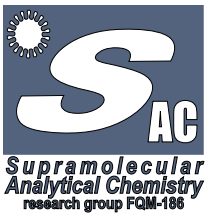
UNIVERSIDAD D CORDOBA

TESIS DOCTORAL

Disolventes Supramoleculares con propiedades de acceso restringido para la extracción de compuestos orgánicos

Supramolecular solvents with restricted access properties for the extraction of organic compounds



NOELIA CABALLERO CASERO

TITULO: *DISOLVENTES SUPRAMOLECULARES CON PROPIEDADES DE ACCESO RESTRINGIDO PARA LA EXTRACCIÓN DE COMPUESTOS ORGÁNICOS*

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Tesis Doctoral:

Disolventes Supramoleculares con propiedades de acceso restringido para la extracción de compuestos orgánicos

Trabajo presentado, para optar al grado de doctor, por

Noelia Caballero Casero

que lo firma en Córdoba, a 25 de noviembre de 2015

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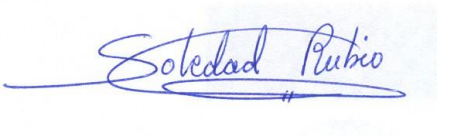

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Mediante la defensa de esta memoria se opta a la obtención de la mención de "Doctorado Internacional" habida cuenta de que la doctoranda reúne los requisitos exigidos:

1. La doctoranda ha realizado una estancia 3 meses en RIKILT- Institute of Food Safety of the University of Wageningen (Países Bajos), que ha contribuido a su formación.
2. Parte de la Tesis Doctoral, en concreto el objeto, resumen y conclusiones, se han redactado en una de las lenguas oficiales de la Unión Europea distinta a cualquiera de las lenguas oficiales en España.
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CERTIFICA: Que la citada Tesis Doctoral *"Disolventes Supramoleculares con propiedades de acceso restringido para la extracción de compuestos orgánicos"* se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba y que, a mi juicio, reúne todos los requisitos exigidos a este tipo de trabajos.

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Catedrática del Departamento de Química Analítica de la Universidad de Córdoba

Agradezco al Ministerio de Economía y Competitividad (MINECO) la concesión de una ayuda de Formación de Personal Investigador con referencia BES-2012-052170, que me ha facilitado la plena dedicación a esta Tesis Doctoral, así como al Instituto de Estudios de Postgrado (IDEP) por concederme la ayuda a la Movilidad con referencia MHE2011-00115.

TÍTULO DE LA TESIS: DISOLVENTES SUPRAMOLECULARES CON PROPIEDADES DE ACCESO RESTRINGIDO PARA LA EXTRACCIÓN DE COMPUESTOS ORGÁNICOS

DOCTORANDO/A: NOELIA CABALLERO CASERO

INFORME RAZONADO DE LA DIRECTORA DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

En esta Tesis Doctoral se ha evaluado la capacidad de los disolventes supramoleculares con propiedades de acceso restringido (SUPRAS-RAM), basados en ácidos carboxílicos alifáticos y alcanoles, para la integración de las etapas de extracción de compuestos y purificación de la muestra en análisis químico.

El primer objetivo de las investigaciones desarrolladas fue la aplicación de SUPRAS-RAM a la determinación de componentes o multicomponentes en una matriz compleja. En este contexto se han desarrollado dos metodologías analíticas para la determinación de ocratoxina A (OTA) en pasas y catorce hidrocarburos policíclicos aromáticos en musgo (PAHs). Ambos métodos se han validado internamente y cumplen los criterios de funcionamiento requeridos para el control de OTA en pasas, a los niveles máximos permitidos por la UE, y para la biomonitorización de la deposición atmosférica de PAHs.

En una segunda etapa, el objetivo fue la aplicación de SUPRAS-RAM a la determinación de un número reducido de componentes en diferentes tipos de matrices (multi-matrices). Para ello, se han desarrollado métodos generalizados de tratamiento de muestras que son independientes de la composición de la matriz. Las propiedades RAM de los SUPRAS han permitido la extracción eficiente de OTA en todos los tipos de especias que la

legislación europea contempla y, curcuminoides en diferentes tipos de alimentos.

Finalmente, se ha evaluado la capacidad de SUPRAS-RAM para la extracción de multicomponentes en multi-matrices. Se ha desarrollado una metodología para la determinación de 21 bisfenoles, derivados clorados y diglicidil éteres en múltiples fuentes de exposición humana a estos alteradores endocrinos, así como los fluidos biológicos (orina y suero) utilizados para biomonitorización.

La simplicidad y universalidad de los métodos de extracción basados en SUPRAS-RAM desarrollados en el curso de esta Tesis, ha propiciado su utilización en diferentes áreas, como el control de la calidad alimentaria, la biomonitorización y los estudios poblacionales.

Los resultados de las investigaciones realizadas se han materializado en 5 artículos científicos (3 publicados en revistas científicas indexadas situadas en el primer cuartil y 2 enviados a publicación). Asimismo, la doctoranda ha participado, por invitación, en la publicación de 1 artículo de revisión en una revista internacional situada en el primer decil y 1 artículo de divulgación. Los resultados obtenidos se han presentado por la doctoranda en 3 contribuciones orales a congresos (2 nacionales y 1 internacional) y 8 carteles (5 nacionales y 3 internacionales)

En base a la originalidad de las investigaciones desarrolladas y expuestas en esta Memoria así como la amplia formación científica adquirida por Dña. Noelia Caballero Casero, autorizo la presentación de esta Tesis Doctoral.

Córdoba, 25 de noviembre de 2015

Firma del/de los director/es

Fdo.: SOLEDAD RUBIO BRAVO

Mi agradecimiento…

A Sole, por darme esta oportunidad, por creer en mí y por ser mucho más que mi directora de Tesis. A las profesoras Lola y Loreto por sus consejos. A Diego, Juani y Rafa por su amable disposición.

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A Sica y Pancho,
que cada día me dan más de
lo que podré devolverle jamás

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que siempre me ha dado fuerzas

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porque nuestras charlas hasta el amanecer
me descubrieron la Ciencia, y son la razón
de que eligiera este camino

A Javier, por su apoyo incondicional,
por estar siempre a mi lado
y por hacerme reír cada día.
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OBJETO

La simplificación de la etapa de tratamiento de muestras en análisis químico, así como la reducción del disolvente orgánico consumido en la misma, continúa siendo un área prioritaria en I+D+I. La simplificación es esencial en áreas en la que se requiere el procesamiento de un número elevado de muestras tales como el control de sustancias reguladas en alimentos, campañas de monitorización de la contaminación ambiental, estudios epidemiológicos, etc. Aspectos tales como la integración de las diferentes etapas implicadas (ej. extracción/purificación) y el incremento de la eficiencia del extractante tienen un impacto positivo en el número de muestras procesadas por unidad de tiempo y en los costes del análisis.

Por otro lado, la reducción del consumo de disolventes orgánicos es una prioridad en las políticas ambientales europeas dado que éstos son responsables de aproximadamente el 25% de los compuestos orgánicos volátiles emitidos a la atmósfera. Entre las estrategias desarrolladas para su reducción en los laboratorios de análisis químico destacan la adsorción de solutos sobre sólidos, la miniaturización y el uso de energías auxiliares. Asimismo se ha prestado atención al uso de disolventes alternativos, principalmente los fluidos supercríticos y, más recientemente, los líquidos iónicos.

El autoensamblaje de compuestos anfifílicos posee un gran potencial para la síntesis de disolventes con características programadas para que cumplan funciones específicas. El desarrollo de la Química Supramolecular ha permitido profundizar en aspectos básicos del autoensamblaje pero prácticamente todos los estudios se han dirigido a la obtención de nanomateriales y apenas se ha prestado atención al desarrollo de disolventes funcionales. En nuestro grupo de investigación hemos desarrollado disolventes supramoleculares con propiedades de acceso restringido (SUPRAS-RAM) a partir de alcanoles y ácidos alquil carboxílicos en mezclas de tetrahidrofurano y agua. Estos disolventes tienen el potencial de extraer eficientemente los analitos y excluir las macromoléculas presentes en la matriz de la muestra mediante mecanismos químicos y físicos, así integrando las etapas de extracción y purificación.

El objeto de las investigaciones que se presentan en esta Memoria ha sido el estudio del potencial de SUPRAS-RAM obtenidos a partir de ácidos alquil carboxílicos y alcanoles para integrar las etapas de extracción de analitos y purificación de muestras, así como reducir o eliminar el consumo de disolvente orgánico. De esta forma se pretende desarrollar metodologías analíticas innovadoras para la determinación de compuestos orgánicos en tres áreas de interés; control de calidad de alimentos, biomonitorización ambiental y estudios epidemiológicos.

Para establecer el ámbito de aplicación de SUPRAS-RAM se explorarán diferentes tipos de problemáticas a las que se enfrentan los laboratorios de análisis químico:

1) Determinación de componentes o multicomponentes en una matriz compleja.
2) Determinación de un número reducido de componentes en diferentes tipos de matrices (multi-matrices).
3) Determinación de multicomponentes en multi-matrices.

Asimismo se investigará la compatibilidad de los SUPRAS-RAM con cromatografía de líquidos acoplada a diferentes detectores (UV-visible, fluorescencia y espectrometría de masas en tándem).

Estos estudios deberían proporcionar una visión aproximada del potencial y ámbito de aplicación de los SUPRAS-RAM en los procesos de extracción y purificación requeridos en análisis químico.

Paralelamente, un objetivo fundamental en el desarrollo de esta Tesis ha sido la formación de la doctoranda a través de actividades complementarias a la labor investigadora, tales como la redacción de artículos científicos, la asistencia y presentación de comunicaciones en congresos nacionales e internacionales, estancias internacionales, etc.

AIM

Reduction of organic solvent consumption and simplification of sample handling continues as a major R&D&I area in chemical analysis. Simplification and reduction of the stages involved in sample treatment is essential in fields where a cost-effective, high sample throughput is required (e.g. food quality control, environmental monitoring campaigns, epidemiological studies, etc.).

Reducing emission of volatile organic compounds (VOCs) from solvents is a priority for European policies aimed at controlling air quality. It is estimated that solvents contribute to around 25% of VOC emissions in Europe. To meet the goals of these policies, chemical analysis labs are progressively incorporating different strategies intended to reduce solvent consumption. Regarding sample treatment, some relatively recent strategies such as the use of solid phase extraction, miniaturization, and auxiliary energies are today in routine use in labs. Great attention has been recently paid to the use of alternative solvents, mainly supercritical fluids and ionic liquids.

The self-assembly of amphiphilic compounds has a great potential for the synthesis of nanostructured, tunable and/or functional solvents. Recent progress in supramolecular chemistry has led to an in-depth understanding of self-assembly processes, which are the basis of the bottom-up approach for the synthesis of smart nano-materials. Unfortunately, this knowledge has been scarcely used for developing nanostructured liquids, a topic in which our research group has been involved in the last two decades.

The research here presented was aimed to study the potential of supramolecular solvents with restricted access properties (SUPRAS-RAM) to both simplify sample treatment and reduce/remove organic solvent consumption in chemical analysis in order to develop innovative methodologies for the determination of organic compounds in three areas; food quality control, environmental biomonitoring and epidemiological studies. For this purpose, SUPRAS-RAM, spontaneously obtained by addition of water to reversed micelles of alkanols and alkyl carboxylic acids in tetrahydrofuran, were explored. These solvents have the potential to efficiently extract organic compounds and exclude the macromolecules

present in the sample matrix, by both chemical and physical mechanisms. In this way, both organic compound isolation and sample purification can be integrated in a single step.

In order to delimit the scope of application of SUPRAS-RAM in this context, common challenges faced by chemical analysis labs were addressed:

1) Determination of single-component or multicomponent in a complex sample.

2) Determination of a few components in different types of samples (multi-matrices).

3) Determination of multicomponents in multi-matrices.

Compatibility of SUPRAS-RAM with liquid chromatography coupled to different detectors (UV-visible, fluorescence and tandem mass spectrometry) were also investigated.

The results obtained in these studies should provide rough guidelines about the suitability of SUPRAS-RAM for integration of extraction and clean-up processes in chemical analysis.

At the same time, a key aim in this Thesis has been the development of a Formation Program for the PhD student. This Program includes, among other activities, the attendance and presentation of contributions in international and national congresses, the writing of scientific papers, international stays, etc.

CONTENIDO

El contenido de la Memoria de esta Tesis Doctoral se ha estructurado en tres bloques, precedidos de una Introducción en la que se discute brevemente la necesidad de desarrollar disolventes alternativos a los disolventes orgánicos y se describen aspectos teóricos y prácticos relacionados con el uso de SUPRAS en procesos de extracción analítica. Los contenidos de los tres bloques se especifican a continuación.

Bloque A: SUPRAS-RAM basados en ácidos alquil carboxílicos para la determinación de componentes/multicomponentes en una matriz compleja.

En este bloque se describen dos metodologías analíticas para la determinación de ocratoxina A (OTA) en pasas (capítulo 1) y catorce hidrocarburos policíclicos aromáticos (PAHs) en musgo (capítulo 2). Ambos métodos se han validado internamente y cumplen los criterios de funcionamiento requeridos para el control de OTA en pasas, a los niveles máximos permitidos por la UE, y para la biomonitorización de la deposición atmosférica de PAHs.

Para el tratamiento de muestras se utilizan dos tipos de SUPRAS obtenidos a partir de disoluciones coloidales de vesículas acuosas (capítulo 1) y micelas inversas (capítulo 2) de ácido decanoico. La coacervación se induce mediante la adición de sales de tetrabutilamonio y agua, respectivamente. En ambos métodos, el tratamiento de muestras se reduce a la microextracción de pasas y musgo con el correspondiente SUPRAS (200-400 μL) mediante agitación durante 5-10 min, centrifugación y análisis directo del extracto mediante cromatografía de líquidos con detección fluorescente.

Bloque B: SUPRAS-RAM basados en ácidos alquil carboxílicos para la determinación de componentes en multi-matrices.

En este bloque se presentan los resultados obtenidos para la determinación de OTA en diferentes tipos de especias (capítulo 3) y curcuminoides en alimentos (capítulo 4). El objetivo principal de las investigaciones aquí incluidas ha sido estudiar el potencial de SUPRAS-RAM para el desarrollo de procedimientos generalizados de tratamiento de muestra para el control de la calidad de alimentos cuando la composición de las matrices de interés varía ampliamente (por ejemplo porcentaje de proteínas,

grasas, hidratos de carbono, agua, etc). Tradicionalmente, estos métodos requieren procedimientos de extracción y/o purificación diferenciados. El uso de procedimientos que sean independientes de la matriz, optimiza y reduce la complejidad de las operaciones requeridas para el control de calidad de los alimentos.

Los SUPRAS-RAM investigados se han sintetizado a partir de disoluciones coloidales de ácido decanoico en THF en las que se induce la coacervación mediante la adición de agua. La combinación de la elevada eficacia de extracción y propiedades RAM de estos SUPRAS ha permitido el desarrollo de procedimientos generalizados para la determinación de OTA en todos los tipos de especias para los que la EU ha establecido valores límites (*Capsicum spp., Piper spp., Myristica fragrans, Zingiber officinale, Curcuma longa*) así como la determinación de curcuminoides en los diferentes grupos de alimentos legislados (aperitivos, gelatina, yogurt, mayonesa, mantequilla, pescado, golosinas). El extracto de SUPRAS, obtenido en todos los casos mediante extracción y centrifugación de las diferentes muestras, se analiza directamente por cromatografía de líquidos, con detección fluorescente en el caso de OTA y detección fotométrica en el caso de curcuminoides.

Bloque C: SUPRAS-RAM basados en alcanoles para la determinación de multicomponentes en multi-matrices.

Las investigaciones que se incluyen en este bloque abordan uno de los retos actuales del análisis químico; la determinación de multicomponentes en multi-matrices. Los analitos objeto de estas investigaciones son mezclas de bisfenoles y derivados (ej. bisfenoles, bisfenoles clorados y bisfenol diglicidil éteres) y las matrices de interés incluyen fuentes de exposición humana por ingestión (alimentos y bebidas) e inhalación (polvo doméstico) y fluidos para biomonitorización (orina y suero).

En el capítulo 5 se realiza una revisión crítica sobre la necesidad de la necesidad de determinar mezclas de bisfenoles y derivados en fuentes de exposición humana y ambiental, y fluidos biológicos, con objeto de obtener la información requerida para establecer medidas legislativas acordes con el *efecto cocktail* de los mismos. El *efecto cocktail* hace referencia a la creciente evidencia de que la acción conjunta de mezclas de compuestos, cada uno de los cuales se encuentra a concentración inferior al nivel máximo permitido, puede ejercer efectos nocivos en humanos y el ambiente. Se revisan las

metodologías analíticas desarrolladas para este fin y se discuten diferentes ítems que requieren un esfuerzo adicional considerable en I+D.

En el capítulo 6 se describe una metodología general para la determinación de 21 bisfenoles y derivados en diferentes tipos de alimentos enlatados (verduras, productos cárnicos, legumbres.), bebidas (tónica, cola, cerveza, té, etc.), agua embotellada, polvo doméstico y fluidos biológicos (orina, suero). La metodología se basa en la extracción de los bisfenoles y derivados utilizando SUPRAS-RAM volátiles obtenidos a partir de disoluciones coloidales de hexanol en THF utilizando como agente coacervante el agua. El procedimiento de tratamiento de muestra consiste en la extracción de los analitos, volatilización del SUPRAS, redisolución de los mismos en la fase móvil y análisis mediante cromatografía de líquidos y espectrometría de masas en tándem.

Finalmente, se incluyen en la Memoria las conclusiones generales derivadas de las investigaciones realizadas y los siguientes anexos:

- Anexo 1: Publicaciones científicas derivadas de la Tesis Doctoral.
- Anexo 2: Comunicaciones realizadas en Congresos nacionales e internacionales.

SUMMARY

The research results included in this Thesis Report are presented in three Parts, which are preceded by an Introduction dealing with the need to develop alternative to conventional organic solvents, and theoretical and practical aspects related to the use of supramolecular solvents in analytical extraction processes. The contents of the three Parts are given below.

PART A: Alkyl carboxylic acid-based SUPRAS-RAM for determination of single component/multicomponents in complex samples.

This Part describes two new analytical methods dealing with the determination of ochratoxin A (OTA) in raisins (Chapter 1) and fourteen polycyclic aromatic hydrocarbons (PAHs) in mosses (Chapter 2), respectively. Both methods have been in-house validated and it has been checked that they meet the performance criteria required for the control of OTA in raisins, at the maximum levels permitted by the EU, and the biomonitoring of the atmospheric deposition of PAHs.

Sample treatment is carried out with two types of SUPRAS, which are obtained from colloidal solutions of aqueous vesicles (Chapter 1) and reverse micelles (Chapter 2) of decanoic acid. The spontaneous coacervation of decanoic acid aggregates is induced by the addition of tetrabutyl ammonium salts and water, respectively. In both methods, sample treatment is greatly simplified; it only involves the stirring of minute quantities of raisin or moss samples with 200-400 µL of SUPRAS for 5-10 min, centrifugation of the mixture, and direct analysis of the extract by liquid chromatography coupled to fluorescence detection.

PART B: Alkyl carboxylic acid-based SUPRAS-RAM for the determination of a few components multi-matrices.

In this Part, two new analytical methodologies dealing with the determination of OTA in different types of spices (Chapter 3) and curcuminoids in food (Chapter 4) are described. These investigations aimed to explore the potential of SUPRAS-RAM for the development of generalized sample treatments in food quality control when matrix composition varies in a wide range (e.g. percentage of protein, fat, carbohydrates, water, etc.).

Traditionally, these matrix-dependent methods require differentiated sample extraction and/or purification procedures. By developing matrix-independent sample treatment procedures, food quality control is greatly simplified.

The SUPRAS-RAM investigated for this purpose were spontaneously synthesized by addition of water to colloidal solutions of reverse micelles of decanoic acid in tetrahydrofuran. The high number of binding sites and mixed-mode mechanisms they offer for solute solubilization, as well as their RAM properties, accounted for the high extraction efficiency and selectivity obtained for the selected compounds. Generalized sample treatment procedures have been proposed for both the determination of OTA in all types of spices regulated by the EU (Capsicum spp., Piper spp., Myristica fragrans, Zingiber officinale, Curcuma longa) and the determination of curcuminoids in seven different food groups (snacks, jelly, yogurt, mayonnaise, butter, fish, sweets). Sample treatment always involved the extraction of minute quantities of food samples with the SUPRAS, centrifugation and direct analysis of OTA or curcuminoids in the extract by liquid chromatography coupled to fluorescence or UV-visible detection, respectively.

PART C: Alkanol-based SUPRAS-RAM for the determination of multicomponents in multi-matrices.

The research included in this Part addresses one of the biggest challenges faced by chemical analysis, namely the determination of multicomponents in multi-matrices. For this purpose, the determination of mixtures of bisphenols and derivatives (i.e. bisphenols, chlorinated bisphenols and bisphenol diglycidyl ethers) in human exposure sources (e.g. food, beverages and house dust) and biological fluids (e.g. urine and serum) was investigated.

In Chapter 5, a critical review dealing with the need to no longer consider bisphenols and derivatives individually for the assessment of bisphenol-related human and environmental exposure and risk, as well as for the development of legislative provisions consistent with the cocktail effect is presented. The cocktail effect refers to the growing evidence that the joint action of compounds mixtures, at concentrations considered safe for the individual chemicals, may have harmful effects on humans and environment. The main chemical, biological and prediction tools developed so far for

evaluation of exposure and risk to mixture of bisphenols are presented and critically discussed. Current knowledge gaps on this topic are outlined.

Chapter 6 describes a general methodology for determination of 21 bisphenols and derivatives in different types of canned food (vegetables, meat products, legumes, etc.), drinks (tonic, cola, beer, tea, etc.), Bottled water, indoor dust and biological fluids (urine, serum). It is based on the extraction of the mixture of bisphenols from the multi-matrices with a volatile SUPRAS-RAM obtained from colloidal solutions of hexanol in tetrahydrofuran, using water as a coacervating agent. The general sample treatment procedure consists in bisphenol extraction, SUPRAS volatilization; bisphenol reconstitution in mobile phase solution and analysis by liquid chromatography tandem mass spectrometry.

Finally, the Thesis Report includes a Conclusion section and two Annexes:

Annex 1: Research articles derived from the Doctoral Thesis.

Annex 2: Contributions to national and international Conferences.

INTRODUCCIÓN

1. Necesidad de alternativas a los disolventes orgánicos

Los disolventes orgánicos, utilizados tanto en el ámbito industrial, donde abarcan la práctica totalidad de los sectores industriales, como en el doméstico, son los responsables de aproximadamente el 25% de los compuestos orgánicos volátiles (COVs) emitidos a la atmósfera [1]. El resto de COVs presentes en la atmósfera tiene origen antropogénico (transporte y producción de energía eléctrica por combustión) y natural (vegetación y animales salvajes y domésticos). Se estima que en la Unión Europea se emiten cada año alrededor de 10 millones de toneladas de COVs procedentes de los carburantes y disolventes.

El término COV agrupa a una gran cantidad de tipos de compuestos químicos, entre los que se incluyen los hidrocarburos alifáticos, aromáticos y clorados así como los aldehídos, cetonas, éteres, ácidos y alcoholes. Alrededor de la mitad de los 189 contaminantes atmosféricos peligrosos regulados por el "Clean Air Act Amendment" en Estados Unidos (1990) son COVs [2].

De acuerdo a su peligrosidad, los COVs se clasifican en tres grupos:

i. Extremadamente peligrosos para la salud (ej. benceno, cloruro de vinilo y 1,2 dicloroetano).
ii. Compuestos de clase A (pueden causar daños significativos al medio ambiente: ej. acetaldehido, anilina, bencilcloruro, tetracloruro de carbono, clorofuorocarbonos, tricloroetileno, triclorotolueno, etc).
iii. Compuestos de clase B (con menor impacto en el medio ambiente: ej. acetona, etanol, etc).

Los efectos negativos de los COVs sobre la salud y el medioambiente derivan fundamentalmente de su carácter volátil y tóxico. Los riesgos mayores para el ser humano se producen por la absorción de éstos compuestos a través de la piel y por inhalación. Los disolventes orgánicos son liposolubles, por lo que ante exposiciones a largo plazo las concentraciones acumuladas pueden ser elevadas y causar lesiones en el hígado, riñones y sistema nervioso central.

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[1]J. Theloke, R. Friedrich, Atmospheric Environ. 41 (2007) 4148.
[2]Public Law No. 101-549, US 101st Congress, Clean Air Act Amendments of 1990 (November 1990).

La exposición a corto plazo puede causar irritación de los ojos y las vías respiratorias, dolor de cabeza, mareo, trastornos visuales, fatiga, pérdida de coordinación, reacciones alérgicas de la piel, náuseas y pérdida de memoria. La emisión de COVs a la atmósfera provoca graves problemas en el medio ambiente, como la degradación del ozono atmosférico y la formación del smog fotoquímico. Como resultado, la industria química está sometida en la actualidad a una gran presión social para que aporte soluciones a los problemas ambientales que ocasionan los disolventes orgánicos.

Con objeto de prevenir y reducir los efectos de las emisiones de COVs al medio ambiente, y los riesgos potenciales para la salud humana, el Consejo de Europa publicó en su día la Directiva 2010/75/UE de 24 de Noviembre de 2010, sobre las emisiones industriales (prevención y control integrados de la contaminación). En el capítulo V, esta directiva establece disposiciones para instalaciones y actividades que utilicen disolventes orgánicos, y en el Anexo VII impone valores límite de emisión de COVs para estas instalaciones [3].

Paralelamente, ha existido un gran interés en la comunidad científica por el desarrollo de disolventes alternativos que cubran las áreas de aplicación de los disolventes orgánicos, proporcionen nuevas funcionalidades, y sobre todo sean menos volátiles, tóxicos e inflamables. Entre las alternativas desarrolladas, destacan los fluidos supercríticos (FSCs), los líquidos iónicos y los disolventes fluorados.

Los FSCs son sustancias que se encuentran por encima de su temperatura y presión crítica. Un FSC posee propiedades de disolvente que se parecen a las de un líquido, pero también exhibe propiedades de transporte parecidas a las de un gas. De esta manera, un FSC no solo puede disolver solutos sino que también es miscible con los gases ordinarios y puede penetrar en los poros de los sólidos. Los FSCs tienen una viscosidad más baja y un coeficiente de difusión más elevado que los líquidos. La densidad de un fluido supercrítico aumenta al aumentar la presión y, al aumentar la densidad, la solubilidad de un soluto en el fluido supercrítico aumenta de forma considerable. El hecho de que las propiedades puedan ajustarse variando la presión y la temperatura tiene ventajas para la aplicación de estos fluidos como agentes de extracción.

[3]Directiva 2010/75/UE del Parlamento Europeo y del Consejo de 24 de noviembre de 2010.

El FSC más utilizado es el dióxido de carbono debido a que es químicamente inerte, tiene bajo coste, no es tóxico, inflamable o corrosivo, sus condiciones críticas son relativamente fáciles de alcanzar, se puede trabajar a baja temperatura y por tanto, se pueden separar compuestos termolábiles y se puede obtener a partir de procesos de fermentación alcohólica. Su utilización en procesos de extracción de solutos a partir de una materia prima supone el reparto del material en el líquido supercrítico, seguido de un cambio de temperatura y presión que tiene como resultado el aislamiento del soluto puro por vaporización del CO_2. Finalmente, el FSC puede reciclarse invirtiendo el cambio en las condiciones de temperatura y presión.

Uno de los campos de aplicación de la tecnología con FSC más desarrollados a nivel industrial es la obtención de ingredientes para la agroindustria, perfumes y cosmética. Las aplicaciones comerciales iniciales de los fluidos supercríticos fueron la obtención de café descafeinado (en 1978) y la extracción del lúpulo (en 1982). Juntos, estos usos representan más de la mitad de los procesos de producción mundial con el uso de fluidos supercríticos. También se usa en la extracción de triglicéridos en patatas fritas y en procesos de limpieza en seco. Sin embargo, debido a los altos costes de las instalaciones y mantenimiento, la tecnología de FSC sólo es rentable para la obtención de productos de alto valor añadido. Por otro lado, la capacidad de CO_2 para extraer compuestos polares es relativamente baja, aunque puede incrementarse con la adición de un modificador como agua, metanol o un tensioactivo. Por otro lado, puede reaccionar con nucleófilos tales como aminas.

Los líquidos iónicos se pueden definir como sales cuya temperatura de fusión está por debajo de 100 ^{0}C. Están constituidos por un catión orgánico, siendo los más comunes los de tetraalquilamonio, tetraalquilfosfonio, N-alquilpiridina y N,N´-dialquilimidazolio y un anión poliatómico entre los que destacan el hexaflorofosfato, tetrafluoroborato, trifluoracetato y trifalto. Dado el gran número de aniones y cationes que potencialmente pueden constituir un líquido iónico pueden formularse un extenso número de ellos, dando lugar así a multitud de líquidos iónicos con propiedades muy distintas que pueden ser usados en aplicaciones concretas.

Son compuestos no volátiles o presentan escasa volatilidad, ya que por su naturaleza iónica tienen una presión de vapor muy baja. Son térmicamente estables, manteniendo su estructura y propiedades hasta 300-450 ^{0}C. La

viscosidad puede modificarse cambiando el anión. Su capacidad de solvatación es muy elevada y presentan un alto grado de reutilización.

Los líquidos iónicos han suscitado un gran interés en los últimos años debido principalmente a que estos materiales, usados inicialmente en aplicaciones electroquímicas, presentan una gran utilidad como medios de reacción tanto en procesos químicos, bioquímicos y como agentes de extracción.

Sin embargo, su repercusión en cuanto a su efecto en los vertidos no están claras, ya que los estudios sobre su toxicidad y biodegradabilidad son todavía escasos. La mayoría de los líquidos iónicos que se han investigado son irritantes y tienen una toxicidad comparable a los disolventes orgánicos convencionales. No obstante, la baja volatilidad reduce las potenciales vías de exposición al contacto directo con la piel e ingestión. Por otro lado, muchos de los iones empleados en la síntesis son tóxicos y el procedimiento sintético es complejo y utiliza reactivos caros. Todo ello ha dificultado la implementación de estos disolventes [4].

Los disolventes perfluorados son disolventes orgánicos en los que los enlaces C-H se han sustituido, total o parcialmente, por enlaces C-F. Los más comunes son perfluoroalcanos, perfluorodialquiléteres o perfluoro trialquilaminas de cadena lineal o ramificada de 6 a 10 átomos de carbono. La fortaleza del enlace C-F les confiere sus propiedades: son químicamente inertes, térmicamente estables, no inflamables y se consideran no tóxicos. Los disolventes fluorados son inmiscibles con muchos disolventes orgánicos y también pueden serlo en agua, dependiendo de la temperatura. Esta propiedad permite su uso en sistemas bisfásicos, así facilitando los procesos de separación. Tienen gran interés para la extracción de compuestos perfluorados. Sin embargo, los disolventes perfluorados son muy caros y existe preocupación por su impacto ambiental a largo plazo.

En esta tesis doctoral abordamos el desarrollo de procesos de extracción analítica basados en el uso de disolventes supramoleculares. Estos disolventes presentan propiedades intrínsecas y operacionales que les convierten en candidatos ideales para sustituir a los disolventes orgánicos en muchos procesos de extracción analítica e industrial. A continuación se

[4]T.P.T. Pham, C-W. Cho, Y-S Yun, Water Research, 44 (2010) 352.

describe la síntesis, propiedades y aplicaciones en procesos de extracción de los disolventes supramoleculares.

2. Síntesis de disolventes supramoleculares

Los disolventes supramoleculares, conocidos por su acrónimo en inglés SUPRAS, se definen como líquidos nanoestructurados generados a partir de una disolución acuosa o hidro-orgánica de moléculas anfifílicas a través de procesos de auto-ensamblaje y coacervación.

El término disolvente supramolecular fue propuesto por vez primera por nuestro grupo de investigación con el objeto de enfatizar el carácter nanoestructurado de estos disolventes en contraposición al carácter iónico y molecular de los líquidos iónicos y disolventes orgánicos, respectivamente. Los términos tradicionales usados para denominar a estas fases líquidas han sido *cloud* point, que hace referencia al enturbiamiento que se produce al aumentar la temperatura en disoluciones de tensioactivos no iónicos antes de la separación de fases, y *coacervado*, que hace referencia al fenómeno de coacervación que dirige la formación de una nueva fase líquida..

El proceso general a través el cual se sintetizan los SUPRAS se muestra en la figura 1 y consta de las siguientes etapas:

i. Las moléculas anfifílicas en disolución forman espontáneamente agregados tridimensionales (micelas acuosas, inversas o vesículas) por encima de la concentración de agregación crítica (fenómeno de autoensamblaje).
ii. Los agregados de la disolución coloidal aumentan de tamaño al modificar las condiciones ambientales y producen microgotitas de aceite a través de un proceso de coacervación [5].
iii. Las microgotas de coacervado se asocian produciendo clústeres.
iv. Los clústeres, con una densidad diferente a la de la disolución que las contiene, se separan en una nueva fase nanoestructurada (SUPRAS) que se encuentra en equilibrio con la disolución de síntesis [6].

[5]IUPAC Compendium of Chemical Terminology 31 (1972) 611
[6]F.M. Menger, B.M. Sykes, Langmuir 14 (1998) 4131

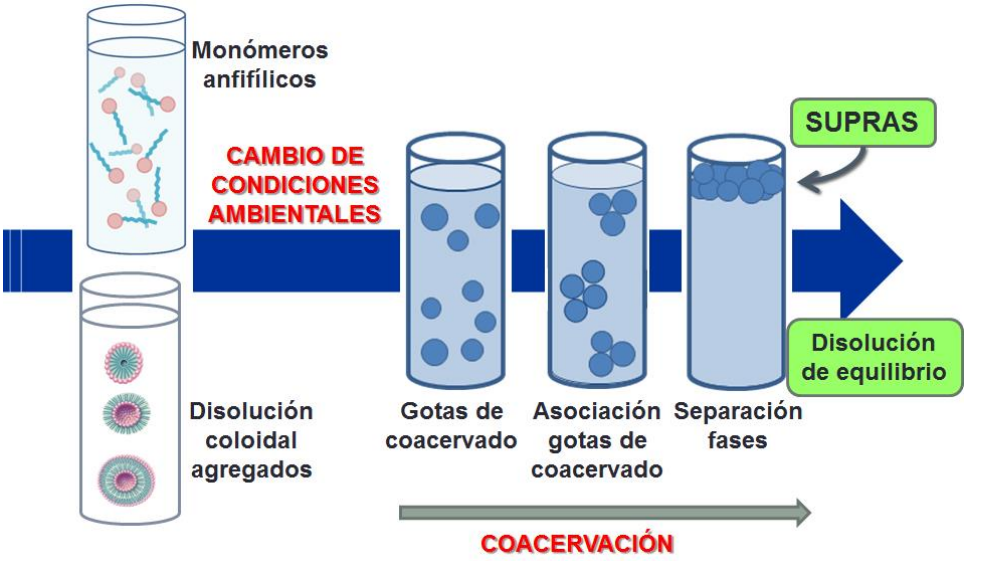

Fig. 1. *Proceso general de formación del SUPRAS*

2.1. Auto-ensamblaje

El auto-ensamblaje es el mecanismo químico a partir del cual dos o más componentes se asocian espontánea y reversiblemente para formar agregados de mayor tamaño, mediante enlaces no covalentes.

La posibilidad de desentrañar los mecanismos del auto-ensamblaje para poder dirigirlo y diseñar las arquitecturas supramoleculares deseadas es uno de los retos más desafiantes para la Química y constituye uno de los pilares fundamentales de la Química Supramolecular [7].

Desde el punto de vista científico, el conocimiento del auto-ensamblaje es fundamental para la comprensión de los sistemas biológicos y el comportamiento de los sistemas coloidales [8,9]. El enorme potencial del auto-

[7]J.M. Lehn, Proc. Natl. Acad. Sci. U.S.A., 99 (2002) 4763.
[8]J.A. Pelesko, Self-assembly: The science of things that put themselves together, Chapman & Hall/ CRC, Boca Raton, FL, 2007.
[9]F. Evans, H. Wennerström, The Colloidal Domain: where physics, chemistry, biology, and technology meet, Wiley-VCH, New York, 1999 2nd Ed.

ensamblaje para la obtención de nuevas materiales avanzados [10] ha abierto nuevos caminos en el campo de la nanotecnología (ej. *the bottom-up approach) [11],* permitiendo el diseño de materiales supramoleculares avanzados y funcionales basados en estructuras de polímeros supramoleculares [12,13], cristales líquidos y vesículas [14-16] o arquitecturas construidas a partir de nanopartículas [17-19], nanotubos [20, 21] o nanovarillas [22, 23]. Sin embargo, este concepto de auto-ensamblaje apenas se ha explorado desde el punto de vista de la Química Analítica para el desarrollo de disolventes funcionales de interés para los laboratorios de análisis y la industria química.

Los procesos de auto-ensamblaje son el resultado de un balance de interacciones repulsivas y atractivas, normalmente débiles y no covalentes (interacciones electrostáticas de tipo Coulomb, interacciones hidrofóbicas e interacciones por puentes de hidrógeno). Sin embargo, muchos investigadores postulan que determinados enlaces covalentes débiles, como por ejemplo los enlaces de coordinación, deberían ser considerados también fuerzas directoras del auto-ensamblaje [24].

El auto-ensamblaje de los compuestos anfifílicos en una disolución se produce cuando los compuestos anfifílicos alcanzan una determinada concentración, denominada concentración de agregación crítica (cac). Al

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[10]K. Ariga, T. Kunitake, Supramolecular Chemistry, Fundamentals and Applications, Springer: Berlin, 2006.
[11]J.W.Steed, D.R. Turner, K.J. Wallace, Core Concepts in Supramolecular Chemistry and Nanochemistry, John Wiley & Sons: Chichester: U.K, 2007.
[12] J.M. Lehn, Prog. Polym. Sci., 30 (2005) 814.
[13]L. Brunsveld, B.J.B. Folmer, E.W. Meijer, R.P. Sijbesma, Chem. Rev., 101 (2001) 4071.
[14]F. M. Benger, K.D. Gabrielso, Angew. Chem. Int. Ed. Engl., 34 (1995) 2091.
[15]C.M. Paleos, Z. Sideratou, D. Tsiourvas, Chem. Bio. Chem., 2 (2001) 305.
[16]V. Marchi-Artzner, T. Gulik-Krzywicki, M.A. Guedeau-Boudeville, C. Gosse, J.M. Sanderson, J.C. Dedieu, J.M. Lehn, Proc.Natl. Acad. Sci. U.S.A., 101 (2004) 15279.
[17]C.A. Mirkin, R.L. Letsinger, R.C. Mucic, J.J. Storhoff, Nature, 382 (1996) 607.
[18]M. Li, H. Schnalblegger, S. Mann, Nature, 402 (1999) 393.
[19]S.J. Park, A. A. Lazarides, C.A. Mirkin, R.L. Letsinger, Angew. Chem. Int. Ed., 40 (2001) 2909.
[20]S. S. Fan, M.G. Chapline, N.R. Franklin, T.W. Tombler, A.M. Casell, H.J. Dai, Science, 283 (1999) 512.
[21]L. Adler-Abramovich, D. Aronov, P. Beker, M. Yevnin, S. Stempler, L. Buzhansky, G. Rosenman, E. Gazit, Nature Nanotechnology , 4 (2009) 849.
[22]M. Yan, H.T. Zhang, E.J. Widjaja, R.P.H. Chang, J. Appl. Phys., 94 (2003) 5240.
[23]A. Salant, E. Amitay-Sadovsky, U. Banin, J. Am. Chem. Soc., 128 (2006) 10006.
[24]B. Olenyuk, J.A. Whiteford, A. Fenchtenkotter, P.J. Stang, Nature 398 (1999) 796.

alcanzarse esta concentración, comienzan a formarse estructuras ordenadas (micelas, vesículas) con el fin de minimizar los efectos solvofóbicos. Esto quiere decir que en la cac, las interacciones entre las moléculas de tensioactivo son más favorables energéticamente que las interacciones tensioactivo-disolvente. Así, estas estructuras surgen como resultado de un delicado balance entre interacciones soluto-soluto y soluto-disolvente [25].

La morfología del agregado supramolecular formado depende de la relación entre el tamaño del grupo polar y la cadena hidrófoba del tensioactivo. La ecuación desarrollada por Israelachvili [26] permite predecir la morfología de los agregados:

$$g = \frac{V}{a_0 l_c}$$

En esta fórmula g es el factor de empaquetamiento, V es el volumen de la cadena hidrófoba, a_0 es el área media de sección que ocupa la cabeza polar en el agregado y l_c es la longitud de la cadena del tensioactivo (Figura 2).

Tal y como se desprende de la ecuación, el parámetro g depende de la geometría molecular del tensioactivo y hace referencia al número de cadenas hidrocarbonadas y de átomos de carbono, al grado de saturación de la cadena y al tamaño y carga de la cabeza polar. Por otra parte, las propiedades de la disolución (pH, fuerza iónica, temperatura, presencia de co-tensioactivo) están implícitamente incluidas en V, a_0 y l_c .

[25]J.W. Steed, D.R. Turner, K.J. Wallace, Core concepts in Supramolecular Chemistry and Nanochemistry, John Wiley & Sons, Chichester (2007), Chapter 1. pp. 1-27.
[26]J.N. Israelachvili, D.J. Mitchell, B.W. Ninham, J. Chem. Soc. Faraday Trans. 72 (1976) 1525.

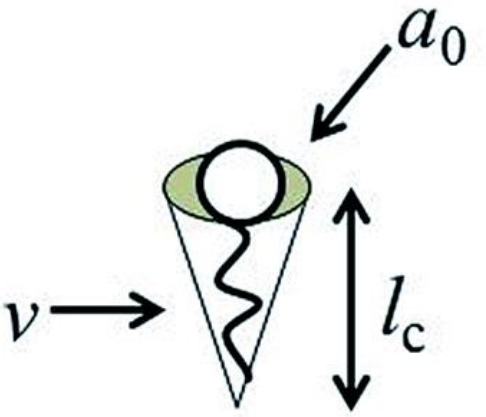
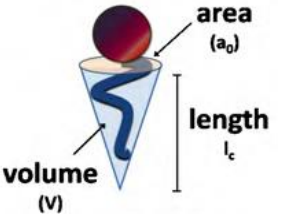
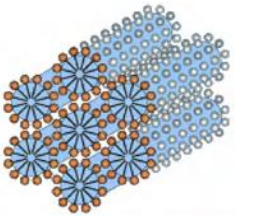
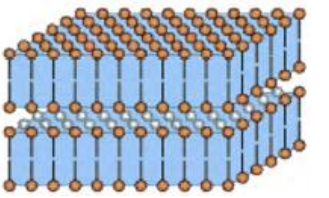
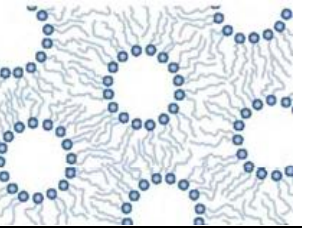


Fig. 2. Parámetros característicos de una molécula de tensioactivo.

Los tensioactivos en disolución forman diferentes estructuras dependiendo del valor del parámetro *g*. La morfología de los agregados depende principalmente de la relación entre la longitud de la cadena hidrocarbonada y el tamaño del grupo polar. Las principales estructuras formadas se muestran en la tabla 1.

Tabla 1. Morfología de los agregados supramoleculares en función del parámetro de empaquetamiento

Tipo de agregado	Parámetro de empaquetamiento	Geometría del anfifilo	Estructura del agregado
Micelas esféricas	$\frac{V}{a_0 l_c} < \frac{1}{3}$		
Micelas cilíndricas	$\frac{1}{3} < \frac{V}{a_0 l_c} > \frac{1}{2}$		
Bicapas o vesículas flexibles	$\frac{1}{2} < \frac{V}{a_o l_c} < 1$		
Bicapas planas	$\frac{V}{a_o l_c} \sim 1$		
Micelas inversas	$\frac{V}{a_a l_c} > 1$		

2.2. Coacervación

La coacervación es definida por la IUPAC como el fenómeno a través del cual se produce la separación de sistemas coloidales en dos fases líquidas [27]. A la fase más concentrada en el componente coloidal se le denomina coacervado; la otra fase, que tiene muy baja concentración en este componente, se denomina fase en equilibrio. El coacervado es por definición inmiscible en la disolución en la que se formó.

El fenómeno de la coacervación fue descrito en 1930 por los químicos holandeses Bungenberg de Jong y Kruyt y desde entonces, los coacervados se han utilizado en múltiples aplicaciones (purificación de proteínas, recuperación de acuíferos contaminados, tratamiento de aguas residuales, etc.). Entre las aplicaciones industriales de los coacervados destaca su uso para el microencapsulamiento de ingredientes activos en la industria farmacéutica, cosmética y agroalimentaria. [28]. El fenómeno de la coacervación también se ha aplicado en procesos de extracción analítica. En este caso los coacervados se generan siempre a partir de agregados moleculares de tensioactivos.

En base al mecanismo por el cual se produce la separación de fases, se distinguen dos tipos de coacervación [29,30]:

i. Coacervación simple: el componente coloidal es una macromolécula neutra o cargada disuelta en agua, o una macromolécula neutra disuelta en un disolvente orgánico. El agente externo que provoca la coacervación puede ser un cambio en el pH o la temperatura, la adición de un electrolito, o bien la adición de un disolvente miscible con el primero, pero en el que la macromolécula es poco soluble. La separación de fases se produce debido fundamentalmente a la desolvatación de las macromoléculas. En una suspensión coloidal, el disolvente interacciona con las macromoléculas a través de enlaces dipolo-dipolo, puentes de hidrógeno y/o fuerzas de Van der Waals; formando una capa alrededor de las macromoléculas que impide o limita la interacción entre las mismas. El agente inductor de la

[27]IUPAC Compendium of Chemical Terminology 31 (1972) 611

[28]Gander, B.; Blanco-Prieto, M.J.; Thomasin, C.; Wandrey, C.; Hunkeler, D. Coacervation/Phase Separation. In Encyclopedy of Pharmaceutical Technology; Swarbrick, J.; Boylan, J.C. (Eds.) Marcel Dekker (2002) New York

[29]Wang, Y.; Kimura, K.; Huang, Q.; Dubin, P.L.; Jaeger, W. Macromolecules 32 (1999) 7128.

[30]Mohanty, B.; Bohidar, H.B. Biomacromolecules 4 (2003) 1080

coacervación simple debe destruir la interacción disolvente-macromolécula favoreciendo de este modo la interacción entre macromoléculas. Los agregados formados son insolubles en la disolución a partir de la que se han generado y se separan de ella produciendo el coacervado.

ii. Coacervación compleja: se produce al añadir a una disolución acuosa de macromoléculas cargadas, una macromolécula de signo opuesto. En este tipo de coacervación el factor electrostático (densidad de carga de macromoléculas, fuerza iónica, etc.) es esencial para la formación del coacervado. Para favorecer la coacervación frente a la precipitación, la densidad de carga superficial no debe ser muy elevada y la distribución de la carga sobre los dos poliiones no debe ser complementaria, es decir, el espaciado entre cargas debe ser asimétrico. Al producirse la interacción, el complejo resultante retiene contraiones y una cantidad considerable de moléculas de disolvente.

La coacervación es un proceso cinético [31] Existen dos modelos para describir la cinética de separación de fases, el modelo de nucleación y crecimiento, y el de descomposición espinodal (Figura 3):

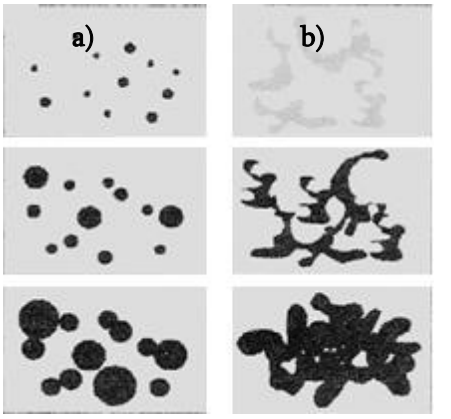


Fig. 3. Modelos de cinética de separación de fases a) Nucleación y crecimiento y b) Descomposición espinodal.

[31]Turgeon, S.L.; Beaulieu, M.; Schmitt, C.; Sánchez, C. *Current Opinión Colloid Interface Sci.* 8 (2003) 401

En el modelo de nucleación y crecimiento se produce la interacción de macromoléculas cercanas que a continuación experimentan coalescencia hasta alcanzar un determinado tamaño, y en ese instante se produce la separación de fases. A escala macroscópica, el coacervado está constituido principalmente por gotas esféricas dispersas en una fase continua, tal y como se puede observar en la imagen 4.

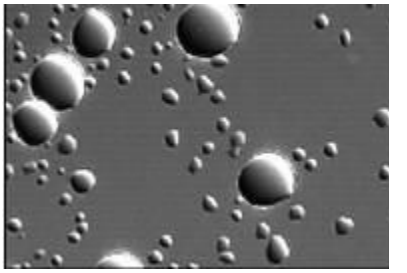


Fig. 4. Coacervado de ácido decanoico en una mezcla de tetrahidrofurano/agua, con estructura globular.

A diferencia del anterior, en el modelo de descomposición espinodal, la interacción implica a muchas macromoléculas en la etapa inicial, y se produce a nivel macroscópico una red tridimensional interconectada, que progresivamente se hace más densa. Esta red interconectada se puede apreciar en la imagen 5.

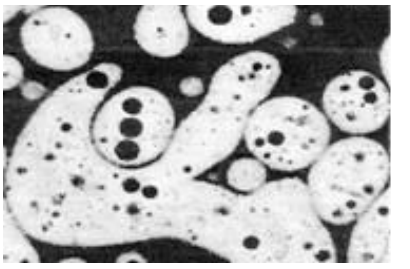


Fig. 5. Coacervado de β-lactoglobulina y goma arábiga, con estructura de red interconectada.

Con objeto de favorecer la interacción entre moléculas anfífilicas para inducir el crecimiento del agregado en coacervación simple deben establecerse las condiciones ambientales que desfavorezcan la interacción disolvente-anfifilo. En la tabla 2 se muestran las principales estrategias usadas dependiendo de la naturaleza del grupo polar de la molécula anfifílica.

Tabla 2. Estrategias para inducir la coacervación.

Tensioactivos Iónicos	Adición de un co-tensioactivo
	Adición de un electrolito
	Cambio de pH
Tensioactivos No Iónicos (C_nE_m)[a]	Adición de disolvente
	Aumento de la temperatura
Tensioactivos Zwiteriónicos	Disminución de la temperatura

[a] *n: longitud de la cadena hidrocarbonada; m: número de unidades de grupo oxietileno.*

Una de las estrategias más usadas para inducir el crecimiento de los agregados supramoleculares a partir de tensioactivos iónicos es la adición de un co-tensioactivo con un grupo polar pequeño, como un alcohol de cadena larga. Sin embargo, esta estrategia es difícil de implementar, ya que con frecuencia se origina una fase lamelar en lugar del disolvente supramolecular. También se puede adicionar un electrolito o un contraión anfifílico. En este caso, la cantidad de sal necesaria es menor cuanto mayor es la longitud de la cadena hidrocarbonada. Por otra parte, el ajuste de pH por debajo de la constante de ionización del grupo polar del tensioactivo se ha aplicado con éxito para la coacervación de alquil sulfatos, alquil sulfonatos, alquil benceno sulfonatos y alquil sulfosucinatos de diferente longitud de cadena hidrocarbonada (C_{10}-C_{14}). El problema que plantea el uso de esta estrategia es que se requiere un ambiente extremadamente ácido para producir la

separación de fases (2-3 M HCl), lo que supone una desventaja en algunas aplicaciones analíticas [32].

En el caso de agregados no iónicos (C_nE_m), la estrategia más efectiva para inducir la coacervación consiste en reducir el número de moléculas de disolvente disponibles para la solvatación, por ejemplo, incrementando la temperatura. Al incrementar la temperatura se produce la pérdida del agua del entorno de los grupos polares, lo que conlleva la reducción del área ocupada por el grupo polar, y se produce el crecimiento micelar. [9]. Por otro lado, las regiones polares de los grupos de oxietileno pertenecientes a distintos agregados se solapan, así potenciando la atracción entre micelas y favoreciendo la coacervación. Otra estrategia interesante para la síntesis de disolventes supramoleculares a partir de tensioactivos no iónicos es la utilización de una mezcla de dos disolventes miscibles, pero en los que el tensioactivo presente muy diferente solubilidad. Con esta estrategia, la desolvatación de los grupos polares se favorece por la interacción disolvente-disolvente que compite con la interacción disolvente-grupos polares. Un ejemplo de SUPRAS obtenido mediante este mecanismo es el de micelas inversas de ácidos carboxílicos en mezclas de tetrahidrofurano:agua, siendo el agente coacervante el agua [33].

La principal estrategia para promover el crecimiento micelar en agregados de tensioactivos zwiteriónicos es la reducción de la temperatura. Cabría esperar, dado su carácter fuertemente polar, que las interacciones electrostáticas sean fundamentales en este proceso; sin embargo, sus efectos son de corto alcance y diferentes a los ocurridos en los tensioactivos iónicos.

Las condiciones ambientales en las que se produce la coacervación se obtienen a partir de la construcción de los correspondientes diagramas de fases. La figura 6 muestra ejemplos de diagramas de fases para tensioactivos iónicos y no iónicos.

[32]I. Casero, D. Sicilia, S. Rubio, D. Pérez-Bendito, Anal. Chem. 71 (1999) 4519.
[33]F.J. Ruiz, S. Rubio, D. Pérez-Bendito, Anal. Chem. 79 (2007) 7473.

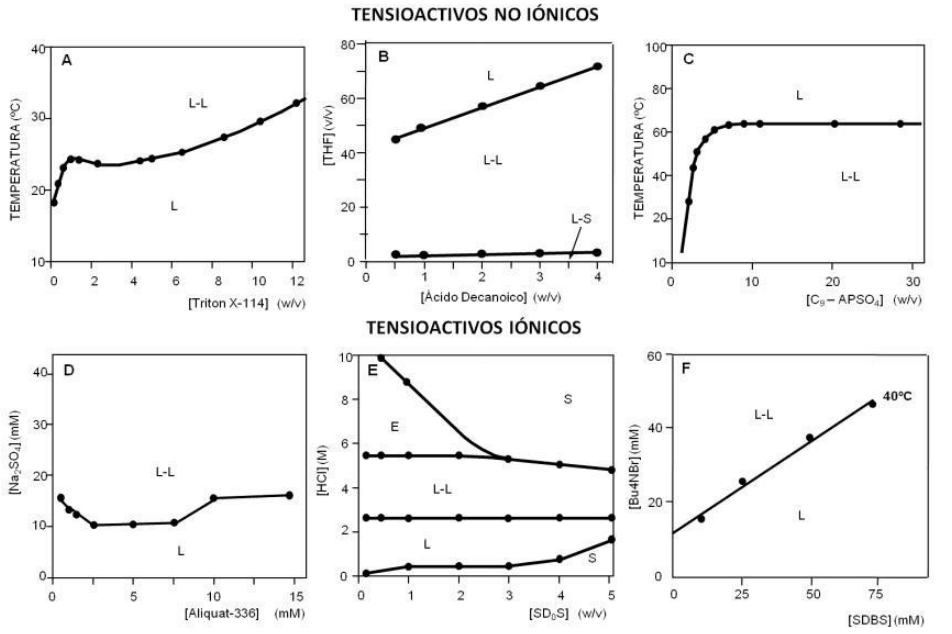


Fig. 6. Diagramas de fases de tensioactivos en diferentes condiciones ambientales: (a) Triton X-114 en función de la temperatura, (b) ácido decanoico en mezclas de THF:agua, (c) 3-(nonildimetilamonio)propil sulfato (C9-APSO4) en función de la temperatura, d) cloruro de tricaprilmetilamonio (Aliquat-336) en presencia de sulfato sódico, e) dodecano sulfonato sódico (SDoS) en presencia de ácido clorhídrico y f) dodecilbenceno sulfonato sódico (SDBS) en presencia de bromuro de tretrabutilamonio. (L): región de líquido homogéneo, (L-L): región de separación de dos fases líquidas; (E): región de emulsión; (S): región líquido-sólido.

Las interfases de estos diagramas se modifican en general, en función de la estructura del tensioactivo y de los aditivos adicionados a la disolución de síntesis. Por ejemplo, en el caso de diferentes homólogos de polioxietileno, se requiere mayor temperatura para la separación de fases a medida que disminuye la longitud de la cadena hidrocarbonada [34]. Para mezclas de dos tensioactivos no iónicos, la temperatura requerida para la síntesis del disolvente supramolecular suele ser intermedia a la de cada uno por separado, mientras que para mezclas de un no iónico y un iónico suele ser más alta que la del primero. Por otro lado, el agua requerida para la coacervación de

[34]P. Taechangam, J.F. Scamehorn, S. Osuwan, T. Rirksomboon, Colloids Surf. A: Physicochem. Eng. Aspects 347 (2009) 200.

micelas inversas de ácidos carboxílicos en tetrahidrofurano es mayor cuanto menor es la longitud de la cadena hidrocarbonada del tensioactivo.

3. Propiedades extractivas de los disolventes supramoleculares

Los SUPRASs presentan propiedades intrínsecas y operacionales que los convierten en candidatos ideales para sustituir a los disolventes orgánicos en procesos de extracción analíticos e industriales. Entre estas propiedades destacan:

1. Capacidad de solubilización simultánea de compuestos polares, apolares y anfifílicos;
2. Versatilidad en las estructuras y en los tipos de interacciones que proporcionan;
3. Elevado número de sustancias anfifílicas naturales y sintéticas comercialmente disponibles a bajo coste;
4. Procesos de síntesis de fácil implementación en cualquier laboratorio o industria;
5. Compatibilidad con las técnicas de detección más comunes.
6. Baja volatilidad e inflamabilidad, lo que permite desarrollar procesos de extracción menos contaminantes y más seguros.

3.1. Solubilización en SUPRAS

Los agregados de moléculas anfifílicas que constituyen los SUPRAS presentan regiones de diferente polaridad, acidez y viscosidad que son los responsables de su solubilización y de los efectos que ejercen en el entorno químico. La capacidad de solubilización de los SUPRASs depende del número de sitios de unión y del tipo enlace que proporcionen al analito. Los SUPRAS contienen elevada concentración del compuesto anfifílico, generalmente 0.1-1 mg/μL, y por lo tanto tienen la capacidad de solubilizar una elevada cantidad de soluto utilizando pequeños volúmenes de disolvente. Por otro lado, los

agregados supramoleculares generalmente ofrecen mecanismos mixtos de solubilización para el soluto. En la Figura 7 se muestra como ejemplo los diferentes microambientes presentes en los agregados de un SUPRAS de alcanol.

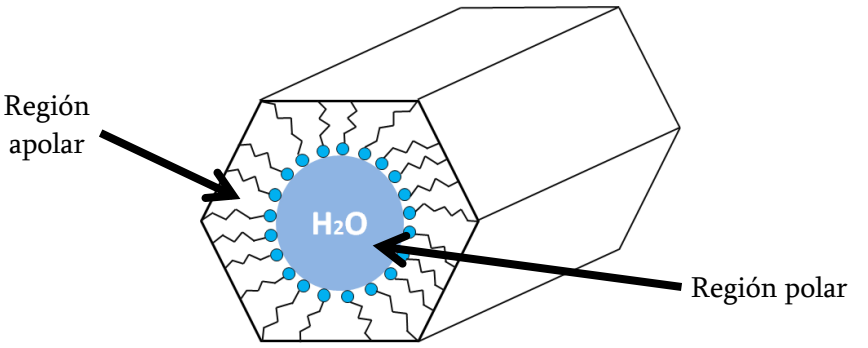


Fig. 7. Agregado hexagonal de micelas inversas de alcanoles formados en mezclas de tetrahidrofurano y agua.

Todos los disolventes supramoleculares, independientemente de la naturaleza del grupo polar de los compuestos anfifílicos que lo constituyen, proporcionan un ambiente apolar en la región hidrocarbonada de los agregados. En esta región, al igual que en los disolventes orgánicos convencionales, la solubilización de los solutos puede predecirse a través de los valores de las correspondientes constantes octanol-agua y está gobernada por interacciones de van der Waals. La solubilización de solutos en la región polar depende del tipo de interacciones que puedan establecerse (iónicas, puentes de hidrógeno, polares, π-catión, etc). Las energías de enlaces de estas interacciones siguen la secuencia iónica > puentes de hidrógeno > dipolo-dipolo > dipolo-dipolo inducido > fuerzas de van der Waals. Los grupos polares más frecuentemente utilizados para la extracción analítica de solutos han sido los óxidos de polietileno, ácidos carboxílicos, sulfatos, sulfonatos, amonio y piridinio.

Una de las propiedades más interesantes de los SUPRAS es su gran capacidad de extracción de compuestos anfifílicos (tensioactivos, drogas, algunos pesticidas, etc.) mediante la formación de agregados mixtos. Un ejemplo de una metodología analítica basada en esta propiedad es la extracción

de tensioactivos catiónicos en muestras sólidas ambientales con un SUPRAS constituido por micelas de dodecilsulfonato [35]. La extracción es rápida (1h) y cuantitativa a pesar de la fuerte interacción de los tensioactivos catiónicos con las matrices ambientales tales como lodos residuales y suelos y supone un gran avance respecto de los métodos tradicionales, que requieren grandes volúmenes de disolvente orgánico y etapas sucesivas que tardan en completarse alrededor de una semana.

La capacidad extractiva de los SUPRAS se define a través del coeficiente de distribución (D):

$$D = \frac{[A]_S}{[A]_{aq}}$$

donde $[A]_S$ y $[A]_{aq}$ son las concentraciones de analito en la fase SUPRAS y fase acuosa, respectivamente.

Por otro lado, la transferencia del soluto hacia el coacervado se puede también considerar como una reacción en la que se origina un producto [36, 37], de acuerdo a la ecuación:

$$Tensioactivo_{SUPRAS} + Soluto_{agua} \longleftrightarrow Tensioactivo - soluto_{SUPRAS}$$

En este caso, la solubilización viene definida por la constante de equilibrio de dicha reacción:

$$K_s = \frac{[Tensioactivo - Soluto]_{SUPRAS}}{[Tensioactivo]_{SUPRAS}[Soluto]_{agua}}$$

[35]F. Merino, S. Rubio, D. Pérez-Bendito, J. Chromatogr. A 998 (2003) 143–154.
[36]S.D. Christian, J.F. Scamehorn, Surfactant Science Series 33 (Surfactant-Bases Sep. Processes) (1989)
[37]W.L. Hinze, D.W. Armstrong, (Eds) Ordered media in Chemical Separations, ACS Symposium Series 342, American Chemical Society (1987) Washintong DC

3.2. Factores de concentración

Los factores de concentración dependen, además de la eficiencia de extracción, de la relación volumen (o cantidad) de muestra/volumen de SUPRAS.

El volumen de SUPRAS obtenido a través de autoensamblaje y coacervación varía linealmente con la cantidad de tensioactivo usado para la síntesis. El valor de la pendiente de la correspondiente recta nos permite predecir el factor de preconcentración teórico que puede alcanzarse en función del tensioactivo utilizado. Este dato es importante, dado que la extracción de solutos en muestras líquidas con SUPRAS siempre implica la formación in situ del mismo.

El volumen de SUPRAS también puede depender de las condiciones ambientales en las que se lleva a cabo la síntesis, especialmente de los factores inductores de la coacervación (ej. temperatura, concentración y tipo de sales, porcentaje de disolvente orgánico, etc). Los SUPRAS son generalmente sensibles a estímulos ambientales y su estructura y/o propiedades pueden modificarse en función de los mismos. En el caso de que haya cambios estructurales en el SUPRAS, lo cual es interesante para su uso como disolvente funcional, debe establecerse la dependencia del volumen de SUPRAS con respecto a la variable ambiental, con objeto de predecir los factores de concentración.

En la figura 8 se muestra, como ejemplo, la dependencia del volumen de SUPRAS obtenido a partir de ácido decanoico en medio THF:agua en función de la cantidad de tensioactivo (dependencia lineal) y porcentaje de THF (dependencia exponencial) utilizados en la síntesis del mismo. Estas dependencias indican que la composición del SUPRAS se mantiene constante cuando las condiciones ambientales de síntesis no varían (ej. relación THF/agua) y que la composición del mismo varía en función del medio de coacervación (ej. relación THF/agua)[38,39] (Figura 8).

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[38]S. García-Fonseca, A. Ballesteros-Gómez, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta 617 (2008) 3–10.
[39]C. Caballo, M.D. Sicilia, S. Rubio, Talanta, 119 (2014) 46-52

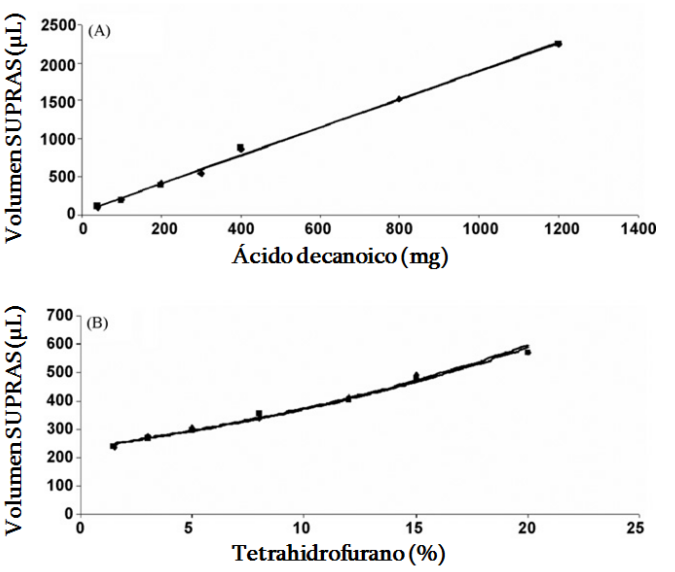

Fig. 8. Volumen de SUPRAS (µL) obtenido como función de (A): la cantidad de tensioactivo (ácido decanoico, mg) y (B): el porcentaje de tetrahidrofurano en muestras de vino tinto.

Se han observado cambios en la composición del SUPRAS y por tanto dependencias no lineales del volumen obtenido con respecto a variables tales como la temperatura [40], concentración de sales [41], concentración de ácido clorhídrico [42] o de co-tensioactivo [43]), etc.

Desde el punto de vista práctico, para la extracción de solutos en muestras líquidas, los mayores factores de preconcentración siempre se obtendrán utilizando SUPRAS generados a partir de bajas cantidades de tensioactivo. La influencia de las condiciones ambientales en los factores de concentración dependerá del agente de coacervación utilizado y debe determinarse para cada SUPRAS. Así, el factor de concentración teórico para solutos extraídos a partir de muestras acuosas utilizando SUPRAS de micelas

[40]R. Carabias,-Martínez, E. Rodríguez-Gonzalo, B. Moreno-Cordero, J.L. Pérez-Pavón, C. García-Pinto, E. Fernández Laespada, J. Chromatogr. A 902 (2000) 251.
[41]M.J. Rosen, Surfactants and Interfacial Phenomena, 3rd edition, John Wiley & Sons, Ltd., Hoboken, 2004, 197.
[42]D. Sicilia, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta, 460 (2002) 13.
[43]E.W. Crick, E.D. Conte, J. Chromatogr. A 877 (2000) 87.

inversas de ácido dodecanoico aumenta de 54 a 203 cuando el tensioactivo usado disminuye de 300 a 50 mg. Por otro lado, el factor de concentración teórico para el mismo SUPRAS aumenta de 38 a 94 cuando el porcentaje de THF en la disolución sintética disminuye del 25 al 12%. [44].

4. Formatos de extracción

La extracción de muestras líquidas con SUPRAS siempre implica la formación *in situ* del mismo. Por lo tanto, la generación del disolvente y la extracción se produce en una etapa única. En la figura 9 se muestra el esquema general más frecuentemente usado.

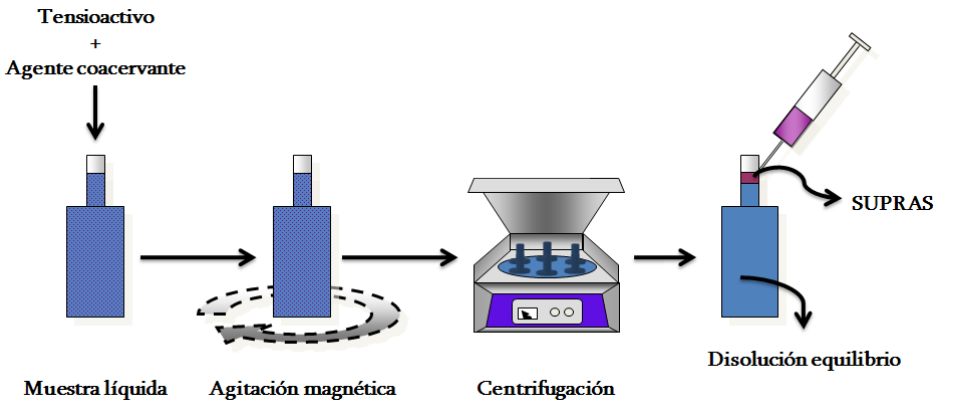

Fig. 9. Modo de operación para la extracción supramolecular de solutos en muestras líquidas.

El procedimiento de extracción generalmente consiste en la adición del tensioactivo (0.25-2%, m/v) a la muestra (10-100 mL) y establecimiento de las condiciones ambientales para que se produzca la coacervación. La formación del SUPRAS es espontánea e instantánea y debido a las microgotas que contiene, el equilibrio de extracción para el soluto se alcanza rápidamente mediante agitación magnética (1-10 minutos). Para favorecer y acelerar la

[44]C. Caballo, M.D. Sicilia, S. Rubio, Anal. Chim. Acta, 761 (2013) 102-108

separación de fases, se incluye una etapa de centrifugación. El extracto de SUPRAS obtenido se recoge y se analiza directamente para los compuestos de interés.

En el caso de SUPRAS cuya densidad sea mayor que la del agua, la recuperación del SUPRAS se facilita enfriando el tubo que contiene la mezcla muestra-SUPRAS una vez centrifugada. De esta forma, aumenta la viscosidad del SUPRAS, y la muestra se separa mediante decantación. La mayoría de los tensioactivos no-iónicos y zwiteriónicos empleados en extracciones analíticas (ej. Triton X-114, Triton X-100, PONPE 7.5, PEG/PPG-18/18 dimeticona, etc.), son más densos que las muestras acuosas. Por otro lado, los SUPRAS sintetizados a partir de tensioactivos catiónicos, aniónicos, ácidos alquil carboxílicos, etc, son menos densos que el agua y no necesitan de una etapa de enfriamiento, ya que flotan sobre la disolución de equilibrio (Figura 10).

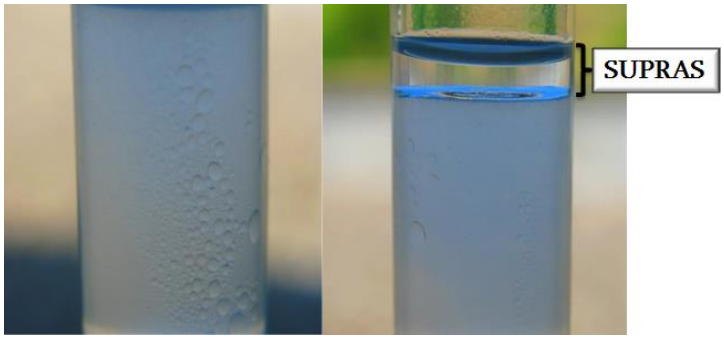

Fig. 10. Gotitas del SUPRAS formado por 1-decanol en tetrahidrofurano/agua antes y después de la etapa de centrifugación.

El volumen obtenido de SUPRAS, necesario para el cálculo de la concentración de soluto en las muestras, puede estimarse a partir de las ecuaciones previamente derivadas, una vez que se ha establecido la dependencia del mismo con respecto a la cantidad de tensioactivo y las condiciones ambientales. En el caso de que no se hayan derivado estas ecuaciones, se puede calcular el volumen de SUPRAS mediante medida directa o por pesada.

Para la medida directa, la extracción se efectúa en tubos cilíndricos de centrífuga. El volumen de SUPRAS generado puede calcularse fácilmente a partir de la expresión:

$$V_{SUPRAS} = \pi\, r^2 h$$

siendo r el radio de la sección de tubo y h la altura de la columna de líquido que el SUPRAS ocupa en el mismo. En el caso de SUPRAS menos densos que el agua, la exactitud de las medidas puede mejorarse usando tubos de centrífuga en los que se ha estrechado la sección en la zona en la que se ubicará el SUPRAS y midiendo la altura con un calibre digital.

Para calcular el volumen de SUPRAS por pesada, una vez que se conoce la densidad del mismo, éste se separa completamente de la muestra utilizando una microjeringa.

La extracción de muestras sólidas con SUPRAS puede realizarse siguiendo dos procedimientos que básicamente se diferencian en la forma en la que se sintetiza el SUPRAS; *in situ* o separadamente. En la figura 11 se muestran los esquemas de ambos procesos.

El procedimiento habitual para la extracción de muestras sólidas con formación in situ del SUPRAS es el siguiente (Fig. 11a): A la muestra sólida (0,1-1 g) se le añade una disolución que contiene el tensioactivo y se establecen las condiciones ambientales requeridas para la coacervación. Al igual que en las muestras líquidas, el proceso de generación del SUPRAS y extracción del soluto ocurre en una etapa simple. Después de la centrifugación se obtienen tres fases; un residuo sólido constituido por los componentes insolubles de la matriz, una disolución de equilibrio acuosa o hidroorgánica y el extracto de SUPRAS. Este procedimiento es muy adecuado para la extracción de compuestos apolares, ya que la solubilización de los mismos ocurre preferencialmente en el SUPRAS [38,45]. Asimismo, la fase intermedia actúa como un medio de eliminación de gran parte de las interferencias polares de la muestra. Este procedimiento, sin embargo, no es adecuado para la extracción de analitos polares, ya que éstos pueden distribuirse entre la

[45]F. J. López-Jiménez, S. Rubio, D. Pérez-Bendito, Food Chem. 121 (2010) 763–769.

disolución de equilibrio y el SUPRAS así disminuyendo el rendimiento de extracción. La formación previa del SUPRAS y adición directa de un volumen del mismo a las muestras obvia este problema (11b).

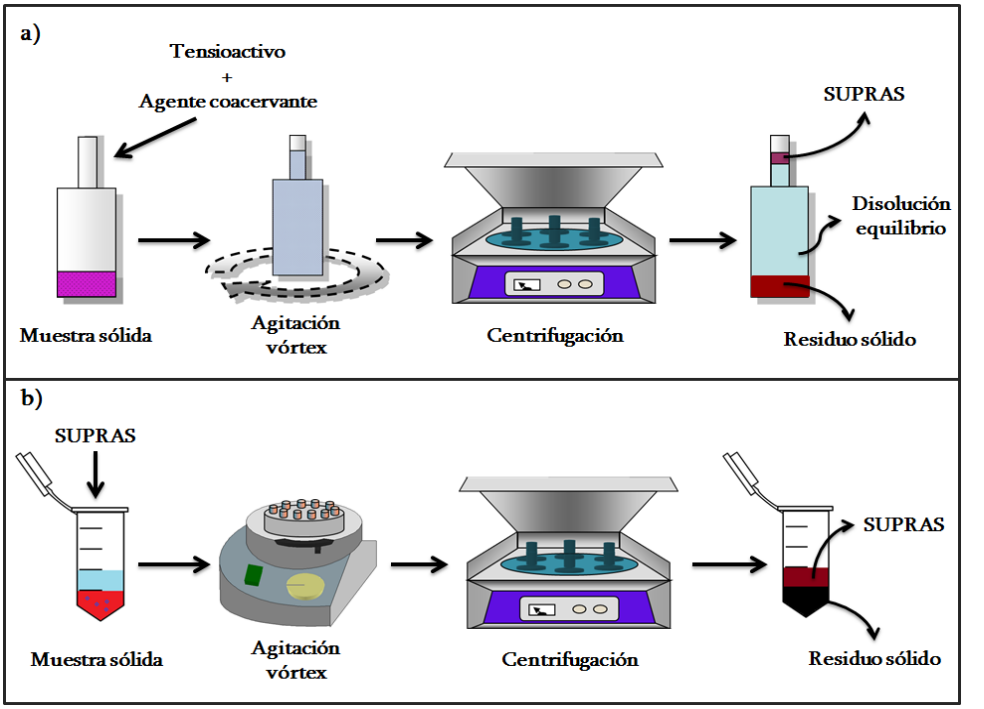

Fig. 11. Esquema de los procesos de extracción en muestras sólidas. a) Formación in situ del SUPRAS; b) Adición de un volumen determinado de SUPRAS previamente sintetizado.

En la figura 11b se muestra el esquema recomendado para la extracción de analitos polares. El procedimiento consiste en la adición de un pequeño volumen de SUPRAS a la muestra (0.1-1 g), agitación de la misma en vortex y centrifugación para separar el residuo no soluble del extracto de SUPRAS.. La relación volumen de SUPRAS (µL)/cantidad de muestra (mg) suele estar comprendida entre 1 y 3. Este formato de extracción se ha aplicado a la determinación de herbicidas quirales (mecoprop y dichlorprop) en suelos [39];

y PAHs en muestras de alimentos [46], obteniendo en ambos casos recuperaciones cercanas al 100%.

Los disolventes supramoleculares también se han empleado en el formato de extracción en una gota (single-drop microextraction, SDME) para la extracción de clorofenoles en aguas ambientales [47]. Para ello se usan SUPRAS constituidos por vesículas de ácidos carboxílicos. En la figura 12a se muestra el dispositivo diseñado para la microextracción.

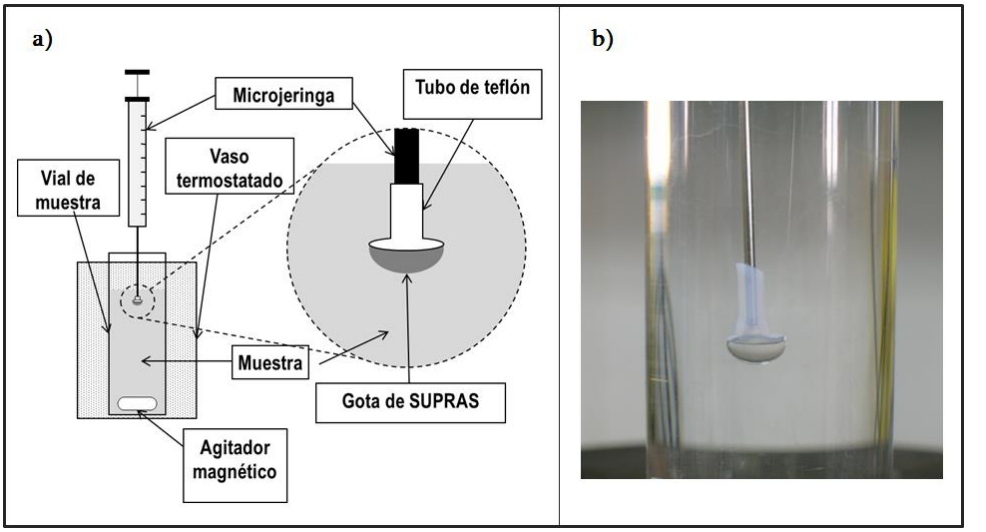

Fig. 12. a) Esquema ilustrativo del dispositivo empleado en la microextracción coacervativa; b) fotografía de la gota esférica empleada para la microextracción.

La formación de gotas esféricas y estables de SUPRAS en la punta de microjeringas convencionales depende del tipo de fuerzas intermoleculares que se establecen entre las cabezas polares del tensioactivo. Los puentes de hidrógeno son suficientemente estables para permitir la formación de gotas esféricas, por lo que los SUPRASs compuestos de ácidos carboxílicos son ideales para esta aplicación. El uso de SUPRASs en SDME constituye una alternativa ventajosa al uso de disolventes orgánicos convencionales en

[46]F.J. López-Jiménez, A. Ballesteros-Gómez, S. RubioFood Chemistry 143 (2014) 341–347
[47]F.J. López-Jiménez, S. Rubio, D. Pérez-Bendito, J. Chromatogr. A 1195 (2008) 25-33.

aplicaciones en las que los analitos son polares o cuando la determinación posterior de los mismos se realiza mediante cromatografía de líquidos.

Además de la miniaturización, otra tendencia en la extracción con SUPRASs es la automatización. Los primeros avances en esta dirección los llevaron a cabo Fang et al. [48] al utilizar un SUPRAS de tensioactivo no iónico en análisis por inyección en flujo (flow injection analysis, FIA). La principal dificultad técnica que presenta la miniaturización es inducir la formación de los agregados supramoleculares y su separación en una nueva fase, pero en este caso la separación de fases se lleva a cabo por adición de una sal en lugar de temperatura, lo cual simplifica el proceso; y se usa una columna de algodón para retener los agregados de tensioactivo del SUPRAS que contienen los analitos. La elución de los mismos se lleva a cabo con un disolvente orgánico. También se han desarrollado acoplamientos en línea a cromatografía de líquidos-fluorescencia (CL-Fl) [49]. Para ello, las muestras acuosas, a las que se les ha adicionado el tensioactivo (Tergitol 15-S-7), se inyectan en la válvula de CL de seis vías y se añade sulfato amónico para inducir la separación de fases. La fase rica en tensioactivo y los analitos (PAHs) se retiene en una columna de gel de sílice. Pasados unos minutos se cambia la válvula a la posición de inyección, de modo que la fase móvil arrastra al analito y al tensioactivo hasta el sistema de CL-Fl.

5. Compatibilidad con técnicas de separación/detección

Los SUPRASs se han usado principalmente en combinación con cromatografía de líquidos (CL) acoplada a detectores de UV-visible, fluorescencia y espectrometría de masas (EM). Los extractos de SUPRAS son compatibles con las fases móviles normalmente empleadas en CL, por lo que tras llevar a cabo la extracción el extracto obtenido se inyecta el disolvente, normalmente sin previa dilución o clean-up. Si en el sistema cromatográfico se emplea una fase móvil hidroorgánica (contenido de disolvente orgánico > 60%), los agregados supramoleculares se destruyen en contacto con ella y producen una elevada concentración de monómeros de tensioactivo que pueden separarse de los analitos y por tanto no influye en su comportamiento

[48]Q. Fang, M. Du, C.W. Huie, Anal. Chem. 73 (2001) 3502.
[49]C. F. Li, J.W.C. Wong, C.W. Huie, M.W.F. Choi, J. Chromatgr. A 1214 (2008) 11.

cromatográfico. Sin embargo, cuando se usan fases móviles con un alto contenido en agua (> 40%), estos agregados se destruyen lentamente a lo largo del sistema cromatográfico, dando así lugar a una pseudofase donde los analitos se pueden distribuir. Este mecanismo de separación adicional, puede beneficiar o perjudicar la resolución cromatográfica de los solutos de interés. En estas condiciones cromatogáficas se aconseja diluir el extracto con disolvente orgánico (por ejemplo dos veces) previa a su introducción en el cromatógrafo de líquidos.

Las propiedades físico-químicas del tensioactivo y su estructura son factores esenciales que deben ser considerados para lograr una adecuada separación cromatográfica y posterior detección del analito. Por ejemplo, si los anlitos a determinar presentan una polaridad media-alta, los tensioactivos no iónicos pertenecientes a las series de Triton X (octil fenoles etoxilados) y PONPE (nonil fenoles etoxilados), no son una buena opción, ya éstos están disponibles comercialmente como una mezcla de homólogos y oligómeros; lo que incrementa las probabilidades de que alguno de los componentes coeluya con los analitos e interfiera con su determinación. Una alternativa para solventar este inconveniente es el uso de tensioactivos iónicos o zwiteriónicos no aromáticos, los cuales eluyen a bajos tiempos de retención, produciendo picos cromatográficos estrechos y cuyos máximos de absorción se encuentran por debajo de los 210 nm.

Cuando se usa espectrometría de masas, como regla general se envía a desecho todo el eluido después de la separación cromatográfica, a excepción de la ventana de elución de los compuestos de interés. De este modo se evita la llegada a la fuente de compuestos que pueden originar el ensuciamiento de la misma.

Los extractos de los SUPRAS desarrollados hasta la fecha no pueden introducirse directamente en cromatografía de gases (CG). Su uso en combinación con esta técnica requiere la eliminación previa del tensioactivo mediante extracción en fase sólida (ej. utilizando dos procesos secuenciales con gel de sílice y Fluorisil para la eliminación de Triton X-114 en la determinación de pesticidas [50]) o re-extracción líquido-líquido del analito (ej. PAHs o difenil éteres polibrominados) en un disolvente inmiscible en

[50]A.M. Faria, R.P. Dardengo, C.F. Lima, A.A. Neves, M.E.L.R. Queiroz, Int. J. Environ. Anal. Chem. 87 (2007) 249.

agua, usando energía de microondas o ultrasonidos [51, 52]. Otra de las estrategias propuestas es la derivatización del tensioactivo (ej. Triton X-114 derivatizado con N,O-bis(trimetilsilil) trifluoroacetamida [53]). De esta forma, se obtiene un pico estrecho que permite la cuantificación exacta y reproducible de una variedad de analitos como PAHs, herbicidas y profenos, e incluso de analitos poco volátiles (ibuprofeno y flurbiprofeno), que son derivatizados al mismo tiempo que el tensioactivo.

Los disolventes supramoleculares también son compatibles con algunos modos de electroforesis capilar (EC), sin embargo el acoplamiento de SUPRAS con CE no ha sido un área fructífera hasta la fecha. Los extractos de SUPRAS son compatibles con las fases móviles usadas en electrocromatografía capilar ya que se trata de una técnica híbrida entre EC y CL. El único tratamiento requerido antes de la inyección es la dilución del extracto con un disolvente orgánico para evitar la obstrucción del capilar. Esta estrategia se ha aplicado a la determinación de PAHs, dibenzo-p-dioxinas policloradas (PCDDs) y ftalatos usando fases estacionaria de C18 [40, 54]. Se debe tener en cuenta que aunque las separaciones son reproducibles, los tiempos de migración de los estándares y los analitos en el SUPRAS pueden diferir debido al recubrimiento dinámico de la fase estacionaria con el tensioactivo.

También se han obtenido resultados satisfactorios combinando extractos de SUPRAS y electroforesis capilar de zona usando medios no acuosos, lo que evita la adsorción del tensioactivo sobre la pared del capilar [55, 56]. Cuando se usan disoluciones reguladoras acuosas se produce la adsorción de una elevada cantidad de tensioactivo sobre la pared del capilar de sílice, lo que conlleva una marcada pérdida de eficiencia y reproducibilidad de los tiempos de migración y los picos electroforéticos [57]. La combinación de SUPRAS con cromatografía micelar electrocinética también es posible diluyendo el SUPRAS con disolvente orgánico para reducir su viscosidad. Con

[51]T.I. Sikalos, E.K. Paleologos, Anal. Chem. 77 (2005) 2544
[52]A.R. Fontana, M.F. Silva, L.D. Martínez, R.G. Wuilloud, J.C. Altamirano, J. Chromatogr. A 1216 (2009) 4339.
[53]Y. Takagai, W.L. Hinze, Anal. Chem. 81 (2009) 7113.
[54]S.R. Sirimanne, J.R. Barr, D.G. Patterson, J. Microcol. Sep. 11 (1999) 109.
[55]R. Carabia-Martínez, E. Rodríguez-Gonzalo, J. Domínguez-Álvarez, J. Hernández-Méndez, Anal. Chem. 71 (1999) 2468.
[56]R. Carabia-Martínez, E. Rodríguez-Gonzalo, J. Domínguez-Álvarez, C. García Pinto, J. Hernández-Méndez, J. Chromatogr. A 1005 (2003) 23.
[57]Y.W. Wu, Y.Y. Jiang, T.X. Xiao, H.L. Zhang, J. Sep. Sci. 31 (2008) 865.

esta estrategia se han analizado con éxito compuestos fenólicos en aguas [58] y verde de malaquita en pescado [59]. Por último, se han abordado estrategias para eliminar el tensioactivo antes de su introducción en CE, bien mediante re-extracción de los analitos en disolución acuosa, previa derivatización de los mismos para conferirles carácter hidrófilo o basándose en sus características ácido-base [60].

6. Aplicaciones analíticas

Los disolventes supramoleculares se han empleado extensamente en las últimas décadas, para la extracción de compuestos orgánicos y metales a partir de muestras ambientales, agroalimentarias y biológicas. A continuación se indican algunas características generales de los diferentes SUPRAS cuando se aplican a la determinación de compuestos orgánicos.

6.1. SUPRAS formados a partir de micelas acuosas de tensioactivos no-iónicos

Los SUPRASs constituidos por agregados de tensioactivos no iónicos, obtenidos mediante incremento de la temperatura de la disolución, han sido los más usados en extracciones analíticas, a las que se le ha denominado técnica de punto de nube (*cloud point extraction*). Esto ha sido principalmente debido a la escasa atención que se ha prestado al desarrollo de nuevos SUPRAS. Los tensioactivos no iónicos más utilizados han sido alquil fenol etoxilados Triton X-114, Triton X-100 y Genapol X-080, a concentraciones aproximadas del 1%, aunque para algunas aplicaciones (ej. determinación de compuestos bioactivos en muestras biológicas) se requieren concentraciones mayores (3-10%). Los factores de concentración obtenidos con este tipo de SUPRAS no son generalmente elevados (1-10), aunque se pueden incrementar los mismos modificando la composición del SUPRAS

[58]P.W. Stege, L.L. Sombra, G.A. Messina, L.D. Martínez, M.F. Silva, Anal. Bioanal. Chem. 394 (2009) 567.
[59]X. Luo, X. Jiang, X. Tu, S. Luo, L. Yan, B. Chen, Electrophoresis 31 (2010) 688.
[60]X.B. Yin, J.M. Guo, W. Wei, J. Chromatogr. A 1217 (2010) 1399.

mediante la variación de las condiciones ambientales (ej. adicionando sales o incrementando la temperatura). De esta forma, se han obtenido factores de concentración en el intervalo 73-152 para la extracción de estriol, estradiol, estrona y progesterona con Triton X-114 (0.25%) adicionando una sal (0,4M Na_2SO_4) a 45 ºC [61]. Otro ejemplo es el uso de SUPRAS formados con 0.1% de Triton X-114 y 0.2M de NaCl a 60ºC, para la extracción de residuos de filtros UV (Eusolex 232, Benzophenone-3, Eusolex 6300, Eusolex 9020, Eusolex 2292), con un factor de concentración de 500 [62].

6.2. SUPRAS formados a partir de micelas acuosas de tensioactivos iónicos

Entre los SUPRASs formados por tensioactivos iónicos los más usados en extracciones analíticas han sido los producidos en medio ácido. Esto se debe a que la principal desventaja que presentan, la formación bajo condiciones extremas (3-4M HCl o 4g NaCl), puede ser muy ventajosa en ciertas aplicaciones, como la extracción de tensioactivos catiónicos a partir de muestras de suelo, lodo y sedimentos [63]. El medio ácido favorece la desorción del tensioactivo de la matriz mediante intercambio iónico.

Otras ventajas que han favorecido su utilización es que proporcionan elevados factores de concentración a bajas concentraciones de tensioactivo (140 con 0.1% of dodecano sulfonato [64]) y un comportamiento cromatográfico adecuado para su uso en combinación con espectrometría de masas. Los tensioactivos aniónicos, a diferencia de los no-iónicos, están disponibles comercialmente como un único homólogo por lo que proporcionan un único pico cromatográfico estrecho, facilitando el enviar a desecho la fracción en la que eluye.

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[61]L. Wang, Y.Q. Cai, B. He, C.-G. Yuan, D.-Z. Shen, J. Shao, G.-B. Jiang, Talanta 70 (2006) 47.
[62]D.L. Giokas, V.A. Sakkas, T.A. Albanis, D.A. Lampropoulou, J. Chromatogr. A 1077 (2005) 19.
[63]M. Cantero, S. Rubio, D. Pérez-Bendito , J. Chromatogr. A 1046 (2004) 147.
[64]D. Sicilia, S. Rubio, D. Pérez-Bendito, N. Maniasso, E. A. G. Zagatto, Anal. Chim. Acta 392 (1999) 29.

Por otra parte, se han descrito aplicaciones muy interesantes con SUPRASs constituidos por tensioactivos catiónicos, como la extracción de clorofenoles de muestras acuosas [65].

6.3. SUPRAS formados a partir de micelas acuosas mixtas

La formación de micelas con mezclas de tensioactivos iónicos y no iónicos es una excelente estrategia para la extracción de compuestos iónicos. Habitualmente la mezcla se compone de Triton X-114 y los tensioactivos dodecilsulfato sódico, cetiltrimetil amonio o cloruro de cetilpiridinio. Este tipo de SUPRAS se ha aplicado a la extracción de pesticidas, colorantes y ácidos húmicos y fúlvicos, a partir de muestras ambientales y biológicas [66-68]. Por otro lado, la formación de micelas con mezclas de dos tensioactivos no iónicos se ha propuesto para la extracción de PAHs, ya que al parecer presentan una mayor superficie activa comparada con las micelas no iónicas simples; y proporcionan una mayor selectividad. La mayor desventaja de estas micelas es la elevada temperatura de trabajo (78ºC) que se recomienda [69,70].

6.4. SUPRAS formados a partir de micelas inversas

Los SUPRASs formados a partir de micelas inversas se han aplicado a la extracción de una amplia variedad de compuestos orgánicos como PAHs, colorantes, alteradores endocrinos, micotoxinas o sustancias bioactivas, en muestras ambientales y agroalimentarias [71 - 77]. La principal ventaja que

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[65]X. Jin, M. Zhu, E.D. Conte, Anal. Chem. 71 (1999) 514.
[66]A.R. Zarei, Anal. Biochem. 369 (2007) 161-167.
[67]N. Pourreza, M. Zareian, J.Hazard. Mater. 165 (2009) 1124-1127.
[68]J.C.A. Wuilloud, R.G. Wuilloud, B.B.M. Sadi, J.A. Caruso, Analyst 128 (2003) 453-458.
[69]B.Delgado, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Anal. Chim. Acta 518 (2004) 165-172.
[70]B.Delgado, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Analyst 130 (2005) 571-577.
[71]A. Ballesteros-Gómez, S. Rubio, D. Pérez-Béndito, J. Chromatogr. A 1216 (2009) 530-539.
[72]A. Ballesteros-Gómez, F.J. Ruiz, S. Rubio, D. Pérez-Béndito, Anal. Chim. Acta 603 (2007) 51-59.

presentan estos SUPRAS respecto a los demás, es la elevada concentración de anfifilos que contienen (> 0,75 mg/μL), lo cual permite obtener grandes factores de concentración en muestras líquidas. La extracción de compuestos polares es muy efectiva debido a la posibilidad que tienes estos SUPRAS para establecer puentes de hidrógeno e interacciones polares con los solutos.

6.5. SUPRAS formados a partir de vesículas

Los SUPRAS formados a partir de vesículas de ácidos alquil carboxílicos contienen la mayor concentración de anfifilos (1 mg/μL) de todos los SUPRAS descritos hasta la fecha, lo que ha permitido la extracción de solutos con factores de concentración superiores a 700. Además ofrecen una serie de ventajas adicionales como los diferentes tipos de interacciones que pueden establecer (iónicas, puentes de hidrógeno, π-catión e hidrofóbicas), la elevada estabilidad cinética de los agregados que conforman el SUPRAS, así como la gran fuerza de cohesión entre las moléculas que constituyen los agregados. Se han aplicado con éxito a la extracción de pesticidas, alteradores endocrinos y fenoles en muestras de alimentos y ambientales [38,78-80].

7. Disolventes supramoleculares con propiedades de acceso restringido (SUPRAS-RAM)

Todos los SUPRAS son, por su naturaleza intrínseca, sensibles a estímulos ambientales. Ello se debe a que las fuerzas que dirigen el proceso de

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[73]A. Ballesteros-Gómez, S. Rubio, D. Pérez-Béndito, J. Chromatogr. A 1203 (2008) 168-176.
[74]A. García-Prieto, L. Lunar, S. Rubio, D. Pérez-Béndito, Anal. Chim. Acta 617 (2008) 51-58.
[75]A. García-Prieto, L. Lunar, S. Rubio, D. Pérez-Béndito, Food Addit. Contam. Part. A 26 (2009) 265-274.
[76]A. García-Prieto, L. Lunar, S. Rubio, D. Pérez-Béndito, Anal. Chim. Acta 630 (2008) 19-27.
[77]S. García-Fonseca, A. Ballesteros-Gómez, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta 617 (2008) 3-10.
[78]A.Moral, M.D. Sicilia, S. Rubio, Anal, Chim. Acta 650 (2009) 207-213.
[79]F.J. Ruiz, S.Rubio, J. Chromatogr. A 1163 (2007) 269-276.
[80]A.Moral, M.D. Sicilia, S. Rubio, J. Chromatogr. A 1216 (2009) 3740-3745.

auto-ensamblaje y coacervación son siempre no covalentes y por tanto reversibles. Los agregados, en los diferentes niveles, se forman mediante un balance de fuerzas de atracción entre las cadenas hidrocarbonadas y de repulsión entre los grupos polares. Si las condiciones ambientales eliminan la repulsión se favorece el crecimiento de los agregados y por tanto la coacervación. En el caso de que se favorezca la repulsión, el coacervado se destruye y se forman agregados de menor tamaño. Podemos por tanto manipular el tamaño de los agregados formados y existe el potencial para manipular las estructuras y propiedades de los SUPRAS para que cumplan funciones específicas (SUPRAS funcionales).

Nuestro grupo de investigación ha descrito por primera vez SUPRAS funcionales que presentan propiedades de acceso restringido [81]. Estos SUPRAS-RAM se sintetizan espontáneamente a partir de disolución de alcanoles (C_6-C_{14}) en tetrahidrofurano (THF) mediante adición de agua. Están formados por agregados hexagonales inversos que contienen cavidades acuosas rodeadas por los grupos alcoholes mientras las cadenas hidrocarbonadas están disueltas en THF (Figura 13A).

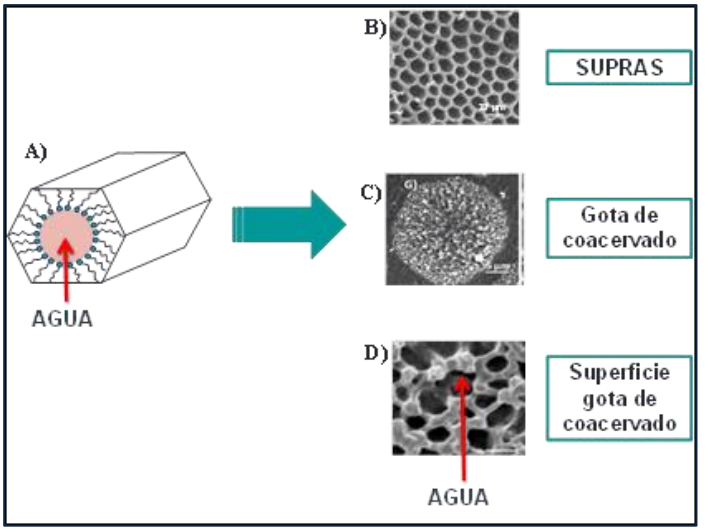

Fig. 13. Diagrama esquemático de los agregados de alcanol en el SUPRAS (A), y microfotografías obtenidas mediante crio-SEM para el SUPRAS (B), una de las gotitas que lo conforman (C) y estructura de la superficie de la misma (D).

[81] A. Ballesteros-Gómez, F.J. Ruiz, S. Rubio, D. Pérez-Béndito, Anal. Chem. 81 (2009) 21, 9012-9020

En la figura 13 también se muestran fotografías, obtenidas mediante crio-microscopía electrónica de barrido, del SUPRAS (Fig. 13B), una gotita de coacervado (Fig. 13C) y la superficie porosa de la misma (Fig. 13D). Seleccionando el porcentaje de THF y agua en la disolución de síntesis, pueden controlarse la composición del SUPRAS (es decir, la cantidad relativa de alcanol, agua y THF que contiene), el tamaño de las gotitas que lo constituyen y el tamaño de las cavidades acuosas de las estructuras hexagonales invertidas en las que se auto-ensamblan los alcanoles [81].

Como un ejemplo, en las figuras 14A-C, obtenidas mediante microscopia óptica, se aprecia cómo aumenta el tamaño de las gotitas que forman el SUPRAS a medida que aumenta el porcentaje de THF en la disolución de síntesis. Las nanoestructuras formadas son reversibles y sus características se modifican espontáneamente cuando se producen cambios en el medio en el que se generan. El auto-ensamblaje sigue rutas predecibles, de forma que tanto la composición como el volumen de coacervado pueden predecirse a partir de modelos matemáticos.

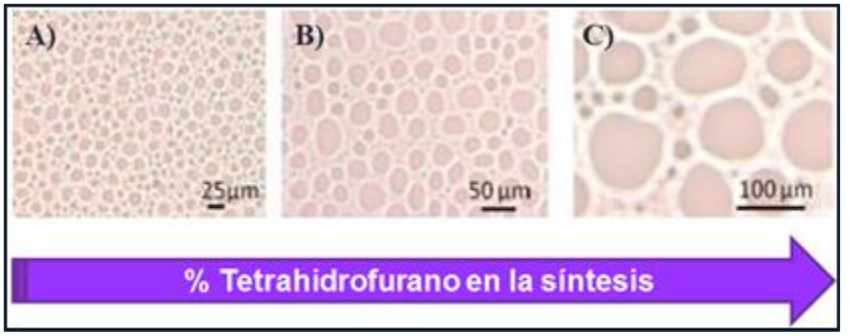

Fig. 14. Microfotografías obtenidas mediante microscopía óptica de las gotitas que forman el SUPRAS, sintetizadas a partir de diferentes porcentajes de tetrahidrofurano: (A) 20%, (B) 50% y (C) 60%.

El aspecto más interesante de estos SUPRAS es que el tamaño de las cavidades acuosas de los agregados hexagonales inversos aumenta cuando lo hace el porcentaje de THF en la disolución de síntesis. Ya que existen en los agregados dos microambientes (polar en la cavidad acuosa y apolar en las cadenas hidrocarbonadas disueltas en THF, Fig. 13A), estos SUPRAS pueden

utilizarse como materiales de acceso restringido para sustancias iónicas y/o polares.

En la Tabla 3 se muestran los porcentajes de recuperación para tres compuestos iónicos de diferente peso molecular (Ocratoxina A, OTA, PM= 403.8; Rojo ácido 97, PM=698.6; y Azul brillante G, PM=825,9), extraídos a partir de cereales infantiles (OTA) y lodos residuales (Rojo ácido 97 y Azul Brillante G), en función del porcentaje de THF en la disolución de síntesis. Se observa que a medida que aumenta el porcentaje de THF, y por tanto el tamaño de las cavidades acuosas, aumenta la recuperación para los tres compuestos. Si se comparan las recuperaciones para un determinado SUPRAS (ej. obtenido con el 30% de THF), se observa claramente que la recuperación depende del PM del compuesto. Por lo tanto, estos SUPRAS-RAM pueden excluir de la extracción a macromoléculas y tienen el potencial de extraer analitos a partir de muestras líquidas y sólidas sin extraer proteínas, polisacáridos, ácidos húmicos y fúlvicos, etc, con los que se lleva a cabo la limpieza simultánea de la muestra.

Tabla 3. Recuperaciones (%) para la extracción de Ocratoxina A en cereales infantiles y colorantes en lodo residual.

Tetrahidrofurano (%)	Recuperación (%)		
	Ocratoxina A (PM 403,5)	Rojo ácido (PM 698,6)	Azul brillante (PM 825,9)
10	23	-	-
20	45	18	-
30	80	36	50
50	109	100	54

Hemos comprobado que la exclusión de macromoléculas por los SUPRAS-RAM puede realizarse a través de mecanismos químicos y físicos. Así las proteínas se eliminan por precipitación inducida por la disminución de la constante dieléctrica en presencia de THF y la formación de complejos con alcanoles. Tanto los polisacáridos como los ácidos húmicos y fúlvicos no se extraen debido a fenómenos de exclusión de tamaño en la cavidad acuosa del SUPRAS-RAM.

Un ejemplo de la aplicación de los SUPRAS-RAM para simplificar el tratamiento de muestra en métodos analíticos es la determinación de alteradores endocrinos en sedimentos [82].

Los alteradores endocrinos contienen grupos hidroxilo y cetónicos y por tanto su extracción se lleva a cabo mediante mecanismos mixtos: interacción por puentes de hidrógeno y fuerzas de Van der Waals. El peso molecular de los mismos es inferior a 300 y las recuperaciones obtenidas con el SUPRAS-RAM son cuantitativas para porcentajes de THF entre 20 y 60%. Los principales componentes orgánicos de la matriz (ácidos húmicos y fúlvicos) no se extraen y el tratamiento de muestra consta de una única etapa; microextracción con 400 µL de SUPRAS durante 10 min e inyección directa del extracto en CL-MS/MS para cuantificación.

Similarmente a los alcanoles, los ácidos alquilcarboxílicos originan SUPRAS en mezclas de THF y agua [33] y los resultados obtenidos hasta la fecha indican que estos también tienen propiedades de acceso restringido.

La coacervación de ácidos alquilcarboxílicos saturados (C_8-C_{16}) e insaturados (C_{18}) ocurre en una amplia variedad de mezclas de agua y disolventes orgánicos, tanto próticos (etilenglicol, metanol, etanol, 1-propanol) como apróticos (tetrahidrofurano, NN-dimetilformamida, acetonitrilo, acetona y dioxano).

Los diagramas de fases obtenidos para mezclas ternarias de ácidos alquilcarboxílicos con diferente longitud de cadena hidrocarbonada (C8-C18), tetrahidrofurano y agua presentan tres regiones (Figura 15).

[82] López-Jiménez, F.J., Rosales-Marcano, M., Rubio, S., J. Chromatogr. A, 1303 (2013) 1-8

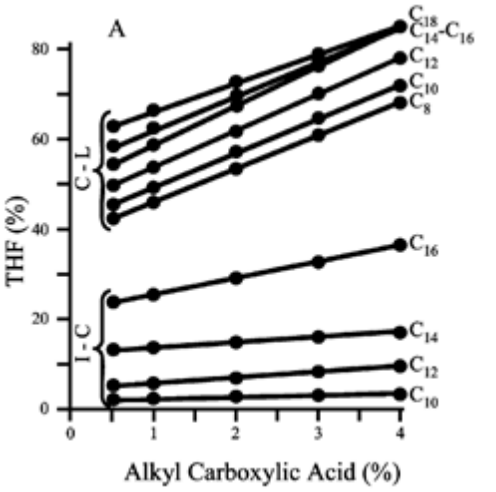

Fig. 15. Diagrama de fases para mezclas ternarias de ácido alquilcarboxílicos (C8-C18), THF y agua.

La región inferior (I) corresponde a la insolubilización de los ácidos alquilcarboxílicos. La región intermedia (C) corresponde a la formación del SUPRAS, mientras que en la superior (L), el contenido de THF en la mezcla es muy elevado y el disolvente supramolecular es miscible con la fase hidroorgánica. Las tres regiones del diagrama de fases de los tensioactivos sólidos a temperatura ambiente (decanoico, dodecanoico, tetradecanoico y hexadecanoico) son reconocibles visualmente. Sin embargo, la interfase I-C no es detectable para los ácidos octanoico y oleico a simple vista ya que son líquidos inmiscibles con agua a temperatura ambiente (punto de fusión a 16 y 14 ºC, respectivamente). En cualquier caso, la composición de la mezcla hidroorgánica que define esta interfase I-C depende de la longitud de la cadena del ácido, ya que el porcentaje de THF requerido para la solubilización del ácido y, por tanto, para la coacervación del mismo aumenta con su hidrofobicidad. La región de formación del disolvente es en todos los casos lo suficientemente amplia para su uso en aplicaciones analíticas.

La formación del SUPRAS requiere que los ácidos alquilcarboxílicos estén protonados (pKa R-COOH= 4,8 ± 0,2); por lo tanto su uso en extracciones analíticas implica que éstas deben llevarse a cabo a valores de pH inferiores a 4.

Los ácidos alquilcarboxílicos también forman SUPRAS a partir de disoluciones coloidales de vesículas en presencia de sales de tetrabutilamonio [83]. Las vesículas se forman en disoluciones acuosas que contienen mezclas equimoleculares de ácido alquilcarboxílicos y alquilcarboxilatos. El catión tetrabutilamonio actúa como agente inductor de la coacervación al neutralizar las cargas del alquilcarboxilato. De esta forma, se favorece la agregación entre vesículas.

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BLOQUE A

SUPRAS-RAM basados en ácidos alquil carboxílicos para la determinación de componentes/multicomponentes en una matriz compleja.

CAPÍTULO 1

Vesicular aggregate-based solventless microextraction of Ochratoxin A in dried vine fruits prior to liquid chromatography and fluorescence detection

Vesicular aggregate-based solventless microextraction of Ochratoxin A in dried vine fruits prior to liquid chromatography and fluorescence detection

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Talanta 89 (2012) 377– 382

Abstract

A solventless microextraction was proposed for the development of a simple, fast, low-cost and environmental friendly sample treatment for the determination of Ochratoxin A (OTA) in dried vine fruits. The objective was to offer an alternative to conventional sample treatments, which invariably involve extractions with large solvent volumes followed by clean-up with expensive, not recyclable and limited storage stability immunoaffinity sorbents. The method involved the stirring of 300 mg of dried vine fruit subsamples with 400 μL of a supramolecular solvent (SUPRAS) made up of decanoico acid/tetrabutylammonium decanoate vesicles. Then, the sample was centrifuged for 15 min and OTA was quantified in the extract by liquid chromatography/fluorescence detection against solvent-based calibration curves. Neither dilution nor further clean-up steps of extracts were needed. Quantitation of OTA was interference-free and recoveries ranged between 95% and 101%. The precision of the method, expressed as relative standard deviation (RSD), was about 3%. The limit of quantification (5.3 μg kg^{-1}) was below the threshold limit established for OTA in dried vine fruits by EU directives (10 μg kg^{-1}). Representativity of subsamples was proven. The method was successfully applied to the analysis of several dried vine fruits (sultanas and muscatels) purchased in local supermarkets in Córdoba (South of

Spain). OTA was not detected in any of the analyzed samples. This solventless sample treatment allows quick and simple microextraction of OTA, while delivering accurate and precise data, and extends the range of eco-friendly methods in labs.

1. Introduction

Regulatory agencies and quality control laboratories are continuously demanding faster, simpler and cheaper methods for the analysis of trace contaminants in food. Development of more general and valuable sample preparation procedures that meet the demanding regulatory limits established, minimize the number of steps required and decrease organic solvent consumption continues as the strongest priority in food chemical analysis [1]. Some strategies intended to reduce solvent consumption (e.g. solid-phase extraction [2], miniaturization [3], accelerated solvent extraction [4] and supercritical fluids extraction [5]) play currently an important role in sample handling for the analytical control of food in labs.

Supramolecular solvents (SUPRASs) have a great potential to develop organic solvent free, single-step sample treatments in food analysis. They are water-immiscible liquids made up of supramolecular assemblies of amphiphiles dispersed in a continuous phase. Aggregation occurs through a sequential self-assembly process. First, amphiphilic molecules spontaneously form threedimensional aggregates (aqueous or reverse micelles or vesicles) above a critical aggregation concentration. Then, the generated nanostructures self-assemble in larger aggregates by the action of an external stimulus (e.g.

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temperature, electrolyte, pH, solvent) and separate from the bulk solution as an immiscible liquid by a phenomenon named coacervation.

Two properties of SUPRASs render them ideal in analytical extractions. First, the aggregates making up SUPRASs have regions of different polarity that provide a variety of interactions for analytes. The type of interaction may be tuned varying the hydrophobic or the polar group of the amphiphile and in theory one may design the most appropriate SUPRAS for a specific application because amphiphiles are ubiquitous in nature and synthetic chemistry. Second, the concentration of amphiphiles in the solvent is very high (typically 0.1–1 mg μL^{-1}) which is an ideal platform for amplification of solute binding. Additional properties of SUPRASs include the use of self-assembly based synthetic procedures that are within everyone's reach and non-volatility and non-flammability which permit the implementation of safer processes. To date, SUPRASs from a variety of surfactant aggregates, i.e. non-ionic [6], zwitterionic [7], cationic [8] and anionic [9] aqueous micelles, reversed micelles [10] and vesicles [11] have been successfully used for the extraction of pollutants in the environment [12 –19] and, more recently, in foodstuffs [20–23].

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This paper evaluates the capability of a supramolecular solvent made up of decanoic acid/tetrabutylammonium decanoate for the eco-friendly single-step extraction/clean-up of Ochratoxin A (OTA) in dried vine fruits. OTA is a widespread contaminant in both raw and processed food commodities [24] that exerts nephrotoxic, immunosuppressive and neurotoxic effects [25]. The International Agency for Research on Cancer (IARC) has considered it as a possible carcinogen to humans (Group 2B) [26]. Its toxicity along with its worldwide occurrence [27] has fostered the development of international regulations to set maximum levels for OTA in a variety of commodities [28–31].

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[28]Commission Regulation (EU) N◦ 105/2010 of 5 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A.
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Dried vine fruits are progressively becoming great demand products in the health food market [32]. Environmental conditions both during post-harvest storage and the whole grape drying process cause the growth of *Aspergillus carbonarius*, the main responsible for OTA production in grapes and dried vine fruits [24]. The contribution of these products to the total human dietary intake of OTA has been reported in different surveys [27, 33 – 42]. Some maximum levels found for OTA were 35 μg kg^{-1} in Swedish raisins [37], 26 and 54 μg kg^{-1} in Canadian [35] and Turkish [42] sultanas, respectively and 54 μg kg^{-1} in English currants [34]. The European Union has established a maximum residue level (MRLs) for OTA in dried vine fruits of 10 μg kg^{-1} [28].

Few methods have been reported for the quantitation of OTA in dried vine fruits so far and, although most of them are straightforward and provide reliable results, unresolved issues remain in both sampling and sample treatment. Sampling concerns mainly derive from the random nature of fungal contamination and thus the uneven distribution of OTA in raw and processed dried fruits. Statistically based sampling plans for mycotoxins are available [43]. Samples from the bulk lot needs to be minced and slurried and then mixed for a reasonable period to obtain homogeneity prior to sub-sampling for

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analysis [44]. Sampling of retail products is less problematic because representative data are best obtained through analysis of large numbers of varied samples. Slurries are usually made with water [34–36] or aqueous bicarbonate [37, 38].

Solvent extraction with methanol is by far the most used strategy for isolation of OTA from dried vine fruits. In order to improve recoveries samples need to be acid- [34] or alkali-extracted [37], with the latter giving better recoveries. The volume of organic solvent consumed per sample is relatively high (50–100 mL). Conventional solid-phase extraction with C18 was used in the past for sample clean-up however, over the past ten years, most laboratories have moved towards using antibody-based affinity column (IAC) clean-up since it provides sample extracts generally free of interferences [34–40]. A major problem of the use of IACs in routine analysis is their cost, which demands for cheaper alternatives.

Liquid chromatography coupled to fluorescence detection (LC-FL), which provides quantitation limits between 0.1 and 10 μg kg^{-1} is by far the most used technique for OTA determination [34–42]. LC with mass detection (MS) provides unequivocal identification of OTA [45,46], however owing to its potential, this technique is more appropriate for multitoxin analysis. Fourier infrared spectroscopy attenuated total reflection has been also proposed for the detection of OTA in dried vine fruits, however, matrix matched calibration was required for quantitation [47].

In this paper, the supramolecular solvent-based extraction/cleanup is combined with LC-fluorescence for the determination of OTA in dried vine fruits with the aim of simplifying sample preparation while keeping method sensitivity below the legislative limits and enough selectivity. The SUPRASs

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made up of decanoic acid/tetrabutylammonium decanoate vesicles was selected on the basis of the different types of interactions it provides for OTA solubilization (i.e., hydrogen bonding, ionic, π–cation and dispersion forces) and the high amphiphile concentration in the solvent (around 0.96 mg μL^{-1}), all of which should give high extraction efficiencies. Parameters affecting sample representativity, extraction efficiency and detection and quantification limits were optimized and the method was successfully applied to the determination of OTA in different varieties of dried vine fruits.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Decanoic acid and tetabutylammonium hydroxide (Bu_4NOH) were obtained from Sigma-Aldrich (Barcelona, Spain), HPLC-grade acetonitrile, methanol and acetic acid glacial were supplied by Panreac (Sevilla, Spain). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Ochratoxin A was purchased from Sigma (St. Louis, MO, USA). A stock standard solution of 10 mg L^{-1} of OTA was prepared in methanol and stored under dark conditions at –20 ºC. Working solutions were prepared by dilution of the stock solution with methanol.

2.2. Apparatus

The liquid chromatographic system used consisted of a Thermo-Quest spectra system (San Jose, CA, USA) furnished with a SCM 1000 vacuum membrane degasser, a P2000 binary pump, an AS3000 autosampler and a

FL3000 fluorescence detector. In all experiments a PEEK Rheodyne 7125NS injection valve with a 20 μL sample loop was used (ThermoQuest, San Jose, CA, USA). The analytical column was a Hypersil ODS C8 (5 μm 150 mm × 4.6 mm) from Analysis Vínicos (Tomelloso, Spain). A Robot300 food chopper from Taurus (Berlin, Germany), a homogenizer-disperser Ultra-Turrax T25 Basic from Ika (Werke, Germany) a vortex-shaker REAX Top equipped with an attachment (ref. 549-01000-00) for 10 microtubes from Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R equipped with an angle rotor 36 × 2.2/1.5 mL (ref. 11462) from MPW Med-Instruments (Warschaw, Poland) were used for sample preparation. A digitally regulated centrifuge Mixtasel equipped with an angle rotor 4 × 100 mL (ref. 7001326) from JP-Selecta (Abrera, Spain) was used for supramolecular solvent production.

2.3. Supramolecular solvent production

The following procedure was routinely followed for the production of the supramolecular solvent (Fig. A.1).

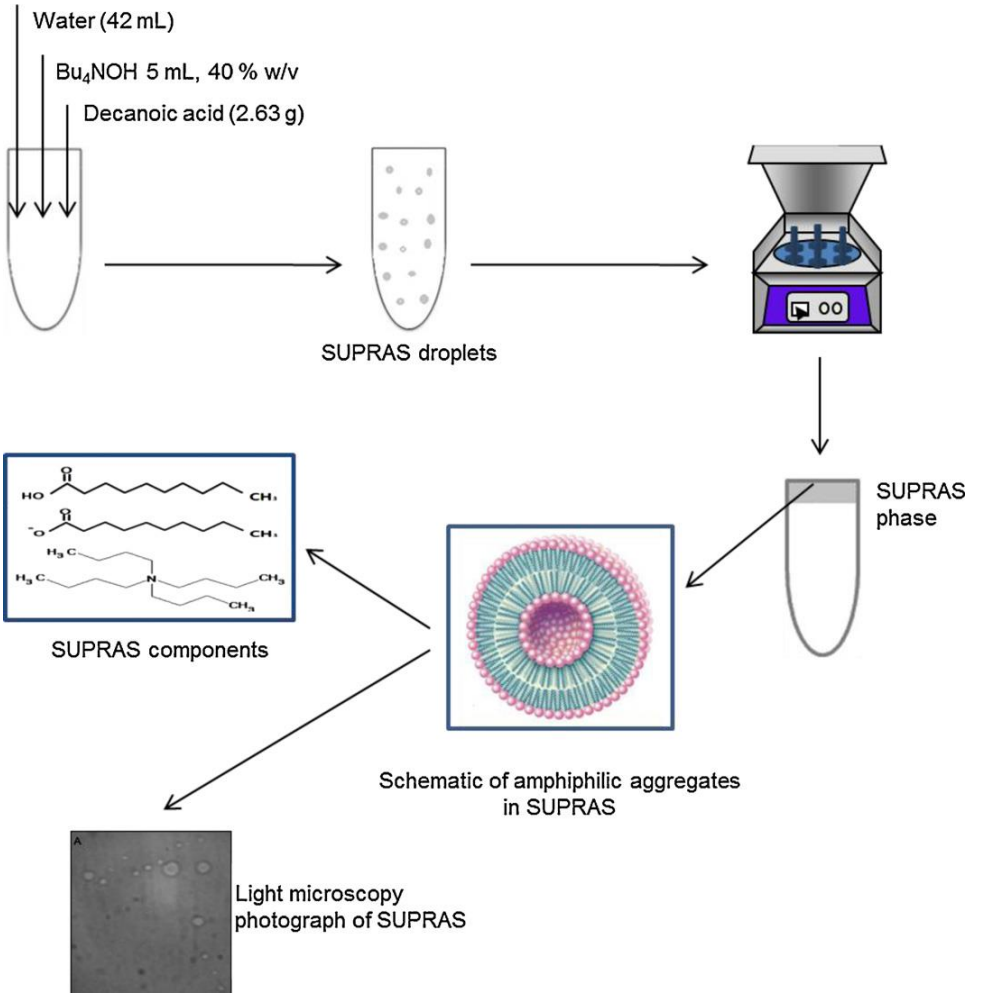

Fig. A.1. Schematic of supramolecular solvent production and composition.

In a 50 mL-glass centrifuge tube were placed in sequence, distilled water (42 mL), decanoic acid (2.63 g) and Bu4NOH (5 mL of 40%, w/v). The mixture was centrifuged at 3500 rpm (1860×g) for 8 min to speed solvent separation up, which is less dense than water. Next, it was withdrawn using a 10 mL-syringe, transferred to a hermetically close storage glass vial and stored at 4 ºC. Under these conditions, the solvent produced was stable for at least one month. The volume of solvent obtained can be adjusted at will by choosing an appropriate, constant decanoic acid/Bu_4NOH/water proportion.

2.4. Determination of OTA in dried vine fruits

2.4.1. Sample preparation

Five dried vine fruit varieties (sultana, white sultana, Málaga muscatel, muscatel and Chile muscatel) were purchased in supermarkets in Córdoba (South of Spain) and were stored at 4 ºC until analysis. Sultana and Málaga muscatel were produced in the South of Spain, while the origin of muscatel, Chile muscatel and white sultana was South America. The whole fruit content in consumer size-packages was used for sample treatment (typically 250 g). Samples were subsequently chopped, mixed with aqueous hydrochloric acid (pH 4) at a water (mL): sample (g) ratio of 4:5, and homogenized with a high-speed ultraturrax for 5 min. Then, portions of 300 mg were taken for analysis and recovery experiments, which were performed in triplicate. Spiking of samples was made by adding the corresponding volume of the working standard solution (100 μg L^{-1} of OTA) to the 300 mg-subsamples to give a final concentration of 10 or 20 μg kg^{-1} and they were allowed to stand at room temperature for 90 min before analysis. OTA was stable in the samples during this period of time.

2.4.2. Supramolecular solvent-based microextraction

In a 2 mL-microtube Safe-Lock from Eppendorf Ibérica (Madrid, Spain) were placed 300 mg-subsample and 500 μL of the vesicular supramolecular solvent. Four little glass balls (3 mm diameter) were introduced in the microtube to favor sample dispersion during extraction, which was made by sample vortex-shaken at 2500 rpm for 10 min. Then, the mixture, thermostated at 20 ºC, was centrifuged at 15,000 rpm for 15 min to

separate the solvent from the solid residue. SUPRAS aliquots were taken using a microsyringe, microfiltered through 0.45 μm nylon filters (Análisis Vínicos S.L. Tomelloso, Spain) to remove possible suspended solids and injected into the liquid chromatographic system. A schematic diagram of this procedure is illustrated in Fig. A.2.

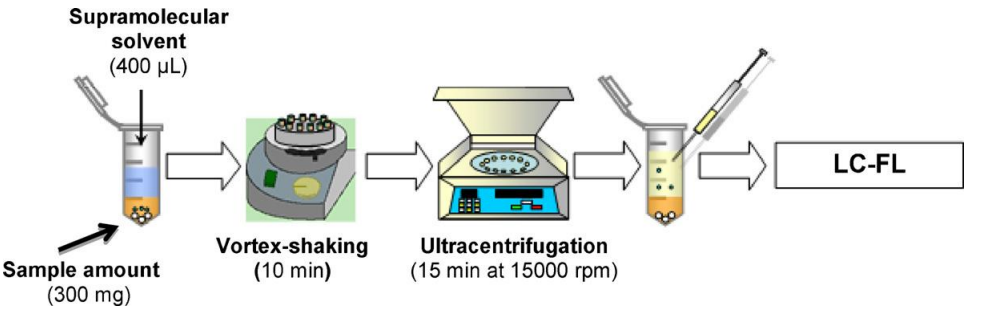

Fig. A.2. Schematic of the method proposed for OTA determination.

2.4.3. Liquid chromatography/fluorescence detection

OTA was separated from matrix components by liquid chromatography using isocratic elution. The mobile phase consisted of water/acetonitrile (45:55), both containing 1% glacial acetic acid, and was pumped at a constant flow rate of 1 mL min^{-1}. The volume of injection of samples and standards was 20 μL. OTA was monitored at λ_{ex} 334 nm and λ_{em} 460 nm. Under these conditions the analyte was eluted at approximately 10 min. Calibration curves were run from standards dissolved in methanol. No differences were detected in peak areas or retention times for the analyte njected in the SUPRASs or organic solvent. Quantitation was performed by measuring peak areas. Calibration curves for OTA were constructed in the range 2.5–30 μg L^{-1}. The precision of the method was evaluated by extracting eleven independent samples of sultana (n = 5) and muscatel (n = 6), fortified

with 10 μg kg^{-1} of OTA. The decision limit (CCα, the limit at and above which it can conclude with an error probability of α that a sample is not-compliant) was established by analyzing 20 blank sultana samples fortified with OTA at the permitted limit (i.e. 10 μg kg^{-1}), and it was calculated from the concentration at the permitted limit plus 1.64 times the standard deviation of the blank samples measurements (α - 5%).

3. Results and discussion

3.1. Sample homogenization

The great difficulty in preparing suitably homogeneous analytical samples of dried vine fruits has been identified by different researchers [34, 35]. Inhomogeneity primarily arises from the random nature of fungal contamination and is of particular importance in compliance testing where the analysis of a sample may result in the acceptance or refusal of a large, valuable lot of dried fruits. Analytical control of fruit contamination in consumer-size packages is easier because their whole content is usually taken for analysis.

Because of microextraction methods rely on the analysis of minute amounts of subsamples (e.g. 300 mg are here proposed instead of the 10–50 g-subsamples usually analyzed) thorough sample homogenization studies were carried out to determine if these subsamples were representative of the whole sample. To our knowledge, no certified reference materials for OTA in dried vine fruits are commercially available.

Formation of proper slurries was found essential for sample homogenization. Slurries were obtained by mixing the minced sample (250 g) with 0.01 M aqueous hydrochloric acid (at solution (mL): sample (g) ratios

from 1:5 to 5:5) and OTA (at the level of 10 or 20 μg kg^{-1}) using a high-speed ultraturrax. Subsamples (300 mg) were analyzed in triplicate and recoveries, along with their corresponding standard deviations, found.

Insufficient homogenization was obtained at solution: sample ratios below 4:5 as inferred from both sample appearance (it was heterogeneous in texture and included visible particles of different color) and standard deviations values. Although mean values for recoveries kept practically constant in the range evaluated, the corresponding standard deviations clearly increased as the volume of solution decreased. Thus, they were 14, 10 and 7% for solution: sample ratios of 1:5, 2:5 and 3:5, respectively. As previously found by other authors [34, 35], mixing four parts of aqueous solution with five parts of dried fruit was enough to reach subsampling representativity (standard deviations between 0.7 and 3%). The ultraturrax allowed a thorough blending of the aqueous solution and dried vine fruits and the slurry was homogenous enough for taking representative 300 mg subsamples.

3.2. Supramolecular solvent-based microextraction of OTA

3.2.1. Solvent description

The supramolecular solvent used for OTA microextraction consisted of unilamellar vesicles of decanoic acid/tetrabutylammonium decanoate dispersed in a continuous phase (Fig. A.1) [11]. This water immiscible liquid was less dense than water and its viscosity was 97.5 mPa s at 25 ºC.

The solvent was produced through a sequential self-assembly process involving two steps. Firstly, decanoic acid and decanoate formed vesicles in an aqueous solution. The amount and stability of these aggregates were maximal at decanoic acid/decanoate molar ratios of ca. 1 since, in addition to

hydrophobic forces, hydrogen bonds between the polar groups of carboxylic and carboxylate molecules were the major force driving their self-assembly. Secondly, aggregate growth was promoted by reduction of ionic head group repulsion with the counterion tetrabutylammonium. The aggregates thus produced separated from the bulk solution through coacervation.

From a practical point of view, the sequential self-assembly process was simplified by the addition of tetrabutylammonium hydroxide (Bu_4NOH) to aqueous decanoic acid suspensions, at a Bu_4NOH/decanoic acid molar ratio of 0.5. In this way, tetrabutylammonium decanoate and decanoic acid, at a molar ratio of ca. 1 were produced, and the vesicular coacervate formed instantaneously without the need for sonication. Since the amount of vesicles, and accordingly of supramolecular solvent, is maximal in the pH range 7 ± 1, where 7 is the (pK_a) apparent of decanoic acid molecules as inserted in the vesicle, extractions should be carried out at neutral or slightly acid or basic pH values.

The volume of SUPRAS produced linearly depended on the amount of decanoic acid initially present in the bulk solution, thus indicating that its composition kept constant. The value of the slope of this linear relationship gave the microliters of solvent obtained per mg of surfactant (1 mg μL^{-1}).

3.2.2. Optimization

Optimization was carried out by extracting 300 mg of sultana blank samples fortified with 20 μg kg^{-1} of OTA. Experiments were made in triplicate. Selection of the optimal conditions was based on the recoveries (R) and the method quantitation limits (MQLs) obtained. MQLs were calculated from the instrumental quantitation limits, the volume of SUPRASs used for extraction, the recoveries obtained and the sample weight used for analysis. The variables investigated were: volume of extractant (200–700 μL), pH for the sample

slurry (1.5–5.5), time required to reach equilibrium conditions (1–60 min) and time of centrifugation necessary to obtain free-particle extracts (1–30 min). After sample centrifugation, three phases were always observed; namely, the insoluble sample matrix components at the bottom; an intermediate aqueous solution arising from the slurry and probably containing very polar matrix components, and the SUPRASs extract containing OTA and other solutes from the sample.

The pH of the aqueous solution used for the formation of the sample slurry influenced both OTA recoveries and the precision of the results, as shown in Table B.1.

Table B.1

Mean percent recoveries and standard deviations obtained for OTA microextraction as a function of the pH of the aqueous solution used for slurry formation.

pH	Recovery ± SD[a] (%)
2.5	76±10
3	86±16
3.5	87.8±0.4
4	90±3
4.5	91±4
5	66.5±0.7

Fortified with OTA at a concentration of 20 μg kg^{-1}.

[a]*Standard deviation; n = 3; liquid:solid sample proportion: 4:5.*

Values of pH below 3.5 affected SUPRAS stability (the apparent pK_a for decanoic acid in the vesicular structure is around 7) provoking some destruction of the vesicular structures and consequently some solubilization of the solvent into the aqueous phase. This resulted in lower recoveries and irreproducible results (see Table B.1). Maximal recoveries were obtained at

pHs around 4, which were selected as optimal. This result indicated that the neutral form of OTA was preferentially extracted (pK_a for the OTA acid group = 4.4). Major interactions expected to be the driving forces for extraction were: (1) hydrophobic interactions between the hydrocarbon chain of decanoic acid and the aromatic rings of OTA (the octanol–water partition coefficient, log K_{ow}, for OTA is 4.74), (2) hydrogen bonds between the carboxylate/ carboxylic acid groups of vesicles and the hydrogen bond donors and acceptors of the mycotoxin, and (3) π–cation interactions between the aromatic rings of OTA and the quaternary ammonium group of Bu_4N^+.

Table B.2 shows the recoveries obtained, along with their respective standard deviations, as a function of the volume of SUPRASs used for microextraction.

Table B.2

Mean percent recoveries, along with their respective standard deviations, and quantitation limits obtained for OTA as a function of the volume of SUPRAS used for microextraction.

Supramolecular solvent (μL)	Recovery ± SD[a] (%)	Quantitation limits (μg kg^{-1})
200	72±4	3.7
300	88±3	4.5
400	100±1	5.3
500	103.5±0.7	6.4
600	105±3	7.5
700	104.5±0.7	8.8

[a]*Standard deviation, n = 3; fortified with OTA at a concentration of 20 μg kg^{-1}.*

Quantitative recoveries were obtained from SUPRAS volume (L)/sample amount (mg) ratios above 1.3. So, a volume of 400 μL was selected

as optimal because it provided quantitative recoveries and limits of quantitation below the threshold limit established for OTA by EU directives (10 μg kg^{-1}).

The time used for extraction of samples (vibration motion = 1500 rpm) influenced the recoveries obtained for OTA (Fig. A.3).

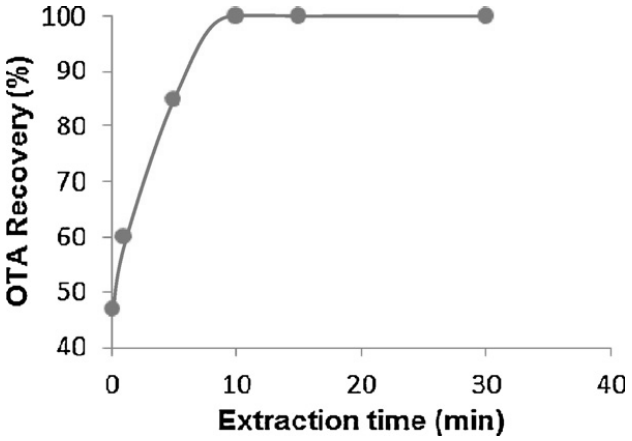

Fig. A.3. Dependence of OTA recovery as a function of the extraction time.

Equilibrium conditions were reached after 10 min of vortex shaking-assisted extraction and this time was selected as optimal. The minimal centrifugation time required to achieve an effective separation of the supramolecular extract from sample particles was 15 min.

3.3. Analytical performance

Calibration curves were run using standard solutions in methanol. No differences in peak areas or retention times were observed for the analyte injected in the SUPRAS or methanol. Correlation between peak areas and OTA concentration (2.5–30 μg L^{-1}) was determined by linear regression and was 0.991, indicating a good fit. The slope of the calibration curve was 77 ± 4 absorbance units L μg^{-1} (n = 6). The instrumental quantitation (LOQ) and detection (LOD) limits were calculated from blank determinations by using a signal-to-noise ratio of 10 and 3, respectively, and were 2.2 and 0.7 μg L^{-1}. The method LOD and LOQ were estimated from the respective instrumental LOD and LOQ taking into account the actual amount of sample analyzed (166 mg), the recoveries obtained (~100%) and the volume SUPRAS used for extraction (400 μL). The value of LOD was 1.7 and LOQ 5.3 μg kg^{-1}. The precision of the method, expressed as relative standard deviation (RSD), was about 3%. The decision limit (CCα) obtained for OTA was 10.25 μg kg^{-1} which proves the suitability of the proposed method for establishing compliance with the legislation for OTA in dried vine fruits [48].

Possible interferences from matrix components that could coelute with OTA were assessed by comparison of the slopes of the calibration curves (n = 7) obtained from standard solutions with those obtained from dried vine fruits fortified with known amounts of OTA (1–12 ng to 300 mg subsamples) and run using the whole procedure. The difference between both slopes (77 ± 4 for standard solutions and 71 ± 4 for spiked samples) was found to be not statistically significant by applying an appropriate Student's t-test [49]. The calculated t-value (1.21) was below the critical t-value (2.20), being significance established at the 0.05 level. Therefore matrix components were not expected to interfere in the determination of OTA.

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[48] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.
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3.4. Analysis of dried vine fruits

Five types of dried vine fruits were analyzed in order to prove the suitability of the proposed method for the routine control of OTA. None of them contained OTA at detectable levels. Table B.3 shows the recoveries obtained after spiking the samples at two levels of concentration (10 and 20 μg kg^{-1}).

Table B.3

Mean percent recoveries and standard deviations obtained in the determination of OTA in different varieties of fortified dried vine fruits.

Ochratoxin A (μg kg^{-1})	Recovery ± SD[a] (%)				
	Sultana	White sultana	Muscatel	Chile muscatel	Málaga muscatel
10	98.7±0.6	101±2	100±4	98±1	95±3
20	100±1	98±1	99±2	97±2	97±2

[a]*Standard deviation; n = 3.*

Recoveries were expressed as the mean value of three independent determinations along with their corresponding standard deviations. Their values were between 95 and 101% with standard deviations ranging from 0.6 to 4%. No interference from matrix components was detected for any of the samples analyzed. Fig. A.4 shows the chromatograms obtained for a blank and two-level fortification muscatel samples.

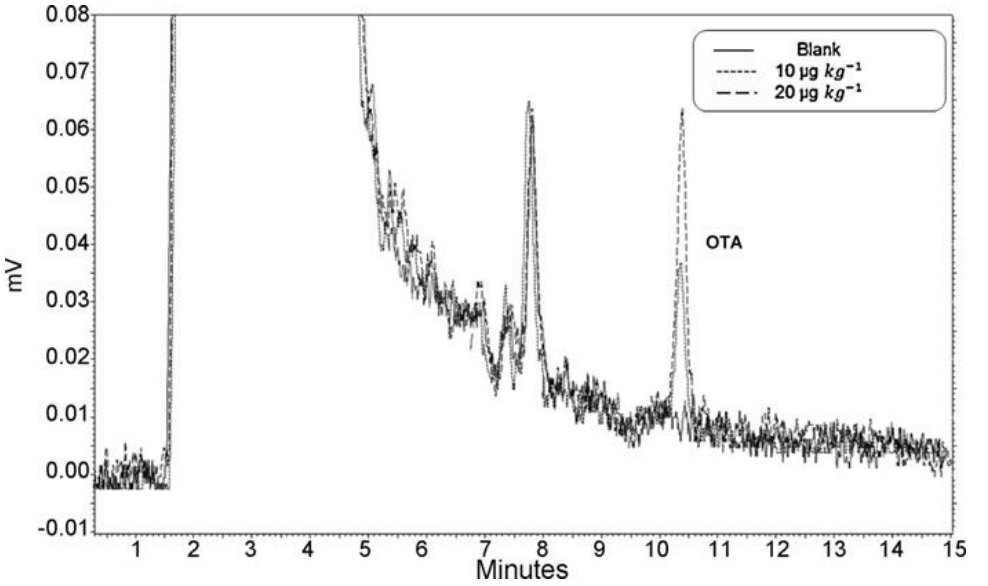

Fig. A.4. Chromatograms obtained from a muscatel blank sample and a muscatel sample fortified at 10 and 20 µg kg^{-1}.

4. Conclusions

Supramolecular solvents consist of amphiphilic nanostructures that provide multiple binding sites and regions of different polarity. These outstanding properties make them suitable to extract a variety of analytes with high efficiency and render them ideal for microextractions. In this research, a supramolecular solvent made up of decanoic acid/tetrabutylammonium decanoate vesicles is proposed as a valuable tool for the microextraction of OTA from dried vine fruits. The sample treatment proposed offers a simple, inexpensive and rapid alternative to conventional sample preparation methods, which combine high solvent consumption with the need for

immunoaffinity column-based clean-up. Valuable assets of the proposed sample treatment are: it takes about 30 min and several samples can be simultaneously treated; it requires a low sample amount (0.3 g fruit), which is made representative of the bulk by strong homogenization, and a low eco-friendly SUPRAS volume (400 μL); it features low cost and conventional equipment in labs is used. The method can be used for the routine control of OTA in dried vine fruits below the tolerance level permitted by the European Directives.

Acknowledgments

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CAPÍTULO 2

Nanostructured alkyl carboxylic acid-based restricted access solvents:

Application to the combined microextraction and cleanup of polycyclic aromatic hydrocarbons in mosses

Nanostructured alkyl carboxylic acid-based restricted access solvents: Application to the combined microextraction and cleanup of polycyclic aromatic hydrocarbons in mosses

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Abstract

Alkyl carboxylic acid-based nanostructured solvents, synthesized in mixtures of tetrahydrofuran (THF) and water through self-assembly and coacervation, were proved to behave as restricted access liquids. Both physical and chemical mechanisms were found responsible for exclusion of macromolecules such as proteins and polysaccharides. The potential of these solvents for extracting small molecules from complex solid samples, without interference from large biomolecules, was here evaluated. For this purpose, they were applied to the extraction of 14 priority polycyclic aromatic hydrocarbons (PAHs) from mosses prior to their separation by liquid chromatography and fluorescence detection (LC-FLD). Sample treatment involved the vortex shaking of 200 mg of moss with 200 mL of decanoic acid-based solvent for 5 min, subsequent centrifugation for 8 min and analysis of the extract by LC-FLD using external calibration. Proteins precipitated during extraction because of both the decrease of the dielectric constant of the solution caused by THF and the formation of macromolecular complexes with decanoic acid. Polysaccharides were not solubilized in the aqueous cavities of the solvent because of their size exclusion. In-house method validation was performed according to the recommendations of the European Commission Decision 202/657/EC. Method detection and quantification limits for the different PAHs were in the ranges 0.04-0.24 and 0.14-0.80 μg kg^{-1}, respectively. The method was applied to the determination of different moss species collected in both polluted and unpolluted sites in the South of Spain.

Recoveries were within the range 71-110%. The results obtained show that solvents with restricted access properties have the potential to expand the scope of application of restricted access materials to areas other than biological fluids because of their suitability to combine analyte isolation and sample cleanup of solid samples in a single step.

1. Introduction

Restricted access materials (RAMs) have been widely used in the past two decades as sorbents for the enrichment of drugs, endogenous substances and xenobiotics in biological fluids without interference from proteins and other matrix components that are excluded by physical, chemical or physicochemical means [1]. The use of RAMs prior to liquid chromatography eliminates multiple sample pretreatment steps (e.g. precipitation, centrifugation, solvent evaporation, residue dissolution, etc.) and that favors increased sample throughput [2,3]. The latest advances in this field have been mainly related to the development of simpler procedures for the synthesis of RAMs [4,5] and the design of more selective materials [6,7].

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Recently, alkanol-based supramolecular solvents (SUPRASs) have been proved to behave as restricted access liquids [8]. These nanostructured solvents have the potential for combined analyte enrichment and cleanup of both liquid and solid samples, which expands the scope of application of RAMs. They are made-up of inverted hexagonal aggregates of alkanols, that spontaneously form in a THF:water solution through self-assembly processes, and offer an external hydrophobic phase, made up of hydrocarbon chains extended and surrounded by tetrahydrofuran (THF), and internal aqueous cavities, whose size can be tailored by controlling the THF:water ratio for alkanol self-assembly. Analytes in a wide polarity range can be solubilized in the two microenvironments offered by these SUPRASs. Exclusion of proteins is carried out by their precipitation induced by both THF and alkanols [9], while polysaccharides and humic acids are not incorporated to the internal aqueous cavities due to size exclusion. These properties have been used for application of RAMs to samples other than biological fluids such as carcinogenic chlorophenols in natural water samples [8] and estrogenic disruptors in sediments [10].

Alkyl carboxylic acid-based SUPRASs [11] have also the potential to behave as restricted access liquids thus expanding the number of available solvent-based RAMs. So, this research was intended to explore the restricted access properties of these solvents as applied to the analyte extraction and cleanup of complex solid samples. The extraction of polycyclic aromatic hydrocarbons (PAHs) from mosses was selected for this aim due to the current interest of the European Monitoring and Evaluation Programme (EMEP) in using these organisms for the evaluation of the atmospheric

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precipitation of these pollutants [12]. Application of conventional RAM sorbents to the extraction and cleanup of complex solid matrices is an almost unexplored area owing to the need for previous solvent extraction of analytes.

The EMEP monitors and models emission and deposition of persistent organic pollutants (POPs) in Europe [12]. In 2012, there were 34 EMEP monitoring sites measuring POPs, mainly PAHs, in air and/or precipitation. The number of EMEP sites has been gradually increasing over the last few years however, spatial coverage is not sufficient yet. An alternative to conventional precipitation analysis is biomonitoring and mosses are among the organisms most frequently used for this purpose [13]. The moss technique is easier and cheaper than conventional precipitation analysis as it avoids the need for deploying large numbers of precipitation collectors with an associated long-term programme of routine sample collection and analysis. Therefore, a much higher sample density can be achieved. At present, the European moss biomonitoring network is actively investigating the suitability of mosses as biomonitors of POPs at the regional scale [14-16]. Also, the EU directive on air quality recommends the use of biomonitoring for assessing the impact of PAHs on ecosystems [17].

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Although sixteen PAHs have been included in United States Environmental Protection Agency (US-EPA) priority pollutant list [18] and seven PAHs have been classified as potentially carcinogenic [19], only benzo[a]pyrene, (BaP) is regulated by the European Commission, which sets a target limit value of 1 ng m^{-3} in the ambient air based on its total concentration in the PM_{10} fraction averaged over a year [17]. To model PAH pollution, EMEP uses four indicators BaP, benzo[b]fluoranthene (Bb), benzo[k]fluoranthene (BkF) and indeno[1,2,3-c,d]pyrene (IcdP) [12]. Decline of PAH4 pollution levels in the EMEP countries from 1990 to 2012 is accounted for 30% on the average, however, in some of the countries of the Central and Eastern Europe changes were small and levels of pollution in 2012 were still significant (2 ng m^{-3} and higher). On the other hand, the amount of people exposed to BaP air concentrations exceeding the EU target value still remains considerable (about 16.5 million people) [12]. Complementary analysis of regular measurements of PAH concentrations in mosses can provide additional insights into the spatial and temporal variations of pollution levels within the EMEP region thus improving the quality of official emission data.

Quantification of PAHs in mosses has been mainly carried out by gas chromatography coupled to mass spectrometry (GC-MS) [20], and liquid chromatography combined with fluorescence detection (LC-FLD) [21-23].

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Sample treatment has traditionally involved Soxhlet [24,25] or Soxtec [26] apparatus which demand for long extraction times (3-24 h) and high volumes of organic solvents (100-200 mL of hexane, dichloromethane, acetonitrile, etc). So, recent research has focused on the simplification of moss sample treatment and techniques such as extraction assisted by ultrasounds [23,27] or microwaves [20,28] and accelerated solvent extraction [22,29] have been proposed as alternatives to the conventional ones, with extractions taking on average 30 min with 20-30 mL of solvent. Purification of extracts is a subsequent required step in order to achieve proper selectivity. Sample cleanup is commonly carried out by SPE with Florisil [20,22], alumina [29] or silica [30]. In all cases, solvent evaporation is required to get enough sensitivity, which increases analysis time. Method quantification limits for the different PAHs are usually within the range 0.1-2.5 and 0.1-35 mg kg^{-1} using LC-FLD [22,31] and GC-MS [20,24,30], respectively. Moss amount used for analysis is usually in the range 1-5 g.

The potential restricted access properties of alkyl carboxylic acid-based SUPRASs along with their high solubilizing properties, derived from

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the outstanding number of binding sites they contain (e.g. concentration of alkyl carboxylic acid in the SUPRAS can be so high as 0.75 mg mL^{-1}) provide excellent opportunities to simultaneously extract PAHs and exclude macromolecules coming from the moss matrix. On the other hand, only conventional lab equipment (e.g. vortex, centrifuge, etc.) is required for sample treatment. Below, the most salient results of this study are described and discussed.

2. Experimental

2.1. Chemicals

Reagents (analytical grade) and solvents were used as received. Tetrahydrofuran (THF) was obtained from Panreac (Barcelona, Spain), acetonitrile from VWR-Prolabo (Bois, France), octanoic, decanoic and dodecanoic acids from SigmaeAldrich (Steinheim, Germany) and ultra-high quality water from a Milli-Q water purification system (Millipore, Madrid, Spain). A certified standard solution of 16 PAHs; Naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHY), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene, (DahA), benzo[ghi]perylene (BghiP), and indeno[1,2,3-cd]pyrene (IcdP) in acetonitrile (10 mg mL^{-1} of each compound) was provided by Supelco (Steinheim, Germany). A stock standard solution containing 1 mg mL^{-1} of each PAH was prepared in acetonitrile and stored at 4ºC. Working solutions were obtained by appropriate dilution of the stock standard solution with acetonitrile.

2.2. Apparatus

PAHs were separated and quantified by using a Waters System liquid chromatograph (Milford, MA, USA) consisting of a 1525 binary pump, a column thermostatic oven, a 717 auto-sampler and a 2475-multiwavelength fluorimetric detector. All data were acquired and processed using the Empower software. The stationary phase for the analytical column was a Supelcosil-LC PAH (5 μm, 250 x 4.6 mm) from Supelco (Bellefonte, PA, USA). A Waters Symmetry C18 guard column (5 μm, 3.9 x 20 mm) was used to protect the analytical column. A vortex shaker from Reax Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R from MPW Medical Instruments (Warschaw, Poland) were used for the microextraction of PAHs from mosses. A Basicmagmix magnetic stirrer from Ovan (Barcelona, Spain) and a digitally regulated centrifuge Mixtasel equipped with an angle rotor 4x100mL from JP-Selecta (Abrera, Spain) were used for production of the SUPRASs.

2.3. Supramolecular solvent production

The following procedure, which permits to obtain a SUPRAS volume (5.3 mL) able to treat 26 moss samples, was routinely followed. Decanoic acid (0.75 g) was dissolved in THF (15 mL) in a 50 mL glass centrifuge tube. Then, 15 mL of 0.01 mol L^{-1} aqueous hydrochloric acid were added. After sealing the tube with parafilm to avoid THF evaporation, the mixture was magnetically stirred at room temperature for 5 min, time in which the SUPRAS spontaneously formed into the bulk solution. The mixture was centrifuged at 2400 g for 10 min to accelerate the separation of the SUPRAS from the decanoic acid-poor bulk solution (i.e. equilibrium solution). Then, the SUPRAS, which is less dense than the bulk solution, was withdrawn using a glass syringe, and transferred to a hermetically closed storage glass vial and stored at 4 ºC. The equilibrium solution, used in the extraction process as a sample wetting agent, was also saved and maintained hermetically closed at 4 ºC. The volume of SUPRAS obtained can be adjusted by choosing an

appropriate, constant decanoic acid/THF/water proportion. Fig. 1 shows a diagram of the procedure to get the SUPRAS.

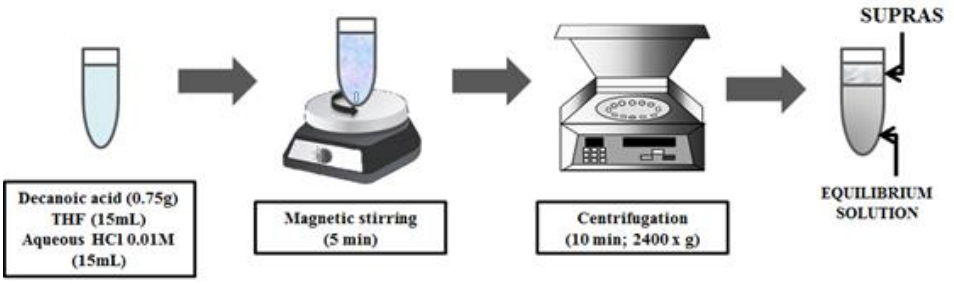

Fig. 1. Schematic of SUPRAS preparation in the bulk solution.

2.4. Determination of PAHs in mosses

2.4.1. Sample collection and preparation

Moss samples were collected from March to May 2014 in mountain areas of Córdoba and Jaen provinces (South of Spain) and urban sites of Córdoba city. The samples growing on open ground, rocks and tree barks were taken randomly. The main objective was to optimize and/or validate the method using different species of mosses growing in the South of Europe, both near and far from sources of pollution. A short description of all sampling sites and species of mosses identified is as follows: *Fissidens crassipes* Wilson ex Brunch & Schimp., *Plagiomnium undulatum* (Hedw.) T. J. Kop., and *Leucodon sciuroides* (Hedw.) Schwägr. were collected from different sampling points of Santa María de Trassierra, a mountain region in Córdoba province far from heavy traffic and industrial areas. This site has some scattered houses and farms crossed by streams, and it is open to touristic activities. The moss species *Distichium capillaceum* (Hedw.) Brunch & Schimp. was sampled from La Pandera, a limestone mountainous region (maximum altitude 1872 m) in Jaén province. This steep terrain is used for

cycling activities. The moss samples of *Pleurochaete squarrosa* (Brid.) Lindb. were collected in Córdoba city near a cement factory. In this case, sampling points were closer than 100 m from one of the main roads of Córdoba, so samples were expected to be also influenced from traffic emissions. In the laboratory, mosses were air-dried at room temperature and cleaned from all dead material and attached litter. Only the green shoots were considered for analysis. They were ground to a fine powder (particle size < 0.5 mm) in a Retsch MM301 mixer mill and stored at -18 ºC in aluminum foil until analysis. All the experiments involved in SUPRAS selection and subsequent optimization studies were carried out by using a composite sample (around 33 g, dry weight) of *Leucodon sciuroides*. Around 0.2 g moss subsamples were spiked with all target PAHs at concentrations in the range 2-100 μg kg^{-1} each by adding minute volumes (10-20 μL) of PAH standard solutions in acetonitrile. Spiked samples were left to stand at room temperature until acetonitrile evaporation. No changes in recoveries for PAHs were detected in the interval 1-12 h. No blank moss samples were found, so in recovery studies, the concentrations of PAHs in non-spiked moss samples were subtracted from those found in the spiked ones. Optimal conditions were selected on the basis of the recoveries and precision obtained.

2.4.2. SUPRAS-based microextraction of PAHs

Moss subsamples (~0.2 g) were weighted into Safe-Lock 2 mL microtubes from Eppendorf Iberica (Madrid, Spain). Four spherical glass balls (3 mm of diameter) were introduced in the microtube to favor sample dispersion during extraction. Then, 900 mL of the synthesis equilibrium solution and 200 mL of SUPRAS were added as wetting agent and extractant, respectively. Afterwards, the mixture was vortex-shaken at 2500 rpm for 5 min and then centrifuged (8500 x g, 8 min, 20 ºC). Finally, the supramolecular extract was withdrawn with a microsyringe and transferred to a glass vial of the LC-FLD autosampler. Fig. 2 shows a schematic of the sample treatment and microextraction procedure.

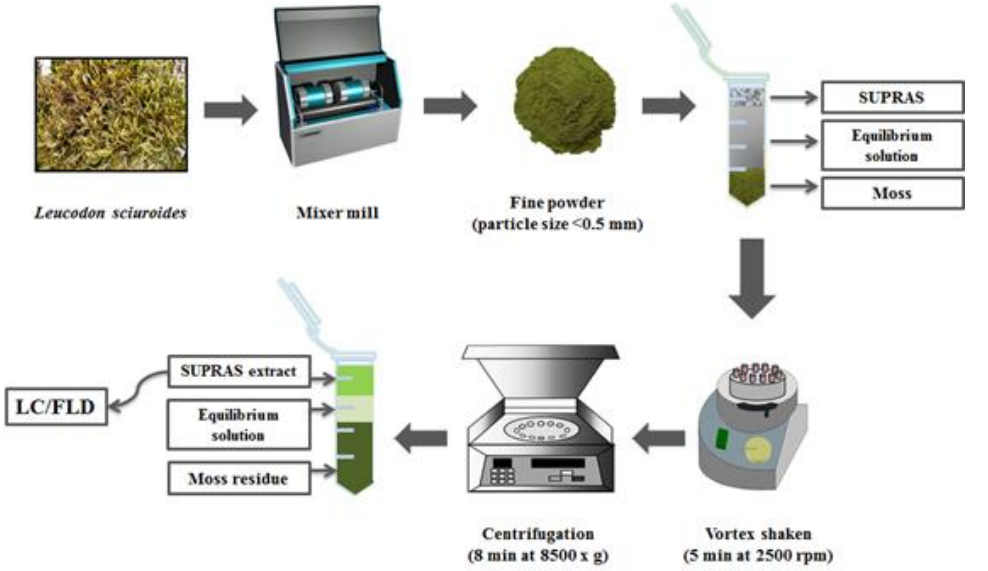

Fig. 2. Schematic of the sample treatment proposed for the extraction of PAHs from mosses.

2.4.3. Liquid chromatography-fluorescence detection

The mobile phase consisted of acetonitrile and water at a flowrate of 2.0 mL min^{-1} and the gradient elution was programed as follows: 50% acetonitrile for 5 min and then 100% of acetonitrile in 25 min. Before another injection, the column was permitted to re-equilibrate under initial conditions for 10 min. The temperature in the column thermostatic oven and the auto-sampler were set at 35 ºC and 15 ºC, respectively. The fluorescence excitation and emission wavelengths were changed during the chromatographic separation in order to obtain better sensitivity and minimal interference. When PAHs eluted close to each other, wavelength switching could not be carried out between these peaks and a wavelength pair appropriate for both compounds was selected. The excitation/emission wavelengths were set as follows: 280/356 nm (ACE, and FLU), 260/392 nm (PHE and ANT), 260/432 nm (FLT), 260/ 392 nm (PYR, BaA, and CHY) 260/432 nm (BbF, BkF, BaP, and DahA), and 300/466 nm (BghiP and IcdP). Naphthalene and acenaphthylene were not considered for this study because of the presence of interfering compounds and low fluorescence intensity, respectively. These

compounds have either not been considered in previous determinations of PAHs in mosses using LC-FLD [e.g. 22, 31] or in the moss pilot survey conducted in 2010 within the EMEP region [15]. Calibrations were run by injecting 20 µL of standard solutions containing a mixture of PAHs at concentrations in the range instrumental quantification limit-250 µg L^{-1} (see Table 1). A volume of 20 µL was also used for analysis of samples.

Table 1

Analytical figures of merit of the proposed method.

PAH	[a]Linear range (µg L^{-1})	Slope ± S (L μg^{-1})	[b]r	[c]MDL (µg kg^{-1})	[d]MQL (µg kg^{-1})
ACE	0.11–250	42.7±0.15	0.99995	0.05	0.17
FLU	0.74–250	12.9±0.04	0.99993	0.24	0.78
PHE	0.11–250	14.3±0.08	0.9998	0.08	0.26
ANT	0.21–250	56.9±0.24	0.99994	0.07	0.23
FLT	0.39–250	7.9 ±0.04	0.99994	0.14	0.45
PYR	0.22–250	27.2±0.14	0.99990	0.08	0.27
BaA	0.19–250	51.8±0.05	0.99999	0.08	0.25
CHY	0.18–250	57.6±0.06	0.99999	0.10	0.32
BbF	0.18–250	39.1±0.15	0.99995	0.12	0.39
BkF	0.10–250	100.5±0.31	0.99996	0.04	0.14
BaP	0.13–250	80.0±0.18	0.99998	0.05	0.15
DahA	0.54–250	5.3 ±0.02	0.99994	0.24	0.80
BghiP	0.25–250	20.9±0.10	0.99992	0.19	0.65
IcdP	0.15-250	28.2±0.28	0.9996	0.10	0.30

[a]*Instrumental quantification limit calculated by using a signal-to-noise ratio of 10.*

[b]*Correlation coefficient.*

[c]*Method detection.*

[d]*Method quantification limits for the determination of PAHs in* Leucodon sciuroides

3. Results and discussion

3.1. Protein and polysaccharide exclusion

Alkyl carboxylic acids spontaneously self-assemble and coacervate as inverted hexagonal aggregates in THF:water mixtures [11]. The new liquid phase produced (SUPRAS) is in equilibrium with a THF:water solution containing alkyl carboxylic acid below the critical aggregation concentration. Fig. 3 shows, as an example, a representative phase diagram depicting the boundaries (solid lines) for the region where separation of two isotropic liquids from ternary mixtures of decanoic acid, THF and aqueous hydrochloric acid (0.01 M) occurs. Beyond these boundaries, decanoic acid gives a single isotropic solution or becomes insoluble.

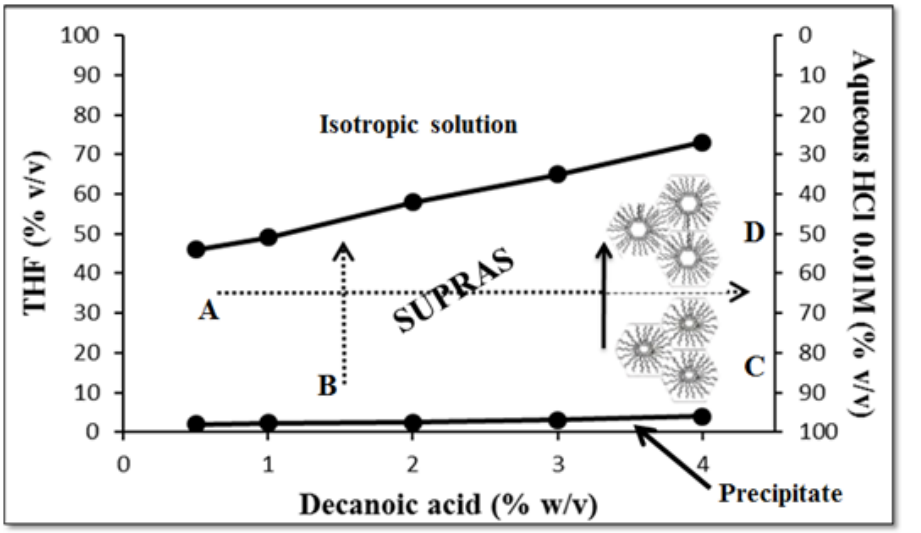

Fig. 3. Phase diagram for ternary mixtures of decanoic acid, THF and aqueous hydrochloric acid (0.01 M).

The volume of solvent produced in the self-assembly process is linearly dependent on the amount of alkyl carboxylic acid and exponentially

dependent on the percentage of THF in the bulk solution according to the equation:

$$y = (1.03 \pm 0.02)a\ e^{(0.0473 \pm 0.0009)b}$$

where y is the volume (mL) of SUPRAS, a the amount (g) of alkyl carboxylic acid and b the THF percentage (v/v). This means that the composition of the SUPRAS synthesized from variable concentrations of alkyl carboxylic acid and constant THF/water ratios (e.g. dotted line A in Fig. 3) remains unchanged. On the other hand, the SUPRASs obtained from a constant concentration of alkyl carboxylic acid and increasing THF/water ratios (dotted line B in Fig. 3) gradually contain more THF and water and consequently become increasingly diluted with respect to the surfactant (e.g. the concentration of alkyl carboxylic acid in the SUPRAS varies from 0.76 to 0.38 mg μL^{-1} for percentages of THF in the bulk solution in the range 5-20%). So, both the global composition of the SUPRAS and the size of the inverted hexagonal aggregates making up it can be tailored by controlling the environment (specifically, the THF:water ratio in the bulk solution) for alkyl carboxylic acid self-assembly. Thus, the higher content of water in the SUPRAS with increasing THF/water ratios also causes the aqueous core of the inverted hexagonal aggregates to increase (compare the size of the aqueous cavities in the hexagonal aggregates in Fig. 3C and D) and opens the possibility of using these liquids as restricted access materials.

The ability of alkyl carboxylic acid-based SUPRASs to exclude proteins and polysaccharides by both chemical and physical means, and consequently to achieve sample cleanup, was examined. Experiments were conducted by extracting 20 mg of pure standards of typical proteins and polysaccharides in 200 µL of SUPRAS obtained according to the procedure specified in section 2.3. The exclusion of proteins was qualitatively checked by their precipitation. For polysaccharides, the remaining solutions after extraction were quantitatively analyzed by spectrophotometry; starch by complexation with triiodide [32] and chitosan by the ninhydrin test [33].

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[32]E. Magel, Qualitative and quantitative determination of starch by a colorimetric method, Starch 43 (1991) 384-387.

[33]M.A. Lago, A. Rodríguez Bernaldo de Quirós, R. Sendon, A. Sanches-Silva, H.S. Costab, D.I. Sánchez-Machado, J. López-Cervantes, H. Soto Valdez, G.P. Aurrekoetxea, I. Angulo, P.

Under SUPRAS extraction, aqueous solutions of albumin from bovine serum, albumin from chicken egg white and gluten from wheat gave a white precipitate that separated from the solvent as a thin layer upon centrifugation. Both THF and decanoic acid were considered responsible for protein precipitation owing to the decrease of the dielectric constant of the solution [34] and the formation of macromolecular complexes [35], respectively. An identical white layer was clearly observed between the insoluble matrix components and the SUPRAS after extraction and centrifugation of moss samples. So, removal of proteins from mosses was carried out during extraction of PAHs.

The behavior of polysaccharides during extraction was evaluated by using chitosan and starch, which play structure-related and storage-related roles, respectively. Neither chitosan nor starch was dissolved by the SUPRAS. Extractions were also carried out under experimental conditions known to increase the solubility of both polymers (e.g. 1% acetic acid for chitosan and 40 ºC for starch) but no extraction in the SUPRAS was observed. Therefore, the SUPRAS has also the ability to act as a RAM for polysaccharides, probably because of size exclusion of these hydrophilic polymers from the aqueous cavity.

3.2. Optimization of PAH extraction

3.2.1. Selection of alkyl carboxylic acid

Selection of a proper SUPRAS to extract PAHs from mosses involved two steps. First, SUPRASs from different alkyl carboxylic acids, namely octanoic, decanoic and dodecanoic acids were synthesized and tested as extractants. Then, the most suitable alkyl carboxylic acid was selected and a

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Paseiro Losada, Compilation of analytical methods to characterize and determine chitosan, and main applications of the polymer in food active packaging, J. Food 9 (2011) 319-328.
[34]K.S. Schwenzer, S.E. Magic, Precipitation of proteins, Patent US4171204A.
[35]V. Morais, H. Massaldi, A model mechanism for protein precipitation by caprylic acid: application to plasma purification, Biotechnol. Appl. Biochem. 59 (2012) 50-54.

variety of SUPRASs were synthesized under different environmental conditions (i.e. different THF:water ratios).

Both the chromatographic behavior and extraction efficiency of the alkyl carboxylic acid (C8-C12) used as an ingredient of the SUPRAS was investigated. The respective SUPRASs were synthesized in bulk solutions containing 30% of THF according to the procedure specified in Section 2.3 and moss samples were directly extracted with 800 mL of SUPRASs. Decanoic acid eluted before PAHs and no differences in peak areas or retention times were observed for the PAHs injected into the SUPRAS or acetonitrile. Both octanoic and dodecanoic acids also eluted before PAHs but their chromatograms showed a peak at around 14 min, probably because of reagent impurities despite working with analytical grade products. The intensity of this peak was much higher for dodecanoic acid, that hindering the quantification of PHE and ANT. Recoveries of PAHs for octanoic and decanoic acid were similar and within the range 89-108%. We decided to select decanoic acid to make up the SUPRAS because of its better chromatographic behavior.

It was observed in these preliminary experiments that a significant fraction of SUPRAS (e.g. around 500 μL) was necessary as a wetting agent for the sample, that rendering this fraction unavailable for analysis. So, the possibility of adding a sample wetting solution was evaluated. The equilibrium solution obtained during self-assembly of the alkyl carboxylic acid (Fig. 1) was selected for this purpose on the basis of its immiscibility with the SUPRAS. In this way, both the global composition of the SUPRAS and the size of the inverted hexagonal aggregates making up it kept unchanged during the extraction process. Additionally, the equilibrium solution could act as a sink for matrix polar compounds, which should increase selectivity. A volume of equilibrium solution equal or above 400 mL was firstly selected for subsequent experiments.

3.2.2. Study of THF/water ratios

The influence of SUPRAS composition and structure on PAHs recovery was investigated by synthesizing SUPRASs from a constant

concentration of decanoic acid (5%, v/v) and variable water/ THF ratios from 90/10 to 40/60 (v/v). A volume of 400 mL of both SUPRAS and equilibrium solution were used as extractant and wetting agent, respectively. Table 2 shows the recoveries obtained and the corresponding standard deviations.

Table 2

Mean recoveries obtained for PAHs as a function of the water/THF ratio for self-assembly, volumes of the SUPRAS and equilibrium solution and vortex shaken time.

Variable	[a]Recovery±[b]S (%)													
	ACE	FLU	PHE	ANT	FLT	PYR	BaA	CHY	BbF	BkF	BaP	DahA	BghiP	IcdP
[c]Water/THF ratio (v/v)														
90/10	77 ± 1	74 ± 3	78 ± 2	89 ± 3	82 ± 8	95 ± 6	84 ± 5	83 ± 4	81 ± 3	81 ± 3	74 ± 3	77 ± 4	84 ± 4	68 ± 5
80/20	86 ± 3	81 ± 3	84 ± 3	90 ± 3	91 ± 3	92 ± 3	92 ± 4	89 ± 6	81 ± 3	85 ± 4	79 ± 4	78 ± 5	89 ± 2	69 ± 4
70/30	89 ± 1	85 ± 5	83 ± 2	93 ± 2	89 ± 5	94 ± 2	96 ± 1	94 ± 1	85 ± 2	89 ± 2	84 ± 1	81 ± 2	90 ± 1	72 ± 3
60/40	90 ± 1	87 ± 4	82 ± 1	94 ± 2	90 ± 2	94 ± 5	94 ± 3	95 ± 2	94 ± 2	98 ± 2	92 ± 3	84 ± 3	92 ± 2	80 ± 1
50/50	97 ± 3	87 ± 3	89 ± 4	96 ± 3	91 ± 4	97 ± 3	104 ± 3	101 ± 4	99 ± 2	97 ± 4	102 ± 3	88 ± 3	100 ± 3	92 ± 5
40/60	98 ± 2	85 ± 2	94 ± 1	93 ± 2	100 ± 1	106 ± 2	96 ± 3	95 ± 3	91 ± 1	91 ± 2	85 ± 3	92 ± 3	95 ± 3	88 ± 3
[d]Volume of equilibrium solution (µL)														
400	97 ± 3	87 ± 3	89 ± 4	96 ± 3	91 ± 4	97 ± 3	104 ± 3	101 ± 4	99 ± 2	97 ± 4	102 ± 3	88 ± 3	100 ± 3	92 ± 5
500	91 ± 4	85 ± 2	86 ± 5	91 ± 5	87 ± 3	90 ± 2	99 ± 6	97 ± 4	96 ± 3	95 ± 4	99 ± 5	86 ± 4	96 ± 4	91 ± 5
600	93 ± 1	86 ± 4	96 ± 2	93 ± 1	86 ± 6	93 ± 5	99 ± 1	97 ± 1	97 ± 1	96 ± 1	99 ± 1	90 ± 6	92 ± 5	95 ± 5
700	91 ± 1	84 ± 3	90 ± 5	92 ± 1	89 ± 4	90 ± 2	98 ± 2	96 ± 2	95 ± 2	93 ± 2	96 ± 3	85 ± 5	93 ± 3	89 ± 2
800	94 ± 2	93 ± 1	93 ± 7	97 ± 1	94 ± 5	92 ± 2	96 ± 1	96 ± 1	96 ± 1	93 ± 1	90 ± 1	89 ± 1	91 ± 1	91 ± 5
900	99 ± 1	91 ± 2	89 ± 4	95 ± 1	92 ± 4	95 ± 1	95 ± 1	94 ± 1	95 ± 1	92 ± 1	92 ± 1	91 ± 1	92 ± 1	95 ± 1
[e]Volume of SUPRAS (µL)														
200	94 ± 2	84 ± 2	89 ± 3	88 ± 3	87 ± 1	98 ± 4	95 ± 3	94 ± 3	91 ± 2	89 ± 2	95 ± 3	97 ± 2	103 ± 1	90 ± 4
300	97 ± 1	89 ± 1	76 ± 7	93 ± 1	90 ± 2	91 ± 2	93 ± 2	92 ± 2	91 ± 2	91 ± 2	90 ± 2	91 ± 3	93 ± 5	95 ± 2
400	99 ± 1	91 ± 2	89 ± 4	95 ± 1	92 ± 4	95 ± 1	95 ± 1	94 ± 1	95 ± 1	92 ± 1	92 ± 1	91 ± 1	92 ± 1	95 ± 1
500	103 ± 2	95 ± 3	84 ± 4	100 ± 2	97 ± 4	86 ± 1	95 ± 2	94 ± 2	92 ± 1	93 ± 2	92 ± 2	89 ± 2	97 ± 4	97 ± 5
600	102 ± 2	94 ± 1	80 ± 3	98 ± 2	99 ± 6	88 ± 1	92 ± 2	92 ± 1	90 ± 2	91 ± 2	91 ± 2	90 ± 1	94 ± 6	95 ± 2
[f]Vortex shaken time (min)														
0	47 ± 6	45 ± 5	46 ± 4	49 ± 5	49 ± 6	46 ± 2	53 ± 5	51 ± 5	51 ± 5	53 ± 4	53 ± 5	54 ± 3	54 ± 4	54 ± 4
1	92 ± 2	81 ± 1	91 ± 7	86 ± 4	89 ± 5	113 ± 6	104 ± 2	103 ± 2	97 ± 2	93 ± 2	100 ± 7	105 ± 1	113 ± 7	92 ± 1
5	94 ± 2	84 ± 2	89 ± 3	88 ± 3	87 ± 1	98 ± 4	95 ± 3	94 ± 3	91 ± 2	89 ± 2	95 ± 3	97 ± 2	103 ±1	90 ± 4
10	95 ± 3	95 ± 2	80 ± 1	93 ± 2	95 ± 5	92 ± 2	105 ± 1	102 ± 2	103 ± 2	101 ± 2	106 ± 3	105 ± 4	105 ± 1	100 ± 2
15	90 ± 5	88 ± 1	83 ± 5	92 ± 4	93 ± 2	91 ± 9	103 ± 5	101 ± 5	100 ± 6	98 ± 6	102 ± 6	94 ± 3	96 ± 5	96 ± 3
30	88 ± 5	85 ± 1	81 ± 8	91 ± 7	93 ± 6	87 ± 6	99 ± 7	97 ± 8	98 ± 6	96 ± 7	99 ± 6	96 ± 1	97 ± 6	93 ± 6

SUPRAS volume: [c,d]400 µL, [f]200 µL; Volume of equilibrium solution: [c]400 µL, [e,f]900 µL.

[a]0.2 g of Leucodon sciuroides spiked with 100 µg kg[-1] of each PAH.

[b]Standard deviation, n=3.

Recoveries for PAHs gradually increased for SUPRAS obtained from bulk solutions containing increasing percentages of THF and they were equal or above 87% for water/THF ratios of 50/50. So, this composition of the bulk solution was selected for self-assembly of decanoic acid. Standards deviations were in the range 3-5%.

Use of different volumes of equilibrium solution, in the interval 400-900 μL, did not affected extraction recoveries for PAHs (Table 2), that indicating their preferential solubilization in the SUPRAS. Because of the main function of this equilibrium solution was the wetting of moss samples, a volume high enough to give three phases in the final step of the extraction procedure, namely a moss solid residue, an intermediate equilibrium solution and a SUPRAS extract (Fig. 2), was selected. According to this criterion, 900 μL of equilibrium solution were chosen for further experiments. In this way, the SUPRAS volume used for extraction was completely recovered.

The influence of the volume of SUPRAS on the extraction efficiency of PAHs is shown in Table 2. Because of the high number of solubilizing sites available in this extractant, recoveries were quite similar in the interval investigated (200-600 mL). Values for recoveries at the minimal volume tested (200 μL) ranged from 84 to 103% and this volume was selected as optimal in order to keep detection limits as low as possible.

Although the SUPRAS here selected has to be synthesized in 50/50 water/THF solutions, in theory, the percentage of decanoic acid used for self-assembly could be chosen at will since the composition of the SUPRAS remains unchanged as the concentration of decanoic acid varies (dotted line A in Fig. 3). In the application here developed we used both SUPRAS (200 μL) and equilibrium solution (900 μL) for the extraction of PAHs from mosses. So, we selected a percentage of decanoic acid (2.5%) to give an equilibrium solution/SUPRAS volume ratio (25.7 mL/6.3 mL = 4.8, section 2.3) similar to that necessary to treat a moss sample (900 μL/200 μL = 4.5). In this way we were able to treat 26 moss samples with the minimum waste of equilibrium solution or SUPRAS.

3.2.3. Optimization of extraction conditions

The extraction procedure has two steps, namely vortex shaken and centrifugation (Fig. 2). First, the time required to reach extraction equilibrium conditions, under vortex shaken of the moss sample/equilibrium solution/SUPRAS mixture at 2500 rpm, was investigated. Table 2 shows the results obtained, expressed as recoveries for PAHs, for vortex shaken times from 0 to 30 min. Low recoveries were obtained for samples only subjected to manual shaking for a few seconds (i.e. 0 min of vortex shaken). Equilibrium extraction conditions were rapidly reached (i.e. around 1 min) as the sample was vortex shaken, that indicating that transferring of PAHs from mosses to SUPRAS was very fast. An extraction time of 5 min was selected as optimum on the basis of a global improvement in the precision.

Centrifugation was necessary in order to obtain three distinguishable phases (solid moss sample, equilibrium solution, and SUPRAS) in the extraction tubes. Effective separation was reached after centrifugation at 8500 x g for 8 min.

3.2.4. Subsample representativity study

To evaluate the representativity of the amount of moss sample used for analysis, the variances obtained for the measurement of PAHs in 200 mg moss subsamples (n = 6) fortified with 50 μg kg^{-1} of each PAH were compared with those obtained from the measurement of 200 mg aliquots (n = 6) taken from a 4 g moss sample fortified at the same concentration level. No statistical significant differences between both variances were observed by applying a Fischer test [36]. The experimental F-values were in the interval 7.763-1.023 and were below the critical F-value (9.364, $n_1 = n_2 = 6$, significant level = 0.05).

[36]J.N. Miller, J.C. Miller, Statistic and Chemometrics for Analytical Chemistry, fifth ed., Pearson Education, Harlow, 2005, p. pp.49.

3.3. In-house method validation

The developed method was subjected to validation according to the guidelines established by the European Commission decision 2002/657/EC [37], which provides admissible performance criteria to evaluate if an analytical method is fit for the purpose.

3.3.1. Linearity and sensitivity

Calibration parameters and method detection (MDLs) and quantitation (MQLs) limits for the determination of PAHs are shown in Table 1. Calibration curves were run by analyzing standard solutions in acetonitrile containing the analytes at nine concentration levels. The maximum concentration tested was 250 μg L^{-1}. Correlation between peak areas and PAH concentrations was determined by linear regression. Correlation coefficients were in the range 0.9996-0.99999 indicating good fits. The MDLs and MQLs were calculated from six independent complete analyses (experimental procedure in Section 2.4) of moss samples by using a signal to noise ratio of 3 and 10, respectively. Since no blank moss samples could be obtained, an estimate of the background signal was made at a representative part of the readout, adjacent to the analyte signal. MDLs and MQLs were in the ranges of 0.04-0.24 and 0.14-0.80 μg kg^{-1} respectively, so quantification of PAHs at the low content they are usually present in moss samples can be performed.

3.3.2. Selectivity

The possible interference from matrix components was investigated by comparison of the slopes of the calibration curves obtained from standards

[37]Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. Eur. Comm. L (2002) 8.

in acetonitrile with those run from *Leucodon sciuroides* spiked with known amounts of PAHs within the linear range (Table 1) analyzed using the whole recommended procedure. The slopes of the calibration curves obtained for the moss and those obtained from standards in acetonitrile were not statistically different by applying a Student's t-test [38]. The experimental tvalues were in the interval 0.45-2.88 and were below the critical tvalue (3.25, significant level = 0.01).

3.3.3. Trueness

As no certified reference materials for PAHs in moss were available, the trueness for the method here developed was investigated by repetitive analysis (n = 6), of *Leucodon sciuroides* samples fortified at two concentration levels; i.e. 2 μg kg^{-1} and 50 μg kg^{-1}. Because of the absence of blank moss samples, recoveries for each PAH was calculated by subtracting the analyte concentrations found in fortified and unfortified samples. The recoveries at 2 μg kg^{-1} and 50 μg kg^{-1} were in the ranges 71-110% and 88-105%, respectively. These results were consistent with the 2002/657/EC decision [37] which considers that the recoveries should be in the interval 70-110% and 80-110% for analyte concentrations lower and higher than 10 μg kg^{-1}, respectively.

3.3.4. Precision

Precision was studied in terms of repeatability and within-laboratory reproducibility. For this purpose, moss samples spiked with PAHs at two concentration levels 2 μg kg^{-1} (n = 18) and 50 μg kg^{-1} (n = 18) were analyzed in three consecutive days (six samples each) using freshly prepared SUPRAS equilibrium solution, mobile phases and standard solutions. The repeatability, expressed as standard deviation, was calculated as the square root of the average value of the intra-day variances obtained and, the within laboratory

[38]L.C. Rodriguez, A.M.G. Campana, F.A. Barrero, C.J. Linares, M.R. Ceba, Validation of an analytical instrumental method by standard addition methodology, J. AOAC Int. 78 (1995) 471-476.

reproducibility as the square root of the mean intra-day variance plus the inter-day variance. The relative standard deviations under repeatability and reproducibility conditions varied within the intervals 1.7-11.8% and 1.2-10.0% at 2 μg kg^{-1}, and 3.5-7.8% and 2.5-8.4% at 50 μg kg^{-1}, respectively, which is in compliance with the 2002/657/EC Commission Decision (i.e. the relative standard deviations for within-laboratory reproducibility conditions should not exceed 20% for analyte concentrations in the range >10-100 μg kg^{-1}) [37].

3.4. Analysis of moss samples

The proposed method was applied to the determination of PAHs in different moss species taken randomly in different locations of the South of Spain (see section 2.4.1). Pleurocarpous mosses are the species more frequently used as biomonitors and their use has been encouraged in the 2015 European moss survey for PAHs biomonitoring [16]. For this reason, a pleurocarpous moss (i.e. *Leucodon sciuroides*) was used in this research for method optimization. However, pleurocarpous mosess are very sensitive to pollution and dryness, and therefore are rare, sometimes absent, in urban areas and dry regions [13]. So, acrocarpous mosses, which grow on stone or brick walls and are more commonly used for studying urban environments and dry regions, were also analyzed.

Both native and fortified (5 μg kg^{-1} each PAH) moss samples were quantified. Table 3 shows the mean values obtained for the concentration (μg kg^{-1}) and recoveries (%) found for PAHs from three independent determinations, besides their corresponding standard deviations and variation coefficient, respectively. All the results were expressed on a dry weight basis. Table 3 also includes the sum of the concentrations of all PAHs, as well as the sum of the four PAHs used by EMEP to model PAH pollution (i.e. BaP, BbF, BkF and IcdP).

Table 3

Mean concentrations and recoveries obtained for the determination of PAHs in unfortified and fortified mosses, respectively.

	Concentration found[a] ± S (µg kg^{-1} dry weight)[b] (Recoveries[c] ± RSD, %)[d]					
PAHs	[e]*Fissidens crassipes*	[e]*Fissidens crassipes*	[e]*Plagiomnium undulatum*	[e]*Pleurochaete squarrosa*	[e]*Distichium capillaceum*	[f]*Leucodon sciuroides*
ACE	ND (82 ± 3)	1.32 ± 0.05 (74 ± 4)	2.53 ± 0.05 (84 ± 10)	2.01 ± 0.03 (84 ± 3)	0.21 ± 0.02 (75 ± 4)	ND (72 ± 2)
FLU	0.81 ± 0.06 (98 ± 6)	1.2 ± 0.1 (95 ± 8)	3.0 ± 0.3 (95 ± 2)	2.83 ± 0.09 (77 ± 5)	2.4 ± 0.2 (89 ± 2)	ND (75 ± 3)
PHE	7.8 ± 0.6 (76 ± 7)	12.2 ± 0.2 (95 ± 6)	9.3 ± 0.6 (110 ± 6)	20.0 ± 0.8 (98 ± 8)	5.5 ± 0.1 (88 ± 10)	7.5 ± 0.7 (83 ± 3)
ANT	0.62 ± 0.01 (100 ± 4)	ND (104 ± 4)	0.75 ± 0.04 (80 ± 3)	1.44 ± 0.07 (104 ± 6)	0.73 ± 0.03 (104 ± 3)	0.81 ± 0.03 (109 ± 3)
FLT	0.61 ± 0.05 (97 ± 5)	2.9 ± 0.1 (85 ± 4)	0.71 ± 0.04 (71 ± 3)	ND (74 ± 4)	ND (92 ± 6)	ND (98 ± 7)
PYR	1.6 ± 0.1 (103 ± 3)	3.84 ± 0.06 (106 ± 3)	2.1 ± 0.1 (84 ± 7)	10.5 ± 0.7 (86 ± 6)	0.82 ± 0.05 (87 ± 9)	2.2 ± 0.1 (84 ± 8)
BaA	0.33 ± 0.01 (100 ± 5)	1.8 ± 0.1 (105 ± 3)	0.43 ± 0.03 (77 ± 4)	1.82 ± 0.03 (98 ± 9)	ND (105 ± 2)	0.33 ± 0.02 (80 ± 2)
CHY	0.52 ± 0.05 (102 ± 3)	2.53 ± 0.06 (104 ± 1)	2.2 ± 0.2 (83 ± 7)	10.9 ± 0.6 (86 ± 4)	0.81 ± 0.04 (91 ± 1)	3.2 ± 0.1 (83 ± 4)
BbF	0.93 ± 0.09 (102 ± 2)	3.8 ± 0.1 (94 ± 5)	1.84 ± 0.06 (86 ± 1)	10.3 ± 0.7 (80 ± 11)	0.81 ± 0.03 (107 ± 7)	2.4 ± 0.1 (86 ± 4)
BkF	0.62± 0.01 (99 ± 1)	1.43 ± 0.02 (92 ± 1)	0.93 ± 0.05 (79 ± 2)	2.6 ± 0.1 (105 ± 5)	0.75 ± 0.02 (107 ± 2)	1.01 ± 0.06 (89 ± 1)
BaP	0.57 ± 0.02 (106 ± 2)	1.15 ± 0.06 (108 ± 3)	0.65 ± 0.03 (73 ± 2)	5.6 ± 0.2 (107 ± 8)	0.54 ± 0.02 (107 ± 5)	1.04 ± 0.04 (86 ± 1)
DahA	ND (84 ± 3)	ND (83 ± 3)	ND (76 ± 5)	ND (100 ± 4)	ND (99 ± 2)	ND (105 ± 3)
BghiP	1.62 ± 0.09 (103 ± 2)	3.26 ± 0.08 (101 ± 4)	1.01 ± 0.08 (100 ± 9)	13.5 ± 0.8 (83 ± 5)	ND (101 ± 5)	2.2 ± 0.2 (88 ± 4)
IcdP	1.0 ± 0.1 (71 ± 3)	2.71 ± 0.03 (82 ± 2)	0.62 ± 0.04 (78 ± 7)	15.2 ± 0.5 (76 ± 2)	ND (80 ± 4)	1.8 ± 0.1 (84 ± 4)
∑PAH14	17 ± 1	38.1 ± 0.6	26.1 ± 0.3	97 ± 2	12 ± 1	22 ± 1
[g]∑PAH4	3.1 ± 0.1	9.1 ± 0.2	4.04 ± 0.09	34 ± 1	2.10 ± 0.06	6.3 ± 0.3

[a] Mean of three independent determinations.; [b] Standard deviation.; [c] Spike level for each analyte: 5 µg kg^{-1}.; [d] Relative standard deviation (n = 3); [e] Acrocarpous moss species; [f] Pleurocarpous moss species; [g] Sum of the concentrations of BbF, BkF, BaP and IcdP.; ND (not detected).

Recoveries for analytes in the samples were all within the range 70-110%, as recommended for the 2002/657/EC decision for analyte concentrations <10 μg kg^{-1} [37]. Fig. 4 shows, as an example, the chromatograms obtained from three species of moss samples, both unfortified and fortified.

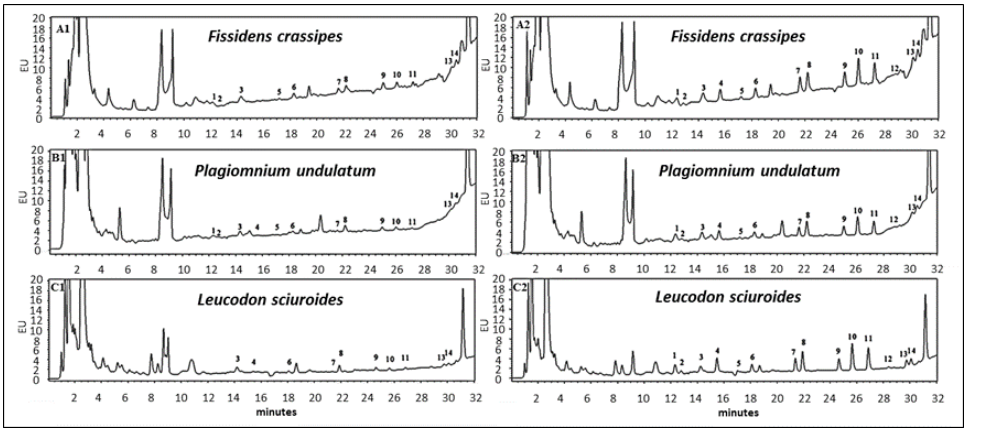

Fig. 4. LC-FLD chromatograms corresponding to (1) unfortified (2) fortified (5 μg kg^{-1} of each PAH) samples of (A) Fissidens crassipes, (B) Plagiomnium undulatum, and (C) Leucodon sciuroides. Peak assignment: (1) ACE, (2) FLU, (3) PHE, (4) ANT, (5) FLT, (6) PYR, (7) BaA, (8) CHY, (9) BbF, (10) BkF, (11) BaP, (12) DahA, (13) BghiP, (14) IcdP.

Confirmation of the presence of each PAH was performed by co-chromatography [37]. For this purpose, the retention time and the peak width at half-maximum height obtained for each analyte from non-spiked and spiked samples were compared. The peak width at half-maximum height should be within the 90e110% range and retention times should be identical within a margin of 5%. Differences between retention times and peak width for analytes measured from spiked and non-spiked samples were lower than 2% and within the range 94-108%, respectively, in all cases.

On the whole, the total concentration of PAHs found in the different moss species located in various sites of the South of Spain were within the values previously reported in the literature [13,15,20,22] and showed a clear spatial pattern, with the highest and the lowest levels at the industrial (97 μg

kg^{-1}) and remote (12 97 μg kg^{-1}) sites, respectively. In concordance with previous reports, phenanthrene was the dominant PAH in the moss samples analyzed [13]. Regarding PAH4, their concentrations accounted for 35.0% of the total PAHs found in the industrial site and were in the range 15.5-27.8% for mosses collected from unpolluted areas. To the best of our knowledge, only an evaluation of the correlation between the modeled and moss PAH4 concentrations found at several European sites (i.e. small regions with quite dense distributions of monitoring points located in France, Norway, Poland, Slovenia, Spain, and Switzerland) has been reported [12]. Both significant and low correlation between the modeled PAH4 concentrations and measurement in mosses were found depending on the sites and specific PAH. These results demand for further studies involving a wider coverage of countries.

4. Conclusions

Restricted access materials have found wide acceptance for analysis of drugs, drug metabolites, peptides and other analytes in matrices such as plasma, serum, whole bood and urine. Application to matrices other than biological fluids has been more limited [1-3]. The ability of some supramolecular solvents to act as restricted access materials for macromolecules, by both chemical and physical mechanisms, opens the possibility to conveniently extend these materials to the extraction and purification of complex solid samples. The research here presented was intended to contribute to this aim.

Regarding the application here developed, PAHs are being currently measured at only 22 stations of the 34 available sites in the EMEP monitoring network. So, moss biomonitoring of PAHs can greatly help to increase spatial coverage of precipitation measurements and a great deal of effort is being made in this respect [14-16]. In our opinion, one of the factors that can help to the successful implementation of moss biomonitoring of PAHs is the availability of simple, low cost and widely accessible analytical methods. The one here developed was intended for this purpose and mainly tried to overcome major problems associated to sample treatment. An overview of the state of art in the analysis of PAHs in mosses has been recently reported [20]. Compared to these methods, the one presented here is simpler (i.e. no cleanup

or solvent evaporation is required), faster (extraction and centrifugation time are 5 and 8 min, respectively) and more environmentally friendly (a substantial reduction of solvent consumption is achieved). In addition, sample treatment features low cost, several samples can be simultaneously treated and it requires conventional equipment that is within everyone's reach. Regarding analytical features, MQLs (0.14-0.80 μg kg^{-1}) were at the same (e.g. 0.1-2.5 μg kg^{-1} [22,31], 0.3-1 μg kg^{-1} [24], 0.1-1.7 μg kg^{-1} [20]) or lower (3.3-7.8 μg kg^{-1} [29], 7-35 μg kg^{-1} [39]) level than those previously reported for LC-FLD- and LC-MS-based methods while recoveries were improved thanks to the high solubilization properties of the SUPRAS [20].

As it has been discussed by Harmens et al. in an excellent review on the use of mosses as biomonitors of atmospheric POPs pollution [13], to further establish the suitability of mosses for this purpose it will be essential their sampling at sites where atmospheric POPs concentrations and/or deposition fluxes are determined, for example at EMEP or national POPs monitoring sites, in order to establish proper correlations.

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BLOQUE B

SUPRAS-RAM basados en ácidos alquil carboxílicos para la determinación de componentes en multi-matrices.

CAPÍTULO 3

Restricted access supramolecular solvents for the simultaneous extraction and cleanup of ochratoxin A in spices subjected to EU regulation

Restricted access supramolecular solvents for the simultaneous extraction and cleanup of ochratoxin A in spices subjected to EU regulation

Noelia Caballero-Casero, Sergio García-Fonseca and Soledad Rubio

Abstract

A supramolecular solvent (SUPRAS) made up of inverted hexagonal aggregates of decanoic acid was here proposed for the simultaneous microextraction of ochratoxin A (OTA) and sample cleanup in spices regulated by the European Union. Macromolecules were excluded from extraction by chemical and physical mechanisms. The method involved the stirring of the sample (0.2 g of spice) with 0.4 mL of SUPRAS for 10 min, subsequent centrifugation and direct analysis of the crude extract by liquid chromatography/fluorescence detection. Extracts were clean enough to reliable quantitation of OTA in paprika, nutmeg, black pepper, ginger and turmeric using solvent based calibration. Method validation proved that it met performance criteria in terms of sensitivity, recoveries, precision, selectivity and trueness set by EU guidelines for quantitation of OTA. So, the method can be recommended for enforcement and surveillance programs that monitor OTA in spices.

1. Introduction

Contamination of spices with Ochratoxin A (OTA) has been well documented in several surveys of retail markets in Brazilian [1], China [2], Italy [3], Malaysia [4], Pakistan [5], Poland [6], Spain [7] and Turkey [8], among others. In 2013, spices contaminated with OTA represented around 15% of the RASFF notifications related to the unacceptable presence of OTA in food [9] and according to a report of the Directorate of the General Health and Consumer Protection [10], the contribution of spices to the dietary intake of OTA by the European population is around 8%.

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In 2010, the EU set a maximum tolerable limit of 30 μg/kg for OTA in spices until mid-2012, when it was reduced to 15 μg/kg [11]. The spices under regulation included chilies, chili powder, cayenne, paprika, white and black pepper, nutmeg, ginger and turmeric, as well as mixtures containing one or more of the abovementioned spices. Because of the difficulty of the producing regions to achieve the projected lower maximum level for OTA in Capsicum spices on a consistent basis, the tolerable limit for these spices was kept at 30 μg/kg until 31.12.2014 and then reduced to 15 μg/kg [12].

Spices are mainly produced in India (˜74% of the global market), followed by Bangladesh (˜7%), Turkey (˜7%) and China (˜5%) [13]. Contamination by OTA occurs prior to harvest, or more commonly during storage, due to the growth of Penicillium verrucosum and Aspergillus ochraceu, which is favored by environmental conditions, such as temperature and humidity, and by poor manufacturing in the production regions. OTA has been identified as nephrotoxic, hepatotoxic, immunotoxic and teratogenic [14], and classified as possible carcinogen for humans by the International Agency for Research of Cancer [15]. Human exposure to OTA has been confirmed by chemical analysis of biological samples such as blood, urine and

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milk from randomly selected healthy humans [10], and high levels in serum have been found in patients showing symptoms of ochratoxicosis [16].

Driven by regulatory authorities (e.g. EC, 2010 [11] and EC, 2012 [12]), extensive analytical efforts have been addressed in the last few years to enable fast and reliable analysis of OTA in spices for surveillance and monitoring purposes [17-19]. Table 1 summarizes sample treatment procedures, separation and detection techniques and analytical features for some of the most representative methods reported for the determination of OTA in spices. Only results concerning the analysis of spices regulated by the EU have been included in this table.

Due to the strong native fluorescence of OTA, LC-FD is considered a good technique for routine analysis of spices since it offers excellent sensitivity [1-5,7,8]. Triple quadrupole MS, often coupled to ultra performance liquid chromatography (UPLC), has been also reported for this purpose [6,20,21]. Usually, spices are extracted with mixtures of acetonitrile/water or methanol/water containing additives like sodium hydrogen carbonate or sodium chloride to enhance solubility and extraction efficiency of OTA. Both,

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solvent volume (mL) to sample size (g) ratios and extraction times vary in a wide range (e.g. 2-20 and 1 min-1h, respectively), however good recoveries are obtained for OTA in the spices investigated under most of the reported experimental conditions (see Table 1 and the references included herein).

Table 1

Comparison of LC methods for the determination of OTA in spices

Sample type (size)	Sample treatment	Separation / detection	Recovery (%)	Method quantitation limit(µg/kg)	Ref.
Chili, turmeric, curry (5 g)	Solvent extraction (20 mL methanol:water 80:20, v/v, and 0.5 g sodium chloride) for 30 min. Centrifugation, cleanup on IAC (AflaOChra HPLC™ multi-mycotoxins cartridges), OTA elution (1 mL methanol) and dilution (1 mL 0.1% acetic acid).	HPLC-FD (External calibration)	71.9-94.2	0.4	(Ainiza et al., 2015)
Pepper, chili, nutmeg, curry (4 g)	Solvent extraction (20 mL methanol:water, 80:20, v/v) for 5 min under ultrasonic stirring. Centrifugation, cleanup on IAC (OtaClean), OTA elution (3 mL methanol), evaporation to dryness and reconstitution in methanol (1 mL).	HPLC-FD (Standard addition method)	71-102.1 (Apparent recoveries)	0.1-2.61	(Prelle et al., 2014)
Chili (25 g)	Solvent extraction (100 mL acetonitrile:water 84:16, v/v, and 5 g of NaCl) for 1 min. Filtration, cleanup on IAC (OchraTest), OTA elution (1.5 mL methanol), evaporation to dryness and reconstitution in acetonitrile:water:acetic acid (47:51:2, v/v/v, 1 mL).	HPLC-FD (External calibration)	92-93	0.18	(Iqbal et al., 2013)
Red chili flake, red chili powder, black pepper, turmeric (25 g)	Solvent extraction (200 mL acetonitrile:water, 60:40, v/v) for 1 min. Filtration, cleanup on IAC (OchraTest), OTA elution (1 mL methanol), evaporation to dryness and reconstitution in acetonitrile:water:acetic acid (47:51:2, v/v/v, 1 mL).	HPLC-FD (External calibration)	86.6-95.7	0.19	(Ozbey et al., 2012)
Chili (25 g)	Solvent extraction (100 mL methanol:$NaHCO_3$ 1%, 70:30, v/v) for 3 min. Centrifugation, cleanup on IAC (OchraTest), OTA elution (1.5 mL methanol), evaporation to dryness and reconstitution in methanol:water (0.5 mL 50:50, v/v).	HPLC-FD (External calibration)	103.4	0.06	(Jalili et al., 2012)
Pepper, chili, curry (25 g)	Solvent extraction (100 mL methanol:water, 60:40, v/v) for 30 min. Filtration, cleanup on IAC (OchraTest™), OTA elution (2 mL methanol).	HPLC-FD (External calibration)	75-102	0.5	(Zhao et al., 2014)

Paprika (5 g)	Solvent extraction (100 mL methanol:NaHCO3 1%, 50:50, v/v) for 3 min. Filtration and cleanup on IAC (OchraprepR), OTA elution (2 mL of methanol–acetic acid mixture, 49:1, v/v). Evaporation to dryness and reconstitution in mobile phase.	HPLC-FD External calibration	92.5-108	0.80	(Shundo et al., 2009)
Paprika, chili (5 g)	Solvent extraction (100 ml of 1% (v/v) sodium bicarbonate-deionised water) for 20 min. Filtration, cleanup on IAC (Ochraprep), OTA elution (1.5 ml of acetic acid–methanol, 2:98, v/v, and then 1.5 mL distilled, and both eluates combined).	HPLC-FD (External calibration)	73.3-83.8	0.1	(Santos et al., 2010)
Hot and sweet paprika (2 g)	QuEChERS extraction (20 mL acetonitrile:water, 50:50, v/v, 4 g magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dehydrate and 0.5 g disodium hydrogen citrate sesquihydrate) for 10 min. Centrifugation, frozen out at -80 °C for 30 min and centrifugation. Evaporation to dryness and reconstitution in methanol:water (0.5 mL, 50:50, v/v).	UPLC-Orbitrap-HRMS (Matrix-matched calibration)	100	1.7	(Reinholds et al., 2016)
Black and white pepper, curry nutmeg, cayenne, turmeric (10g)	Solvent extraction (30 mL acetonitrile:water, 60:40, v/v) for overnight. Cleanup on IAC (OchraTest), OTA elution (1.5 mL methanol), evaporation to dryness, reconstitution in methanol (1 mL).	UPLC-QQQ-MS (External calibration)	95.6-101.3 (black pepper)	0.3	(Waskiewicz et al., 2013)
Black and white pepper, red chili (1 g)	5 mL water for 1 min vortex-shaking, and soaking 30 min. Solvent extraction (5 mL water) for 30 min and then (5 mL acetonitrile:formic acid, 99:1, v/v) for 20 min. Addition of 2 g magnesium sulphate, and 0.5 g sodium chloride. Centrifugation and filtration.	HPLC-QQQ-MS (Matrix-matched calibration)	98-104 (apparent recoveries)	4.2-13	(Yogendrarajaha et al. 2013)
Ginger (20 g)	Solvent extraction (40 mL acetonitrile:water, 60:40, v/v) for 20 min by sonication. Cleanup on AFFINIMIP-based SPE, OTA elution (2 mL methanol:acetic acid, 98:2, v/v). Evaporation to dryness and reconstitution (1 mL methanol/0.5% aqueous acetic acid (65/35, v/v).	UPLC-QQQ-MS (External calibration)	91.1-92.3	0.09-0.3	(Cao et al. 2013)
Red pepper powder, white and black pepper (5 g)	Solvent extraction (20 ml acetonitrile/water, 86:14, v/v) for 1 h. Filtration and 4-fold dilution with mobile phase eluent.	UHPLC-QQQ-MS (Matrix matched calibration)	112 (red pepper)	2.7 (red pepper) 144 (black pepper)	(Skrbi et al., 2013)

In general, and independently of the applied detection technique, there is a need for careful sample cleanup and analyte enrichment [22]. Spices are classified within the group difficult or unique commodities [23]. They are considered as complex matrices due to their high content in pigmentation and essential oil compounds, which interfere with the extraction of mycotoxins [24,25]. In addition, they contain high concentration of carbohydrates and some of them, high fat content (e.g. nutmeg). Immunoaffinity columns (IACs) are now in routine use in OTA protocols involving LC-FL detection (Table 1 and references included herein). These materials exclusively retain OTA, thus producing cleaner extracts with a minimum level of interfering matrix components and excellent signal to noise ratios compared to less selective SPE sorbent materials [22]. IACs are also a good selection for the highly selective LC-MS detection in order to avoid signal suppression/enhancement [6]. However, IACs are expensive and less feasible to multitoxin analysis since they are highly specific for only one target or class mycotoxin. So, methods intended to simultaneously determine aflatoxins (AFs) and OTA in spices (maximum limits have been also set for AFs by the EU) use two parallel sample treatment protocols (i.e. different solvent extractions and immunoaffinity materials) as well as different chromatographic runs [1,3-5,7,8]. Recently, a multi-mycotoxin immunoaffinity column has been

[22]Zöllner, P., Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography–atmospheric pressure ionisation mass spectrometry. *J. Chromatogr. A, 1136,* 123-169.

[23]EC. (2014). Commission Regulation No 519/2014 of 16 May 2014 amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis. Official Journal of the European Union, L 147, 29-43.

[24]Bircan, C. (2005). The determination of aflatoxins in spices by immunoaffinity column extraction using HPLC. Int. J. Food Sci. Tech., 40, 929-934.

[25]O'Riordan, M. J., Wilkinson, M. G. (2009). Comparison of analytical methods for aflatoxin determination in commercial chilli spice preparations and subsequent development of an improved method. Food Control, 20, 700-705.

proposed for spice matrix cleanup in order to determine AFs and OTA simultaneously [26].

Despite the higher selectivity of LC-MS compared to LC-FD, direct analysis of solvent extracts from spices using both high [27] and low [21,28] resolution MS, requires the use of matrix-matched calibration in order to achieve reliable quantitation of OTA. In some matrices such as black pepper, complete signal suppression has been found for OTA when analyzed in crude extracts, that significantly deteriorating the accuracy, precision, sensitivity and linearity of the method [21]. The use of molecularly imprinted polymer-based SPE prior to LC-MS has been proposed as an alternative to IAC for sample cleanup [20]. Independently of the technique and the method used, concentration of OTA is commonly carried out by evaporation to dryness of the eluates coming from SPE.

This research was intended to develop a cost-effective, high-throughput procedure for the routine determination of OTA in the spices included in EU regulations. For this purpose, the suitability of a supramolecular solvent with restricted access properties (SUPRAS-RAM) to simultaneous extract OTA and remove interferences from spice matrices was explored. The aim was to obtain crude extracts clean enough to be directly analyzed by LC-FD. In this way, by removing the need for using IACs, time and costs can be considerably reduced. Below, the most salient results obtained in the optimization of the extraction and cleanup of OTA from spices using a decanoic acid-based SUPRAS, as well as the results found in method validation and application of the proposed method to the analysis of spices, are presented and discussed.

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[26]Ainiza, W.M.W., Jinap S., Sanny M. (2015). Simultaneous determination of aflatoxins and ochratoxin A in single and mixed spices. Food Control, 50, 913-918.

[27]Reinholds, I., Pugajeva, I., Bartkevics V. (2016). A reliable screening of mycotoxins and pesticide residues in paprika using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry. Food Control, 60, 683-689.

[28]Yogendrarajaha P., Pouckeb C., Meulenaera B., Saeger S. (2013). Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices. J. Chromatogr. A, 1297, 1-11.

2. Experimental

2.1 Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Decanoic acid was obtained from Sigma-Aldrich (Barcelona, Spain). Tetrahydrofuran (THF), HPLC-gradient acetonitrile and acetic acid glacial were supplied by Panreac (Sevilla, Spain). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Ochratoxin A was purchased from Sigma (St. Louis, MO, USA). A stock standard solution of 10 mg/L of OTA was prepared in acetonitrile and stored under dark conditions at -20ºC. Working solutions were prepared daily by dilution of the stock standard solution with acetonitrile:water (1:1 v/v).

2.2 Apparatus

The liquid chromatographic system used consisted of a ThermoQuest spectra system (San Jose, CA, USA) furnished with a SCM 1000 vacuum membrane degasser, a P2000 binary pump, an AS3000 autosampler, a Spectra System UV6000 LP detector and a FL3000 fluorescence detector. In all experiments a PEEK Rheodyne 7125NS injection valve with a 20 μL sample loop was used (ThermoQuest, San Jose, CA, USA). The analytical column was a Kromasil C8 (5 μm, 250 mm × 4 mm) from Analysis Vínicos (Tomelloso, Spain). It was thermostated at 35 ºC. A Retshc MM301 mixer mill, a vortex-shaker REAX Top equipped with an attachment (ref. 549-01000-00) for 10 microtubes from Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R equipped with an angle rotor 36x2.2/1.5 mL (ref. 11462) from MPW Med-Instruments (Warschaw, Poland) were used for

sample preparation. A digitally regulated centrifuge Mixtasel equipped with an angle rotor 4x100 mL (ref. 7001326) from JP-Selecta (Abrera, Spain) was used for SUPRAS production.

2.3. Supramolecular solvent production

The following procedure, which permits to obtain a volume of SUPRAS (10.4 mL) able to treat 25 spice samples, was routinely followed. Decanoic acid (1.5 g) was dissolved in THF (12 mL) in a 50 mL glass centrifuge tube. Then, 18 mL of 0.01 M aqueous hydrochloric acid were added. After sealing the tube with parafilm to avoid THF evaporation, the mixture was magnetically stirred for 1 min, time in which the SUPRAS spontaneously formed into the bulk solution, and then it was centrifuged at 2400g for 10 min to speed solvent separation up. The SUPRAS, which is less dense than water, was withdrawn using a 1 mL glass-syringe, transferred to a hermetically closed glass vial and stored at 4 °C until use. Under these conditions, the SUPRAS produced was stable for at least one month. The volume obtained can be adjusted at will by choosing an appropriate, constant surfactant/THF/aqueous hydrochloric acid ratio. Figure 1A shows a schematic of the procedure followed to get the SUPRAS.

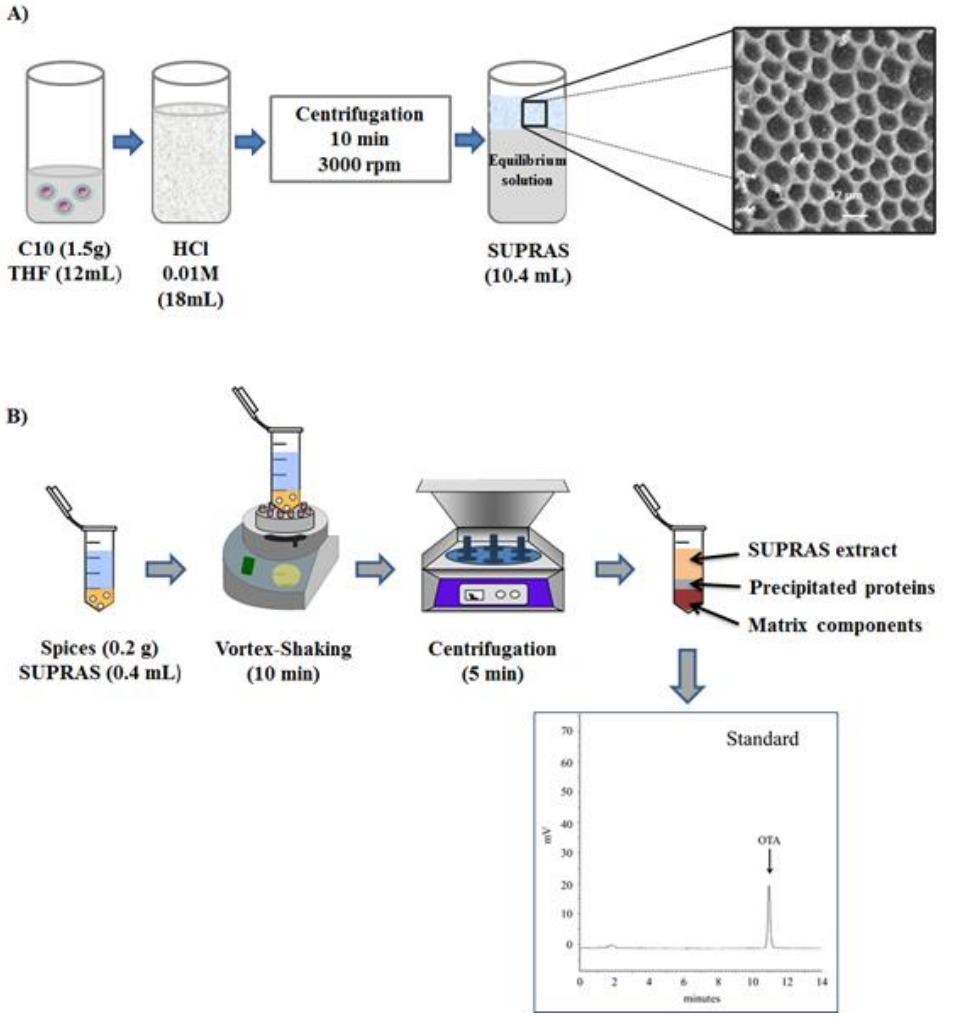

Fig. 1. Schematics of the procedures followed for (A) the synthesis of the SUPRAS, including a micrograph obtained by cryo-SEM that shows the hexagonal inverted aggregates making it up, and (B) the determination of OTA in spices, including a chromatogram for a standard solution of OTA.

2.4. Determination of OTA in spices

2.4.1 Sample collection and preparation

Spice samples (black pepper, nutmeg, ginger, turmeric and paprika) were purchased in local supermarkets in Córdoba (South Spain) and stored in their original containers at 4 ºC, under dark conditions, until analysis. The whole content of consumer-size packages was used for sample treatment. Where the retail pack was much less than 100 g (e.g. around 50 g for nutmeg, ginger, black pepper and turmeric) one sample consisting of two mixed retail packs was taken for analysis, according to EU regulations [29]. Samples were ground and blended in a mixer mill (particle size < 0.5 mm). Paprika, usually supplied as a fine powder, was used as supplied. Aliquots of 200 mg were taken for analysis and recovery experiments, which were performed in triplicate. The subsamples were fortified with OTA (concentration in samples = 15 μg/kg) by adding a minute volume (<20 μL) of a standard solution in acetonitrile-water. Fortified samples were left to stand at room temperature and dark conditions for 60 min before analysis. OTA in samples was stable during this period of time. For subsample representativity studies, the whole content of the sample was fortified.

2.4.2 SUPRAS-based microextraction

In a 2 mL Safe Lock microtube from Eppendorf Ibérica (Madrid, Spain) were mixed 200 mg of spice sample and 0.4 mL of SUPRAS. Four little glass balls (3 mm diameter) were introduced in the microtube to favor sample dispersion during extraction. The mixture was vortex-shaken at 2500 rpm for 10 min and then centrifuged at 14160 g for 5 min to separate the SUPRAS

[29]EC. (2006). Commission Regulation (EU) No 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L 70, 12.

from the solid residue. Finally, the SUPRAS extract was transferred to a glass vial, from which 20 μL was injected into the chromatographic system. A schematic of the procedure followed for sample treatment is shown in Figure 1B.

2.4.3. Liquid chromatography/ fluorescence detection

Quantification of OTA was carried out by LC-FD. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 1% glacial acetic acid. The elution program was as follows: isocratic conditions for 10 min (55% solvent B), then linear gradient for 3 min until reaching 52% of solvent B and finally, linear gradient for 5 min until reaching 100% of solvent B. The conditioning of the column took 7 min. The mobile phase was pumped at a constant flow rate of 1 mL/min. OTA eluted at approximately 11.2 min and was monitored at excitation and emission wavelengths of 334 and 460 nm, respectively. UV-visible spectra were also obtained in the range 200-700 nm for identification purposes. Calibration curves were run by injecting 20 μL of standards dissolved in acetonitrile:water (1:1 v/v) in the range of concentrations 1.2-500 μg/L.

3. Results and discussion

3.1. Solubilization and restricted access properties of decanoic acid-based SUPRASs

Molecules of decanoic acid spontaneously self-assemble and coacervate as inverted hexagonal aggregates in mixtures of THF and acid

aqueous solutions, with the polar groups surrounding aqueous cavities and the hydrocarbon chains dissolved in THF [30]. The pH of the water used in the synthesis is kept below 4 to prevent decanoic acid from deprotonation (pKa = 4.8±0.2), and consequently, from solubilization in the hydro-organic phase in equilibrium with the SUPRAS.

Both, the two microenvironments and mixed-mode mechanisms offered by the hexagonal aggregates make them suitable to solubilize analytes in a wide polarity range. The expected driving forces for microextraction of OTA are Van der Waals interactions between the hydrocarbon chains of the decanoic acid and the hydrocarbon framework of OTA, and hydrogen bonds between the acceptor and donor groups of OTA and the polar head-groups of the surfactant and/or water in the aqueous cavity.

The volume of SUPRAS produced in the self-assembly process was linearly dependent on the amount of decanoic acid and exponentially dependent on the percentage of THF in the bulk solution according to the equation 1:

$$y = (1.03\pm0.02)\, a\, e^{(0.0473\pm0.0009)\, b} \qquad (1)$$

where y is the volume (mL) of SUPRAS, a the amount (g) of decanoic acid and b the THF percentage (v/v). This equation indicated that both the global composition of the SUPRAS and the size of the aqueous cavities can be tailored by controlling the THF:water ratio in the solution where the molecules of decanoic acid self-assemble. Thus, the SUPRASs obtained from a constant concentration of decanoic acid and increasing THF/water ratios gradually contained more THF and water and consequently became

[30]Ruiz, F.J., Rubio, S., Pérez-Bendito, D. (2007). Water-induced coacervation of alkyl carboxylic acid reverse micelles: phenomenon description and potential for the extraction of organic compounds. Anal. Chem. 79, 7473-7484.

increasingly diluted with respect to the surfactant. The higher content of water in the SUPRAS with increasing THF/water ratios also caused the aqueous core of the inverted hexagonal aggregates to increase and it opens the possibility of using these liquids as restricted access materials.

Regarding the EU regulated spices, they consist of 6.2-11.4 percent water, 5.8-14.8 percent proteins, a high percent of carbohydrates (49.3-71.6) and a wide range of fat content (4.2-36.3%) (Table 2). Considering the carbohydrate fraction, fiber represents a major component while the content of sugars is very different for the target spices (e.g. 0.6% for black pepper and 28.5% for nutmeg).

Table 2

Composition of the spices regulated for OTA by the European Union

Spices	Nutritional composition (g/100 g)					
	Water	Protein	Total Carbohydrate	Fiber	Sugars	Fat
Paprika	9.5	14.8	55.7	37.4	10.3	11.6
Black pepper	10.5	10.9	64.8	26.5	0.6	3.12
Nutmeg	6.2	5.8	49.3	20.8	28.5	36.3
Ginger	9.9	9.0	71.6	14.1	3.4	4.2
Turmeric	11.4	7.8	64.9	21.1	3.2	9.8

The ability of decanoic acid-based SUPRASs to exclude proteins and carbohydrates by both chemical and physical means, and consequently to achieve sample cleanup, was examined. Experiments were conducted by extracting 20 mg of pure standards of typical proteins and carbohydrates in 0.4 mL of SUPRAS obtained according to the procedure specified in section 2.3.

Under SUPRAS extraction, aqueous solutions of albumin from bovine serum, albumin from chicken egg white and gluten from wheat gave a white precipitate that separated from the solvent as a thin layer upon centrifugation.

Both THF and decanoic acid were considered responsible for protein precipitation owing to the decrease of the dielectric constant of the solution and the formation of macromolecular complexes [31]. An identical white layer was clearly observed between the insoluble matrix components and the SUPRAS after extraction and centrifugation of spices. So, removal of proteins from spices was carried out during extraction of OTA.

The behavior of polysaccharides during extraction was evaluated by using starch. It was checked that this polymer did not incorporate to the SUPRAS. Extractions were also carried out under experimental conditions known to increase the solubility of starch (e.g. 40ºC) but no extraction in the SUPRAS was observed. Therefore, the SUPRAS has the ability to act as a RAM for polysaccharides, probably because of size exclusion of these hydrophilic polymers from the aqueous cavity.

3.2. Optimization of the microextraction of OTA

Optimization was carried out by extracting 200 mg of hot paprika fortified with OTA at the maximum level set by the EU for this toxin in spices (i.e. 15 μg/kg;[12]). Paprika was selected because its composition regarding water, proteins, carbohydrates, fiber, sugars and fat was considered appropriate for studying effects of the different matrix components making up the regulated spices (Table 2). Paprika samples were fortified 1 h before extraction. It was checked that equilibration between OTA and paprika matrix for a longer period of time (i.e. overnight) did not change the extraction efficiency. Experiments were made in triplicate. Selection of the optimal conditions was based on recoveries (R) and estimated method quantitation limits (MQLs). Estimation was carried out from the instrumental

[31]Morais, V., Massaldi, H. (2012). A model mechanism for protein precipitation by caprylic acid: Application to plasma purification. Biotechnol. Appl. Biochem., 59, 50-54.

quantitation limit, the volume of SUPRAS used for extraction, the recoveries obtained and the weight of sample used for analysis.

The influence of SUPRAS composition and structure on OTA recovery was investigated by synthesizing SUPRASs from a constant amount of decanoic acid (1.5 g) and variable water/THF ratios from 95/5 to 40/60 (v/v). Aliquots of 500 μL of each type of SUPRAS were used for extraction. Table 3 shows the composition of the SUPRASs investigated, expressed as mass fraction, as well as the composition of the bulk solution from which they were synthesized.

Table 3

Mean recoveries and standard deviations obtained for ochratoxin A as a function of decanoic acid-based SUPRAS of different composition.

% THF in the bulk solution (v/v)	Bulk composition (mass fraction)			SUPRAS composition (mass fraction)			Recovery[a] ± S[b] (%)	MQL[c] (µg/kg)
	Decanoic acid	THF	Water	Decanoic acid	THF	Water		
5	0.05	0.04	0.91	0.80	0.16	0.04	42 ± 12	7.1
10	0.05	0.09	0.86	0.68	0.26	0.06	69 ± 6	4.3
20	0.05	0.18	0.77	0.50	0.42	0.08	55 ± 5	5.5
30	0.05	0.27	0.68	0.35	0.54	0.11	78 ± 1	3.8
40	0.05	0.36	0.59	0.24	0.62	0.14	101.2 ± 0.9	2.9
50	0.05	0.44	0.51	0.18	0.65	0.16	102 ± 1	2.9
60	0.05	0.53	0.42	0.15	0.66	0.19	103.4 ± 0.8	2.9

[a] *Mean of three determinations from extraction of 0.2 g of paprika, spiked with 15 µg/kg of OTA, with 0.5 mL of SUPRAS;* [b]*Standard deviation;* [c]*Estimated method quantitation limit*

Both the water and THF content in the SUPRASs increased as the percentage of THF in the bulk solution did. As a result, the solvent became increasingly diluted with respect to decanoic acid despite nearly all the surfactant was always transferred from the bulk solution to the SUPRAS during the self-assembly process. The recoveries were strongly dependent on the water/THF ratio in the synthetic solution and hence on the size of the aqueous cavities of the hexagonal inverted aggregates making up the SUPRASs formed. The size of these vacuoles increased with increasing content of THF in the solution. Quantitative recoveries for OTA were only obtained with SUPRAS synthesized from 40% THF (Table 3), which gave vacuoles large enough to allow OTA to efficiently diffuse through them. Extracts were clean enough to inject them directly in the chromatographic system and no interference for OTA was detected from matrix components. So, the decanoic acid-based SUPRAS both extracted efficiently OTA and acted as a restricted access material for macromolecules which were excluded from extraction. Pigments extracted from paprika could be chromatographically separated from OTA. A SUPRAS made up of 24% decanoic acid, 62% THF and 14% water, obtained from a bulk solution containing 40% THF was selected as optimal for extraction and sample cleanup (Table 3). The value of MQL obtained under these experimental conditions was 2.9 μg/kg, which was low enough to control OTA at the levels set by EU regulations.

The influence of the volume of SUPRAS on the extraction efficiency of OTA was investigated in the interval (0.1-0.6 mL). A volume of 0.5 mL of the equilibrium solution obtained in the synthesis of the SUPRAS (Figure 1A) was used as wetting agent in this experiment in order to be able to investigate the extraction efficiency of the lowest SUPRAS volume (e.g. 100-200 μL). So, three phases were obtained, namely a paprika solid residue, an intermediate equilibrium solution and a SUPRAS extract. It was previously checked that OTA was preferentially solubilized in the SUPRAS. The recoveries obtained for OTA, along with their respective standard deviations, were 36±7%, 65±6%, 87±3%, 101.4±0.8%, 101.2±0.9% and 101.3±0.1% for volumes of SUPRAS of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL, respectively. Both recoveries and

precision progressively increased and quantitative recovery was obtained for SUPRAS volumes equal or above 0.4 mL, which was selected as optimal. The estimated MQL for OTA under these optimal conditions was 2.4 μg/kg. Because 0.4 mL of SUPRAS was enough for both sample wetting and efficient extraction, the equilibrium solution was removed for further experiments.

The extraction procedure has two steps, namely vortex shaken and centrifugation (Figure 1B). First, the time required to reach extraction equilibrium conditions, under vortex shaken of the paprika/SUPRAS mixture at 2500 rpm, was investigated in the interval 0-30 min. Equilibrium conditions were reached after 10 min; recoveries kept constant at higher extraction times, with standard deviations below 0.5%, and decreased at the lower ones (e.g. 66±85% and 85±4% at extraction times of 1 and 5 min). The centrifugation time required to achieve an effective separation of the supramolecular extract from sample particles was 5 min at 14160 g.

To evaluate the representativity of the amount of spice subsample used for analysis, the variance obtained for the measurement of OTA in 200-mg paprika subsamples (n=6) fortified with 15 μg/kg of OTA was compared with that obtained from the measurement of 200-mg aliquots (n=6) taken from a 5 g paprika sample fortified at the same concentration level. No statistically significant differences between both variances were observed by applying a Fischer test [32]. The experimental F-value, 1.78, was below the critical F-value (5.05, n1=n2=5, significant level=0.05).

[32]Miller, J.N., Miller, J.C. (2005). Statistic and Chemometrics for Analytical Chemistry. (fifth ed.), Harlow: Pearson Education, (page 49).

3.3. In-house method validation

The developed method was subjected to validation according to the guidelines established by the European [23,33], which provides admissible performance criteria to evaluate if an analytical method is fit for the purpose.

3.3.1. Linearity and Sensitivity

Calibration curves for OTA were run by analyzing standards in water:acetonitrile (1:1 v/v) at nine concentration levels in the range 1.2–500 μg/L. Correlation between peak areas and OTA concentration was determined by linear regression and was 0.9999, indicating a good fit. The instrumental detection (LOD) and quantitation (LOQ) limits were calculated from blank determinations by using a signal-to-noise ratio of 3 and 10, respectively, and they were 0.4 and 1.2 μg/L. The MDLs and MQLs were calculated from six independent complete analyses of paprika by using a signal to noise ratio of 3 and 10, respectively and were 0.7 and 2.3 μg/kg OTA, so the sensitivity of the method is high enough to quantify this toxin in spices at the maximum levels set by EU regulations [12] without the need for extract evaporation.

3.3.2. Selectivity

Possible interferences from matrix components that could co-elute with OTA were assessed by comparison of the slopes of the calibration curves

[33]EC. (2002b). Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Union, L8.

(n = 6) obtained from standard solutions with that obtained from hot paprika fortified with known amounts of OTA within the linear range and run using the whole procedure. No differences were detected in peak areas or retention times for the analyte injected in the SUPRASs or organic solvent. The slopes of the calibration curves obtained for paprika and standards were not statistically different by applying an appropriate Student's t-test [34]. The calculated t-value (3.03) was below the critical t-value (3.17), being significance established at the 0.01 level. Therefore matrix components were not expected to interfere in the determination of OTA.

3.3.3. Trueness

Trueness was investigated by repetitive analysis (n=6) of hot paprika fortified with OTA at concentrations equivalent to 0.5, 1 and 2 times the maximum allowable limit (i.e. 15μg/kg). The recoveries for OTA at 7.5, 15 and 30 μg/kg were 103%, 100% and 101%, respectively. These results were consistent with the performance criteria set by the EU for concentrations of OTA above 1 μg/kg (i.e. recoveries should be in the interval 70-110% [23]).

3.3.4. Precision

Precision was studied in terms of repeatability and within-laboratory reproducibility. For this purpose, hot paprika, fortified with OTA at concentrations equivalent to 0.5, 1 and 2 times the permitted limit (15 μg/kg) were analyzed in three consecutive days (n=18). The relative standard

[34]Rodríguez, L.C., Campana, A.M.G., Barrero, F.A., Linares, C.J., Ceba, M.R. (1995). Validation of an analytical instrumental method by standard addition methodology. J. AOAC Int. 78, 471-476.

deviation (%), under repeatability (RSD_r) and reproducibility (RSD_R) conditions were 0.36% and 0.35%, 0.32% and 0.35%, and 0.34% and 0.31% for 7.5, 15 and 30 μg/kg of OTA, respectively, All these values were far below the performance criteria set for concentrations of OTA above 1 μg/kg (i.e. RSDr ≤20% and RSD_R ≤30% [23]).

3.4. Analysis of spices

Six commodities belonging to the five groups of spices regulated for OTA by the EU were analyzed in order to check the suitability of the method proposed for the control of this toxin at the maximum level permitted [12]. Both native and fortified (15 μg/kg of OTA) spice samples were quantified. All the samples analysed, except hot paprika, contained OTA at detectable levels, although the concentrations found were below the maximum permitted threshold. Table 4 shows the mean values obtained for OTA and the recoveries found from three independent determinations, besides their corresponding standard deviations.

Table 4

Mean concentrations found and recoveries, along with the respective standard deviations, for OTA in spices.

Spice	**Commodity**	**Concentration found[a] ± S[b] (μg/kg)**	**Recovery[c] ± S[b] (%)**
Capsicum spp.	Hot paprika	N.D.	100.7 ± 0.4
	Sweet paprika	8.4 ± 0.5	98 ± 6
Piper spp	Black pepper	3.5 ± 0.4	87 ± 5
Myristica fragrans	Nutmeg	4.5 ± 0.4	95 ± 3
Zingiber officinale	Ginger	6.7 ± 0.2	99.5 ± 0.9
Curcuma longa	Turmeric	9.8 ± 0.3	90 ± 5

[a]Mean of three independent determinations of spice samples; [b]Standard deviation; [c]Sample spiked with 15 μg/kg of OTA; N.D. not detected.

Recoveries for OTA in spices were all within the range 70–110%, with relative standard deviations below 20% as recommended by EC regulations [23].

Figure 2 shows the chromatograms obtained from the different spices analysed. Among these spices, there are matrices characterized by (see Table 2) high fiber and protein content (e.g. paprika), high fat content (e.g. nutmeg), high carbohydrate content (e.g. ginger) and high content of pigmentation and essential oil compounds (e.g. black pepper).

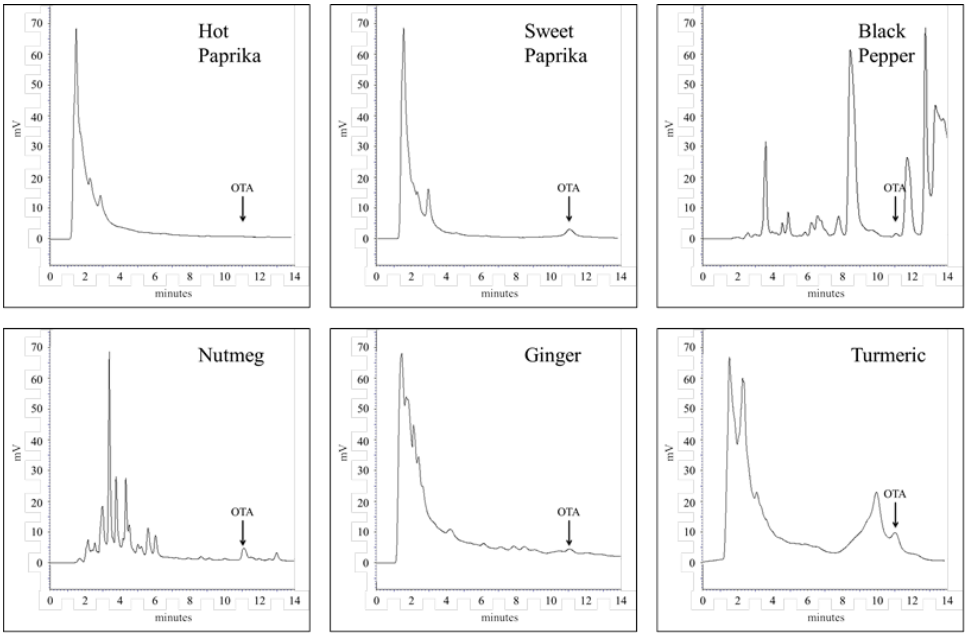

Fig. 2. LC-FD chromatograms obtained for spice samples.

4. Conclusions

The results here obtained prove the capability of the decanoic acid-based SUPRAS to efficiently extract OTA from the spices under regulation in the EU and simultaneously remove macromolecules such as carbohydrates and proteins. The most valuable asset of the method here reported is the absence of matrix effects without the need for IACs cleanup, that permitting reliable quantitation using external calibration and reduction of costs. Additional advantages include fast extraction (~ 10 min) and the low solvent volume/sample amount ratio used (~ 2). This study shows that the sample treatment proposed was effective for the five groups of legislated spices, and reliable results were obtained independent of matrix composition. The method validation also yielded results that were within the acceptable range set by the EU. So, the method can be recommended for enforcement and surveillance programs that monitor OTA in spices.

Regarding the method quantitation limits reported for OTA in spices (Table 1), many of them are below 1 μg/kg as a result of both the sample size used for analysis (5-25g) and the evaporation of IAC eluates. Because the MQL of the method was well below the maximum permitted level for OTA, we selected no to evaporate extracts in order to increase sample throughput in monitoring programs. Further research is necessary in order to extend the method to the determination of aflatoxins in spices.

Acknowledgments

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CAPÍTULO 4

Quick supramolecular solvent-based microextraction for quantification of low curcuminoid content in food

Quick supramolecular solvent-based microextraction for quantification of low curcuminoid content in food

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Abstract

There is a need to monitor the consumption of curcuminoids, an EU-permitted natural colour in food, to ensure that acceptable daily intakes are not exceeded, especially by young children. This paper describes a sensitive method able to quantify low contents of curcumin (CUR), demethoxycurcumin (DMC) and bis-demethoxycurcumin (BDMC) in foodstuffs. The method was based on a single step extraction by use of a supramolecular solvent (SUPRAS) made up of reverse aggregates of decanoic acid, and direct analysis of the extract by use of liquid chromatography–photodiode array (PDA) detection. The extraction involved the stirring of 200 mg foodstuff with 600 μL SUPRAS for 15 min. No cleanup or concentration of the extracts was required. Curcuminoid solubilisation occurred via dispersion and hydrogen bonding. The method was used for the determination of curcuminoids in different types of foodstuff (snack, gelatine, yoghurt, mayonnaise, butter, candy and fish products) that encompassed a wide range of protein, fat, carbohydrate, sugar and water contents (0.85–11.04, 0–81.11, 0.06–75, 0.06–79.48, and 10.08–85.10 g, respectively, in each 100 g of food). Method quantification limits for the foodstuffs analysed were in the ranges 2.9–7.7, 2.8–11.2 and 3.3–9.0 μg kg^{-1} for CUR, DMC and BDMC, respectively. The concentrations of curcuminoids detected in the foodstuffs and the recoveries obtained from fortified samples were in the ranges ND–284, ND–201 and ND–61.3 μg kg^{-1}, and 82–106, 89–106 and 90–102 %, for CUR, DMC and BDMC, respectively. The relative standard deviations were in the range 2–7 %. This method enabled quick and simple microextraction of curcuminoids with minimal solvent consumption, while delivering accurate and precise data.

1. Introduction

Modern analysis of food samples involves analytical and operational challenges that require innovative strategies. Accurate results must be delivered at set regulatory limits, and quality control laboratories are continuously demanding faster, simpler, safer and cheaper analytical methods.

Sample handling still has a basic function in the development of comprehensive methods for food analysis. Much effort has been devoted in the last two decades to reducing solvent organic consumption and simplifying sample extraction and cleanup. In solid food sample treatment, the use of auxiliary energy to enhance solvent extraction (e.g. pressurised liquid extraction (PLE) [1], microwave-assisted extraction (MAE) [2], ultrasound-assisted extraction, (UAE) [3]) and the use of alternative solvents (e.g. supercritical fluids [4]) have become familiar in many analytical labs. These techniques are faster and more environmentally friendly than traditional solvent extraction, but problems related to the need for dedicated equipment, cost, and matrix-dependent results still remain. In the last few years, some attention has been focused on other alternative solvents, including room temperature ionic liquids [5] and supramolecular solvents (SUPRAS) [6].

Supramolecular solvents are nano-structured liquids generated spontaneously from aqueous or hydro-organic solutions of amphiphiles via a self-assembly process. This process is induced by changes in the temperature

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[1]Mustafa A, Turner C (2011) Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. Anal Chim Acta 703(1):8–18

[2]Tanongkankit Y, Sablani SS, Chiewchan N, Devahastin S (2013) Microwave-assisted extraction of sulforaphane from white cabbages: effects of extraction condition, solvent and sample pretreatment. J Food Eng 117:151–157

[3]Kamalijit V (2008) Applications and opportunities for ultrasound assisted extraction in the food industry—A review. Innov Food Sci Emerg Technol 9:161–169

[4]Ávila M, Zougaghb M, Escarpa A, Ríos A (2011) Determination of Sudan dyes in food samples using supercritical fluid. J Supercrit Fluid 55:977–982

[5]Ruiz-Aceituno L, Sanz ML, Ramos L (2013) Use of ionic liquids in analytical sample preparation of organic compounds from food and environmental samples. Trends Anal Chem 43:121–145

[6]Ballesteros-Gómez A, Sicilia D, Rubio S (2010) Supramolecular solvents in the extraction of organic compounds. A review. Anal Chim Acta 677:108–130

[7] or the pH of the solution [8], or by the addition of salts [9] or a nonsolvent for the amphiphile [10]. Both the special arrangement and high concentration of amphiphiles in the ordered aggregates render SUPRAS very attractive for the development of generalised sample treatments able to deal with a range of food types and analytes. The ordered aggregates have regions of different polarity that provide a variety of interactions for analytes; the type of interaction can be tuned by varying the hydrophobic or the polar group of the amphiphile; and in theory, because amphiphiles are ubiquitous in nature and synthetic chemistry, we could design the most appropriate SUPRAS for a specific application. This last property is very attractive for multiresidue analysis, as shown in a recent report dealing with the determination of sulfonamides in meat [11]. Another benefit is that the high concentration of amphiphiles in the SUPRAS, and therefore the large number of binding sites they offer, means these solvents have the potential to extract analytes from a variety of food types with an efficiency that should be matrix-independent. Reported uses of SUPRAS for extracting contaminants [12–14] from solid food samples, although scarce so far, have proved that SUPRAS enable development of efficient, fast, simple, cheap and safe sample treatments.

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[7]Carabias R, Martínez E, Rodríguez-Gonzalo B, Moreno-Cordero JL, Pérez-Pavón C, García-Pinto E, Laespada F (2000) Surfactant cloud point extraction and preconcentration of organic compounds prior to chromatography and capillary electrophoresis. J Chromatogr A 902: 251–265

[8]Casero I, Sicilia D, Rubio S, Pérez-Bendito D (1999) An acidinduced phase cloud point separation approach using anionic surfactants for the extraction and preconcentration of organic compounds. Anal Chem 71:4519–4526

[9]Ruiz FJ, Rubio S, Pérez-Bendito D (2006) Tetrabutylammoniuminduced coacervation in vesicular solutions of alkyl carboxylic acids for the extraction of organic compounds. Anal Chem 78:7229–7239

[10]Ruiz FJ, Rubio S, Pérez-Bendito D (2006) Water-induced coacervation of alkyl carboxylic acid reverse micelles: phenomenon description and potential for the extraction of organic compounds. Anal Chem 79:7473–7484

[11]Costi EM, Sicilia D, Rubio S (2010) Multiresidue analysis of sulfonamides in meat by supramolecular solvent microextraction, liquid chromatography and fluorescence detection and method validation according to the 2002/657/EC decision. J Chromatogr A 1217:6250–6257

[12]Ballesteros-Gómez A, Rubio S, Pérez-Bendito D (2009) Potential of supramolecular solvents for the extraction of contaminants in liquid foods. J Chromatogr A 1216:530–539

[13]Costi EM, Sicilia D, Rubio S (2010) Supramolecular solvents in solid sample microextractions: application to the determination of residues of oxolinic acid and flumequine in fish and shellfish. J Chromatogr A 1217:1447–1454

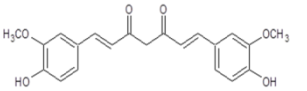
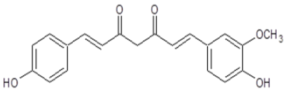
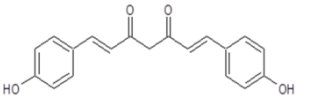
[14]Moral A, Sicilia D, Rubio S (2009) Determination of benzimidazolic fungicides in fruits and vegetables by supramolecular solvent-based microextraction/liquid chromatography/fluorescence detection. Anal Chim Acta 650:207–213

There is no need for dedicated equipment, and only minute volumes of solvent, obtained by means of easily accessible synthetic procedures, are required.

This paper deals with the development of a generalized sample treatment for extracting curcuminoids from different foodstuffs before LC–PDA. Curcuminoids are the major bioactive constituents of Curcuma species. The ground rhizomes of turmeric (Curcuma longa L.) contain 3–15 % curcuminoids, namely curcumin (CUR), demethoxycurcumin (DMC) and bis-demethoxycurcumin (BDMC) [15]. Commercial curcumin, obtained by means of solvent extraction and purification of turmeric rhizomes, is widely used as a natural food colour additive in Europe and North America [16]. It consists of a mixture of CUR, DMC and BDMC (e.g. 71.5 %, 19.4 % and 9.1 %, respectively [17]). Table 1 shows the chemical structures of curcuminoids, their ionisation constants, and their ability to form hydrogen bonds.

Table 1.

Chemical structure, ionisation constants, and number of proton acceptors and donors for curcuminoids

Compound name	Stucture	pKa[a]	H^+ acceptors/donors [a]
Curcumin		8.11 ± 0.46	6 / 2
Demethoxycurcumin		8.31 ± 0.46	5 / 2
Bis-Demethoxycurcumin		8.50 ± 0.46	4 / 2

[a] *Source: https://scifinder.cas.org*

[15]Li S, Yuan W, Deng G, Wang P, Yang P, Aggarwal BB (2011) Chemical composition and product quality control of turmeric (Curcuma longa L.). Pharm Crops 2:28–54

[16]NATCOL (2010) NATCOL-Natural Food Colours Association. http://www.natcol.org

[17]Pfeiffer E, Höhle S, Solyom AS, Metzler M (2003) Studies on the stability of turmeric constituents. J Food Eng 56:257–259

The acceptable daily intakes (ADI) for curcumin set by the European Food Safety Authority (EFSA) and the Joint FAO–WHO Expert Committee on Food Additives (JECFA) are 3 and 0–3 mg, respectively, per kg body weight [18]. European Directive 94/36/EC [19] prescribes maximum limits for curcumin of between 20 and 500 mg kg^{-1}. Suitable analytical methods are required to ensure ADIs are not exceeded, especially by young children, and to comply with EU legislation. Analysis of foodstuffs permitted to contain curcumin quantum satis may be also required to ensure labelling compliance.

Analytical methods for curcuminoids have been mainly developed for turmeric roots and biomatrices other than food, to study their pharmacological properties and biological effects [20,21]. Scotter has written an excellent review of the methods available for the determination of permitted added natural colours in food, including those for curcumin [22]. As pointed out by this author, methods for determining individual curcuminoids in food are limited. As far as we are aware, only three methods have dealt with the determination of individual curcuminoids in food [23–25], and because there was until very recently a lack of commercially available pure standards, much effort has been devoted to the preparation of standards from turmeric extracts, which is very time consuming. LC–electrospray–mass spectrometry (LC–ESI–MS) has been proposed for the

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[18]Scotter MJ (2011) Emerging and persistent issues with artificial food colours: natural colour additives as alternatives to synthetic colours in food and drink. Qual Assur Saf Crop 3:28–39

[19]EU (1994) Directive 94/35/EC of European parliament and of the council of 30 June 1994 on sweeteners for use in foodstuffs. Off J Eur Union L237:13–29

[20]Metzler M, Pfeiffer E, Schulz SI, Dempe JS (2013) Curcumin uptake and metabolism. Biofactors 39:14–20

[21]Gupta SC, Kismali G, Aggarwal B (2013) Curcumin, a component of turmeric: from farm to pharmacy. Biofactors 39:2–13

[22]Scotter MJ (2011) Methods for the determination of European union permitted added natural colours in foods: a review. Food Addit Contam A 28:527–596

[23]Inoue H, Hamasaki S, Yoshimura Y, YamadaM, NakamuraM, Ito Y, Nakazawa H (2003) Validation of LC/Electrospray-MS for determination of major curcuminoids in foods. J Liq Chromatogr R T 26:53–62

[24]Scotter MJ (2009) Synthesis and chemical characterisation of curcuminoid colouring principles for their potential use as HPLC standards for the determination of curcumin colour in foods. Food Sci Technol 42:1345–1351

[25]Lee JH, Choung M (2011) Determination of curcuminoid colouring principles in commercial foods in HPLC. Food Chem 124:1217– 1222

determination of curcuminoids in food and validated for tea and candies [23]. Sample treatment involved extraction with methanol assisted by ultrasonication. For samples with low expected curcuminoid content, the methanolic extract was dispersed in water and then subjected to SPE cleanup, methanol elution, evaporation to dryness, and methanol reconstitution in a small volume. Two more methods for curcuminoid determination have been reported, both based on LC–PAD detection [24, 25]. In the first [24], a comprehensive chromatographic separation of curcuminoids and annatto was reported, and this was used in a single spiking experiment involving mince fish (average recovery 85 %). Sample treatment included grinding with Celite and HCl in the presence of ascorbyl palmitate, followed by a hexane wash to remove oils and extraction into acetonitrile in the presence of antioxidants. The water content of the samples was known to significantly affect the separation of curcuminoids. The second method [25] was applied to a range of foodstuffs. Sample treatment involved the extraction of 0.5 g of dried food with 10 mL methanol for 6 h, and, although global recoveries for curcuminoids were in the range 84.7–98.9 % for solid samples, no recoveries for individual curcuminoids were given. No quantitation limits were reported, but our estimation from spiking experiments gives values of approximately 2 mg kg^{-1}, which is not low enough to analyse foodstuffs with low curcuminoid content.

The research here presented evaluated the ability of a SUPRAS consisting of reverse aggregates of decanoic acid to efficiently extract curcuminoids from food, using low solvent volumes. Foodstuffs were selected to cover the different food categories for which the addition of these natural yellowish colorants is authorised [19] and to encompass a wide range of protein, fat, carbohydrate, water and sugar contents. The objective was to develop a simple and fast method able to deal with both high and low curcuminoid content. The variables affecting the efficiency of the SUPRAS-based microextraction were studied, the analytical features of the LC–PDA method were evaluated, and the method developed was used for the determination of curcuminoids in non-spiked and spiked foodstuffs.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade and were used as supplied. Decanoic acid and HPLC grade acetonitrile were obtained from Sigma-Aldrich (Barcelona, Spain). Tetrahydrofuran (THF), hydrochloric acid and acetic acid glacial were supplied by Panreac (Sevilla, Spain). Ultra-highquality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Standards for curcumin, demethoxycurcumin and bis-demethoxycurcumin were purchased from Sigma (St. Louis, MO, USA). Stock individual solutions of curcuminoids at a concentration of 1 g L^{-1} were prepared in methanol and stored under dark conditions at –20 ºC in an amber flask. Working solutions were prepared by proper dilution of the stock solutions with methanol.

2.2. Apparatus

The liquid chromatographic system used consisted of a ThermoQuest spectra system (San Jose, CA, USA) equipped with a SCM 1000 vacuum membrane degasser, a P2000 binary pump, an AS3000 autosampler and a Spectra System UV6000 LP detector. In all experiments, a PEEK Rheodyne 7125NS injection valve with a 20 μL sample loop was used (ThermoQuest, San Jose, CA, USA). The analytical column was an Ultrabase C18 (5 μm, 250 mm×4.6 mm) from Analisis Vínicos (Tomelloso, Spain). A homogenizer-disperser Ultra-Turrax T25 Basic fromIka (Werke, Germany), a vortex-shaker REAX Top equipped with an attachment (ref. 549-01000-00) for 10 microtubes fromHeidolph (Schwabach, Germany), and a high speed brushless centrifuge MPW-350R equipped with an angle rotor 36 × 2.2/1.5 mL (ref. 11462) from MPW Med-Instruments (Warschaw, Poland) were used for sample preparation. A digitally regulated centrifuge Mixtasel equipped with an angle rotor 4 × 100 mL (ref. 7001326) from JP-Selecta (Abrera, Spain) was used for supramolecular solvent production.

2.3. Supramolecular solvent production

The following procedure was used to obtain a SUPRAS volume (~10 mL) able to treat 16 food samples. Decanoic acid (0.9 g), tetrahydrofuran (15 mL) and hydrochloric solution 0.01 mol L^{-1} (15 mL) were mixed in a 50 mL glass centrifuge tube. The mixture was centrifuged at 3500 rpm (2400 g) for 10 min. The SUPRAS formed was less dense than water, and was separated as a new liquid phase at the top of the solution. It was extracted by use of a syringe, and stored in a closed glass vial until use. Under these conditions, it was stable at room temperature for at least one month. Figure 1 shows a schematic of the synthetic procedure.

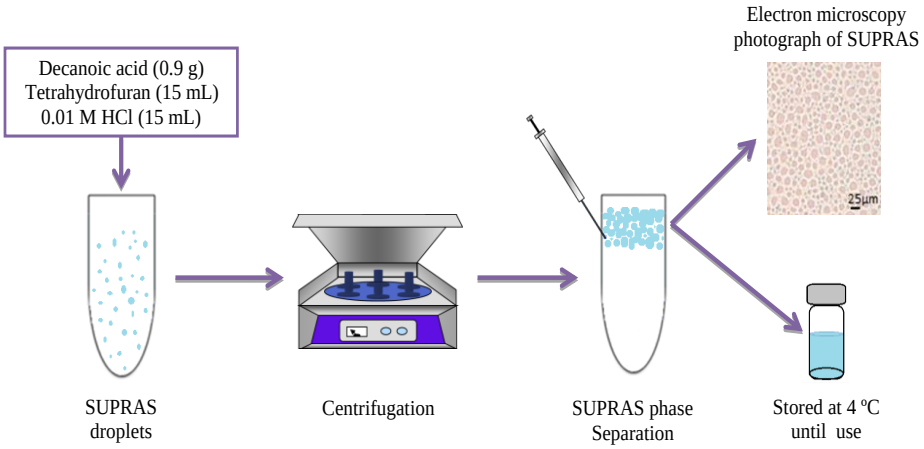

Fig. 1 Schematic of the procedure for synthesising decanoic acid-based SUPRAS

2.4. Determination of curcuminoids in food samples

2.4.1. Sample preparation

Samples belonging to different food categories (snack, gelatine, yoghurt, mayonnaise, processed fish, butter and candy) were purchased in local supermarkets in Córdoba (South Spain) and were stored under dark conditions at 4 ºC until analysis. The whole content of consumer-size packages was used for sample treatment. Samples, except candies, were homogenised by use of a high-speed Ultra-Turrax for 5–10 min. Because of the high sugar content of candies the addition of ultra-high quality water at a sample/water ratio (w/w) of two was required, before homogenisation by use of the Ultra-Turrax for 10 min. After homogenisation, 200 mg subsamples were taken for analysis and recovery experiments, which were performed in triplicate. The subsamples were fortified at different concentration levels (0.1–2 mg kg^{-1}) by adding minute volumes (20–40 μL) of curcuminoid standard solutions in methanol. Fortified samples were left to stand at room temperature and in dark conditions for 90 min before analysis. Curcuminoids in samples were stable during this period of time. For subsample representativity studies, the whole content of the sample was fortified.

2.4.2. Supramolecular solvent-based microextraction

Subsamples (200 mg) were mixed with 600 μL SUPRAS in a safe-lock 2 mL microtube from Eppendorf Ibérica (Madrid, Spain). Four or five 3 mm diameter glass balls were introduced in the microtube to improve sample dispersion during extraction, which was performed by means of vortex-shaking at 2500 rpm for 15 min. The mixture was then centrifuged at 15000 rpm for 5–10 min to separate the solvent from the solid residue. Finally, the SUPRAS containing the target analytes was transferred to a glass vial, from which 20 μL was injected into the chromatographic system. A schematic of the sample treatment procedure is shown in Fig. 2.

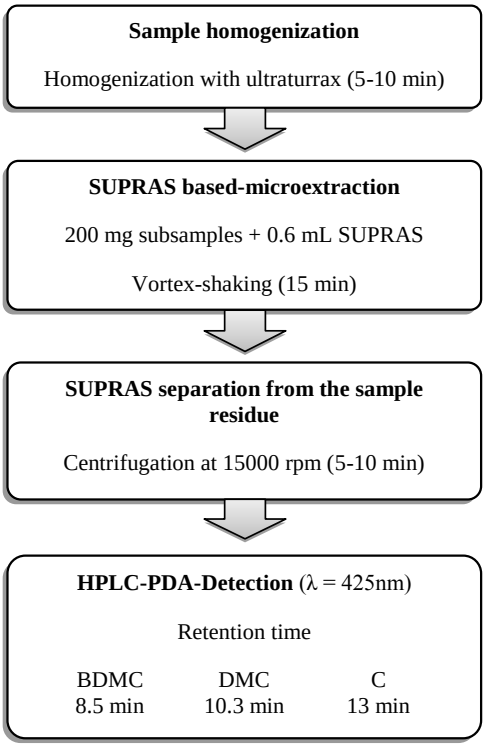

Fig. 2 Schematic of the procedure for determination of curcuminoids in food

2.4.3. Liquid chromatography–photodiode array detection

The three curcuminoids were separated and quantified by use of LC with PDA detection. The mobile phase consisted of an aqueous solution of acetic acid 2 % (A) and acetonitrile (B), and was pumped at a constant flow of 1 mL min^{-1}. A linear gradient from 45 % A to 65 % A in 15 min was used for separation. Reconditioning of the column took approximately 5 min. The column temperature was set at 40 ºC. Calibration curves were run from standards dissolved in methanol, in the concentration ranges 0.8 μg L^{-1} – 80 mg L^{-1}, 0.7 μg L^{-1} – 80 mg L^{-1} and 0.5 μg L^{-1} – 80 mg L^{-1} for CUR, DMC and BDMC, respectively. Quantitation was performed by means of measuring peak areas at 425 nm.

3. Results and discussion

3.1. Supramolecular solvent production and binding capabilities

Decanoic acid, like other alkyl carboxylic acids, is slightly soluble in water (0.15 g L^{-1} at 20 ºC) and very soluble in THF, where it forms reverse aggregates via a sequential-type self-association model [26]. Addition of decanoic acid (typically at concentrations below 8 %, w/v) to a hydroorganic solution of water and THF (water/THF (v/v) ratios between 1.5 and 50) causes the formation of oily droplets that flocculate and produce conglomerates of individual droplets. These conglomerates separate from the bulk solution as a new liquid phase, named SUPRAS. The process does not occur for decanoate, so the pH of the solution should be below four to ensure the presence of decanoic acid (pK_a = 4.8 ± 02). Figure 1 shows a microphotograph of the individual droplets that make up the SUPRAS. These consist of decanoic acid, THF and water, in proportions that depend on the THF/water ratio (v/v) of thesynthetic solution. The volume of solvent produced was a linear and an exponential function of the amount of decanoic acid and THF, respectively, in the synthetic solution. The following equation was derived:

$$y = (1.04 \pm 0.02)\ a\ e^{(0.0473 \pm 0.0009)b}$$

where y is the volume of SUPRAS in μL, a the amount of decanoic acid in mg, and b the percentage of THF (v/v). Decanoic acid-based SUPRAS are expected to efficiently extract curcuminoids by use of low solvent volumes, on the basis of:

[26] Pileni MP (1989) Structured and reactivity in reverse micelles. Elsevier, Amsterdam

1. the types of interaction they can establish, namely dispersion forces between the hydrocarbon chain of the acid and the aromatic structure of curcuminoids, and hydrogen bonds between the polar groups of the surfactant and analytes; and

2. the large concentration of surfactant in the SUPRAS (e.g. 0.2–0.8 mg μL^{-1}).

Curcuminoids are only stable at acidic pH, rapidly decomposing at pHs above neutral, so the SUPRAS is a convenient medium for analyte stabilisation.

3.2. Optimisation of the supramolecular solvent-based microextraction

Optimisation was performed by extracting 200 mg of snack fortified with 2 mg kg^{-1} of each curcuminoid. Unfortified samples were analysed for curcuminoids, and the concentration found subtracted from those of fortified samples. Experiments were performed in triplicate. Selection of the optimum conditions was on the basis of recoveries and method quantitation limits (MQLs) obtained. MQLs were calculated from the instrumental quantitation limits (0.8, 0.7 and 0.5 μg L^{-1} for CUR, DMC and BDMC, respectively), the volume of SUPRAS used for extraction, the recoveries obtained and the sample weight used for analysis. The variables investigated were SUPRAS composition and volume, and time required to reach equilibrium conditions. After sample centrifugation, two phases were always observed: the insoluble sample matrix components at the bottom, and the SUPRAS extract containing the three curcuminoids and other solutes from the sample. Table 2 shows the results obtained.

Table 2.

Mean recoveries and method quantification limits obtained for curcuminoids as a function of the composition and volume of SUPRAS and the extraction time

Variable	CUR		DMC		BDMC	
	[a]Recovery ± [b]S (%)	MQL μg kg^{-1}	[a]Recovery ± [b]S (%)	MQL μg kg^{-1}	[a]Recovery ± [b]S (%)	MQL μg kg^{-1}
THF (%)						
10	42±2	5.6	47±1	4.4	40±3	3.7
20	55±3	3.6	50±2	4.1	53±2	2.8
30	67±2	3.5	70±2	2.9	65±2	2.3
40	80±1	2.9	77±1	2.7	90±1	1.6
50	99±1	2.4	100±1	2.1	98±1	1.5
60	84±1	2.8	92±1	2.3	88±1	1.7
70	76±2	3.1	80±1	2.6	80±1	1.8
SUPRAS volume (ml)						
200	-		-		-	
400	-		-		-	
600	101±2	2.3	98±2	2.1	102±1	1.4
800	98±1	3.2	99±1	2.8	100±2	2.0
Extraction time (min)						
5	88±2	2.7	84±3	2.5	87±1	1.7
10	95±2	2.5	88±2	2.4	88±2	1.7
15	100±1	2.3	98±1	2.1	98±1	1.5
20	99±1	2.4	103±1	2.0	102±1	1.4
30	102±1	2.3	99±1	2.1	101±1	1.4

[a] *200 mg of snack spiked with 2 mg kg^{-1} of each curcuminoid*

[b] *Standard deviation, n=3*

As commented above, the decanoic acid, THF and water content of the SUPRAS depends on the THF/ water ratio in the synthetic solution. SUPRAS composition remains invariable when the amount of decanoic acid in the synthetic solution is increased at constant THF/water ratios. Both the water and the THF content of the SUPRAS increase as the THF/water ratio does, so the decanoic acid in the SUPRAS becomes more diluted. Table 2 shows the recoveries obtained, and the MQL estimated for curcuminoids, by

use of 600 μL SUPRAS synthesised in solutions containing THF in the range 10–70 %. Maximum recoveries and minimum MQL were obtained by use of SUPRAS synthesised in 50 % THF and 50 % water, so this SUPRAS was selected for further experiments. The water solution used for synthesis was adjusted to pH 2, so the pH of the water group of the reverse aggregates in the SUPRAS was acidic and was therefore a suitable medium to stabilise the curcuminoids.

The effect on recovery of the volume of SUPRAS used for the microextraction of curcuminoids was assessed in the range 200–800 μL. Volumes below 600 μL were not able to completely wet the subsamples of snacks, candies, fish products and some other foodstuffs. The minimum volume enabling quantitative recoveries (i.e. 600 μL) was selected, to keep MQL values as low as possible (Table 2).

Only 15 min vortex shaking (vibration motion=2500 rpm) was necessary to reach equilibrium conditions (Table 2). This is a consequence of the high extraction efficiency of the SUPRAS, probably caused by the different types of interaction it offers and the high number of binding sites.

Representativity of the food subsamples used for analyses was investigated by spiking 50 g aliquots of snack with 2 mg kg^{-1} of each curcuminoid, and then analysing six 200 mg subsamples. The standard deviation for the different curcuminoids was in the range 2–3 %, so the subsamples used were believed representative of the whole sample.

3.3. Analytical performance

3.3.1. Sensitivity and linearity

Calibration curves were run using standard solutions in methanol. No differences in peak areas or retention times were observed for the curcuminoids injected into the SUPRAS or methanol. The linear range was confirmed by visual inspection of the plot residuals versus analyte amount; the residuals were randomly scattered within a horizontal band, and a

random sequence of positive and negative residuals was obtained. Linearity was achieved in the ranges 0.8 μg L^{-1}–80 mg L^{-1}, 0.7 μg L^{-1}–80 mg L^{-1} and 0.5 μg L^{-1}–80 mg L^{-1} for CUR, DMC and BDMC, respectively. Correlation coefficients were in the range 0.996–0.999, indicating good fits.

Practical quantification limits [27] were estimated from six independent complete determinations of curcuminoids in typical low-level foodstuffs. When these foodstuffs could not be obtained, an estimate of the background signal was made at a representative part of the readout adjacent to the curcuminoid signal in the curcuminoid-containing sample. Quantification limits were calculated by use of a signal-tonoise ratio of 10. The values obtained for the different types of foodstuff analysed are shown in Table 3, and were in the ranges 2.9–7.7, 2.8–11.2, and 3.3–9.0 μg kg–1 for CUR, DMC, and BDMC, respectively, enabling the determination of low curcuminoid content in food.

Table 3.

Mean concentration and recovery, with their respective standard deviations, obtained for the determination of curcuminoids in non-fortified and fortified samples, and practical method quantitation limits obtained for each type of foodstuff analysed.

[27]Thompson M, Ellison SLR, Wood R (2002) Synthesis and chemical characterisation of curcuminoid colouring principles for their potential use as HPLC standards for the determination of curcumin colour in foods. Pure Appl Chem 74:835–855

Foodstuff	CUR			DMC			BDMC		
	[h]Concentration found ± [i]S (μg kg^{-1})	[k]Recovery ± [i]S (%)	[l]MQL (μg kg^{-1})	[h]Concentration found ± [i]S (μg kg^{-1})	[k]Recovery ± [i]S (%)	[l]MQL (μg kg^{-1})	[h]Concentration found ± [i]S (μg kg^{-1})	[k]Recovery ± [i]S (%)	[l]MQL (μg kg^{-1})
[a]Snack	7.5±0.1	103±4	2.9	3.41±0.07	99±5	2.8	ND	102±3	3.3
[b]Gelatine	284±9	101±2	3.5	201±5	102±4	5.6	61.3 ± 0.2	98±5	6.0
[c]Yogurt	46±1	106±7	4.0	34.1±0.6	99±4	4.5	7.69 ± 0.02	101±3	4.2
[d]Mayonnaise	83±2	91±6	4.3	70±1	80±2	7.0	21.62 ± 0.06	90±7	8.7
[e]Butter	7.0±0.4	102±5	4.2	ND	106±6	3.5	ND	97±7	2.5
[f]Candy	ND	91±4	4.0	ND	87±3	8.5	ND	90±4	3.9
[g]Fish	28±1	82±5	7.7	12±1	89±7	11.2	12 ± 1	90±5	9.0

ND, not detected

Nutritional composition: (g per 100 g food): [a]Water: 5.44; fat: 2.97; protein: 8.03; carbohydrate: 75.00; sugar: 9.38; [b]Water: 84.39; fat: 0; protein: 1.22; carbohydrate: 14.19; sugar: 13.49; [c]Water: 85.10; fat: 0.39; protein: 10.19; carbohydrate: 3.60; sugar: 3.24; [d]Water: 54.30; fat: 19; protein: 0.9; carbohydrate: 23.9; sugar: 4.34; [e]Water: 15.87; fat: 81.11; protein: 0.85; carbohydrate: 0.06; sugar: 0.06; [f]Water: 10.08; fat: 0.08; protein: 5.97; carbohydrate: 12.7; sugar: 79.48; [g]Water: 53.08; fat: 13.25; protein: 11.04; carbohydrate: 21.18; sugar: 2.50

[h]Mean for three independent determinations from the analyses from unfortified samples

[i]Standard deviation

[k]Sample spiked with 0.1 mg kg-1 of each curcuminoid

[l]Calculated from six independent complete determinations

3.3.2. Selectivity

Possible interference from matrix components that could co-elute with the three curcuminoids was assessed by comparison of the slopes of the calibration curves (n = 6), obtained from standard solutions in methanol, with those obtained from 200 mg of different foodstuffs fortified with known amounts of curcuminoids (0.1–2 mg kg^{-1} to 200 mg subsamples) and run using the whole procedure. No statistically significant difference between these slopes was revealed by use of a student's t-test [28]. The experimental t-values were in the interval 1.8–2.9, and were below the critical t-value (3.36, significance level=0.01). Therefore, matrix components were not expected to interfere with the determination of curcuminoids.

[28]Cuadros-Rodríguez L, García-Campaña A M, Alés-Barrero F, Jiménez-Linares C, Román-Ceba, M (1995) Validation of an analytical instrumental method by standard addition methodology. J AOAC Int 78:471–476

3.3.3. Precision

The precision of the method was evaluated by extracting eleven independent samples of snack fortified with 2 mg kg^{-1} curcumin. The precision, expressed as relative standard deviation (RSD), was between 3 and 7 for the three curcuminoids.

3.4. Analysis of foodstuffs

The method here proposed was used to determine CUR, DMC and BDMC in fortified and unfortified foodstuffs bought in different local supermarkets in Córdoba (Spain). Spiking of samples at 0.1 mg kg^{-1} was performed for each curcuminoid. The protein, fat, carbohydrate, sugar, and water contents of the selected foodstuffs, expressed as g per 100 g of food, were in the ranges 0.85–11.04, 0–81.11, 0.06–75, 0.06–79.48 and 10.08–85.10, respectively, and thus encompassed a wide range of compositions (see Table 3 for detailed composition). It is reasonable to expect that the results here obtained can be extrapolated to other foodstuffs within these composition ranges.

Table 3 shows the concentrations found and the recoveries obtained, with the corresponding standard deviations, for the three curcuminoids in the different foodstuffs analysed. The concentrations of CUR, DMC and BDMC were in the ranges ND–284 μg kg^{-1}, ND–201 μg kg^{-1} and ND–61.3 μg kg^{-1}, respectively. The content of individual curcuminoids in most samples analysed was below 100 μg kg^{-1}, thus revealing the need for sensitive methods to evaluate ADI values for curcuminoids, especially the values prescribed for young children, and to ensure labelling compliance. Recoveries ranged between 82–106, 89–106 and 90–102 % for CUR, DMC and BDMC, respectively, with relative standard deviations in the range 2–7 %.

Figure 3 shows the chromatograms obtained for some of the unfortified foodstuffs analysed.

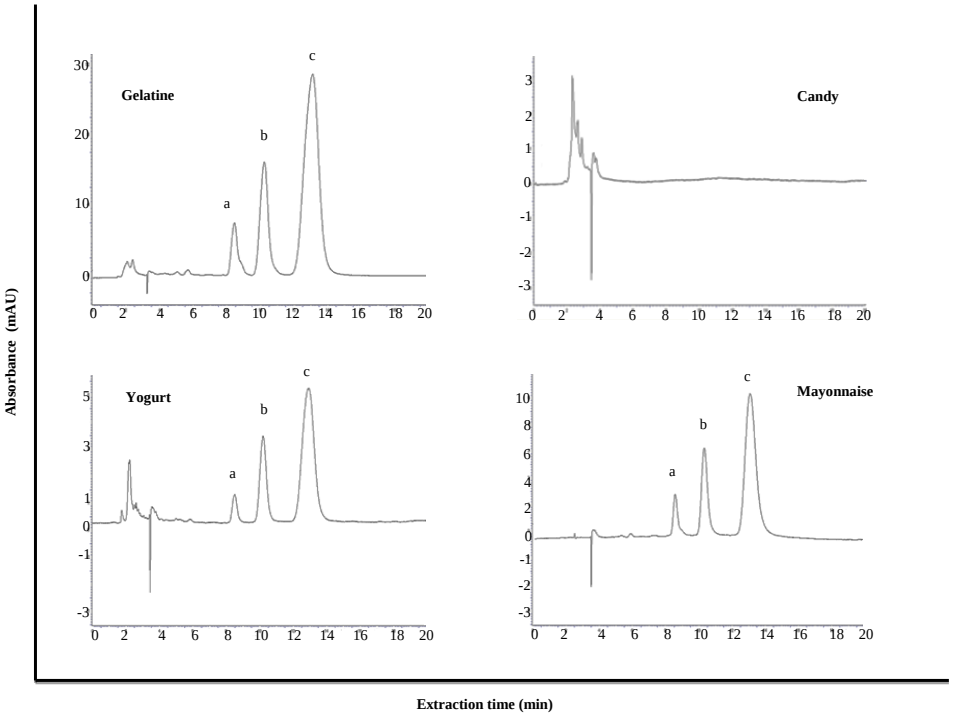

Fig. 3 Chromatograms obtained for the determination of curcuminoids in gelatine, candy, yoghurt and mayonnaise. The concentrations obtained for (a) BDMC, (b) DMC and (c) CUR are given in Table 3

No coelution of other matrix components was observed for curcuminoids in any of the samples analysed, except for BDMC in foodstuffs including gelatine and mayonnaise (peak a in Fig. 3). The shoulder observed in the chromatographic peak for these samples corresponded to a clear peak at 370 nm. Therefore, for exact quantification, the right side of the chromatographic peak should be extrapolated to the baseline.

Differences between the retention times measured for the analytes from standards and fortified samples were lower than 0.7 % in all cases, and

the repeatability obtained for consecutive measurements of this variable from samples, expressed as relative standard deviations (n =6), was 0.35, 0.43 and 0.5 % for CUR, DMC and BDMC, respectively.

4. Conclusion

A SUPRAS made up of reverse aggregates of decanoic acid was here proposed for the quick and inexpensive microextraction of curcuminoids from foodstuffs belonging to different categories (snack, gelatine, yoghurt, mayonnaise, butter, candy and fish products) and featuring a wide range of protein (0.85–11 %), fat (0–81.11 %), carbohydrate (0.06–75 %), sugar (0.06–79.48 %) and water (10.08–85.10 %) contents. The two structure-derived extraction properties of SUPRAS, namely the different polarity regions able to provide several types of interaction, and the large number of binding sites, make SUPRAS excellent candidates for use as general extractants in food analysis. Valuable assets of the matrix-independent sample treatment here developed are: it takes approximately 15 min and several samples can be simultaneously treated; it requires only a small amount of sample (0.2 g), made representative of the bulk by homogenisation, and a small, eco-friendly SUPRAS volume (600 μL); it is low in cost; and conventional lab equipment is used.

As commented in the Introduction, only three methods have been previously reported for simultaneous determination of CUR, DMC and BDMC in food [23–25]. The main disadvantages of these methods are the complex sample treatments required (extraction, SPE cleanup or hexane wash, evaporation to dryness, etc [23, 24]), the time-consuming procedures involved (e.g. extractions take 6 h [25]), the inability to provide validation for a wide range of foodstuffs [23, 24] or the individual determination of curcuminoids in food [25], and/or the high LODs obtained, making them unsuitable for the determination of low curcuminoid contents in food [25].

The analytical method here proposed is sensitive enough to determine low curcuminoid contents in food and is suitable for estimating acceptable daily intakes and for monitoring labelling compliance, using sample treatment and quantification equipment available to most enforcement laboratories.

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BLOQUE C

SUPRAS-RAM basados en alcanoles para la determinación de multicomponentes en multi-matrices

CAPÍTULO 5

Assessing human and environmental exposure and risk to a cocktail of bisphenols and derivatives. A review

Assessing human and environmental exposure and risk to a cocktail of bisphenols and derivatives. A review

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Abstract

Bisphenol A (BPA), a high-production volume chemical, is ubiquitous in humans and the environment. Its potential adverse effects through genomic and non-genomic pathways, mainly in vulnerable populations (e.g. human fetuses, neonates and infants), has fostered BPA replacement by bisphenol analogs that, unfortunately, exert similar adverse effects. Many of these analogs, as well as their derivatives, have already found in humans and the environment and major concerns have arisen over their low dose- and cocktail-related effects. This review aims to strength the need to no longer consider bisphenols and derivatives individually for the assessment of bisphenol-related human and environmental exposure and risk, as well as for the development of the respective legislative provisions. The main chemical, biological and prediction tools developed so far for evaluation of exposure and risk to mixture of bisphenols will be here presented and critically discussed. Chemical methods intended to determine the composition of such mixtures in exposure sources and biological samples have been mainly based on liquid chromatography tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS) and, in less extent, LC with fluorescence detection (LC-FD). Main characteristics, advantages and drawbacks of these methods will be comparatively discussed. Although at an early stage, some approaches for the assessment of the risk to mixtures of bisphenols, mainly based on the combination of chemical target analysis and toxicity evaluation, have been already applied and they will be here presented. Current knowledge gaps hindering a reliable assessment of human and environmental risk to mixtures of bisphenols and derivatives will be outlined.

1. Introduction

1.1. Bisphenols: Uses and properties

Bisphenol A (BPA) is a high-production volume industrial chemical mainly used as a monomer in the production of polycarbonate plastics (~80%) and epoxy resins (~18%) [1-3]. Polycarbonate plastics are used in reusable food and drink containers, in tableware, and in water pipes. Other uses include housings of electrical/electronic devices, optical storage media, building materials and medical equipment. Epoxy resins are used as inner coatings of cans and lids of glass jars and bottles for food and beverages and wastewater tanks and pipes. They are also used for paints, adhesives or electrical laminates. Other applications of BPA (~2%) include their utilization as color developer in thermal paper, as an additive of polyvinyl chloride to avoid oxidation, as intermediate in the production of polyurethane and as monomeric building block of fire resistant polymers (e.g. tetrabromobisphenol A) [1-3]. Following ECHA (European Chemical Agency), BPA has been registered according to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), in the tonnage band more than 1 million tons/year [4] and, according to ICIS Chemical Business, the European capacity amounted to 1.4 million tons/year in 2011 [5]. The

[1]M. Klar, D. Gunnarsson, A. Prevodnik, C..Hedfors, U. Dahl, Everything you (don't) want to know about plastics, Swedish Society for Nature Conservation, 2014, Available in http://www.naturskyddsforeningen.se/sites/default/files/dokument-media/rapporter/Plastic-Report.pdf, last accessed 07/09/2015.

[2]R. U. Halden, Plastics and Health Risks, Annu. Rev. Publ. Health, 31(2010) 179-194.

[3]Å. Bergman, J. J. Heindel, S. Jobling, K. A. Kidd, R. T. Zoeller, State of the Science of Endocrine Disrupting Chemicals – 2012, United Nations Environment Programme and World Health Organization, 2013, Available in http://www.who.int/ceh/publications/endocrine/en/, last accessed 08/09/2015.

[4]European information center on bisphenol A, http://www.bisphenol-a-europe.org/, last accessed 08/09/2015.

[5]ICIS (ICIS Chemical Business) (2011) European chemical profile: bisphenol A, available at: http://www.icis.com/Articles/2011/05/09/9457899/european+chemical+profile+bisphenol+a.html, last accessed 08/09/2015.

volume of the world BPA production is predicted to surpass the 5.4 million tonnes mark by 2015 [6].

BPA can be released from consumer products to the surroundings. No complete polymerization and/or polymer degradation account for leaching of BPA monomers from BPA-based polymers. Migration from products containing BPA as additive is even easier because it is usually not bound to the polymer. The serious concerns about the adverse effects of BPA on human health and aquatic life have led to industry to replace it with other bisphenols in some applications (Table 1) [7-9]. Thus, epoxy resins for food contact materials have been produced from bisphenol F (BPF), bisphenol E (BPE), bisphenol P (BPP), bisphenol AP (BPAP) and bisphenol S (BPS), and infant feeding bottles and children´s tableware, made up of polyethersulfone, have used BPS as the starting monomer. Likewise, BPS is widely used as color developer in thermal paper, BPAP is used as a cross-linker in fluoroelastomers and bisphenol Z (BPZ) is a component of cosmetics, personal care products and highly heat resistant plastics.

[6]Bisphenol A (BPA): 2015 World Market Outlook and Forecast up to 2019, Market Publishers, 2015.

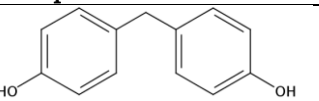
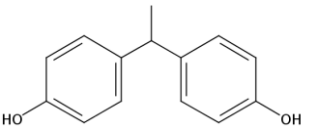
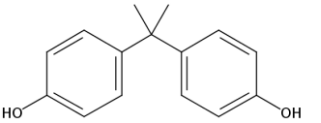
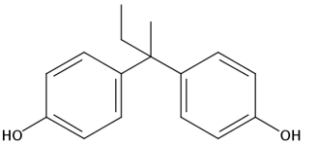
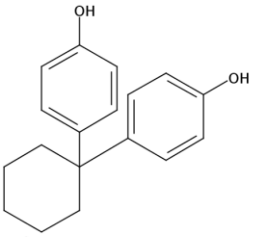
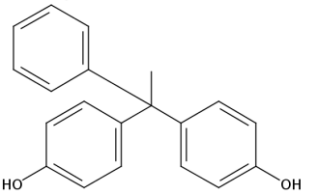
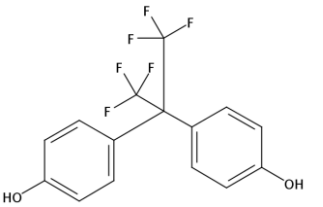
[7]Bisphenol A alternatives in thermal paper, Environmental Protection Agency, US, 2014, Available at http://www2.epa.gov/sites/production/files/2014-05/documents/bpa_final.pdf, last accessed on 08/09/2015.

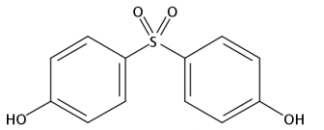
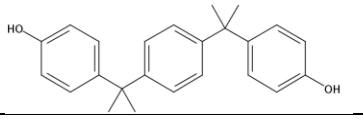
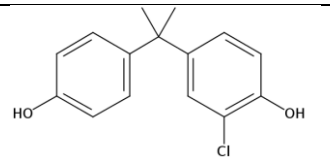
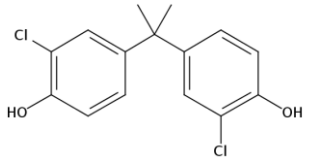
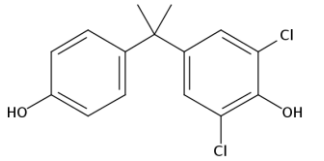
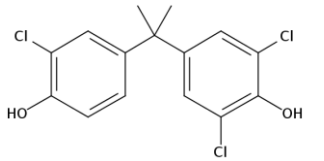
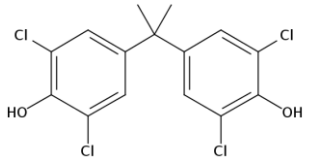
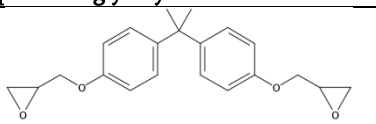
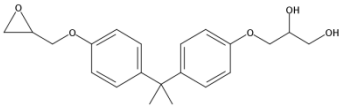
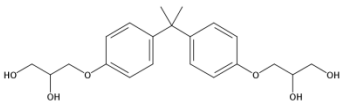
[8]SUBSPORT Specific Substances Alternatives Assessment – Bisphenol A, 2013, Available at http://www.subsport.eu/wp-content/uploads/data/bisphenol_A.pdf, last accessed on 08/09/2015.

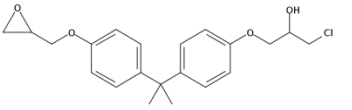
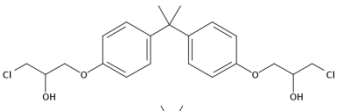
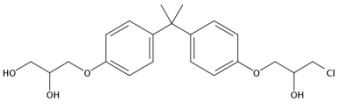
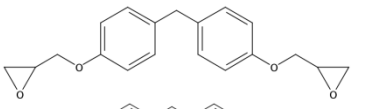
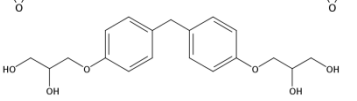
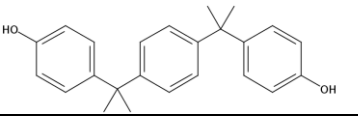
[9]OPINION of the French Agency for Food, Environmental and Occupational Health & Safety on the assessment of the risks associated with bisphenol A for human health, and on toxicological data and data on the use of bisphenols S, F, M, B, AP, AF and BADGE, 2013, available at https://www.anses.fr/en/system/files/CHIM2009sa0331Ra-0EN.PDF, last accessed on 08/09/2015.

Table 1

Chemical structures, octanol-water partition coefficients (log $K_{o/w}$) and ionization constants (pK_a) for bisphenols, bisphenol A chlorinated derivatives and bisphenols diglycidyl ethers

Chemical name	Chemical Structure	CAS Number	log K_{ow}	pK_a
Bisphenols				
4,4'-Dihydroxydiphenylmethane (BPF)		87139-40-0	2.76	9.91
4,4'-Ethylidenebisphenol (BPE)		2081-08-5	3.23	10.10
2,2′-Bis(4-hydroxyphenyl)propane (BPA)		80-05-7	3.64	10.29
2,2′-Bis(4-hydroxyphenyl)butane (BPB)		77-40-7	4.15	10.27
1,1′-Bis(4-hydroxyphenyl)-cyclohexane (BPZ)		843-55-0	4.87	9.91
1,1′-Bis(4-hydroxyphenyl)-1-phenyl-ethane (BPAP)		1571-75-1	4.33	10.22
2,2′-Bis(4-hydroxyphenyl)hexafluoropropane (BPAF)		1478-61-1	3.98	8.74

Bis(4-hydroxyphenyl)sulfone (BPS)		80-09-1	2.14	7.64
1,4-Bis(2-(4-hydroxyphenyl)-2-propyl)benzene (BPP)		2167-51-3	6.56	10.31
Bisphenol A chlorinated derivatives				
2-Chloro-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (MCBPA)		74192-35-1	4.33	9.79
4,4'-(1-Methylethylidene)bis[2-chlorophenol (DCBPA)		79-98-1	5.03	9.44
2,6-Dichloro-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (DCBPA)		14151-65-6	5.03	8.98
2,6-Dichloro-4-[1-(3-chloro-4-hydroxyphenyl)-1-methylethyl]phenol (TCBPA)		40346-55-2	5.72	8.93
4,4'-(1-methylethylidene)bis[2,6-dichloro-Phenol (TeCBPA)		79-95-8	6.41	8.59
Bisphenol diglycidyl ethers				
2,2´-Bis(4-glycidyloxyphenyl)propane (BADGE)		1675-54-3	3.71	-
2-[4-(2,3-Dihydroxypropyloxy)phenyl]-2-[4-(glycidyloxy)phenyl]propane (BADGE·H_2O)		76002-91-0	3.19	13.53
2,2´-Bis[4-(2,3-dihydroxypropoxy)phenyl]propane (BADGE·2H_2O)		5581-32-8	2.52	13.23

2-[4-(3-Chloro-2-hydroxypropyloxy)pheny]-2-[4-(glycidyloxy)phenyl]propane (BADGE·HCl)		13836-48-1	4.02	13.33
2,2´-Bis[4-(3-chloro-2-hydroxypropoxy)phenyl]propane (BADGE·2HCl)		4809-35-2	4.34	12.83
2-[4-(3-Chloro-2-hydroxypropyloxy)phenyl]-2-[4-(2,3-dihydroxypropyloxy)phenyl] propane (BADGE·HCl·H_2O)		227947-06-0	3.50	13.13
Bis[4-(glycidyloxy)phenyl]methane (BFDGE)		2095-03-6	2.449	-
Bis[4-(2,3-dihydroxypropoxy)phenyl]methane (BFDGE·2H_2O)		72406-26-9	1.254	13.23
Bis[4-(3-chloro-2-hydroxypropoxy)phenyl]methane (BFDGE·2HCl)		374772-79-9	3.093	13.52

Because of the similarity in structure to BPA, the same or improved technical product properties as BPA can be obtained with the bisphenols included in Table 1. Unfortunately, toxicity profiles are also similar to BPA, with the disadvantage of being even less well-known within the scientific community. The presence of bisphenols other than BPA in human and environmental samples has just started to be detected. Thus, human urine from eight different countries showed the presence of BPS in 81% of the population [10]. So, risk assessment to bisphenols should not focus exclusively on BPA, as currently in force, but exposure to the entire mixture of bisphenols should be considered.

Many other bisphenol-related compounds will undoubtedly contribute to the human and environmental exposure to bisphenols, namely chlorinated derivatives and bisphenol diglycidyl ethers. Chlorinated

[10]C.Liao, F. Liu, H. Alomirah, V. D. Loi, M. A. Mohd, H. Moon, H. Nakata, K. Kannan, Bisphenol S in urine from the United States and seven Asian countries: occurrence and human exposures, Environ. Sci. Technol. 46 (2012) 6860-6866.

derivatives mainly result from the reaction of bisphenols with sodium hypochlorite, used as bleaching agent in paper factories and water disinfection (Table 1 shows as an example the chlorinated derivatives of BPA). Bisphenol diglycidyl ethers are the primary chemical building blocks for epoxy resins, epoxy-based lacquers or vinylic organosol (PVC) resins. The structures of bisphenol A and bisphenol F diglycidyl ethers (BADGE and BFDGE, respectively), as well as their hydrolytic and chlorinated derivatives, generated when the coating comes into contact with water and hydrochloric acid of the foodstuff during heat stabilization and storage, are also shown in Table 1 [11,12].

Properties of interest for the extraction, separation and detection of mixtures of bisphenols and derivatives (e.g. octanol-water partition coefficients and acid dissociation constants) are included in this table. Most of bisphenols are in the neutral form in samples and their mixtures encompass a wide range of polarity (eg. log K_{ow} 1.254-6.564).

1.2. Levels for human and environmental exposure to bisphenols

Human and environmental exposure to bisphenols and their derivatives can be assessed either from estimated daily intakes [13 - 20] or

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[11]J. Simal-Gándara, S. Paz-Abuín, L. Ahmé, A critical review of the quality and safety of BADGE-based epoxy coatings for cans: implications for legislation on epoxy coatings for food contact, Crit. Rev. Food Sci. Nutr. 38(1998) 8, 675-688.

[12]L. Wang, Y. Wu, W. Zhang, K.Kannan, Widespread occurrence and distribution of bisphenol A diglycidyl ether (BADGE) and its derivatives in human urine from the United States and China, Environ. Sci. Technol. 46(2012) 12968-12976.

[13]J. Muncke, Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? Sci. Total Environ. 407 (2009) 4549-4559.

[14]C. Liao, F. Liu, K. Kannan, Bisphenol S, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues, Environ. Sci. Technol. 46 (2012) 6515-6522.

[15]C. Liao, K. Kannan, Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure, J. Agric. Food Chem. 61(2013) 4655-4662.

[16]Joint FAO/WHO Expert Meeting to Review Toxicological and Health Aspects of bisphenol A, 2010. Food and Agriculture Organization / World Health Organization (FAO/WHO).

biomonitoring [21-23]. The last strategy gives more exact information on actual exposure because it does not require identification of all exposure sources. Table 2 shows the concentrations found in the literature for bisphenols, chlorinated derivatives of BPA and diglycidyl ethers of BPA and BPF in human exposure sources, grouped according to the route of exposure (e.g. ingestion, dermal, inhalation), environmental compartments and biological samples. Only studies including more than one bisphenol or derivative, analyzed in at least 10 samples, have been considered for calculation of the results reported in Table 2 [14,15,23-62]. These results are expressed as arithmetic or geometric mean depending on the data reported in the respective studies. Also the whole range of concentrations found for the target compounds as well as their frequency of detection are shown in the table. When available, both free and total bisphenol concentrations have been included. These results are expected to give a rough picture of current human and environmental exposure to mixture of bisphenols.

Available from http://www.who.int/foodsafety/chem/chemicals/BPA_Summary2010.pdf, last accessed on 5/10/2015.

[17]Opinion of the Scientific Committee on Food on Bisphenol A, May 3, 2002. European Commission (EC). Available from http://ec.europa.eu/food/fs/sc/scf/out128_en.pdf, last accessed on 5/10/2015.

[18]C. Liao, F. Liu, Y. Guo, H. Moon, H. Nakata, Q. Wu, K. Kannan, Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: implications for human exposure. Environ. Sci. Technol. 46 (2012) 9138-9145.

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[20]L. Wang, C. Liao F. Liu, Q. Wu, Y. Guo, H. Moon, H. Nakata, K. Kannan, Occurrence and human exposure of p-hydroxybenzoic acid esters (parabens), bisphenol A diglycidil ether (BADGE), and their hydrolysis products in indoor dust from the United States and three East Asian countries, Environ. Sci. Technol. 46(2012) 11584-11593.

[21]A.G. Asimakopoulos, L.Wang, N. S. Thomaidis, K. Kannan, Benzotriazoles and benzothiazoles in human urine from several countries: A perspective on occurrence, biotransformation, and human exposure, Environ. Int.59 (2013) 274-81.

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Table 2

Levels of bisphenols, bisphenol A chlorinated derivatives and bisphenol diglycidyl ethers in human exposure sources (ingestion, dermal, inhalation) environmental and biological samples

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Analyte		Ingestion			Dermal		Inhalation	Environmental		Biological						Ref.
		Packed food (ng g^{-1})	Beverages (ng mL^{-1})	Drinking water (ng mL^{-1})	Personal care (ng g^{-1})	Paper (ng g^{-1})	Dust (ng g^{-1})	Sediments (ng g^{-1})	Sewage sludge (ng g^{-1})	Urine (ng mL^{-1})	Serum (ng mL^{-1})	Placental tissue (ng g^{-1})	Breast milk (ng mL^{-1})	Adipose tissue (ng g^{-1})	Colostrum (ng mL^{-1})	
								Bisphenols								
BPA	n	871	97	62	36	16	284	172	76	50[c] 40	58	59	33	20	21	[14,15, 24-29,31, 33-38, 41-44, 46-49, 53-56, 58, 60-62]
	Mean[a]/GM[b]	21.2[a]	1.92[a]	0.02[a]	20.9[a]	10511[a]	1000[a]	117[a]	459[a]	1.06[ac] 4.47[a]	2.91[a]	7.49[a]	0.43[a]	5.83[a]	1.87[a]	
	Range	nd-521	nd-46.4	nd-0.128	nd-88.3	nd-70000	9.6-32000	nd-13370	6.48-4700	nd-4.3[c] nd-40	nd-7.12	nd-34.9	<0.01-3.29	nd-12.01	nd-6.12	
	F (%)	66.4	43.3	95.2	55.6	88	100	84.9	100	40[c] 57.5	51.7	37.3	100	55	90.5	
BPB	n	686	76		29		284	172	76	20[c] 20	58					[15,24, 25, 27-29, 37,38, 43,44, 49,58, 60-62]
	Mean[a]/GM[b]	17.4[a]	0.09[a]		0.01[a]		<1[a]	0.06[a]	0.97[a]	nd[c] 0.68[a]	5.15[a]					
	Range	nd-85.7	nd-0.16		nd-0.014		<1-8.4	nd-10.6	nd-5.6	nd[c] nd-1.15	nd-11.94					
	F (%)	4.6	19.7		3.45		1	0.6	1.4	0[c] 10	27.6					
BPE	n	16	10													[25,60]
	Mean[a]/GM[b]	nd	nd													
	Range	nd	nd													
	F (%)	0	0													
BPF	n	641	67		29		284	172	76							[15,24, 25,34, 36,49, 55,56, 60-62
	Mean[a]/GM[b]	3.37[a]	0.16[a]		0.13[a]		1000[a]	69.7[a]	17.2[a]							
	Range	nd-623	nd-0.26		nd-0.79		<1-110000	nd-9650	nd-242							
	F (%)	17.3	7.5		5		83	62.2	68.0							

BPP	n	464	35	29		284	172	76				15,24, 49,61, 62]
	Mean[a]/GM[b]	1.15[a]	0.025[a]	nd		<2[a]	nd	1.06[a]				
	Range	nd-73.1	nd-0.03	nd		<2-9.4	nd	nd-6.4				
	F (%)	5.4	2.8	0		nr	0	4.2				
BPS	n	500	31	29	8	284	172	76		30		[14,15, 24,25, 41,49, 59,61, 62]
	Mean[a]/GM[b]	6.3[a]	0.007[a]	0.04[a]	11025[a]	220[a]	12.37[a]	34.5[a]		0.23[a]		
	Range	nd-175	nd-0.007	nd-0.04	1000-60000	<2-21000	nd-1970	nd-1480		nd-0.23		
	F (%)	25.8	3.2	13.8	100	100	28.5	84		3.3		
BPZ	n	474	45	29		284	172	76				[15,24, 36,49, 61,62]
	Mean[a]/GM[b]	0.69[a]	0.09[a]	nd		<0.5[a]	0.37[a]	1.81[a]				
	Range	nd-2.6	nd-0.09	nd		<0.5	nd-63.3	nd-66.7				
	F (%)	3.6	2.2	0		nr	0.6	1.4				
BPAF	n	464	35	29		284	172	76				[15,24, 49,54, 61,62]
	Mean[a]/GM[b]	0.02[a]	0.006[a]	0.03[a]		3.1[a]	0.05[a]	6.01[a]				
	Range	nd-0.76	nd-0.009	nd-0.07		<0.1-54	nd-4.23	nd-72.2				
	F (%)	12	6.45	10.3		73	4.1	46				
BPAP	n	464	35	29		284	172	76				[15,24, 49,61, 62]
	Mean[a]/GM[b]	0.89[a]	nd	0.19[a]		0.38[a]	1.71[a]	nd				
	Range	nd-127	nd	nd-1.01		<0.5-4.5	nd-252	nd				
	F (%)	9.5	0	31		1	7.6	0				
Bisphenol A chlorinated derivatives												
MCBPA	n		62				31	59	33	20	21	[41,42, 45-48, 59]
	Mean[a]/GM[b]		0.005[a]				0.055[b]	9.45[a]	nd	3.05[a]	nd	
	Range		0.0002-0.027				nd-1.68	nd-21.4	nd	nr	nd	
	F (%)		100				16.1	40.7	0	15	0	

DCBPA	n	62	31	59	33	20	21	[41,42, 45-48, 59]
	Mean[a]/GM[b]	0.001[a]	0.048[b]	23.3[a]	2.53[a]	9.21[a]	1.96[a]	
	Range	nd-0.006	nd-1.06	nd-58.8	<0.4-4.13	nr	<0.4-4.13	
	F (%)	98.4	19.4	40.7	100	80	100	
TCBPA	n	62	31	59	33	20	21	[41,42, 45-48, 59]
	Mean[a]/GM[b]	0.002[a]	0.047[b]	11.3[a]	0.68[a]	0.74[a]	0.17[a]	
	Range	nd-0.008	nd-0.675	nd-31.2	nd-0.68	nr	<0.4-0.68	
	F (%)	58.1		39.0	33.3	10	11	
TeCBPA	n	62		59		20		[42, 47, 48]
	Mean[a]/GM[b]	0.0005[a]		nd		nd		
	Range	nd-0.005		nd		nd		
	F (%)	48.4		0		0		
Bisphenol diglycidyl ethers								
BADGE	n	134	158	127[c] 303				[23, 30-33, 35,39, 40, 50-53, 57,60, 61]
	Mean[a]/GM[b]	32.63[a]	3.47[b]	0.116[bc] 2.63[b]				
	Range	nd-440	nd-7750	0.027-0.497[c] nd-295				
	F (%)	36	91.1	100[c] 68				
BADGE·H_2O	n	129	158	127[c] 227				[23, 30-33, 35,39, 40, 50-52 57,60, 61]
	Mean[a]/GM[b]	48.33[a]	40.5[b]	0.06[bc] 0.221[b]				
	Range	nd-179	nd-8850	nd-0.37[c] nd-3.7				
	F (%)	14	99.4	79.5[c] 57.7				

BADGE·2H$_2$O	n	110	158	127[c] 303	[23, 30-33 35,39, 40,50, 51,57, 60,61]
	Mean[a]/GM[b]	104.3[a]	1310.7[b]	0.537[b,c] 6.59[b]	
	Range	nd-675	35-59900	nd-5.24[c] nd-1450	
	F (%)	64	100	99.2[c] 91.2	
BADGE·HCl	n	129		100	[23, 30-33, 35,39, 40, 50-52, 60]
	Mean[a]/GM[b]	21.43[a]		<2	
	Range	nd-74.42		nd - <2	
	F (%)	13		3	
BADGE·2 HCl	n	128			[30-33, 35, 50-52, 60]
	Mean[a]/GM[b]	42.4[a]			
	Range	nd-810			
	F (%)	39			
BADGE·HCl·H$_2$O	n	128	158	127[c] 227	[23, 30-33 35,39, 40, 50-52 60,61]
	Mean[a]/GM[b]	39.1[a]	229.1[b]	0.042[b,c] 0.138[b]	
	Range[c]	nd-533	5-24300	nd-1.265[c] nd-3.412	
	F (%)	64	100	44.1[c] 52.8	
BFDGE	n	73			[30,35 50-52, 60]
	Mean[a]/GM[b]	81[a]			
	Range	nd-314			
	F (%)	21.9			

BFDGE·H_2O	n	10	[51]
	Mean[a]/GM[b]	nd	
	Range	nd	
	F (%)	0	
BFDGE·$2H_2O$	n	19	[30,35, 51]
	Mean[a]/GM[b]	0.6[a]	
	Range	nd-0.81	
	F (%)	26	
BFDGE·HCl	n	10	[51]
	Mean[a]/GM[b]	nd	
	Range	nd	
	F (%)	0	
BFDGE·2HCl	n	57	[30,35, 50,51, 60]
	Mean[a]/GM[b]	69.5[a]	
	Range	nd-120	
	F (%)	3.5	
BFDGE·HCl·H_2O	n	10	[51]
	Mean[a]/GM[b]	nd	
	Range	nd	
	F (%)	0	

n: number of samples; F: frequency of detection; nd: not detected; nr: not reported; [a] arithmetic mean; [b] geometric mean; [c] free bisphenols;

Human exposure to bisphenols occurs primarily through ingestion of canned food and beverages [15, 24-28,30-38,50-57,60] but also through skin absorption [14,63] and inhalation of dust [61] (Table 2). Migration of bisphenols from epoxy-based linings of cans into foods are influenced by different parameters including the time and temperature of contact, the type of foodstuff and the nature of the packaging material [64-67]. Skin absorption of bisphenols from personal hygiene and cosmetic products derives from their migration from polycarbonate plastics containers or degradation of bisphenols-based components as silicones in hair conditioner or urethane thickening agents [68,69]. Paper products (e.g. office paper, tissue, packaging, advertisement brochures, tickets, bills, newspaper, thermal receipts and so on) are another important via of dermal exposure to bisphenols, which are used as color developers [14]. Intake of dust shows a similar pattern to ingestion of food and exposure can be affected by factors such as occupation (soil cultivation), lifestyle, type of residential area and application of electronic equipment [18-20]. According to the results shown in Table 2, BPA continues being the bisphenol at the highest concentration and detection rate in foodstuffs, but other bisphenols as BPP show mean concentrations nearly those of BPA. Dust is, at present, an important source for BPA, BPF and BPS.

Human exposure to chlorinated derivatives of bisphenols occurs primarily through the consumption of tap water (Table 2). Drinking water reservoirs are coated with bisphenol-based epoxy paints and traditional

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[63]J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba, Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC-MS, J. Pharm. Biomed. Anal. 78-79 (2013) 255-260.
[64]K. Bhunia, S. S. Sablani, J. Tang, B. Rasco, Migration of Chemical Compounds from Packaging Polymers during Microwave, Conventional Heat Treatment, and Storage, Compr. Rev. Food Sci. 12 (2013) 523-545.
[65]N. Lin, Y. Zou, H. Zhang, Kinetic migration studies of bisphenol-A_related compounds from can coatings into food simulant and oily foods, Eur. Food Res. Technol. 237 (2013) 1009-1019.
[66]I. S. Arvanitoyannis, K. V. Kotsanopoulos, Migration phenomenon in food packaging. Food-package interactions, mechanisms, types of migrants, testing and relative legislation- A review, Food Bioprocess Technol. 7 (2014) 21-36.
[67]A. Guart, F. Bono-Blay, A. Borrell, S. Lacorte, Effect of bottling and storage on the migration of plastic constituents in Spanish bottled waters, Food Chem. 156 (2014) 73-80.
[68]H. Sebag, A. Zysman, Polyquaternarypolysiloxane polymers, US Patent4533714 (1985).
[69]A. Ohtta, H. Itayama, M. Tomioka, Hair treatment and method of treating hair using compounded resin containing urethane resin, US Patent 6641804B1 (2003).

potable water distribution is being replaced by polymer pipes [70,71]. So, chlorinated derivatives mainly arise from the chlorination of these bisphenol-contaminated waters [26]. Concerning bisphenol diglycidyl ethers and derivatives, ingestion of canned food continues being the highest route for human exposure (Table 2). Among the analyzed diglycidyl ethers, BADGE.2H2O and BFDGE occur at the highest concentration.

In general, bisphenols and derivatives mainly enter the environment through wastewater [8]. Soils can become contaminated by farmland fertilization with sewage sludge. Both, wastewater and leaching from landfill sites containing these compounds are the major sources of contamination of ground waters, rivers, streams and, eventually, drinking water [72,73]. Bisphenols have low vapor pressure and only very low levels (<1%) are thought to occur in the atmosphere, where they are believed to photo-oxidize and breakdown rapidly [74]. Levels found for bisphenols in sediments and sewage vary over a wide range as it can be seen in Table 2.

Biomonitoring of bisphenols and derivatives requires the knowledge of their transformation and distribution in the body. After ingestion, they are quickly metabolized in the gut wall and liver to predominantly give glucuronides and, in a less extent, sulfates. These metabolites are more soluble in water than the parent compounds and, therefore are rapidly cleared from blood by the kidneys and excreted in the urine with half-lives of less 6 h after oral administration. The measurement of total bisphenols (unconjugated and their glucuronidated and sulphated conjugates) in urine has been the most used parameter for their biomonitoring, although differential measurements

[70]J. Romero, F. Ventura, M. Gomez, Characterization of paint samples used in drinking water reservoirs: identification of endocrine disruptor compounds, J. Chromatogr. Sci. 40(2002) 191-197.

[71]A.J. Whelton, T. Nguyen, Contaminant Migration from polymeric pipes used in buries potable water distribution systems: A review, Crit. Rev.Environ. Sci. Technol. 43 (2013) 879-751.

[72]N. Dorival-Garcia, A. Zafra-Gomez, A. Navalon, J.L. Vilchez, Improved sample treatment for the determination of bisphenol A and its chlorinated derivatives in sewage sludge samples by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry, Talanta, 101 (2012) 1-10.

[73]N. Dorival-Garcia, A. Zafra-Gomez, A. Navalon, J.L. Vilchez, Analysis of bisphenol A and its chlorinated derivatives in sewage sludge samples, Comparison of the efficiency of three extraction techniques, J. Chromatogr. A, 1253 (2012) 1-10.

[74]I.T. Cousins, C.A. Staples, G.M. Klecka, D. Mackay, A multimedia assessment of the environmental fate of bisphenol A. Hum. Ecol. Risk Assess. 8 (2002) 1107-1135.

for free bisphenols and metabolites have been also reported. Table 2 shows the levels reported for the target bisphenols and derivatives in urine. Other biological fluids such as serum have been less used for biomonitoring purposes.

On the other hand, there is a great concern about exposure of human fetuses, neonates, and infants to bisphenols because of the sensitivity of the developing organs and brain to endocrine disrupting chemicals. Considering that bisphenols can cross the placental barrier, the fetus remains exposed to these compounds. Also, neonates and infants are exposed to bisphenols through breast milk. The evaluation of "in utero exposure" to bisphenols, through the analysis of biological fluids from pregnant or nursing mother (i.e. blood, urine, breast milk, colostrum), the fetus or newborn infant (i.e. meconium, umbilical cord blood, neonatal urine), and from both the fetus and the mother (i.e. placental tissues, amniotic fluid), would allow for a better understanding and a more concrete picture into the exposure of the most vulnerable segment of the human population. The reported levels of bisphenols and BPA chlorinated derivatives in some of these types of samples are shown in Table 2.

1.3. Dose-related effects of bisphenols

Concerning the effects of bisphenols and derivatives on human health and the environment, those produced by BPA have been so far the most investigated [75-77]. BPA exhibits both estrogenic and antiandrogenic effects

[75]A. Kortenkamp, O. Martin, M. Faust, R. Evans, R. McKinlay, F. Orton, E. Rosivatz, State of the art assessment of endocrine disrupters, 2012, Available in http://ec.europa.eu/environment/chemicals/endocrine/pdf/sota_edc_final_report.pdf, last accessed 17/09/2015.

[76]Scientific Opinion of European Food Safety Authority (EFSA) on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: Executive summary. EFSA Journal 13 (2015) 3978.

[77]Scientific Opinion of European Food Safety Authority (EFSA) on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: PART II - Toxicological assessment and risk characterization, EFSA Journal 13 (2015) 3978.

[78] and many studies have examined correlations between elevated levels of BPA and negative impacts on reproduction, neurobehavioral development, and metabolic diseases (e.g. obesity, diabetes, heart disease, thyroid and liver function) [79]. A lot of information is also available in the literature on the adverse effects on animals (viz. rats and mice), following exposure to low doses of BPA [80,81]. These health effects are not fully understood yet and there are strong controversies in their interpretation, even governmental decisions are sometimes contradictory [75]. On the other hand, non-monotonic effects of BPA that are inconsistent with the traditionally expected dose-response curves, based on extrapolation of high dose data predicting a decreasing effect for lower doses, are quite frequent (e.g. U-shaped dose response of Sertoli cells to BPA [82] or inverted U-shaped dose response for BPA stimulation of JKT-1 cell proliferation [83].

So, there is a growing need to uncover potential mechanisms of bisphenol A at low doses in order to improve model systems, modeling approaches and biomarker development [75]. Also the mechanistic actions how endogenous hormone levels/background estrogen levels influence the effects of low doses of bisphenol A are widely unknown. Mechanistic studies published since 2010 continue to support the conclusion that BPA affects a number of receptor-dependent and independent signalling pathways,

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[78]S. Kitamura, T. Suzuki, S. Sanoh, R. Kohta, N. Jinno, K. Sugihara, S. Yoshihara, N. Fujimoto, H. Watanabe, S. Ohta, Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds, Toxicol. Sci. 84 (2005) 249–259.

[79]J.R. Rochester, Bisphenol A and human health: a review of the literature, Reprod. Toxicol. 42 (2013) 132–155.

[80]J. A. Taylor, F. S. Saal, W. V. Welshons, B. Drury, G. Rottinghaus, P. A. H, P. Toutain, C. M. Laffont, C. A. Vande-Voort, Similarity of bisphenol A pharmacokinetics in rhesus monkeys and mice: relevance for human exposure, Environ. Health. Perspect. 119 (2011) 4, 422-430.

[81]K. Maruyama, M. Nakamura, S. Tomoshige, K. Sugita, M. Makishima, Y. Hashimoto, M. Ishikawa, Structure-activity relationships of bisphenol A analogs at estrogen receptors (ERs): Discovery of an ERα-selective antagonist, Bioorg. Med. Chem. Lett. 23 (2013) 4031-4036.

[82]M. Gualtieri, J. Øvrevik, J. A. Holme, M. G. Perrone, E. Bolzacchini, P. E. Schwarze, M. Camatini, Differences in cytotoxicity versus pro-inflammatory potency of different PM fractions in human epithelial lung cells, Toxicol In Vitro, 24 (2010) 1, 29-39.

[83]A. Bouskine, M. Nebout, F. Brucker-Davis, M. Benahmed, P. Fenichel, Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor, Environ. Health. Perspect. 117 (2009) 1053-1058.

resulting in effects on hormone homeostasis and gene expression as well as in cytogenetic and epigenetic effects [84,85].

The impacts of BPA-related endocrine disruptions in wildlife are much less well known and studies have demonstrated few clear trends [74]. Terrestrial wildlife is likely to experience low exposures of BPA, however, some invertebrate, fish and amphibian species appear to be susceptible to low exposures of BPA, and benthic organisms may be exposed to higher concentrations of BPA because of elevated sediment levels. A recent aquatic hazard assessment has lowered the predicted non-effect concentration from 100 μg L^{-1} to 0.06 μg L^{-1}, which indicates that development, reproduction, and survival of wildlife is likely to be impacted at current environmental ranges [86].

Regarding the effects of bisphenols, other than BPA, the information is quite scarce but the few studies reported confirm that human and environmental toxicity and exposure pathways are similar to those of BPA [81,87]. Thus, in an recent report on BPA alternatives in thermal paper, the human health effects and aquatic toxicity of BPF, BPC, BPAP, BPS and 2,4-BPS have been assessed and compared with those known for BPA [88]. These bisphenols showed identical toxicological hazard for human health in terms of carcinogenicity and reproductive and neurological effects. BPS and 2,4-BPS presented lower hazard regarding developmental effects and aquatic toxicity, but genotoxicity increased. All bisphenols showed higher persistence in the environment than BPA.

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[84]A. Wendler, E. Baldi, B.J. Harvey, A. Nadal, A. Norman, M. Wehling, Position paper: Rapid responses to steroids: current status and future prospects, Eur J Endocrinol. 162 (2010) 825-830.

[85]Exploring novel endpoints, exposure, low-dose- and mixture-effects in humans, aquatic wildlife and laboratory animals, Contract No. QLK4-CT2002-00603, Endocrine Disruptors Research (2007).

[86]M. Wright-Walters, C. Volz, E. Talbott, D. Davis, An update weight of evidence approach to the aquatic hazard assessment of bisphenol A and the derivation a new predicted no effect concentration (Pnec) using a non-parametric methodology, Sci. Total Environ. 409 (2011) 676-685.

[87]A. K. Rosenmai, M. Dybdahl, M. Pedersen, B. M. A. Vugt-Lussenburg, E. B. Wedebye, C. Taxvig, A. M. Vinggaard, Are structural analogues to bisphenol A safe alternatives? Toxicol. Sci. 139 (2014) 1, 35–47.

[88]United States Environmental Protection Agency, Bisphenol A alternatives in thermal paper final report (2014).

Studies involving BPA by-products suggest that they could be even more cytotoxic than BPA [89]. Thus, MCBPA and DCBPA have a higher human α-estrogen receptor affinity [89] and TCBPA is a stronger human pregnane X receptor agonist [90]. On the other hand, bisphenol diglycidyl ethers have lower estrogenic potency compared to bisphenols but some studies indicate that they seem to induce adverse effects in humans at estimated intake levels [91].

On the whole, the effects reported for bisphenols and derivatives, mainly those in the low dose range, as well as their simultaneous presence in different sources, strength the need to no longer consider them individually for both human and environmental exposure and legislative provisions.

1.4. International and national provisions regarding bisphenols and derivatives

The European Union (EU) has set food migration limits for BPA (0.6 mg Kg^{-1} [92]), BADGE and its hydrolysis products (9 mg Kg^{-1}, [93]) and chlorinated BADGE byproducts (1 mg Kg^{-1}, [93]). In 2011, on the basis of the

[89]J.H. Kang, D. Asai, Y. Katayama, Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms, Crit. Rev. Toxicol. 37 (2007) 607–625.

[90]M. Song, D. Liang, Y. Liang, M. Chen, F. Wang, H. Wang, G. Jiang, Assessing developmental toxicity and estrogenic activity of halogenated bisphenol A on zebrafish (Danio rerio), Chemosphere, 112 (2014) 275-81.

[91]R. Chamorro-García, S. Kirchner, X. Li, A. Janesick, S.C. Casey, C. Chow, B. Blumberg, Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stomal cells through a peroxisome proliferator-activated receptor gamma-independent mechanism. Environ. Health Perspect. 120 (2012) 984-989.

[92]Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. Official J. Eur. Union (2011) L 12, 1-89.

[93]European Commission Regulation (EC) No1895/2005 of 18 November 2005 on the restriction of use of certain epoxy derivatives in materials and articles intended to come into contact with food (2005) L302/28, 1-5.

precautionary principle, the EU restricted the production and sale of BPA-based polycarbonate baby bottles [94].

Since 2006, the European Food Safety Authority (EFSA) has conducted several scientific assessments on BPA. The last scientific opinion, published in January 2015, consists of three separate documents, namely Executive Summary [76]; Part I-Exposure assessment [95] and Part II-Toxicological assessment and risk characterization [77]. In this opinion, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids has established a temporary Tolerable Daily Intake (t-TDI) of 4 μg Kg^{-1} body weight per day and it has concluded that there is no consumer health risk from BPA exposure. Nevertheless, bans on the use of BPA for food packaging intended for young children (zero to three years old) have been proposed by several EU Member States (viz. Denmark, Sweden, France, Belgium and Austria) [95].

The U.S. Environmental Protection Agency (EPA) and U.S. Food and Drug Administration (FDA) have chosen not to regulate BPA due to insufficient scientific evidence of adverse human health effects at low-levels of exposure [96,97], despite provisions for its elimination from some products such as baby bottles [97]. The National Sanitation Foundation (NSF) recommends a BPA drinking water criterion of 0.1 mg L^{-1} total allowable concentration and 0.01 mg L^{-1} single-product allowable concentration [98]. BADGE is not regulated by the EPA or FDA but the NSF recommends a drinking water criterion of 1 mg L^{-1} total allowable concentration and 5 mg L^{-1} short term exposure level [98].

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[94]EC regulation No 321/2011 of 1 April 2011 amending Regulation (EU) No 10/2011 as regards the restriction of use of Bisphenol A in plastic infant feeding bottles, Official Journal of the European Union, L87/1.

[95]Scientific Opinion of European Food Safety Authority (EFSA) on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. PART I: Exposure assessment, EFSA Journal 13 (2015) 3978.

[96]USEPA, U.S. Environmental Protection Agency. Bisphenol A Action Plan (2010) http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa_action_plan.pdf, last accessed on 08/09/2015.

[97]USFDA, U.S. Food and Drug Administration Bisphenol A (BPA): Use in Food Contact Application, (2013) http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm, last accessed on 08/09/2015.

[98]NSF, 2010 NSF International Standard/American National Standard NSF/ANSI 61-2010a Drinking Water System Components - Health Effects NSF International, Ann Arbor, Michigan (2010).

1.5. Cocktail effect of chemical mixtures

It is becoming increasingly evident that, in combination, some chemicals can cause harmful effects in wildlife species, laboratory animals, and humans, at concentrations considered safe for the individual chemicals [99]. This is of special concern for mixtures of endocrine disrupter chemicals (EDCs), for which determinants of additivity have been characterized and are now well understood [3,75,100]. Where EDCs act in concert with endogenous hormones, significant additional effects may result under certain circumstances [101]. In view of this evidence, the traditional chemical-by-chemical approach to risk assessment is hard to justify, and the ground is prepared to seriously consider group-wise regulation of chemicals [102].

Guidance for conducting cumulative risk assessments has been published by EPA [103], the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment [104], the Norwegian Scientific Committee for Food Safety [105], and the German CVUA [106]. A framework for the risk assessment of combined exposures to multiple chemicals has been proposed by the WHO/IPCS [107]. General support for this framework and

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[99]A. Kortenkamp, M. Faust, M. Scholze, T. Backhaus, Low-Level Exposure to Multiple Chemicals: Reason for Human Health Concerns? Environ. Health Perspect. 115 (2007) 106-114.
[100]A. Kortenkamp, Ten Years of Mixing Cocktails: A Review of Combination Effects of Endocrine-Disrupting Chemicals, Environ. Health Perspect. 115 (2007) 98-105.
[101]A. Kortenkamp, Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology, Int. J. Andrology, 31 (2008) 233-240.
[102]T. Backhaus, M. Faust, A. Kortenkamp, Cumulative risk assessment: A European perspective on the State of the Art and the necessary next steps forward, Integrated Environ. Assessment and Management, 9 (2013) 547–548.
[103]USEPA, Supplemental guidance for conducting health risk assessment of chemical mixtures as a supplement to the EPA's guidelines for the health risk assessment of chemical mixtures (USEPA, 1986) (2002).
[104]COT, UK committee on toxicity of chemicals in food, consumer products and the environment. Risk assessment of mixtures of pesticides and similar substances (2002).
[105]VKM, Norwegian Scientific Committee for Food Safety. Combined toxic effects of multiple chemical exposures. Report (2008).
[106]CVUA, Chemisches und Veterinäruntersuchungsamt Stuttgart/Germany. Toxikologische Bewertung von Mehrfachrückständen in Obst und Gemüse. Report (2007).
[107]WHO, World Health Organization. Harmonization Project. DRAFT Document for Public and Peer Review. Risk Assessment of Combined Exposures to Multiple Chemicals: A WHO/IPCS Framework (2009b).

associated terminology was expressed at an OECD-WHO-ILSI-HESI Workshop in 2011 [108]. At EU level, the European Council of Environmental Ministers published its conclusion on combination effects toward the end of 2009, requesting the European Union (EU) Commission to step up its chemical management and research efforts in the area [109]. This resulted in the mixture opinion of the European Scientific Committees in 2011 [110] and finally the Communication of the European Commission back to the Council in 2012 [111]. In this Communication, the Scientific Committees provided extensive guidance regarding the identification of chemical mixtures of highest concern and the methodologies for assessing/predicting the toxicity of such mixtures. Also, they drew attention to the many data and knowledge gaps that impede a more systematic and effective application of these methodologies.

Regarding the number of bisphenols and derivatives used in the market as monomers or additives of plastic materials and other consumer products (and also the many possible byproducts generated), and considering the strong previous experimental evidence that EDCs of relatively low potency and at low exposure levels can still work together to produce significant combination effects when they are present in sufficient number, it seems that the current approach of assessing human and environmental risk to bisphenols through the t-TDI set for BPA (the only legislated bisphenol) may underestimate the actual risk to these compounds.

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[108]OECD, WHO OECD ILSI/HESI International Workshop on Risk Assessment of Combined Exposures to Multiple Chemicals. Paris, France, OECD Environment Directorate. OECD Environment, Health and Safety Publications. Series on Testing and Assessment (2011).

[109]Council conclusions on combination effects of chemicals, 2988th ENVIRONMENT Council meeting, Brussels, 22 December 2009, Available at http://www.consilium.europa.eu/uedocs/cms_data/docs/pressdata/en/envir/112043.pdf, last accessed 17/09/2105.

[110]Opinion of the European Scientific Committees (Consumer Safety; Emerging and Newly Identified Health Risks; and Health and Environmental Risks) on Toxicity and Assessment of Chemical Mixtures, 2011, Available at http://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf, last accessed 30/09/2015.

[111]Communication from the Commission to the Council, The combination effects of chemicals. Chemical mixtures. Brussels, 31.5.2012, COM (2012) 252. Available at http://www.parliament.bg/pub/ECD/118563COM_2012_252_EN_ACTE_f.pdf, last accessed 30/09/2015.

Bisphenol mixtures meet several of the criteria set by the EC to be considered as a mixture of potential concern [111]. Thus, exposure of the human population and the environment to bisphenols is widespread; they are pseudo-persistent; there is potential for adverse effects to the likely exposure levels; as EDCs, there is scientific base to predict that they will probably act similarly; and, except for BPA, threshold limits for the effects of mixture components have been not established.

2. Assessing human and environmental exposure to a cocktail of bisphenols and derivatives

Chemical analysis is essential in the assessment of wildlife and humans exposure to a cocktail of bisphenols. The components to be analyzed differ greatly in their physical and chemical properties (Table 1) and are generally present at trace concentrations in complex matrices (Table 2), that requiring highly selective and sensitive analytical methods for their quantification. Adding greatly to the complexity, and to the number of bisphenols in our environment, are the unknown potential byproducts that can be formed via human and environmental transformations.

Table 3 gives an overview of the analytical methods reported in the literature for the determination of mixtures of bisphenols and derivatives in human and environmental exposure sources and biological samples. Packed food, aquatic environment and biological fluids have been the areas receiving the greatest attention, although interesting methods involving other human exposure sources such as beverages, drinking water, personal care products, paper and dust, have been also reported. There have been substantial differences among the mixture of bisphenols or derivatives selected for analysis depending on the matrix. Thus, separate mixtures of bisphenols or diglycidyl ethers have been preferentially analyzed in packed food, while bisphenols and BPA chlorinated derivatives have been jointly analyzed in aquatic environments. Methods reported for biological samples have been mainly devoted to the determination of mixtures of BPA plus chlorinated derivatives and BPA plus diglycidyl ethers. Very few methods have included BPE as a component in the mixtures investigated.

Table 3

Analytical methods reported for the determination of mixtures of bisphenol and derivatives

Sample type (size)	Analytes	Sample treatment	Separation / detection	R (%)	Detection limits	Ref.
		Packed food				
Canned vegetables and fruits (10 g, total content for fruits; solid portion for vegetables)	BPA, BPB	Sample + water, 5 mL; stirring and centrifugation QuEChERS extraction: AcN, 10 mL; $MgSO_4$, 4 g; NaCl, 1 g; shaking 15 min Centrifugation DLLME: 1mL AcN extract as dispersive solvent and 50 µL T4CE as extractive solvent; add to 3 ml of water and shake Centrifugation	GC-EI-MS(SIM) Derivatization agent: AA Column: DB-5MS (30m x 0.25mm, 0.25µm)	69-104	MDL= 0.1-0.6 ng g^{-1} MQL= 1 ng g^{-1}	[27]
Canned seafood (10 g, total content except for tuna)	BPA, BPB	Fat removal: sample +n-heptane, 5 mL+ water, 10 mL; stirring and centrifugation QuEChERS extraction: AcN, 10 mL; $MgSO_4$, 4 g; NaCl, 1 g; shaking 15 min; centrifugation; AcN extract+ $MgSO_4$, 1.2 g+C18, 120 mg + GCB, 50 mg; stirring Centrifugation DLLME: 1 mL AcN extract (dispersive solvent)+K_2CO_3, 5% to pH>10+ 50 µL T4CE (extractive solvent); add to 4 ml of water and shake Centrifugation	GC-EI-MS(SIM) Derivatization agent: AA Column: DB-5MS (30m x 0.25mm, 0.25µm)	68-104	MDL= 0.2-0.4 ng g^{-1} MQL= 1 ng g^{-1}	[28]
Canned peeled tomatoes (20 g, total content)	BPA, BPB	Extraction: AcN, 2x150 mL Clean up: n-hexane, 3x60 mL (fat removal); solvent evaporation; reconstitution water: AcN (90:10), 6 mL;SPE,C-18 Strata E; elution MeOH 4x5mL, solvent evaporation, reconstitution hexane: EA (96:4), 6 mL; SPE, Forisil; elution EA 4x5mL Solvent evaporation Reconstitution AcN, 20 mL	LC-UV-FD (λ 228 nm) (λex 273nm, λem 300nm) Mobile phase: AcN/water (isocratic) Column: Synergi Fusion-RP80Å (250 x 4.6mm; 4µm)	94.3–95.7	UV MDL= 15.4-20 ng g^{-1} MQL= 51.3–66.9 ng g^{-1} FD MDL= 0.7–1.1 ng g^{-1} MQL= 2.3–3.7 ng g^{-1}	[37]

Canned powdered infant (10 mL)	BPA, BPB	Reconstitution with water Precipitation of protins and fats : TCA 10% in MeOH, 5mL; stirring; centrifugation Upper layer at pH≥10 with potassium carbonate, 5 %; take 10 mL Extraction: DLLME (extractive solvent: T4CE, 30 µL; dispersive solvent: AcN, 440 µL)	Heart cutting-GC-EI-MS(SIM) Derivatization agent: AA Column 1: DB-5HT (5m x 0.32mm, 0.1µm) Column 2: DB-5MS (15m x 0.25mm, 0.25µm)	68 -114	MDL=$3\cdot10^{-2}$ - $6\cdot10^{-2}$ ng g^{-1} MQL= 0.1 - 0.2 ng g^{-1}	[38]
Canned fish, meat, pasta, fruits, vegetables, etc. (20 g, total content)	BPA, BPF	Extraction: AcN:n-heptane (fat removal) (50:50), 40 mL; n-heptane washing with AcN, 20 mL; AcN extrats combinated and treated with anhydrous sodium sulphate Solvent evaporation to 5 mL Dilution: water, 50 mL Derivatization Extraction: n-heptane, 5 mL	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	61-128	MDL= 2-10 ng g^{-1}	[34]
Canned infant formula (10 g)	BPA, BPF	Extraction: AcN, 20 mL+10 mL Solvent evaporation to 5 mL Dilution: water, 50 mL Derivatization Extraction: n-heptane, 5 mL	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	61-128	MDL= 2-10 ng g^{-1}	[34]
Canned tomato paste and corn (10 g, total content)	BPA, BPF	Extraction: AcN:water (90:10), 15 mL Preconcentration: HS-SPME (SWCNT) of the extract and derivatization	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	79-86	LOD=0.1 ng g^{-1} LOQ=0.3 ng g^{-1} MDL=0.045 ng g^{-1}	[55]
Canned corn, tomato paste, stew, tuna fish (10 g, total content)	BPA, BPF	Extraction: AcN:water (90:10), 15 mL Preconcentration: HS-SPME (-) of the extract and derivatization	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	79-86	LOD=0.1 ng g^{-1} LOQ=0.3 ng g^{-1} MDL=0.045 ng g^{-1}	[56]

Milk, lactic acid milk drinks and carbonated drinks (10 mL)	BPA, BPAF	Sample+26 mg magnesium sulphate/centrifugation Extraction: SUPRAS (octanol in THF), 500 µL Dilution of SUPRAS to 1 mL with AcN	LC-UV/Vis (λ 240nm) Mobile phase: AcN/water (gradient) Column: C18 (150 x 4.6 mm; 5µm)	91-105	MDL=0.14-0.32 ng mL^{-1} MQL=0.41-1.02 ng mL^{-1}	[54]
Canned vegetables (solid portion, 2 g; liquid portion, 10mL)	BPS, BPA	Solid portion + water, 10 mL Liquid portion + water, 4 mL Extraction: SPME (PA fiber, 85 µm) Desorption in the injection port at 280 °C	GC-EI-MS(SIM) Derivatization agent: BSTFA Column: SLB-5MS (30m x 0.25mm, 0.25µm)	84-112	MDL= 0.0025-0.016 ng mL^{-1} MQL= 0.0083-0.055 ng mL^{-1}	[118]
Canned vegetables (2 mL, liquid portion)	BPA, BPF, BPZ	Dilution to 10 mL with water Extraction: SBSE (PDMS) Desorption: thermal desorption unit equipped with an autosampler	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	86-122	LOD=$0.9 \cdot 10^{-3}$–$2.5 \cdot 10^{-3}$ ng mL^{-1} LOQ=$3.2 \cdot 10^{-3}$ - $8.4 \cdot 10^{-3}$ ng mL^{-1}	[36]
Cereals and cereal products, meat and meat products, fish and seafood, eggs, bean products, fruits, vegetables, cookies/snacks, cooking oils,etc (1-4 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Samples were freeze-dried Extraction: AcN, 2x6 mL; shaking; centrigufation; solvent evaporation; reconstitution DCM:hexane (10:90), 2 mL Clean up: SPE, Strata NH2; elution MeOH:acetone (80:20), 5 mL Solvent evaporation Reconstitution: MeOH, 0.5 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Betasil C18 (100 x 2.1 mm; 5µm)	62-126	MQL= 0.01-0.05 ng g^{-1}	[24] [15]
Milk and milk products (3 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Extraction: AcN, 6 mL (sample:acetonitrile= 1:2, v/v); shaking; centrifugation; solvent evaporation to 4 mL Dilution to 10 mLwith formic acid, 0.2% (pH=2.5) Clean up: SPE, Oasis MCX Concentration of eluate to 0.5 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Betasil C18 (100 x 2.1 mm; 5µm)	62-126	MQL= 0.01-0.05 ng g^{-1}	[24] [15]
Fish, meat (5 g, total content)	BADGE, BADGE·$2H_2O$, BADGE·H_2O	Extraction: pentane 3x2 mL/ solvent evaporation Clean up: MeOH, 3x5mL Solvent evaporation Reconstitution: mobile phase, 0.5 mL	LC-FD (λ_{ex} 275nm, λ_{em} 305nm) Mobile phase: CH/TBME (for BADGE) and AcN/water/MeOH (for hydrolysis products) Column: Hibar Lichrosorb Si60	90-114	LOD: 10 ng g^{-1}	[57]

			(250 x 4.6mm; 5µm) for BADGE and Hibar Lichrosorb Diol (250 x 4.6mm; 5µm) for hydrolysis products			
Canned tuna (10 g, total content)	BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl,	Extraction: AcN, 40 mL; stirring; add NaCl and stirring Centrifugation	LC-FD (λ_{ex} 275nm, λ_{em} 305nm) Mobile phase: AcN/water (gradient) Column: Spherisorb ODS2 (100 x 4.6mm; 5µm)	95-99	MDL= 10-20 ng g^{-1} MQL= 30-50 ng g^{-1}	[32]
Fish and meat (2 g)	BADGE, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE	Extraction: MAE (hexane, 5 mL+acetone, 3 mL; 20 min at 105°C and 200 W)/Centrifugation Clean up: supernatant washing with AcN, 2x5 mL/solvent evaporation/redissolution with water:MeOH (90:10), 3 mL/ SPE, PS-DVB; elution AcN, 5mL Solvent evaporation Reconstitution: MeOH, 1 mL	LC-FD (λ_{ex} 230nm, λ_{em} 301nm) Mobile phase: AcN/water (gradient) Column: Lichrospher C18 (150 x 2.1mm; 5µm)	70.46-103.44	LOD=0.79-3.77 ng g^{-1} LOQ=2.75-10.92 ng g^{-1}	[52]
Canned fruits and vegetables (3 g, total content)	BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·2H_2O, BFDGE·2HCl	Extraction: EA, 6 mL; 5 mL to dryness Reconstitution: MeOH: water (50:50), 1 mL	LC-ESI(+)-MS/MS(SRM) Mobile phase: MeOH/ 25mM formic acid-ammonium formate buffer (pH 3.75) (gradient) Column: Fused Core™Ascentis Express C18 (50 x 2.1mm; 2.7µm)	60-90	MQL= 1-4 ng g^{-1}	[112]
Fish and meat (10 g, total content)	BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·2HCl	Sample +diatomaceous earth Extraction: PLE (hexane-acetone (4:1), 100°C, 1500 psi and 5 min static time and two cycles) Concentration to 10 mL under nitrogen Clean up: AcN, 3x2 mL; SPE (C18+NH2), elution AcN-MeOH (1:1) Solvent evaporation	LC-APCI(+)-MS/MS(SRM) Mobile phase: AcN/water (isocratic) Column: Kromasil C18(100 x 2.1mm; 3.5µm)	82-101	LOD=0.25-1 ng g^{-1} LOQ=0.8-3.5 ng g^{-1}	[50]

		Reconstitution: AcN: water (1:1), 0.2 mL				
Canned fish (2 g, total content)	BADGE, BADGE·H_2O, BADGE·2H_2O, BADGE·HCl•H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·2H_2O, BFDGE·2HCl	Extraction: AcN:n-hexane (1:1), 20 mL; stirring; centrifugation Solvent evaporation of 1 mL of AcN layer Reconstitution: ammonium formate (0.01M), 0.5 mL	LC-ESI(+)-MS/MS(MRM) Mobile phase: MeOH/ammonium formate (0.01M) (gradient) Column: Synergy MAX-RP (100 x 2mm; 2.5µm)	90 - 110	MDL= 0.5 – 3.1 ng g^{-1} MQL= 1.8 – 10.3 ng g^{-1}	[30]
Fish, meat, vegetables and peanut butter (5 g)	BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·2H_2O, BFDGE·H_2O, BFDGE·HCl·H_2O, BFDGE·2HCl, BFDGE·HCl	Extraction: MAE (hexane, 10 mL+ acetone, 5 mL; 10 min at 105°C; 5 min 800 W) Centrifugation Clean up: AcN, 2x5 mL Solvent evaporation Reconstitution: AcN: water (50:50), 1 mL	UPLC-ESI(+)-MS/MS(MRM) QTRAP Mobile phase: AcN/ formic acid solution (0.2 %) (gradient) Column: ACQUITY UPLC™ BEH C18 (100 x 2.1 mm, 1.7µm)	65.4-101.8	LOD=0.19-1.27 ng g^{-1} MDL= 0.24-1.84 ng g^{-1}	[51]
Milk (10 mL)	BPA, BADGE	Sample+MeOH, 10 mL and sonication (emulsion destabilization) Dilution to 100 mL (water) Extraction: SPE, C18 Clean up: SPE, Forisil Solvent evaporation Reconstitution: EA, 0.3 mL	GC-EI-MS(SIM) Column: HP-5MS (30m x 0.25mm, 0.25µm)	81-119	MDL: 0.15-0.36 ng mL^{-1}	[53]
Milk (10 mL)	BPA, BPB, BPF, BADGE,BFDGE	Sample+MeOH,10 mL and sonication (emulsion destabilization) Dilution to 100 mL (water) Extraction: SPE, Chromabond C18; elution AcN, 5 mL	LC-FD (λ 228 nm) (λex 273nm, λem 300nm) Mobile phase: AcN/water (isocratic) Column: SynergiFusion-RP (250 x 4.6mm; 4µm)	93-106	LOD= 0.3-4.2 ng mL^{-1} LOQ = 1-14 ng mL^{-1}	[119]

Canned peas, tuna, olives, maize, artichokes and palm hearts (10 ml, liquid portion)	BPA, BADGE, BFDGE BADGE·diol, BADGE·2HCl, BFDGE·diol	Sample +0.75 g of sodium chloride Extraction: SPME (carbowax, 65 µm) Desorption: 150 µL mobile phase or in the desorption chamber of SPME-LC interface	LC-FD (λ 228 nm) (λex 275nm, λem 305nm) Mobile phase: AcN/water (isocratic) Column: XTerra MS C18 (100 x 4.6mm; 5µm)	7–65	LOD= 0.7–2.4 ng mL^{-1} LOQ= 2.5–7.2 ng mL^{-1}	[120]
Canned vegetables, sauces, fish, others (2-5 g, total content)	BPA BADGE, BADGE·H_2O, BADGE·$2H_2O$, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl	Extraction: AcN - Clean up: SPE, Oasis HLB Solvent evaporation Reconstitution: AcN: water (40:60), 1 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: AcN/water (gradient) Column: Acquity BEH C18 (50 x 2.1mm; 1.7µm) LC-ESI(+)-MS/MS(MRM) Mobile phase: MeOH/ammonium acetate (5 mM) (gradient) Column: Acquity BEH C18 (50 x 2.1mm; 1.7µm)	93(BPA) 69 - 103	LOD= 0.3 (BPA) ng g^{-1} MQL= 0.39 – 0.69 ng g^{-1}	[31]
Canned fish, meat, fruits and vegetables (5 g)	BPA, BADGE, BADGE·$2H_2O$, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl	Extraction: AcN, 40 mL Clean up: n-hexane, 75 mL; n-hexane washing with AcN, 30+20 mL; solvent evaporation; reconstitution MeOH: water (5:95), 3 mL; SPE, Oasis HLB; elution MeOH 2x2mL, MeOH: EA (50:50) 2 mL, EA 2 mL Solvent evaporation Reconstitution: AcN: water (90:10), 1 mL	LC-FD (λ_{ex} 235nm, λ_{em} 317nm) Mobile phase: AcN/water (gradient) Column: Nucleosil-100 C18 (250 x 4mm; 5µm)	86.07-114.06	MDL= 4.5-7.9 ng g^{-1} MQL= 13.7-24.1 ng g^{-1}	[33]
Canned fish and meat (1.25 g, total content)	BPA, BADGE, BADGE·$2H_2O$, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·$2H_2O$, BFDGE·2HCl	Sample + 10 g anhydrous sodium sulphate Extraction: DCM 2x30 mL; solvent evaporation; reconstitution DCM:CH (50:50), 25 mL Clean up: (2 mL) Gel permeation chromatography(Bio-Beads S-X3); elution DCM:CH (50:50) Eluate evaporation Reconstitution: AcN, 0.5 mL	LC-FD (λex 233nm, λem 310nm) Mobile phase: AcN/water (gradient) Column: LiChrospher250-4 (250 x 4 mm; 5µm)	75-92	MDL= 3 ng g^{-1} MQL= 10 ng g^{-1}	[35]

Canned vegetables, fruits, fish, grains and meat (0.2 g)	BPA, BPB, BPE, BPF, BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl, BADGE·HCl·H_2O, BADGE·2HCl, BFDGE, BFDGE·2HCl	Extraction: 0.6 mL SUPRAS (tetradecanoic acid, 40% THF, v/v) stirring. Centrifugation	LC-FD (λex 276nm, λem 303nm) Mobile phase: acetonitrile/water (gradient) Column: Ultrabase C18 (250 x 4.6mm; 5µm)	80-110	MDL= 0.9-3.5 ng g^{-1} MQL= 0.3-1.1 ng g^{-1}	[60]
			Beverages			
Canned cola, energetic drinks, carbonated drink and no carbonated flavor drink (10 mL)	BPA, BPB	Sample at pH≥10 with potassium carbonate, 5 % Extraction: DLLME (extractive solvent: T4CE, 30 µL; dispersive solvent: AcN, 440 µL)	Heart cutting-GC-EI-MS(SIM) Derivatization agent: AA Column 1: DB-5HT (5m x 0.32mm, 0.1µm) Column 2: DB-5MS (15m x 0.25mm, 0.25µm)	82-111	MDL=$2\cdot10^{-5}$ –$5\cdot10^{-5}$ ng mL^{-1} MQL=$7\cdot10^{-3}$ – $1\cdot10^{-2}$ ng mL^{-1}	[38]
Canned soft drinks (50 mL)	BPA, BPF	Degasification Derivatization Extraction: n-heptane, 5 mL	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	61-128	MDL= 2-10 ng mL^{-1}	[34]
Canned soda, tonic water, beer, sports drink, tea and cola (2 mL)	BPA, BPF, BPZ	Degasification Centrifugation Dilution to 10 mL with water+ derivatization agent Extraction: SBSE (PDMS), 3 h Desorption: unit of thermal desorption connected to the autosampler	GC-EI-MS(SIM) Derivatization agent: AA Column: HP-5MS (30m x 0.25mm, 0.25µm)	86-122	LOD=$0.9\cdot10^{-3}$ – $2.5\cdot10^{-3}$ ng mL^{-1} LOQ=$3.2\cdot10^{-3}$ - $8.4\cdot10^{-3}$ ng mL^{-1}	[36]
Canned soda, tonic water, beer, sports drink, tea, cola and beer (1 mL)	BPA, BPF, BPE, BPB,BPS	Extraction: SPE on-line, C18; loading solvent MeOH:water (5:95)	LC-ESI(-)-MS/MS(SRM) Mobile phase: MeOH/water (gradient) Column: : Fused Core™Ascentis Express C18 (50 x 2.1mm; 2.7µm)	93-98	MDL=$5\cdot10^{-5}$ –$5\cdot10^{-2}$ ng mL^{-1} MQL=$1.5\cdot10^{-2}$ – 0.167 ng mL^{-1}	[25]
Juice, liquor, coffee drinks, bottled water, soft drink, fruit juice, beer and wine	BPA, BPB, BPF BPAF, BPAP, BPP, BPS, BPZ	Extraction: EA, 2x6 mL; solvent evaporation; Reconstitution DCM:hexane (10:90), 2 mL Clean up: SPE, Strata NH2; elution MeOH:acetone (80:20), 5 mL Solvent evaporation	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Betasil C18 (100 x 2.1 mm; 5µm)	78-122	MQL= 0.01-0.05 ng g^{-1}	[24] [15]

(5g)		Reconstitution: MeOH, 0.5 mL				
Canned soda, beer, cola, tea and tonic water (3 mL)	BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE, BFDGE·2H_2O, BFDGE·2HCl	Degasification by sonication Extraction: SPE, Oasis HLB; elution MeOH, 4 mL Solvent evaporation Reconstitution: MeOH:water (50:50), 1 mL	LC-ESI(+)-QQQ(SRM) Mobile phase: MeOH/ 25mM formic acid-ammonium formate buffer (pH 3.75) (gradient) Column: Fused Core™Ascentis Express C18 (50 x 2.1mm; 2.7µm)	70-95	MQL= 0.13-1.6 ng mL^{-1}	[112]
			Drinking water			
Tap water (100 mL)	BPA, BPF, BPB,BPS	Extraction: SPE (C18); elution AcN, 5 mL Solvent evaporation Reconstitution: MeOH (70%), 1 mL	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/ water (gradient) Column: ACQUITY BEH C8 (50 x 2.1 mm, 1.7µm)	89.2-106.9	MDL=0.25-1 ng mL^{-1}	[122]
			Personal care products			
Cosmetic and personal hygiene products (0.12 g)	BPA, BPF, BPZ	Sample+water, 10 mL+NaCl, 1 g and sonication Extraction: SBSE (EG-silicone), 3 h at 900 rpm Water washing Desorption: unit of thermal desorption connected to the autosampler	GC-EI-MS(SIM) No derivatization step Column: HP-5MS (30m x 0.25mm, 0.25µm)	89-114	MDL=8-8.7 ng g^{-1} MQL=26.7-29.2 ng g^{-1}	[121]
			Paper			
Paper (0.12 g)	BPA, BPS	Air-drying in an oven at 105°C for 24 hours	Py-GC-EI-MS(SIM) Derivatization agent: TMAH Column: DB5 fused silica (30m x 0.25mm, 0.25µm)	n.r.	LOD= $0.5 \cdot 10^3$- $0.6 \cdot 10^3$ ng g^{-1} LOQ=$1.3 \cdot 10^3$-$1.4 \cdot 10^3$ ng g^{-1}	[123]
			Dust			
Houses, offices and laboratories (0.05-0.1 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Extraction: MeOH:water (5:3), 5+3 mL; shaking; centrifugation Solvent evaporation to ~ 4 mL Dilution to 10 mL with formic acid (0.2 %, pH=2.5) Clean up: SPE (Oasis MCX); elution MeOH, 5	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Betasil C18 (100 x 2.1mm)	51 - 137	MQL= 0.5 - 2 ng g^{-1}	[18]

		mL Solvent evaporation to 1 mL				
Houses, offices, cars, air conditioner, and laboratories (0.1 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Extraction: [MeOH:water (5:3), 5 mL; shaking; centrifugation] x 3 times. Solvent evaporation to ~ 4 mL Dilution to 10 mL with formic acid (0.2 %, pH=2.5) Clean up: SPE (Sep-Pack C18); elution MeOH, 4 mL+ THF:MeOH (4:6), 3 mL+ THF, 3 mL Solvent evaporation to 1 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (0.1% ammonium) (gradient) Column: Betasil C18 (100 x 2.1mm)	78.3-104.8	LOD= 0.05- 0.2 ng g^{-1} LOQ=0.5-2 ng g^{-1} MDL=0.07-1 ng g^{-1}	[61]
Houses, offices and laboratories (0.05-0.1 g)	BPA, BADGE, BADGE·H_2O, BADGE·HCl·H_2O, BFDGE·$2H_2O$	Extraction: MeOH:water (5:3), 5+3 mL; shaking; centrifugation Solvent evaporation to ~ 4 mL Dilution to 10 mL with formic acid (0.2 %, pH=2.5) Clean up: SPE (Oasis MCX); elution MeOH, 5 mL Solvent evaporation to 1 mL	LC-ESI(+)-MS/MS(MRM) Mobile phase: MeOH/MeOH:ammonium acetate 2mM 1:9 v/v (gradient) Column: Betasil C18 (100 x 2.1mm)	86-115	MQL= 0.2 ng g^{-1}	[20]
			Environmental			
Lake water (100 mL)	BPA, BPF, BPB,BPS	Extraction: SPE (C18); elution AcN, 5 mL Solvent evaporation Reconstitution: MeOH (70%), 1 mL	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/ water (gradient) Column: ACQUITY BEH C8 (50 x 2.1 mm, 1.7µm)	89.2-106.9	MDL=0.25-1 ng mL^{-1}	[122]
River water (250 mL)	BPA, BPE, BPF, BPM,TCBPA	Filtration Extraction: SPE (MISPE, BPE-MIP) Elution: HAc (5% in MeOH), 4 mL Solvent evaporation Reconstitution: AcN, 0.1 mL	LC-UV-VIS (λ 232 nm) Mobile phase: NH_4Ac (10mM, pH 5.8)/methanol (gradient) Column: Capcell Pack C18 (250 x 4.6 mm; 5µm)	86 - 97	MQL= $2.5 \cdot 10^{-3} - 5 \cdot 10^{-3}$	[129]
River water (1 mL)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Extraction: SPE on-line, Hypersil Golg C18	LC-ESI(-)-MS/MS(SRM) Mobile phase: AcN/MeOH/water (gradient) Column: Fused Core™Ascentis Express C18 (50 x 2.1mm; 2.7µm)	85 - 100	MQL= $5.7 \cdot 10^{-3}$ - 0.18 ng mL^{-1}	[126]
River water	BPA, BPF, BPB,BPS,	pH=3-7 with HCl (1 M)	UPLC-ESI(-)-MS/MS(MRM)	80.6-	MDL=0.02-0.45 ng mL^{-1}	[127]

(300 mL)	BPAF, TCBPA	Extraction: SPE (Oasis HLB); elution ammonia (2% in MeOH), 6 mL Clean up: (extract+1.5 mLwater) SPE (Oasis MAX); elution formic acid (2% in MeOH), 6 mL Solvent evaporation Reconstitution: MeOH:water (50:50), 1 mL	Mobile phase: MeOH/ water (gradient) Column: ACQUITY BEH C18 (100 x 2.1 mm, 1.7µm)	107.3	MQL=0.05-1.49 ng mL^{-1}	
River water (10.8 mL)	BPA, BPF, BADGE, BFDGE	Extraction: SUPRAS (decanoic acid, 60 mg in THF, 1.2 mL)+sample at pH=2; stirring Centrifugation	LC-FD (λex 280nm, λem 306nm) Mobile phase: AcN/water (gradient) Column: Hipersil ODS C18 (150 x 4.6mm; 5µm)	79-95	MDL=0.03-0.035 ng mL^{-1}	[125]
Sewage sludges (0.1-0.2 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Extraction: [MeOH:water (5:3), 5 mL; shaking; centrifugation] x 2 times. Solvent evaporation to ~ 4 mL Dilution to 10 mL with formic acid (0.2 %, pH=2.5) Clean up: SPE (Oasis MCX); elution MeOH, 5 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (1% ammonium hydroxide, v/v) (gradient) Column: Betasil C18 (100 x 2.1mm, 5µm)	78-103	MQL=78-103 ng g^{-1}	[62]
Sewage sludges (1 g)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Dried samples Extraction: PLE (EA), 100°C, 1000 psi , preheating period 2 min, 8 min static time, 3 cycles; final volume 15 mL Solvent evaporation Reconstitution: mobile phase, 0.5 mL Centrifugation	LC-APCI(-)-MS/MS(MRM) Mobile phase: MeOH (ammonia 0.025% v/v)/ 0.025% (v/v) ammoniacal aqueous solution (gradient) Column: Gemini C18 (100 x 2.0mm; 3µm)	97.7-100.6	MDL=0.004-0.008 ng g^{-1} MQL=0.014-0.026 ng g^{-1}	[72]
Sewage sludges (1 g)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Dried samples Extraction: PLE (EA), 100°C, 1000 psi , preheating period 2 min, 8 min static time, 3 cycles; final volume 15 mL Solvent evaporation Reconstitution: mobile phase, 0.5 mL Centrifugation	LC-APCI(-)-MS/MS(MRM) Mobile phase: MeOH (ammonia 0.025% v/v)/ 0.025% (v/v) ammoniacal aqueous solution (gradient) Column: Gemini C18 (100 x 2.0mm; 3µm)	97.7-100.6	MDL=4-8 ng g^{-1} MQL=14-26 ng g^{-1}	[73]
Sewage sludges (1 g)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Dried samples Extraction: EA, 10 mL; stirring; sonication Centrifugation Solvent evaporation	LC-APCI(-)-MS/MS(MRM) Mobile phase: MeOH (ammonia 0.025% v/v)/ 0.025% (v/v) ammoniacal aqueous solution	97.9-103.1	MDL=2-4 ng g^{-1} MQL=8-14 ng g^{-1}	[73]

		Reconstitution: mobile phase, 0.5 mL Centrifugation	(gradient) Column: Gemini C18 (100 x 2.0mm; 3µm)			
Sewage sludges (1 g)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Dried samples Extraction: MAE (EA, 10 mL+water, 0.4 mL); 10 min (10 min for holding) at 90°C; 1000 W Centrifugation Solvent evaporation Reconstitution: mobile phase, 0.5 mL centrifugation	LC-APCI(-)-MS/MS(MRM) Mobile phase: MeOH (ammonia 0.025% v/v)/ 0.025% (v/v) ammoniacal aqueous solution (gradient) Column: Gemini C18 (100 x 2.0mm; 3µm)	97-101.4	MDL=6-9 ng g^{-1} MQL=20-30 ng g^{-1}	[73]
Sediments and sludges (0.5 and 0.2 g)	BPA, BPF, BPB,BPS, BPAF, TCBPA	Dried samples+MeOH:acetone (50:50), 2x10 mL and sonication Centrifugation Solvent evaporation to 1 mL Dilution to 10 mL with water Extraction: SPE (Oasis HLB); elution ammonia (2% in MeOH), 6 mL Clean up: (extract+1.5 mLwater) SPE (Oasis MAX); elution formic acid (2% in MeOH), 6 mL Solvent evaporation Reconstitution: MeOH:water (50:50), 1 mL	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH / water (gradient) Column: ACQUITY BEH C18 (100 x 2.1 mm, 1.7µm)	57.1-103.2	MDL=0.02-0.86 ng g^{-1} MQL=0.06-2.83 ng g^{-1}	[127]
Sediments (0.2-0.5 g)	BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ	Extraction: MeOH:water (5:3), 3x5 mL; shaking; centrifugation, extract combination Solvent evaporation to ~4 mL Dilution: formic acid (2%, pH=2.5), 10 mL Clean up: SPE (Oasis MCX); elution MeOH, 5 mL Solvent evaporation to 1 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH /water (gradient) Column: Betasil C18 (100 x 2.1 mm; 5µm)	82 - 138	MQL= 0.25 - 1 ng g^{-1}	[49]
Influent and effluent of sewage treatment plant (400 mL)	BPA, BPF	Sample containing TBAC 5mM Extraction: SPE (hemimicelles of SDS on alumina) Elution: MeOH, 1 mL	LC-FD (λex 278nm, λem 306nm) Mobile phase: AcN/water (gradient) Column: Waters Nova-Pack C18 (150 x 3.9mm; 4µm)	96-106	MDL=0.01-0.015 ng mL^{-1}	[124]
Dringking water treatment plants	BPA, MCBPA, DCBPA, TCBPA	Sample + sodium thiosulphate (20 mg L-1)for the removal of residual chlorine	UPLC-APCI(-)-MS/MS(MRM) Mobile phase: MeOH/water	88-108	MDL= 0.3 $\cdot 10^{-3}$ – 2.3 $\cdot 10^{-3}$ ng mL^{-1}	[128]

(250 mL)		Sample+ MeOH 1.25 mL Extraction: SPE (C18 upti-clean)); elution hexane:DCM (1:1), 2x2 mL+MeOH:acetone:EA (2:2:1), 2x2 mL Solvent evaporation Reconstitution: water:MeOH (50:50), 0.250 mL	(gradient) Column: Supercosil ABZ® (150 x 4.6 mm; 3µm)		MQL= 1 $\cdot 10^{-5}$ – 6.8 $\cdot 10^{-5}$ ng mL^{-1}	
Influent (100 mL) and effluent (300 mL) of sewage treatment plant	BPA, BPF, BPB,BPS, BPAF, TCBPA	pH=3-7 with HCl (1 M) Extraction: SPE (Oasis HLB); elution ammonia (2% in MeOH), 6 mL Clean up: (extract+1.5 mLwater) SPE (Oasis MAX); elution formic acid (2% in MeOH), 6 mL Solvent evaporation Reconstitution: MeOH:water (50:50), 1 mL	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/ water (gradient) Column: ACQUITY BEH C18 (100 x 2.1 mm, 1.7µm)	75.8-114.3	MDL=0.02-0.82 ng mL^{-1} MQL=0.06-1.65 ng mL^{-1}	[127]
Industrial effluent (paper recycling plant) (1 mL)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Extraction: SPE on-line, Hypersil Golg C18	LC-ESI(-)-MS/MS(SRM) Mobile phase: AcN/ MeOH/water (gradient) Column: Fused Core™Ascentis Express C18 (50 x 2.1mm; 2.7µm)	85 - 100	MQL= 5.7 $\cdot 10^{-3}$ - 0.18 ng mL^{-1}	[126]
DWTPs (100 mL)	BPA, MCBPA, DCBPA, TCBPA,TeCBPA	Sample + sodium thiosulphate (20 mg L-1)for the removal of residual chlorine Extraction: SPE (Oasis HLB); elution MTBE:MeOH (1:1), 4 mL Derivatization Reconstitution: AcN, 0.5 mL	UPLC-ESI(+)-MS/MS(MRM) Mobile phase: AcN/water (0.1% formic acid) (gradient) Column: AcquityUPLC BEH C18 (100 x 2.1 mm; 1.7µm)	102-109	LOD= 0.001 – 0.03 ng mL^{-1} MQL= 2 $\cdot 10^{-5}$ - 5 $\cdot 10^{-5}$ ng mL^{-1}	[26]
Influent and effluent of sewage treatment plant (10.8 mL)	BPA, BPF, BADGE, BFDGE	Extraction: SUPRA (decanoic acid, 60 mg in THF, 1.2 mL)+sample at pH=2; stirring Centrifugation	LC-FD (λex 280nm, λem 306nm) Mobile phase: acetonitrile/water (gradient) Column: Hipersil ODS C18 (150 x 4.6mm; 5µm)	78-96	MDL=0.03-0.035 ng mL^{-1}	[125]
			Biological samples			
Urine (5 mL)	BPA, BPB (free and total concentration)	Sample* +K2CO3, 5% to pH>10 DLLME: 1.325 mL AcN (dispersive solvent)+50 µL T4CE (extractive solvent)+0.125 µL derivatization agent; inject into the sample; shaking; centrifugation	Heart cutting-GC-EI-MS(SIM) Derivatization agent: AA Column 1: DB-5HT (5m x 0.32mm, 0.1µm) Column 2: DB-5MS (15m x	71 - 93	MDL= 0.03 - 0.05 ng mL^{-1} MQL= 0.1 ng mL^{-1}	[43]

		*for total concentration, previous deconjugation using β-glucuronidase/sulfatase	0.18mm, 0.18µm)			
Urine (0.1 mL)	BPA, BPF, BPS (free and total concentration)	Sample* to 1 mL with formic acid 0.1 M; stirring; centrifugation Extraction: SPE on-line, LiChrosfer RP-18 ADS; injection volume 0.350 mL *for total concentration, previous deconjugation using β-glucuronidase/sulfatase	LC-APCI(-)-MS/MS Mobile phase: MeOH/10mM ammonium acetate (gradient) Column: Chromolith® C18 (100 x 4.6mm)	91-107	MDL=0.03-1 ng mL^{-1}	[132]
Urine (0.3 mL)	BPA, MCBPA, DCBPA, TCBPA, TeCBPA (free concentration)	Sample+ACN, 0.6 mL; shaking; +ammonium formate 10 M, 0.150 mL; stirring Centrifugation Solvent evaporation of 0.4 mL organic layer Reconstitution: water (0.1 mL)	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Acquity CSH™ C18 (100 x 2.1 mm, 1.7µm)	33-45	MDL= 0.009 – 0.048 ng mL^{-1}	[44]
Urine (5 mL)	BPA, MCBPA, DCBPA, TCBPA, TeCBPA, BPS (free and total concentration)	Sample* to 10 mL with NaCl 10%; pH= 2 with HCl 0.1 M DLLME: 0.5 mL acetone (dispersive solvent)+ 0.750 µL TCM (extractive solvent); inject into the sample; shaking; centrifugation Solvent evaporation of organic layer Reconstitution: MeOH (0.1% ammonia): water (0.1% ammonia) (60:40) (0.1 mL) *for total concentration, previous deconjugation using β-glucuronidase/sulfatase	UPLC-ESI(+,-)-MS/MS(MRM) Mobile phase: MeOH (ammonia 0.1% v/v)/ 0.1% (v/v) ammoniacal aqueous solution (gradient) Column: Acquity UPLC® BEH C18 (50 x 2.1 mm, 1.7µm)	94-106	MDL=0.1-0.6 ng mL^{-1}	[58]
Urine (0.5 mL)	BPA, BPADS, BPADG, MCBPA, DCBPA, TCBPA,	Sample+ EI, 0.05 mL+ammonium acetate buffer (1 M, pH=5),1 mL+formic acid buffer (1 M, pH=1), 0.24 mL+water, 1.21 mL For BPA and BPA chlorides: Extraction: SPE (Oasis HLB); elution MeOH, 5 mL Solvent evaporation to 0.5 mL For BPAG and BPADS Extraction: SPE (Strata NH2+Sep-Pak C18 in series) Elution Strata NH2: NH_4OH (5% in MeOH), 3 mL; pH to 7 with formic acid; concentration to 1 mL (fraction containing BPADS) Elution Sep-Pak C18: MeOH), 5 mL; concentration to 1 mL (fraction containing	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/10mM ammonium acetate (gradient) Column: Betasil C18 (100 x 2.1mm, 3µm)	76-129	LOD= 0.003 – 0.02 ng mL^{-1} LOQ= 0.01 – 0.05 ng mL^{-1}	[45]

		BPAG)				
Urine (0.2 mL)	BADGE, BADGE·H_2O, BADGE·$2H_2O$	Extraction: EA:hexane (1:1), 2 mL; stirring; centrifugation Solvent evaporation Reconstitution: initial mobile phase, 0.2 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH (5mM ammonium acetate)/water (5mM ammonium acetate) (gradient) Column: Luna C18 (50 x 2mm, 3µm)	51 - 114	MQL= 0.05 – 0.2 ng mL^{-1}	[132]
Urine (0.5 mL)	BADGE, BADGE·H_2O, BADGE·HCl·H_2O, BFDGE·$2H_2O$ (free and total concentration)	Sample*+ammonium acetate 1 M, 0.3 mL Extraction: EA, 3x3 mL; centrifugation; combination of extracts Wash: water, 1 mL Solvent evaporation Reconstitution: MeOH (0.5 mL) *for total concentration, previous deconjugation using β-glucuronidase/sulfatase	LC-ESI(+)-MS/MS(MRM) Mobile phase: MeOH/ MeOH:ammonium acetate 2mM 1:9 v/v (gradient) Column: Betasil C18 (100 x 2.1mm)	72 – 107	LOQ= 0.01 – 0.03 ng mL^{-1}	[23]
Urine (0.5 mL)	BADGE, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·$2H_2O$, BADGE·HCl (total concentration)	Deconjugation using β-glucuronidase/sulfatase Extraction: EA, 3x3 mL; centrifugation; combination of extracts Wash: water, 1 mL Solvent evaporation Reconstitution: MeOH (0.5 mL)	LC-ESI()-MS/MS(SRM) Mobile phase: Column: Betasil C18 (100 x 2.1mm; 5µm)	nr	LOQ= 0.5 – 2 ng mL^{-1}	[39]
Urine (0.5 mL)	BADGE, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·$2H_2O$, BADGE·HCl, BADGE·2HCl, BFDGE, BFDGE·2HCl, BFDGE·$2H_2O$, BPA, BPAP, BPAF, BPP, BPS, BPZ (total concentration)	Deconjugation using β-glucuronidase/sulfatase Extraction: EA, 3x3 mL; centrifugation; combination of extracts Wash: water, 1 mL Solvent evaporation Reconstitution: MeOH (0.5 mL)	LC-ESI(+)-MS/MS(MRM) Mobile phase: MeOH/water: MeOH (9:1) (1.5% ammonium acetate) (gradient) Column: Betasil C18 (100 x 2.1mm; 5µm) LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water: MeOH (9:1) (1.5% ammonium acetate) (gradient) Column: Betasil C18 (100 x 2.1mm; 5µm) LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH /water (gradient) Column: Betasil C18 (100 x 2.1mm; 5µm)	nr	MQL= 0.01-2 ng mL^{-1}	[40]

Serum (0.3 mL)	BPA, BPB (free concentration)	Sample+mobile phase, 0.150 mL+ perchloric acid (25%), 0.150 mL(for protein precipitation); stirring Centrifugation	LC-FD (λ_{ex}273nm, λ_{em} 300nm) Mobile phase: AcN/phosphate buffer (pH 6) (isocratic) Column: Onyx Monolithic C18 (100 x 4.6mm)	86 - 88	LOD= 0.15 – 0.18 ng mL^{-1} LOQ= 0.5 – 0.6 ng mL^{-1}	29
Serum (0.5 mL)	BPA, BPADS, BPADG, MCBPA, DCBPA, TCBPA,	Sample+ EI, 0.05 mL+ammonium acetate buffer (1 M, pH=5),1 mL+formic acid buffer (1 M, pH=1), 0.24 mL+water, 1.21 mL Extraction: SPE (Strata NH2+ Oasis MCX in series) Elution Strata NH2: NH4OH (5% in MeOH), 3 mL; pH to 7 with formic acid; concentration to 1 mL (fraction containing BPADS) Elution Oasis MCX: MeOH, 5 mL; concentration to 1 mL (fraction containing BPA,BPAG and BPA chlorides)	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/10mM ammonium acetate (gradient) Column: Betasil C18 (100 x 2.1mm, 3µm)	76 - 129	LOD= 0.003 – 0.02 ng mL^{-1} LOQ= 0.01 – 0.05 ng mL^{-1}	[45]
Plasma (5 mL)	MCBPA, DCBPA, TCBPA, TeCBPA	Dilution to 15 with water Proteins precipitation: $ZnSO_4$ (10%), 1 ml+NaOH (0.1 M), 1 mL, centrifugation, supernatant filtration Acidification: HCl (0.1 M), pH=3-3.5; water to 20 mL Extraction: SPME (PA 85 µm) Desorption: thermal desorption Derivatization in the GC injector port	GC-EI-MS(SIM) Derivatization agent: BSTFA Column: HP1 (30m x 0.25mm, 0.25µm)	94-107.2	MDL=0.5-3 ng mL^{-1} MQL=0.8-5 ng mL^{-1}	[131]
Plasma (venous blood and umbilical cord blood) (0.2 mL)	BADGE, BADGE·H_2O, BADGE·$2H_2O$	Extraction: EA:hexane (1:1), 2 mL; stirring; centrifugation Solvent evaporation Reconstitution: initial mobile phase, 0.2 mL	LC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH (5mM ammonium acetate)/water (5mM ammonium acetate) (gradient) Column: Luna C18 (50 x 2mm, 3µm)	51 - 114	MQL= 0.05 – 0.2 ng mL^{-1}	[132]
Placental tissue (1.5 g)	BPA, MCBPA, DCBPA, TCBPA	Homogenization: sample+water, 1.5 mL; stirring Extraction: EA,3 mL; stirring; centrifugation Solvent evaporation of the organic layer Reconstitution: amonia in MeOH (0.1%), 1 mL (containing the I.E.)+ amonia in water (0.1%), 1 mL; shaking Centrifugation	LC-APCI(-)-MS/MS(MRM) Mobile phase: ammoniacal aqueous (0.1%)/ ammonia in MeOH (0.1%) (gradient) Column: Gemini C18 (100 x 2 mm, 3µm)	97 - 105	MDL= 0.2 – 0.6 ng g^{-1} MQL= 0.5 - 2 ng g^{-1}	[47]

Placental tissue (0.250 g)	BPA, MCBPA, DCBPA, TCBPA, TeCBPA	Lyophilized tissue+silica, 0.75 g; manual grounding Extraction: MSPD (mixture into a polypropylene cartridge containing PSA, 0.75 g as clean up sorbent); extraction MeOH, 12.5 mL Solvent evaporation Reconstitution: MeOH:water (containing ammonia 0.1%) (60:40), 0.1 mL; stirring; centrifugation	UPLC-ESI(+/-)-MS/MS(SRM) Mobile phase: ammoniacal aqueous (0.1%)/ ammonia in MeOH (8.5mM) (gradient) Column: Acquity BEH C18 (50 x 2.1 mm, 1.7µm)	97 - 105	MDL= 0.1 ng g^{-1} MQL= 0.3 – 0.4 ng g^{-1}	[42]
Human breast milk (3 g)	BPA, BPB, BPAP, BPAF, BPBP, BPC, BPC12, BPE, BPPH, BPS, BPF, DHDPE, BPFL, BPZ, BPM, BPP, BP4,4´,BIS2 (total concentration)	Deconjugation using β-glucuronidase/sulfatase Protein precipitation: Acetone, 5+3 mL Evaporation of organic layer Extraction: SPE, CHROMABOND HR-X; elution AcN, 14 mL Clean up: SPE, MIP(MACHEREY BPA); elution MeOH, 10 mL Solvent evaporation Reconstitution AcN, 0.1 mL Derivatization	GC-EI-MS(SRM) Derivatization agent:MSTFA Column: Optima®-17-MS (30m x 0.25mm, 0.25µm)	90-109	MDL= 0.001 – 0.03 ng g^{-1} MQL= 0.003 – 0.1 ng g^{-1}	[41]
Human breast milk (0.5 mL)	BPA, MCBPA, 2,6-DCBPA, 2,2´-DCBPA, TCBPA,	Stirring Extraction: MeOH,4 mL; stirring; sonication; centrifugation Solvent evaporation of supernatant Reconstitution: water:MeOH (70:30), 1 mL Clean up: SPE on-line, Xbridge C8; injection volume 0.050 mL; loading solvent MeOH:water (20:80)	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH /water (gradient) Column: Acquity CSH™ C18 (100 x 2.1 mm, 1.7µm)	81-119	MDL=0.01-0.09 ng mL^{-1} MQL=0.4 ng mL^{-1}	[59]
Adipose tissue (0.2 g)	BPA, MCBPA, DCBPA, TCBPA	Homogenization: sample+n-hexane, 6 mL; Extraction: AcN, 2 mL; shaking Separation of phases Solvent evaporation Reconstitution water, 15 mL Clean up: SPE, AccuBONDII ODS-C18; elution DEE:MeOH:DCM (9:1), 3 mL Solvent evaporation Reconstitution EA, 0.120 mL Derivatization	GC-EI-MS(SIM) Derivatization agent: BSTFA/TMCS (1:1) Column: ZB-5MS Zebron(30m x 0.25mm, 0.25µm)	95–105	LOD= 0.5 - 3 ng g^{-1}	[48]

Colostrum (0.5 mL)	BPA, MCBPA, 2,6-DCBPA, 2,2´-DCBPA, TCBPA	Extraction: MeOH,4 mL; stirring; sonication; centrifugation Solvent evaporation of supernatant Reconstitution: water:MeOH (50:50), 1 mL Clean up: SPE on-line, Xbridge C8; injection volume 0.050 mL; loading solvent MeOH:water (20:80)	UPLC-ESI(-)-MS/MS(MRM) Mobile phase: MeOH/water (gradient) Column: Acquity CSH™ C18 (100 x 2.1 mm, 1.7µm)	80-120	LOD= 0.01 – 0.09 ng mL^{-1} LOQ= 0.4 ng mL^{-1}	[46]

Abbreviations: AA: acetic anhydride; AcN: Acetonitrile; APCI: atmospheric-pressure chemical ionization; BSTFA: bis-(trimethylsilyl)trifluoroacetamide); CH: cyclohexane; DCM: dichloromethane; DEE: diethyl ether; DLLME: dispersive liquid–liquid microextraction; EA: ethylacetate; EI: electron impact; ESI: electronspray ionization; FD: fluorescence detection; GC: gas chromatography; GCB: graphitized carbon black; HAc: acetic acid; HLB: hydrophilic-lipophilic balance; HS-SPME: headspace solid-phase microextraction; LC: liquid chromatography; LOD: instrumental detection limit; LOQ: instrumental quantitation limit; MAE: microwave-assisted extraction; MAX: mixed-mode anion-exchange; MCX: mixed-mode cation-exchange; MDL: method detection limit; MeOH: methanol; MIP: molecularly imprinted polymer; MISPE: molecularly imprinted solid-phase extraction; MQL: method quantitation limit; MRM: multiple reaction monitoring; MS: mass spectrometry; MSTFA: N-methyl-N(trimethylsilyl)-trifluoroacetamide); PA: polyacrylate; PDMS: polydimethylsiloxane; PLE: pressurized liquid extraction; PSA: poly secondary amine; PS-DVB: polystyrene-divinylbenzene; Py: pyrolysis; QTRAP: hybrid triple quadrupole/linear ion trap mass spectrometer; SBSE: stir bar sorptive extraction; SDS: dodecilsulfato sódico; SIM: single ion monitoring; SLB-5MS: poly (5%-diphenyl, 95%-methyl siloxane); SPE: solid-phase extraction; SPME: solid-phase microextraction; SRM: selected reaction monitoring; SWCNT: single wall carbon nanotubes; T4CE: tetrachloro ethylene; TBAC: tetrabutylammonium chloride; TBME: tert-Butylmethylether; TCA: trichloroacetic acid; TCM: Trichloromethane; THF: tetrahydrofuran; TMAH: trimethylammonium hydroxide; TMCS: trimethylchloro silane; TMSH: trimethylsulphonium hydroxide; UPLC: ultra performance liquid chromatography.

Common steps in sample treatment for most of the analytical methods reported for mixtures of bisphenols and derivatives include sample pretreatment, extraction of analytes from the matrix, cleanup of the extracts to remove interferences, and concentration to achieve the desired sensitivity. Analyte separation and quantification has been almost exclusively carried out by LC-MS/MS or GC-MS, in the last case prior derivatization, but LC-fluorescence detection has also found some applications. Below, we summarize the state-of-the-art of the analytical methodologies developed, including strategies for removal of background contamination, sample preparation and separation and detection of mixtures of bisphenols and derivatives.

2.1. Sources and removal of background contamination

Background contamination of bisphenols occurs at ng-μg L^{-1} levels during sample collection, preservation, handling and/or quantitation. Bisphenols are inherently ubiquitous in the lab environment due to the widespread use of polycarbonate plastics and epoxy resins in lab materials and equipment. The random pattern of this contamination makes it difficult to identify specific sources, often compromising the accurate quantification of bisphenols. As a general rule, contact of the sample with material susceptible of leaching bisphenols should be avoided. Otherwise, contamination has to be controlled or eliminated using appropriate laboratory procedures and designated work areas. Procedural blanks should be conducted for each sample batch to account for background contamination

Commonly reported specific sources of contamination for bisphenols include, among others, labware, gloves, plastic and microcentrifuge tubes, SPE cartridges, syringe metal needles (owing to the epoxy-resin based adhesive used to fix the needle to the glass syringe), solvents, reagents, ultra-high-quality water (contamination arises from the purification system), pipes and connections in instruments [26, 112-114].

[112]H. Gallart-Ayala, E. Moyano, M.T. Galceran, Fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives in canned food and beverages, J.Chromatogr. A, 1218 (2011) 1603-1610.

Where possible, replacement of bisphenol-leaching materials (e.g. use of glassware, nitrile gloves, metallic connections, etc) is the most adequate strategy to deal with background contamination. Before use, glassware must be rinsed several times with organic solvents and treated at high temperature (400-500 ºC) during 2-4 hours [27,28,115]. Biological samples (e.g.blood) are susceptible to be contaminated during collection, so it is recommended to use vacuum containers as Vacu-test® [29]. The contamination arising from SPE cartridges (e.g. Oasis HLB) can be effectively removed by their pre-washing with an organic solvent such as methanol [26]. Filtration of the ultra-high-quality water through a hydrophobic membrane (Empore disk) has proved efficient to obtain bisphenol-free water [116].

Organic solvents used as mobile phases in LC represent an important source of contamination of bisphenols. An effective approach for removing or minimizing this contamination is the use of a C18 pre-column between the LC pump and the injection valve. In this way, bisphenols coming from the solvents making up the mobile phase are retained in the precolumn and elute after the respective bisphenols coming from the sample [117].

2.2. Sample treatment

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[117]X. Ye, X. Zhou, R. Hennings, J. Kramer, A. Calafat, Potential external contamination with bisphenol A and other ubiquitous organic environmental chemicals during biomonitoring analysis: an elusive laboratory challenge, Environ. Health Persp.121 (2013) 3, 283-286.

2.2.1. Packed food

Sample treatment is somewhat different for solid and liquid foodstuffs. Solid foodstuffs are usually homogenized and sometimes freeze-dried [15,24]. Canned food containing both liquid and solid portions (e.g. vegetables) can be filtered and treated separately [118] or the whole sample taken for analysis [34]. The amount of food required for analysis is usually in the range 1-20 g for solid samples [e.g. 15, 34], and 10 mL for liquid samples such as bottled milk [54] and the liquid portion of canned vegetables [118]. Many methods have been developed for specific food matrices such as seafood [28], vegetables [36,118], infant formula [34,38], tuna [32], milk [53,119] etc, but various of them are applicable to a range of samples [24,51,60].

Solvent extraction is the most common technique for the isolation of mixtures of bisphenols and diglycidyl ethers from solid foodstuffs (Table 3). Among solvents, acetonitrile is usually preferred for the extraction of bisphenols [24,34,55] while pentane [57], hexane-acetone [50,52] and ethyl acetate [112], in addition to acetonitrile [32], have been proposed for the extraction of diglycidyl ethers. Typically, conventional solvent extraction using stirring or sonication is applied and repeated extractions are usually necessary to ensure the complete isolation of both bisphenols and diglycidyl ethers. Other strategies reported include the application of the QuECHERS method to the extraction of mixtures of bisphenols [27,28], and the use of auxiliary energies, such as microwave [51,52] and pressure [50], in the extraction of diglycidyl ethers. Benefits of using microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) include increased extraction rates and considerable reduction in solvent consumption [50-52]. On the other hand, very low enrichment factors have been achieved for the extraction of mixtures of bisphenols using the QuECHERS approach [27,28]. To overcome this problem, dispersive liquid-liquid microextraction (DLLME), using tetrachloroethylene as extractant and acetonitrile as dispersive solvent,

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[118]P. Viñas, N. Campillo, N. Martínez-Castillo, M. Hernández-Córdoba, Comparison of two derivatization-based methodsfor solid-phase microextraction–gas chromatography–mass spectrometric determination of bisphenol A, bisphenol Sand biphenol migrated from food cans, Anal. Bioanal. Chem. 397 (2010) 115–125.

[119]L. Grumetto, O. Gennari, D. Montesano, R. Ferracane, A. Ritieni, S. Albrizio, F. Barbato, Determination of five bisphenols in commercial milk samples by liquid chromatography coupled to fluorescence detection, J. Food Protec. 76 (2013) 9, 1590–1596.

has been applied to the QuECHERS extracts, after removal of interferences by dispersive solid-phase extraction with C18 and graphitized carbon black.

Supramolecular solvents (SUPRAS), as an alternative to organic solvents, have been also proposed for the extraction of mixtures of bisphenols plus diglycidyl ethers from foodstuffs [60]. SUPRAS have outstanding properties for microextraction of a wide polarity range of compounds, mainly arising from the mixed-mode mechanisms and high number of binding sites they offer to solutes. Because of the restricted access properties of some SUPRAS, isolation of analytes and sample cleanup can be simultaneously performed, this greatly simplifying sample treatment [60].

Although some methods report the direct analysis of solvent extracts using LC and GC separation techniques [60, 32], in most cases additional sample cleanup and concentration steps are required to achieve the desired selectivity and sensitivity. Thus, removal of lipids from the extract is essential for samples of animal origin (e.g. fish, meat) since they can significantly reduce the analytical performance of LC and GC. Lipidic material affects the active surface of the stationary phase in LC and degrades the resolution power of the column. In GC/MS, lipids accumulate in the injection port, column and ion source. Fat removal is mainly made by liquid-liquid extraction with n-heptane [28,34] and n-hexane [37].

Solid phase extraction (SPE) cleanup is the most commonly reported technique to achieve selectivity in the determination of mixtures of both bisphenols and diglycidyl ethers in solid foodstuffs. Gel permeation chromatography [35] and solvent extraction [51] have been also proposed. Non-selective sorbents such as chemically bonded reversed-phase silica, C18 [28,37,50], magnesium silicate, Florisil [37], strata NH2 [24,50], mixed-mode cation exchange OASIS MCX [15], polystyrene-divinylbenzene, PS-DVB [52] and divinylbenzene/N-vinylpyrrolidone copolymer, OASIS HLB [31,33] have been used, singly or sequentially, for SPE cleanup. After removal of interferences, evaporation of eluates, reconstitution with organic solvent and filtration complete sample treatment.

A variety of techniques have been reported for isolation of mixtures of bisphenols and diglycidyl ethers from liquid foodstuffs (e.g. bottled milk and the liquid portion of canned vegetables, Table 3). They include SPE with non-selective sorbents such as C18 [53, 119], solid-phase microextraction, SPME,

on fibers of polyacrylate [118] or carbowax [120], stir bar sorptive extraction, SBSE, on fibers of polydimethyl siloxane [36], DLLME with tetrachloroethylene-acetonitrile [38] and SUPRAS-based microextraction using inverted hexagonal aggregates of octanol [54]. Prior to SPE, milk samples are usually mixed with methanol and sonicated for emulsion destabilization and then diluted with water to reduce viscosity. In this way, a better flow rate is achieved during SPE [53,119]. Sample cleanup is also preferentially carried out using SPE [15,24, 53, 119].

On the whole, except for some methods based on sorbent [36,118,120] or solvent [54,60] microextraction, the overall consumption of solvent, including analyte isolation and cleanup, is high and more green sample treatments should be developed for analysis of foodstuffs. Recoveries for both bisphenols and diglycidyl ethers are in the range 61-126%.

2.2.2. Beverages

Among methods reported for analysis of beverages (including juice, soft, carbonated and alcoholic drinks), those related to the determination of mixtures of bisphenols predominate (Table 3). Both solvent-based extraction (e.g. heptane [34], ethylacetate [15,24], DDLME with tetrachloroethylene-acetonitrile [38]) and sorbent-based extraction (e.g. SPE on C18 [25] or OASIS HLB [112], SBSE on polydimethyl siloxane [36]) have been proposed for analyte isolation. Degasification of carbonated drinks by sonication is required before extraction [112,34,36]. Sorbent-based extraction methods [25,36,112] usually provide eluates clean enough and no further sample treatment is necessary for interference removal. On the contrary, many of the extracts obtained from solvent-based extractions require further cleanup (usually by SPE [15,24]) for accurate determination of bisphenols. Recoveries were in the range 61-128%.

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[120]C. Nerín, M. R. Philo, J. Salafranca, L. Castle, Determination of bisphenol-type contaminants from food packaging materials in aqueous foods by solid-phase microextraction-high-performance liquid chromatography, J. Chromatogr. A, 963 (2002) 375-380.

2.2.3. Other human exposure sources

Insufficient attention has been paid to the development of methods for the determination of mixtures of bisphenols and diglycidyl ethers in human exposure sources other than foodstuffs and beverages (e.g. drinking water, personal care products, paper and dust, Table 3). Minute amounts of samples are used for analysis of personal care products (e.g. 120 mg [121]) and dust (e.g 50-100 mg [18,20]). Sample treatment for tap water is simple; it involves C18-based SPE, solvent evaporation and reconstitution in 70% methanol [122]. Dust is a more complex matrix and it requires extraction with a mixture of methanol:water (5:3, v/v), cleanup on SPE (OASIS MCX) and solvent evaporation [18,20]. Analysis of paper only required its pyrolysis after air-drying in an oven at 105ºC [123]. SBSE on EG-silicone has been proposed for the extraction of BPA, BPF and BPZ from cosmetic and personal care products once the sample is dispersed in sodium chloride- containing water by sonication [121]. General recoveries reported for samples were good except for bisphenols in dust, which were in the range 51-137% [18].

2.2.4. Environmental samples

Analytical methods reported for the determination of bisphenols in samples of environmental interest have included natural waters (e.g. rivers and lakes), residual waters (e.g. domestic and industrial influents and effluents), sediments and sewage sludge. Although some of these methods were intended to determine mixtures of bisphenols [122,124] or bisphenols

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[121]J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba, Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC-MS, J. Pharm. Biomed. Anal. 78-79 (2013) 255-260.

[122]X.M. Shan, D.H. Shen, B.S. Wang, B.B. Lu, F.Y. Huang, Simultaneous Determination of Bisphenols and Alkylphenols in Water by Solid Phase Extraction and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry, Biomed. Environ. Sci., 27 (2014) 471-474.

[123]V. Becerra, J.Odermatt, Detection and quantification of traces of bisphenol A and bisphenol S in paper samples using analytical pyrolysis-GC/MS, Analyst, 137 (2012) 2250–2259.

[124]A. Moral, M.D. Sicilia, S. Rubio, D. Perez-Bendito, Determination of bisphenols in sewage based on supramolecular solid-phase extraction/liquid chromatography/fluorimetry, J. Chromatogr. A, 1100 (2005) 8-14.

plus diglycidyl ethers [125], most of them involved the determination of mixtures of BPA and chlorinated-BPA derivatives (Table 3). Minute amounts of samples were taken for analysis of sediments (0.2-05 g) and sewage sludge (1g). The volume of water samples required for analysis was in the range 100-400 mL except for those methods using on-line SPE (1 mL, [126]) or SUPRAS-based extraction (around 11 mL, [125]).

SPE has been the primary technique for the extraction of mixture of bisphenols in both natural and residual waters. Mixed-mode sorbents such as OASIS HBL [26,127] and hemimicelles/admicelles [124] are particularly valuable for this application, although other sorbents such as C18 [122,126,128], both in on-line [126] and off-line [122,128] modes, and molecular imprinted polymers [129] have been also proposed. The double retention mechanism (e.g. hydrophilic and lipophilic interactions) provided by OASIS HLB permitted the extraction of a mixture of seven bisphenols and chlorinated derivatives (log K_{ow} in the range 1.2-7.2) from river and residual water with overall recoveries in the range 75.8-114.3% [127]. Interferences causing ion suppression in LC-MS/MS were removed by SPE-based sample cleanup on OASIS MAX (a mixed-mode reversed-phase/strong anion-exchange sorbent).

Hemimicelles and admicelles (i.e. surfactant-coated mineral oxides) also offer different microenvironments for solute solubilization [130]. Hemimicelles of dodecyl sulphate adsorbed onto alumina have been proposed

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[125]A. Ballesteros-Gomez, F.J. Ruiz, S. Rubio, D. Perez-Bendito, Determination of bisphenols A and F and their diglycidyl ethers in wastewater and river water by coacervative extraction and liquid chromatography-fluorimetry, Anal. Chim. Acta, 603 (2007) 51-59.

[126]H. Gallart-Ayala, E. Moyano, M.T. Galceran, On-line solid phase extraction fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A and its chlorinated derivatives in water samples, J. Chromatogr. A, 1217 (2010) 3511-3518.

[127]Y. Yang, L. Lu, J. Zhang, Y. Yang, Y. Wu, B. Shao, Simultaneous determination of seven bisphenols in environmental water and solid samples by liquid chromatography-electrospray tandem mass spectrometry, J. Chromatogr. A, 1328 (2014) 26-34.

[128]A. Dupuis, V. Migeot, A. Cariot, M. Albouy-Llaty, B. Legube, S. Rabouan, Quantification of bisphenol A, 353-nonylphenol and their chlorinated derivatives in drinking water treatment plants, Environ. Sci. Pollut. Res. 19 (2012) 4193-4205.

[129]J. Yin, Z. Meng, Y. Zhu, M. Song, H. Wang, Dummy molecularly imprinted polymer for selective screening of trace bisphenols in river water, Anal. Methods, 3 (2011) 173-180.

[130]F.Merino, S.Rubio, M.D.Pérez-Bendito, Evaluation and optimization of an on-line admicelles-based extraction-liquid chromatography approach for thr analysis of ionic organic compounds, 76 (2004) 3878-3886.

for the SPE of BPA and BPF from sewage influents and effluents [124]. Formation of mixed aggregates between dodecyl sulphate and tetrabutylammonium provided a mixed-mode retention mechanism for bisphenols (e.g. dispersion and π-cation interactions), that permitting their quantitative extraction (i.e. recoveries 96-106%) with preconcentration factors of 400 without the need for solvent evaporation.

Similarly, SUPRASs offer different microenvironments for solute solubilization and a SUPRAS made up of hexagonal inverted aggregates of decanoic acid has been proposed for the extraction of BPA, BPF, and their corresponding diglycidyl ethers (BADGE and BFDGE) from wastewater and river water (~11 mL), prior to their determination by LC-FD [125]. The procedure is robust (extractions are no dependent on the ionic strength, temperature or matrix components), simple (treatment of samples only require the extraction of bisphenols for 5 min and no clean-up of extracts or solvent evaporation are necessary) and fast (each complete extraction procedure takes about 15–20 min and several samples can be simultaneously extracted. The method achieves actual concentration factors in the range 87–102 and the recoveries in samples ranged between about 80 and 92%.

Relatively few analytical methods have been reported for the determination of mixtures of bisphenols in sewage sludge [127,72,73] and sediments [48,127]. Mixtures of BPA and BPA-chlorinated derivatives are efficiently extracted with ethylacetate assisted by auxiliary energies such as sonication, microwaves and pressure [72,73]. Recoveries were in the range around 97-107%. On the other hand, extraction of mixtures of bisphenols from sediments and sludge can be carried out with methanol-acetone [127] or methanol-water [49] but recoveries in these cases decrease (e.g. 57-103% [127] and 82-138% [49]), and one-two steps SPE-based sample cleanup, using mixed-mode mechanism sorbents (e.g. OASIS HBL-OASIS MAX [127] and OASIS MCX [49]), is required to achieve selectivity.

2.2.5. Biological samples

Although urine has received the highest attention regarding biomonitoring of mixtures of bisphenols, some analytical methods involving

other biological samples such as serum, plasma, placenta, human breast milk, colostrum and adipose tissue have been also reported (Table 3). Most of these methods involved the determination of mixtures of BPA plus BPA-chlorinated derivatives, and only a few dealt with mixtures of bisphenols or diglycidyl ethers.

In biological samples, bisphenols and derivatives can exist in both the conjugated and the unconjugated form. Both glucuronides and sulphates are the most common conjugates, the first one being the predominant. Usually, both free and total concentration of bisphenols is calculated. So, methods to determine total concentration require an enzymatic deconjugation using a mixture of β-glucuronidase and sulphatase. Even if a study is focused only on free bisphenols, the information on total or conjugated bisphenols is needed for quality control purposes. Additional quality criteria include the information on extraction recovery and the use of surrogate standards to monitor the extent of the deconjugation reaction.

Different strategies have been proposed for the extraction of mixtures of BPA+BPA chlorinated derivatives. They include extraction with an organic solvent such as methanol [42,46,59,], mixtures of hexane-acetonitrile [48] or ethylacetate [47], salting-out assisted liquid-liquid extraction (SALLE) using acetonitrile as an extractant and 10 M ammonium formate as a salting-out reagent [44], DLLE with acetone as a dispersive solvent and trichloromethane as an extractant [58], and SPME on a fiber of polyacrylate, after removal of proteins with zinc sulphate in a basic medium [131]. One advantage of using acetonitrile as the extraction solvent is the simultaneous precipitation of endogenous proteins in the matrix. The use of hexane is particularly useful for lipid removal. Sample cleanup, as required, is carried out by SPE on C18 sorbents [48,59]. On the whole, reported recoveries were good, except for SALLE (33-45%) [44].

The methods reported for the determination of mixtures of BPA free, BPA conjugates (i.e. glucuronide, BPAG, and sulphate, BPADS) and BPA chlorinated derivatives are a bit more complicated [45]. Their determination in urine requires the use of three SPE sorbents; BPA and chlorinated

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[131]M. del Olmo, A. Zafra, B. Suarez, A. Gonzalez-Casado, J. Taoufiki, J.L. Vilchez, Use of solid-phase microextraction followed by on-column silylation for determining chlorinated bisphenol A in human plasma by gas chromatography-mass spectrometry, J. Chromatogr. B, 817 (2005) 167-172.

derivatives are retained on OASIS HLB and eluted with methanol while BPAG and BPADS are subjected to extraction with Strata NH_2 and C18 in series. Elution of BPADS from Strata NH_2 is carried out with ammonia in methanol while BPAG is eluted from C18 with methanol. The determination of the same mixture of bisphenols in serum uses two SPE cartridges in series (i.e. Strata NH_2 and OASIS MCX). Similarly to urine, elution of BPADS from Strata NH2 is carried out with ammonia in methanol while BPA, BPAG and BPA chlorinated derivatives are eluted from OASIS MCX with methanol. Recoveries in both urine and serum were in the range 76-129% [45].

Determination of mixtures of diglycidyl ethers has mainly involved urine [23,39,40,132] and the procedures reported for sample treatment are quite similar (i.e. repetitive extraction with ethylacetate, washing of the extract with water, evaporation and reconstitution in methanol [23,39,40]). Extraction with mixtures of ethylacetate-hexane gave poor recoveries for some diglycidyl ethers (e.g. 51% for BADGE.2H2O) [132].

Scarce attention has been paid so far to the biomonitoring of mixtures of bisphenols [29,41,43,132]. Among the methods reported, it is worth mentioning the one described for the simultaneous determination of 18 bisphenols in human breast milk [41]. After protein precipitation with acetone, the sample is subjected to SPE with CHROMABOND HR-X followed by cleanup with SPE BPA-MIP and solvent evaporation. Recoveries for all bisphenols were in the range 90-109%.

2.3. Separation and detection

LC-MS/MS has become the first choice for separation and quantitation of mixtures of bisphenols, diglycidyl ethers and chlorinated derivatives (Table 3). GC-MS continues as a valuable alternative for determination of mixtures of bisphenols but application to mixtures of diglycidyl ethers and chlorinated derivatives has been occasional. LC-MS/MS is generally preferred to analyze

[132]X. Zhou, J.P. Kramer, A.M. Calafat, Y. Ye, Automated on-line column-switching high performance liquid chromatography isotope dilution tandem mass spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11 other phenols in urine, J. Chromatogr. B, 944 (2014) 152-156.

these compounds, because generic and fast methods for screening purposes can be developed, and the derivatization steps needed for a proper GC-MS analysis can be omitted [133]. A more simple technique, LC-FD, has found interesting applications related to the determination of mixtures of bisphenols and/or diglycidyl ethers in food, environmental and biological samples. Detailed information about chromatographic and detection conditions for determination of mixtures of bisphenols and derivatives is summarized in Table 3.

For both GC-MS and LC-MS methods of analysis, isotope-dilution mass spectrometry, based on stable isotope-labelled (^{2}H or ^{13}C) surrogates as internal standards, is considered as the most specific, selective, accurate and precise detection method for measuring trace levels of bisphenols and derivatives. When labeled standards are not available, matrix effects and recoveries can be corrected using matrix-matched calibration or the standard addition method. However, the standard addition method is not very convenient when a high number of samples must be analyzed.

2.3.1. Liquid chromatography tandem mass spectrometry

LC-MS/MS analysis of bisphenols and derivatives usually involves the use of reversed-phase C18 columns, atmospheric pressure ionization (API) interfaces and triple quadrupole analyzers operating in the multiple reaction monitoring (MRM) mode. Among API interfaces, electrospray ionization (ESI) is usually preferred because it provides better sensitivity for bisphenols and derivatives than atmospheric pressure chemical ionization (APCI). However, APCI has been successfully applied to the determination of chlorinated derivatives in sewage sludge [73,128] and biological samples [47,132].

Regarding bisphenols, their LC-MS/MS analysis is carried out using ESI in the negative mode and methanol-water as the mobile phase. Responses

[133]J.Regueiro, A. Breidbach, T. Wenz, Derivatization of bisphenol A and its analogues with pyridine-3-sulfonyl chloride: multivariate optimization and fragmentation patterns by liquid chromatography/Orbitrap mass spectrometry, Rapid Commun. Mass Spectrom. 29 (2015) 1473-1484.

for bisphenols decrease in the presence of acetonitrile, probably because the lower surface tension of methanol that favors the desolvation of the electrospray droplets [133]. The addition of buffers or additives (e.g. acetic acid, ammonium acetate) to the mobile phase causes ion suppression and should be avoided [126]. Derivatization of bisphenols with pyridine-3-sulfonyl chloride has been proposed to enhance the detection capability of these compounds by ESI-MS/MS [133]. This derivatization reagent gives MS/MS transitions involving analyte-specific product ions rather than reagent-specific product ions. In this way, interferences arising from matrix components are reduced.

The ESI(-) full-scan MS spectrum of bisphenols only show the isotopic cluster corresponding to the deprotonated molecule $[M–H]^-$ [134]. For those bisphenols containing an alkyl chain in the central carbon (BPA, BPE, BPB, Table 1), their MS/MS spectra show the fragment $[M–H–CH3]^-$ for BPA and BPE and $[M–H–C2H5]^-$ for BPB, as a base peak. Additionally, the MS/MS spectra of bisphenols show the product ion resulting from the cleavage of the hydroxyl-benzyl group, $[M–H–C6H6O]^-$ for BPA, BPF, BPE and BPB and $[M–H–C6H5O]^-$ for BPS [25]. Other product ions are due to the cleavage of the hydroxyphenyl-alkyl bond that yields the ion at m/z 93 $[C6H5O]^-$ for BPF and BPB and the ion at m/z 92 $[C6H4O]^-$ for BPS [25].

The behavior of BPA chlorinated derivatives in ESI(-) depends on the chlorination degree [126]. In mobile phases consisting of methanol and water, the corresponding full-scan MS spectrum of MCBPA shows only the isotopic cluster corresponding to the deprotonated molecule $[M–H]^-$, however, the spectra of DCBPA, TCBPA and TeCBPA show a double-charged ion that corresponds to the deprotonation of both hydroxyl groups $[M–2H]^{2-}$. The relative abundance between the mono-charged and double-charged ions increases with the number of chlorine atoms, probably favored by the lower pK_a values of the highly chlorinated derivatives of BPA. Addition of acetonitrile to the mobile phase causes double-charged ion suppression and, although the response for MCBPA decreases, acetonitrile-water [26] and ternary mixtures of water-acetonitrile-methanol [126] have been successfully used for determination of BPA chlorinated derivatives. The two most abundant product ions in the MS/MS spectra of these compounds are [M-H-

[134]H. Gallart-Ayala, E. Moyano, M.T. Galceran, Liquid chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives, Rapid. Commun. Mass Spectrom. 21 (2007) 4039-4048.

$CH3\text{-}HCl]^-$ and $[M\text{-}H\text{-}C2H4OCl]^-$, which have been used as quantifier and qualifier ions for their determination [126,134].

Quantification of BPA chlorinated derivatives by APCI, in negative mode, is better suited in mobile phases containing basic additives (e.g. aqueous ammonia-methanol [47,72,73]) in order to get improved ionization. In APCI(-), even under mild conditions, BPA chlorinated derivatives undergo in-source fragmentation to yield ions in agreement with those obtained in MS/MS. This behavior is more pronounced for the highly halogenated BPAs [133]. Nevertheless, as APCI(-) is selected as the ionization source, mixtures of BPA chlorinated derivatives are commonly fragmented under MS/MS [47,72,73,128].

Bisphenol diglydicyl ethers show a high tendency to form clusters of $[M+Na]^+$, $[M+K]^+$, $[M+NH4]^+$ and $[M+ACN]^+$ ions under electrospray conditions [135]. Some of these clusters (e.g. $[M+Na]^+$) are very stable and do not produce fragmentation in MS/MS, but efficient fragmentation can be obtained from $[M+NH4]^+$ adducts. In order to enable the formation of ammonium adducts and ensure signal reproducibility, formic acid/ammonium formate buffer is generally used as an additive in the mobile phase (usually water-methanol) for analysis of bisphenol diglycidyl ethers in the ESI(+) [20,23,30,31,40,132]. Concentrations above 0.1% of formic acid are necessary to help the ionization process, but concentrations exceeding 0.3% have been known to produce ionization suppression [136]. Responses decrease for mobile phases consisting of acetonitrile-water, probably due to the fact that quasi-moleculars ions clusters between analytes and acetonitrile are not properly formed [30]. The fragmentation of $[M+NH4]^+$ adducts starts with the cleavage of the phenyl-alkyl bond, which is followed by the α-cleavage of the ether group to generate the characteristic product ions at m/z 135, $[C_9H_{11}O]^+$, and m/z 107, $[C_7H_7O]^+$ [137].

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[135]H. Gallart-Ayala, O. Núñez, P. Lucci, Recent advances in LC-MS analysis of food-packaging contaminants, Trends Anal. Chem. 42 (2013) 99-124.

[136]H. Zhang, M. Xue, Y. Zou, Z. Dai, Simultaneous determination of NOGE-related and BADGE-related compounds in canned food by ultra-performance liquid chromatography–tandem mass spectrometry, Anal. Bioanal, Chem. 398 (2010) 3165-3174.

[137]H. Gallart-Ayala, E. Moyano, M.T. Galceran, Multiple-stage mass spectrometry analysis of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their derivatives, Rapid Commun. Mass Spectrom. 24 (2010) 3469-3477.

Ion suppression due to sample-matrix components is a general problem in the analysis of bisphenols and derivatives by LC-(ESI)MS/MS and that requires the use of proper sample cleanup strategies to get accurate results, as it has been discussed in section 2.2. Ion suppression due to buffer additives hinders the sensitive simultaneous determination of bisphenols and their diglycidyl ethers because the ammonium salts required to form the $[M+NH_4]^+$ adducts with diglycidyl ethers reduce drastically the signal for bisphenols [40,134,137,138 ,139].

2.3.2. Gas chromatography-mass spectrometry

GC-MS analysis of bisphenols involves their derivatization by acetylation or silylation, ionization with electron impact (EI) and ion separation in single quadrupole analyzers operating in the single ion monitoring (SIM) mode (Table 3). For years, this approach has been the most popular due to its high capabilities and sensitivity [133].

Derivatization of the free hydroxyl functional groups of bisphenols leads to sharp peaks, thus improving their separation, and enhanced sensitivity and accuracy. Acetylation with acetic anhydride has been the most frequent procedure to obtain bisphenol derivatives for GC-MS [27,28,34,36,38,43,55,56]. Silylation is also very common because the reaction is fast and quantitative and yields thermally stable and highly volatile derivatives [133]. The most popular silylation reagents for this application have been N,O-bis(trimethylsily)trifluoroacetamide, BSTFA [131], N,N-methyl-(trimethylsilyl)trifluoroacetamide, MSTFA [41] and BSTFA containing trimethylchlorosilane, TMCS [48]. The addition of TMCS favors the formation of single derivatives, since the reaction of BSTFA with analytes having different hydroxyl groups, such as bisphenols, can generate several derivatives, that reducing the sensitivity and selectivity of the analysis.

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[138]T. Soeborg, S. H. Hansen, B. Halling-Sorensen, Determination of bisphenol diglycidyl ethers in topical dosage forms, J. Pharm. Biomed. Anal. 40 (2006) 322-330.

[139]S. Chu, G. D.Haffner, R. J. Letcher, Simultaneous determination of tetrabromobisphenol A, tetrachlorobisphenol A, bisphenol A and other halogenated analogues in sediment and sludge by high performance liquid chromatography-electrospray tandem mass spectrometry, J. Chromatogr. A, 1097 (2005) 25-32.

Electron ionization spectra of bisphenols after silylation show as the base peak the loss of a methyl group from the cleavage of the isopropyl group. The molecular ion, with a relatively low intensity, is used for confirmation purposes [133].

Derivatization of deuterated internal standards has been found essential to maintain its isotopic purity during chromatographic separation on a fused-silica capillary column [140] since it degrades owing to the deuteron-proton (^{2}H-H) exchange in the aromatic portion of the molecule. This phenomenon has been attributed to the aromatic electrophilic exchange of deuterium atoms with active hydrogen atoms located on the internal surface of the column.

Linear quadrupoles have been the most used analyzers for GC-MS analysis of bisphenols [27,28,34,36,38,43,55,56]. They feature low cost, compactness and simplicity of operation. The last developments, related to the stability of mass calibration and higher scan-speed and sensitivity, continue to make these analyzers attractive.

2.3.3. Liquid chromatography-fluorescence detection

Both bisphenols and their respective diglycidyl ethers show native fluorescence in the solvents more frequently used in LC mobile phases, namely water, acetonitrile and methanol. LC-FD is well suited for the determination of mixtures of bisphenols and/or diglycidyl ethers in packed food [32,33,35,37,52,57,60,119,120] and environmental samples [124,125].

Reversed-phase LC is always used for the analysis of bisphenols and/or diglycidyl ethers. The composition of the mobile phase has an important effect on the elution order of diglycidyl ethers. Thus, the order of elution of

[140]P. Varely, D. Balafas, Preparation of 4,4 '-(1-[H-2(6)]methylethylidene)bis-[2,3,5,6-H-2(4)]phenol and its application to the measurement of bisphenol A in beverages by stable isotope dilution mass spectrometry, J. Chromatogr. A, 883 (2000) 163-170.

BADGE.H_2O and BADGE.HCl.H_2O changes in methanol compared to acetonitrile due to the different hydrophobicity of both solvents [141,142].

The identification of bisphenols and diglycidyl ethers in the sample is only based on retention times, so the possibility of false positives should be always considered.

3. Assessing human and environmental risk to a cocktail of bisphenols and derivatives

Risk assessment has long been the tool for science-based decision-making and has become an integral part of EU policy development. Common applied approaches for risk assessment of chemical mixtures are based on the combination of chemical target analysis and toxicity assessment, which is carried out by either whole-mixture or component-based methods. In whole-mixture methods, the mixture is considered as a single entity and toxicity is assessed by biotesting. Component-based methods rely on toxicological data and exposure information for individual mixture constituents. The mixture toxicity is then assessed in terms of expectable additive or interactive actions of mixture components [143]. Effect-directed analysis (EDA) and toxicity identification evaluation (TIE), widely used for identifying anthropogenic contaminants causing toxicological effects on environmental samples, are two examples of approaches that combine chemical target analysis and toxicity assessment [144].

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[141]J. Lintschinger, W. Rauter, Simultaneous determination of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their hydrolysis and chlorohydroxy derivatives in canned foods, Eur. Food Res. Technol. 211 (2000) 211-217.

[142]N. Leepipatpiboon, O. Sae-Khow, S. Jayanta, Simultaneous determination of bisphenol-A-diglycidyl ether, bisphenol-F-diglycidyl ether, and their derivatives in oil-in-water and aqueous-based canned foods by high-performance liquid chromatography with fluorescence detection, J. Chromatogr. A, 1073 (2005) 331-339.

[143]A. Kortenkamp, T. Backhaus, M. Faust, State of the Art Report on Mixture Toxicity, 2009, available at http://ec.europa.eu/environment/chemicals/effects/pdf/report_mixture_toxicity.pdf, last accessed on 08/10/2015.

[144]R.M. Burgess, K.T. Ho, W. Brack, M. Lamoree, Effects-directed analysis (EDA) and toxicity identification evaluation (TIE): Complementary but different approaches for diagnosing causes of environmental toxicity, Environ. Toxicol. Chem. 32 (2013) 1935–1945.

Regarding bisphenols and derivatives, risk assessment is difficult at present due to major knowledge gaps related to their occurrence (e.g. where, how often and to what extent humans and the environment are exposed to mixtures of bisphenols and how exposure may change overtime) and mode of action (the molecular basis behind the deleterious effects of bisphenols and derivatives is poorly understood, especially at low doses).

Considering that the harmful effects caused by bisphenols mainly stem from their endocrine disruptors properties, a factor to have in mind is that other non-bisphenols EDCs will be almost always present in the target samples. So, risk assessment to bisphenols and derivatives will be only possible after determining their concentration in the target samples. Then, biotesting of standard solutions of these compounds, at the same composition found in samples, or risk estimation by component-based methods, may be applied.

Because assessment of human and environmental risk to a cocktail of bisphenols and derivatives is at an early stage, below some of the tools available for this assessment are briefly discussed with the aim of fostering research in this field. In addition to the estimation of risk, expected benefits from this research are estimation of safe levels and margins of exposure, ways to prioritize the mixtures of bisphenols and derivatives of major concern and guidelines to decrease the negative impact of plastic industry in both human health and the environment [143].

3.1. Biotesting

Among mechanisms reported to account for the deleterious effects of bisphenols and derivatives on human health and the environment, cellular response through nuclear-associated estrogen receptors (ERs), ERα and ERβ, has been considered the most important. Other nuclear-associated receptors have been also known to be activated (e.g. pregnane X receptor, estrogen-related receptor γ (ERRγ), the peroxisome proliferator activated receptor γ) or inhibited [e.g. androgen receptor (AR), thyroid hormone receptor (TR)] by bisphenol A or its halogenated derivatives [145]. Another important

[145]V. Delfosse, M. Grimaldi, J.L. Pons, A. Boulahtouf, A. le Maire, V. Cavailles, G. Labesse, W. Bourguet, P. Balaguer, Structural and mechanistic insights into bisphenols action provide

mechanism, proven for low BPA concentrations, is the stimulation of cellular responses through membrane-associated estrogen receptors (G protein-coupled ER) [146].

Assays based on receptor signaling are usually defined in terms of receptor activation using recombinant yeast or mammalian systems; cell proliferation in receptor-competent cells; and physiological responses (e.g. proliferation of uterine tissue in rodents and induction of vitellogenin in fish for the estrogen receptor, and the Hershberger assay for the androgen receptor) [75]. Most of these assays have been widely applied to the identification of endocrine disrupters in environmental samples [147,148].

Reporter gene systems based on yeast have been mainly used for the identification of estrogens (yeast estrogen screen, YES) and androgens (yeast androgen screen, YAS). In YES, genetically modified yeast cells (Saccharomyces cerevisiae), which contain the gene for the human estrogen receptor coupled with a so-called reporter gene (LacZ), indicate estrogenic effects. If an estrogenically active substance binds to the estrogen receptor in the cell, the corresponding gene and then the reporter gene are read. The latter gene encodes for an enzyme (beta-galactosidase), which converts chlorophenol red b-D-galactopyranoside and thus induces a color reaction, which is directly correlated to the existence of estrogenically active substances. After 3-day exposure to chemicals or environmental samples, the estrogenicity of the analyzed samples can be measured based on color induction. The same assay principle applies to the detection of anti-estrogenic substances in YES, as well as androgenic and anti-androgenic substances in YAS [149]. As microtiter plate-based test systems they meet the criteria of high-throughput applications and require small amounts of samples [150].

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guidelines for risk assessment and discovery of bisphenol A substitutes, Proc. Natl. Acad. Sci. USA, 109 (2012) 14930-14935.

[146]S. Dong, S. Terasaka, R. Kiyama, Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. Environ. Pollut. 159 (2011) 212-218.

[147]M.G. Weller, A unifying review of bioassays-guided fractionation, effect-directed analysis and related techniques, Sensors 12 (2012) 9181-9209.

[148]W. Brack, H.J.C. Klamer, M. López de Alda, D. Barceló, Effect directed analysis of key toxicants in European river basins. A Review, Env. Sci. Pollut. Res. 14 (2007) 30-38.

[149]M. Bistan, M. Podgorelec, R. M. Logar, T. Tisler, Yeast Estrogen Screen Assay as a Tool for Detecting Estrogenic Activity in Water Bodies, Food Technol. Biotechnol. 50 (2012) 427–433.

[150]J. Rajasarkka, M. Virta, Miniaturization of a Panel of High-Throughput Yeast-Cell-Based Nuclear Receptor Assays in 384- and 1536-Well Microplates, Comb. Chem. High Throughput Screen, 14 (2011) 47-54.

The specific mixture related problems that can be encountered during the application of such assays have been discussed by Frische et al. [151].

Reporter gene systems based on mammalian cells, mainly breast cancer cell lines (e.g. MCF-7), are available, among others, for estrogen, androgen and thyroid hormone receptors [75]. Mammalian reporter-gene assays include the CALUX (Chemically Activated LUciferase gene eXpression) type assays [152]. In these assays, when the EDC binds to the receptor, the receptor complex migrates toward the nucleus of the cell where it binds to a responsive element in the DNA. After transcription and translation of the reporter gene, an enzyme is formed that induces luminescence.

Cell proliferation-based assays have been reported for ER (E-screen), AR (A-screen) and TR (T-screen) [75]. The E-screen assay compares the cell number achieved by similar inocula of MCF-7 human breast cancer cells in the absence of estrogens (negative control) and in the presence of 17β-estradiol (E2, positive control) and a range of concentrations of chemicals suspected to be estrogenic. A-Screen assay is dependent on a suppressive effect of AR on ER signalling which requires co-exposure to estrogen and therefore the assay does not indicate AR activity in isolation. Because these assays are not engineered, the expressed receptor and subtypes depends on the endogenous expression of the cell. This can complicate interpretation of results since the cell may not have been exhaustively characterized for expression of receptor types other than the one of interest, and indeed could potentially alter expression in response to chemical stimuli.

In general, similar responses are observed as different bioassays are applied on the same sample, however notable differences are obtained in some cases. Thus, the E-screen gave higher estrogenicity (~6.51 ng g^{-1} fat) than the YES (<LOQ == 1.08 ng g^{-1} fat) for adipose tissues from fish with ovotestis [153]. Possible reasons may be the different extraction methods

[151]T. Frische, M. Faust, W. Meyer, T. Backhaus; Toxic Masking and Synergistic Modulation of the Estrogenic Activity of Chemical Mixtures in a Yeast Estrogen Screen (YES), Environ Sci Pollut Res 16 (2009)593–603.

[152]H. Witters, A. Freyberger, K.Smits, C. Vangenechten, W. Lofink, M. Weimer, S. Bremer, P.H.J. Ahr, P. Berckmans, The assessment of estrogenic or anti-estrogenic activity of chemicals by the human stably transfected estrogen sensitive MELN cell line: Results of test performance and transferability, Reproductive Toxicol. 30 (2010) 60-72.

[153]A. Kortenkamp, Exploring Novel Endpoints, Exposure, Low-dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animals, 2007, Available on

which did not remove all agents that may inhibit the bioassays or the fact that the E-screen also allows for stimulation of cell proliferation via cell signalling pathways not present in the YES. Anyway, the major difficulty with these simplified models is the extrapolation from effects in a cell culture to potential risks to human health.

Different studies based on in vitro assays have been recently conducted to assess the human and environmental risk to mixtures of bisphenols. Thus, the estrogenic potencies of eight BPs have been evaluated using a bioluminescence YES assay [154]. Although all BPs exhibited estrogenic activity, there were significant differences between the potency of individual chemicals. BPAF had the highest activity, followed by TCBPA, BPF, BPA, BPE and BPS.

The ER-CALUX assay has been applied to the evaluation of the estrogenic activity of drinking water after BPA removal by chlorination [155]. Targeted measurements first evidenced a fast removal of BPA (>99%) by chlorination with sodium hypochlorite (0.8 mg L-1) within 10 min. Twenty one chlorination products of BPA were detected. The estrogenic activity of water samples, evaluated by the ER-CALUX assay, was found to significantly decrease after 10 min of chlorination, thus confirming that chlorination was effective at removing BPA in drinking water and that the generated compounds had significantly lower estrogenic activity.

The E-screen assay has been used to help in the design of non-estrogen active BPA analogs [156]. It was proved that the actions of the environmental hormones BPA analogs stemmed from two chemical properties; (i) 'stereo structure-controlled' and (ii) 'electronic structure-

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http://ec.europa.eu/research/endocrine/pdf/eden_final_report_en.pdf, last accessed on 11/10/2015.

[154]T. Ruan, D. Liang, S. Song, M. Song, H. Wang, G. Jiang, Evaluation of the in vitro estrogenicity of emerging bisphenol analogs and their respective estrogenic contributions in municipal sewage sludge in China, Chemosphere 124 (2015) 150–155.

[155]M. Bourgin, E. Bichon, J.-P. Antignac, F. Monteau, G. Leroy, L. Barritaud, M. Chachignon, V. Ingrand, P. Roche, B. Le Bizec, Chlorination of bisphenol A: Non-targeted screening for the identification of transformation products and assessment of estrogenicity in generated water, Chemosphere 93 (2013) 2814–2822.

[156]S. Kobayashi, H. Shinohara, K. Tabata, N. Yamamoto, A. Miyai, Stereo Structure-Controlled and Electronic Structure-Controlled Estrogen-Like Chemicals to Design and Develop Non-estrogenic Bisphenol A Analogs Based on Chemical Hardness Concept, Chem. Pharm. Bull. 54 (2006) 1633-1638.

controlled' estrogen-like chemical activities. The results obtained constitute a valuable guide for the design and synthesis of BPA analogs without estrogen activity.

The effects of BPA, BPF and BPS on several major endocrine factors involved in testicular functioning have been examined [157]. Results from recombinant yeast cells expressing the AR or glucocorticoid receptor (GR) clearly showed that BPA and BPF were GR and AR antagonists with IC50 values of 67 and 60mM for GR, and 39 and 20mM, for AR, respectively, whereas BPS did not affect receptor activity. In addition, murine MA-10 Leydig cells exposed to BPF and BPS showed altered testicular steroidogenesis. Because of the fetal-like characteristics of MA-10 Leydig cells, the assay covered fetal steroidogenesis, which is highly relevant for studies of masculinization of male fetuses.

In vivo assays are considered to reflect receptor activity relatively directly. In mammals, there are well established in vivo assays for estrogen (rat uterotrophic bioassay) and androgen (Hershberger assay) receptor activity. In fish, the induction of vitellogenin (an egg yolk precursor protein) is controlled by ER, and this response can be used to monitor exposure to estrogenic chemicals in juvenile or male fish. [100]. Using vitellogenin induction in fish, the potential estrogenic effects of several bisphenols on medaka and common carp have been studied [158]. The order of in vivo estrogenic potencies of BPs was as follows: BPC˜BPAF>BPB>BPA⋙BPP. Likewise, in vivo vitellogenin assay on zebrafish embryos/larvae proved that BPAF showed a stronger estrogenic activity than BPA [159].

[157]M.J. Roelofs, M. van den Berg, T.F. Bovee, A.H. Piersma, M.B. van Duursen, Structural bisphenol analogues differentially target steroidogenesis in murine MA-10 Leydig cells as well as the glucocorticoid receptor, Toxicol. 329 (2015) 10–20.

[158]A. Yamaguchi, H. Ishibashi, K. Arizono, N. Tominaga, In vivo and in silico analyses of estrogenic potential of bisphenol analogs in medaka (Oryzias latipes) and common carp (Cyprinus carpio), Ecotoxicol. Environ. Safety 120 (2015) 198–205.

[159]M. Song, D. Liang, Y.Liang, M. Chen, F. Wang, H. Wanga, G. Jiang, Assessing developmental toxicity and estrogenic activity of halogenated bisphenol A on zebrafish (Danio rerio) Chemosphere 112 (2014) 275–281.

3.2. Component-based methods

The available evidence shows that EDs produce combination effects in a dose additive manner [100]. Where deviations from expected additivity occur, the differences between anticipated and observed effects are small. Thus, it is safe to say that for regulatory purposes, the concept of dose addition (DA) is sufficiently accurate for predicting combination effects of groups of EDs with similar effects.

DA is based on the idea that all components in the mixture behave as if they are simple dilutions of one another, which is often taken to mean that the concept describes the joint action of compounds with an identical mechanism of action. When these chemicals interact with an identical, well-defined molecular target, it is thought that one chemical can be replaced totally or in part by an equal fraction of an equi-effective concentration (e.g. an EC50) of another, without changing the overall combined effect.

There are various risk assessment methods for evaluating combined exposures in practice. Without exception, these methods are derived from the DA concept. Perhaps the best known of these are the Hazard Index (HI), the Point of Departure Index (PODI) and the toxic equivalence factor (TEF) [143]. As input values, all these methods require knowledge on the qualitative and quantitative mixture composition as well as effect concentrations of all components, relating to an identical effect level, biological system (bioassay) and endpoint.

The HI sums up ratios of exposure levels and reference doses over chemicals. If HI > 1, the total concentration (or dose) of mixture components exceeds the level considered to be acceptable. The reference doses can be arrived at by utilizing different uncertainty factors for each mixture component. If this is perceived to be a problem, the PODI method can be used. In contrast to the HI, exposure levels of chemicals in a mixture are not expressed in PODI as fractions of individually acceptable levels but as fractions of their respective points of departure (PODs), such as no observed adverse effect level (NOAELs) or Benchmark concentrations or doses (BMD). Extrapolation issues (e.g. animal to human) are then dealt with by using one overall uncertainty factor. In the toxic equivalence factor (TEF), the total toxicity of the mixture is assessed in terms of the toxicity of an equivalent

concentration of an index compound. The total equivalent quantity (TEQ) is then estimated by summation of the concentrations (or doses) of mixture components multiplied by the respective TEF. The application of TEF only holds when the underlying dose-effect relationships are linear. If this pre-condition is violated, TEFs vary with the effect level that is considered for analysis.

The total estradiol equivalent quantities (EEQs) of eight bisphenol (BPA, BPAF, BPAP, BPB, BPF, BPP, BPS, BPZ) in sludge from US [160] and China [154] have been recently reported. Values for EEQs in sludge from US wastewater treatment plants (WWTPs) were in the range 0.26-90.5 pg g^{-1} dw, with BPA accounting for 81% of the total estrogen equivalents, followed by BPAF (13%), BPS (3.5%) and BPF (1.9%). EEQs for bisphenols were 1000 times lower than the estrogenic activity contributed by natural estrogens present in the sewage sludge samples [160]. Similar EEQs were obtained in sludge from China WWTPs (2.16–49.13 pg g^{-1}dw), that accounting for 0.05–1.47% of the total EEQs in sewage sludge samples [154]. These results indicated that BPs made a minor contribution to the total estrogenic activity of the investigated sewage sludge. However, non-genomic mechanisms should be also taken into account in further experiments.

4. Conclusion

Because of their endocrine disruptor properties, low-dose effects and non-monotonic dose-response curves, understanding the effects of the mixtures of bisphenols to which humans and wildlife are exposed is increasingly important. A great deal of information exists about the concentrations of BPA in human biological samples and the environment, but data concerning the levels of multiple bisphenols and derivatives in the same samples are still scarce and more information on typical exposure situations needs to be compiled and systematized. Regarding human biomonitoring, comprehensive measurements of all exposure events during a lifetime,

[160]X. Yu, J. Xue, H. Yao, Q. Wu, A.K. Venkatesan, R.U. Halden, K. Kannan, Occurrence and estrogenic potency of eight bisphenol analogs in sewage sludge from the U.S. EPA targeted national sewage sludge survey, J. Hazard. Mat. 299 (2015) 733-739.

particularly during critical life stages such as fetal development, early childhood and the reproductive years, should be considered for a reliable assessment of human risk to mixtures of bisphenols.

Collecting such huge volume of human and environmental exposure data will require dedicated, targeted monitoring strategies and LC-MS/MS seems to be, at present, the most versatile technique for this purpose. Given the breadth and complexity of this task, monitoring methods involving simpler, generalized sample treatments, able to simultaneously quantify all the bisphenols and derivatives of interest in a range of matrices, are still lacking.

Other knowledge gaps hinder, at present, a reliable assessment of human and environmental risk to mixtures of bisphenols. They include the poor understanding of the mechanisms of low dose actions (including epigenetics and non-genomic pathways) and pharmacokinetics for the different components of the mixture. This knowledge would offer the possibility of strictly regulating, or even eliminating those bisphenols that are shown to have the greatest impact on a combination effect. It would also provide information about the feasibility of using certain index chemicals as surrogates for exposure measurements. And, last but not least, regulation on bisphenol-based plastic materials and other consumer products would be made in a more reliable way.

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CAPÍTULO 6

Volatile restricted access supramolecular solvents for generalized sample treatments in multi-matrix multicomponent liquid chromatography tandem mass spectrometry detection

Volatile restricted access supramolecular solvents for generalized sample treatments in multi-matrix multicomponent liquid chromatography tandem mass spectrometry detection

Noelia Caballero-Casero and Soledad Rubio

Abstract

In recent years there has been an increasing focus on the effects on human health and on the environment arising from exposure to many different chemicals (*cocktail effects),* that demanding for a better understanding of these effects through the use of extensive monitoring. In this work, we report a generalized sample treatment for the determination of twenty one bisphenols and derivatives in the major human exposure sources (canned foodstuffs and beverages, and indoor dust) and the biological fluids used for their biomonitoring (urine and serum). The method was based on the use of a volatile supramolecular solvent made up of inverted aggregates of hexanol that showed restricted access properties. Efficient analyte extraction and multi-matrix interference removal was simultaneously achieved by stirring of the liquid or solid samples with minute volume of SUPRAS (180-200 μL). Quantification was carried out by liquid chromatography, electrospray tandem mass spectrometry. Method validation was carried out according to the recommendations of the European Commission Decision 2002/657/EC. Eight samples, including canned food and beverages, indoor dust, urine and serum, were investigated for matrix effects and recoveries. No signal suppression or enhancement was observed at the selected experimental conditions and absolute recoveries for the analytes in the different samples were in the range 72-114%. Quantitation limits for bisphenols in liquid and solid samples were in the interval 0.019-0.24 μg L^{-1} and 0.12-0.81 μg kg^{-1}. The method was applied to the analysis of the target analytes in fifteen representative samples encompassing a wide range in macromolecules content (e.g. protein, fat, carbohydrates, etc.). Only four bisphenols out of twenty one were not found in the analyzed samples. The analytical and operational

characteristics of this method make it suitable for monitoring programs intended for the assessment of human exposure to a cocktail of bisphenols.

INTRODUCTION

It is becoming increasingly evident that, in combination, some chemicals can cause harmful effects in wildlife species, laboratory animals, and humans, at concentrations considered safe for the individual chemicals (cocktail effect) [1]. This is of special concern for mixtures of endocrine disrupter chemicals (EDCs), for which determinants of additivity have been characterized and are now well understood [2-4]. In view of this evidence, the traditional chemical-by-chemical approach to risk assessment is hard to justify, and the ground is prepared to seriously consider group-wise regulation of chemicals [5]. Guidance for conducting cumulative risk assessments has been published by EPA [6], the WHO/IPCS [7] and the Union (EU) Commission [8].

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[1]Kortenkamp, A.; Faust, M.; Scholze, M.; Backhaus, T.; Low-Level Exposure to Multiple Chemicals: Reason for Human Health Concerns? Environ. Health Perspect. 2007, 115, 106-114.

[2]Bergman, Å.; Heindel, J. J.; Jobling, S.; Kidd, K. A.; Zoeller, R. T.; State of the Science of Endocrine Disrupting Chemicals – 2012, United Nations Environment Programme and World Health Organization, 2013, Available in http://www.who.int/ceh/publications/endocrine/en/, last accessed 08/09/2015.

[3]Kortenkamp, A.; Martin, O.; Faust, M.; Evans, R.; McKinlay, R.; Orton, F.; Rosivatz, E.; State of the art assessment of endocrine disrupters, 2012, Available in http://ec.europa.eu/environment/chemicals/endocrine/pdf/sota_edc_final_report.pdf, last accessed 17/09/2015.

[4]Kortenkamp, A. Ten Years of Mixing Cocktails: A Review of Combination Effects of Endocrine-Disrupting Chemicals, Environ. Health Perspect., 2007, 115, 98-105.

[5]Backhaus, T.; Faust, M.; Kortenkamp, A.; Cumulative risk assessment: A European perspective on the State of the Art and the necessary next steps forward, Integrated Environ. Assessment and Management, 2013, 9, 547–548.

[6]USEPA, Supplemental guidance for conducting health risk assessment of chemical mixtures as a supplement to the EPA's guidelines for the health risk assessment of chemical mixtures (USEPA, 1986) (2002).

[7]WHO, World Health Organization. Harmonization Project. DRAFT Document for Public and Peer Review. Risk Assessment of Combined Exposures to Multiple Chemicals: A WHO/IPCS Framework (2009b).

[8]Communication from the Commission to the Council, The combination effects of chemicals. Chemical mixtures. Brussels, 31.5.2012, COM (2012) 252. Available at

Human and environmental exposure to a cocktail of chemicals can be assessed either from estimated daily intakes or biomonitoring. The knowledge about the extent and magnitude of this exposure, which is a highly challenging task, is essential for the development of the respective legislative provisions. Assessment of human exposure to a chemical cocktail always involves multicomponent analysis in a wide variety of sample matrices (multi-matrices). Additional challenges are the wide range of polarity and extremely low concentrations of most of toxic molecules in real samples (parts per billion or even parts per trillion levels), and the interferences from sample matrices (e.g., large molecules and salts). These interferences can greatly suppress the analytical responses of the target small molecules. On the other hand, exposure to most of chemicals is widespread and epidemiological studies or occurrence levels in exposure sources always involve the analysis of a huge number of samples, which demands for methods capable of high-throughput quantification.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) has become a standard technique for multicomponent analysis of chemicals encompassing a wide polarity range. Sample treatment, involving several tedious and time-consuming steps (e.g. repetitive or sequential extractions, sample cleanup, concentration, etc.), continues being the most challenging task in the global analytical process and the main reason for low-throughput analysis. Most of sample treatments are matrix-dependent, far from the needs for simple and rapid methods in the assessment of human exposure to a cocktail of chemicals.

Here we report a method for high-throughput and rapid quantification of mixtures of bisphenols, chlorinated bisphenols and bisphenol diglycidyl ether in human exposure sources and biological fluids, based on sample treatment with volatile restricted access supramolecular solvents (V-SUPRAS-RAM) followed by liquid chromatography-tandem mass spectrometry analysis.

Bisphenol mixtures meet several of the criteria set by the EC to be considered as a mixture of potential concern [8]. Thus, exposure of the human population and the environment to bisphenols is widespread; they are pseudo-persistent; there is potential for adverse effects to the likely exposure levels; as

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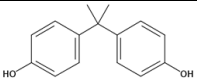
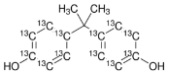
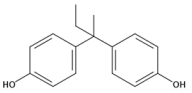
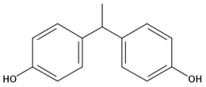
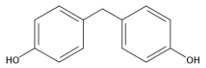
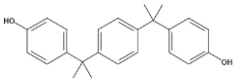
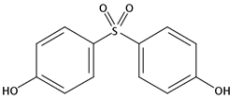
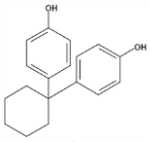
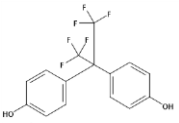
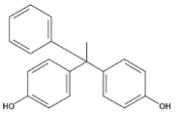
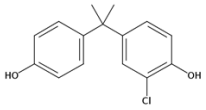
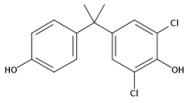
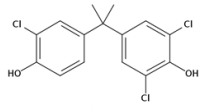
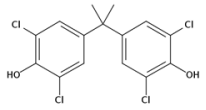
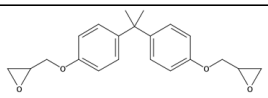
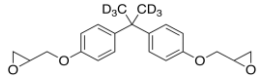
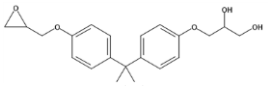
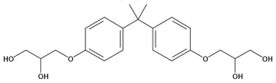
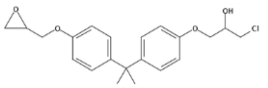
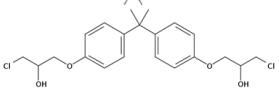
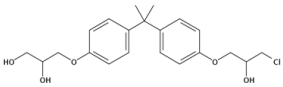
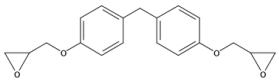
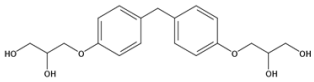
http://www.parliament.bg/pub/ECD/118563COM_2012_252_EN_ACTE_f.pdf, last accessed 30/09/2015.

EDCs, there is scientific base to predict that they will probably act similarly; and, except for BPA, threshold limits for the effects of mixture components have been not established. Table 1 shows the chemical structure of the most common bisphenols and derivatives found in human exposure sources (e.g. food and drink commodities and indoor dust) and biological fluids (serum and urine). They encompass a wide polarity range (e.g. values for log $K_{o/w}$ were in the interval 1.254-6.564).

Table 1.

Chemical structures, molecular weight (MW), octanol-water partition coefficient (log $k_{o/w}$), ionization constants (pKa), precursor, product ions and tandem MS parameters used in the analysis of the bisphenols/chlorinated derivatives and bisphenols diglycidyl ethers.

Compound	Chemical Structure[a]	MW[a]	Log Ko/w[a]	pKa[a]	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)
BPA		228.29	3.641	10.29	227	212.0 133.0	-100 -100	-25 -40	-15 -5
BPA-12C[13]		240.20	-	-	239	224.1 138.8	-95 -95	-26 -40	-5 -5
BPB		242.31	4.150	10.27	241	212.0 93.0	-50 -50	-25 -70	-15 -15
BPE		214.26	3.230	10.10	213	198.0 119.0	-50 -50	-25 -40	-15 -15
BPF		200.23	2.764	9.91	199	93.0 105.0	-50 -50	-25 -25	-15 -15
BPP		346.46	6.564	10.31	345	329.9 133.0	-100 -100	-40 -70	-15 -25
BPS		250.27	2.139	7.64	249	108.0 92.0	-100 -100	-40 -55	-5 -5
BPZ		268.35	4.870	9.91	267	172.9 144.9	-100 -100	-40 -55	-15 -5
BPAF		336.23	3.975	8.74	335	264.9 69.0	-100 -100	-40 -70	-15 -15
BPAP		290.36	4.331	10.22	289	273.9 195.0	-100 -100	-25 -40	-15 -15
MCBPA		262.73	4.335	9.79	261	182.0 245.8	-100 -100	-40 -25	-15 -15
DCBPA		297.18	5.027	8.98	296	244.0 216.1	-100 -100	-24 -28	-5 -5
TCBPA		331.62	5.721	8.93	330	251.8 279.8	-85 -85	-44 -36	-11 -21
TeCBPA		366.07	6.413	8.59	365	313.7 285.6	-85 -85	-36 -44	-17 -17

BADGE		340.41	3.710	-	359	190.9 135.0	52 51	21 43	10 24
BADGE-d6		346.45	-	-	364	197.2 141.2	51 51	19 47	18 8
BADGE·H_2O		358.43	3.185	13.53	376	208.9 191.1	56 56	19 27	12 10
BADGE·$2H_2O$		376.44	2.515	13.23	394	209.0 135.0	61 61	23 45	12 22
BADGE·HCl		376.87	4.025	13.33	394	226.9 135.1	56 56	19 43	12 22
BADGE·2HCl		413.33	4.340	12.83	430	227.1 135.1	56 56	21 49	12 22
BADGE·HCl·H_2O		394.89	3.500	13.13	412	135.0 208.9	61 56	45 19	22 12
BFDGE		312.36	2.449	-	330	163.1 133.0	51 51	19 23	8 22
BFDGE·$2H_2O$		348.39	1.254	13.52	366	181.1 107.1	51 51	21 41	10 18

[a]: *Obtained from Scifinder Scholar. Available from: https://scifinder.cas.org*

Volatile supramolecular solvents with restricted access properties (V-SUPRAS-RAM) have the potential to simplify sample treatment in evaluating the cocktail effect of bisphenols. They are nanostructured solvents made up of inverted hexagonal aggregates of hexanol, obtained spontaneously in mixtures of tetrahydrofuran and water by self-assembly and coacervation processes. V-SUPRAS-RAM have unique properties for the development of generalized, matrix-independent, sample treatment procedures, namely: (A) The inverted hexagonal aggregates have regions of different polarity where solutes spanning wide polarity range can be solubilized; (B) They offer mixed-mode mechanisms for solute solubilization (acceptor-donor hydrogen bond, polar interaction, van der Waals interaction) and multiple sites for binding (the concentration of hexanol in the SUPRAS is in the range 7.4 - 0.15 mg μL^{-1}), that fostering efficient extraction using minute volumes of solvent; (C) The global composition of the solvent, the size of the coacervate droplets that form

it, and the aqueous cavities of the inverted hexagonal arrangement of the hexanol can be tailored by controlling the THF:water ratio in the bulk solution. By controlling the size of the vacuoles, the SUPRAS shows RAM properties (i.e. it has the potential to selectively enrich low-mass compounds from complex samples meanwhile excluding the interferences from large molecules) and this means that it is possible to combine efficient extraction and cleanup in a single step; (D) Hexanol is volatile and its removal after extraction is advantageous for two reasons; to avoid coelution in multicomponent analysis of chemicals encompassing a wide polarity range and to prevent the ionization source from getting dirty.

Below, the most salient results obtained from the application of V-SUPRAS-RAM to the extraction of mixtures of bisphenols and derivatives in human exposure sources and biological fluids are outlined.

EXPERIMENTAL SECTION

Chemicals

All chemicals were of analytical grade and used as supplied: 2,2´-bis(4-hydroxyphenyl)propane (Bisphenol A, BPA); 4,4'-dihydroxydiphenylmethane (bisphenol F, BPF); 2,2´-Bis(4-glycidyloxyphenyl)propane (bisphenol A diglycidyl ether, BADGE); 2-Chloro-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (mono-chlorobisphenol A, MCBPA), 4,4'-(1-Methylethylidene)bis[2-chlorophenol (di-chlorobisphenol A, DCBPA) and 2,6-Dichloro-4-[1-(3-chloro-4-hydroxyphenyl)-1-methylethyl]phenol (tri-chlorobisphenol A, TCBPA) were obtained from Aldrich (St. Louis, USA). Labeled isotopically bisphenol A ($12C^{13}$-BPA) was supplied by Cambridge isotope laboratories (Uk). Labeled isotopically BADGE (BADGE-d6) was supplied by Toronto Research Chemicals (Toronto, Canada). 2-[4-(2,3-Dihydroxypropyloxy)phenyl]-2-[4-(glycidyloxy)phenyl]propane (BADGE·H_2O, ≥95%); 2,2´-Bis[4-(2,3-dihydroxypropoxy)phenyl]propane (BADGE·2H2O, ≥97%); 2-[4-(3-Chloro-2-hydroxypropyloxy)pheny]-2-[4-(glycidyloxy)phenyl]propane (BADGE·HCl, ≥90%); Bis[4-(glycidyloxy)phenyl]methane (BFDGE, ≥95%); 2-[4-(3-Chloro-2-hydroxypropyloxy)phenyl]-2-[4-(2,3-dihydroxypropyloxy)phenyl] propane

(BADGE·H2O·HCL, ≥95%) and 2,2´-Bis[4-(3-chloro-2-hydroxypropoxy)phenyl]propane (BADGE·2HCL, ≥97%) were supplied by Fluka Chemika (Bush, Switzerland) and Bis(4-hydroxyphenyl)sulfone (BPS, 98%); 2,2´-Bis(4-hydroxyphenyl)hexafluoropropane (BPAF, 97%); 1,1´-Bis(4-hydroxyphenyl)-1-phenyl-ethane (BPAP, 99%); 1,4-Bis(2-(4-hydroxyphenyl)-2-propyl)benzene (BPP, 99%); 1,1´-Bis(4-hydroxyphenyl)-cyclohexane (BPZ, 98%) and Bis[4-(2,3-dihydroxypropoxy)phenyl]methane (BFDGE·2H_2O, ≥95%) by Sigma Aldrich (Steinheim, Germany). 2,2´-Bis(4-hydroxyphenyl)butane (BPB, 98%) and 4,4'-Ethylidenebisphenol (BPE, 98%) were obtained from TCI Europe (Zwijndrecht, Belgium) and 4,4'-(1-methylethylidene)bis[2,6-dichloro-Phenol (TeCBPA) from Tokyo chemical industries (Tokyo, Japan). Methanol (100%), 1-hexanol (100%), and tetrahydrofuran (98%) were obtained from VWR-Prolabo (Bois, France), ammonium formate (≥99%) from Sigma Aldrich (St. Louis, USA), formic acid (98%) from Panreac Química, (Barcelona, Spain), and ultra-pure quality water from a milli-Q water purification system (Millipore, Madrid, Spain) and Lichrosolv® water supplied by Merck KGaA (Darmstadt, Germany).

Stock solutions were prepared for individual bisphenols or internal standards in methanol at concentrations between 1–2.5 g L^{-1} and stored at -20 ºC until their use. Intermediate solutions of bisphenols mixtures were prepared in methanol at a concentration of 10 mg L^{-1}. Working solutions were prepared daily by appropriate dilution of the intermediate solutions with methanol:water (50:50, v/v) for bisphenols/chlorinated derivatives and, methanol:ammonium formate/formic acid buffer (12.5 mM, pH 3.75) (50:50 v/v) for bisphenols diglycidyl ethers.

Instrumentation

A liquid chromatograph (Agilent HP 1200 series, Palo Alto, CA, USA) equipped with a binary solvent pumping system and an autosampler was used for separation, which was performed at 35 ºC on a reverse-phase analytical column ACE 3 C18-PFP (150 mm × 3.0 mm, 3.5 µm, ACE, UK). The analytical column was preceded by a C18 Guard Cartridge ACE 3 C18-PFP (3.0 mm × 4.6 mm, 4 µm, ACE, UK). A Symmetry C18 Column (75 mm × 4.6 mm ID, 3.5 µm, Waters) was placed between the LC pump and the injection valve to retain potential bisphenols coming from the solvents used as mobile phases or leached from the plastic components of the LC system. In this way, these

bisphenols elute after the respective ones coming from the sample. Mass spectrometry analysis was accomplished by using a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex 4000QTRAP, Foster City, CA, USA) equipped with a TurboIonSpray (TIS) interface.

A vortex shaker from Reax Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R from MPW Medical Instruments (Warschaw, Poland) were used for the microextraction of bisphenols from both liquid and solid samples. A Basicmagmix magnetic stirrer from Ovan (Barcelona, Spain) and a digitally regulated centrifuge Mixtasel equipped with an angle rotor 4x100 mL from JP-Selecta (Abrera, Spain) were used for production of the SUPRASs.

Determination of bisphenols in multi-matrices

Sample collection. A variety of samples representative for the assessment of human exposure sources to bisphenols were taken for analysis, namely canned food (sweet corn, lentils, chickpeas, sausages and meat balls), beverages (tonic, te, cola, beer and bottled water) and environmental (indoor dust) samples. Biological samples representative for human biomonitoring were also analyzed (i.e. human urine and serum). Canned foods and beverages were purchased in local supermarkets in Córdoba (South Spain) and were stored under dark conditions at 4 °C until analysis. The whole solid content of can food was homogenized using a high-speed Ultra-Turrax for ~5min. Carbonated beverages were degasified with nitrogen. Dust was collected from domestic carpets and furniture with a vacuum-cleaner. Serum samples were obtained in vacuum containers (Vacu-test®) to avoid possible contamination during collection. Samples were taken by laboratories Análisis Clínicos José Cruz Ortega (Córdoba, Spain). Samples were frozen at -20 ºC until analysis. Urine samples were collected from healthy volunteers in Córdoba (Spain) from June to August of 2015. Samples were centrifuged for 20 min at 3000 rpm to eliminate the possible sediments and kept frozen at -20 ºC until analysis. The University of Córdoba obtained the approval for the analysis of human specimens according to the Spanish Regulation R.D. 1716/2011.

Control of background contamination. Sample preparation was performed in a separate room designated exclusively for bisphenol handling. Due to the ubiquitous presence of bisphenols in the environment, laboratory

reagents and glassware, working benches were cleaned with methanol on a daily basis at least twice. The benches were covered with aluminum foil over which clean materials were left to dry. Nitrile gloves were worn at all times and were kept out of contact with equipment or material outside the sample preparation room. Reagents used in the preparation of solutions and SUPRAS were checked for the presence of bisphenols migrating from the environment or as components of such materials and reagents. Glassware for preparing solutions or storage and other material to be used in preforming runs in the LC-MS system were washed first twice with soap and tap water, twice with distilled water and twice with methanol and left on clean aluminium foil to dry before use. Methanol was then rinsed through each flask, shaken and afterward 250µL transferred to an insert in a chromatographic vial for analysis to determine the presence of any.

A quick elution programme was developed to elute and detect the presence of any contaminants in the reagents and solvents or washings from apparatus and material. Whenever a signal considerably higher than noise was observed, the material was re-subjected to the cleaning protocol and afterwards retested again. The elution programme consisted of a linear gradient from 90% to 98%. The flow rate was 300µL min^{-1}. Further experiments were performed after ascertaining that contamination was completely eliminated or kept at a very low level in materials and reagents. Other materials to be used such as pipette tips and microtubes were also rinsed twice with methanol and left to dry at room temperature on clean aluminium foil before use.

SUPRAS-based extraction/clean-up of bisphenols in liquid samples. A volume (1050 µL) of sample (beverage, urine or serum) was added to a 2 mL-microtube Safe-Lock from Eppendorf Iberia (Madrid, Spain) containing hexanol (75 µL) dissolved in THF (375 µL). The water content present in the liquid samples promoted the self-assembly of hexanol and caused the instantaneous *in-situ* formation of the SUPRAS. The mixture was vortex-shaken at 2500 rpm for 5 min to favor analyte extraction, and then centrifuged at 14160 g for 30 min. Two aliquots of the SUPRAS extract (75 µL each) were transferred to 10 mL-glass centrifuge tubes and evaporated to dryness under a gentle nitrogen stream at 60 ºC and then, the residues were reconstituted in 300 µL of methanol:water (50:50 v/v) for determination of bisphenols/chlorinated derivatives and 300 µL of methanol: ammonium

formate/ formic acid (12.5 mM, pH 3.75) buffer (50:50 v/v) for quantitation of diglycidyl ethers.

SUPRAS-based extraction/clean-up of bisphenols in solid samples. The SUPRAS used for the extraction of solid samples was obtained by mixing hexanol (3 mL), tetrahydrofuran (6 mL) and water (21 mL) in a 50 mL glass centrifuge tube. The SUPRAS formed instantaneously under addition of water and the mixture was centrifuged at 2400 g for 30 min to speed up liquid-liquid phase separation. Then, the SUPRAS was separated from the equilibrium solution with a syringe and stored in a closed glass vial until use. Under these conditions, it was stable at room temperature for at least one month and a SUPRAS volume (~6.2 mL) able to treat 31 samples was obtained.

The SUPRAS (200 μL) and the homogenized solid subsamples (200 mg) were mixed in safe-lock 2 mL microtubes. Wetting of dust samples required the addition of 300 μL of the equilibrium solution obtained in the synthesis of the SUPRAS. Four glass balls (3 mm diameter) were introduced in the microtube in all the cases to improve sample dispersion during extraction, which was performed by means of vortex-shaking at 2500 rpm for 20 min. The mixture was then centrifuged at 14460 g for 15 min to separate the solvent from the solid residue. Two aliquots (75 μL) of the SUPRAS extract were evaporated to dryness and reconstituted according to the procedure above described for liquid samples.

Liquid chromatography-mass spectrometry analysis. Separation and quantification of bisphenol/chlorinated derivatives and diglycidyl ethers were carried out by LC-ESI(±)-MS/MS. The mobile phase consisted of water (A) and methanol (B), and the elution program, identical for separation of both group of compounds, consisted in sequential linear gradients as follows: from 50% to 60% solvent B for 2 min, then from 60% to 80% B for 2 min, next from 80% to 90% B for 18 min and finally, from 90% to 100% B for 1 min. Column equilibration at the initial mobile phase conditions took 5.5 min. The injection volume was 10 μL and the flow rate 0.3 mL min^{-1}.

Mass spectrometry analysis of target analytes was carried out in both positive (ammonium diglycidyl ethers) and negative (bisphenols and their chlorinated derivatives) ESI modes with the following settings: probe vertical y-axis position, 2 mm; probe horizontal y-axis position, 6 mm; curtain gas (N_2), 10 psig; nebulizer gas, 40 psig; turbo gas, 60 psig; temperature of the

turbo gas, 650 ºC; ion spray voltage: ± 4500 V. Collision gas 3.0 x10^{-5} Torr. All data were acquired and processed using the Analyst 1.5.1 Software (Applied Biosystems). Quantitative analyses were carried out using two specific combinations of a precursor-product ion transition for each compound, with a dwell time set up at 100 ms. Compound specific MS/MS parameters for each compound are shown in Table 1.

RESULTS AND DISCUSSION

Liquid chromatography-mass spectrometry behavior of bisphenols and derivatives

LC-MS/MS has become the first choice for separation and quantitation of mixtures of bisphenols, diglycidyl ethers and chlorinated derivatives. The LC-MS/MS analysis of mixtures of bisphenols and chlorinated derivatives is usually carried out using mobile phases consisting of methanol:water, ionization by electrospray in the negative mode (ESI-) and detection by triple quadrupole analyzers. Methanol is preferred over acetonitrile because it provides higher responses for bisphenols [9-11], probably because the lower surface tension of methanol that favors the desolvation of the electrospray droplets [12]. The addition of buffers or additives (e.g. acetic acid, ammonium

[9]Gallart-Ayala, H.; Moyano, E.; Galceran, M.T.; Liquid chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives; Rapid. Commun. Mass Spectrom. 2007, 21, 4039-4048.
[10]Chu, S.; Haffner, G.D.; Letcher, R.J.; Simultaneous determination of tetrabromobisphenol A, tetrachlorobisphenol A, bisphenol A and other halogenated analogues in sediment and sludge by high performance liquid chromatography-electrospray tandem mass spectrometry, J. Chromatogr. A 2005, 1097, 25-32.
[11]Maragou, N.C.; Lampi, E.N.; Thomaidis, N.S.; Koupparis, M.A. J. Chromatogr. A 2006, 1129, 165.
[12]Regueiro, J.; Breidbach, A.; Wenz, T.; Derivatization of bisphenol A and its analogues with pyridine-3-sulfonyl chloride: multivariate optimization and fragmentation patterns by liquid chromatography/Orbitrap mass spectrometry Rapid Commun. Mass Spectrom. 2015, 29, 1473-1484.

acetate) to the mobile phase causes ion suppression and should be avoided [9,10,13].

In order to get the highest possible sensitivity in the LC-MS/MS analysis of bisphenols and chlorinated derivatives, we selected mobile phases consisting of methanol: water. Under the optimized experimental conditions (see Experimental), the ESI(-) full-scan MS spectrum of bisphenols only showed the isotopic cluster corresponding to the deprotonated molecule $[M–H]^-$. For those bisphenols containing an alkyl chain in the central carbon (BPA, BPE, BPB, Table 1), their MS/MS spectra showed the fragments $[M–H–CH_3]^-$ for BPA and BPE and $[M–H–C_2H_5]^-$ for BPB, as a base peak. Additionally, the MS/MS spectra of bisphenols showed the product ion resulting from the cleavage of the hydroxyl-benzyl group, $[M–H–C_6H_6O]^-$ for BPA, BPF, BPE and BPB and $[M–H–C_6H_5O]^-$ for BPS. Other product ions were due to the cleavage of the hydroxyphenyl-alkyl bond that yields the ion at m/z 93 $[C_6H_5O]^-$ for BPF and BPB and the ion at m/z 92 $[C_6H_4O]^-$ for BPS. All these results were in agreement with previously described fragmentation patterns [14].

Regarding BPA chlorinated derivatives, the corresponding full-scan MS spectra using ESI(-) showed only the isotopic cluster corresponding to the deprotonated molecule $[M–H]^-$ (see Table 1), despite it has been described the presence of double-charged ion that corresponds to the deprotonation of both hydroxyl groups $[M–2H]^{2-}$ in methanol:water mobile phases [15]. The two most abundant product ions in the MS/MS spectra of these compounds were $[M\text{-}H\text{-}CH_3\text{-}HCl]^-$ and $[M\text{-}H\text{-}C_2H_4OCl]^-$, which were used as quantifier and qualifier ions for their determination. Figure 1a shows the total ion chromatogram obtained for bisphenols and chlorinated derivatives by LC-MS/MS under the conditions described in the Experimental section.

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[13]Guerra, P.; de la Torre, A.; Martinez, M.A.; Eljarrat, E.; Barcelo, D. Rapid Commun. Mass Spectrom. 2008, 22, 916.

[14]Gallart-Ayala, H.; Moyano, E.; Galceran, M.T.; Analysis of bisphenols in soft-drinks by on-line solid phase extraction fast liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta, 2011, 683, 227-233.

[15]Gallart-Ayala, H.; Moyano, E.; Galceran, M.T.; On-line solid phase extraction fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A and its chlorinated derivatives in water samples, J. Chromatogr. A, 2010, 1217, 3511-3518.

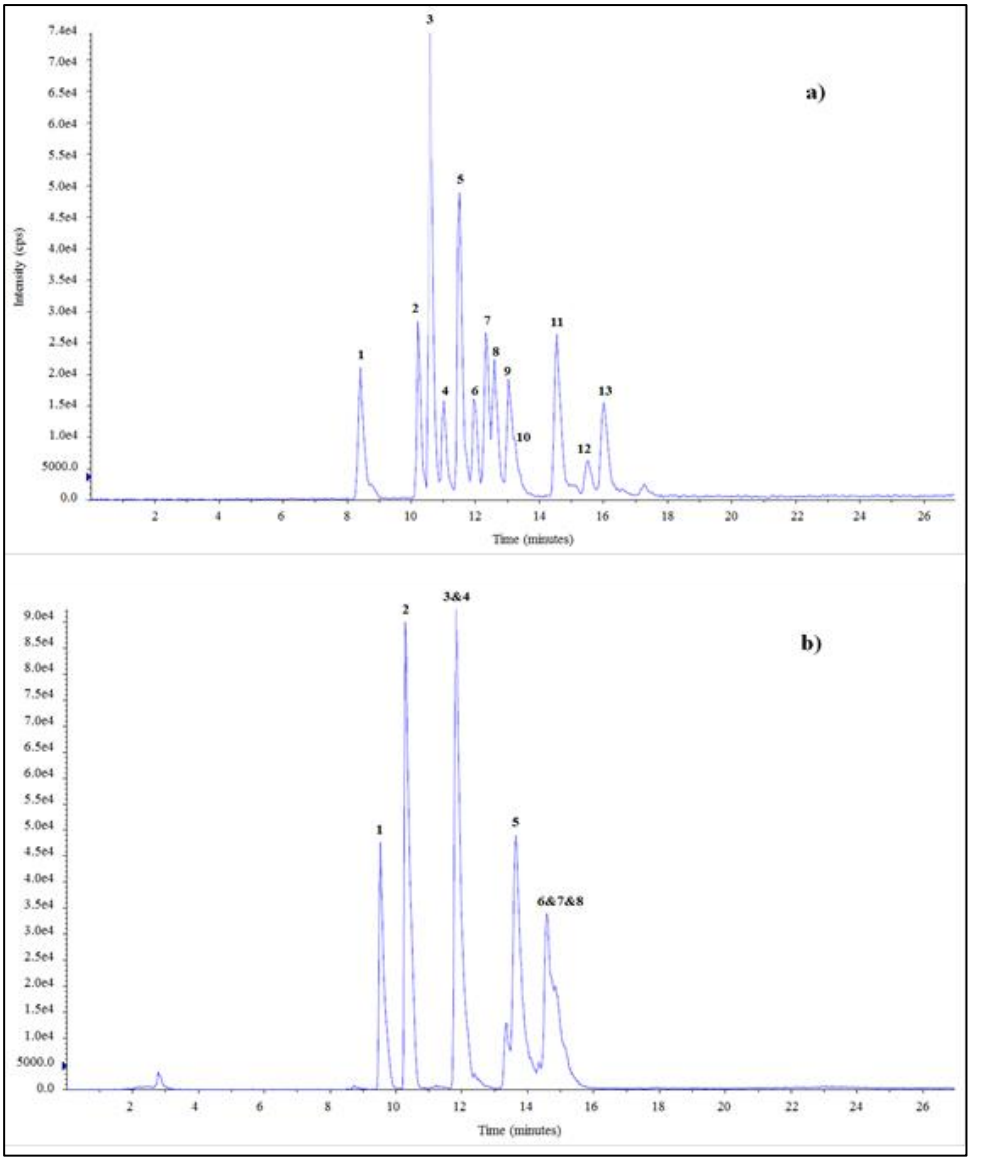

Fig. 1. LC-ESI-MS/MS total ion chromatogram obtained a) from a mixture of bisphenols/chlorinated derivatives standards (20 μgL^{-1}). The compounds named 1;BPS, 2;BPF, 3;BPE, 4;BPA, 5;BPB, 6;MCBPA, 7;BPAP, 8;BPZ, 9;BPAF, 10; DCBPA, 11;TCBPA, 12;BPP, 13;TeCBPA and b) from a mixture of diglycidyl ethers standards (20 μgL^{-1}). The analytes names 1; BFDGE·2H$_2$O, 2; BADGE·2H$_2$O, 3; BADGE·H$_2$O, 4; BADGE·H$_2$O·HCL, 5; BFDGE, 6; BADGE·2HCL, 7; BADGE·HCL, 8; BADGE.

For LC-MS/MS analysis of bisphenol diglydicyl ethers, it is important to take into consideration that they show a high tendency to form clusters of $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$and $[M+ACN]^+$ ions under electrospray conditions [16]. Some of these clusters (e.g. $[M+Na]^+$) are very stable and do not produce fragmentation in MS/MS, but efficient fragmentation can be obtained from $[M+NH_4]^+$ adducts. In order to enable the formation of ammonium adducts and ensure signal reproducibility, formic acid/ammonium formate buffer is generally used as an additive in the mobile phase (usually water-methanol) for analysis of bisphenol diglycidyl ethers in the ESI(+) mode [17- 22]. The fragmentation of $[M+NH_4]+$ adducts starts with the cleavage of the phenyl-alkyl bond, which is followed by the α-cleavage of the ether group to generate the characteristic product ions at m/z 135, $[C_9H_{11}O]^+$, and m/z 107, $[C_7H_7O]_+$ [23].

The need and absence of mobile phase buffer additives required for the ESI-MS analysis of diglycidyl ethers and bisphenols/chlorinated

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[23]Gallart-Ayala, H.; Moyano, E.; Galceran, M.T.; Multiple-stage mass spectrometry analysis of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their derivatives, Rapid Commun. Mass Spectrom. 2010, 24, 3469-3477.

derivatives, respectively, hinders their simultaneous determination. And, what it is worse, sequential analysis is only possible after thorough cleaning of the chromatographic system since the presence of only traces of ammonium salts drastically reduces the response for bisphenols [9,10,21-24]. This cleaning is time consuming and no cost-effective.

In an attempt to solve this problem and speed up the sequential analysis of both groups of bisphenols, we tried to form the $[M+NH_4]^+$ adducts in the sample aliquot intended to determine bisphenol diglycidyl ethers. Chromatographic separation was investigated by applying the same mobile phase and elution program than that selected for bisphenols and chlorinated derivatives. Table 2 shows the response obtained for diglycidyl ethers (1μg L^{-1}each) as a function of the concentration of ammonium formate/formic acid buffer (pH 3.75) added to the sample.

Table 2.

The response of diglycidyl ethers (1 μg L^{-1}) as a function of the concentration of ammonium formate/formica cid buffer (pH 3.75) (%) ± standard deviation.

[24]Soeborg, T.; Hansen, S.H.; Halling-Sorensen, B.; Determination of bisphenol diglycidyl ethers in topical dosage forms, J. Pharm. Biomed. Anal. 2006, 40, 322-330.

Buffer concentration (mM)	BADGE	BADGE·H_2O	BADGE·$2H_2O$	BADGE·HCl	BADGE·2HCl	BADGE·HCl·$2H_2O$	BFDGE	BFDGE·$2H_2O$
2.5	42±9	48±12	37±6	67±4	50±6	40±2	40±2	40±5
5	45±3	49±11	46±3	69±3	59±6	59±5	45±3	46±6
7.5	53±3	54±8	65±4	68±3	64±3	64±5	53±3	50±1
10	89±2	94±1	87.3±0.7	92±7	74±4	89±3	89±6	85±2
12.5	98±3	98±2	95±4	101±3	108 ± 5	99±3	98±4	101±4

Responses are expressed as percentages of those obtained by using the buffer in the mobile phase. They were maximal at a concentration of 12.5 mM and thus, this concentration was selected for further experiments. Figure 1b shows the total ion chromatogram obtained for diglycidyl ethers by LC-MS/MS under the same experimental conditions than those used for bisphenols and chlorinated derivatives. It was checked that sequential analysis of both groups of bisphenols was carried out without the need for any intermediate cleaning step.

Description and properties of volatile hexanol-based SUPRAS-RAM. Hexanol,

Similarly to other alkanols of higher hydrocarbon chain (i.e. C_8 and C_{14} [25]), self-assembles in tetrahydrofuran and coacervates under addition of water. The coacervation occurs on the whole pH range. The density of the new liquid phase, named SUPRAS, is slightly lower than that of the solution in which it formed.

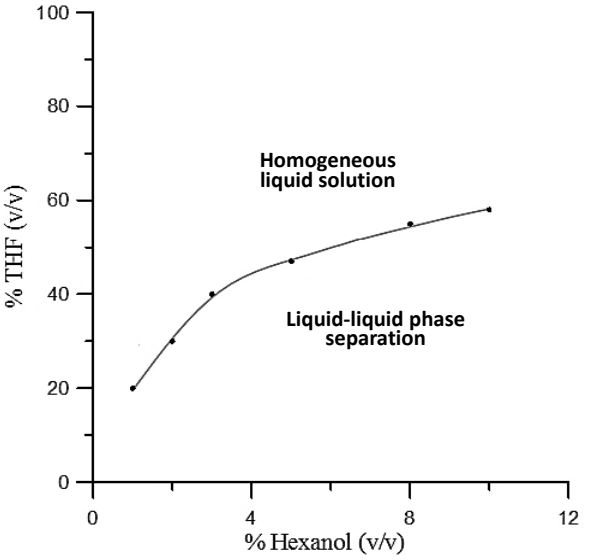

Fig. 2. Phase diagram of hexanol-based SUPRAS

[25]Ballesteros-Gómez, A; Rubio, S.; Environment-responsive alkanol-based supramolecular solvents: characterization and potential as restricted access property and mixed-mode extractants, Anal. Chem., 2012, 84, 342-349.

Figure 2 shows the phase diagram obtained for mixtures of hexanol, water and THF. Only two regions are clearly visible to the naked eye, namely that corresponding to the formation of a homogenous liquid solution and the one for liquid-liquid phase separation. The boundary between them depends on the amphiphile concentration. The region for liquid-liquid phase separation includes both SUPRAS formation and separation of pure hexanol from water at very low percentages of THF (both liquids are immiscible). The boundary for these two liquid phases is not discernible to the naked eye.

The volume of SUPRAS produced is linearly dependent on the percentage of amphiphile in the bulk solution, at least for concentrations below 12% that is the range of analytical interest. This linearity indicates that the composition of the SUPRAS is hexanol-independent provided that the THF/water ratio in the solution remains unchanged. On the other hand, the volume of SUPRAS is exponentially dependent on the percentage of THF for a constant percentage of hexanol and this means that the composition of the SUPRAS changes as a function of the environmental conditions for self-assembly.

The following equation predicts the volume of SUPRAS produced (μL mL^{-1} of solution), as a function of the percentage of hexanol (H, v/v) and THF (v/v) in the bulk solution:

$$V_{SUPRAS}=(10.7\pm0.3).H.e^{(0.0330\pm0.0007)THF}$$

It permits us to estimate the maximum concentration factors that can be achieved under given experimental conditions.

The THF/water ratio for self-assembly of hexanol not only influences SUPRAS composition but also the size of the aqueous cavities in the hexagonal inverted aggregates making up them. So, vacuoles size can be tailored as a function of the self-assembly environment, that opening up the possibility of using these SUPRAS as restricted access materials in the extraction of both liquid and solid samples.

Exclusion of macromolecules by SUPRAS-RAM includes both physical and chemical mechanisms. Thus, proteins are removed by precipitation in the presence of THF while other macromolecules (humic acids, carbohydrates, etc.,) are not extracted into the aqueous cavities in the SUPRAS by size exclusion.

Solubilization of bisphenols and derivatives in SUPRAS-RAM is expected to be based on donor/acceptor hydrogen bonds, polar interactions and dispersion interactions.

Microextraction of bisphenols and derivatives with hexanol-based SUPRAS-RAM

The ability of the hexanol-based SUPRAS-RAM to simultaneously extract the mixture of target bisphenols and derivatives (Table 1) and remove matrix components from samples representative for human exposure and biomonitoring, was evaluated. For this purpose, the following samples were investigated; canned food (lentils, chickpeas, sausages and meat balls), beverages (tonic), environmental (indoor dust) and biological fluids (human urine and serum). Both selectivity and recoveries were studied as a function of the percentage of THF in the solution where hexanol self-assembled. So, SUPRAS of different composition and vacuole size were tested.

First, the solubilization of the target bisphenols from the residues obtained after evaporation to dryness of the SUPRAS extracts was investigated. For this purpose, two aliquots of SUPRAS (75 μL) were fortified with 4μg L^{-1} of each bisphenol and chlorinated derivative, or bisphenol diglycidyl ethers, respectively. Then, the extracts were evaporated to dryness under a nitrogen stream at 60 ºC, reconstituted in solvent mixtures of different composition and the recoveries for each target bisphenol calculated. Quantitative extraction was obtained for bisphenols and chlorinated derivatives in 300 μL of methanol:water (50/50, v/v); recoveries were in the range 97-115%. Similarly, reconstitution of the second aliquot with 300 μL of methanol: ammonium formate/ formic acid buffer, 12.5mM, pH 3.75 (50:50, v/v) was optimal for solubilization and adduct formation of bisphenol diglycidyl ethers; recoveries were in the range 95-110%.

For selectivity and recovery studies, SUPRAS of different composition were synthesized according to the procedure specified in the Experimental section. For liquid samples, the SUPRAS were synthesized *in situ.* The percentage of hexanol was kept constant (8%) while the percentage of THF ranged in the interval 10-40%. The selection of the concentration of hexanol was determined by the minimal volume of SUPRAS required for determination of the target compounds (i.e. two aliquots of SUPRAS extract, 75 μL each, were required to determine mixtures of bisphenols plus chlorinated derivatives and bisphenol diglycidyl ethers, respectively). For solid samples, SUPRAS from 10% of hexanol and percentages of THF in the range 10-40% were prepared in distilled water and a fixed volume of SUPRAS (200 μL) was used for the extraction of the samples (200 mg). Selectivity studies were carried out by measuring signal suppression or enhancement (SSE) from SUPRAS extracts spiked with the target analytes at concentrations of 1 μg L^{-1} each and the appropriate internal standard, 5 μg L^{-1} (i.e. $12C^{13}$-BPA for analysis of bisphenols and chlorinated derivatives and d8-BADGE for bisphenol diglycidyl ethers). Then the SUPRAS extracts were subjected to evaporation and reconstitution as it has been above described. The parameter SSE was calculated by measuring analyte concentration from calibration of standards in methanol:water (50:50). The SSE calculated in this manner may be referred to as an absolute matrix effect; percentages higher than 100 indicate ion enhancement, while percentages lower than 100 are indicative of ion suppression.

Table 3 shows the SSE values obtained for three of the eight analyzed samples (i.e. lentils, tonic and urine) as a function of the percentage of THF and therefore, of the composition and vacuole size of the SUPRAS.

Table 3.

Values of SSE (%) obtained for bisphenols and derivatives in lentils, tonic and urine as a function of the percentage of THF, along with their corresponding standard deviations.

	THF (%)	BPA	BPB	BPE	BPF	BPP	BPS	BPZ	BPAF	BPAP	MCBPA	DCBPA	TCBPA	TeCBPA	BADGE	BADGE ·H_2O	BADGE ·$2H_2O$	BADGE ·HCl	BADGE ·2HCl	BADGE ·HCl·H_2O	BFDGE	BFDGE ·$2H_2O$
Lentils	10	85±9	87±6	79±4	88±6	83±5	88±1	85±4	98±1	89±1	84±1	88±3	80±7	82±8	106±9	100±9	104±8	101±2	102±9	100±1	108±5	105±3
	20	88±5	91±1	85±4	102±3	90±1	108±5	93±4	102±6	86±1	89±1	88±3	82±2	85±3	106±7	102±4	108±7	103±6	109±3	98±4	106±5	108±3
	30	93±1	89±2	89±6	62±6	70±3	103±6	70±5	111±1	71±7	75±9	90±4	105±1	85±1	81±1	98±6	108±9	107±3	106±1	103±6	84.7±0.3	100±2
	40	115±9	88±7	85±7	60±2	65±6	107±8	66±4	100±3	90±7	80±3	89±5	81±1	84±5	88±1	94±8	106±6	102±2	102±2	105±9	87±2	105±6
Tonic	10	101±3	93±6	93±4	112±10	98±7	93±3	101±3	96±7	106±2	105±2	93±7	94±9	88±12	111±2	107±1	100±4	101±1	109±4	102±2	104±4	103±6
	20	96±4	92±3	93±2	81±10	88±7	95±2	88±4	91±3	102±4	98±7	95±5	100±3	83±16	100±3	95±2	93±2	86±4	100±2	95±5	109±8	99±6
	25	97±2	95±5	89±8	104±3	100±6	101±5	106±8	103±7	103±9	109±1	101±6	96±4	94±1	105±2	108±4	86±1	111±2	78±3	107±1	109±5	109±9
	30	109±6	92±4	95±3	95±7	88±8	98±4	104±4	100±6	107±7	111±3	98±4	101±6	92±1	104±1	109±8	88±4	104±8	58±5	95±4	84±8	92±3
	40	108±4	95±6	104±1	103±2	93±7	95±5	107±7	105±1	106±2	95±1	98±3	107±4	83±6	103±6	107±12	101±2	80±5	49±2	98±7	90±10	97±6
Urine	10	102±7	101±9	99±6	114±4	91±6	113±3	111±3	98±1	104±4	98±9	113±5	96±2	95±4	108±2	98±17	83±10	104±8	95±4	85±1	102±2	104±1
	20	89±2	109±3	103±2	94±2	125±9	97±6	100±4	107±4	103±8	110±3	97±3	96±1	103±6	103±2	101±3	88±9	101±5	116±3	88±8	107±8	109±3
	25	100±6	98±1	102±7	100±2	92±2	108±2	100±2	109±3	106±3	107±1	108±6	106±6	113±2	104±4	81±2	96±3	102±2	100±2	90±5	101±7	108±3
	30	87±9	110±4	104±2	92±2	108±4	103±4	102±6	102±3	108±1	103±1	103±3	109±3	104±4	101±8	91±5	95±5	105±7	98±2	98±2	109±3	100±5
	40	97±10	94±6	83±4	99±2	103±8	105±1	102±5	108±7	108±9	103±4	105±5	101±9	100±2	97±4	107±7	96±7	106±9	105±1	100±2	105±4	103±2

The behavior was very similar in all the samples investigated; the signal was within the interval recommended for analysis of contaminants (e.g. 70-120%) or suppression was observed for some analytes at percentages of THF above or equal to 30% (e.g. BPF, BPP and BPZ in lentils). Under the experimental conditions selected (see the Experimental Section), all the analyzed samples gave SSE values in the range 74-112%, proving thus the suitability of the method to effectively remove matrix interferences from a wide variety of samples (table 4).

Table 4.

Values of SSE (%) and standard deviations obtained for bisphenols and derivatives in samples analyzed under the selected experimental conditions.

Sample	BPA	BPB	BPE	BPF	BPP	BPS	BPZ	BPAF	BPAP	MCBPA	DCBPA	TCBPA	TeCBPA	BADGE	BADGE ·H_2O	BADGE ·$2H_2O$	BADGE ·HCl	BADGE ·2HCl	BADGE ·HCl·H_2O	BFDGE	BFDGE ·$2H_2O$
[a]Lentils	88±5	91±1	85±4	102±3	90±1	108±5	93±4	102±6	86±1	89±1	88±3	82±2	85±3	106±10	102±4	108±7	103±6	109±3	98±4	106±5	108±3
[a]Chickpeas	110±3	83±1	82±2	80±7	93±4	94±2	88±3	97±5	75±3	80±2	95±5	98±6	81±1	102±7	97±6	102±4	90±3	95±1	109±4	108±6	109±2
[a]Sausages	100±9	99±4	104±4	116±2	88±7	99±8	100±3	95±7	107±2	107±1	93±5	108±5	89±7	92±3	105±4	95±3	100±3	11±1	100±6	93±2	109±4
[a]Meatballs	96±1	95±2	99±1	116±1	90±1	107±3	98±7	78±4	82±9	91±1	100±3	89±6	74±1	108±1	110±2	101±5	112±3	109±7	97±2	106±5	107±3
[b]Tonic	97±2	95±5	89±8	104±3	100±6	101±5	106±8	103±7	103±9	109±1	101±6	96±4	94±1	105±2	108±4	86±1	111±2	78±3	107±1	109±5	109±9
[a]Dust	110±4	93±1	105±1	85±4	98±1	111±9	100±2	106±3	100±6	106±2	88±4	96±2	90±3	90±2	86±8	109±8	94±8	101±2	117±3	91±5	91±4
[b]Urine	100±6	98±1	102±7	100±2	92±2	108±2	100±2	109±3	106±3	107±1	108±6	106±6	113±2	104±4	81±2	96±3	102±2	100±2	90±5	101±7	108±3
[b]Serum	105±3	86±1	105±3	90±2	85±5	111±1	85±1	96±3	109±1	103±2	102±2	90±3	78±3	96±3	105±3	98±3	106±3	104±2	100±1	109±5	109±6

a: SUPRAS composition: 10% hexanol; 20% THF; 70% Water. b:SUPRAS composition: 5% hexanol; 25% THF; 70% Sample

Absolute recoveries for the target bisphenols were investigated from the analysis of fortified samples subjected to the whole analytical process. Solid and liquid samples were spiked with the whole mixture of analyes to give a final concentration of 4 μg kg^{-1} and 2.5 μg L^{-1} each. The concentrations added of $12C^{13}$-BPA and d6-BADGE to the solid and liquid samples were 20 μg kg^{-1} and 3.5 μg L^{-1}, respectively. Table 5 shows the recoveries obtained, along with the respective standard deviations (n=3), for the different analytes and samples investigated. Recoveries were in the range 72-114% with relative standard deviations in the interval 0.3-10%. So, good extraction efficiency was obtained for all the analytes in the different matrices evaluated.

Table 5.

Recoveries (%) and standard deviation (%) obtained for bisphenols plus chlorinated derivatives and bisphenols diglycidyl ethers in different solid and liquid samples.

Sample	BPA	BPB	BPE	BPF	BPP	BPS	BPZ	BPAF	BPAP	MCBPA	DCBPA	TCBPA	TeCBPA	BADGE	BADGE ·H_2O	BADGE ·$2H_2O$	BADGE ·HCl	BADGE ·2HCl	BADGE ·HCl·H_2O	BFDGE	BFDGE ·$2H_2O$
Lentils[a]	86±4	75.5±0.9	82±4	87±2	82±1	106±5	90±5	107±6	114±4	87±1	101±6	97±3	70±2	76±7	90±3	102±4	99±6	100±6	98±8	77±5	100±4
Chickpeas[a]	107±3	74.7±0.3	89±2	77±4	78±4	95±1	79±3	92±5	83±10	78±2	86±4	78±6	79±3	84±9	80±5	102±2	82±4	92±5	100±5	99±5	103±1
Sausages[a]	87±4	87±3	87±1	112±2	77±7	110±5	84±2	94±6	82±2	80±1	93±8	114±6	79±7	88±2	83±4	99±2	96±4	85±1	85±5	76±2	84±4
Meatballs[a]	97±1	98±2	93±1	94±7	84±3	111±2	76±7	75±8	80±8	85±2	79±5	79±5	88±3	90±6	82±9	87±6	88±2	107±3	105±8	77±6	101±3
Tonic[b]	103±5	102±3	104±6	103±2	111±4	99±8	99±4	103±8	97±5	83±5	90±8	74±8	110±8	75±3	97±8	85±1	73±2	75±8	107±2	109±5	109±7
Dust[a]	182±2	87±5	105±9	101±2	84±2	107±9	90±3	86±3	82±9	81±2	77±3	100±10	89±3	86±7	83±7	97±6	74±8	100±2	86±4	82±4	84±6
Urine[b]	100±1	107±3	101±2	98±9	83±7	105±7	92±9	91±7	108±4	103±7	99±9	105±4	97±5	97±9	108±4	76±4	78±2	79±9	88±5	100±3	88±3
Serum[b]	107±9	93±5	100±1	113±10	87±3	85±4	72±7	80±9	77±8	88±6	76±5	82±8	92±8	104±4	104±4	95±7	107±5	74±1	97±6	88±8	91±2

[a]: SUPRAS composition: 10% hexanol; 20% THF; 70% Water. [b]:SUPRAS composition: 5% hexanol; 25% THF; 70% Sample

Method performance.

In addition to the study of matrix effects and absolute recoveries, above described, the determined method performance parameters included the working range, method detection and quantification limits, repeatability, reproducibility and trueness. These parameters were evaluated following the guidelines of Commission Decision 2002/657/EC [26].

Quantification for all the target analytes was based on calibrations from standards in methanol:water (50/50) for bisphenols plus chlorinated derivatives, and methanol: ammonium formate/ formic acid buffer, 12.5mM, pH 3.75 (50:50, v/v) for bisphenol diglycidyl ethers, by using the internal standard approach. A single internal standard ($12C^{13}$-BPA or d6-BADGE) was used in each chromatographic run, in order to correct for process variability. Calibration graphs for all target analytes were linear over the whole concentration range tested. This linear range was confirmed by visual inspection of the plot residuals versus analyte amount; the residuals were randomly scattered within a horizontal band and a random sequence of positive and negative residuals was obtained. Correlation between peak area ratios and analyte concentrations was determined by linear regression and 1/x weighted calibration. Correlation coefficients were in the range 0.997-0.9999. Table 6 shows the elution times, working range and estimated method quantification limits for both liquid and solid samples. Sensitivity was low enough to determine common concentrations found in real-world samples.

Table 6

Analytical features of the method for determination of bisphenols plus halogenated derivatives and diglycidyl ethers.

[26]Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

Compound	t_R (min)	Linearity (μg L^{-1})	MQL[a] (μg kg^{-1})	MQL[b] (μg L^{-1})
BPA	11.03	0.04-250	0.16	0.035
BPB	11.50	0.02-250	0.12	0.019
BPE	10.50	0.04-250	0.21	0.029
BPF	10.20	0.09-250	0.39	0.091
BPP	15.58	0.04-250	0.18	0.031
BPS	8.79	0.03-250	0.12	0.023
BPZ	12.63	0.03-250	0.14	0.036
BPAF	13.05	0.04-250	0.23	0.035
BPAP	12.34	0.04-250	0.21	0.040
MCBPA	11.98	0.08-250	0.36	0.084
DCBPA	13.24	0.05-250	0.24	0.056
TCBPA	14.56	0.03-250	0.16	0.032
TeCBPA	16.01	0.04-250	0.24	0.041
BADGE	14.78	0.02-250	0.21	0.024
BADGE·H_2O	11.83	0.01-250	0.56	0.049
BADGE·$2H_2O$	10.29	0.09-250	0.48	0.19
BADGE·HCl	14.57	0.13-250	0.54	0.11
BADGE·2HCl	14.37	0.13-250	0.61	0.12
BADGE·HCl·H_2O	11.87	0.1-250	0.52	0.094
BFDGE	13.62	0.15-250	0.81	0.13
BFDGE·$2H_2O$	9.53	0.11-250	0.74	0.17

a: Method quantitation limit for solid samples; b: method quantitation limit for liquid samples.

Precision was studied in terms of repeatability and within-laboratory reproducibility. For this purpose, six aliquots of a urine sample spiked at 0.1, 0.2 and 1 μg L^{-1} for bisphenols plus chlorinated derivatives and 0.3 and 0.6 y 3 μg L^{-1} for bisphendiglycidyl ethers were analyzed in three days (six aliquots each) using freshly prepared mobile phases and standard solutions. The repeatability, expressed as standard deviation, was calculated as the square

root of the average value of the intra-day variances obtained and, the within laboratory reproducibility as the square root of the mean intra-day variance plus the inter-day variance. The relative standard deviations under repeatability conditions were in the ranges 5.0-16.2%, 5.0-18.5% and 1.2-10% for concentrations of bisphenols and chlorinated derivatives of 0.1, 0.2 and 1 μg L^{-1}, respectively, and in the intervals 4.3-14.2%, 0.7-13.1% y 0.5-10% for concentrations of bisphenol diglycidyl ethers of 0.3 and 0.6 y 3 μg L^{-1}, respectively. The reproducibility was in the ranges 6-20%, 6-13.5% and 7-12% for 0.1, 0.2 and 1 μg L^{-1} of bisphenol and chlorinated derivatives, respectively, while it varied in the ranges 4-15%, 5-15% and 3-12% for 0.3 and 0.6 y 3 μg $L^{-1}$of bisphenol diglycidyl ethers, respectively.

Due to the absence of certified reference materials for the target analytes, the trueness of the proposed method was assessed by measuring the recoveries of spiked urine samples at the same concentration level than that used for precision studies. Recoveries were in the ranges 90-106%, 97-107% and 95-110% for concentrations of bisphenols and chlorinated derivatives of 0.1, 0.2 and 1 μg L^{-1}, respectively, and in the intervals 90-111%, 93-107 and 91-105 for concentrations of bisphenol diglycidyl ethers of 0.3 and 0.6 y 3 μg L^{-1}, respectively.

All the values obtained for the method performance parameters investigated were consistent with the criteria established by the 2002//657/EC decision.

Analyses of bisphenols in human exposure sources and biological fluids.

The method here proposed was used to determine bisphenols and derivatives in different canned food commodities, beverages, indoor dust, urine and serum samples. The content of protein (0-23%), carbohydrate (0.5-14%), fat (0.5-20%) and sugars (0-10%) in the selected foodstuffs, expressed as g per 100 g of food, encompassed a wide range. Table 7 shows the results obtained, expressed as the mean value of three determinations (μg L^{-1} or μg kg^{-1}), along with the corresponding standard deviations.

Table 7.

Found concentrations of bisphenols/chlorinated derivatives and bisphenols diglycidyl ethers in canned food, beverages, dust, urine and serum samples.

	BPA	BPB	BPE	BPF	BPP	BPS	BPZ	BPAP	TCBPA	BADGE	BADGE ·H_2O	BADGE ·$2H_2O$	BADGE ·HCl	BADGE ·2HCl	BADGE ·HCl·H_2O	BFDGE	BFDGE ·$2H_2O$
Sweet Corn[a]	2.6±0.4	nd	nd	nd	nd	nd	nd	0.63±0.05	nd	0.32±0.01	nd	nd	nd	nd	nd	nd	nd
Lentils[a]	2.88±0.08	<LQ	nd	nd	0.198±0.001	nd	nd	nd	nd	0.23±0.04	1.4±0.2	84±7	nd	2.8±0.4	61± 6	0.81±0.04	0.9±0.2
Chickpeas[a]	5.1±0.6	nd	nd	nd	nd	nd	nd	nd	nd	0.216±0.004	0.75±0.0.3	36.4±0.5		2.08±0.04	9.4±0.4	nd	nd
Sausages[a]	nd	nd	nd	0.42±0.02	nd	nd	nd	nd	nd	nd	nd	1.12±0.04	nd	nd	nd	nd	nd
Meatballs[a]	3.11±0.01	nd	0.071±0.003	0.60±0.01	nd	nd	nd	nd	nd	nd	nd	45±7	nd	0.77±0.01	24±3	nd	nd
Tonic[b]	1.2±0.1	0.43±0.09	0.81±0.04	1.02±0.03	0.46±0.05	0.88±0.05	1.0±0.1	1.08±0.05	nd	nd	nd	nd	nd	nd	nd	nd	nd
Bottled water[b]	8±1	0.07±0.01	0.052±0.002	nd	0.82±0.04	nd	nd	0.36±0.05	nd	1.12±0.02	nd	nd	nd	nd	nd	nd	nd
Tea[b]	7.2±0.1	0.061±0.003	nd	nd	0.56±0.01	nd	nd	nd	nd	0.87±0.02	nd	nd	3.1±0.2	nd	nd	nd	nd
Cola[b]	6.4±0.1	nd	nd	nd	0.285±0.005	nd	nd	nd	nd	1.051±0.009	nd	nd	nd	nd	nd	nd	nd
Beer[b]	9.1±0.8	nd	nd	nd	0.95±0.04	nd	nd	1.10±0.02	nd	0.09±0.02	nd	nd	0.24±0.02	nd	nd	nd	nd
Dust[b]	5.3±0.5	nd	nd	1.52±0.08	0.18±0.04	0.183±0.002	nd	nd	0.28±0.05	nd	nd	nd	nd	nd	nd	nd	nd
Urine[a]	1.0±0.1	0.32±0.02	1.12±0.04	1.21±0.05	nd	nd	0.82±0.07	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Serum[a]	0.75±0.05	nd	0.60±0.04	1.2±0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

a: Found concentration (μg kg^{-1}); b: Found concentration (μg L^{-1})

Only four bisphenols were not found in the analyzed samples (BPAF, MCBPA, DCBPA and TeCBPA). As it was expected, BPA showed the highest occurrence and some BADGE derivatives the highest concentration. The method proved its suitability to determine the target analytes in a wide variety of human exposure sources and biological fluids.

CONCLUSIONS

The effect of bisphenols and derivatives on human health, especially on vulnerable populations such as infants, young children, pregnant and breastfeeding women, continues as a matter of debate. The cocktail effect of these compounds, an unexplored area so far, as well as their low dose-effects, demands for dedicated efforts to know how often and to what extent humans and the environment are exposed. There is a need to better understand human and environmental exposures, both through the use of monitoring and modeling.

Monitoring of a cocktail of chemicals originating from multiple sources and through multiple pathways is a challenging task and there is a lack of matrix-independent methods able to deliver accurate results through simple and fast procedures. The method here developed has valuable analytical and operational assets for the determination of major bisphenols and derivatives in human exposure sources and biological fluids. Thus, it provides generalized sample treatments for the intended analytes, in both liquid and solid samples based on the ability of the hexanol-based SUPRAS for efficient extraction and matrix macromolecule removal.

From a practical point of view, the sample treatment here proposed features low cost, it is fast, several samples can be simultaneously treated and it requires conventional lab equipment. So, it meets the requirements to be used in monitoring campaigns intended to know the occurrence in the different human exposure sources or epidemiological studies.

ACKNOWLEDGMENTS

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CONCLUSIONES

Las conclusiones obtenidas a partir de las investigaciones recogidas en la Memoria de esta Tesis Doctoral derivan de:

1. El análisis de 28 matrices alimenticias, dos tipos de fluidos biológicos (orina y suero) y dos tipos de muestras ambientales (polvo y musgo). Entre los alimentos analizados se incluyen productos enlatados, bebidas, especias, productos lácteos y derivados, pasas, golosinas, aperitivos, gelatina, etc. El porcentaje de agua (0,06-94), hidratos de carbono (0,06-75), proteínas (0,3-84,4) y grasas (0,08-82) para estos alimentos varía en un amplio intervalo. La orina humana es una matriz acuosa (95% de agua) que contiene niveles elevados de residuos nitrogenados (ej. urea) y sales (NaCl, KCl) que son excretados de forma constante, independientemente del volumen de orina, por lo que la osmolaridad de la misma fluctúa ampliamente. La concentración de proteínas en suero es muy elevada (> 600 mg/mL) y también contiene una elevada concentración de azúcares (> 1 mg/mL). Los hongos pertenecen al reino vegetal, y por tanto sus células poseen una pared celular formada por polisacáridos como la celulosa, pudiendo contener en algunos casos quitina.

2. La determinación de 39 compuestos, incluyendo 1 micotoxina, 3 curcuminoides, 14 PAHs y 21 bisfenoles y derivados. La polaridad de estos compuestos (expresada como log de los coeficientes de partición octanol/agua varía en un amplio intervalo (1,25-6,58).

3. El tratamiento de muestra con tres tipos de SUPRAS-RAM obtenidos a partir de disoluciones coloidales de vesículas y micelas inversas de ácido decanoico y micelas inversas de hexanol.

4. El análisis directo de los extractos utilizando cromatografía de líquidos con tres tipos de detectores; UV-visible, fluorescencia y espectrometría de masas en tándem.

5. El desarrollo y validación de 5 metodologías analíticas, relacionadas con el control de calidad de los alimentos, biomonitorización ambiental y estudios de exposición humana a mezclas de contaminantes.

CONCLUSIONES

1. Los SUPRAS derivados de ácidos alquil carboxílicos y alcanoles tienen la capacidad de extraer eficazmente analitos en un amplio intervalo de polaridad (log P 1,25-6,58) tanto de muestras líquidas como sólidas. Los porcentajes de recuperación obtenidos en nuestros estudios se encuentran en el intervalo 71-114%. Esta capacidad es consecuencia de la elevada concentración de compuesto anfifílico en los SUPRAS investigados (0.14-0.55 mg/μL), los diferentes microambientes de polaridad presentes en los agregados, y los mecanismos mixtos de interacción ofrecidos por el SUPRAS para solubilización de los compuestos (interacciones de van der Waals, puentes de hidrógeno, interacciones polares, y en el caso de vesículas de ácido decanoico, interacciones π-catión).

2. Los SUPRAS-RAM obtenidos a partir de ácido decanoico y hexanol, en medio THF:agua, tienen la capacidad de eliminar proteínas a través de un doble mecanismo; el THF disminuye la constante dieléctrica y el compuesto anfifílico forma un complejo con las mismas que flocula en el medio de extracción. La eliminación de macromoléculas tales como los polisacáridos ocurre a través de efectos de exclusión por tamaño en las cavidades. Las propiedades RAM de los SUPRAS se ha demostrado tanto en disoluciones de macromoléculas puras como en macromoléculas que forman parte de la matriz de las muestras analizadas.

3. La combinación de la elevada eficacia de extracción y propiedades RAM de los SUPRAS ha permitido el desarrollo de 5 metodologías para el análisis de una amplia variedad de muestras líquidas y sólidas que cumplen con los criterios de funcionamiento requeridos, en términos de sensibilidad, selectividad, precisión, etc, para la determinación de los compuestos para los que se han validado. Estas metodologías permiten la determinación de componentes y multicomponentes en muestras complejas y multi-matrices, e incluyen el desarrollo de procedimientos generalizados de tratamiento de muestras en el caso de análisis de multi-matrices.

4. Los SUPRAS-RAM tienen una elevada capacidad para integrar las etapas de extracción de solutos y purificación de muestras y como consecuencia de simplificar el proceso analítico. Los procedimientos de extracción desarrollados para las diferentes muestras analizadas son rápidos (5-20 min) y utilizan bajos volúmenes de disolvente (la relación volumen de SUPRAS (μL)/cantidad de muestra (mg) está comprendida entre 1 y 3). Son por tanto idóneos para reducir los tiempos de análisis y costes en tres áreas; control de calidad de los alimentos, campañas de monitorización ambiental y estudios epidemiológicos.

5. Todos los procedimientos de extracción pueden ser implementados utilizando material comúnmente presente en cualquier laboratorio de análisis químico (agitadores vórtex, centrífugas, etc.) y los SUPRAS se obtienen espontáneamente a través de procedimientos sintéticos muy simples.

6. Todos los SUPRAS investigados son compatibles con cromatografía de líquidos acoplada a detectores UV-visible y fluorescentes.

7. Los SUPRAS volátiles tales como hexanol, son idóneos para aplicaciones en las que se analizan multicomponentes en un amplio intervalo de polaridad mediante cromatografía de líquidos acoplada a espectrometría de masas ya que su eliminación reduce los problemas de coelución cromatográfica con los componentes a determinar y como consecuencia, los problemas de supresión de la señal en la fuente de ionización del espectrómetro de masas.

CONCLUSIONES ESPECÍFICAS

1. SUPRAS-RAM basados en ácidos alquilcarboxílicos para la determinación de componentes/multicomponentes en una matriz compleja

1.1. Determinación de ocratoxina A

1. Se ha desarrollado un método para determinar OTA en pasas, basado en la extracción con un SUPRAS vesicular de mezclas equimoleculares de ácido decanoico y tetrabutil amonio decanoato y el análisis directo del extracto mediante cromatografía de líquidos con detección fluorescente.

2. El método de extracción desarrollado es una alternativa ventajosa a los métodos tradicionales, que requieren el uso combinado de elevados volúmenes de disolvente orgánico para extracción y columnas de inmunoafinidad para purificación de la muestra. La metodología propuesta es más simple, los que permite reducir el tiempo y los costes del análisis.

3. La sensibilidad obtenida para OTA (límite de cuantificación del método: 5,3 $\mu g\ kg^{-1}$) permite su determinación en pasas por debajo de los niveles máximos permitidos por la legislación europea.

1.2. Determinación de 14 PAHs en musgo

1. Los materiales con propiedades de acceso restringido (RAM) tienen gran aplicación en el análisis de fluidos biológicos como plasma, orina o suero; pero no así en matrices biológicas sólidas, dado que se requiere una extracción previa con disolvente orgánico. El elevado rendimiento y selectividad obtenida en la extracción de PAHs en musgos con un SUPRAS-RAM constituido por micelas inversas de

ácido decanoico, amplia el campo de aplicación de los materiales RAM.

2. El análisis directo de los extractos del SUPRAS-RAM mediante cromatografía de líquidos acoplada a un detector de fluorescencia, sin necesidad de incluir una etapa previa de evaporación para incrementar la sensibilidad, reduce considerablemente el tiempo de análisis. El procedimiento global de tratamiento de muestras requiere 13 minutos en total, 5 minutos para la extracción de PAHs y 8 minutos para la separación del extracto y el residuo mediante centrifugación.

3. La extracción de PAHs es cuantitativa, con rendimientos de extracción comprendidos en el intervalo entre 71% y 110%. Los límites de cuantificación del método varían entre 0,14 y 0,80 μg kg^{-1}, respectivamente. Las características analíticas son adecuadas para el uso de este método en el estudio de la deposición atmosférica de PAHs.

2. SUPRAS-RAM basados en ácidos alquilcarboxílicos para la determinación de componentes en multi-matrices

2.1. Determination of ocratoxina A en especias

1. Se ha evaluado la capacidad de SUPRAS-RAM de agregados inversos de ácido decanoico, para la determinación de OTA en los cinco grupos de especias incluidos en la actual legislación europea.

2. La principal ventaja de este método es la combinación de la extracción de OTA y purificación de la muestra en una etapa simple. La capacidad del SUPRAS-RAM investigado para excluir los carbohidratos y proteínas evita el uso de columnas de inmunoafinidad, con la consiguiente reducción de costes y tiempo del análisis. La

selectividad alcanzada permite la cuantificación de OTA usando calibración externa.

3. El procedimiento de tratamiento de muestras desarrollado es independiente de la matriz de la misma y por tanto puede aplicarse al control de OTA en todas las especias legisladas por la UE. Tanto el límite de cuantificación (2,9 μg kg^{-1}) como los rendimientos de extracción (87-101%) cumplen con los criterios de funcionamiento establecidos por la legislación europea.

2.2. Determinación de curcuminoides en alimentos

1. El disolvente supramolecular constituido por agregados inversos de ácido decanoico, THF y agua es un extractante eficiente de los curcuminoides presentes en diferentes tipos de alimentos lo que ha permitido el desarrollo de un método generalizado de tratamiento de muestra.

2. Las escasas metodologías existentes para la determinación individual de los tres curcuminoides utilizados como aditivos en alimentos no están en general validadas, usan tratamientos de muestra complejos y tienen baja sensibilidad. El método basado en SUPRAS-RAM es rápido (el tratamiento de muestras se realiza en aproximadamente 15 min y se pueden tratar varias muestras simultáneamente), requiere una cantidad de muestra (0.2g) y volumen de SUPRAS (600 μL) pequeño y es económico.

3. La combinación de SUPRAS-RAM con cromatografía de líquidos acoplada a un detector fotométrico de diodos en fila, permite la determinación simultánea de los tres curcuminoides con elevada sensibilidad.

3. SUPRAS-RAM basados en alcanoles para la determinación de multicomponentes en multi-matrices

3.1. Determinación de mezclas de bisfenoles en multi-matrices.

1. Se ha desarrollado un tratamiento de muestra generalizado basado en el uso de un SUPRAS-RAM volátil, constituido por agregados hexagonales inversos de hexanol, para la determinación de mezclas de 21 bisfenoles y derivados en fuentes de exposición humana y muestras biológicas.

2. El método es selectivo y extrae eficientemente los bisfenoles y derivados en una etapa simple. Los valores de porcentaje de recuperación absolutos obtenidos se encuentran en el intervalo 72-114% y los límites de cuantificación para muestras líquidas y sólidas están en el intervalo 0.019-0.24 μg L^{-1} y 0.12-0.81 μg kg^{-1}, respectivamente.

3. El método cromatográfico desarrollado permite por vez primera la determinación secuencial de los bisfenoles/derivados clorados y los bisfenol diglicidil éteres utilizando idénticas condiciones cromatográficas. Para ello, los aductos de bisfenol diglicidil éteres se forman mediante adición de sales de amonio al extracto de la muestra.

4. El método cumple los criterios de funcionamiento para la determinación de bisfenoles en una amplia variedad de fuentes de exposición humana (alimentos, bebidas, polvo) y fluidos biológicos (orina y suero) y por lo tanto para su aplicación al control de la exposición humana a estos compuestos y a estudios poblacionales.

CONCLUSIONS

The research here presented enables some general conclusions to be drawn. They arise from the results obtained from the following studies:

1. The analysis of 28 food matrices, two types of biological fluids (serum and urine) and two types of environmental samples (dust and moss). The food samples analyzed included canned foodstuffs, drinks, spices, milk products, raisings, candy, snacks, gelatin, etc. The content of water (0.06-94%), carbohydrates (0.06-75%), proteins (0.3-84.4%) and fats (0.08-82%) for the selected food encompassed a wide range. The rest of samples analyzed included aqueous matrices with a high content in nitrogen compounds and salts (e.g. urine), high protein concentration (e.g. >600 mg/mL in serum) or high content in polysaccharides (e.g. cellulose in moss).

2. The determination of 39 organic compounds, including 1 mycotoxin, 3 curminoids, 14 PAHs and 21 bisphenols and derivatives. They encompassed a wide polarity range (viz. the logarithm of their partition coefficient was in the range 1.25-6.58).

3. The development of sample treatment procedures using three types of SUPRAS, obtained by coacervation of colloidal solutions made up of vesicles or inverted micelles of decanoic acid and inverted micelles of hexanol.

4. The direct analysis of SUPRAS extracts by liquid chromatography coupled to diode array, fluorescence or mass spectrometric detection.

5. The development and validation of five analytical methodologies intended for determining toxicants or additives in food quality control, pollutants in environmental biomonitoring and human exposure to a chemical cocktail.

GENERAL CONCLUSIONS

1. Alkyl carboxylic acids and alkanol-based SUPRAS are able to efficiently solubilize organic chemicals in a wide polarity range (log P 1.25-6.58) from both solid and liquid samples. The recoveries obtained in our studies were all in the interval 71-114%. This excellent extraction efficiency was the result of the high concentration of amphiphile in the SUPRAS investigated (0.14-0,55 mg/μL), the different polarity regions in the aggregates making up the SUPRAS and the mixed-mode mechanisms offered by them for solute solubilization (van der Waals, ionic, hydrogen bonding, polar and π-cation interactions).

2. SUPRAS-RAM obtained from alkyl carboxylic acids or alkanols in mixtures of THF and water have the potential to behave as restricted access liquids (SUPRAS-RAM). Proteins exclusion is carried out by both the decrease of dielectric constant by THF and the formation of macromolecular complexes with the amphiphiles. Macromolecules such as polysaccharides are not solubilized in the SUPRAS because of size exclusion phenomena. The ability of SUPRAS-RAM to exclude proteins and macromolecules has been proved from both pure standards and sample matrices.

3. The combination of both the high extraction efficiency and restricted access properties of SUPRAS enabled us the development of five methods for the analysis of a wide variety of solid and liquid samples that meet the performance criteria regarding sensitivity, selectivity, precision, etc., for the intended compounds. These methods are suitable for the determination of single components and multicomponents in complex samples and multi-matrices, and include the development of generalized, matrix-independent sample treatment methods.

4. SUPRAS-RAM simplifies sample treatment procedures because of their ability to integrate analyte extraction and sample cleanup in a single step. Extractions are fast (5-15 min) and require minute volume of solvent (SUPRAS volume (μL)/sample amount (mg) ratios are

between 1 and 3). So, they are ideal to save time and costs in food quality control, environmental monitoring campaigns and epidemiological studies.

5. SUPRAS are generated through spontaneous self-assembly processes and extractions are carried out using conventional equipment in analytical labs, so implementation of SUPRAS-based sample treatment is at the everyone´s reach.

6. Direct analysis of SUPRAS-RAM extracts is compatible with LC and UV and fluorescence detection.

7. The use of volatile SUPRAS-RAM, such as those obtained from hexanol, is recommended in multicomponent analysis by liquid chromatography tandem mass spectrometry. Removal of the SUPRAS before analysis reduces interferences arising from the coelution of solutes with the amphiphile making up the solvent aggregates.

SPECIFIC CONCLUSIONS

1. Alkyl carboxylic acid-based SUPRAS-RAM for determination of single component/multicomponents in complex samples.

1.1. Determination of ochratoxin A

1. A new analytical method has been developed for the determination of OTA in raisings. It is based on its extraction with a SUPRAS made up of equimolecular decanoic acid/tetrabutylammonium decanoate vesicles and direct analysis of the extract by liquid chromatography and fluorescence detection.

2. The extraction procedure surpasses the previously described ones, which require the use of high solvent volumes for extraction and immunoaffinity columns for sample purification. The procedure here described combines both analyte extraction and sample purification in a single step making sample treatment simpler and more cost-effective.

3. OTA can be reliably quantified (method quantitation limit: 5,3 μg kg^{-1}) below the maximum permitted levels set by European provisions.

1.2. Determination of 14 PAHs in mosses

1. Restricted access materials (RAM) have been widely applied to the sample treatment of biological fluids such as serum, plasma and urine. However, application of RAM to solid biological matrices has been almost inexistent owing to the need for previous solvent extraction of analytes. The study here reported extends the scope of RAM to biological matrices. It deals with the application of a SUPRAS-RAM, based on decanocid acid reversed micelles, to the extraction of PAHs in mosses. Both high extraction efficiency and selectivity were

obtained for PAHs, this proving that SUPRAS-RAM fit for the purpose.

2. SUPRAS-RAM extracts are directly analyzed by liquid chromatography and fluorescence detection with high sensitivity for PAHs. There is no need for solvent evaporation, that speeding up analysis. The whole sample treatment takes around 13 min (5 min for extraction and 8 min for separation of SUPRAS-RAM extracts from sample residue).

3. Extraction of PAHs in quantitative; recoveries are in the interval 71-110%. Method quantitation limits range from 0,14 to 0,80 μg kg^{-1}, respectively. Method analytical performance meets the criteria for the biomonitoring of the atmospheric deposition of PAHs.

2. Alkyl carboxylic acid-based SUPRAS-RAM for the determination of a few components in multi-matrices.

2.1. Determination of ochratoxin A in spices

1. SUPRAS-RAM consisting of inverted aggregates of decanoic acid have been successfully applied to the extraction of OTA in the five groups of spices under European regulation.

2. A valuable asset of this method is the integration of analyte extraction and sample purification in a single step. The ability of SUPRAS-RAM to exclude macromolecules such as proteins and carbohydrates makes it unnecessary the use of immunoaffinity columns, that reducing analysis time and cost. Quantification of OTA using external calibration was possible on the basis of the selectivity achieved.

3. The sample treatment here developed is matrix-independent and therefore suitable for the determination of OTA in all the spices under European regulation. Both method quantitation limit (2,9 μg kg^{-1}) and absolute recoveries (87-101%) meet the performance criteria set by the EU provisions.

2.2. Determination of curcuminoids in food

1. A generalized sample treatment for the determination of curcuminoids in different types of foodstuffs has been developed. It is based on their extraction with a SUPRAS-RAM consisting of inverted aggregates of decanoic acid in THF and water.

2. The features of the treatment procedure here developed surpass those of the procedures previously reported, which have not been usually validated. Thus, the SUPRAS-RAM based method is matrix-independent, that simplifying tasks in labs, it is faster (the whole sample treatment takes 15 min and several samples can be simultaneously processed), it requires minute volume of solvent (600 μL), it is more cost-effective and the sensitivity for curcuminoids is enough without the need for solvent evaporation.

3. Simultaneous determination of the three curcuminoids at high sensitivity is achieved by combination of SUPRAS-RAM extraction and liquid chromatography diode array detection

3. Alkanol-based SUPRAS-RAM for the determination of multicomponents in multi-matrices.

3.1. Determination of mixtures of bisphenols in multi-matrices.

1. A generalized sample treatment for the determination of 21 bisphenols and derivatives in human exposure sources and biological fluids has been developed. It is based on the simultaneous extraction of bisphenols and sample cleanup with a volatile SUPRAS-RAM made up of inverted hexagonal aggregates of hexanol.

2. Both extraction efficiency and selectivity are high; absolute recoveries were in the interval 72-114%. Quantification limits for liquid and solid samples were 0.019-0.24 μg L^{-1} y 0.12-0.81 μg kg^{-1}, respectively.

3. A chromatographic method has been developed for the sequential analysis of bisphenols/chlorinated derivatives and bisphenol diglycidyl ethers by using identical chromatographic conditions. For this purpose, ammonium adducts of bisphenol diglycidyl ethers were produced in the sample extract.

4. The method meets the performance criteria for the determination of a *cocktail* of bisphenols in a wide variety of human exposure sources (e.g. food, drinks, dust, etc) and biological fluids (urine, serum). So, it is suitable for application to the assessment of human exposure to these compounds and epidemiological studies.

ANEXO 1

Publicaciones científicas derivadas de la Tesis Doctoral

Publicaciones científicas derivadas de la Tesis Doctoral

1. **Vesicular aggregate-based solventless microextraction of Ochratoxin A in dried vine fruits prior to liquid chromatography and fluorescence detection**
   Noelia Caballero-Casero, Sergio García-Fonseca, Soledad Rubio
   Talanta 89 (2012) 377– 382

   Factor de impacto (posición revista/nº total revistas); Área Química Analítica, 2012 (JCR): 3,498 (12/75, 1º Cuartil)

2. **Quick supramolecular solvent-based microextraction for quantification of low curcuminoid content in food**
   Noelia Caballero-Casero, M. Ocak, Ü. Ocak, Soledad Rubio
   Analytical Bioanalytical Chemistry 406 (2014) 2179–2187

   Factor de impacto (posición revista/nº total revistas); Área Química Analítica, 2014 (JCR): 3.436 (13/74, 1º Cuartil)

3. **Nanostructured alkyl carboxylic acid-based restricted access solvents: Application to the combined microextraction and cleanup of polycyclic aromatic hydrocarbons in mosses**
   Noelia Caballero-Casero, H. Çabuk, G. Martínez-Sagarra, J.A. Devesa, S. Rubio
   Analytica Chimica Acta 890 (2015) 124-133

   Factor de impacto (posición revista/nº total revistas); Área Química Analítica, 2014 (JCR): 4.513 (5/74, 1º Cuartil)

4. **Assessing human and environmental exposure and risk to a cocktail of bisphenols and derivatives. A review**
   N. Caballero-Casero, L. Lunar, S. Rubio
   Enviado a *Analytica Chimica Acta* (Septiembre 2015)

5. **Restricted access supramolecular solvents for the simultaneous extraction and cleanup of ochratoxin A in spices subjected to EU regulation**
   Noelia Caballero-Casero, Sergio García-Fonseca and Soledad Rubio

6. **Volatile restricted access supramolecular solvents for generalized sample treatments in multi-matrix multicomponent liquid chromatography tandem mass spectrometry detection**
   Noelia Caballero-Casero and Soledad Rubio

ANEXO 2

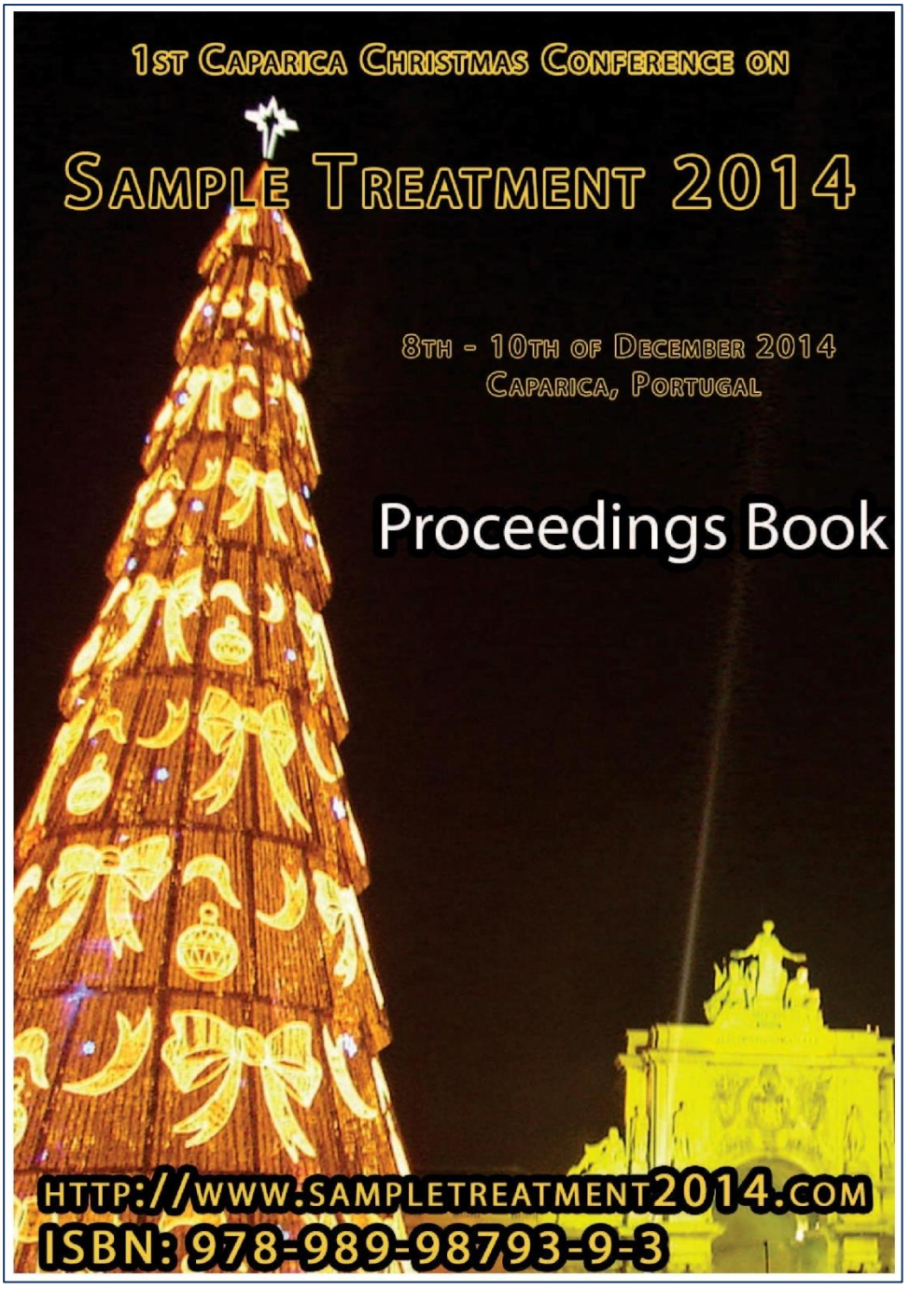
Comunicaciones realizadas en Congresos nacionales e internacionales

Oral internacional

Supramolecular solvents: efficient and green nanostructured liquids for the extraction of organic compounds

1st Caparica Christmas Conference on Sample Treatment 2014

Caparica, Portugal. Del 8 al 10 de diciembre de 2014


1st Caparica Christmas Conference on
Sample Treatment 2014
8th - 10th of December 2014
Caparica, Portugal
Proceedings Book
http://www.sampletreatment2014.com
ISBN: 978-989-98793-9-3

O 41 - Supramolecular Solvents: Efficient and Green Nanostructured Liquids for the Extraction of Organic Compounds

Noelia Caballero-Casero; Soledad Rubio

Department of Analytical Chemistry. Institute of Fine Chemistry and Nanochemistry. Edificio Anexo Marie Curie. Campus de Rabanales, 14071-Córdoba, Spain

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Abstract

The increasing pressure to decrease organic solvent usage in laboratories is fostering the search for alternative solvents. Supramolecular solvents are nanostructured liquids generated from aqueous or hydro-organic solutions of amphiphiles through self-assembly processes. Self-assembly based synthetic procedures are within everyone´s reach and provide unique opportunities to obtain tailored solvents with advanced functional features.

This talk will focus on the most significant developments concerning both theoretical and practical aspects related to the use of supramolecular solvents in analytical extractions. Theoretical aspects will include those advances allowing a better understanding of the mechanisms of solvent production and solvent structure and composition. A comprehensive understanding of these fluids is no doubt essential with a view to their rational exploitation in a number of fields. Practical aspects will include the consequences of the unique array of physico-chemical properties of supramolecular solvents on extraction efficiency, suitability for extracting solutes spanning a wide polarity range and potential for developing generic sample treatments and multiresidue analysis. In this context, recent developments in formats and strategies making supramolecular solvents compatible with separation and detection techniques and the most outstanding applications reported so far will be discussed. Trends and topics deserving development in this area will be finally outlined.

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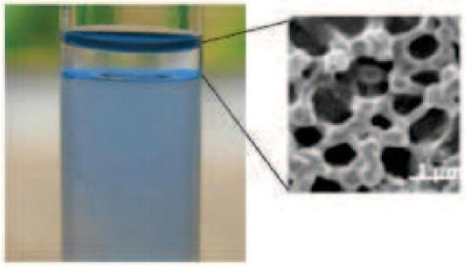
[2] A. Ballesteros-Gómez, S. Rubio. Analytical Chemistry, 2012, 84, 342-349

[3] F.J. Ruiz, S. Rubio, D. Pérez-Bendito. Analytical Chemistry, 2007, 79, 7473-7485

[4] F.J. Ruiz, S. Rubio, D. Pérez-Bendito. Analytical Chemistry, 2006, 78, 7229-7239

Figures



Cartel Internacional

Quick supramolecular solvent-based microextraction for quantification of low curcuminoid content in food

6th International Symposium on Recent Advances in Food Analysis

Praga, República Chequeca. Del 5 al 8 de noviembre de 2013

Determination of Ochratoxin A in dried wine fruits by solventless microextraction with vesicular aggregates, liquid chromatography and fluorescence detection

13as Jorandas de Análisis Instrumental

Barcelona, España. Del 14 al 16 de noviembre de 2011

BOOK OF ABSTRACTS

6th International Symposium on

RECENT ADVANCES IN FOOD ANALYSIS

November 5–8, 2013
Prague, Czech Republic

Jana Pulkrabová, Monika Tomaniová, Michel Nielen and Jana Hajšlová
Editors




INSTITUTE OF CHEMICAL TECHNOLOGY PRAGUE

RIKILT WAGENINGEN UR

H-67

QUICK SUPRAMOLECULAR SOLVENT-BASED MICROEXTRACTION FOR QUANTIFICATION OF LOW CURCUMINOID CONTENT IN FOOD

Noelia Caballero-Casero[1], Miraç Ocak[2], Ümmühan Ocak[3], Soledad Rubio[4*]

[1 4] University of Córdoba, Córdoba, Spain
[2 3] Karadeniz Technical University, Trabzon, Turkey
**Corresponding author – E-mail: qa1rubrs@uco.es, Phone: 34957218644*

There is a need to monitor the consumption of curcuminoids, an EU-permitted natural colour in food, to ensure the acceptable daily intakes are not exceeded, especially by young children. A sensitive method able to quantify low contents of curcumin (CUR), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) in foodstuffs was here described. It was based on a single-step extraction with a supramolecular solvent (SUPRAS) made up of reverse aggregates of decanoic acid and direct analysis of the extract by liquid chromatography-photodiode array (PDA) detection. The extraction involved the stirring of 200 mg of foodstuff with 600 µL of SUPRAS for 15 min. No cleanup or concentration of the extracts was required. Driving forces for curcuminoid solubilisation were dispersion and hydrogen bond. The method was applied to the determination of curcuminoids in different types of foodstuffs (i.e. snack, gelatine, yogurt, mayonnaise, butter, candy and fish products) tha t encompassed a wide range of protein, fat, carbohydrate, sugar and water content (i.e. 0.85–11.04, 0–81.11, 0.06–75, 0.06–79.48 and 10.08-85.10 g/100 g of food, respectively). Method quantification limits for the foodstuffs analyzed were in the ranges 2.9–7.7, 2.8–11.2 and 3.3–9.0 µg kg^{-1} for CUR, DMC and BDMC, respectively. The concentration of curcuminoids found in the foodstuffs and the recoveries obtained from fortified samples were in the ranges ND-284, ND-201 and ND–61.3 µg kg^{-1} and 82–106, 89–106 and 90–102% for CUR, DMC and BDMC, respectively. The relative standard deviations were from 2 to 7%. This method allowed the quick and simple microextraction of curcuminoids with minimal solvent consumption while delivering accurate and precise data.

Keywords: *Curcuminoids, supramolecular solvent, microextraction, food*

13as JORNADAS DE ANÁLISIS INSTRUMENTAL

RECINTO GRAN VIA. 14-16 NOVIEMBRE 2011

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EuCheMS · DIVISION OF ANALYTICAL CHEMISTRY

PROGRAMA CIENTÍFICO

DETERMINATION OF OCHRATOXIN A IN DRIED VINE FRUITS BY SOLVENTLESS MICROEXTRACTION WITH VESICULAR AGGREGATES, LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

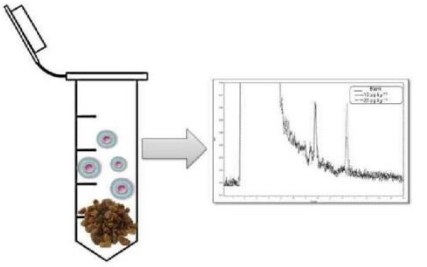
N. Caballero-Casero[a], S. García-Fonseca[a], S. Rubio[a]
[a] Department of Analytical Chemistry. Institute of Fine Chemistry and Nanochemistry. Edificio Anexo Marie Curie. Campus de Rabanales, 14071-Córdoba, Spain

A solventless microextraction is proposed for the development of a simple, fast, low-cost and environmentally friendly sample treatment for the determination of Ochratoxin A (OTA) in dried vine fruits. The objective is to offer an alternative to conventional sample treatments, which invariably involve extractions with large solvent volumes followed by clean-up with expensive, not recyclable and limited storage stability immunoaffinity sorbents.

The method involves the stirring of 300 mg of dried vine fruit subsamples with 400 µL of a supramolecular solvent (SUPRAS) made up of decanoico acid/tetrabutylammonium decanoate vesicles. Then, the sample is centrifuged for 15 minutes and OTA is quantified in the extract by liquid chromatography/fluorescence detection against solvent-based calibration curves. Neither dilution nor further clean-up steps of extracts were needed.

The method was successfully applied to the analysis of several dried vine fruits (sultanas and muscatels) purchased in local supermarkets in Córdoba (South of Spain). Quantitation of OTA was interference-free and recoveries ranged between 95 % and 101 %. The precision of the method, expressed as relative standard deviation (RSD), was about 3 %. The limit of quantification (5.3 µg kg^{-1}) was below the threshold limit established for OTA in dried vine fruits by EU directives (10 µg kg^{-1}). Representativity of subsamples was proven.

This solventless sample treatment allows quick, inexpensive and simple microextraction of OTA, while delivering accurate and precise data. Thus, it takes about 30 minutes and several samples can be simultaneously treated. In addition, it requires a small sample amount and a low eco-friendly SUPRAS volume. The proposed method fulfils analytical and legal requirements to be used for the routine monitoring OTA in dried vine fruits.



Tipo de comunicación: Poster - Calidad y seguridad alimentaria

Oral Nacional

Disolventes supramoleculares: líquidos nanoestructurados sensibles a estímulos ambientales para la extracción de compuestos orgánicos

XX Reunión de la Sociedad Española de Química Analítica

Santiago de Compostela, España. Del 1 al3 de julio de 2015

Materiales Supramoleculares Multifuncionales y Específicos en Procesos de Extracción Analíticos e Industriales

IV Congreso científico de Investigadores en formación de la Universidad de Córdoba

Córdoba, España. Del 18 al 19 de noviembre de 2014

XX Reunión de la Sociedad Española de Química Analítica

Santiago de Compostela 1-3 de julio de 2015

CERTIFICADO DE PARTICIPACIÓN

El Comité Organizador certifica que:
N. Caballero-Casero, S. Rubio

Han presentado la comunicación en formato ORAL **"DISOLVENTES SUPRAMOLECULARES: LÍQUIDOS NANOESTRUCTURADOS SENSIBLES A ESTÍMULOS AMBIENTALES PARA LA EXTRACCIÓN DE COMPUESTOS ORGÁNICOS"** en la XX Reunión de la Sociedad Española de Química Analítica, celebrada en Santiago de Compostela, del 1-3 de julio de 2015

Y para que así conste, se expide el presente certificado
En Santiago de Compostela, a 3 de julio de 2015

Dra. Pilar Bermejo Barrera
Presidenta del Comité Organizador

O1

DISOLVENTES SUPRAMOLECULARES: LÍQUIDOS NANOESTRUCTURADOS SENSIBLES A ESTÍMULOS AMBIENTALES PARA LA EXTRACCIÓN DE COMPUESTOS ORGÁNICOS

Noelia Caballero-Casero, Soledad Rubio

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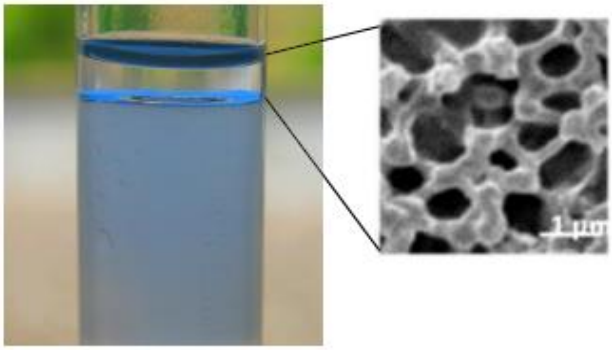
El desarrollo de disolventes alternativos a los disolventes orgánicos ha despertado un enorme interés en los ámbitos industrial y científico, sectores en los que se deben atender las exigencias de las políticas medioambientales y sociales. Los disolventes supramoleculares son líquidos nanoestructurados generados a partir de una disolución acuosa o hidro-orgánica de moléculas anfifílicas a través de un proceso espontáneo de autoensamblaje. Estas fases líquidas nanoestructuradas proporcionan una oportunidad única para el diseño y síntesis de disolventes funcionales con la eficiencia y selectividad requerida para el desarrollo de estrategias innovadores en procesos de extracción analítica e industrial.

En esta comunicación se presentarán algunos de los avances obtenidos en los últimos años en relación a la síntesis, caracterización y aplicación en procesos de extracción analítica de los disolventes supramoleculares. El desarrollo de la Química Supramolecular y, consecuentemente, la mejor comprensión de los mecanismos de autoensamblaje molecular, ha proporcionado las bases para el diseño de disolventes con propiedades programadas para cumplir funciones específicas. La caracterización de las nanoestructuras que los conforman sigue siendo un gran reto debido a la labilidad que se deriva del tipo de interacciones que conducen el proceso de autoensamblaje. El uso de técnicas de preparación de muestras como vitrificación está permitiendo la obtención de excelentes microfotografías mediante TEM Y SEM (ver figura). Las propiedades intrínsecas de los disolventes supramoleculares (ej. regiones de distinta polaridad en la nanoestructuras, diferentes tipos de interacciones, elevada concentración de compuestos anfifílicos) los convierten en ideales para procesos de extracción eficientes en análisis multiresiduo, el desarrollo de procesos generales de extracción, en estudios epidemiológicos y campañas de monitorización ambiental.



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[2] A. Ballesteros-Gómez, S. Rubio. Analytical Chemistry, 2012, 84, 342-349

La Coordinadora Académica del Campus de Excelencia Internacional en Agroalimentación ceiA3

ACREDITA que :

NOELIA CABALLERO

ha presentado **la COMUNICACIÓN ORAL** que lleva por título :

MATERIALES SUPRAMOLECULARES MULTIFUNCIONALES Y ESPECÍFICOS EN PROCESOS DE EXTRACCIÓN ANALÍTICOS E INDUSTRIALES

en el **III Congreso Científico de Investigadores en Formación en Agroalimentación ceiA3,** organizado por la Escuela Internacional de Doctorado en Agroalimentación eidA3, celebrado en Córdoba los días 18 y 19 de noviembre de 2014.

Y para que así conste, se expide y firma este certificado en Córdoba, a 19 de noviembre de 2014

Fdo: JULIETA MÉRIDA GARCÍA

Coordinadora Académica del ceiA3

Rectorado de la Universidad de Córdoba – Avd. Medina Azahara 5, 14071 CÓRDOBA

Título:

Materiales Supramoleculares Multifuncionales y Específicos en Procesos de Extracción Analíticos e Industriales

Objetivos:

1. Síntesis y caracterización de nuevos disolventes supramoleculares sensibles a estímulos ambientales.
2. Desarrollo de metodologías analíticas para la multideterminación de compuestos perjudiciales para la salud, en el sector agroalimentario.

Método de trabajo:

1. Disolventes supramoleculares:

Los disolventes orgánicos se han empleado como extractantes en un sinfín de procesos analíticos e industriales durante décadas. Sin embargo, su elevado consumo y sus efectos perjudiciales para la salud y el medioambiente han fomentado la búsqueda de nuevos disolventes más respetuosos con el ambiente.

Los disolventes supramoleculares son líquidos nanoestructurados generados a partir de disoluciones acuosas o hidroorgánicas de moléculas anfifílicas a través de un proceso de autoensamblaje. Los agregados supramoleculares originados durante este proceso presentan una morfología y funcionalidad dependientes de la concentración de moléculas anfifílicas y el ambiente donde tiene lugar el autoensamblaje. Así, una modificación del medio puede dar lugar a una nueva fase líquida nanoestructurada.

Para la consecución de los objetivos propuestos, la estrategia seguida es la neutralización de la carga de las moléculas de tensioactivo mediante un agente coacervante (adición de una sal o modificación del pH), que reduce la repulsión entre sus grupos polares y favorece el aumento del número de agregación hasta que la densidad del agregado difiere de la del medio de autoensamblaje. Esta diferencia de densidad provoca la aparición de una nueva fase, inmiscible con la disolución, denominada coacervado o disolvente supramolecular (SUPRAS).

Las propiedades intrínsecas de los SUPRASs los convierten en excelentes sistemas extractantes para un amplio intervalo de compuestos en diferentes matrices. Estas propiedades dependen del medio en el que ha sido producido el disolvente, por lo que, en principio, podrían ser seleccionadas y/o predichas por el investigador. Sin embargo, la naturaleza no covalente de los enlaces intermoleculares de los agregados que forman el SUPRAS ha dificultado enormemente la dilucidación de estas estructuras, impidiendo el desarrollo de nuevos SUPRASs basados en modelos predictivos.

La síntesis, estudio y caracterización de nuevos SUPRAS, permitirá profundizar en el conocimiento de las nanoestructuras permitiendo el diseño de disolventes funcionales de acuerdo a sus propiedades. La metodología general que se seguirá consistirá en: (1) selección del tensioactivo y condiciones ambientales adecuadas para generar el material supramolecular con las propiedades y funciones requeridas; (2) síntesis de los materiales, utilizando procesos de autoensamblaje, y estudio de las propiedades físico-químicas generales de los mismos así como de las propiedades singulares para las que fueron programados; y (3) caracterización estructural de los materiales y determinación del tamaño y polidispersión de los agregados que los constituyen.

La caracterización estructural de los materiales supramoleculares se llevará a cabo mediante microscopía electrónica de barrido (SEM), de transmisión (TEM) y dispersión de rayos X de ángulo pequeño (SAXS). Debido a que los enlaces intermoleculares de los agregados son de carácter no covalente, las condiciones de operación utilizadas en estas técnicas provocan cambios morfológicos en estas estructuras; dificultando el estudio de las mismas. Para preservar estas nanoestructuras se emplearán técnicas de preparación de muestras como la vitrificación o el enfriamiento a gran velocidad (crio-SEM y crio-TEM).

1. **Multideterminación de compuestos en agroalimentación. Calidad alimentaria y bisfenoles:**

Los bisfenoles son un grupo de compuestos orgánicos con dos grupos hidroxifenil unidos, en la mayoría de los casos, por una cadena alquílica. Esta estructura es la que les confiere la propiedad de alterador endocrino, al unirse a los receptores del estrógeno. El bisfenol A fue el primero en ser sintetizado y utilizado por la industria, aunque en las últimas décadas han irrumpido con gran fuerza en la industria, alternativas al bisfenol A como pueden ser el bisfenol S o el F.

La principal aplicación de estos bisfenoles y de sus derivados glicidil éteres, es la producción de policarbonatos o resinas epoxy. Estos materiales son empleados en la fabricación de diversos productos como juguetes, envases alimenticios, selladores dentales, etc. La principal fuente de exposición humana son los alimentos, debido a su migración desde los recubrimientos de las latas y envases que los contienen, aunque se han descrito otras muchas fuentes de exposición.

Actualmente, la legislación europea sólo establece valores máximos permitidos e ingesta diaria tolerable (IDT) para algunos bisfenoles, de forma individual. Sin embargo, existe una preocupación creciente por las consecuencias sobre la salud humana del efecto combinado de estos compuestos. Así, en 2012, la Comisión Europea indicó la necesidad de realizar estudios sobre el nivel de exposición humana y ambiental a mezclas de estos compuestos, con el fin de sustentar el desarrollo de nuevas políticas de seguridad alimentaria.

Los SUPRAS han sido empleados para la multideterminación de compuestos orgánicos en muestras biológicas y ambientales con éxito, por el grupo de investigación donde

esta Tesis se está desarrollando. Con el uso de SUPRAS se están estableciendo metodologías de extracción para la evaluación de la exposición humana a bisfenoles. El esquema general seguido es: (1) selección del tensioactivo y condiciones ambientales para sintetizar el SUPRAS con las propiedades requeridas empleando procesos de autoensamblaje; (3) determinación de las propiedades extractivas (recuperaciones, factores de concentración, selectividad, etc.) para los solutos y aplicaciones de interés; (4) desarrollo de las correspondientes metodologías de extracción en el formato idóneo para cada aplicación y (5) validación de los métodos analíticos desarrollados.

Aportaciones de la Tesis Doctoral:

1. **Diseño, síntesis y caracterización de disolventes supramoleculares:**

La Tesis se centra en la síntesis de nuevos disolventes supramoleculares sensibles a estímulos ambientales. Para el diseño de las nanoestructuras en cada uno de ellos se contemplarán dos factores: el parámetro crítico de empaquetamiento, que determinará el tipo de agregado formado durante la etapa de autoensamblaje, y el tipo de estímulo ambiental y su aplicación durante la etapa de extracción.

La caracterización de estas estructuras permitirá el desarrollo y mejora de las aplicaciones analíticas e industriales de estos nuevos materiales.

2. **Multideterminación de bisfenoles en muestras alimenticias:**

Utilizando SUPRAS se desarrollará un proceso de extracción generalizado que con mínimas modificaciones permita la extracción de todos los bisfenoles y derivados a partir de una amplia variedad de matrices y que sea compatible con LC-MS/MS. Hasta el momento he desarrollado una metodología analítica para el evaluación de la exposición humana a los principales bisfenoles y sus derivados glicidil éteres a través de la alimentación.

El método propuesto contempla la determinación simultánea de doce bisfenoles y derivados en alimentos enlatados. El método se basa en la microextracción de los analitos presentes en 200mg de alimento con 600 μL de un disolvente supramolecular constituido por agregados inversos de tetradecanol. Para facilitar la transferencia de los compuestos de interés hacia el SUPRAS se aplica agitación vibracional (tipo Vórtex) durante 15 minutos, tras lo cual se centrifuga a 15000 revoluciones por minuto para acelerar la separación de fases (el extracto con los analitos, proteínas y otras macromoléculas insolubles, y la muestra). El extracto es introducido directamente para su análisis en el sistema de cromatografía líquida acoplada a un detector de fluorescencia.

Las características analíticas del método lo hacen ideal para la biomonitorización de la exposición humana a bisfenoles a través de la alimentación.

Cartel Nacional

Microextracción de curcuminoides en alimentos con disolvente supramolecular y determinación por LC-DAD

XVIII Reunión de la Sociedad Española de Química Analítica

Úbeda, España. Del 16 al 19 de junio de 2013

Microextracción de Ocratoxina A en especias con disolventes nanoestructurados

NANOUCO IV. Encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces

Córdoba, España. Del 7 al 8 de febrero de 2013

Microextracción de Ocratoxina A en pimentón basada en el uso de disolventes supramoleculares

Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica

Málaga, España. Del 7 al 8 de junio de 2012

Microextracción supramolecular de Ocratoxina A en pasas previa a su determinación por cromatografía/fluorescencia

NANOUCO III. Encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces

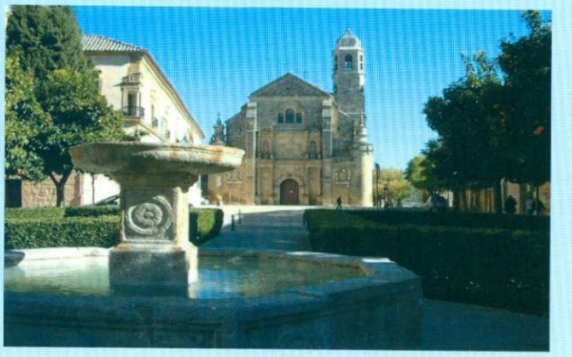
Córdoba, España. Del 10 al 11 de febrero de 2011

XVIII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

SEQA 2013

2013 SEQA

VI REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE ESPECTROMETRÍA DE MASAS



Úbeda, 16-19 junio 2013

SEQA

S.E.E.M

MICROEXTRACCIÓN DE CURCUMINOIDES EN ALIMENTOS CON DISOLVENTE SUPRAMOLECULAR Y DETERMINACIÓN POR LC-DAD

Noelia Caballero-Casero[a], Miraç Ocak[b], Ümmühan Ocak[b], Soledad Rubio[a]

[a]Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, España
[b]Department of Chemistry. Faculty of Art and Science, Trabzon, Turkey
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La curcumina es un término general para designar a un colorante de origen natural, muy apreciado como aditivo alimentario, que se extrae del rizoma de *Curcuma longa*. El colorante está compuesto por curcumina y dos análogos metoxilados: demetoxicurcumina (DMC) y bis-dimetoxicurcumina (BDMC). Numerosos estudios han puesto de manifiesto las propiedades antioxidantes, anti-inflamatorias, antimicrobianas y anticancerígenas de estos curcuminoides, especialmente del BDMC. El uso de curcumina como aditivo para una amplia variedad de alimentos está regulado por la Directiva europea 94/36/EC.

En los últimos años se han desarrollado diferentes métodos analíticos para la determinación de curcumina en biomatrices, dado el gran interés que ha despertado las propiedades nutricionales de este colorante natural. Sin embargo, no existen metodologías analíticas simples y generales para la determinación de los tres curcuminoides en la amplia variedad de alimentos en los que el uso de este colorante está regulado.

En este trabajo se evalúa la capacidad de los disolventes supramoleculares para el desarrollo de una metodología general para la extracción de los tres curcuminoides en diferentes tipos de alimentos. El principal objetivo es disponer de un método de tratamiento de muestras que sea eficiente, simple y rápido y que combinado con cromatografía de líquidos y detección con diodos en fila nos proporcione datos exactos.

El disolvente supramolecular (SUPRAS) seleccionado está constituido por micelas inversas de ácido decanoico. El procedimiento para el tratamiento de muestras es muy simple; Después de triturar y homogenizar el alimento, se toma una alícuota de 0.2 g del mismo y se mezcla con 0,6ml de SUPRAS en un microtubo. La mezcla se agita en vórtex durante 15 minutos a 2500 rpm y después se centrifuga para separar los componentes insolubles de la matriz. Una alícuota del extracto se analiza directamente mediante CL-DAD. No se requiere ninguna etapa de limpieza de la muestra. Las recuperaciones obtenidas para muestras fortificadas a un nivel de concentración de 2 $mg \cdot kg^{-1}$ son cuantitativas.

La cuantificación es exacta y no está afectada por los componentes de la matriz. Los límites de cuantificación del método son 7, 5 y 5 $\mu g \cdot kg^{-1}$ para curcumina, DMC y BDMC respectivamente, y por lo tanto este método presenta la sensibilidad requerida para evaluar si la concentración añadida de curcumina se ajusta a la regulación de la Unión Europea. La precisión del método, expresada como desviación estándar relativa fue del 5.7 %. La validez del método se comprobó mediante el análisis de los curcuminoides en matrices representativas de los grupos de alimentos incluidos en la legislación europea: gelatina, gominolas, productos de pescado, yogurt, mayonesa y aperitivos de maíz.

IV Workshop

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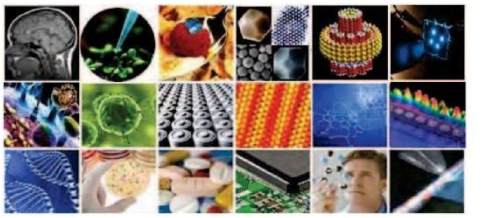

Córdoba, 7 y 8 de Febrero 2013

UNIVERSIDAD D CORDOBA

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MICROEXTRACCIÓN DE OCRATOXINA A EN ESPECIAS CON DISOLVENTES NANOESTRUCTURADOS

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Determinados hongos como *Penicillum* o *Aspergillus* producen micotoxinas en un amplio intervalo de condiciones. Las micotoxinas están ampliamente distribuidas en alimentos tales como cereales, café, uvas pasas, vino, especias, etc.

La Ocratoxina A (OTA) es una de las principales micotoxinas y presenta características nefrotóxicas, neurotóxicas e inmunosupresoras. Además la Agencia Internacional de Investigación contra el Cáncer (IARC) considera a la OTA como un posible cancerígeno humano, clasificándolo en el grupo 2b. Debido a sus propiedades tóxicas y su amplia distribución, son numerosos los estudios dedicados al desarrollo de métodos de extracción y detección de OTA. Sin embargo, son muy pocos los métodos desarrollados para la cuantificación de OTA en especias, ya que la complejidad de estas matrices hace que a pesar de obtener resultados precisos y reproducibles, los métodos requieran costosos y complicados tratamientos de muestra.

Con esta investigación se pretende desarrollar un método para la determinación de OTA que sea simple, rápido y sostenible tanto económica como ambientalmente. Además el método analítico debe alcanzar los requerimientos de límites máximos de OTA establecidos por la Unión Europea (15 μg kg^{-1}).

El método desarrollado está basado en la microextracción de OTA en pimentón con un disolvente supramolecular (SUPRAS) constituido por micelas inversas de 1-decanol. La OTA se re-extrae con una disolución básica para eliminar el SUPRAS. La separación y cuantificación de la micotoxina se realiza mediante cromatografía de líquidos acoplada a un detector de fluorescencia. El procedimiento de extracción es simple y rápido; y no se requiere ningún tratamiento de muestra previo. 0,2 g de pimentón se agitan en vórtex con 0,5 mL de SUPRAS durante 15 minutos a 2500 r.p.m. Después de la separación de los componentes insolubles de la matriz, el sobrenadante se recoge y mezcla con una disolución de amoniaco (0,05 M); tras lo cual una alícuota se analiza directamente.

La cuantificación es exacta y no está afectada por los componentes de la matriz. Las recuperaciones para muestras fortificadas con OTA para un nivel de concentración de 10 μg kg^{-1} se encontraron en el intervalo comprendido entre el 82 y el 104%. El límite de cuantificación del método es de 2,4 μg kg^{-1}, suficientemente bajo para cumplir con las regulaciones de la Unión Europea. La precisión del método, expresada como desviación estándar relativa fue 3.65 %. La validación del método se realizó mediante el análisis de pimentón de diferentes procedencias y marcas comerciales.


XIII
GRASEQA 2012
Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica
MÁLAGA,
7 y 8 de junio de 2012
SEQA

MICROEXTRACIÓN DE OCRATOXINA A EN PIMENTÓN BASADA EN EL USO DE DISOLVENTES SUPRAMOLECULARES

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Resumen:

La Ocratoxina A (OTA) es una micotoxina ampliamente distribuida en alimentos tales como cereales, café, uvas pasas, vino y especias. Además de considerarse un posible cancerígeno humano por la Agencia Internacional de Investigación contra el Cáncer (IARC), OTA presenta características nefrotóxicas, neurotóxicas e inmunosupresoras. La Unión Europea ha establecido límites máximos para su presencia en alimentos en el intervalo 0.50 – 80 µg/Kg.

La determinación de OTA a nivel de rutina se realiza mediante cromatografía de líquidos con detección fluorescente, aunque en el caso de que se realice una determinación multiresiduo de micotoxinas se prefiere espectrometría de masas. Debido a la complejidad de las matrices y a la baja concentración permitida, el tratamiento de muestras requiere del uso de varias etapas que incluyen la extracción con un disolvente orgánico, la limpieza de la muestra, generalmente con inmunoadsorbentes, y la concentración de la misma mediante evaporación. Problemas asociados a estos tratamientos son el elevado volumen de disolvente orgánico utilizado y el coste de las columnas de inmunoafinidad.

En esta investigación se propone un método simple y rápido para la microextracción de OTA en pimentón, una matriz especialmente compleja, para la que la Unión Europea ha establecido un límite máximo permitido de 15 µg/Kg que entrará en vigor a partir del 1 de julio de 2012.

El método desarrollado utiliza como extractante un disolvente supramolecular (SUPRAS) constituido por micelas inversas de ácido decanoico. El tratamiento de la muestra se reduce a la agitación en vórtex de 0.2 g de pimentón con 0,5ml de SUPRAS durante 15 minutos a 2700 rpm. A continuación se separa el extracto de los componentes insolubles de la matriz y una alícuota del mismo se analiza directamente mediante cromatografía de líquidos y detección fluorescente.

La cuantificación es exacta, sin interferencias por parte de los componentes de la matriz, y los porcentajes de recuperación para muestras fortificadas con OTA a un nivel de concentración de 10 µg/Kg se encontraron en el intervalo comprendido entre el 82 y el 104%. El límite de cuantificación del método es de 2,3 µg/Kg, suficientemente bajo para cumplir con la regulación de la Unión Europea. La precisión del método, expresada como desviación estándar relativa fue de 3.6 %. Empleando el método desarrollado se analizó pimentón de diferentes procedencias y marcas comerciales. El método cumple los requisitos analíticos y operacionales para utilizarse como un método de rutina en los laboratorios de análisis agroalimentario.

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NANOUCO III

Encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces

Córdoba, 10 y 11 de febrero de 2011

MICROEXTRACCIÓN SUPRAMOLECULAR DE OCRATOXINA A EN PASAS PREVIA A SU DETERMINACIÓN POR CROMATOGRAFÍA/FLUORESCENCIA

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La Ocratoxina A (OTA) es una micotoxina presente en numerosos alimentos como cereales, café, uvas pasas, vino, especias, etc.; debido a la acción de distintas especies de hongos (*Penicillum*, *Aspergillus*) bajo condiciones ambientales de elevadas temperatura y humedad. La Agencia Internacional de Investigación contra el Cáncer (IARC) considera la OTA como un posible cancerígeno humano (grupo 2b). Además son numerosos los estudios que ponen de manifiesto el carácter nefrotóxico, inmunosupresivo y neurotóxico de la OTA. La Unión Europea ha establecido niveles máximos de concentración de OTA en los numerosos alimentos en los que se puede encontrar.

Con esta investigación se pretende desarrollar un método para la determinación de OTA de forma simple, rápida y sostenible tanto económica como ambientalmente. Además el método analítico debe alcanzar los requerimientos de límites máximos de OTA establecidos por la Unión Europea. Para las pasas el límite está establecido en 10µg/Kg.

El método desarrollado está basado en la microextracción de la OTA con un disolvente supramolecular constituido por vesículas de ácido decanoico y decanoato de tetrabutilamonio y la separación y cuantificación de la micotoxina mediante cromatografía de líquidos y detección fluorescente. El procedimiento de extracción es simple y rápido; la muestra (0.3 g) se agita magnéticamente con 0,4ml de disolvente durante 10 minutos a 2700 r.p.m. Después de la separación de los componentes insolubles de la matriz, se analiza directamente una alícuota del sobrenadante.

La cuantificación es exacta y no está afectada por los componentes de la matriz. Las recuperaciones para muestras fortificadas con OTA a dos niveles de concentración (10µg/Kg y 20µg/Kg) se encontraron en el intervalo comprendido entre el 96 y el 99%. El límite de cuantificación del método es de 5,4µg/Kg, suficientemente bajo para cumplir con las regulaciones de la Unión Europea. La precisión del método, expresada como desviación estándar relativa fue del 3%. El método es válido para la determinación de los diferentes tipos de pasas: sultana, sultana blanca, moscatel. Moscatel de Chile y moscatel de Málaga.