibrations points), and the linearity obtained indicates that the method is appropriate for quantification of these compounds (Table III.3). Detection limits (DLs), (defined as signal three times the height of the noise level) were calculated in accordance with American Chemical Society [36] and are shown in Table III.3. The lower DL corresponds to BADGE, and although in other works [6,17,22,28] have reached lower levels employing fluorescence detection, in this case, no positive confirmation is necessary. Additionally, for the substances with a higher DL, it has been observed

that if other ions are syntonized it could be reached a lower DL (about three times less in case of BADGE.H_2O.HCl).

The method was also evaluated in water instead of 90% acetonitrile. For all substances correlation coefficients were higher than 0.99 and the DLs were similar except for BADGE.$2H_2O$ and BADGE.H_2O.HCl, which were 0.1 mg/l for both substances.

In both cases (for 90% acetonitrile and water), comparing the established SMLs in the European Legislation [8], the detection limits provide well enough performance, although with other techniques lower values can be obtained.

Precision was estimated as relative standard deviation (RSD %) analysing six independent solutions at the level of 1mg/l, obtaining for all compounds a value ≤ 5% (Table III.3).

	Linear range (mg/l)	Linearity (r^2)	Detection Limit (mg/l)	RSD (%)
BADGE	0.1-2.4	0.9975	0.05	3.9
BADGE.HCl	0.4-2.4	0.9970	0.1	4.1
BADGE.2HCl	0.4-2.4	0.9902	0.1	3.9
BADGE.H_2O	0.4-2.4	0.9980	0.1	4.3
BADGE.H_2O.HCl	0.8-2.4	0.9903	0.4	5.0
BADGE.$2H_2O$	0.4-2.4	0.9975	0.1	4.0

Table III.3: Method validation parameters.

Once the operating conditions had been optimised, screening analyses were performed with empty cans (eight different samples) analysing them by duplicate and extracting them with acetonitrile as it has been described previously. No peaks of interest were detected in any case, which demonstrates the safety of the cans tested. A similar extracting procedure has been used by other authors [22] to test empty cans. Acetonitrile helps to test this type of materials and seems to be a good extractor [27], once this simple sample procedure could give an idea of the security of cans prior its use as a packaging material for foodstuffs.

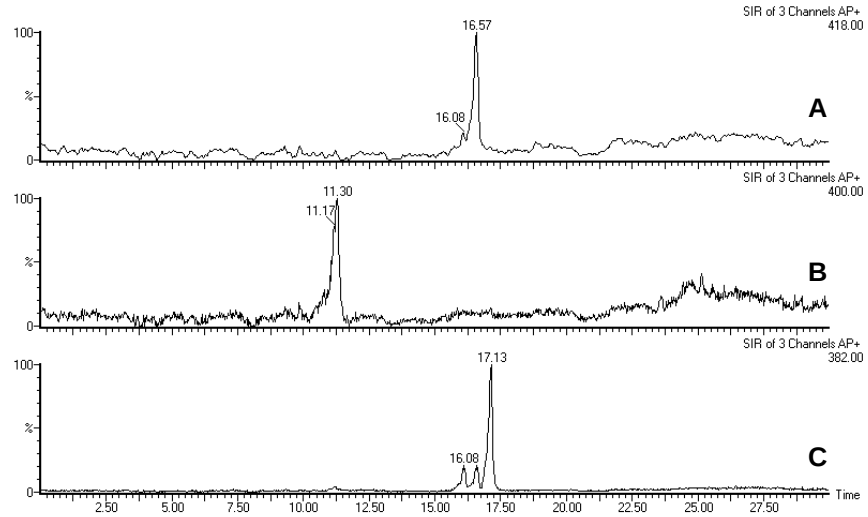


Figure III.3: MS chromatograms in APCI (+) of BADGE.HCl (**A**), BADGE.H_2O (**B**) and BADGE (t_R: 17.3) and BADGE.2HCl (t_R: 16.0) (**C**) at a concentration of 0.8 mg/l.

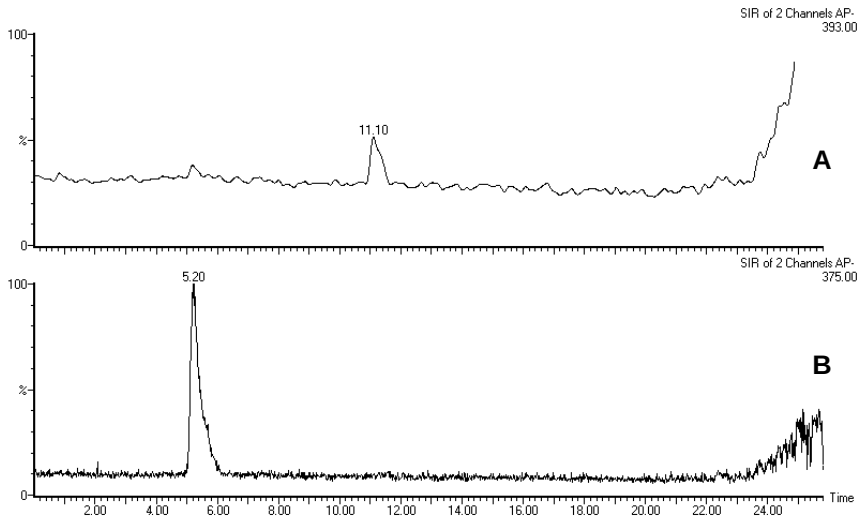


Figure III.4: MS chromatograms in APCI (-) of BADGE.H_2O.HCl (**A**) and BADGE.$2H_2O$ (**B**) at a concentration of 0.8 mg/l.

In conclusion, the developed method is appropriate for the simultaneous analysis of all these compounds in acetonitrile extracts and in water (official food simulant). This procedure is also a powerful technique that allows the positive confirmation of the presence of these compounds, not being necessary another technique for its quantification.

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CAPÍTULO IV: DETERMINATION OF BISPHENOL F DIGLYCIDYL ETHER AND ITS RELATED COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Enviado para su publicación

Presentado como póster en: 8th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers. Brujas, Bélgica, 4-6 de febrero, 2004.

ABSTRACT

A reversed phase high performance liquid chromatographic method combined with mass spectrometry detection (HPLC-MS) using atmospheric pressure chemical ionisation (APCI) is developed for the separation, quantification and identification of bisphenol F diglycidyl ether (BFDGE) and other derivatives (epoxy and chlorohydroxy related compounds). These compounds are employed in formulations intended to be used to cover inner surfaces of food cans. Quantification of the analytes was carried out in the single ion recording mode (SIR), once their characteristic masses were selected from their full spectra, by using an external calibration. Novolac glycidyl ether (NOGE) oligomers can be also identified by this method.

KEYWORDS

Bisphenol F diglycidyl ether (BFDGE); Novolac glycidyl ethers (NOGE), food can coatings, HPLC-MS.

IV.1.- INTRODUCTION

The internal surface of food cans and other types of food containers are usually coated with an epoxy resin. One type of these resins are epoxy novolac formulations which are made by condensing formaldehyde with phenolic substances in an acid medium, followed by epoxidation [1]. NOGEs (Novolac glycidyl ethers) are complex mixtures of two or more aromatic rings and therefore with a variable weight composition. Bisphenol F diglycidyl ether (BFDGE) is the lowest molecular weight component of NOGE series and moreover, there are other oligomers of various means of molecular weights [2]. BFDGE and NOGE are often confused. The technical product used for food can coatings is NOGE (Figure VI.1).

n	Name	Mw
0	BFDGE	312.4
1	NOGE 3 rings	474.5
2	NOGE 4 rings	636.7
3	NOGE 5 rings	798.4
4	NOGE 6 rings	961.1

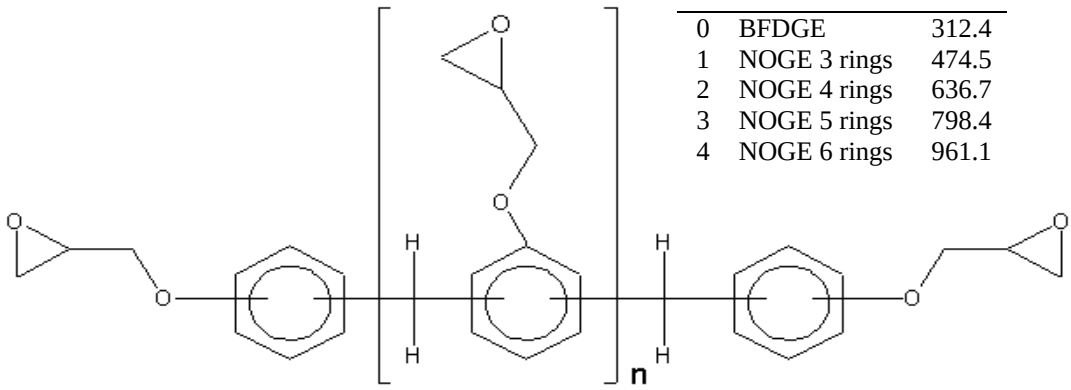


Figure IV.1: Compounds of low molecular weight in NOGE series.

NOGE has been also used as additive to polyvinyl based dispersions (organosols) containing mainly polyvinyl chloride and vinyl acetate as monomers [3]. In these materials they serve as scavengers for the hydrochloric acid formed during the heat treatment of the coating procedure, which results in formation of chlorohydroxy derivatives. If it is added only as an additive and therefore not crosslinked in the finished coating, it can more easily migrate into food matrix and may easily hydrolyse in contact with aqueous and acidic food.

European Legislation related on the use of certain epoxy derivatives as food contact materials establishes that the sum of the migration levels of BFDGE, and its hydrolysis and chlorohydroxy derivatives (Figure IV.2) shall not exceed the limit of 1 mg/kg in foodstuffs or in food simulants [4, 5] or 1 mg/6dm^2 in the material tested [6], (conventional conversion factor that expresses the relationship between the surface area of materials and the quantity of foodstuffs in contact therewith). Concerning to the use of NOGE for food contact materials, it establishes since March 2003, the quantity of NOGE components with more than two aromatic rings and at least one epoxy group, as well as their derivatives containing chlorohydrin functions and having a molecular mass less than 1000 Daltons, should not be detectable at levels higher than 0.2 mg/6dm^2.

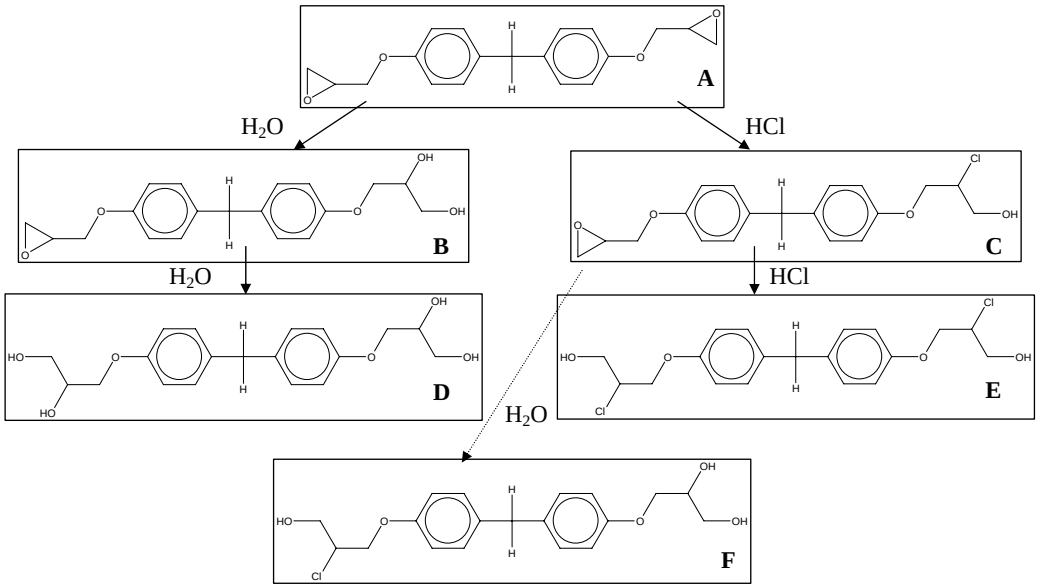


Figure IV.2: Chemical structures of **A:** *p,p* bisphenol F diglycidyl ether (BFDGE, Mw: 312,36); **B:** *p,p* bisphenol F (2,3-dihydroxypropyl) glycidyl ether (BFDGE.H_2O, Mw: 330,37); **C:** *p,p* bisphenol F (3-chloro-2-hydroxypropyl) glycidyl ether (BFDGE.HCl, Mw: 348,82); **D:** *p,p* bisphenol F bis(2,3-dihydroxypropyl) ether (BFDGE.2H_2O, Mw: 348,39); **E:** *p,p* bisphenol F bis(3-chloro-2-hydroxypropyl) ether (BFDGE.2HCl, Mw: 385,28); **F:** *p,p* bisphenol F (3-chloro-2-hydroxypropyl) (2,3-dihydroxypropyl) ether (BFDGE.H_2O.HCl, Mw: 366,84).

NOGE formulations, and other glycidyl ethers as bisphenol A diglycidyl ether (BADGE) are allowed to be used as starting substances for materials to be in contact with food by EU legislation. Its use is authorized until 31th December 2005 [5]. In the case of NOGE, it is not allowed its use as additive.

Great attention has been given to BADGE once it was detected at concentrations exceeding some national legal limits [2], but only in the last years, scientific works were focused on NOGE. Thus, the most widely used techniques for the analysis of BFDGE and its hydroxy and chlorohydroxy derivatives are normal and reverse phase high performance liquid chromatography (NPLC and RP-HPLC) with fluorescence detection [3, 7-14]. Several researches have applied gas chromatography (GC) coupled with mass spectrometry to determine these compounds [8, 15, 16]. Most of the times this technique was used just to confirm positively the presence of these compounds after HPLC separation and quantification with fluorescence detection [7-9, 11, 12].

Some works are focused on the identification of these substances (NOGE with two or more aromatic rings) and other oligomers or derivatives using HPLC with MS detection by atmospheric pressure chemical ionisation mode (APCI) [3, 17-19], thermospray ionisation (TSP) [7] and electrospray ionisation (ESI) [19].

The objective of this work is to develop a method that allows both, the quantification and positive identification of NOGE and BFDGE derivatives using an RP-HPLC/APCI-MS technique, after its majority characteristic masses were selected. As far as we know, nobody has tempted to quantify BFDGE and its related compounds applying this useful technique.

IV.2.- EXPERIMENTAL

IV.2.1. Chemicals and reagents

Acetonitrile was HPLC grade, supplied by Merck (Darmstadt, Germany) and ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). Standards of Bisphenol F diglycidyl ether: BFDGE, CAS No. [2095-03-6], total of three isomers (~97%): *ortho-ortho, para-para, ortho-para;* Bisphenol F bis (2,3-dihydroxypropyl) ether (BFDGE.2H_2O, CAS No. [72406-26-9], total of three

isomers (≥97%): *ortho-ortho, para-para, ortho-para*); Bisphenol F bis (3-chloro-2-hydroxypropyl) ether (BFDGE.2HCl, CAS No. not yet established, total of three isomers (~95%): *ortho-ortho, para-para, ortho-para*) were all purchased from Fluka Chemie AG (Buchts, Switzerland); Poly (phenyl glycidyl ether)-*co*-formaldehyde (NOGE, CAS No. [28064-14-4] Average M ca. 345) was from Sigma-Aldrich (Steinheim, Germany).

Individual stock solutions of all compounds containing 1000 mg/L were prepared in acetonitrile and were kept at -20ºC. Intermediate standards solutions in 90% (v/v) acetonitrile and water were prepared by dissolving appropriate amounts of all compounds to yield a concentration of 10 mg/L. Calibration solutions ranged between 0.2 and 2.0 mg/L in water and 90% (v/v) acetonitrile were prepared from these intermediate solutions. Intermediate solutions in 90% acetonitrile were kept at 4ºC and intermediate solutions in water were kept at -20ºC.

BFDGE.H_2O, BFDGE.HCl and BFDGE.H_2O.HCl for qualitative purposes were prepared as described previously [14].

IV.2.2. Equipment

The HPLC–MS system comprised a Spectra-Physics Series P2000 pump, a Spectra-Physics Series SCM1000 degasser and a Spectra-Physics Series AS3000 autosampler, (all from SP Thermo Separation Products, Altrincham, UK) coupled to a Navigator II AQA single quadrupole mass spectrometer with an APCI interface (ThermQuest, Finnigan, Manchester, UK). All system was controlled by Xcalibur (version 1.2) software. The column was a Kromasil 100 C 18 (15 × 0.4 cm I.D., 5μm particle size) (Teknokroma, Barcelona, Spain).

IV.2.3. Chromatographic and spectrometric conditions

Elution conditions were developed in a previous article [20]. The mobile phase was acetonitrile-water 30:70 (v/v) in an isocratic mode for two min, followed by a gradient to 80% acetonitrile for eighteen min, another gradient to 100% acetonitrile for three min and finally an isocratic elution during seven min. The flow rate was 1.0 mL/min.

During the operation of mass spectrometer both positive or negative atmospheric pressure chemical ionisation (APCI) were used, probe temperature was set to 350ºC, corona pin discharge was 3.0 kV, electron multiplier voltage was 700 V, cone voltage +15 or -5 V, and nitrogen as nebulizer and sheath gas. Spectra were acquired in full scan mode between 100–800 *m/z* and selected ion recording (SIR) to quantify are shown in Table IV.1.

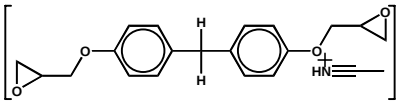
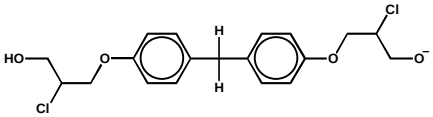
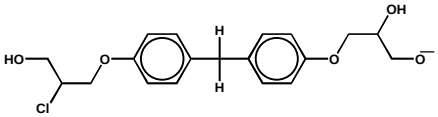
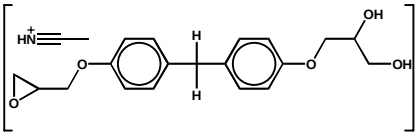
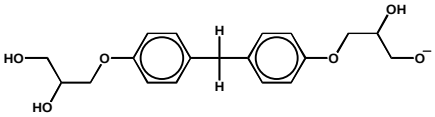
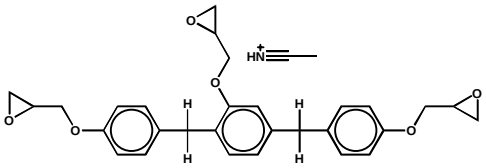
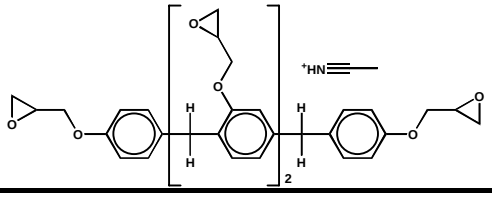
Analyte	Structure	*m/z*	Mode
BFDGE		354	APCI (+)
BFDGE.HCl	Unidentified fragment	390	APCI (+)
BFDGE.2HCl		383 and 385	APCI (-)
BFDGE.H_2O.HCl		365	APCI (-)
BFDGE.H_2O		372	APCI (+)
BFDGE.$2H_2O$		347	APCI (-)
NOGE 3 rings		516	APCI (+)
NOGE 4 rings		678	APCI (+)

Table IV.1: Chemical structures of selected ions for quantification purposes (only *para, para* isomers are showed).

IV.3.- RESULTS AND DISCUSSION

IV.3.1. Mass Spectrometry

First of all, the objective of this work was to establish the optimal spectrometric conditions to achieve the most selective ions for each compound, thus, ions that were not common for different molecules. Analysis by this technique is greatly influenced by the cone voltage, which determines the degree of fragmentation of analyte ions. Cone voltages of 5, 10, 15 and 20 V, in (+) and (-) mode, were evaluated for each substance, in order to set a cone voltage that allowed to obtain selective and also sensitivity ions for each compound. Probes temperatures of 300, 350 and 400ºC were tested and, although it did not greatly affect the degree of fragmentation, it was finally set at 350ºC to achieve a better sensitivity for all the compounds.

Although full scan mode gives more information, it was chosen the SIR mode for the tentative quantification, since this way the sensitivity was improved.

Once detector conditions were optimised, and to establish the SIR parameters for the quantification, initial tests on concentrate standard solutions of 10 mg/L were carried out using the full scan mode to obtain the maximum information from their mass spectra (Figures IV.3 A, IV.3 B and IV.3 C).

In the production of BFDGE, condensation of phenol and formaldehyde may occur in the *ortho* as well as in the *para* position of the phenol. This is why this compound consists of three isomers and it is always accompanied by compounds of three and more rings. Each class of a given ring number exist as numerous isomers [21]. Consequently all BFDGE derivatives are also mixtures of three isomers.

Therefore, it is important to point out that only three individual commercial standards are available and they correspond to BFDGE, BFDGE.2H_2O and BFDGE.2HCl, and apart from that, the existence of isomers of each one, makes the chromatographic separation and ulterior identification and quantification more difficult.

For those which no commercial standards are available, it is possible to obtain mixtures containing these substances (BFDGE.H_2O, BFDGE.HCl and BFDGE.H_2O.HCl), but to achieve an acceptable chromatographic separation becomes complicated and makes individual identification very hard. Thus, three different mixtures were prepared following a procedure previously reported [14]. One

containing BFDGE.H_2O and BFDGE.$2H_2O$, prepared by storage of a dissolution of BFDGE in water at 60ºC; other containing BFDGE, BFDGE.HCl and BFDGE.2HCl obtained by addition of HCl to a stock solution of BFDGE; and the last one with all BFDGE derivatives (BFDGE.H_2O, BFDGE.$2H_2O$, BFDGE.2HCl, BFDGE.HCl and BFDGE.H_2O.HCl) by dissolution in water of an amount of the stock solution containing HCl and storing it a 60ºC (Figure IV.2).

Thus, it was possible, once chromatographic separation was achieved, to identify individually each compound on the basis of its mass spectra.

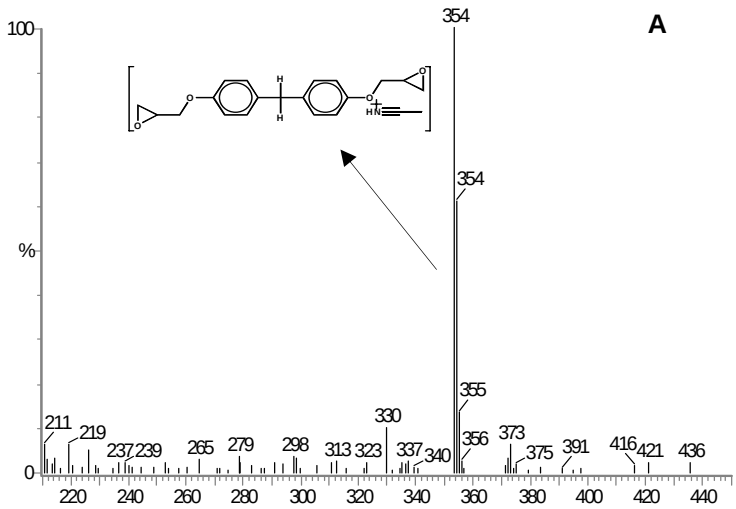


Figure IV.3 A: APCI mass spectra of BFDGE at a concentration of 10 mg/L.

BFDGE fragmentation observed follows a similar profile determined previously for BADGE [22]. Moreover, BFDGE was characterized by other authors [17] using the APCI interface. Thus, APCI (+) was used, and fragment corresponding to the cluster comprising this analyte and a molecule of acetonitrile were observed to be the most abundant ($[M+CH_3CNH]^+$) (Figure IV.3 A). So this fragment was selected for quantification. For BFDGE.$2H_2O$, APCI (-) was used and the fragment corresponding to the $[M-H]^-$ was selected to quantify due to its selectivity and abundance (Figure IV.3 B). In case of BFDGE.2HCl, APCI (-) was also selected and two fragments were chosen to quantify, which correspond to the most abundant

isotopic ionisation forms $[M-H]^-$. This presence of several ionisations forms is caused by the existence of two atoms of chloride in the molecule (Figure IV.3 C). The *m/z* selected fragments are shown in Table IV.1.

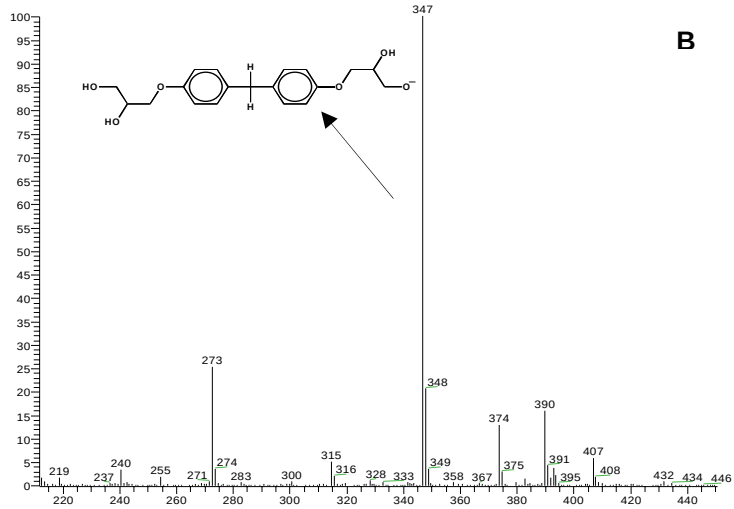


Figure IV.3 B: APCI mass spectra of BFDGE.2H_2O and at a concentration of 10 mg/L.

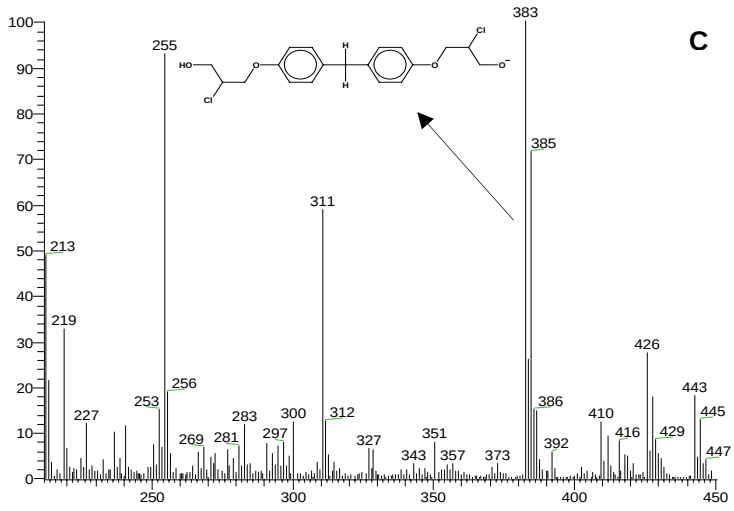


Figure IV.3 C: APCI mass spectra of BFDGE.2HCl at a concentration of 10 mg/L.

In case of the intermediate derivatives, as BFDGE.H_2O, the fragment corresponding to the cluster $[M+CH_3CNH]^+$ was selected to identify it due to its selectivity comparing with the other fragments observed (Table IV.1, Figure IV.4 C).

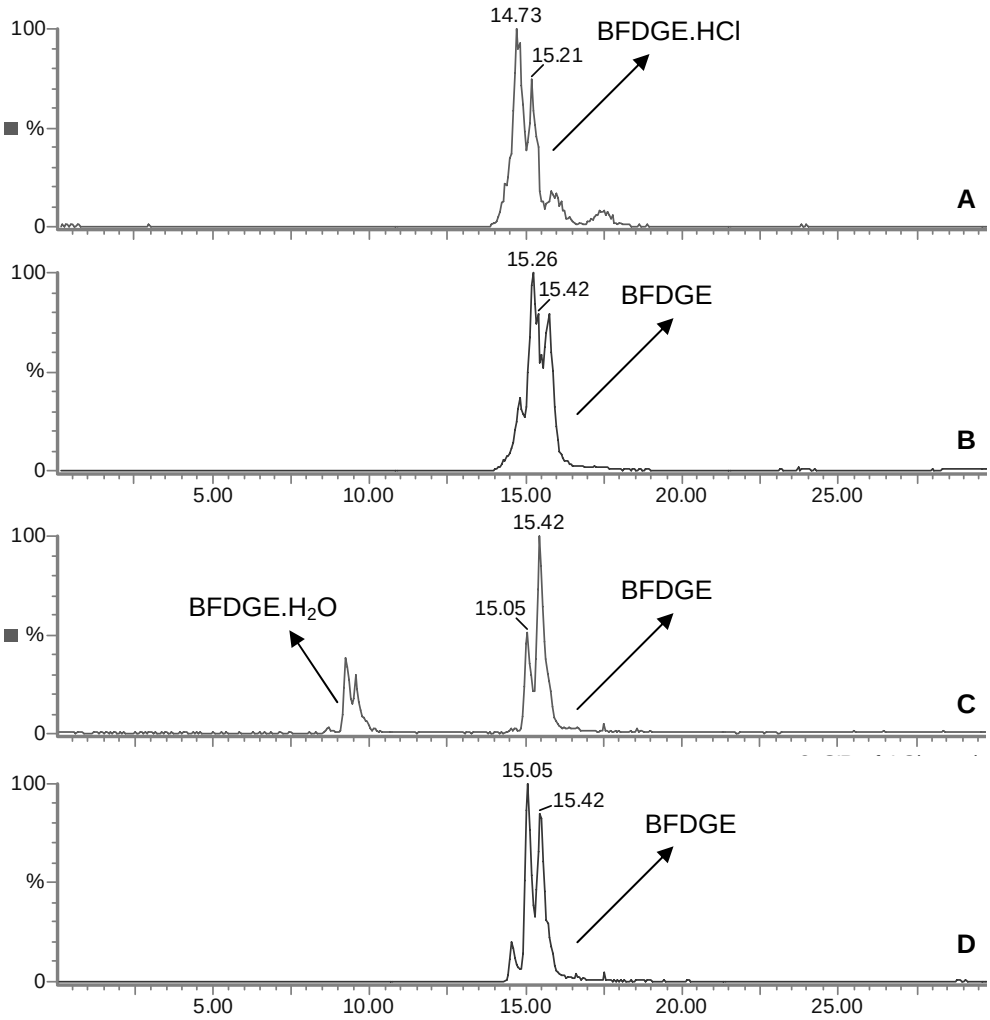


Figure IV.4: MS chromatograms using APCI (+) of a mixture of BFDGE.HCl and BFDGE (**A** and **B**) using *m/z* 390 and 354 respectively; and a mixture of BFDGE.H_2O and BFDGE (**C** and **D**) using m/z 372 and 354 respectively, at a concentration of 20 mg/L (expressed as the sum of all derivatives).

For BFDGE.H_2O.HCl, APCI (-) was chosen, and the more characteristic fragment observed was $[M-H]^-$ (Table IV.1, Figure IV.5 B).

Ionization of BFDGE.HCl has showed an *m/z* ion that was not identified (Figure IV.4 A). Similar difficulty was found in the analysis of BADGE and its related compounds employing the same technique [23].

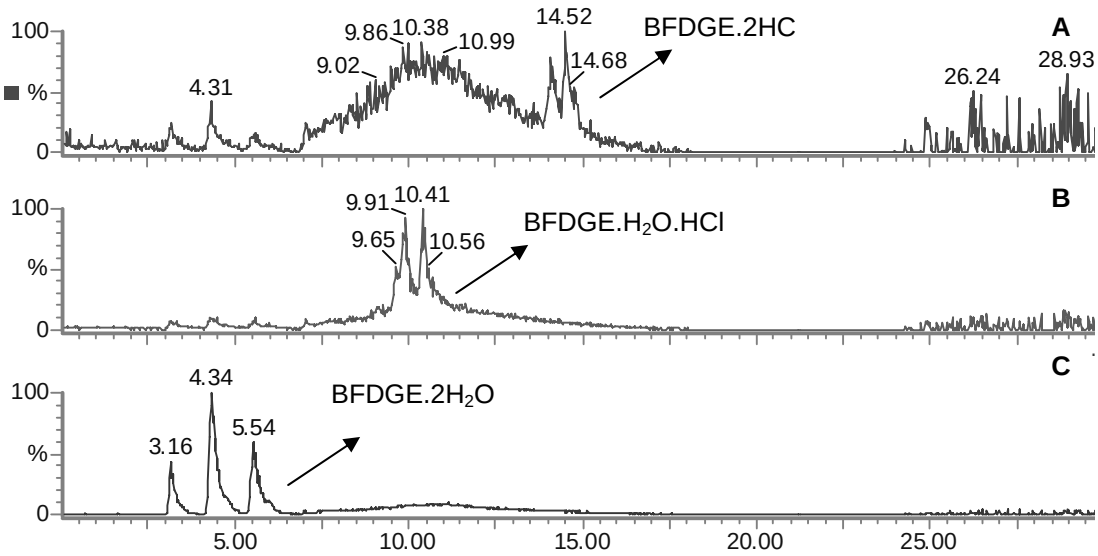


Figure IV.5: MS chromatograms using APCI (-) of a mixture of BFDGE.2HCl (**A**), BFDGE.H_2O.HCl (**B**) and BFDGE.$2H_2O$ (**C**) using *m/z* 383+385, 365 and 347 respectively, at a concentration of 20 mg/L (expressed as the sum of all derivatives).

As has been explained before, the 2-ring product of NOGE is bisphenol F diglycidyl ether (BFDGE). There are three isomers of BFDGE, seven of 3-ring NOGE and 27 of 4-ring NOGE [18].

Regarding its analysis, the chromatographic separation of the individual components with more than four rings has not achieved due to the large number of isomers. For the compounds with three and four rings, APCI in positive mode was chosen. Fragments considering more characteristic were analogous as for BFDGE, thus, clusters formed with a molecule of acetonitrile (Table IV.1, Figure IV.6). Comparable ammonium adducts were found by other author for BFDGE, NOGE 3-

rings and NOGE-4 rings [8], but other authors [17] have observed that these strong ions only are formed by compounds of molecular weight less than 400 Daltons and with more than one epoxy group (such as BFDGE). Figure IV.6 shows the chromatogram obtained for a solution of NOGE, in which the fragments analyzed correspond to the clusters formed with a molecule of acetonitrile.

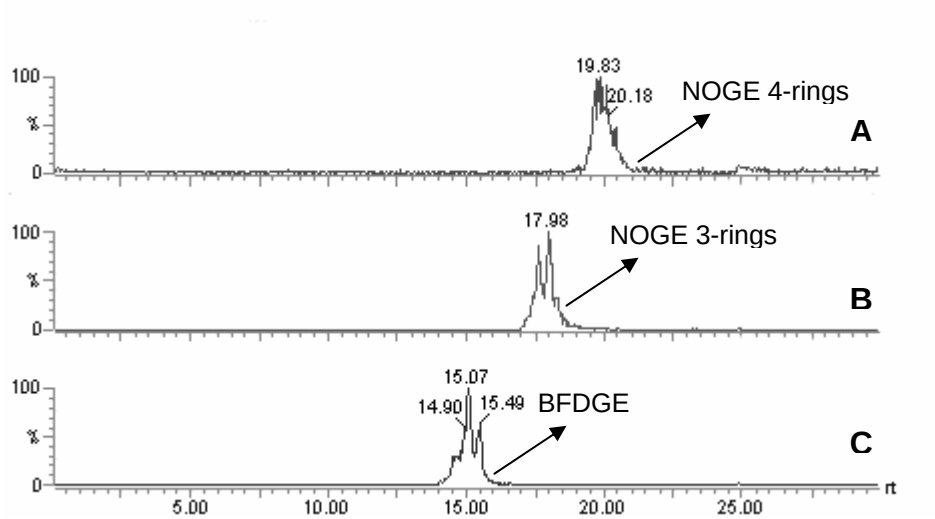


Figure IV.6: MS chromatograms in APCI (+) of a mixture of NOGE (commercial standard); NOGE 4-rings using *m/z* 678 (**A)**, NOGE 3-rings, using *m/z* 516 (**B**), and BFDGE, using *m/z* 354 (**C**) at a concentration of 50 mg/L (expressed as the sum of all compounds).

IV.3.2. Chromatography

Chromatographic protocol has been optimised previously in order to improve the separation of these substances. Not all BFDGE derivatives have response in the same ionisation mode, so two injections were always necessary in negative and positive mode. Figures IV.4 and IV.5 show chromatograms acquired in both positive and negative mode.

The same chromatographic conditions were employed to analyze BADGE, its chlorohydroxy derivatives and oligomers [23], although spectrometric conditions were different, the employ of RP-HPLC coupled to a MS spectrometer via its APCI

interface seems to be a powerful tool to quantify simultaneously BADGE and BFDGE related compounds.

IV.3.3. Method validation

As all individual standards are not available, only for those that were commercially available, the calibration lines were constructed. Thus, standards in 90% acetonitrile (in case of BFDGE and BFDGE.2HCl) and water (in case of BFDGE.2H_2O) of known concentrations were used. The relationship between known concentrations and measured areas (expressed as the sum of the three isomers of each compound) was assessed by linear regression (four calibrations points), and the linearity obtained indicates that the method would be appropriate for quantification of these compounds (Table IV.2). Detection limits (DLs), (defined as signal three times the height of the noise level) were calculated in accordance with American Chemical Society [24] and are shown in Table IV.2. The lower DL corresponds to BFDGE and BFDGE.2H_2O, and although lower levels can be reached employing fluorescence detection, in this case, no positive confirmation would be necessary. Nevertheless, comparing the established SMLs in the European Legislation [4], the detection limits obtained for the three compounds provide well enough performance.

	Linear range (mg/L)	Linearity (r^2)	Detection Limit (mg/L)
BFDGE	0.2-2.0	0.9950	0.05
BFDGE.2HCl	0.2-2.0	0.9945	0.1
BFDGE.2H_2O	0.2-2.0	0.9929	0.05

Table IV.2: Method validation parameters.

European Legislation establishes the migration limit for NOGE compounds at the detection limit of 0,2 mg/6dm² [4]. Taking into account that the products used for food cans typically contained 30-40% of 2-ring NOGE (BFDGE) and decreasing proportions of 3-ring to 8-ring components it is achievable to quantify NOGE up to

four rings in BFDGE. The only limitation of this procedure would be the detection limit of these compounds that could be solved using a previous concentration step.

Another advantage of this method is that to employ characteristic *m/z* makes possible to differentiate complex mixtures.

In conclusion, the optimised method is appropriate to determine the chloro and hydroxy derivatives of BFDGE in a range between 0.2 mg/L– 2 mg/L. Calibration rate is appropriate to guarantee the compliance of these coatings with food packaging European Legislation. NOGE (2, 3 and 4-rings) could be also quantified using this method. This procedure is a potential technique that allows the positive confirmation of the presence of these compounds, not being necessary another technique for its quantification.

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CAPÍTULO V: *MIGRATION SURVEY OF BISPHENOL A DIGLYCIDYL ETHER AND BISPHENOL F DIGLYCIDYL ETHER FROM CANNED FISH AND SEAFOOD, IN SPANISH MARKET DURING LAST YEARS.*

ABSTRACT

Epoxy resins are the main coatings used in food industry. Bisphenol A diglycidyl ether (BADGE) is the lower molecular weight compound of bisphenol A epoxy resins and bisphenol F diglycidyl ether (BFDGE) is the lower molecular weight compound of novolac epoxy phenolic resins.

This study evaluates the contamination of canned fish and sea products regarding BADGE, BFDGE and related compounds during the last six years. The canned fish and sea food products samples (n=389) were supplied by different Spanish industries. In 1999-2001 only 1 % of samples exceeded the specific migration limit and in the last three years (2002-2004) the amount of all migrants analyzed was negligible. The results obtained are compared with previous surveys in Spain and in Europe. It could be concluded that the concentration of these compounds is quite lower that those found at the end of 90's.

KEYWORDS

BADGE, BFDGE, migration, can coatings, canned fish

V.1.- INTRODUCTION

Epoxy resins, polyvinyl chloride (PVC) containing organosols or polyester lacquers are used in food industries to coat the internal surface of food cans (sea products, vegetables, beer, soft drinks, powder milk) or big vessels for wine, water, and other foodstuffs. The function of these coatings is to provide chemical and corrosion resistance.

Food can lacquers supplied into Europe are mainly consisted of epoxy phenolic resins (70%) (Dionisi and Oldring, 2002). Main epoxy phenolic resins used are Bisphenol A epoxy resins and/or Epoxy Novolacs resins; both are a complex mixture of chemical species. Bisphenol A diglycidyl ether (BADGE) is the lower molecular weight compound of bisphenol A epoxy resins and bisphenol F diglycidyl ether (BFDGE) is the lower molecular weight compound of novolac epoxy phenolic resins.

They are starting substances used to manufacture surface coatings. If reaction process or cure conditions were not applied in a proper way, and/or finished coating is not correctly crosslinked, components of Bisphenol A epoxy resins or Epoxy Novolacs resins and its reaction products could migrate to food (Sendón et al., 2003).

Epoxy resins are the main coatings used in food industry but, in addition, these substances (BADGE and BFDGE) can be added to organosol PVC based lacquers to scavenge the hydrochloric acid formed during the heat treatment of the coating process, which results in formation of chloroderivatives that could also migrate into food.

Although in vivo studies in laboratory mammals do not indicate that BADGE causes adverse effects on reproduction, and other studies reflects the lack of oncogenic potential (Poole et al., 2004), BADGE and related compounds have been proved to be mutagenic (Sueiro et al., 2001), allergenic (Kanerva et al, 1991) and able to induce both cytotoxic and genotoxic effects (Suárez et al., 2000). Its hydrolysis products present oestrogenic activity (Nakazawa et al., 2002.), but other studies did not show any affinity of BADGE for the oestrogen receptor in different experiments (Perez et al., 1998; Ogata et al., 2001). BFDGE is also proved to induce mutagenic and genotoxic effects (Sueiro et al., 2003). As far as we know, NOGE (novolac glycidyl ethers) of higher molecular weight have not been yet toxicologically evaluated.

The toxicological properties have been considered by European Union and regarding the available data, it was established a migration limit of 1 mg/kg into food for BADGE and BFDGE, which include its hydrolysis and chlorohydroxy derivatives (European Commission, 2002), and the use of these substances has been authorized until the end of 2005 (European Commission, 2004). For NOGE, and due to the lack of toxicological information available, a specific migration limit of 0.02 mg/kg was established (European Commission, 2002).

Canned foods represent an important section of food consumed in Europe. Spain is the main producer in Europe of canned fish and one of the most important producers of canned food together with UK, France and Italy (Dionisi and Oldring, 2002). It is necessary to consider that all data available at the moment does not include the new countries recently incorporated to the EU. Spain has produced 285000 Tm of canned fish and sea products in 2003 (Ministry of Agricultural, Fisheries and Food, 2004). The per capita consumption of canned foods at EU countries was estimated by Dionisi and Oldring (2002) in around 1.1 cans/person/week or 62 g/person/day.

Most of epoxy migrants of low molecular weight found by different researchers in canned foods are bisphenol A diglycidyl ether and their respective clorohydroxy derivatives (Hammarling et al., 2000; Berger and Oehme, 2000), bisphenol F diglycidyl ether and its derivatives (Rauter and Lintschinger, 2000), and also bisphenol A (Munguia-López and Soto-Valdez, 2001) and bisphenol F (Goodson et al., 2002).

During the past few years, several studies were focused in studying the migration levels of these compounds in different type of foods. Canned milk products and vegetables oils were evaluated by Theobald et al. (1999). Canned fish in oil from different European countries (Spain was not included) were investigated by Simoneau et al. (1999) and Hammarling et al. (2000) have evaluated the safety of fish in oil canned from the Swedish market, although they have included other types of canned foods. All these researches were developed regarding BADGE. Concerning BFDGE, Theobald et al. (2000) have evaluated its level in fish canned in oil, in samples from the European Union and from Switzerland.

This work evaluates the level of BADGE, BFDGE and derivatives in different types of both, canned fish and sea food products, supplied by Spanish

companies during the last six years. A comparison with the levels found in European countries was also done.

V.2.-MATERIALS AND METHODS

V.2.1. Samples

The canned fish and seafood products samples (n=389) were supplied by different Spanish industries. The diverse products analyzed along these years include, besides those showed in Table V.1, various types of seafood products in different sauces.

V.2.2. Reagents and solvents

Acetonitrile and heptane were HPLC grade and were supplied by Merck (Darmstadt, Germany) and purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standards of Bisphenol A diglycidyl ether (BADGE, CAS No: [1675-54-3], (≥97%)) and Bisphenol F diglycidyl ether (BFDGE, CAS No: [2095-03-6], total of three isomers (≥97%): ortho-ortho, para-para, ortho-para) were purchased from Fluka Chemie AG (Buchts, Switzerland).

Individual stock solutions of all compounds containing 1000 mg/L were prepared in acetonitrile and were kept at -20ºC. Intermediate standards solutions in 90% (v/v) acetonitrile and water were prepared by dissolving appropriate amounts of all compounds to yield a concentration of 10 mg/L. Calibration solutions in 90% (v/v) acetonitrile and water (ranged between 0.1 and 2.4 mg/L) were prepared from these intermediate solutions. Intermediate solutions in 90% acetonitrile were kept at 4ºC and intermediate solutions in water were kept at -20ºC.

Other standards were used for identification purposes. They were: Bisphenol A (2,3-dihydroxypropyl) glycidyl ether (BADGE.H_2O, CAS No. [76002-91-0], (≥97%)); Bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE.$2H_2O$, CAS No. [5581-32-8], (≥97%)); Bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether (BADGE.HCl, CAS No. [13836-48-1], (95%)); Bisphenol A bis(3-chloro-2-

hydroxypropyl) ether (BADGE.2HCl, CAS No. [4809-35-2], (≥99%)); Bisphenol A (3-chloro-2-hydroxypropyl) (2,3-dihydroxypropyl) ether (BADGE.H_2O.HCl, CAS No. [227947-06-0], (≥98%)); Bisphenol F bis (2,3-dihydroxypropyl) ether (BFDGE.2H_2O, CAS No. [72406-26-9], total of three isomers (≥97%): ortho-ortho, para-para, ortho-para) and Bisphenol F bis (3-chloro-2-hydroxypropyl) ether (BFDGE.2HCl, total of three isomers (~95%): ortho-ortho, para-para, ortho-para). All were all purchased from Fluka Chemie AG (Buchts, Switzerland).

BFDGE.H_2O, BFDGE.HCl and BFDGE.H_2O.HCl, also for qualitative purposes, were prepared as described previously (Sendón et al., 2003).

Poly (phenyl glycidyl ether)-co-formaldehyde (NOGE, CAS No. [28064-14-4] Average M ca. 345) and Poly (Bisphenol A -co- epichlorohydrin) glycidyl end-capped (BADGE oligomers, CAS No. [25036-25-3] Average M ca. 1075) were from Sigma-Aldrich (Steinheim, Germany).

V.2.3. Sample preparation

The rapid extraction of BADGE and BFDGE from food samples was based on a previous validated method described by Cortizas Castro (1999). A brief abstract is given: the surrounding oil or sauces of samples were separated from the solid portion. 5 g of the liquid portion were accurately weighed in a centrifuge tube, and 5 mL of heptane were added; after mixing carefully, 10 mL of acetonitrile 90 % (v/v) were added. This mixture was hand shaken vigorously for 1 min and was centrifuged for 5 min at 1500 rpm. Acetonitrile phase was taken up, filtered through a PTFE 0.45 µm 13 mm syringe filter and analyzed.

V.2.4. Analytical method

Chromatographic measurements were performed with a Hewlett Packard system (Waldbronn, Germany) comprised of a HP1100 liquid chromatograph fitted with a quaternary pump, an autosampler, a fluorescence scanning detector and HP Chemstation data analysis software (version A.06.01). A Kromasil 100 C18 column (15 × 0.4 cm I.D., 5µm particle size) from Teknokroma (Barcelona, Spain) was used for the separation.

Chromatographic conditions were developed in a previous article (Paseiro et al., 1997). The mobile phase consisted of acetonitrile/water 30:70 (v/v) in an isocratic mode for two minutes, followed by a gradient to 80% acetonitrile for eighteen min, another gradient to 100% acetonitrile for three min and finally an isocratic elution during seven min. The flow rate was 1 mL/min. The injection volume was 50 µL. Fluorescence detection was performed with excitation and emission wavelengths of 225 nm and 305 nm, respectively.

V.3.- RESULTS AND DISCUSSION

All samples were supplied by industries to test their safety regarding BADGE and BFDGE, but due to the presence, in some cases, of other related compounds, an identification of them was carried out. As it is shown in Table V.1 the major quantity of samples corresponds to tuna in oil, which is the most canned fish product manufactured in Spain (Ministry of Agricultural, Fisheries and Food, 2004).

Table V.1 shows the results found in 1999, 2000 and 2001 respectively. As can be observed, in 1999 and with respect to BADGE, no sample exceeded the specific migration limit of 1 mg/kg established by the European Legislation (European Commission, 2002), although an important percentage of samples exhibited the presence of BADGE, almost 50 % contained BADGE above and below the quantification limit. Regarding to BFDGE, four samples exceeded the legal limit for this compound. The Figure V.1 shows a chromatogram of one of these samples. It can be observed that apart from BFDGE, there are chloroderivatives and NOGE in a similar concentration range.

Year	Type of sample	BADGE			BFDGE		
		% samples > 0.1 mg/kg	**Range** mg/kg	**% samples** < 0.1 mg/kg	**% samples** > 0.1 mg/kg	**Range** mg/kg	**% samples** < 0.1 mg/kg
1999	Tuna in oil (*n*=44)	3.7	0.2-0.5	12.8	0	-	0
	Sardines in oil (*n*=15)	11.9	0.1-0.5	1.8	0,9	0.1-0.2	0
	Mussels in pickled sauce (*n*=12)	3.7	0.1-0.5	-	3,7	1.3-3.1	0
	Other (*n*=38)	3.7	0.1-0.5	9.2	0	-	0
	Total of samples (*n*=109)	**22.9**		**23.9**	**4,6**		**0**
2000	Tuna in oil (*n*=38)	0.9	1.0-1.5	1.8	0	**-**	0
	Anchovies in olive oil (*n*=24)	0	**-**	0	0	**-**	0
	Other (*n*=38)	0	**-**	0	0	**-**	0
	Total of samples (*n*=114)	**0.9**		**1.8**	**0**		**0**
2001	Tuna in oil (*n*=38)	0	**-**	0	1,6	0.4-0.5	0
	Other (*n*=89)	0	**-**	6.3	0	**-**	0
	Total of samples (*n*=127)	**0**		**6.3**	**1.6**		**0**

Table V.1: Summary of results of BADGE and BFDGE survey.

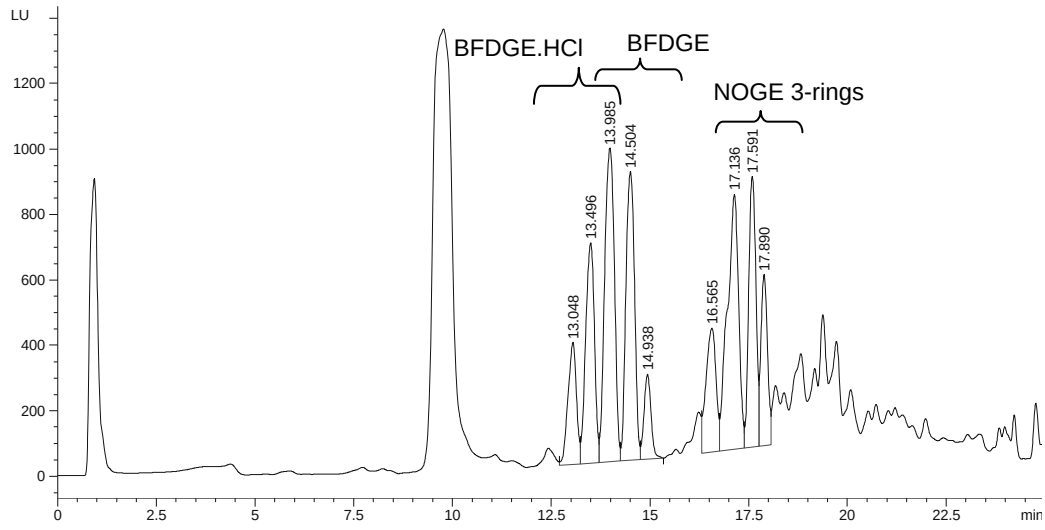

Figure V.1: Chromatogram of mussels in pickled sauce with a concentration of 1.43 mg BFDGE /kg of foodstuff.

In 2000, only one sample exceeded the SML of BADGE, its chromatogram is showed in Figure V.2. Otherwise, the presence of BFDGE was negligible in the samples tested.

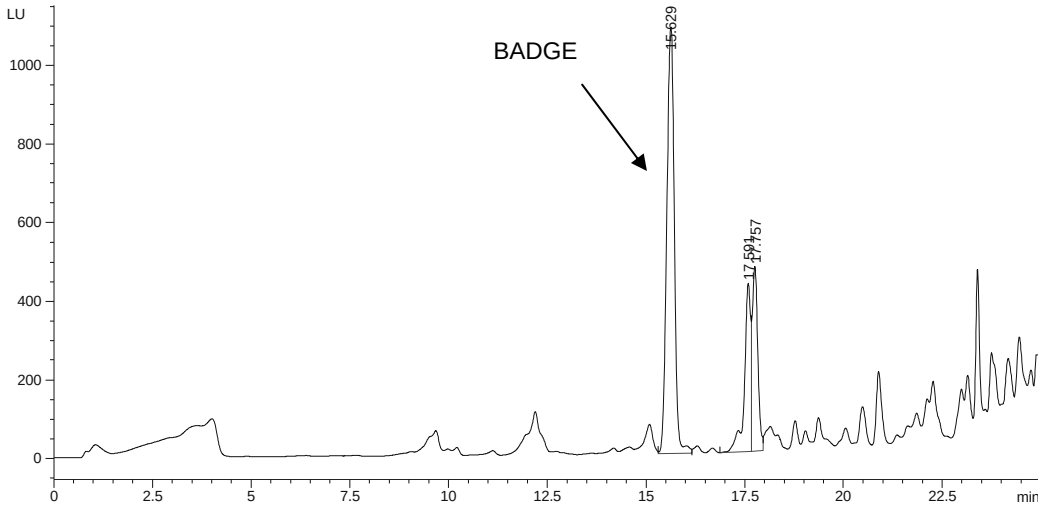

Figure V.2: Chromatogram of tuna in oil with a concentration of 1.2 mg BADGE /kg of foodstuff.

Concerning 2001, and considering only the presence of BADGE, none of samples showed a concentration higher than 0.1 mg/kg and only in a low percentage of samples this compound was detectable, and most corresponding to samples of mussels in pickled sauce. However, in one sample, the chloroderivatives of BADGE were found (Figure V.3). In this case the value is around 0.1 mg/kg, expressed as mg BADGE /kg of foodstuff. BFDGE was found only in one sample.

In 2002, 2003 and 2004 the number of canned products tested decreased (n=29) due to producers started to supply empty cans to test its safety before to use them as food cans. Nevertheless, in all food products tested along the last three years no sample has exceeded the quantification limit of 0.1mg/kg and only two

samples had results of BADGE below 0.1 mg/kg. BFDGE was not detectable in any sample.

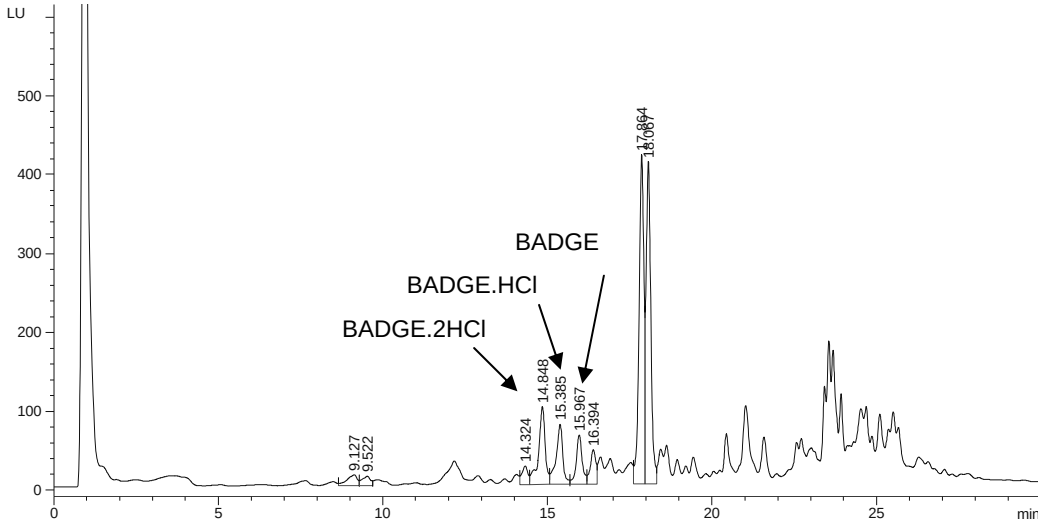

Figure V.3: Chromatogram of mussels in pickled sauce with a concentration of all BADGE derivatives of 0.16 mg/kg foodstuff.

This evolution is in accordance with previous studies carried out in our laboratory. Data obtained between 1996 and 1998 (Cortizas Castro, 1999) showed a worse scenario regarding BADGE and BFDGE, but with a decrease of the concentrations found at the end of this period. In this study, the mean values found at the end of 1996 were 2.14 mg/kg for BADGE and 2.17 mg/kg for BFDGE. At the beginning of 1997 the mean concentrations found were 0.17 mg/kg and 3.25 mg/kg for BADGE and BFDGE respectively. Hammarling et al. (2000), in a previous survey done in Swedish in 1997, found also higher values for BADGE (up to 5.1 mg/kg). Simoneau et al. (1999) reported, in a survey in the European Union that about 3% of canned fish in oil exhibited a concentration higher than the SML established by the European Legislation, which is a higher value than the one found in this study. Theobald et al. (2000) ascertained similar results in a BFDGE survey.

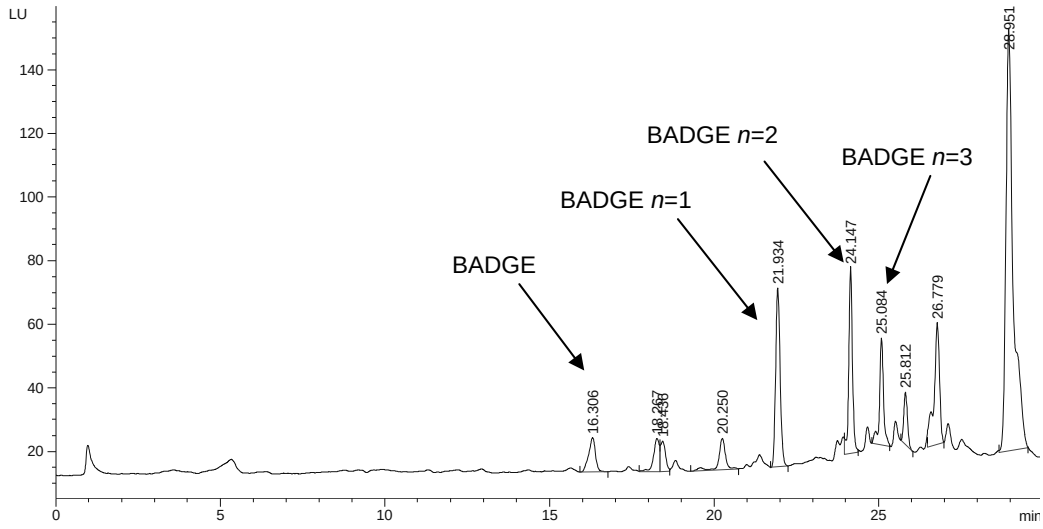

Figure V.4: Chromatogram of tuna in oil with a concentration of BADGE below 0.1 mg/kg of foodstuff, but with oligomers of high molecular weight.

The cases found these last years, for which the migration is higher, generally come from one sample containing a high concentration rather than a larger number samples with lower concentrations.

Moreover, BADGE oligomers (which are not included in European Legislation) were found in some samples (Figure V.4). These oligomers were already found in canned oily food by Biedermann et al. (1997).

So, it is necessary to emphasize that although migration levels of BADGE and BFDGE (and also its related compounds of low molecular weight) are nowadays lower than last years, new problems are arising. Figures V.2, V.3 and V.5 showed that new substances (retention times between 17.5 and 18.0 minutes) are migrating to foods.

Although these unknown compounds are much less than those observed in other previous works (Grob et al., 1999), it should be marked that research on this field should continue to try to identify new migrants from food packaging materials and hence, to ensure consumers' safety.

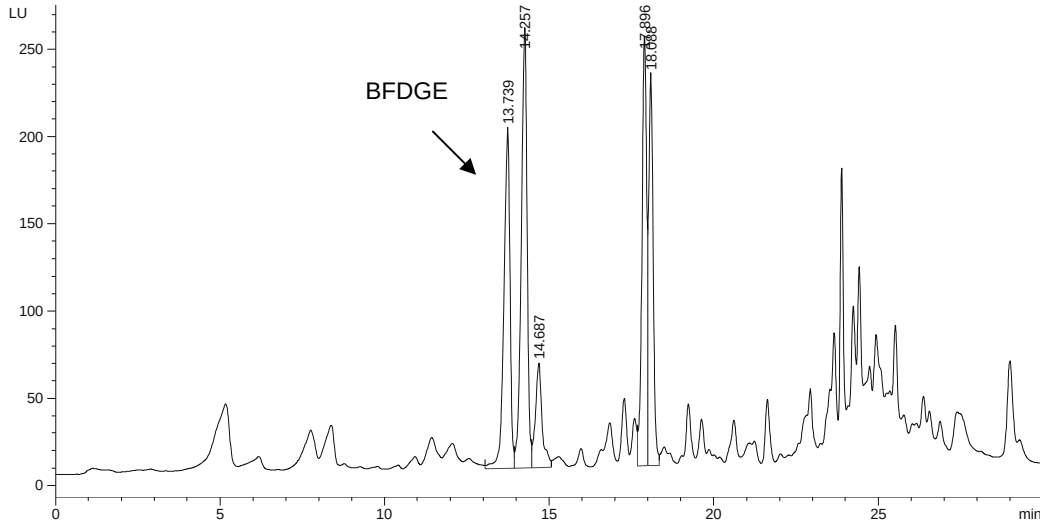

Figure V.5: Chromatogram of tuna in oil with a concentration of BFDGE of 0.4 mg/kg of foodstuff.

This study shows the evolution of the contamination of canned food regarding these epoxy compounds in the last years, and it could be concluded that the concentration of these compounds is quite lower than those found at the end of 90's. This means that both, can and fish food producers are more concerned to guarantee the consumers' safety, regarding the migration compounds from epoxy resins in canned foods.

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CAPÍTULO VI: COMPILATION OF ANALYTICAL METHODS TO DETERMINE MIGRATION OF ADDITIVES AND MONOMERS FROM PLASTICS TO FOODSTUFFS

Presentado como póster en: ILSI Europe 3rd International Symposium on Food Packaging: Ensuring the Safety, Quality and Traceability of Foods. Barcelona, España, 17-19 de noviembre, 2004.

ABSTRACT

Reference plastics as well as model migrants of different chemical and physical properties have been selected to carry out a study of the migration kinetics from plastics to foodstuffs. On the basis of their representativeness and their relevance for food contact materials, Irganox 1076®, diphenylbutadiene, Chimassorb 81®, Uvitex OB®, caprolactam, benzophenone, diphenyl phthalate and bis (2-diethylhexyl) adipate (DEHA) have been selected for this study.

Available literature on analytical methods in polymers, food simulants and foodstuffs was reviewed in detail, although most of the times it was scarce, and more information was found on analysis in simulants and polymers than on food analysis.

Preliminary experiments were performed in order to evaluate interesting spectroscopic properties, as these properties are generally unavailable in the ordinary bibliography and they are a key point to begin the development of analytical methods.

General guidelines for the determination of the selected migrants in foods were suggested and discussed taking in account all the properties of each molecule.

KEYWORDS

Migration, foodstuffs, plastics, migrants, food packaging.

VI.1.- INTRODUCTION

Plastic additives such as antioxidants, stabilizers and plasticizers have a major influence in the processing and shelf-life of plastics and are responsible for many of the properties of these materials (Haider and Karlsson 1999). Plastics additives, which are present in small amounts in plastics (generally ranging from 0.1-1%- Dilettato *et al.* 1991), are dispersed in the polymer matrix and avoid effects like the thermo-oxidative deterioration, which initiates scission and cross-linking of the macromolecular chains and, consequently, the deterioration of the polymer (Dilettato *et al.* 1991). Chain scission causes loss of molecular mass and decreases toughness, while cross-linking increases molecular mass and toughness in the early stage (Wang 2000). As plastic additives have, generally, low molecular weights, they are often related with migration mechanisms into foods. Moreover, in polymeric matrices it is possible to find monomers and oligomers that have not reacted in the polymerization reactions.

The European Commission has adopted the policy of using restrictions (mostly specific migration limits, SML's) to control the safety of food contact materials and articles. There are several hundred SML's in Directive 2002/72/EC (European Commission, 2002) and amendments which have been assigned to plastics monomers and additives. To demonstrate compliance with SML's, migration tests are conducted and the quantity of the restricted migrant must be determined in food or food simulants. A small number of analytical methods have been validated for measuring migration of substances (EN 13130 parts 2, 3, 5 and 7) although most of these only apply to food simulants owing to the complexity of foods.

While most of the restricted substances still have no analytical method for the determination of their migration levels, mathematical models are of great interest since they are able to predict migration of additives and other low molecular components from plastic packaging materials. Despite being a good alternative to time-consuming and expensive migration experiments, especially when it is necessary to test a new packaging, sometimes these models do not adjust to the real materials behaviour (Helmoroth *et al.* 2002).

Due to the importance of migration in consumer protection and food safety, the European Community decided to finance a project, as well as it has done in the near past (Feigenbaum *et al.* 2002, Franz 2002), with the acronym

FOODMIGROSURE. This provides a novel and cost efficient tool for estimation of consumer exposure from food contact materials. Included in this context, our laboratory has carried out an extensive bibliographic research of the information available of eight of the substances chosen by the project as model migrants.

For each of the compounds studied in this paper, our laboratory has compiled their information regarding the chemical and physical properties, as well as available methods for extraction from simulants or foodstuffs and chromatographic analysis. Also, some of the incomplete data found have been completed with information obtained experimentally.

The determination of plastic additives and monomers in food matrices is associated with two main difficulties. The first is the low detection level required, once these substances are added in small amounts. The second one is the diversity of potential interferences present in different foodstuffs (Quinto-Fernandez *et al.* 2003). General recommendations for the analytical determination of each model migrant in foods are suggested in the paper, based on physical and chemical properties, the preparation of the sample and the determination stage.

VI.2.- EXPERIMENTAL

VI.2.1 Chemicals and Standards Solutions

Irganox 1076®, DPBD, Chimassorb 81®, caprolactam, benzophenone, and diphenyl phthalate standards were purchased from Sigma-Aldrich (Quimica SA, Madrid, Spain). Uvitex OB® and DEHA were supplied by Fluka Chemie AG (Buchts, Switzerland). Ethanol was of analytical grade (purity >99,9%) and supplied by Merck (Darmstadt, Germany). Irganox 1076, Chimassorb 81 and Uvitex OB are trademarkets of CIBA Specialty Chemicals Inc.

Independent standard stock solutions containing Irganox 1076, DPBD, Chimassorb 81, Uvitex OB, caprolactam, benzophenone, diphenyl phthalate and DEHA at 1000 mg l^{-1} in ethanol were prepared and stored at 4ºC. Intermediate solutions were prepared by appropriate dilutions of existing stock solutions in order to obtain a concentration of 10, 1 and 0.1 mg l^{-1}.

VI.2.2. Extraction from olive oil

In order to calculate the remaining fraction in the acetonitrile phase after one extraction with an equal volume of olive oil, standard solutions of 10 mg $l^{-1}$of each model migrant were prepared in acetonitrile. Then to 10 g of olive oil, 10 ml of each standard solution were added and shaken vigorously for three minutes in a centrifuge tube of 40 ml. After phase separation, 0.1 ml of acetonitrile phase was filtered and 50 µl were injected in the HPLC-UV chromatograph.

VI.2.3. Apparatus and Conditions

VI.2.3.1. Ultraviolet-Visible Spectrophotometer

A Cary 3E UV-Visible double-beam spectrophotometer was used to perform single scans of all solutions from 200 to 400 nm. For all compounds solutions of 10 mg l^{-1} were used except for diphenylbutadiene where a solution of 1 mg l^{-1} was employed. The software Cary Win UV was used for data acquisition.

VI.2.3.2. Fluorescence Spectrometer

A Perkin Elmer LS 50 spectrometer fitted with a xenon flash lamp, Monk Gillieson monochromators and 1 cm quartz cuvettes was used to perform spectrofluorimetric measurements. Spectral data acquisition and processing were carried out using the software FL Winlab on a PC serially interfaced to the LS 50.

Initially, a pre-scan was carried out for all migrants. Scans were recorded between 200 and 800 nm for excitation and 200 and 900 nm for emission. The scan speed was 240 nm min^{-1}. Then, maximum values for excitation wavelengths were selected, only for those compounds that showed fluorescence, and emission scans were completed. In the same way, maximum values for emission wavelengths were selected and excitation scans were completed.

For all compounds, solutions of 10 mg l^{-1} were used except for diphenylbutadiene and uvitex OB, where solutions of 0.1 mg l^{-1} and 0.01 mg l^{-1} were use, respectively.

VI.2.3.3. HPLC-UV-Fluorescence

Chromatographic measurements were performed with a HP 1100 (Waldbronn, Germany) liquid chromatograph fitted with a quaternary pump, an autosampler, a diode array detector, a fluorescence scanning detector and HP Chemstation data analysis software (version A.06.01). A Kromasil 100 C18 column (150 mm x 40 mm, 5µm) was employed. The mobile phase consisted of acetonitrile/water 75:35 (v/v) in an isocratic mode for 2 minutes, followed by a gradient to 100% acetonitrile for 8 min, and finally an isocratic elution during 20 min. Flow rate was 1 ml min^{-1}. The injection volume was 50 µl. The column oven temperature was kept at room temperature. UV detection was performed with 205, 225 and 255 nm, and fluorescence detection was performed with excitation and emission wavelengths of 250 nm and 306 nm, respectively.

VI.2.3.4. LC-MS

A Spectra Physics series P200 liquid chromatograph equipped with a Rheodyne loop (50 µl) and a mass detector (Fisons VG Platform) (VG Biotech, Altrincham, UK) was used to record APCI (negative and positive mode) spectra. The Masslynx TM data system was used to control the system. Total ion chromatograms and selected-ion chromatograms of the analytes were obtained by direct injection in flow, the mobile phase was 70:30 acetonitrile/water at a flow rate of 1ml min^{-1}. Injections were monitored using real time chromatogram updates. Full scan spectra (*m/z* 70-700) were acquired every second, with a scan delay of 0.10 s. In SIR mode, the dwell time of each ion was set at 0.10 s.

The following instrument parameters were applied: probe temperature: 450ºC and 250ºC, ionisation source temperature: 130ºC, cone voltage: ± 30 VI, electron multiplier voltage: 700 VI, sheath gas nitrogen at 200 l h^{-1} and drying gas nitrogen at 250 l h^{-1}.

VI.2.3.5. GC-FID

Initial experiments were performed with a Fisons 8000 series gas chromatograph equipped with a flame ionization detector (FID; 260ºC), an auto sampler (AS 800) and a split-splitless injector. The column used was a 30 m x 0.32 mm, 5 µm film thickness Supelcowax 10 from Supelco. To improve the column life a

50 cm pre-column (deactivated fused silica 0.32 mm i.d.) was used. Helium was used as carrier gas flowing at 1 ml min^{-1}. The injector temperature used was 280ºC and the split ratio selected was 1:30.

In a first test the column temperature was held at 40ºC for 2 min during injection, and then increased at 7ºC min^{-1} to 260ºC, then held for 10 min. In a second test the column temperature was held at 150ºC for 2 min, and then increased at 5ºC min^{-1} to 260ºC, then held for 20 min.

VI.3.- RESULTS AND DISCUSSION

A bibliographic review of the physical and chemical properties of model migrants was carried out. Tables VI.1 to VI.8 summarize the available information of chemical and physical properties. Due to the incomplete data found, some of spectroscopic properties were evaluated in our laboratory. The results are shown in the table VI.9.

An extensive research of the available literature was also made. The conclusions of the revised literature on analytical methods in polymers, food simulants and foodstuffs can be summarized in three points: (1) the literature on this subject is scant, and there is more emphasis on the results concerning migration in simulants and amounts in polymers than on food analysis; (2) analytical methods are still in the research and development stage, and no particular set of methods has attracted widespread agreement. It seems that few studies agree on the same procedure to determine a given compound in a given type of sample; (3) some of the protocols found in the literature are not described in sufficient detail to allow confident replication by other laboratories. This is a common situation in the early stage of the development of the analytical methodology.

VI.3.1. Physical and chemical properties

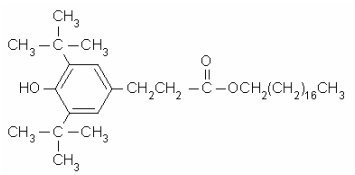
Due to the importance of these properties on the definition of the best analytical approach for the determination of each selected migrant in foodstuffs, data were collected from different databases (Epi Suite, Lide 1997, Budavari 2001,

Properties of Organic Compounds, 1996) and documents like the Synoptic document (European Commission 2003). Those data which were not available in the literature were estimated using the Epiwin software (ver 3.11).

VI.3.1.1. Irganox 1076

Irganox 1076 is a well-known antioxidant added to polymeric materials, especially to polyolefins. Table VI.1 summarizes the main physical and chemical properties. The chemical structure shows that Irganox 1076 contains a hindered phenol unit with a long aliphatic hydrocarbon chain. It is thermostable and presents relative low boiling point (Wang 2000). As antioxidants, prevent the process of oxidation often generalized like "degradation" or "aging" (Agüí *et al.* 1995).

Table VI.1: Chemical and physical properties of Irganox 1076.

Irganox 1076	
Ref. Nº	68320
CAS Nº	2082-79-3
Formula	C35H62O3
Chemical Name	Benzenepropanoic acid-3, 5- bis (1,1-dimethylethyl) -4-hydroxyoctadecylester
MW	531
mp (ºC)	50-55
Bp/Fp (ºC)	-/273
Water solubility (mg l^{-1})	<0,03
Solubility (g x 100g solution^{-1})	Acetone: 19; benzene: 57; chloroform: 57; cyclohexane: 40; ethanol: 1.5; ethylacetate: 38; n-hexane: 32; methanol: 0.6; toluene: 50; water: < 0.01
Application	Antioxidant and thermal stabilizer blend
Structure	
SML (mg kg^{-1})	6
TDI (mg kg^{-1})	0.1

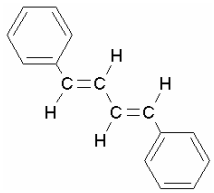
In order to carry out their function, antioxidants are consumed during the polymer degradation process, and the amount of unreacted antioxidant in the polymeric material is an indicator of the state of the oxidation process. Since the concentration of antioxidants added is low (usually between 100-1000 ppm depending on the application and processing conditions) (Wang 2000) and tends to decrease with time in the polymeric materials, there is a need for analytical methods which have very low detection limits (Molander *et al.* 1999). Several chromatographic methods have been described for the identification and quantification of phenolic antioxidants. Tables VI.11 to VI.13 (pages 131-132) resume some of the methods available in the bibliography. The most used methods are gas chromatography and liquid chromatography. However, supercritical fluid chromatography (SFC) has also been used in order to avoid limitations of conventional techniques. Less common methods like electrochemical (Agüí *et al.* 1995), pyrolysis-gas chromatography (Py-GC) (Wang 2000) or coupled techniques like HPLC-NMR-MS (high-performance-liquid chromatography- nuclear magnetic resonance- mass spectrometry) and HPLC-FT-IR (high performance liquid chromatography- Fourier transform-IR spectroscopy) (Ludlow *et al.* 1999) have also been reported.

VI.3.1.2. DPBD

Diphenylbutadiene (DPBD) is a fluorescent whitening agent added to a material to absorb ultraviolet rays in sunlight and release them as blue rays. These blue rays will then interact with the yellowish color and give the plastic the appearance of being whiter. DPBD is a light yellow crystalline powder, soluble in many organic solvents, with high lipophilicity (Calculated Log Kow (octanol-water) = 5.29) (EPI).

DPBD has been incorporated into polyethylene film to act as a reference material BCR.593 in the test method for the determination of fatty contact (EN 14481). In order to determine the DPBD migration level from plastics to foodstuffs, an HPLC-UV method was developed in three selected foods that represent three types of foodstuffs with different physical and chemical properties: aqueous and acidic food (orange juice), non fatty food (chicken breast), and fatty food (gouda cheese) (Sendón García *et al.* 2004).

Table VI.2: Chemical and physical properties of DPBD.

DPBD	
CAS Nº	538-81-8
Formula	C16H14
Chemical Name	Diphenylbutadiene
MW	206
mp (ºC)	153
Bp/Fp (ºC)	350/-
Water solubility (mg l^{-1})	0.5-1.2
Solubility	very soluble in: benzene, ethanol and ether
Application	Fluorescent additive
Structure	
Spectral Data	UV: 319 nm (cyhex) Fl: Ex:320, Em:375 nm (hex)

VI.3.1.3. Chimassorb 81 and Uvitex OB

Other two substances selected by the FOODMIGROSURE project are Chimassorb 81, an ultraviolet absorber, and Uvitex OB, an optical brightening agent. These chemical additives are used to enhance the lifetime of plastics, since they minimize photo-chemical processes and, therefore, polymer degradation (Vargo and Olson 1985). Like other additives/monomers targeted in this project, they were chosen because they present different active groups, have different functions within the polymer and are used in food contact materials (polystyrenes and polyolefins) (Quinto-Fernandez *et al.* 2003).

Regarding the analytical methods for determination of these additives, thin-layer chromatography (Lawrence and Ducharme 1980) has been substituted by high performance liquid chromatography (HPLC). Table VI.14 presents several HPLC-UV methods used to determine these additives. Owing to the advantages of HPLC technique, like the higher resolution, capacity to handle larger samples, reliable quantification detection, no need of clean-up step or internal standards to correct for poor recovery or instrumental errors, this is the most used method (Quinto-Fernandez *et al.* 2003).

Table VI.3: Chemical and physical properties of Chimassorb 81.

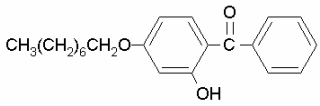
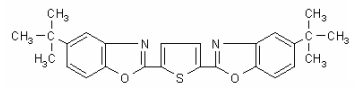
Chimassorb 81	
Ref. Nº	61600
CAS Nº	1843-05-6
Formula	C21H26O3
Chemical Name	2-Hydroxy-4-n-octyoxybenzophenone
MW	326
mp (ºC)	48-49
Bp/Fp (ºC)	>400/>200
Water solubility (mg l^{-1})	< 0.03
Solubility (g x 100g solution^{-1})	Acetone: 43; benzene:72; chloroform:61; ethanol: 3.5; ethyl acetate: 44; n-hexane: 12; methanol: 1.7; MEK: 65; methylene chloride: 67; toluene: >50; water: < 0.01
Application	UV absorber/ stabiliser
Structure	
Spectral Data	UV: 290, 243 nm (ethanol) MS peaks: 213 (100), 214 (39) 326(19) 325 (14) 43 (11) 77 (10) 41 (10) 215 (7).
SML (mg kg^{-1})	6
TDI (mg kg^{-1})	0.1

Table VI.4: Chemical and physical properties of Uvitex OB.

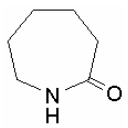
Uvitex OB	
Ref. Nº	38560
CAS Nº	7128-64-5
Formula	C26H26N2O2S
Chemical Name	2,5- Bis(5-tert-butyl-2-benzoxazolyl thiophene
MW	431
mp (ºC)	196-203
Bp/Fp (ºC)	-/>350
Water solubility (mg l^{-1})	<0.03
Solubility (g x 100g solution^{-1})	Acetone: 0.2; dioxane: 2; DMF: 0.8; ethanol: 0.1; ethyl acetate:1 ; n-hexane: 12; methanol: 0.05; MEK: 1; toluene. 5; tetrahydrofuran:5; xylene: 5; water: < 0.01
Application	UV stabiliser; optical brightener; fluorescent white agent
Structure	
Spectral Data	Fl: Ex: 375; Em: 435 nm
SML (mg kg^{-1})	0.6
TDI (mg kg^{-1})	0.01

Uvitex OB, has also been determined by spectrofluorimetry thanks to its high and characteristic excitation and emission wavelengths (Quinto-Fernandez *et al.* 2003). The determination of these compounds has also been performed by gas chromatography (Table VI.15).

VI.3.1.4. Caprolactam

Caprolactam is a monomer of polyamide (nylon). Table VI.5 summarises the properties of this molecule. During the reaction of polymerization of caprolactam, about 90% of the monomer is consumed, leaving a residue of monomer and low molecular weight oligomers. The residue can either be removed by filtration or vacuum treatment of the polymer melt, although there is always a small % that remains in the polymer (Barkby and Lawson 1993). Several chromatographic methods have been used to quantify the amount of free caprolactam and its oligomers in the polymer. Most of them include LC methods with UV (Barkby and Lawson 1993, Krajnik *et al.* 1982) or RI detection (Bonifaci *et al.* 1991).

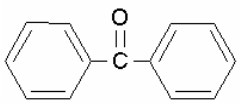
Table VI.5: Chemical and physical properties of ε-caprolactam.

ε-Caprolactam	
Ref. Nº	14200
CAS Nº	105-60-2
Formula	C6H11NO
Chemical Name	Aza-2-cycloheptanone
MW	113
mp (ºC)	70-72
Bp/Fp (ºC)	267/125
Water solubility (mg l^{-1})	> 700000
Solubility (g x 100g solution^{-1})	soluble in water, ethanol, benzene, chloroform, cyclohexane, acetone and dimethylsulfoxide
Application	Monomer
Structure	
Spectral Data	MS peaks: 55(100) 113 (87) 30(81) 56(66) 84(60) 85(57) 42(51) 41(33). UV: 198 nm (H_2O)
SML (mg kg^{-1})	15
TDI (mg kg^{-1})	0.25

VI.3.1.5. Benzophenone

Benzophenone is the most commonly employed photo-initiator for inks and varnishes cured with ultraviolet light. Also it is a wetting agent as it increases the flow of inks by acting as a reactive solvent (Anderson and Castle 2003, Papilloud and Baudraz 2002, Choi *et al.* 2002). Although benzophenone is applied to the outside of the packaging material (often paper or cartonboard), owing to its low molecular weight, it can penetrate through the packaging material structure and migrate to foods packaged with these materials (Anderson and Castle 2003). Frequently, the paper packaging materials are coated with polymeric materials (like polyethylene, polypropylene or polyethylene terephthalate) which act as a barrier against moisture and food contamination (Song *et al.* 2003). However, the efficiency of this coating depends on its thickness and storing packaging conditions (Choi *et al.* 2002). Several papers have already determined this polar, water soluble surrogate. GC- (FID or MS) has been the most used technique (see table VI.15).

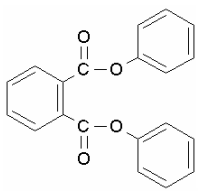
Table VI.6: Chemical and physical properties of benzophenone.

Benzophenone	
Ref. Nº	38240
CAS Nº	119-61-9
Formula	C13H10O
Chemical Name	Diphenylmetanone
MW	182
mp (ºC)	48
Bp/Fp (ºC)	305/-
Water solubility (mg l^{-1})	137
Solubility (g x 100g solution^{-1})	water:1; ethanol:4; diethyl ether: 4; acetone:4; benzene: 3; chloroform:4; acetic acid:4; methanol:3; carbon sulphur:4
Application	Light stabiliser
Structure	
Spectral Data	MS peaks: 105 (100) 77(49) 182 (32) UV: 33, 252 nm (methanol)
SML (mg kg^{-1})	0.01
TDI (mg kg^{-1})	0.6

VI.3.1.6. Diphenyl Phthalate and DEHA

According to the International Union of Pure and Applied Chemistry (IUPAC), a plasticizer is a substance or material incorporated in a material (plastic, for instance) to increase its flexibility, workability or stretchability (Cano *et al.* 2002, Page and Lacroix 1995). However, plasticizers can also act as lubrificants, flame retardants or thermal stabilizers (Wang 2000). Plasticizers like phthalates, adipates, phosphates, citrates and sebacates are widely used in food packaging materials (Van Lierop and Van Veen 1988), often in significant amounts (sometimes exceed 20% by weigh of the polymer) (Startin *et al.* 1987, Goulas *et al.* 1998). The properties of the plasticized polymer depend on the chemical nature and concentration of the plasticizer (Cano *et al.* 2002).

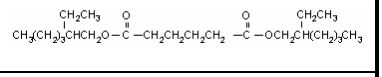
Table VI.7: Chemical and physical properties of diphenyl phthalate.

Diphenyl Phthalate	
CAS Nº	84-62-8
Formula	C20H14O4
Chemical Name	1,2-Benzenedicarboxylic acid, diphenyl ester
MW	318
mp (ºC)	73
Bp/Fp (ºC)	405/224
Water solubility (mg l^{-1})	0.08
Solubility (g x 100g solution^{-1})	water:1; ethanol:2; diethyl ether:2; ete:2
Application	Plasticizer
Structure	
Spectral Data	MS peaks: 94(100) 28(83) 225(32) UV: 225nm (methanol)

Generally plasticizers have low molecular weight, therefore, they tend to migrate from packaging material to the wrapped food and become indirect food additives (Goulas *et al.* 1998). The specific migration limit of DEHA is 3 mg dm^{-2} (Petersen *et al.* 1997). Regarding diphenyl phthalate, which is an alkyl-substituted

phthalate, its identification by mass spectra is not an easy task, once all the substances which belong to this family do not give an intense parent ion and present common major fragments (Wang 2000).

Table VI.8: Chemical and physical properties of DEHA.

DEHA	
Ref. Nº	31920
CAS Nº	103-23-1
Formula	C22H42O4
Chemical Name	Adipic acid, bis(2-ethylhexyl) ester
MW	371
mp (ºC)	<-70
Bp (ºC)	417
Water solubility (mg l^{-1})	0.78
Application	Plasticizer
Structure	
Spectral Data	MS peaks: 129, 57, 112, 147.
SML (mg kg^{-1})	18

The key to the correct analytical approach of plasticizers requires not only the knowledge of these plastics additives but also the comprehensive understanding of the polymer, once the plasticizers are homogeneously blended with the polymer molecules (Wang 2000). Tables VI.16 and VI.17 refer to GC methods for the determination of DEHA.

VI.3.2. Sample preparation for the analysis of model migrants

VI.3.2.1. In polymer materials

In general, the first step for the determination of migrants in plastics is their separation from the polymeric matrix. The two most widely methods used are the extraction with solvents and the use of solvents that swell and/or dissolve both, selected compound and polymer.

VI.3.2.1.1. Extraction with solvents

It is broadly accepted that the solvent used should dissolve the target compound and also swell the polymeric matrix. Polymer swelling data are widely available in the literature, but the solubilities of the selected migrants in extraction solvents are incompletely documented and must be estimated on the basis of the nature of analyte and extractant. Several combinations solvent/analyte/polymer has been used. Dichloromethane has been used to determine Irganox 1076 and DEHA in multilayer materials (PP/EVA/EVOH) and PVC (polyvinylchloride); acetonitrile for Chimassorb 81 and Irganox 1076 in PET (polyethylene terephthalate), tetrahydrofuran for Chimassorb 81 and Uvitex OB in HDPE (high density polyethylene) and PP (polypropylene); iso-octane for DEHA in PVC and diethyl ether for Irganox 1076 and DEHA. Extraction procedures are usually carried out by hand shaking, although sometimes ultrasonic maceration or Soxhlet are used to improve the process (Vargo and Olson 1985, Ulsaker and Teien 1992, Nerin *et al.* 1996, O' Brien *et al.* 1997, Marque *et al.* 1998, Monteiro *et al.* 1998, Specific migration, Petersen *et al.* 2004). Regarding other food contact materials, benzophenone was extracted from paper and board with absolute ethanol (Summerfield and Cooper 2001, Triantafyllou *et al.* 2002). Extractions with supercritical fluid or microwave-assisted were used for Irganox 1076 and chimassorb 81 in PE (Salafranca *et al.* 1999), and DEHA in PVC (Cano *et al.* 2002, Guerra *et al.* 2002).

VI.3.2.1.2. Use of solvents that swell and/or dissolve both selected compound and polymer, followed by precipitation of the polymer

Toluene has been used for polyolefins containing Irganox 1076 followed by precipitation of the polymer with methanol (Specific Migration) while DEHA was determined by total dissolution of polymers with chloroform and then precipitating with methanol (O' Brien *et al.* 1997).

VI.3.2.2. In food simulants

VI.3.2.2.1. Aqueous official food simulants

Usually they are directly injected when the analysis is carried out by reverse phase- HPLC (RP-HPLC). DEHA and Irganox 1076 were determined following this methodology (O'Brien *et al.* 1997, Howe and Borodinsky 1998), and

Chimassorb 81 and caprolactam after dilution with miscible organic solvents (Spyropoulos 1998, Specific migration).

Sometimes a concentration step or change of solvent is necessary before the chromatographic analysis. For instance, Irganox 1076 and DEHA were extracted with hexane (Simoneau and Hannaert 1999), Chimassorb 81 with dichloromethane (Nerin *et al.* 1996), Uvitex OB with chloroform and DEHA with heptane (O' Brien *et al.* 1997). Benzophenone was extracted using an activated silica-bound-phase cartridge, which was eluted with a methanolic solution (Papillound and Baudraz 2002).

VI.3.2.2.2. Official fatty food simulants

In some cases the fat simulant is diluted with a solvent followed by direct injection in a chromatographic system. For instance, acetone was used in the determination of the the migration of Irganox 1076 (Helmroth *et al.* 2002; O'Brien *et al.* 1997), dichloromethane or tetrahydrofuran were used to determine Chimassorb 81 (Nerin *et al.* 1996, Spyropoulos 1998) and heptane and acetonitrile were the solvents when migration of DEHA was evaluated (O' Brien *et al.* 1997, Specific migration).

Extraction with immiscible solvents can also be performed. For example, acetonitrile was used to extract DEHA and Irganox 1076 (Simoneau and Hannaert 1999), and methanol to extract Chimassorb 81 (Spyropoulos 1998), although very poor recovery was achieved.

For volatile substances specific techniques were applied. A Tenax adsorption column followed by extraction with hexane has been employed to determine DEHA (Van Lierop and Van Veen 1988). Also, DEHA was extracted with ethyl acetate/toluene and extracts were injected in a GPC column (Biobeads S-X3) to proceed to the clean-up step (Petersen and Breindahl 1998).

VI.3.2.2.3. Substitute fat simulants

Regarding this type of simulants, direct injection of the simulant it is commonly used in a chromatographic system, after migration tests. This method has been used to determine the migration of Irganox 1076, Chimassorb 81, Uvitex OB and ε-caprolactam (Specific migration) and Chimassorb 81 after dilution with tetrahydrofuran (Spyropoulos 1998). On the other hand, evaporation of the simulant

to dryness followed by dissolution in chloroform or acetonitrile, was used to determine Irganox 1076 (Garde *et al.* 2001) and benzophenone (Papillound and Baudraz 2002), respectively and extraction with hexane/water was used to determine DEHA and Irganox 1076 in ethanol 95% (Simoneau and Hannaert 1999).

VI.3.2.3. Foodstuffs

Due to the complexity of food samples, the procedure more broadly used with simulants, by direct injection, is not the most appropriate to evaluate migration in foods. In spite of the lack of abundant bibliographic support, the available information related with the study of migration into foodstuffs reveals that the scientific community focuses mainly on the three approaches: extraction with solvents, solid phase extraction and size exclusion chromatography or gel permeation chromatography.

VI.3.2.3.1. Extraction with solvents

This procedure is one of the most generally used, as much for fatty foods as non-fatty foods. For instance, DEHA was extracted from several food items with cyclohexane-dichloromethane (1:1) or hexane-dichloromethane (10:1) (Lau and Wang 1996, Page and Lacroix 1995). Benzophenone was extracted from several foods with a mixture of acetonitrile and dichloromethane (1:1).

Occasionally, after solvent extraction a clean-up step, like solid phase extraction, size exclusion chromatography or gel permeation chromatography may be necessary.

VI.3.2.3.2. Solid phase extraction

It was used in sample preparation of DEHA and other plasticizers in fatty food (animal tissues, fats and high fat content cheese) after blending with dichloromethane and passing the extract through a Florisil column (Page and Lacroix 1995). It was also used to analyse DEHA in water samples (Brossa *et al.* 2002).

VI.3.2.3.3. Size exclusion chromatography or gel permeation chromatography

These techniques were used to clean up fatty matrices containing non polar analytes. DEHA in homogenized foods was extracted with acetone/hexane, extracts were dried, evaporated to dryness and redissolved in dichloromethane/cyclohexane and a Biobeads S-X3 column was used to clean-up (Startin *et al.* 1987, Oi-Wah and Siu-Kai 1996). Also DEHA was extracted from cheese with acetone/pentane (1:1), the solvent was decanted, filtered and dried and then, re-dissolved in ethyl acetate/toluene (3:1). A Biobeads S-X3 was used to clean-up (Petersen *et al.* 1995). GPC has also been applied for the extraction of DEHA from baby food and infant formulae (Petersen and Breindahl 2000).

DEHA migration was determined in fatty food by extracting samples with diethyl ether in a Soxhlet apparatus and then using Tenax (Van Lierop and Van Veen 1988). Also DEHA was determined in cheese with hexane in a Soxhlet apparatus (Goulas *et al.* 2000).

Volatile compounds can be separated by volatilization with or without concentration step (static or dynamic headspace, solid phase microextraction (SPME) or Tenax). DEHA and several phthalates were extracted from water using a polyacrylate fiber (SPME) (Peñalver *et al.* 2000).

VI.3.3. Chromatographic determination of model migrants

In the literature many chromatographic methods were found. Tables VI.11 to VI.17 summarize the most significant characteristic of these methodologies employed.

VI.3.3.1. HPLC

Most of analyses were performed on reversed phase, with C18 columns and using appropriate gradient and composition of mobile phase, mainly acetonitrile/water or alcohols/water, with or without modifiers. Some analyses were performed with normal phase on silica columns, and then mobile phases used were hexane (Specific migration), hexane/2-propanol/dichloromethane (Mountfort *et al.* 1997), and dichloromethane/hexane (Nerin *et al.* 1996).

VI.3.3.2. Gas Chromatography

Most of analyses were carried out on apolar columns, type 5% phenyl-95%dimethylpoysiloxane, with a length of 15 to 30 m, and a phase ratio of intermediate values, although in some cases other types of columns were used. Split/splitless injection is the widely used technique.

VI.3.4. General guidelines for the analysis of model migrants

The general guidelines to determine the studied model migrants are shown in table VI.10.

Irganox 1076, Chimassorb 81 and Uvitex OB have a very low solubility in water (< 0.03 mg l^{-1}), therefore it can be expected that migration to non-fatty food, especially aqueous food, also will be very low. Both extraction and concentration steps are necessary, extraction with weakly or non polar solvents seems better choice than polar solvents, in which they are not very soluble.

Although data on fat solubility are not available, the migration is expected to be much higher than into water. In this case, the better approach seems to be the extraction with weakly or non polar solvents, followed by a GPC-SEC clean-up step to separate bulk lipids followed by a concentration step. The extraction with polar solvents immiscible with fat seems more difficult, because it will involve a high number of extraction steps and/or big extraction volumes, although laborious it can be a simpler way for analytic laboratories.

Regarding the determination, the HPLC-fluorescence combination is the best for Uvitex OB and HPLC-(UV or APCI(+)) and GC-(FID or MS) are possible for the three compounds.

Diphenylbutadiene, benzophenone and diphenylphthalate**,** can be extracted from foods with polar solvents immiscible with fat, although DPBD with some difficulty. All the migrants exhibit good properties in the ultraviolet region and furthermore DPBD shows very good response in fluorescence. Therefore HPLC with UV-FI detection seems an appropriate determination technique. Except for diphenylbutadiene, HPLC coupled with APCI-MS detector can be also used. GC-(FID or MS) is also a common possible technique for most of them.

DEHA, has a low solubility in water and it presents a problem similar to the first group. It does not show interesting properties in UV or fluorescence and it needs to be determined by GC-(FID or MS) or by HPLC only with an APCI-MS detector.

For all migrants and depending of each combination analyte/food matrix, more purification steps could be necessary, usually using SPE or liquid-liquid extraction. But this can only be discussed in specific cases and it is outside of the scope of this compilation.

VI.4.- CONCLUSIONS

To sum up, at present it is not possible to draw up reliable analytical protocols for the determination of all the selected compounds in food. For most migrant/food combinations, any protocol drawn up on the basis of the literature using general analytical principles and experience is necessarily a proposal which requires exhaustive evaluation. Taking in account all of the compiled data, our laboratory has suggested guidelines to prepare analytical procedures to determine migrants in foods.

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Table VI.9: Results of preliminary experiments.

MODEL MIGRANT	Spectroscopic Properties				GC-FID analysis	Aacetonitrile /olive oil
	Fluorescence	Ultraviolet	MS-APCI$^+$	MS-APCI$^-$		
Irganox 1076		λmax (ε): 204 (33400), 218 (10600), 273 (4000)	475, 419, 167			
DPBD	λex: 330 λem: 375	λmax (ε):206 (47300), 232 (45000), 315 (92200), 328 (105900), 344(69600)			Yes	0.32
Chimassorb 81		λmax (ε):206 (23700), 243 (13000), 288 (16600), 324 (11100)	327, 439			0.18
Uvitex OB	λex: 374 λem: 432	λmax (ε):207 (41600), 263 (12100), 355 (42100), 373 (47300)	431		Yes	0.24
Caprolactam		λmax (ε):204 (5900), 224 (2200)	114		Yes	
Benzophenone		λmax (ε):208 (27100), 251 (19900)	183, 105		Yes	0.58
Diphenyl Phthalate	λmax (ε):206 (45800), 227 (17100)		225, 149, 319, 177	144, 121, 148, 169		0.87
DEHA			129, 147, 241, 259, 113, 371		Yes	

Table VI.10: Guideline suggested for the analysis of eight selected model migrants.

Compounds	Food	Sample Preparation	Analysis procedure
Irganox 1076	Non Fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used (***) • Extraction/dilution with polar solvents (*)	• GC-FID or MS (**). Column type DB5, with medium to high phase ratio (β), on column or splitless injection mode. • HPLC-UV or APCI (+) (**). Column type C8 is better than a C18. Mobile phase: acetonitrile, Detection at λ= 205 or 218 nm by UV and 475 (*m/z*) by MS.
	Fatty	• Extraction with weakly non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used (***) • Extraction with polar solvents not miscible with fat (*)	
DPDB	Non fatty	• Extraction with polar to non polar solvents (**)	• HPLC-UV-or FL (***). Column type C18. Mobile phase: acetonitrile, Detection at λ= 328 nm by UV and λex= 330 nm, λem = 375 nm by fluorescence.
	Fatty	• Extraction with polar solvents not miscible with fat (**)	
Chimassorb 81	Non fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used (***) • Extraction/dilution with polar solvents (*)	• HPLC-UV or APCI (+) (***). Column type C18. Mobile phase: acetonitrile/water, Detection at. λ= 288 nm by UV and 327 (*m/z*) by MS • GC-FID or MS (*). Column type polar, with medium to high phase ratio (β), on column or splitless injection mode.
	Fatty	• Extraction with weakly non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used (***) • Extraction with polar solvents not miscible with fat (*)	
Uvitex OB	Non fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used (***) • Extraction/dilution with polar solvents (*)	• HPLC-UV or FI or APCI (+) (***). Column type C18. Mobile phase: acetonitrile, Detection at λ= 373 nm by UV, λex= 374 nm, λem= 432 nm by fluorescence and 431 (*m/z*) by MS.
	Fatty	• Extraction with weakly non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used (***) • Extraction with polar solvents not miscible with fat (*)	
Caprolactam	Non fatty	• Extraction/dilution with polar solvents (***)	• GC-FID or MS (***). Column type polar, with medium to high phase ratio (β), split or splitless injection mode. • HPLC-UV or APCI (+) (**). Column type C18. Mobile phase: acetonitrile/water, Detection at. λ= 224 nm by UV and 114 (*m/z*) by MS.
	Fatty	• Extraction with polar solvents not miscible with fat (***)	
Benzophenone	Non fatty	• Extraction with polar to non polar solvents (**)	• GC-FID or MS (***). Column type apolar, with medium to high phase ratio (β), on column or splitless injection mode. • HPLC-UV or APCI (+) (***). Column type C18. Mobile phase: acetonitrile/water, Detection at λ= 251 nm by UV and 183 (*m/z*) by MS.
	Fatty	• Extraction with polar solvents not miscible with fat (**)	
Diphenyl phthalate	Non fatty	• Extraction with polar to non polar solvents (**)	• GC-FID or MS. To check. HPLC-UV or APCI (+) or (-) (***). Column type C18. Mobile phase: acetonitrile, Detection at λ= 227 nm by UV and 225 (*m/z*) by APCI(+) or 144 (*m/z*) APCI(-).
	Fatty	• Extraction with polar solvents not miscible with fat (**)	
DEHA	Non fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used (***) • Extraction/dilution with polar solvents (*)	• GC-FID or MS (***). Column type apolar, with medium to high phase ratio (β), on column or splitless injection mode. • HPLC-APCI (+) (***). Column type C18. Mobile phase: acetonitrile/water, Detection at 129 (*m/z*) by MS.
	Fatty	• Extraction with weakly non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used (***) • Extraction with polar solvents not miscible with fat (*)	
(***) Appropriate option; (**) Possible option; (*) Difficult option			

Table VI.11: HPLC methods for Irganox 1076 analysis

Detector	Analytical column	Mobile phase	Detector conditions	Type of sample	References
UV	Hypersil 5 ODS	Tetrahydrofuran/ acetonitrile	λ = 275 nm	Aqueous simulants	O'Brien *et al.* 1997
	Spherisorb ODS1	Acetonitrile/water	λ = 275 nm	Polymer	Marque *et al.* 1998
	Hypersil ODS-2	Ethanol/water	λ = 230 nm	Polyolefins	Specific Migration
	EC 25/3 Nucleosil 100-5	Ethanol 95%/water	λ = 230 nm	Polyolefins: Simulant: 95% ethanol	
	Spherisorb ODS	Ethyl acetate/methanol/ water	λ = 230 nm	Polyolefins:	Salafranca *et al.* 1999
	Homemade (50 cm x 320 µm, 5 µm)	Acetonitrile	λ = 280 nm	LDPE	Molander *et al.* 1999
	C18 (150 x 3.9 mm, 5 µm)	Acetonitrile/water	λ = 200 nm	LDPE	Zhou *et al.* 1999
FI	Hypersil 5 ODS	Prop-2-ol/ Acetonitrile	λex = 282 nm λem = 308 nm	Olive oil	O'Brien *et al.* 1997
	Phenomenex Sphereclone ODS2 (250 x 4.6 mm)	Propan-2-ol, acetonitrile, tetrahydrofuran	λex = 282 nm λem = 342 nm	Sunflower oil	Specific Migration
MS		Acetonitrile/water	Mass range: 300-1300	Polymer	Lawson *et al.* 1994

Table VI.12: GC methods for Irganox 1076 analysis

Detector	Analytical column	Oven program	Type of sample	References
FID	DB5-MS	From 1ºC under the boiling point of the solvent to 310ºC	Simulants:ethanol; isooctane; ethyl acetate; 2-propanol; ciclohexane; dichloromethane; olive oil; tricaprylin; tributyrin	Helmroth *et al.* 2002
	SPB5	From 300ºC to 350ºC	Fatty food simulants: - 95% ethanol - n-heptane	Garde *et al.* 2001
	HP5	From 100ºC to 350ºC	Aqueous simulants	Simoneau and Hannaert 1999
	DB5 HT	From 100ºC to 365ºC	Oil simulant	
	SPB-5	From 40ºC to 270ºC	Polymer: - Polyethylene - Polypropylene	Dilettato *et al.* 1991
	DB-5	From 50ºC to 320ºC		Wang 2000
MS	DB-5	From 40ºC to 320ºC		Wang 2000
	DB-5	From 50ºC to 325ºC	PVC and others strech films	Petersen *et al.* 2004

Table VI.13: SPE (Supercritical Fluid Chromatography) methods for Irganox 1076 analysis

Analytical column	Mobile phase	Detector conditions	Type of sample	Reference
Nucleosil 120	Methanol/water Acetonitrile/water	λ = 330 nm	Simulants: -15% aq ethanol - 95% aq ethanol - 3% aq acetic acid - isooctane -Olive oil	Spyropoulos 1998 (Chimassorb 81)
PL-Gel 50 A	Dichloromethane/ hexane	λ = 280 nm	Simulants: - water - acetic acid 3% - ethyl alcohol 15% - rectified olive oil	Nerin *et al.* 1996 (Chimassorb 81)
ODS	Acetonitrile/water/ tetrahydrofuran	λ = 330 nm	Sunflower oil	Specific Migration (Chimassorb 81 & Uvitex OB)
Hypersil ODS-2	Acetonitrile 1% acetic acid/water/ tetrahydrofuran,	λ = 330 nm	HDPE and PP	
ODS	Acetonitrile	λ = 280 nm Note: LC-UV-MS	Polymers	Vargo and Olson 1985 (Chimassorb 81 & Irganox 1076)
Hypersil ODS	Tetrahydrofuran; 1% VI/VI acetic acid/water	λ = 280 nm		Quinto-Fernandez *et al.* 2003 (Chimassorb 81 & Uvitex OB)

Table VI.14: HPLC-UV methods for Chimassorb 81 and Uvitex OB analysis

Detector	Analytical column	Oven program	Type of sample	Reference
FID		80ºC		Doehl *et al.* 1987
	cross-linked 5% phenyl methylpolysiloxane (10 m x 50 µm, 0.4 µm)		Polymer: - Polyethylene - Polypropylene	Dilettato *et al.* 1991
	Deltabond cyano column (100 x 1.0 mm, 5 µm)			Zhou *et al.* 1999
	C18 nucleosil (20 cm x 1.0 mm. 5 µm)	60ºC or 150ºC	Polystyrene	Ashraf-Khorassani *et al.* 1991
MS	SB-Biphenyl-30 (10 m x 50 µm, 0.25 µm)	120ºC		Leuker *et al.* 1994

Table VI.15: GC methods for Chimassorb 81, Uvitex OB and benzophenone analysis

Detector	Analytical column	Oven program	Type of sample	Reference
FID	DB-5	From 120ºC to 260ºC	PE	Choi *et al.* 2002 (Benzophenone)
	DB-5	From 60ºC to 280ºC	PP	Song *et al.* 2003 (Benzophenone)
MS	DB 1701	From 100ºC to 280ºC	PET	Monteiro *et al.* 1998 (Chimassorb 81)
	Optima Delta 6	From 50ºC to 250ºC	Simulants: - water - acetic acid aq 3% - ethanol aq 15% - rectified olive oil	Papilloud and Baudraz 2002 (Benzophenone)
	Chrompack CPSil5CB	From 40ºC to 260ºC	PET	Nerin *et al.* 2003 (Benzophenone)
	Restek Rtx-1	From 60ºC to 270ºC	PET	
	Restek Rtx-1	From 50ºC to 280ºC	Several foods	Anderson and Castle 2003 (Benzophennone)

Table VI.16: GC-FID methods for DEHA analysis.

Analytical column	Oven program	Type of sample	Reference
BPX5	From 240ºC to 300ºC	Polymers	O'Brien *et al.* 1997
BPX5	From 100ºC to 360ºC	Aqueous simulants	
DB-5	From 60ºC to 295ºC	Fatty and non-fatty food	Page and Lacroix 1995
DB-5	From 50ºC to 320ºC	PVC	Wang 2000
SPB-5	From 110ºC to 260ºC	PVC	Cano *et al.* 2002
DB-1	From 130ºC to 320ºC	PVC	Specific Migration
10% SE-30 stationary phase on Anachrom ABS, 60-80 mesh (1.9 m x 6.35)	165ºC	Cheese	Goulas *et al.* 2000
HP-5 and NB-1701 A		Cheese	Petersen *et al.* 1995
HP-5	From 100ºC to 300ºC	Food simulants: 95% ethanol; 15% ethanol; 3% acetic acid	Simoneau and Hannaert 1999
DB5-HT	From 100ºC to 365ºC	Olive oil	
HP-5	From 150ºC to 250ºC	Cap-sealing resins for bottled foods	Hirayama. *et al.* 2001
SPB-5	From 110ºC to 260ºC	PVC	Guerra *et al.* 2002

Table VI.17: GC-MS methods for DEHA analysis

Analytical column	Oven program	Type of sample	Reference
DB-5	From 60ºC to 300ºC	Fat and fatty food	Van Lierop and Van Veen 1998
CPSIL 5 CB	230ºC	Food	Startin *et al.* 1987
DB-5	From 100ºC to 300ºC	Olive Oil Food samples	Lau and Wang 1996
DB-5	From 40ºC to 320ºC	PVC	Wang 2000
HP-5MS	From 100ºC to 300ºC	PVC	Specific migration
Rtx-1	From 50ºC to 300ºC	Olive oil	
HP-5MS	From 60ºC to 280ºC	Water	Peñalver *et al.* 2000
HP-5	From 40ºC to 270ºC	Water	Brossa *et al.* 2002
Restek XTI-5	From 90ºC to 280ºC	Isooctane Olive oil	Petersen and Breindahl 1998
Restek XTI-5	From 90ºC to 280ºC	Baby food Infant formulae	Petersen and Breindahl 2000
DB-1701	From 50ºC to 280ºC	Cap-sealing resins for bottled foods	Hirayama *et al.* 2001

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***CAPÍTULO VII*: STATE OF THE ART OF ANALYTICAL STRATEGIES TO EVALUATE DIFFERENT MIGRANTS FROM FOOD PACKAGING MATERIALS**

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ABSTRACT

Integrated in the FOODMIGROSURE project (QLRT-2001-2390), and in order to carry out a compilation of the available information on analytical methods for determination of some model migrants in selected foodstuffs an extensive bibliographic review was done.

Model migrants of different chemical structures, polarities, lipophilicity and molecular weight have been selected: styrene, Bisphenol A, 1-octene, limonene, di-isopropylnaphthalene (DIPN), laurolactam, triacetin, tri-n-butylacetyl citrate (ATBC), butylated hidroxytoluene (BHT) and Triclosan.

In spite of the lack of abundant bibliographic support, the available information related to the study of migration into foodstuffs reveals that the scientific community addresses the problem in different ways depending of each combination analyte/food matrix. Any protocol drawn up on the basis of the literature, general analytical principles and experience is necessarily a tentative proposal requiring exhaustive evaluation and preliminary experiments.

Important properties of these migrants such as ultraviolet and fluorescence were also evaluated to help to decide the approach for the development of analytical methods that allow determining these compounds in foodstuffs. Several techniques were also checked (HPLC, GC).

KEYWORDS

Food packaging, food contact materials, migration.

VII.1.- INTRODUCTION

Lately food packaging has gained a widespread importance in food safety due to the possibility of migration of chemicals from food contact materials. The term "migration" usually describes a diffusion process, which may be strongly influenced by an interaction of components of the food with the packaging material (Arvanitoyannis and Bosnea 2004).

Although much work has been undertaken to help reduce the extent of analytical testing required through simplifying testing or use of mathematical modelling, within different EU projects during last years, further work was proposed before finally agreed models could be put forward for its adoption (Gilbert and Rossi 2000).

The current approach for the authorisation and control of substances used in food contact materials is cautious in estimating the potential exposure of the consumer to these substances. Approaches, which take better account of the actual exposure of the consumer to food contact materials in risk assessment, are under discussion. The use of mathematical modelling to predict migration, which can reduce the amount of tests to be undertaken, has been recently introduced into legislation. Practical examples for the application of this new concept are described in the Practical Guide (European Commission 2003a).

To estimate dietary exposure to a substance migrating from a food packaging material, information is needed on the types of food packaged, the nature of the packaging material, migration data, packaging usage factors and food consumption (ILSI Europe 2001).

Migration testing using food simulants is the normal procedure for checking compliance of a food packaging material against specific migration limits (SMLs). However, as Feigenbaum *et al.* (2002) point out, this is not practical for several reasons. Industries that put packaged foods or materials intended for food contact on the market do not know the identities of the potential migrants; sometimes, those who manufacture and sell these raw materials do not know the processing conditions or the final application; and even if the identities of the migrants were known it may be difficult to analyze them.

Within this framework, the project FOODMIGROSURE (QLRT-2001-2390) (Modelling migration from plastic materials into foodstuffs as a novel and cost efficient

tool for estimation of consumer exposure from food contact materials) aims to find tools that allow evaluate consumer exposure to these chemicals.

The above mentioned project carried out the selection of these model migrants being based on several approaches: the measurement of the substance on foodstuffs should be possible at low detection limits; migrants should be stable in foods; should cover a molecular weights a chemical groupings and should be relevant to food contact materials, including environmental aspects.

Once model migrants of different chemical structures, polarities, lipophilicity and molecular weight have been selected (styrene, Bisphenol A, 1-octene, limonene, di-isopropyl naphthalene (DIPN), laurolactam, triacetin, tri-n-butylacetyl citrate (ATBC), butylated hidroxytoluene (BHT) and Triclosan, a bibliographic review for the determination of these substances in different matrices was made.

In order to complete the information available, a review of the physical and chemical properties of these substances was made. For those that were considered significant and no data were found, experimental evaluations were carried out.

VII.2.- CHEMICAL AND PHYSICAL INFORMATION OF MODEL MIGRANTS

Styrene, also known as styrol, vinylbenzene, etc, is a monomer used in the manufacture of numerous types of plastics. Styrene monomer can be in direct contact with food when polymers such as polystyrene and acrylenenitrile-butadiene-styrene are used as packaging materials (Silva *et al.* 2000). It is a low molecular weight substance and a volatile compound.

Bisphenol A (other names are: diphenylolpropane, Ipognox 198, Parabis, etc) is a monomer used to produce polycarbonate plastics (Mountfort *et al.* 1997). It is also used as a raw material in the synthesis of bisphenol A diglycidyl ether (BADGE) (Simal Gandara *et al.* 1993) and as an antioxidant or inhibitor in the manufacture and processing of polyvinyl chloride (PVC) (López-Cervantes and Paseiro-Losada 2003).

To modify the polyethylene polymers' properties, 1-octene is incorporated to these materials as a co-monomer.

Limonene is very commonly found in recovered PET (polyethylene terephthalate) and arises from earlier contact with soft or fruit-flavored drinks (Franz 2002, Nerín *et al.* 2003). It is a volatile compound that is known as a precursor to off-flavour compounds in foods and it can be absorbed also by other packaging materials as low density polyethylene (LDPE) and polycarbonate (PC) (Van Willige *et al.* 2003). Hence, the determination of this substance could be used to ensure that a recycled plastic material is free of contaminants from previous contents.

Diisopropylnaphthalenes (DIPN) are widely employed as solvents in the paper industry in the manufacture of carbonless copy paper and thermal paper, and also for the production of dielectric fluid and thermal oil substituting polychlorinated biphenyls. The presence of this substance in foodstuffs may possibly occur due to the migration from the paperboard packaging, made from recycled fibres originating from feedstock containing carbonless copy paper (Boccacci *et al.* 1999).

In food packaging, nylon 12 is an important polyamide, which is produced by polycondensation of laurolactam. During its production, the monomer laurolactam as well as lower oligomers will remain in the polyamide and may migrate into foods (Stoffers *et al.* 2003).

Triacetin, also known as glycerol triacetate (GTA) is an antifungal agent used in the perfumery and pharmaceutical industries, although it is also used as a plasticizer in cellulose.

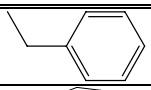
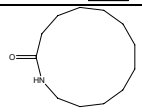
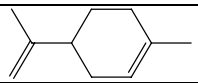
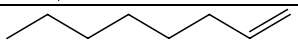
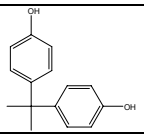
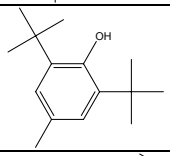
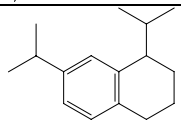
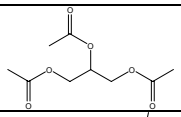
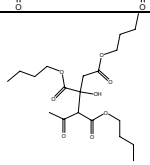
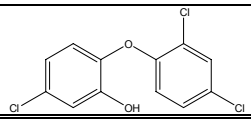
Acetyltributyl citrate (ATBC) is the most widely used plasticizer in cling-films made of polyvinylidene chloride (also known as the trade name of “Saran”) (Castle *et al.* 1988). This type of material is broadly used in microwave oven, especially in home-use applications (Badeka *et al.* 1999).

The phenolic compound, butylated hydroxytoluene (BHT), is used as a food additive, but it has been also used as a common antioxidant in rubber and petroleum products and lately in plastics (Tombesi and Freije 2002). BHT-impregnated film has shown to retard lipid oxidation of a packaged oatmeal cereal through its migration from the product via an evaporation/sorption mechanism (Lee *et al.* 2004). It is a small molecule which exhibits high mobility and therefore has a tendency to migrate rapidly from packaging materials into foods (Wesslilng *et al.* 1998).

Triclosan has been widely used recently as a broad spectrum antimicrobial and antifungal agent. As an additive in plastics it prevents the growth of micro-

organisms, avoiding the formation of stains and odours and, therefore, allows extending the polymer's shelf life. For this reason, it inclusion on food contact polymeric materials has been considered (Sanches-Silva *et al.* 2005).

Table VII.1: Chemical structures.

MIGRANT	CHEMICAL STRUCTURE
Styrene	
Laurolactam	
Limonene	
1-Octene	
Bisphenol A	
BHT	
Diisopropylnaphthlene	
Triacetin	
Tri-n-butylacetyl Citrate	
Triclosan	

These substances cover a wide range of different technical applications in the plastic materials as monomers (styrene, Bisphenol A, 1-octene, laurolactam) and additives (triacetin, ATBC, BHT). All of them, except DIPN, are included in the Synoptic Document (European Commission 2003b). For this reason they could be used in the manufacture of plastics intended to come into contact with foodstuffs. Some of them have a specific migration limit (SML) like laurolactam, 1-octene, BHT and bisphenol A, while those which have not been given a SML, are: substances with an ADI (Acceptable Daily Intake) or TDI (Tolerable Daily Intake) established, like Triacetin; substances without ADI or TDI but their use could be accepted like Triclosan; monomers that could be used but if its residue it is reduced as much as possible (styrene) and substances for which enough data are not available (limonene).

Moreover they have different chemical structures (Table VII.1), polarities, and also their molecular weights are very different, from 104 uma (styrene) to 402 uma (ATBC). Their solubility varies between triacetin (very soluble in water) and DIPN (almost insoluble in water); some of them are considered volatile compounds (1-octene, styrene) while others have very high boiling points. All the physico-chemical properties considered significant, since could help to approach an analytical method for their determination, are summarized in Table VII.2. All date were collected from different databases (Epi Suite, Lide 1997, Budavari 2001, Properties of Organic Compounds, 1996), and those data that were not available from the literature were estimated using the Epiwin software ver. 3.12.

VII.3.- SAMPLE PREPARATION APPROACHES

VII.3.1. Migrants Determination in Polymeric Materials

In general, the first stage in the determination of the migrants in polymers is their separation from the matrix.

Table VII.2: Chemical and physical information.

MIGRANT	Ref. Nº/ CAS Nº	Formula MW	mp bp/Fp (ºC)	Density g/cm^3	Log Kow	Solubility	Spectral Data
Styrene	24610 100-42-5	C8H8 104	-31 145-146/32	0.9	2.95	Soluble in alcohol, ether, acetone, and carbon disulfide. W sol.: 310 mg/L	**MS** Peaks: 104(100) 103(41) 78(32) 51(28) 77(23) **UV** max: 289 281 272 246 nm (cyhex)
Laurolactam SML=5 mg/kg	19490 947-04-6	C12H23NO 167	149-153 -	n. a.	2.92	W sol.: 290 mg/L	**MS** Peaks: 30(100) 55(98) 41(96) 100(64) 98(64) 73(53) 86(51) 43(46)
Limonene	63970 138-86-3	C10H16 136	-95 178/46	0.84	4.57	Soluble in carbon tetrachloride. Miscible in ethanol and diethyl ether. W sol.: 7.6 mg/L	**MS** Peaks: 68(100) 67(64) 93(60) 39(58) 41(46) 79(41)
1-Octene SML=15 mg/kg	22660 111-66-0	C8H16 112	-101 121/-	0.71	4.57	Slightly soluble in carbon tetrachloride; Soluble in diethyl ether, acetone, benzene; Very soluble in organic solvents; Miscible in ethanol. W sol.: 4.1 mg/L	**MS** Peaks: 43(100) 41(82) 55(80) 56(67) 70(54) 29(44) **UV** max: 177 nm (hp)
Bisphenol A SML=3 mg/kg	13480 80-05-7	C15H16O2 228	153 250/-	n. a.	3.32	Soluble in acetic acid; Very soluble in ethanol, diethyl ether; benzene, alkali. W sol.: 120 mg/L	**MS** Peaks: 213(100) 228(26) 119(25) **UV** max: 279, 227 nm (MeOH)
BHT	46640 128-37-0	C15H24O 220	71 265/	0.89	5.10	Soluble in ethanol, acetone, benzene, petroleum ether. W sol.: 0.6 mg/L	**MS** Peaks: 205(100) 220(27) 57(27) **UV** max: 283, 277, 227 nm (Iso)
Diisopropyl naphthlene (DIPN)	38640-62-9	C16H20 212	n. a.	n. a.	6.08*	W sol.: 0.11 mg/L	n. a.
Triacetin Glycerol triacetate (GTA)	57760 102-76-1	C9H14O6 218	-78 259//138	1.16	0.25	Slightly soluble in water, ligroin; Very soluble in acetone; Miscible in ethanol, diethyl ether, benzene, chloroform. W sol.: 58000 mg/L	**MS** Peaks: 43(100) 103(44) 145(34)
Tri-n-butylacetyl Citrate (ATBC)	93760 77-90-7	C20H34O8 402	>330 170/-	1.05	4.29*	W sol.: 5 mg/L	**MS** Peaks: 185(100) 129(57) 259(54) 43(54)
Triclosan	93930 3380-34-5	C12H7Cl3O2 289	56-58 -/223	1.58	4.76	Soluble in acetone and isopropanol. W sol.: 17 mg/L	n. a.

n. a.: not available.
*: Estimated with epiwin ver. 3.11.

When the migrant is a volatile compound, such as styrene, the headspace GC technique is very suitable for the analysis and it has been used in the EU project Specific Migration (Specific Migration), by heating the polystyrene dissolved in dimethylacetamide at 90ºC for 120 min. This methodology is also used by Simal-Gandara *et al.* (2000a) in the analysis of 1-octene, and in the analysis of limonene in LDPE, PC and PET absorbed from orange juice (Van Willige *et al.* 2002, Van Willige *et al.* 2003, Nerín *et al.* 2003).

Otherwise, if the migrant is not a volatile compound it is necessary to use a liquid extraction step. It is widely accepted that the solvent used should both dissolve the target compound well and also swell or dissolve the polymer matrix. Polymer swelling data are readily available in the literature, but the solubilities of the selected substances in plausible extraction solvents are only incompletely documented and, pending determination, they must be estimated on the basis of the nature of analyte and the extracting solvent. Several combinations solvent/analyte/polymer has been used. When the polymer is dissolved in a solvent, the polymer is then usually re-precipitated by addition of a solvent in which the polymer is insoluble.

Discontinuous extraction: Van Willige *et al.* (2003) utilized hexane for the extraction of limonene, and Nerín *et al.* (2003) dichloromethane or ethanol shaking the sample for 50 h; a liquid extraction with toluene from PP or a mixture of toluene and m-cresol from other packaging materials is also used by Caner (2004) for the analysis of limonene. Avison *et al.* (2001) extracted limonene using methanol from a polymer infused with this substance.

In the extraction of ATBC from sealing resins, hexane is the chosen solvent by Hirayama *et al.* (2001).

Acetonitrile (Monteiro *et al.* 1998, Vargo and Olson 1985) was selected for the extraction of BHT. Instead of this, Wessling *et al.* (1998) used a double extraction with heptane and then a concentration step.

Usually extraction procedures are carried out with hand shaking. Nevertheless sometimes ultrasonics (Marque *et al.* 1998) or maceration (Monteiro *et al.* 1998) were employed to improve the process.

Continuous extraction: Gramshaw and Vandenburg (1995) used dynamic head space to extract styrene from thermoset polyester passing through the U-tube nitrogen at 25ml min^{-1} and heating the oven at 200ºC. To analyze styrene dimers and

trimers Soxhlet extraction with dichloromethane was used followed by size exclusion chromatography to clean up the dichloromethane extracts. The Soxhlet extraction is also used by Summerfield and Cooper (2001) to determine DIPN from paper and board employing dichloromethane and ethanol as solvents, and Guerra *et al.* (2002) extracted ATBC from PVC refluxing cyclohexane during 5 h. To extract BHT incorporated in a HDPE film Lee *et al.* (2004) utilized 100 ml of acetonitrile and 24 h of Soxhlet extraction.

VII.3.1.1. Extraction with solvents that swell and/or dissolve both migrant and polymer

Snyder and Breder (1985) determined residual styrene monomer from polystyrene by dissolving a portion of the material in tetrahydrofuran (THF) followed by SEC prior the analysis by RP-HPLC.

For the analysis of Bishphenol A in baby bottles Biles *et al.* (1997) dissolved the polymer with methylene chloride in an ultrasonic bath and then the polymer was precipitated by adding methanol. Similar procedure was used by Mountfourt *et al.* (1997) but employing dichloromethane to dissolve and propan-2-ol to precipitate the polymer, afterwards hexane was added and supernatant was taken to dryness and dissolved in the HPLC mobile phase. Residual Bisphenol A in polycarbonates was determined by Howe and Borodinsky (1998) dissolving the polymer in chloroform and extracting the solution with 0.01 M sodium hydroxide.

Triantafyllou *et al.* (2002) extracted limonene from recycled PET with a mixture of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2-propanol.

For the determination of DIPN from paper and board Summerfield and Cooper (2001) immersed those materials in absolute ethanol in an ultrasonic bath when the analysis method chosen was HPLC.

Supercritical Fluid Extraction (SFE): Nerín *et al.* (2003) analyzed limonene using CO_2 modified with toluene as SFE fluid. ATBC was extracted also by this system (Cano *et al.* 2002; Guerra *et al.* 2002).

Microwave-assisted extraction (MAE): Cano *et al.* (2002) extracted ATBC by a microwave sample preparation system from a ethanol dissolution of the sample.

VII.3.2. Migrants Determination in food simulants

VII.3.2.1. Official aqueous simulants

Varner and Breder (1981) analyzed styrene in distilled water by static headspace GC. But usually, the aqueous simulants are directly injected when analysis is carried out with RP-HPLC technique. Extracts of Bisphenol A in water and different concentrations of ethanol in water (Biles *et al.* 1997) and in acetic acid (Lopez-Cervantes and Paseiro-Losada 2003) were directly injected for its analysis.

Nevertheless, sometimes a concentration step or a change of solvent step is needed prior to chromatographic analysis once the solvent must be compatible with the chromatography conditions used.

For volatiles substances some specific techniques has been applied: Silva *et al.* (2000) employed SPME for the determination of styrene and Tombesi and Freije (2002) for the analysis of BHT. Linssen *et al.* (1993) used thermal desorption extraction (DTD) for the determination of limonene.

VII.3.2.2-Fatty food simulants

The official fatty food simulant is rectified olive oil, but sometimes alternatives simulants are used as synthetic mixtures of triglycerides (e. g. Myglyol) sunflower oil or corn oil. When tests with the food oils are not possible, the European legislation allows substitute tests using isooctane, 95% ethanol and modified polyphenylene oxide (MPPO, Tenax is the registered trademark).

Due to the complexity of olive oil, migration tests are time consuming and difficult to conduct and therefore to approach this problem, many methods of extracting migrants from this simulant were investigated. An exception is when the substance is a volatile compound; an example is the determination of styrene using the static headspace (Specific Migration). Some of the methodologies adopted were:

Extraction with solvents: Lopez-Cervantes and Paseiro-Losada (2003) used acetonitrile 90% (v/v) for the extraction of Bisphenol A.

Continuous steam distillation: Styrene migration into olive oil was determined by Jickells *et al.* (1993) extracting the sample with hexane, using the Linkens-Nikerson apparatus.

SEC (size exclusion chromatography): This procedure, based on the separation of the components using a column and further collection of the eluted fraction of interest, was used by Stoffers *et al.* (2003) in the migration evaluation of laurolactam.

The methodologies applied to simulants, as the synthetic mixtures of triglycerides and sunflower oil, are very similar to those used when olive oil is the fatty food simulant selected. In the analysis of Bisphenol A from Miglyol an extraction step in methanol is required prior to its analysis by HPLC (Biles *et al.* 1997) or dissolution in chloroform (Howe and Borodinsky 1998).

Solid phase extraction with a Tenax adsorption column followed by extraction with hexane has been employed to determine ATBC in these simulants by Van Lierop and Van Veen (1988).

In order to overcome the analytical difficulties found in extracting migrants from olive oil, solvents have been used as substitutes. Commonly, extraction of the migrant from some of these simulants (e.g. ethanol 95%, isooctane) does not present a major problem. So, more undemanding techniques may be applied such as:

Simulant evaporation to dryness followed by dissolution in an appropriate solvent: This method was applied when migration of laurolactam (Stoffers *et al.* 2003) and BHT (Wessling *et al.* 1998) were evaluated. Methanol was the selected solvent in the dissolution step for the first substance (Stoffers *et al.* 2003) and heptane for the last one (Wessling *et al.* 1998).

Extraction with solvents: Diethyl ether is used to extract styrene from Tenax (Specific migration). Absolute ethanol was the selected solvent to extract DIPN (Summerfield and Cooper 2001, Triantafyllou *et al.* 2002), methanol was used to extract BHT according the IUPAC method 2.432 (Dieffenbacher 1998); Yankah *et al.* (1998) used acetonitrile to extract BHT.

VII.3.3. Migrants Determination in foodstuffs

Due to the complexity of food samples, most extraction procedures performed in simulants, especially in aqueous simulants, are not suitable to obtain a suitable analytical method for evaluate migration in food. Although there is a lack of bibliographic support, the few available information related with the study of migration

into foodstuffs reveals that scientific community approaches the problem in two different ways.

There are approaches that try to apply the most undemanding technique. This, commonly coincides with the one used with simulants, usually fat simulants. These methodologies include:

Extraction with solvents

-Discontinuous. This procedure is one of the most widely applied when migration on food is evaluated, especially non fatty food, although has also been applied to fatty foods. ATBC from not fatty foods (such as jelly and candy) was extracted by Lau and Wong (1996) with cyclohexane-dichloromethane by shaking the sample during 2 h and finally dried with sodium sulphate. Summerfield and Cooper (2001) determined DIPN in fatty food (pastry and cake) by extraction with acetonitrile. Nevertheless dry food was extracted by the same authors with absolute ethanol in an ultrasonics bath. Yankah *et al.* (1998) also determined BHT on smoke flavoured sausage by extraction with acetonitrile. Good recoveries were achieved. Munguia-Lopez and Soto-Valdez (2001) extracted bisphenol A from the liquid portion of tuna and jalapeno peppers also with acetonitrile. Jordan *et al.* (2002) utilized methylene chloride to extract limonene from kiwi fruit puree and commercial kiwi essence. Sanches-Silva (in press) extracted triclosan from orange juice, chicken meat and cheese using hexane, this organic phase was evaporated in a rotavapor and then the residue extracted with acetonitrile.

Nevertheless a detail that must be had in account when food is the study matrix, it is the importance of the homogenisation step as well as phases separation and filtration steps. These are critical in chromatographic analysis and establish the column and equipment life as well as the suitability for routinely use of the method.

-Continuous: To extract styrene from cooked pork meat Gramshaw and Vandenburg (1995) used a Likens-Nickerson extractor with pentane for 1 h. The pentane extracts were then evaporated using a Kuderna-Danish evaporator prior the analysis. Otherwise, Van Lierop and Van Veen (1988) determined ATBC migration in fatty food extracting samples with diethyl ether in a Soxhlet apparatus for 6-7 h. Soxhlet extraction was also used by Badeka *et al.* (1999) to extract this compound from cooked meat of different fat contents but followed by a saponification with KOH, which decomposes the ATBC in citric acid and 1-butanol, and this last one is quantified by GC.

In the other hand there are papers which describe newer extraction methodologies to overcome this difficulty. Although being, in general, time consuming and laborious approaches. Some of these alternatives approaches are:

Solid phase extraction (SPE): The use of a column which allows the separation of the substances of interest it is helpful when working samples have a complex composition.

Mountfort *et al.* (1997) analysed an infant feed regarding for bisphenol A content. After mixing some ammonia drops and ethanol to the sample, hexane was added. The lower layer was passed through an SPE cartridge and Bisphenol A was eluted with water and methanol. Yoshida *et al.* (2001) homogenised the solid portion in cans of vegetables and fruits with acetonitrile and sodium sulphate (avoids the formation of an emulsion) followed by an extraction with hexane. The acetonitrile layer was evaporated to dryness and dissolved with acetone-heptane (2.5:97.5). This solution was applied to a Sep-Pak Florisil cartridge and bisphenol A eluted with acetone/heptane (20:80). Good recoveries and relative standard deviation were achieved. Otherwise, the aqueous portions of those food samples were applied to an OASIS HLD extraction cartridge. Bisphenol A was eluted with methanol. The eluate was evaporated to dryness and applied to a Sep-Pak Florisil cartridge. Kang and Kondo (2002) also determined bisphenol A but in a coffee sample. After treatment, sample was passed through an ISOLUTE multimode cartridge. Bisphenol A was eluted with acetonitrile/ water (40:60, v/v). Limonene was determined in water (Linssen, *et al.* 1993) and cheese (Valero *et al.* 2001) purging the sample with purified nitrogen using a Tenax TA to trap the substance of interest. For the determination of ATBC in fatty food Van Lierop and Van Veen (1988) extracted a mixture of the fatty food and dry sodium sulphate with diethyl ether in a Soxhlet apparatus for 6-7 h. After evaporation of ether, the solution was passed through a Tenax absorption column, heated at 210ºC in an oil-bath.

Size Exclusion Chromatography (SEC): Castle *et al.* (1988) determined ATBC (acetyl tributyl citrate) in food (cheese, fruit, vegetables, soups, cakes, puddings and meal dishes) using this procedure. Prior to SEC clean-up, homogenised food was bended with acetone/hexane (1:1). Extraction was repeated and then, once the extracts were combined, were evaporated to dryness and the residue re-dissolved in dichloromethane-cyclohexane (1:1). The column used was a Biobeads S-X3, the mobile phase was dichloromethane- cyclohexane (1:1) flowing at 3.0 ml/min.

Gel Permeation Chromatography (GPC): ATBC content on fatty food (bacon and cheese) was determined by Lau and Wong (1996) applying this method. First, sample was homogenized with cyclohexane-dicloromethane (1:1) for 2 h in an automatic shaker. The extract (5 ml) was dried with anhydrous sodium sulphate, evaporated to dryness and re-dissolved with the same solution. Then, a gel-permeation chromatography was made to clean-up. First 90 ml of the eluent were discarded and the following 40 ml collected and evaporated to 2 ml by heating.

Headspace (static and dynamic): when the migrant is a volatile substance this is an effective analytical procedure. This technique has been applied to the determination of styrene in coffee and tea by Varner and Breder (1981), in sliced potatoes with grated cheese and minced beef fried with tomato sauce by Jickells *et al.* (1993). Otherwise Gramshaw and Vandenburg (1995) could not analyze this compound in meat by dynamic headspace due to the water present in the meat condensing in the trap. The dynamic procedure was applied for the determination of 1-octene from pure beef fat (Umano and Shibamoto 1987) and menhaden fish oil (Horiuchi *et al.* 1998) and for the determination of limonene from cheese (Valero *et al.* 2001; Peres, *et al.* 2002).

Solid phase microextraction (SPME): This technique is very useful when the substance is volatile. Silva *et al.* (2000) determined styrene in drinking water using a fibre with 85 µm polyacrylate coating; Andres *et al.* (2002) determined 1-octene in the ham by this technique using a 75 µm carboxen-poly(dimethylsiloxane) coating fibre. Peres *et al.* (2001) determined limonene using also this procedure attempting 4 different fibres: 100 µm dimethylsiloxane (DVB); 85 µm polyacrylate (PA); 65 µm PDMS/divinylbenzene (PDMS/DVB); 75 µm Carboxen/DVB.

VII.4.- REVIEW OF CHROMATOGRAPHIC METHODS FOR MIGRANTS DETERMINATION

A large variety of chromatographic methods were found in the literature. The following revision was sorted by chromatographic technique and by detector.

VII.4.1. High Performance Liquid Chromatography (Reversed Phase)

VII.4.1.1. Fluorescence Detector

Bisphenol A was analysed employing a C18 column with acetonitrile/water as mobile phase. Fluorescence conditions of λex 275 nm and λem 300 nm were employed (Simal Gandara *et al.* 1993; Simal-Gandara *et al.* 2000b; Kang and Kondo 2002). Other authors used similar chromatographic conditions but changing the fluoresce wavelengths, λex 225 nm and λex 310 nm (Munguia-Lopez and Soto-Valdez 2001) or λex 225 nm and λex 305 nm (Lopez-Cervantes and Paserio-Losada 2003). Different detection conditions (λex: 235 nm and λem: 317 nm) were used for the analysis of this compound by Howe and Borodinsky (1998) and heating the chromatographic column. Similar wavelengths were used by Biles *et al.* (1997) but with a mobile phase of methanol/water/acetonitrile. To carry out the analysis, Mountfort *et al.* (1997) employed a C8 column with methanol/water as mobile phase with similar fluorescence conditions (λex: 285 nm and λem: 300 nm).

Summerfierld and Cooper (2001) analyzed DIPN using a also an ODS column with a mobile phase of water/acetonitrile and λex: 232 nm and λem: 338 nm.

Yankah *et al.* (1998) determined BHT extracted from lipids and smoked-flavoured sausages using a RP-18 column and mixture of water/acetic acid/acetonitrile as mobile phase working with an λex: 280 nm and λem: 310 nm followed by a GC/MS confirmation.

VII.4.1.2. UV Detector

Tawfik and Huyghebaert (1998) determined styrene using a C18 column and employing a mobile phase of acetonitrile/water and setting the detector to 245 nm. Bisphenol A was analysed utilizing a C18 column with acetonitrile/water as mobile phase, setting the detector to 228 nm (Yoshida *et al.* 2001) or to 225 nm (Lopez-Cervantes and Paseiro-Losada 2003). Similar column was used by Howe and Borodinsky (1998) but with a different mobile phase (0.1% acetic acid in water /acetonitrile). This analysis was carried out heating the column to 60-65 ºC and setting the UV detector to 280 nm.

Stoffers *et al.* (2003) determined laurolactam utilizing an ODS column and using water/methanol as mobile phase and fixing the wavelength in 207 nm.

The IUPAC method for BHT uses a Silica RP18 column, a mobile phase of water/acetic acid/acetonitrile and a wavelength of 280 nm. An alternative method comprises just a change in the mobile phase composition: methanol and potassium dihydrogen phosphate buffer 0.02 M (Dieffenbacher 1998). Lee *et al.* (2004) used methanol and water as mobile phase setting the detector to 280 nm to analyze this compound extracted from HDPE with this antioxidant incorporated.

Sanches-Silva *et al.* (2005) analyzed triclosan was using a C18 column with a gradient of acetonitrile water, using three different wavelengths (205, 235 and 280 nm).

VII.4.1.3. MS Detector

Lopez-Cervantes and Paseiro-Losada (2003) confirmed the identity of Bisphenol A using this detector with APCI (Atmospheric Pressure Chemical Ionisation) in negative mode with identical chromatographic conditions as for the quantification by UV. To determine Laurolactam (dimer and trimer) Stoffers *et al.* (2003) also used APCI. The column was an ODS and water/methanol was the mobile phase. Moreover this detector (in negative mode) is one of the selected by Sanches-Silva *et al.* (in press) to confirm triclosan identity extracted from food samples.

VII.4.2.High Performance Liquid Chromatography (Normal Phase)

VII.4.2.1. Fluorescence Detector

Mountfort *et al.* (1997) utilized for the analysis of Bisphenol A, a cyano phase column with hexane/2-propanol/dichloromethane as mobile phase and with the same fluorescence conditions as for the reversed phase HPLC.

VII.4.3-Gas Chromatography

VII.4.3.1. GC-FID

This technique is used by Gramshaw and Vandenburg (1995) to determine styrene, with a BP-1 column setting the oven to 200ºC, and injecting in a cold on-column mode. Silva *et al.* (2000) determined styrene using an HP-5 column and employing lower Tª (from 50ºC to 150ºC), after SPME extraction.

In the analysis of 1-octene from a liquid paraffin matrix carried out by Simal-Gandara *et al.* (2000a), a PLOT fused silica column with a film of PoraPLOT Q was used, injecting the sample in unsplit headspace mode, setting the oven from 70ºC to 200ºC.

Triantafyllou *et al.* (2002) analyzed limonene in recycled PET using a DB-1 column with an oven temperature from 50ºC to 280ºC.

Boccacci *et al.* (1999) determined DIPN extracted from food samples using an OVI column and with hydrogen as carrier gas (oven T from 40 to 200ºC) followed by confirmation by GC-MS. Triantafyllou *et al.* (2002) used a DB-1 column and the same carrier gas (oven T from 60 to 270ºC) followed also by confirmation by GC-MS.

Stoffers *et al.* (2003) analyzed Laurolactam using a DB-624 column and hydrogen as carrier gas (oven T max.: 240ºC) after be separated by SEC. Hirayama *et al.* (2001) determined ATBC extracted from sealing resins using an HP-5 column, injecting the sample in split mode and heating the oven from 150ºC to 250ºC. When this compound was extracted by Cano *et al.* (2002) from different samples by MAE (microwave-assisted extraction) and by SFE, it was determined using an analogous column but setting the oven T from 110 to 260ºC.

For the analysis of Limonene, Linssen *et al.* (1993) used a thermal desorption/cold trap device for transferring the volatile compounds from Tenax to the column (DB-1). The oven program achieved to 250ºC. Furthermore, this compound was analyzed by Van Willige *et al.* (2000) using the large volume injection (LVI), injecting 30 µl of sample; the desolvation column was a MEGA and the analytical column was a DB-1701.

For the separation of 1-octene Umano and Shibamoto (1987) used a DB-WAX column with an oven program from 40 to 200ºC.

VII.4.3.2. GC-MS

Gramshaw and Vandenburg (1995) investigated the presence of sytrene in cooked pork meat using a BP-1 column holding the oven to 50ºC. When the sample is polystyrene, this technique is also used in the EU project Specific Migration, but employing a HP-5MS column and heating the oven from 40ºC to 180ºC, and with helium as carrier gas. If the sample is Tenax, the same project used different oven temperatures (from 60ºC to 100ºC) and a different column (DB17) (Specific Migration).

For the identification of Bisphenol A this technique was chosen by Biles *et al.* (1997), using an Rtx-5 capillary column and setting the oven temperature from 100ºC to 280ºC. Analogous conditions were used by other authors (Munguia-Lopez and Soto-Valdez 2001; Lopez-Cervantes and Paseiro-Losada 2003).

In the determination of 1-octene after a SPME extraction, Andres *et al.* (2002) used a HP-5 column for the separation with a temperature gradient from 40 to 200ºC. Instead of a HP-5, a DB-WAX column was used for the separation with a similar oven programme (Umano and Shibamoto 1987; Horiuchi *et al.* 1998).

A ZB-5MS column was employed to determine limonene with helium as carrier gas. The analysis was carried out with an oven program from 40 to 250ºC (Bentivenga *et al.* 2001, Avison *et al.* 2001, Peres *et al.* 2001, Peres *et al.* 2002, Triantafyllou *et al.* 2002). Analogous oven program was also used by other authors but carrying out the separation in a different column, a cross linked phenyl-methyl siloxane column (Jordan *et al.* 2002), a homemade column (FFAP/OV-1) (Valero *et al.* 2001), and a methyl silicone or phenyl/methyl silicone column (Nerín *et al.* 2003).

DIPN extracted from paper and board was analysed Summerfierld *et al.* (2001) using also a HP5-MS column and employing a ramp temperature from 40 to 300ºC. Boccacci *et al.* (1999) investigated its presence in foodstuffs using a SPB-608 column with helium as carrier gas (oven T from 50 to 240ºC). Triantafyllou *et al.* (2002) also confirmed its presence using a HP-5MS column (oven T from 60 to 270ºC).

Castle *et al.* (1988), after clean up samples by SEC, determined ATBC using a BP-5 column, by on column injection and with helium as carrier gas (oven T from 70 to 210ºC). Other authors also used similar column and an oven programme that heated from 60 to 300ºC (Van Lierop and Van Veen 1988) or from 100ºC to 300ºC (Lau and Wong 1996). Otherwise Hirayama *et al.* (2001) used a DB-1701 column and a ramp temperature from 50ºC to 280ºC to confirm the identity of this substance.

Wessling *et al.* (1998) separated BHT using a 50% phenyl-505 methyl polysiloxane column and helium as carrier gas employing a ramp temperature from 100ºC to 290ºC. Yankah *et al.* (1998) used a Quadrex 65 HT column to confirm its identity.

After a SPME extraction, Tombesi and Freije (2002) determined BHT using a HP-5 column, with helium as carrier gas and with a ramp temperature from 40 to 250ºC.

Sanches-Silva *et al.* (2005) confirmed triclosan identity using a DB-5 column and a temperature gradient from 160ºC to 260ºC.

VII.5.- EXPERIMENTAL PROCEDURE

Due to the lack of some important properties of the substances selected, those which were considered more important were evaluated in our laboratory.

VII.5.1. Solvents and model migrants

Acetonitrile, ethanol, and hexane were HPLC grade; all supplied by Merck (Darmstadt, Germany) and ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). Styrene, laurolactam, limonene, 1-octene, Bisphenol A and BHT were from Sigma-Aldrich (Steinheim, Germany). triacetin, ATBC and Triclosan were supplied from Fluka Chemie AG (Buchts, Switzerland).

Stock solutions of all migrants above cited were prepared in ethanol at the level of 1000 mg l^{-1}. All these solutions were stored in the darkness at 4ºC. Of all of them, intermediate dissolutions were prepared at the levels of 10, 1 and 0.1 mg l^{-1} in ethanol and in acetonitrile.

VII.5.2. Apparatus

VII.5.2.1. Luminescence Spectrometer

All spectrofluorimetric measurements were performed with a Perkin Elmer LS 50 luminescence spectrometer fitted with a xenon flash lamp, Monk Gillieson monochromators and 1 cm quartz cuvettes. Spectral data acquisition and processing were carried out by means of the program FL Winlab on a PC serially interfaced to the LS 50.

In a first measure, a full scan range pre-scan was achieved for all migrants. All scans were recorded between excitation wavelengths 200 and 800 nm and emission wavelengths 200 and 900 nm. The scan speed was 240 nm min^{-1}.

Subsequently maximum values for excitation wavelengths were selected for each migrant (of those that showed fluorescence) and emission scans were completed. In the same way maximum values for emission wavelengths were selected and excitation scans were completed.

For styrene and bisphenol A, solutions of 0.1 mg l^{-1} in ethanol were used.

In the table VII.3, excitation and emission wavelengths maximum detected are showed.

VII.5.2.2. UV-Visible Spectrophotometer

A Cary 3E UV-Visible double-beam spectrophotometer was used to perform single scans of all solutions from 200 to 400 nm. Software Cary WinUV was used for the acquisition of the data. For all compounds solutions of 10 mg l^{-1} in ethanol were used.

Table VII.3 shows the maximum wavelengths for each migrant and its molar absorptivity (ε).

VII.5.2.3. HPLC-MS

APCI (Atmospheric Pressure Chemical Ionization) negative and positive spectra were recorded on a VG Platform (Fisons Instruments) single-quadrupole spectrometer, which has coupled via its APCI interface to a Spectra Physics model P200 HPLC gradient pump. The Masslynx ^{TM} data system was used to control both the APCI LC-MS system, from tuning through data acquisition (in scanning and selected-ion recording (SIR) mode).

Total ion chromatograms (*m/z* 70-700) and selected-ion chromatograms of the analytes were obtained by direct flow injection analysis using the solutions of 1000 mg l^{-1}. The mobile phase was 70:30 acetonitrile:water (flow rate: 1 ml min^{-1}). 50 μl loop injections were monitored using real time chromatogram updates. Full scan spectra (*m/z* 70-700) were acquired every second, with a scan delay of 0.10 sec. In SIR mode, the dwell time of each ion was set at 0.10 sec. The solutions used were of 10 mg l^{-1}.

The following instrument parameters were applied: probe temperature: 450ºC and 250ºC, ionisation source temperature: 130ºC, cone voltage: $\pm$30 V, electron

multiplier voltage: 700 V, sheath gas nitrogen at 200 l h^{-1} and drying gas nitrogen at 250 l h^{-1}.

The table VII.3 shows the majority fragments (in order of abundance) obtained in APCI+ and APCI-.

VII.5.2.4. GC-FID

Initial experiments were performed with a Fisons 8000 series gas chromatograph equipped with a flame ion detector (FID; 260ºC), an auto sampler (AS 800) and a split-splitless injector. The capillary column used was a 30 m × 0.32 mm i.d. × 0.25 μm film thickness Supelcowax 10 from Supelco. To improve the column life a 50 cm pre-column (deactivated fused silica 0.32 mm i.d.) was used.

Helium was used as carrier gas flowing at 1 ml min^{-1}. The injector temperature used was 280ºC and the split ratio selected was 1:30.

In a first test the column temperature was held at 40ºC for 2 min during injection, and then increased at 7ºC min^{-1} to 260ºC, which was held for 10 min. In a second test the column temperature was held at 150ºC for 2 min, and then increased at 5ºC min^{-1} to 260ºC, which was held for 20 min.

Solutions of 1000 mg l^{-1} were injected using these conditions. The table VII.3 shows which compounds displayed response.

VII.5.2.5. HPLC-UV-Fluorescence

Chromatographic measurements were performed with a Hewlett Packard (Waldbronn, Germany) system comprised of a HP1100 liquid chromatograph fitted with a quaternary pump, an autosampler, a column oven, a diode array detector, a fluorescence scanning detector and HP Chemstation data analysis software (version A.06.01). A Kromasil 100 C18 column (15 × 0.4 cm i.d., 5μm particle size) from Teknokroma (Barcelona, Spain) was used for the separation.

The chromatographic conditions employed were: Column: A Kromasil 100 C18 column (5μm i.d., 150mm x 40mm). The mobile phase consisted of acetonitrile/water 75:35 (v/v) in an isocratic mode for two minutes, followed by a gradient to 100% acetonitrile for 8 min, and finally an isocratic elution during 20 min.

The flow rate was 1 ml min^{-1}. The injection volume was 50 µl. The column oven temperature was kept at room temperature. UV detection was performed with 205, 225 and 255 nm, and fluorescence detection was performed with excitation and emission wavelengths of 250 nm and 306 nm, respectively.

Table VII.3: Results of preliminary experiments.

MIGRANT	Spectroscopic Properties				GC-FID analysis	Acetonitrile / Olive oil
	Fluorescence	Ultraviolet	MS-APCI$^+$	MS-APCI$^-$		
Styrene	λex: 250 λem: 305	λmax (ε):208 (14000), 247 (15000)			Yes	0.53
Bisphenol A	λex: 280 λem: 307	λmax (ε): 207 (35000), 227 (33900), 278 (9000)		227		
1-Octene		λmax (ε): 205 (9500), 222 (4200), 274 (1100)			Yes	
Limonene		λmax (ε): 204 (9700)			Yes	
DIPN						
Laurolactam		λmax (ε): 203 (7700), 222 (2900)	198		Yes	
Triacetin			159, 99, 275, 117		Yes	
ATBC			259, 185, 213, 157, 273, 329, 361, 425, 403, 217	341, 211, 111, 139, 267		
BHT		λmax (ε): 204 (25200), 217 (10000), 276 (3300)		219	Yes	0.33
Triclosan		λmax (ε): 206 (43900), 229 (16500), 281 (7000)		289, 253		0.47

VII.5.3. Sample preparation

Assays with spiked olive oil were carried out in order to estimate the partition coefficient of model migrants between olive oil and acetonitrile.

Standard solutions of individual migrants were prepared in acetonitrile. Then to 10 g of olive oil 10 ml of each standard solution were added and these mixtures were shaken vigorously for three minutes in a centrifuge tube of 40 ml. After approximately one hour, when the two phases were separated, 0.1 ml of acetonitrile phase was injected. The areas obtained were compared with the areas obtained for the individual standards.

Partition coefficients were calculated for all signals acquired and are showed in the table VII.3.

VII.6.- CONCLUDING REMARKS

The literature on this subject is, most of the times, scant, and has placed more emphasis on results concerning, migration in simulants and amount in polymers than on food analysis. Moreover, analytical methods seem to be still in the research and development stage, and no particular set of methods has attracted widespread agreement. No two studies on the use of compounds appear to have used the same procedure to determine a given compound in a given type of sample.

Some of the protocols that have been used are not described in the literature in sufficient detail to allow confident replication by other laboratories. This is a common situation in the early stage of the development of the analytical methodology.

In general it is not at present possible to draw up reliable analytical protocols for the determination of all the selected compounds in food. For most migrant/food combinations, any protocol drawn up on the basis of the literature and general analytical principles and experience is necessarily a tentative proposal requiring exhaustive evaluation. However, in view of the bibliographic data available and the preliminary experiments carried out some guidelines could be drawn.

Bisphenol A, BHT and Triclosan can be extracted from foods with polar solvents immiscible with fat, although BHT with some difficulty. All the migrants exhibit good properties in the ultraviolet region and furthermore Bisphenol A shows very good response in fluorescence. Therefore HPLC with UV-FI detection seems an appropriate

determination technique. HPLC coupled with APCI-MS detector can be also used. GC-(FID or MS) is also a common possible technique for most of them.

Triacetin is a compound soluble in water and not very soluble in oils. It can be extracted from foods with polar solvents immiscible with fat. It can be determined by GC-(FID or MS) or by HPLC only with an APCI-MS detector because it does not show interesting properties in fluorescence or ultraviolet region.

Laurolactam and ATBC have low solubility in water and they present similar difficulties to the first group. They do not show interesting properties in UV or fluorescence and they need to be determined by GC-(FID or MS) or by HPLC with only an APCI-MS detector.

Styrene, 1-octene and limonene can be separated from food matrix by volatilization or distillation and it seems the better way to remove them from other food components. Analysis by static or dynamic headspace-GC (FID or MS) is an excellent and common determination for the three compounds. Other ways also are possible as extraction from non fatty foods with weakly polar solvents immiscible with water, or using polar solvents immiscible with fat to extract from fatty foods. Styrene shows a very good response by fluorescence and ultraviolet and can be determined by HPLC using those detectors.

Table VII.4 summarized proposals found more suitable for the separation and quantification of these substances in foodstuffs.

For all migrants and depending of each combination analyte/food matrix, more purification steps could be necessary, usually using SPE or liquid-liquid extraction, again.

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Table VII.4: Suggestions to prepare analytical methods to determine migrants in foods.

MIGRANT	Food	Sample Preparation	Analysis procedure
Styrene	Non fatty	• Separation by volatilization. • Extraction or dilution with polar solvents.	• GC-FID or MS. Column type apolar, with medium to low phase ratio (β), static or dynamic headspace. • HPLC-UV or FI. Column type C18. Mobile phase: acetonitrile, Detection at. λ=247 nm by UV, λex=250 nm, λem = 305 nm by fluorescence.
	Fatty	• Separation by volatilization. • Extraction with polar solvents not miscible with fat.	
Bisphenol A	Non fatty	• Extraction or dilution with polar solvents.	• HPLC-UV or FI or APCI (-). Column type C18. Mobile phase: acetonitrile/water, Detection at. λ = 227 nm by UV, λex=280 nm or 225 nm, λem= 307 nm by fluorescence and 227 (*m/z*) by MS.
	Fatty	• Extraction with polar solvents not miscible with fat.	
1-octene	Non fatty	• Separation by volatilization.	• GC-FID or MS. Column type apolar, with medium to low phase ratio (β), static or dynamic headspace.
	Fatty	• Separation by volatilization.	
Limonene	Non fatty	• Separation by volatilization. • Extraction or dilution with polar solvents.	• GC-FID or MS. Column type polar, with medium to low phase ratio (β), static or dynamic headspace.
	Fatty	• Separation by volatilization. • Extraction with polar solvents not miscible with fat.	
DIPN	Non fatty	• Extraction or dilution with polar solvents.	• GC-FID or MS. Column type apolar, with medium to high phase ratio (β), on column or splitless injection mode. • HPLC-UV or FI or APCI (+) or (-). Column type C18. Mobile phase: acetonitrile/water.
	Fatty	• Extraction with polar solvents not miscible with fat.	
Laurolactam	Non fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used. • Extraction/dilution with polar solvents.	• GC-FID or MS. Column type apolar, with medium to low phase ratio (β), split injection mode. • HPLC-APCI (+). Column type C18. Mobile phase: acetonitrile/water. Detection at 198 (*m/z*).
	Fatty	• Extraction with weakly non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used. • Extraction with polar solvents not miscible with fat.	
Triacetin	Non fatty	• Extraction with polar to non polar solvents.	• GC-FID or MS. Column type apolar, with medium phase ratio (β), split injection mode. • HPLC-APCI. Column type C18. Mobile phase: acetonitrile/water. Detection at 159 (*m/z*).
	Fatty	• Extraction with polar solvents not miscible with fat.	
ATBC	Non fatty	• Extraction with weakly or non polar solvents and concentration step. Solvent change if HPLC is used. • Extraction/dilution with polar solvents.	• GC-FID or MS. Column type apolar, with medium to low phase ratio (β), split injection mode. • HPLC-APCI (+) or (-). Column type C18. Mobile phase: acetonitrile/water. Detection at 259 (*m/z*) by APCI(+) or 341 (*m/z*) APCI(-).
	Fatty	• Extraction with weakly or non polar solvents followed by SEC-GPC clean up and concentration step. Solvent change if HPLC is used. • Extraction with polar solvents not miscible with fat.	
BHT	Non fatty	• Extraction or dilution with polar or on polar solvents.	• GC-FID or MS. Column type apolar, with medium phase ratio (β), split or splitless injection mode. • HPLC-UV or APCI (-). Column type C18. Mobile phase: acetonitrile/water. Detection at λ=217 nm by UV and 219 (*m/z*) APCI(-).
	Fatty	• Extraction with polar solvents not miscible with fat.	
Triclosan	Non fatty	• Extraction or dilution with polar or non polar solvents.	• HPLC-UV or APCI (-). Column type C18. Mobile phase: acetonitrile/water, Detection at λ=229 nm by UV and 289 (*m/z*) APCI(-).
	Fatty	• Extraction with polar solvents not miscible with fat.	

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CAPÍTULO VIII: DETERMINATION OF DIPHENYLBUTADIENE BY LIQUID CHROMATOGRAPHY-UV-FLUORESCENCE IN FOODSTUFFS

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ABSTRACT

Diphenylbutadiene (DPBD) is an optical brightener incorporated into a wide range of polymeric materials. Framed in the FOODMIGROSURE project, it was chosen as a model migrant to study the migration kinetics from polymeric materials in relevant foodstuffs.

An analytical method was developed and optimized for the DPBD determination in foods. The sample preparation procedure uses both hexane and acetonitrile as extraction solvents, followed by HPLC analysis.

HPLC analysis was performed using UV detection at a wavelength of 330 nm, and fluorescence detection achieved with excitation and emission wavelengths of 330 nm and 375 nm, respectively. Good linearity and recovery were achieved. Data are reported.

KEYWORDS

Diphenylbutadiene (DPBD), food packaging materials, high performance liquid chromatography.

VIII.1.- INTRODUCTION

In the last years, food packaging has gained a widespread importance in the food industry. An essential investigation field within food packaging it is focused into the possibility of migration of chemicals from these materials. Food packaging materials must be safe in the sense of not releasing potentially harmful material into the food [1].

In the area of polymer chemistry, additives are often incorporated into polymer systems to improve their physical properties and to enhance their end-use performance. Plastic generally ages rapidly under the effects of light, oxygen and heat, leading to loss of strength, discoloration, scratching, etc. Typical polymer additives include antioxidants, antistatic agents, blowing agents, catalysts, colorants, fillers and reinforcements, flame retardants, impact modifiers, lubricants and slip agents, plasticizers and stabilizers, fluorescent white agents, and a large variety of chemicals with different technical functions.

A fluorescent white agent is a substance that is added to a material to absorb ultraviolet rays in sunlight and release them as blue rays. These blue rays will then interact with the yellowish color and give the plastic the appearance of being whiter. Diphenylbutadiene (DPBD) is an example of these fluorescent white agents.

DPBD (trans, trans-1,4-diphenyl-1,3-butadiene), CAS Nº. 538-81-8, is a light yellow crystalline powder, soluble in many organic solvents, with high lipophilicity (Calculated Log Kow (octanol-water) = 5.29) [2] and with a molecular weight of 206.3.

This compound was selected as a model migrant framed in the FOODMIGROSURE project, to study the migration kinetics from polymeric materials in relevant foodstuffs. Its choice was based on the representativeness for food contact materials migrants, so the results will be obtained can be extrapolated to other substances of similar characteristics, and based on its relevance (use frequency in food contact materials) and its analytical behavior.

The type of contact between the packaging and the food (i.e. aqueous, acidic, alcoholic or fatty) determines along with other factors the nature and the extent of the migration that occurs [3].

In order to determine the migration level into foodstuffs coming into contact with plastics, an analytical method was developed for the determination of DPBD in

three selected foods that represent three types of foodstuffs with different physical and chemical properties: aqueous and acidic food (orange juice), non fatty food (chicken breast), and fatty food (gouda cheese).

VIII.2.- EXPERIMENTAL

VIII.2.1. Samples

Three representative food items were chosen: orange juice (liquid, acid and medium carbohydrates content), chicken breast (solid, high protein content) and Gouda cheese (solid, high fat content). The samples were purchased in local supermarkets.

VIII.2.2. Chemicals and reagents

Acetonitrile, ethanol, and hexane were HPLC grade; all supplied by Merck (Darmstadt, Germany) and ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). Trans, trans-1,4-diphenyl-1,3-butadiene (DPBD, CAS No [538-81-8] average Mr 206.29) was from Sigma-Aldrich (Steinheim, Germany).

A stock standard solution was prepared by dissolving 100 mg DPBD in 100 ml of ethanol and was kept at 4ºC in the refrigerator. Intermediate standard solutions of DPBD were prepared by dissolution of appropriate amounts of stock standard solution in acetonitrile. These solutions were stored in amber bottles at 4ºC in the refrigerator.

VIII.2.3. Extraction of samples

About 10 g of each food sample were accurately weighed in a 40 ml Pyrex tube with a PTFE-lined screw cap. Then, 10 ml of hexane were added and the tubes were shaken manually for 10 min. To separate the organic phase the tubes were centrifuged at 1036 *g* for 10 min. The extraction was repeated with 10 ml of hexane twice. All hexane phases were colleted in a round flask and evaporated to dryness using a rotatory evaporator.

For orange juice and chicken breast, the solid residue was redissolved in 10 ml acetonitrile and the solution obtained was homogenized by ultrasonics. The solution was filtered through a PTFE 0.45 µm, 13 mm syringe filter, and an aliquot was transfer into a HPLC vial and then injected. Regarding Gouda cheese extraction, the fatty liquid residue obtained was extracted twice with 10 ml of acetonitrile by vortex shaking for 5 min. The acetonitrile phases were evaporated to 10 ml. The solution was filtered and then injected.

VIII.2.4. Equipment

VIII.2.4.1. UV-Visible Spectrophotometer

A Cary 3E UV-Visible double-beam spectrophotometer (Varian, Australia) was used to perform single scans from 200 to 400 nm. Software Cary WinUV (version 3.0) was used for the acquisition of the data. A solution of 1 mg/l was used to establish the most sensitive wavelength.

VIII.2.4.2. Luminescence Spectrometer

Preliminary spectrofluorimetric measurements were performed with a Perkin Elmer LS 50 luminescence spectrometer (Buckinghamsiher, England) fitted with a xenon flash lamp, Monk Gillieson monochromators and 1 cm quartz cuvettes. Spectral data acquisition and processing were carried out by means of the program FL Winlab on a PC serially interfaced to the LS 50. In a first measure, a full scan range pre-scan was achieved. All scans were recorded between excitation wavelengths 200 and 800 nm and emission wavelengths 200 and 900 nm. The scan speed was 240 nm/min. Subsequently the maximum value for excitation wavelength was selected and emission scan was completed. In the same way, maximum value for emission wavelength was selected and excitation scan was completed. These measurements were carried out with solutions of 0.1 mg/l.

VIII.2.4.3. High performance liquid chromatography

Chromatographic measurements were performed with a Hewlett Packard (Waldbronn, Germany) system comprised of a HP1100 liquid chromatograph fitted with a quaternary pump, an autosampler, a column oven, a diode array detector, a fluorescence scanning detector and HP Chemstation data analysis software (version A.06.01). A Kromasil 100 C18 column (15 $\times$ 0.4 cm I.D., 5µm particle size) from Teknokroma (Barcelona, Spain) was used for the separation.

VIII.2.4.4. Gas chromatography

Identification of selected compounds was carried out using a gas chromatograph Fisons 8000 series (Manchester, UK) coupled to a mass spectrometer (MD 800) operating in the electron impact ionization mode. The gas chromatograph was equipped with a split/splitless injector. A capillary column 30 m × 0.25 I.D., 1 μm film thickness with DB-5 MS as stationary phase from J&W Scientific (CA, USA) was used. Masslab software (version 1.4) was used for data acquisition.

VIII.2.4.5. Liquid Chromatography-mass spectrometry

An HPLC-MS system comprised a Spectra Physics series P2000 chromatograph equipped with an autosampler and a mass detector Navigator II (all from TermoQuest, Finnigan, Manchester, UK) was used to try to confirm the identity of DPBD The column and mobile phase were as in 2.5. The detector conditions were as follows: negative and positive atmospheric pressure chemical ionisation (APCI) mode; probe temperature: 250ºC, cone voltage: +/– 15 V, electron multiplier voltage 650 V, full scan mode scanning a mass spectrum range of 100-400 amu (two scans for second). The system was computer-controlled using the Xcalibur (version 1.2) software.

VIII.2.4.6. Other equipment

An Ultra-turrax homogenizer (T25 basic, IKA® Labortechnik, Stanfen, Germany) was used to homogenize cheese.

A rotatory evaporator (RE200, Bibby Sterilin, Staffordshire, UK), a centrifuge (Eba 12, Hettich, Krichlenger, Germany) and an ultrasonic bath (5510 Bransonic, Danbury, CT, USA) were also used in the sample preparation procedure.

VIII.2.5. Chromatographic conditions

For the HPLC system the following chromatographic conditions were applied: the mobile phase was acetonitrile-water 65:35 (v/v) in an isocratic mode for two min, followed by a gradient to 100% acetonitrile until seventeen min, and finally an isocratic elution during thirteen min. The flow rate was 1.0 ml/min. The injection volume was 50 µl. The column oven temperature was kept at 30ºC. UV detection was performed at a wavelength of 330 nm. Fluorescence detection was performed with excitation and emission wavelengths of 330 nm and 375 nm, respectively.

In case of GC, the chromatographic conditions were as follows: column temperature program was initiated at 160ºC and held for 1 min, increased at 15ºC/min to reach 260ºC and then held at 260ºC for 54 min. Injections of 1 µL of samples were in split mode and the injector was heated to 260ºC. The carrier gas was helium at 1.0 ml/min. Mass spectra were recorded at 70 eV (EI+), in full scan mode between *m/z* 50 and 300 with a scan time of 0.45 s.

The positive confirmation of DPBD was carried out by comparing the obtained spectrum with those of the Willey Library.

VIII.3. RESULTS AND DISCUSSION

VIII.3.1. Preliminary studies

Important properties of DPBD such as ultraviolet and fluorescence were evaluated to help to decide the approach for the development of the chromatographic method that allowed determining this compound in foodstuffs. Thus, the maximum response obtained in the UV-vis spectrophotometer was at 330 nm, and in the luminescence spectrometer the maximum wavelengths of excitation and emission corresponds to 330 and 375 nm respectively. These conditions were used later to carry out the chromatographic analysis by HPLC (Figure VIII.1).

In order to find an analytical technique that allowed positive identification of this compound HPLC-APCI-MS was checked, both positive and negative mode, and evaluating different probe temperatures (200-400ºC) and cone voltages (10-40), but satisfactory results were not found. In the other hand, good results were achieved using GC-MS. The elution temperature of DPBD was 260ºC. Characteristic mass 206 corresponds to the ionisation form $[M^+]$.

VIII.3.2. Calibration line

The method was calibrated using series of standards in acetonitrile of known concentrations. The relationship between known concentrations and measured areas was assessed by linear regression (seven calibrations points), and

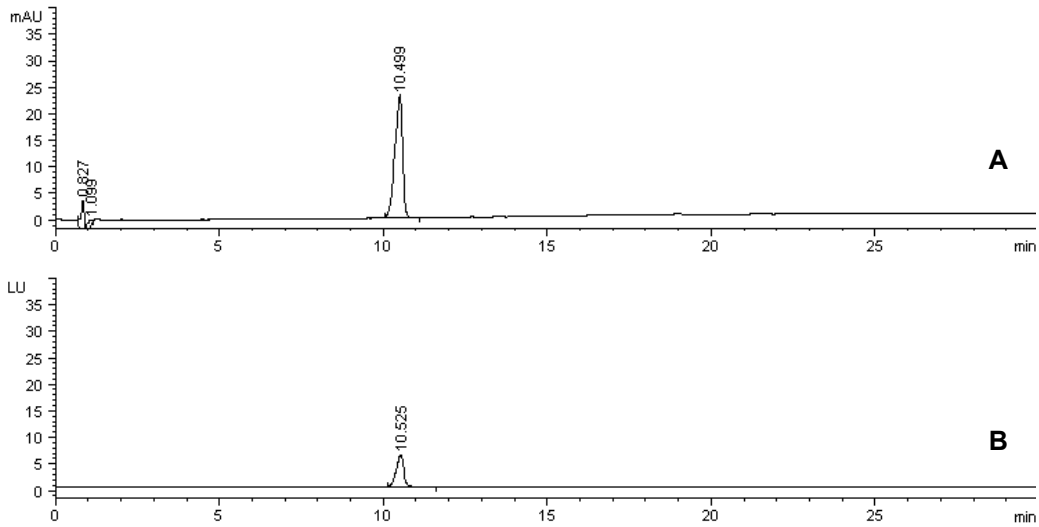

Figure VIII.1: HPLC chromatogram of a DPBD standard solution (1 μl/ml) **A:** Signal of UV detection at a wavelength of 330 nm and **B:** Fluorescence detection at excitation and emission wavelengths of 330 and 375 nm respectively.

the linearity obtained over the concentration range of 0.1-10 μg/ml for selected wavelengths, indicates that the method is appropriate for quantification of this compounds (Table VIII.1). Detection limits (DLs), were calculated in accordance with American Chemical Society [4] and are shown in Table VIII.1.

VIII.3.3. Sample extraction

Trying to cover the analytical complexity of the food matrix, three representative food items were chosen to determine DPBD: orange juice (aqueous and acidic liquid with medium carbohydrates content), chicken breast meat (solid with a high protein content) and Gouda cheese (solid with a high lipid content). These food items were used to complete all analysis.

Parameters of calibration curve	λ = 330 nm	λex = 330 nm λem = 370 nm
Slope	378.84	78.91
Intercept	-1.20	12.74
Correlation coefficient	0.9998	0.9983
Range [a]	0.1-10 µg/ml	0.1-10 µg/ml
Detection limit	25 ng/ml	12 ng/ml

[a]: Seven calibration points in the range indicated.

Table VIII.1: Method validation parameters.

In order to establish a suitable sample amount, distinct quantities were assayed; good results were obtained with 10 g of sample. Besides sample amount, the sample homogenisation is of great importance once can greatly influence the extraction efficiency. Orange juice was directly extracted and it has not raised any analytical difficulty (Figure VIII.2). Chicken was chopped up and due to its consistency, a slurry was obtained and considered suitable for an effective extraction procedure while cheese was homogenised with an ultra-turrax homogeniser.

To the chicken sample, 2 ml of acetonitrile were added directly and homogenised by hand shaking, prior to the first extraction with hexane due to this process originated a compact mass, which not desegregate, and foam, which interfered during phases dryness in the rotary evaporator.

In what concerns to the extraction of DPBD from Gouda cheese, its high fat content provided low recovery using extraction procedure applied to orange juice and chicken. Therefore, a double extraction with pure acetonitrile was investigated; this allowed obtaining acceptable recovery data.

The accuracy of the method for determining this compound in foodstuffs was calculated by performing the extraction procedures with spiked orange juice, chicken breast and Gouda cheese at three different levels (six replicates). Recoveries data were found higher than 83% (Table VIII.2). To orange juice the recovery found

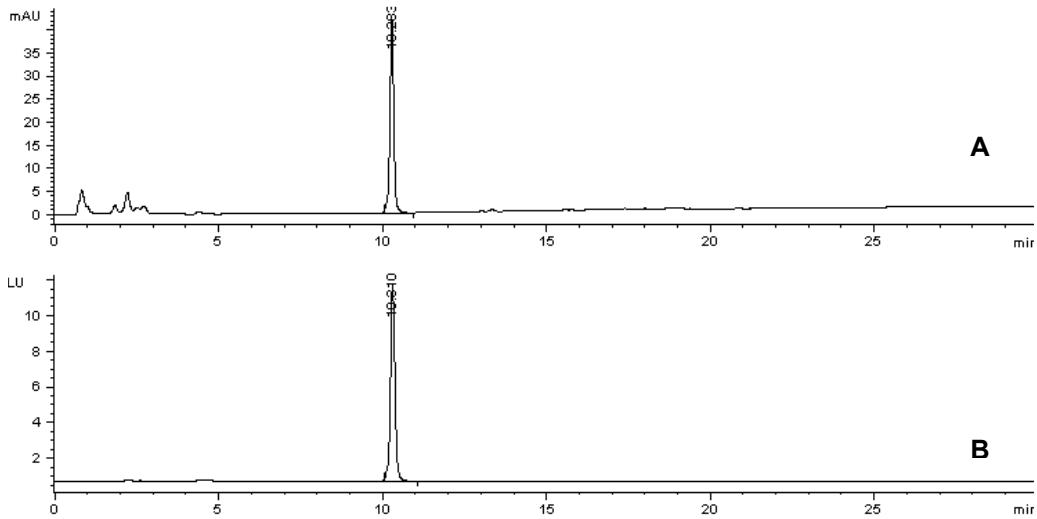

Figure VIII.2: HPLC chromatogram of an orange juice sample spiked with DPBD (1μl/ml) **A**: Signal of UV detection at a wavelength of 330 nm and **B**: Fluorescence detection at excitation and emission wavelengths of 330 and 375 nm respectively.

were higher than 85% and higher than 83% to chicken breast (Table VIII.2), in both cases for UV detection. The recoveries obtained with the fluorescence signal were different (Table VIII.3), which can be explained due to this signal is more affected by light. Regarding to explain this fact, a study on stability of DPBD was carried out.

Several trials were performed in order to study solutions stability. Solutions were stored in three light conditions: presence of sunlight, artificial light and darkness, all of them at room temperature. Solutions stability was studied after two hours comparing the DPBD peak of a fresh prepared stock solution with those maintained under the studied conditions.

DPBD was more prone to degradation reaction in presence of light. It was observed that fluorescence signal was more influenced by light than UV signal. Results showed that, solutions exposed to sunlight, after two first hours, presented a 45% decrease of the DPBD peak area in case of UV signal, while for the fluorescence signal, decrease was 75%.

Matrix	Level (µg/g)	Mean (µg/g)	Recovery (%)	Repeatability (RSDr, %)
Orange juice	5	4.95	98.9	2.0
	1	0.852	85.2	2.3
	0.5	0.468	93.7	3.7
Chicken breast	5	4.18	83.7	8.4
	1	0.856	85.6	5.0
	0.5	0.417	83.4	3.1
Gouda cheese	5	4.20	84.1	2.3
	1	0.864	86.4	7.1
	0.5	0.5	100	9.7

Table VIII.2: DPBD recoveries at different concentrations for orange juice, chicken breast and Gouda cheese obtained with an ultraviolet detector.

Regarding the results obtained under artificial light, although the decrease was not so significant, in case of UV signal, this decrease was 7% and for fluorescence it was 8%. Using amber vials, only slight fluctuations were observed under artificial light as well as sunlight.

Matrix	Level (µg/g)	Mean (µg/g)	Recovery (%)	Repeatability (RSDr, %)
Orange juice	5	5.375	107.5	2.9
	1	0.799	79.9	5.3
	0.5	0.402	80.5	6.1
Chicken breast	5	4.42	88.4	11.0
	1	0.842	84.2	12.7
	0.5	0.359	71.9	9.0
Gouda cheese	5	5.295	105.9	8.6
	1	1.057	105.7	10.7
	0.5	0.538	107.6	12.6

Table VIII.3: DPBD recoveries at different concentrations for orange juice, chicken breast and Gouda cheese obtained with a fluorescence detector.

Therefore sample preparation procedure should be performed under absence of direct sunlight and using amber glass material.

Blanks of others food items were carried out in order to establish the possibility of determine DPBD. The foods analysed were: apple sauce, milk UHT, tomato ketchup, cola, margarine, condensed milk, dark chocolate, toasted bread, wheat flour, rice and honey. In any case no interference was found at the retention time of DPBD, which indicate the suitability of the method for these foodstuffs.

DPBD identity was confirmed by GC-MS. Several assays were performed to achieve best conditions in GC-MS. SIR was selected towards full scan mode due to its sensitivity.

VIII.4.- CONCLUSIONS

As DPBD is light sensitive, extraction procedures should be conducted in absence of direct sunlight, and all the material employed must be amber.

The HPLC-UV method is suitable for the determination of DPBD in foodstuffs in a range of 0.1-10 mg/Kg. However, for foods with a high content of fat, the sample extraction procedure must be slightly different in order to obtain acceptable recovery data. GC-MS is an analytical technique suitable to confirm the presence of this substance.

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CONCLUSIONES

A continuación se recogen las principales conclusiones extraídas de los trabajos que componen esta Tesis Doctoral.

Capítulo II: Se desarrolló un método multirresiduo basado en HPLC-FL, para la separación y cuantificación de BADGE, BFDGE, sus derivados y oligómeros del BADGE y NOGE en simulantes de alimentos.

Debido a que no existen patrones comerciales de los oligómeros del BADGE y NOGE, se han construido dos rectas de calibración usando BADGE y BFDGE, que han sido usadas para cuantificar los oligómeros del BADGE y NOGE respectivamente. Con estas rectas de calibración hechas con los patrones comerciales puros (de BADGE y BFDGE) se obtuvieron en todos los casos coeficientes de correlación mayores a 0,996 y límites de detección de 0.02 mg L^{-1}. Para la cuantificación de los derivados del BADGE y BFDGE se empleó el mismo procedimiento.

Se describe una manera rápida y sencilla para obtener todos los derivados clorados e hidroxilados del BADGE y BFDGE.

Los datos de migración obtenidos mostraron que el recubrimiento epoxídico analizado cumplía la legislación europea. Se observó que cuando se usaba el ensayo alternativo con el acetonitrilo y el recubrimiento no había sido sometido a un tratamiento posterior a alta temperatura, la cantidad total de sustancias migrantes (incluidos los oligómeros del BADGE) era más alta que en el ensayo de migración para el aceite de oliva. Se observó también que para todas las muestras sometidas a un proceso posterior a alta temperatura, se obtuvieron valores de migración menores.

Capítulo III: Se desarrolló un método de cromatografía líquida de alta resolución en fase reversa acoplada a un espectrómetro de masas vía una sonda de ionización química a presión atmosférica (APCI) que permite tanto la cuantificación como la identificación positiva del BADGE y de todos sus derivados.

El método ha sido evaluado en acetonitrilo al 90% (v/v) y en agua. En ambos casos se obtuvieron para todas las sustancias coeficientes de correlación mayores a 0.99 y límites de detección satisfactorios, comparándolos con los límites de migración específica establecidos en la legislación.

Capítulo IV: Se modificó y optimizó el método descrito en el capítulo III para la separación, cuantificación e identificación del BFDGE, sus derivados

hidroxilados y clorados y oligómeros de peso molecular mayor. Se obtuvieron en todos los casos coeficientes de correlación mayores de 0.99 y límites de detección adecuados, teniendo en cuenta el límite de migración específica.

Capítulo V: Se cuantificó la migración de BADGE y BFDGE en muestras reales y se identificaron otros derivados, mediante la aplicación de los métodos cromatográficos anteriormente optimizados. Los resultados de migración mostraron que en cuanto al BADGE, solo en el año 2000 un 1% de las muestras superaron en límite legal para este compuesto. Para el BFDGE se encontraron valores que excedían los límites legales en un 4 % de las muestras en el año 1999. Esto permite concluir que, a lo largo de los últimos seis años, estos compuestos se encontraron en concentraciones inferiores a las que se obtenían a finales de los años 90 en España. Estos valores son del orden de los resultados encontrados en otros estudios realizados en Europa.

Capítulos VI y VII: Se realizó una extensa revisión bibliográfica de las propiedades físicas y químicas de 18 compuestos seleccionados por el proyecto FOODMIGROSURE.. Se llevó a cabo además, una revisión bibliográfica de todos los métodos analíticos disponibles para cada compuesto en polímeros, simulantes y alimentos.

La bibliografía relacionada con este tema es escasa y está más enfocada al estudio del contenido total del migrante en polímeros y a la migración en simulantes, que a la migración en alimentos. Además, los métodos analíticos disponibles están aún en fase de desarrollo, no habiendo por tanto métodos de aprobación general.

En conclusión, actualmente, no están descritos protocolos analíticos para la determinación de la gran mayoría de los migrantes potenciales.

La recopilación extensa de propiedades físico-química junto con esta revisión bibliográfica hizo posible el establecer una guía para cada compuesto con el fin de facilitar el desarrollo de métodos de análisis en alimentos.

Capítulo VIII: Se desarrolló un método basado en la cromatografía líquida de alta resolución en fase reversa con detectores de ultravioleta y fluorescencia, para la determinación de difenilbutadieno. Se obtuvieron en ambos casos coeficientes de determinación mayores a 0.998. Se determinó este compuesto en tres alimentos diferentes, obteniéndose para todos ellos recuperaciones mayores al 83 %. La

identificación positiva de esta sustancia se llevó a cabo utilizando la cromatografía gaseosa con detector de espectrometría de masas.

Se comprobó la ausencia de interferencias al aplicar este método a otros alimentos.

La imagen utilizada como fondo en el diseño de esta Tesis, ha sido tomada de www.foodmigrosure.com