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Departamento de Química Analítica

COACERVADOS Y HEMIMICELAS/ADMICELAS COMO SISTEMAS EXTRACTANTES DE ALTERADORES ENDOCRINOS

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Amalia García Prieto
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TITULO: *Coacervados y hemimicelas/admicelas como sistemas extractantes de alteradores endocrinos*

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COACERVADOS Y HEMIMICELAS/ADMICELAS COMO SISTEMAS EXTRACTANTES DE ALTERADORES ENDOCRINOS

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Trabajo presentado para optar al grado de Doctor en Ciencias Químicas

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CERTIFICAN: Que la citada Tesis Doctoral *"Coacervados y Hemimicelas/Admicelas como Sistemas Extractantes de Alteradores Endocrinos*" se ha realizado en los laboratorios del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Córdoba y que, a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, septiermbre de 2008.

Dª. D. Pérez Bendito

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"La felicidad humana generalmente no se logra con grandes golpes de suerte, que pueden ocurrir pocas veces, sino con pequeñas cosas que ocurren todos los días".

“La Ciencia será siempre una búsqueda, jamás un descubrimiento real. Es un viaje, nunca una llegada”.

K. R. Popper

“La dicha de la vida consiste en tener siempre algo que hacer, alguien a quien amar y alguna cosa que esperar”.

T. Chalmers

Esta Tesis se la dedico a mi madre, Aurora, por ser la mejor del mundo y a mis abuelas Eugenia y Elvira de quienes tanto he aprendido.

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OBJETO

La evaluación de riesgos humana y ambiental a la exposición a alteradores endocrinos requiere, entre otras acciones, el control analítico de los mismos a dos niveles; en muestras biológicas, para determinar el nivel de exposición, y en muestras de alimentos y ambientales, porque constituyen las principales fuentes de contaminación.

Entre las diferentes sustancias que originan alteración de la función endocrina preocupa especialmente el efecto de las hormonas esteroides sexuales, debido a su elevada potencia estrogénica, y el bisfenol A, por su elevada producción y consumo, razones por las que estos compuestos han sido el objeto de nuestras investigaciones.

La determinación de alteradores endocrinos en muestras biológicas, de alimentos y ambientales plantea importantes retos analíticos debido a la complejidad de las matrices implicadas y los bajos niveles a los que estos compuestos ejercen su efecto nocivo. Las metodologías analíticas desarrolladas, aunque proporcionan resultados de calidad, requieren el uso de numerosas etapas, frecuentemente largas y laboriosas, para la preparación de la muestra. De entre estas etapas, la relativa a la extracción y preconcentración de los analitos es sin duda la más crítica y por tanto, se requieren propuestas innovadoras que permitan el desarrollo y validación de métodos para el análisis simple, rápido y exacto de alteradores endocrinos en las matrices de interés.

El objetivo general de las investigaciones que se presentan en esta Memoria ha sido el desarrollo de métodos analíticos para la determinación de hormonas esteroides y bisfenol A en matrices biológicas, de alimentos y aguas naturales y residuales, en los que se simplifique y mejore sustancialmente la etapa de preparación de muestras. Para ello se evalúa la aplicabilidad del uso de extractantes supramoleculares constituidos por agregados de tensioactivos; concretamente coacervados de micelas inversas de ácido decanoico para la extracción líquido-líquido y sólido-líquido de bisfenol A en muestras biológicas y de alimentos y hemimicelas/admicelas para la extracción en fase sólida de hormonas esteroides en muestras acuosas ambientales.

Los extractantes supramoleculares presentan un conjunto de características que son de gran relevancia para procesos de extracción analítica y por tanto los convierten en idóneos para el objetivo planteado. Entre estas propiedades destacan las siguientes: los procedimientos de síntesis se basan en fenómenos de autoensamblaje y por lo tanto al alcance de cualquier laboratorio; el número de compuestos anfifílicos, naturales y sintéticos, es muy elevado; existe la posibilidad de modificar las propiedades del extractante variando el grupo polar o la cadena hidrocarbonada del compuesto anfifílico que lo constituye; los agregados que forman el extractante tienen estructura tridimensional y presentan regiones de diferente polaridad que muestran una excelente capacidad de solvatación para una gran variedad de compuestos orgánicos e inorgánicos

y tienen la capacidad para actuar como multiligandos debido a la presencia de múltiples grupos polares en el agregado supramolecular.

Además del desarrollo de las metodologías señaladas, se pretende profundizar en aspectos teóricos relevantes al proceso de extracción en relación a las interacciones analito:extractante y matriz:extractante que se establezcan en los sistemas investigados. El objetivo es el establecimiento de reglas generales que nos permitan conocer a priori el extractante y las condiciones experimentales más adecuadas para una aplicación concreta así como la aplicabilidad real de estos extractantes en las áreas investigadas: ambiental, biológica y de alimentos.

Paralelamente, la formación de la doctoranda ha sido un objetivo fundamental y se ha desarrollado con actividades complementarias a la labor investigadora que se recogen en la parte final de la memoria: publicaciones científicas derivadas de la Tesis Doctoral (Apéndice A) y asistencia y presentación de comunicaciones a congresos (Apéndice B).

INTRODUCCIÓN GENERAL

1.- TENDENCIAS EN LAS TÉCNICAS DE EXTRACCIÓN USADAS EN ANÁLISIS QUÍMICO

La determinación de contaminantes químicos en áreas tan diversas como Medioambiente, Agroalimentación, Toxicología, etc., es una actividad prioritaria en los laboratorios de análisis dado el ingente volumen de legislación desarrollada para la protección de la salud humana y ambiental. La Unión Europea (UE) ha aprobado una nueva política sobre compuestos químicos, REACH (acrónimo de Registration, Evaluation and Authorisation of Chemicals) que entró en vigor en Junio de 2007 (1). En 2009, la Agencia Europea de Compuestos Químicos (European Chemicals Agency), establecida en Helsinki, realizará una primera recomendación de sustancias prioritarias que requerirán autorización para su uso. Durante la próxima década miles de compuestos químicos que se producen en la UE, o son importados a la misma, tendrán que ser autorizados sobre la base de sus efectos en el hombre y el ambiente y este proceso deberá sustentarse en medidas analíticas fiables que permitan la identificación y cuantificación de los compuestos químicos más peligrosos y la evaluación de su distribución en el ambiente.

El control analítico de contaminantes en matrices ambientales, agroalimentarias, biológicas, etc., plantea importantes retos que derivan de la complejidad de las muestras implicadas, la amplia variedad de estructuras de los residuos orgánicos y contaminantes de interés, y las bajas concentraciones a las que éstos deben ser detectados y/o cuantificados (ng - μg L^{-1} y ng - μg Kg^{-1}) (2). Un aspecto clave en el control analítico de contaminantes es el uso de métodos que proporcionen confirmación inequívoca y cuantificación exacta. Otras características deseables para estos métodos son rapidez, simplicidad y adecuación a los principios de la química verde.

A pesar del gran desarrollo instrumental producido en la última década, son muy pocas las muestras ambientales, agroalimentarias, biológicas, etc., que pueden analizarse de forma directa o después de un tratamiento simple de las mismas. El tratamiento de las muestras continúa siendo la etapa más crítica en un proceso analítico ya que engloba múltiples operaciones las cuales requieren considerable participación humana, son lentas

(1) EC, 2006. Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006, concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Agency, amending Directive 1999/45/EC and Repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93.67/EEC, 93/105/EC and 2000/21/EC. Off. J. Eur. Union (2006) 850.

(2) Richardson S. D., Ternes T. A. *Anal. Chem.* 77(2005) 3807.

(suponen entre el 70 y 90% del tiempo consumido en un proceso analítico global) y constituyen una fuente potencial de errores analíticos. Por otro lado, es la etapa que entraña mayores riesgos personales y medioambientales ya que se utilizan ácidos, disolventes, gases a presión, etc. que pueden afectar tanto a la seguridad e higiene del personal del laboratorio como al medio ambiente. Como consecuencia, ha habido un enorme esfuerzo en I+D en los últimos años para simplificar los procesos de extracción analítica con el objeto de aumentar la capacidad del procesamiento de muestras, mejorar la calidad de los resultados obtenidos y reducir el consumo de disolventes orgánicos.

En la actualidad, l**a extracción/concentración de los contaminantes químicos presentes en muestras líquidas** se realiza preferentemente mediante extracción en fase sólida (SPE) (3-6). En el formato convencional, donde la muestra líquida se filtra en modo *off-line* u *on-line* a través de un cartucho que contiene el adsorbente, las investigaciones en la última década se han centrado en el desarrollo de nuevos materiales con dos objetivos; proporcionar elevada capacidad de retención para compuestos de muy diferente polaridad, orientados fundamentalmente al análisis de multiresiduo, o proporcionar elevada selectividad para la retención de compuestos específicos o grupos de compuestos. Simultáneamente, se han desarrollado nuevos formatos para extracción en fase sólida cuyo objetivo ha sido simplificar, miniaturizar y automatizar el proceso de extracción a la vez que reducir o eliminar el consumo de disolventes orgánicos. Entre los formatos desarrollados destacamos la microextracción en fase sólida (SPME) y la microextracción por adsorción en barras magnéticas agitadoras (SBSE).

Dentro de los materiales adsorbentes que permiten la retención de compuestos en un amplio intervalo de polaridad destacan los siguientes:

a) Copolímeros de divinilbenceno/N-vinilpirrolidona (patentados por Waters, nombre comercial, OASIS HLB). Su elevada área superficial (800 m^2g^{-1}) y la presencia en el polímero del grupo pirrolidona, que es un aceptor de hidrógeno, mejora notablemente la retención de compuestos polares, mientras que el polímero divilnilbenceno permite la retención eficaz de los compuestos hidrófobos. El adsorbente es estable en un amplio intervalo de pH (1-14) y ha encontrado amplia aplicación en el análisis de multiresiduos de drogas y sus metabolitos en fluidos biológicos y contaminantes en muestras acuosas ambientales.

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(3) Wardencki W., Curylo J., Namiesnik J. *J. Biochem. Biophys. Methods* 70 (2007) 275.
(4) Fidalgo-Used N., Blanco-González E., Sanz-Medel A. *J. Chromatogr. A* 590 (2007) 1.
(5) Smith R.M. *J. Chromatogr. A* 1000 (2003) 3.
(6) Hennion M.C. *J. Chromatogr. A* 856 (1999) 3.

b) Adsorbentes que proporcionan mecanismos de retención mixto (ej. Isolute HCX y HAX). Están constituidos por un sólido al que se enlazan cadenas alquilo (C8 y C18) y grupos cambiadores de iones y están especialmente indicados para la SPE de drogas ácidas y básicas en matrices biológicas. El doble mecanismo de retención de estos adsorbentes permite la elución secuencial de las interferencias de la matriz y proporciona extractos fácilmente analizables por LC/MS.

c) Adsorbentes de carbono. Inicialmente, se utilizaron en SPE adsorbentes de carbono no poroso con un área superficial muy baja (por ejemplo ENVI-Carb, comercializado por Supelco), sin embargo, la retención de analitos polares era superior comparada con la correspondiente en silica-C18. En la actualidad se investiga extensamente el uso de fulerenos y nanotubos de carbono en SPE. Ambas formas alotrópicas del carbono presentan una elevada área superficial y se han aplicado hasta la fecha a la extracción de diferentes tipos de compuestos tales como alteradores endocrinos, proteínas, compuestos orgánicos volátiles, iones metálicos, etc. (7,8).

Los materiales adsorbentes selectivos que se han desarrollado para SPE están especialmente indicados para la simultánea extracción, concentración y limpieza de contaminantes concretos o grupos de contaminantes estructuralmente relacionados. En general, el desarrollo de estos materiales es complejo. Los adsorbentes selectivos de mayor interés son:

a) Los materiales de acceso restringido (Restricted Access Materials, RAM). Permiten el acoplamiento en línea de las etapas de limpieza y extracción de muestras biológicas (plasma y suero) con cromatografía de líquidos. Para ello combinan la exclusión por tamaño de proteínas y otras macromoléculas con el enriquecimiento simultáneo de analitos de bajo peso molecular en la superficie de los poros internos del material a través de mecanismos de retención convencionales (interacciones hidrófobas, iónicas, etc) o específicos (9,10).

b) Los inmunoadsorbentes (ISs), constituidos por anticuerpos unidos mediante enlaces covalentes, no covalentes o adsorbidos a un soporte adecuado, extraen selectivamente a los analitos antígenos mediante un proceso de reconocimiento molecular; siendo los

(7) Pyrzynska K. *Anal. Sci*. 23 (2007) 631.
(8) Valcárcel M., Simonet B.M, Cárdenas S., Suárez B. *Anal. Bioanal. Chem.* 382 (2005) 1783.
(9) Mullett W.M. *J.Biochem. Biophys. Methods* 70 (2007) 263.
(10) Souverain S., Rudaz S., Veuthey J.L. *J. Chromatogr. B* 801 (2004) 141.

anticuerpos monoclonales más selectivos y reproducibles que los policlonales. Los ISs han encontrado amplia aplicabilidad en el área medioambiental y biológica (11), siendo su principal limitación el alto coste de la producción de los anticuerpos y la escasa reusabililidad.

c) Los polímeros de impresión molecular (MIPs) se obtienen mediante el ensamblaje de una matriz polimérica alrededor de un analito o molécula diana, de manera que se crea una huella del analito. Una vez obtenido el polímero es posible extraer el compuesto quedando así huecos libres con "memoria" selectiva que reconocerán de forma específica nuevas moléculas del contaminante molde. La afinidad y selectividad del sitio de unión por el analito es muchas veces comparable a la desarrollada por los anticuerpos. Entre las ventajas de estos materiales destacan la sencillez y rapidez de preparación, la elevada estabilidad química, física y térmica y el bajo coste. Presentan el inconveniente de la incompleta eliminación de las moléculas molde lo que origina problemas de contaminación. Los MIPs se han aplicado ampliamente en el área medioambiental y biológica (12-14).

Entre los nuevos formatos de SPE, la microextracción en fase sólida (SPME) es el formato más popular. Se basa en la extracción de los analitos usando una fibra de sílice fundida que está recubierta de un sorbente, en la mayoría de los casos polimérico, seguida de la desorción de los analitos mediante temperatura o un disolvente orgánico (3,5). La técnica es simple, presenta bajo coste, puede ser automatizada, requiere pequeños volúmenes de muestra y puede usarse para todo tipo de matrices; aire, agua y en el espacio de cabeza para sólidos. El principal inconveniente deriva de la limitada capacidad de la fibra, debido a que la cantidad de recubrimiento es muy pequeña, por lo que la sensibilidad alcanzada no es adecuada para muchos contaminantes, sobre todo si la SPME se utiliza combinada con la cromatografía de líquidos.

La extracción por adsorción en barras magnéticas agitadoras (SBSE) se basa en el uso de un imán situado en el interior de una barra de vidrio que a su vez está recubierta de una fibra de polidimetilsiloxano (PDMS). Comparada con SPME, la cantidad de fase estacionaria en la barra de vidrio es 50-250 veces mayor que en la fibra por lo que se obtienen menores límites de detección. Las extracciones se realizan por inmersión de la

(11) Delaunay N., Pichon V., Hennion M.C. *J. Chromatogr. A* 745 (2000) 15-37.

(12) Tamayo F,G., Turiel E. Martín-Esteban A. *J. Chromatogr. A* 1152 (2007) 32.

(13) Pichon V. *J. Chromatogr. A* 1152 (2007) 41.

(14) Stevenson D. *Trends Anal. Chem.* 18 (1999) 154.

barra en la muestra líquida o situando la misma en el espacio de cabeza por encima de la muestra líquida o sólida cuando compuestos volátiles o semivolátiles son de interés (headspace sorptive extraction, HSSE). SBSE se ha aplicado al análisis ambiental y de alimentos y en biomedicina (15). Su principal limitación es que hasta la fecha sólo se comercializan barras recubiertas de PDMS y por tanto sólo son aplicables a la extracción de compuestos apolares. La aplicación de SBSE a compuestos polares y semipolares requiere un paso previo de derivatización del material adsorbente.

La extracción/concentración de los contaminantes químicos presentes en muestras sólidas se lleva a cabo rutinariamente utilizando disolventes orgánicos. Con objeto de alcanzar suficiente sensibilidad y selectividad, el proceso requiere generalmente el tratamiento de una cantidad considerable de muestra, el desarrollo de extracciones repetidas para asegurar el aislamiento completo del analito de interés, una etapa de limpieza del extracto y la evaporación del disolvente. En este contexto, las investigaciones en los últimos años se han centrado en el desarrollo de estrategias que permitan reducir el consumo de disolventes y el tiempo necesario para la extracción. Las estrategias con mayor éxito se han basado en el aumento de la eficacia de la extracción mediante el uso de energías auxiliares [ej. extracción asistida por microondas (MAE), extracción acelerada por ultrasonidos y extracción con líquidos presurizados (PLE)], el uso de disolventes alternativos [ej. fluidos supercríticos (SFE)], o el uso combinado de disolventes y adsorbentes para la simultánea extracción y limpieza de la muestra (dispersión de la matriz en fase sólida, MSPD).

La extracción asistida por microondas se realiza en equipos que difieren en el tipo de energía aplicada a la muestra (multimodo o focalizada) y en el uso o no de sobrepresión. Generalmente, los sistemas cerrados (los que operan a alta presión) son multimodo, es decir la radiación generada se dispersa de forma aleatoria en una cavidad. Los sistemas abiertos (en los que el proceso se lleva a cabo a presión atmosférica) son sistemas focalizados o monomodo, es decir la radiación microondas se confina en un espacio mucho más reducido, donde se sitúa la muestra, y por tanto la radiación que llega a la misma es más intensa que en los sistemas multimodo. Existen numerosas revisiones en las que se describen los equipos utilizados, los principios en los que se basa la técnica y las aplicaciones desarrolladas hasta la fecha (16-18).

(15) David F., Sandra P. *J. Chromatogr*. A 1152 (2007) 54.

(16) Chen L., Song D., Tian Y., Ding L., Yu A., Zhang H. *Trends Anal. Chem.* 27 (2008) 151.

(17) Luque-García J.L., Luque de Castro M.D. *Trends Anal. Chem.* 22 (2003) 90.

(18) Jin Q., Liang F., Zhang H., Zhao L., Huan Y., Song D. *Trends Anal. Chem.* 18 (1999) 479.

En la extracción asistida por ultrasonidos (sonidos con una frecuencia superior a la que el oído humano puede percibir, >20 kHz) se utilizan dos tipos de dispositivos; los baños y las sondas (19). En los primeros, la reproducibilidad es muy baja debido a la falta de uniformidad en la transmisión de los ultrasonidos y la disminución de la potencia con el tiempo, por lo que su uso debería restringirse a operaciones como las de eliminación de gases disueltos, disolución etc., para las que han sido diseñadas. Las sondas ultrasónicas o sonotrodos focalizan su energía en una zona específica y producen resultados más reproducibles, aunque en general son poco robustos y dependen de la composición de la matriz de la muestra y del tamaño de partícula de la misma.

La extracción con líquidos presurizados (PLE), también denominada extracción acelerada con disolventes (ASE), implica el uso de disolventes a elevada temperatura (40-200 ºC) y presión (1000-2500 psi). En estas condiciones los disolventes aumentan el poder de solvatación y se incrementa la velocidad de extracción. La técnica tiene capacidad para realizar múltiples extracciones, puede automatizarse y ha encontrado amplia aplicación en la extracción de contaminantes en muestras ambientales, biológicas y de alimentos (20-23). La USEPA (United States Environmental Protection Agency) ha adoptado esta técnica para el análisis de plaguicidas en suelos (24). Frecuentemente, PLE se combina con SPE o SBSE para preconcentrar los analitos.

Los fluidos supercríticos son extractantes con propiedades intermedias entre los líquidos y los gases. Tienen viscosidad parecida a los gases, densidad parecida a los líquidos y elevada difusividad (25). La densidad del fluido, y por tanto su capacidad de solvatación para el soluto depende de la presión y la temperatura. El fluido más utilizado es el dióxido de carbono (punto crítico 31ºC y 74 bar). Tiene baja polaridad y es ideal para extracción de compuestos apolares. La adición de metanol (1-10%) aumenta la polaridad y permite su aplicación a la extracción de un amplio número de compuestos (26,27).

(19) Luque-García J.L., Luque de Castro M.D. *Trends Anal. Chem.* 22 (2003) 41.

(20) Carabias-Martínez R., Rodriguez-Gonzalo E., Revilla-Ruiz P. Henández-Méndez J. *J. Chromatogr*. A 1089 (2005) 1.

(21) RamosL., Kistenson E.M., Brinkman U.A.Th. *J. Chromatogr*. A 975 (2002) 3.

(22) Smith R.M. *J. Chromatogr. A* 975 (2002) 31.

(23) Björklund E., Nilsson T., *Trends Anal. Chem.* 19 (2000) 434.

(24) EPA Method 3545, US Environmental Protection Agency, Washington, DC, 1996.

(25) Luque de Castro M.D., Valcárcel M., Tena M.T. *Extracción con fluidos supercríticos en el proceso analítico,* Editorial Reverté (1993) Barcelona

(26) Pourmortazavi S.M., Hajimirsadeghi S.S. *J. Chromatogr*. A 1163 (2007) 2.

(27) Nemoto S. *J. Pestic. Sci.* 32 (2007) 328.

La dispersión de la matriz en fase sólida (MSPD) es una técnica adecuada para la extracción de muestras de alimentos y biológicas sólidas, semisólidas y/o de elevada viscosidad (28,29). El procedimiento consiste en dispersar la muestra con un adsorbente tal como C18, C8, sulfato sódico, tierra de diatomeas, etc. hasta conseguir una mezcla homogénea. A continuación se procede al lavado de la mezcla con un pequeño volumen de disolvente o bien ésta se sitúa en una minicolumna de SPE antes de la elución. El procedimiento es simple, versátil y ofrece la posibilidad de llevar a cabo la extracción y la purificación de la muestra en una única etapa con lo que se reduce el tiempo de análisis y el consumo de disolvente orgánico. La MSPD se utiliza ampliamente en la extracción de drogas en tejidos animales y multiresiduos de plaguicidas en frutas y verduras (30).

Las estrategias de extracción que se abordan en esta Tesis, al igual que las anteriormente expuestas, tienen como objetivo simplificar los procesos de extracción y reducir el consumo de disolventes orgánicos. Así, se investiga el uso de hemimicelas y admicelas en formato SPE convencional como materiales adsorbentes con capacidad para la retención de analitos de muy diferente polaridad, basada en las múltiples interacciones que proporcionan en los diferentes microambientes que los constituyen. Por otro lado, la extracción de contaminantes en muestras de alimentos y biológicas se aborda con el uso de coacervados como una alternativa muy ventajosa al uso de disolventes orgánicos.

2.- AGREGADOS MOLECULARES EN PROCESOS DE EXTRACCIÓN ANALÍTICA

2.1. Aspectos generales

Los sistemas supramoleculares son entidades formadas por la asociación de dos o más moléculas unidas mediante enlaces intermoleculares (31). El enlace intermolecular es un término genérico que incluye interacciones muy diversas, tales como interacciones electrostáticas, hidrófobas, enlaces de hidrógeno, enlaces π-π, etc. La característica común de estas interacciones es que no son covalentes y por lo tanto las entidades formadas lo hacen a través de reacciones reversibles aunque existe la idea, cada vez

(28) Barker S.A., *J. Biochem. Biophys. Methods* 70 (2007) 151.

(29) Bogialli S., Di Corcia A. *J. Biochem. Biophys. Methods* 70 (2007) 163.

(30) Anastassiades M. Lehotay S.J. Stajnbaher D., Schenck F.J. *J. AOAC* 86 (2003) 412

(31) Lehn J.M. *Supramolecular Chemistry: Concepts and Perspectives. A Personal Accoun*t, John Wiley & Sons (1995) New York

más generalizada, de que los enlaces covalentes relativamente débiles (por ejemplo los enlaces de coordinación) también pueden producir sistemas supramoleculares (32). Recientemente se ha sugerido que deberían considerarse interacciones intermoleculares todas aquellas con una energía de enlace igual o inferior a 30 Kcal mol^{-1} y en las que el equilibrio cinético se alcance en menos de 24 horas (33). En general, los sistemas supramoleculares son termodinámicamente menos estables, cinéticamente más lábiles y dinámicamente más flexibles que las moléculas (31,34-36).

Los sistemas supramoleculares se clasifican en dos grandes grupos que se denominan supermoléculas y agregados moleculares (37,38). Las supermoléculas son especies oligomoleculares de estructura definida resultantes de la asociación intermolecular de dos o tres componentes. Constituyen los denominados sistemas anfitrión-huésped o receptor-substrato. El anfitrión o receptor es un compuesto macrocíclico (éter corona, éter cripta, ciclodextrina, etc.) que enlaza con el substrato de forma reversible y selectiva, basado en los principios de reconocimiento molecular.

Los agregados moleculares son entidades polimoleculares resultantes de la asociación espontánea de un número indefinido de componentes que constituyen una fase específica con características macroscópicas y organización microscópica más o menos definida, dependiendo de su naturaleza. Los agregados de mayor interés en química analítica son las micelas (acuosas e inversas), las microemulsiones y las vesículas. La característica esencial de estos sistemas supramoleculares es que los componentes que lo integran son moléculas que contienen en su estructura una parte hidrófoba y otra hidrófila y/o contienen centros aceptores o donadores de protones (39). Esta característica va a determinar que en los sistemas supramoleculares existan regiones con diferentes polaridad, acidez y viscosidad las cuales determinarán la región de solubilización del soluto dentro del sistema supramolecular y serán responsables de

(32) Olenyuk B., Whiteford J.A., Fenchtenkotter A., Stang P.J. *Nature* 398 (1999) 796.
(33) Anslyn E. V. *J. Org. Chem. (Perspective)* 72 (2007) 687.
(34) Atwood J.L., Lehn J.M., Davies J.E.D., MacNicol D.D. *Comprehensive Supramolecular Chemistry: 11-Volume Set*, F. Vogtle (Editors), Pergamon Press (1996).
(35) Lehn J.M. *Supramolecular Chemistry: Where it is and where it is going,* Ungaro R., Dalcanale E. (Eds.), Kluwer Dordrecht (1999), pp. 287.
(36) Steed J.W., Atwood J.L. *Supramolecular Chemistry*, John Wiley & Sons (2000) New York.
(37) Fendler J.H. *Membrane Mimetic Chemistry: characterizations and applications of micelles, microemulsions, monolayers, bilayers, vesicles, host-guest systems and polyions.* John Wiley & Sons, (1982) New York
(38) Rubio S., Pérez-Bendito D. *Trends Anal. Chem.* 22 (2003) 470.
(39) Fuhrhop J.H., Koning J. *Membranes and Molecular Assemblies: The synkinetic approach,* The Royal Society of Chemistry (1994) Cambridge

los efectos microambientales que ejercen sobre los sistemas químicos. Estos dos aspectos (solubilización y efectos microambientales) constituyen el fundamento de las aplicaciones analíticas de los sistemas supramoleculares en áreas tan diversas como Espectroscopia, Electroanálisis, Cromatografía, Electroforesis Capilar, Técnicas de Extracción, Análisis Cinético, etc. (38,40) La interfase entre Química Analítica y Química Supramolecular ha dado lugar a un campo interdisciplinar muy fructífero y en continua expansión denominado "Química Analítica Supramolecular" (33,41).

2.2. Metodologías de extracción basadas en el uso de agregados moleculares

El uso de agregados moleculares en procesos de extracción analítica constituye una alternativa prometedora al uso de disolventes orgánicos en extracción líquido-liquido y sólido-líquido y amplía el horizonte de las fases adsorbentes utilizadas en SPE. Los agregados más utilizados hasta la fecha en disolución han sido las micelas acuosas e inversas y sólo recientemente se ha propuesto el uso de vesículas. En fase heterogénea se han usado hemimicelas y admicelas adsorbidas sobre óxidos minerales y, en menor medida, sobre resinas de cambio iónico. En todos los casos, los agregados están constituidos por moléculas de tensioactivos que se asocian de forma espontánea por encima de una concentración crítica. El tipo de agregado producido depende fundamentalmente de su estructura química y de la naturaleza del medio. La Figura 1 muestra una representación simplificada de las estructuras supramoleculares utilizadas como extractantes y la Tabla 1 muestra los tensioactivos más usados hasta la fecha.

Existen diferencias morfológicas y estructurales entre los distintos tipos de agregados producidos en disolución las cuales afectan al proceso de extracción. Así, aunque las micelas acuosas e inversas son entidades esféricas con diámetros muy similares (3-60 y 40-80 Å, respectivamente) las primeras se forman en agua y la segundas en disolventes apolares y de baja constante dieléctrica y por tanto, los medios de extracción utilizados son diferentes. El tamaño de las vesículas es considerablemente mayor (300-5000 Å) y como consecuencia, el número de moléculas de soluto que pueden solubilizarse en las mismas es superior al que se solubilizan en las micelas.

Un aspecto a considerar es que los agregados moleculares son muy estables, una vez formados permanecen inalterados durante semanas. Sin embargo, no son estructuras

(40) Pramauro E., Pelizzetti E. *Surfactants in Analytical Chemistry, Applications of Organized Amphiphilic Media*, Elsevier (1996) New York.

(41) Rubio S., *New Challenges of Supramolecular Chemistry in Environmental Analysis*. 11as Jornadas de Análisis Instrumental (Noviembre 2005) Madrid.

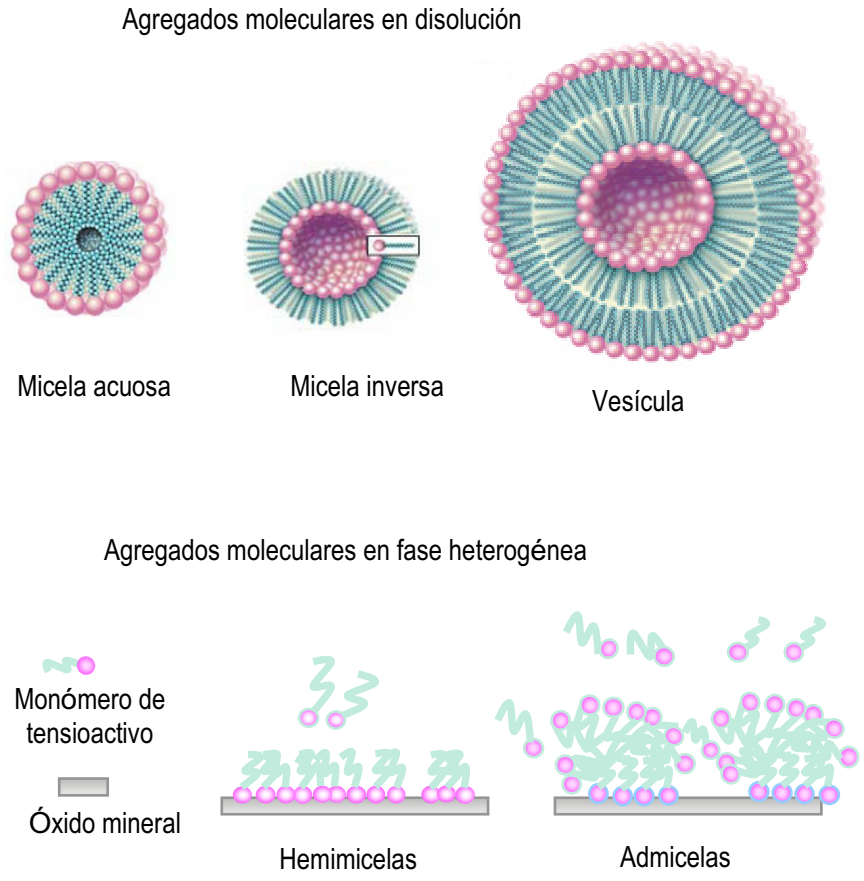


Figura 1. Representación simplificada de diferentes tipos de agregados moleculares

rígidas sino entidades dinámicas en equilibrio con los monómeros de tensioactivo en disolución. La escala de tiempo para la disociación de las micelas, es decir para la liberación de una molécula de tensioactivo y su subsiguiente reincorporación al agregado, es del orden de microsegundos. Por el contrario, los tiempos de residencia del tensioactivo en las vesículas son del orden de minutos a horas y por tanto estos agregados son considerablemente más rígidos.

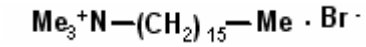
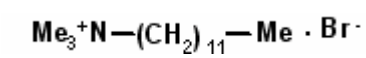
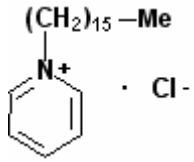
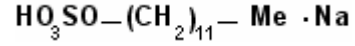
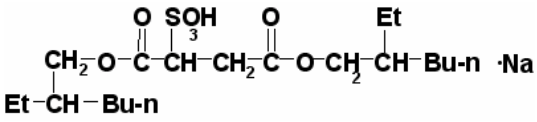
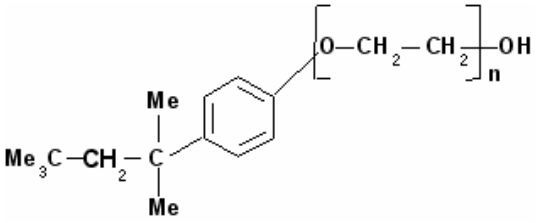
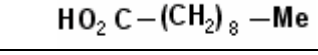
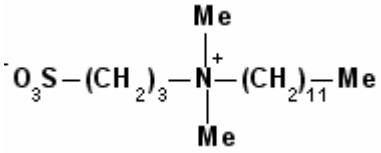
CATIÓNICOS	Bromuro de cetiltrimetil amonio (BrCTA)	
	Bromuro de dodeciltimetil amonio (Br DTA)	
	Cloruro de cetilpiridinio (CPC)	
ANIÓNICOS	Dodecil sulfato sódico (SDS)	
	Bis (2-etilhexil)sulfosuccinato sódico (Aerosol OT)	
NO IÓNICOS	Polioxietileno (9-10) p-teroctil fenol (Tritón X-100)	
	Ácido decanoico	
ANFÓTEROS	3-(dodecildimetilamonio) propano-1-sulfonato (SB-12)	

Tabla 1. Tensioactivos de uso común en extracciones analíticas.

Los agregados hemimicelares se forman cuando moléculas de tensioactivo cargadas se adsorben sobre la superficie de un material (generalmente un óxido mineral) de carga opuesta. La fuerza conductora de la asociación tensioactivo-soporte son las interacciones iónicas, pero la concentración crítica de tensioactivo a partir de la cual comienza la formación de agregados hemimicelares es aquella en la que se establecen interacciones hidrófobas entre las cadenas hidrocarbonadas de las moléculas del tensioactivo adsorbido. La morfología de los agregados resultantes es la de conos invertidos ("teepee" en terminología anglosajona, Figura 1).

Las admicelas se forman cuando toda la superficie del óxido está cubierta por hemimicelas y por tanto la adsorción de nuevas moléculas de tensioactivo se produce a través de interacciones hidrófobas entre las cadenas hidrocarbonadas de los mismos. Su morfología y estructura es semejante a las micelas en disolución y al igual que éstas son estructuras dinámicas en equilibrio con los monómeros de tensioactivo (Figura 1).

Las metodologías de extracción basadas en el uso de agregados moleculares que mayor popularidad han alcanzado son las siguientes:

• *Extracción con micelas acuosas* (M*icellar extraction).* Las disoluciones acuosas micelares se han utilizado como disolventes para la extracción de compuestos orgánicos en matrices sólidas ambientales, fundamentalmente suelos y sedimentos (40,42,43), y para la extracción de una amplia variedad de productos biológicos en membranas celulares y cultivos microbiológicos (44). Más recientemente, las micelas acuosas se están utilizando como disolventes en extracciones asistidas por microondas (microwave assisted micellar extraction) para la solubilización de contaminantes tales como hidrocarburos y plaguicidas en muestras de suelos y alimentos (16)

• *Extracción con micelas inversas (Reverse micellar extraction).* En esta metodología se utiliza como extractante un disolvente apolar que contiene el tensioactivo por encima de la concentración micelar crítica. La metodología ha encontrado amplia aplicación en la extracción de biomoléculas, tales como proteínas y enzimas, debido a la elevada estabilidad de las mismas en el extractante (45). Asimismo, se ha usado para la

(42) Jafvert C.T. *Environ. Sci*. Technol. 25 (1991) 1039

(43) Diallo M.S., Abriola L.M., Weber W.J. Jr. *Environ. Sci*. Technol 28 (1994) 1829.

(44) Hinze W. L *Ordered media in Chemical Separations. ACS Symp. Ser. 342, ACS,* Hinze W.L. and Armstrong D.W. (Eds.) (1987) Washintong DC.

(45) Krishna S.H., Srinivas N.D., Raghavarao K.S., Karanth N.G. *Adv. Biochem. Eng./Biotech.* 75 (2002) 119.

extracción líquido-líquido de metales y compuestos orgánicos en disoluciones acuosas (40). En estas aplicaciones, las micelas inversas constituyen un agente quelatante o formador de pares iónicos que posibilita la extracción de sustancias iónicas y ofrecen un medio con gran capacidad de solubilización debido a las diferentes interacciones que pueden establecer con los analitos.

• *Ultrafiltración micelar (micellar-enhanced ultrafiltration (MEUF))*. Se basa en la adición de agregados moleculares a la muestra. Los analitos se solubilizan en los mismos y seguidamente se separan por ultrafiltración a través de una membrana con un tamaño de poro lo suficientemente pequeño como para bloquear a estos agregados. Esta técnica de separación se ha utilizado principalmente para eliminar contaminantes en aguas, sobre todo metales iónicos (46) y, en menor medida, disolventes orgánicos (47), y sustancias orgánicas, por ejemplo fenoles y colorantes (48,49).

• *Extracción con coacervados.* Se basa en la separación de fases que experimentan los agregados moleculares en disolución mediante la acción de un agente coacervante (cambios en la temperatura de la disolución, el pH, adición de sales, adición de un disolvente en el que los agregados son poco solubles, etc). En el proceso, que ocurre de forma espontánea, se origina una nueva fase de muy bajo volumen (coacervado) que contiene los agregados moleculares y se caracteriza por ser inmiscible con la disolución en la que se originó y por tener una elevada capacidad para solubilizar los analitos. Por tanto, los coacervados pueden aplicarse en extracciones líquido-líquido y sólido-líquido. Durante muchos años, los procesos de extracción analítica basados en coacervación han utilizado casi de forma exclusiva el fenómeno de coacervación de tensioactivos no-iónicos causado al elevarse la temperatura de las disoluciones. Esta metodología, denominada de punto de nube *(cloud-point extraction (CPE))* se ha aplicado fundamentalmente a la extracción de sustancias apolares en muestras acuosas ambientales (38,46, 50-52) y la extracción de quelatos metálicos (53,54). En los últimos

(46) Pramauro E., Prevot A.B., *Recent Res. Develop. in Pure & Appl. Anal. Chem.* 1 (1998) 225.
(47) Christian S.D., Scamehorn J.F., *Surfactant Science Series*, 33 (Surfactant-Bases Sep. Processes) (1989).
(48) Bielska M., Szymanowski J. *Water Res.* 40 (2006) 2027.
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(50) Sosa Ferrera Z., Padron Sanz C., Mahugo Santana C., Santana Rodriguez J.J. *Trends Anal. Chem* 23 (2004) 469
(51) Carabias-Martinez R., Rodriguez-Gonzalo E., Moreno-Cordero B. Perez-Pavon J.L., Fernandez-Laespada E. *J. Chromatogr. A* 902 (2000) 251.
(52) Halko R., Hutta M. *Chemicke Listy*, 94 (2000) 990.
(53) Bezerra M., Arruda M.A., Ferreira S.L. *Applied Spectrosc. Reviews* 40 (2005) 269.
(54) Stalikas C.D. *Trends Anal. Chem.* 21 (2002) 343.

años, se han descrito nuevos procesos de coacervación y se han abordado nuevas aplicaciones que amplían de forma sustancial las prestaciones y las áreas de aplicación de estos extractantes. En la introducción de la Parte I de esta Memoria se describe de forma más exhaustiva esta técnica de extracción.

• *Extracción con hemimicelas y admicelas.* Estos agregados moleculares se han utilizado durante algún tiempo para la extracción en fase sólida de metales pesados en muestras acuosas. Para ello, el metal forma complejos con agentes quelatantes, previamente adsolubilizados en las hemimicelas o admicelas (55-57), o se forma el quelato en la disolución acuosa como un paso previo a la adsolubilización (58). Recientemente, nuestro grupo de investigación ha demostrado las excelentes prestaciones de estos agregados para la SPE de contaminantes orgánicos en muestras acuosas ambientales (59,60). En la introducción de la Parte II de esta memoria se realiza una descripción pormenorizada de los aspectos teóricos y prácticos relacionados con estos agregados.

2.3. Características de los agregados moleculares relevantes para la extracción

Los agregados moleculares presentan un conjunto de características de gran relevancia para su aplicación en procesos de extracción. Entre estas características, destacan las siguientes:

• S*e generan mediante fenómenos de autoensamblaje que están al alcance de cualquier laboratorio.* El autoensamblaje es un proceso en el que se produce la unión espontánea de componentes moleculares, nanoscópicos o macroscópicos en agregados perfectamente estructurados, estables, y unidos mediante interacciones no covalentes (61). El autoensamblaje molecular es un proceso omnipresente en química, en la ciencia de los materiales y en biología, a pesar de que sólo recientemente se ha convertido en un campo de estudio con entidad propia y una nueva estrategia de síntesis (62,63).

(55) Hiraide M., Iwasawa J., Kawaguchi H. *Talanta*, 44 (1997) 231.
(56) Manzoori J.L, Sorouraddin M.H., Shabani A.M. H. J. *Anal. Atomic Spectrometry* 13 (1998) 305.
(57) Manzoori J.L, Sorouraddin M.H., Shabani A.M. H. *Microchem. J.* 63 (1999) 295
(58) Hiraide M., Hori J. *Anal. Sci.* 15 (1999) 1055.
(59) Merino F., Rubio S. Pérez-Bendito D. *Anal. Chem.* 75 (2003) 6799.
(60) Merino F., Rubio S. Pérez-Bendito D. *Anal. Chem.* 76 (2004) 3878.
(61) Atwood J.L., Steed J.W. (Eds) *Encyclopedia of Supramolecular Chemistry,* Marcel Dekker (2004) New York.
(62) Lehn J.M., Ball P. *New Chemistry*, Ed. Hall, N. (Cambridge Univ. Press, Cambridge, U.K.) (2000) pp. 300.
(63) Philip D., Stoddart J.F. *Angew. Chem.* (International Edition in English) 35 (1996) 1155.

• *Están formados por moléculas anfifílicas que son ubicuas en la naturaleza y en la síntesis química.* El número de estructuras disponibles es muy elevado, lo que permite seleccionar la más adecuada para la aplicación de interés. Se pueden obtener agregados a la carta variando el grupo polar o la cadena hidrocarbonada del compuesto anfifílico que lo constituye. La mayoría de estas moléculas son biodegradables y el coste suele ser bajo.

• *Tienen estructura tridimensional y presentan regiones de diferente polaridad* que muestran excelente capacidad de solvatación para una gran variedad de compuestos orgánicos e inorgánicos.

• *Poseen capacidad para actuar como multiligandos* debido a la presencia de múltiples grupos polares en el agregado.

• *Tienen baja volatilidad e inflamabilidad*, lo que hace que los procesos de extracción sean menos contaminantes y más seguros.

• *La mayoría de los agregados son compatibles con los sistemas de separación y detección usados en los procesos analíticos.*

• *Estabilizan los compuestos solubilizados* lo cual se ha aprovechado para simplificar e integrar los procesos de toma de muestra, transporte, conservación, extracción y concentración de contaminantes químicos en el ambiente (64,65).

3.- ALTERADORES ENDOCRINOS

3.1. Conceptos básicos

Dentro del Programa Internacional de Protección frente a los Productos Químicos (PIPPQ), en el que participan la Organización Mundial de la Salud (OMS), el Programa de las Naciones unidas para el Medio Ambiente (PNUMA) y la Organización Internacional del Trabajo (OIT), se ha acordado, en colaboración con especialistas de Japón, EE.UU., Canadá, la Organización para la Cooperación y Desarrollo Económico (OCDE) y la Unión Europea, definir a los alterados endocrinos como aquellas sustancias exógenas o

(64) Luque N., Rubio S., Pérez-Bendito D. *J. Chromatogr. A* 1094 (2005) 17.
(65) Luque N., Rubio S., Pérez-Bendito D. *Anal. Chim. Acta* 584 (2007) 181.

combinaciones de ellas, que alteran las funciones del sistema endocrino y, por lo tanto, tienen efectos perjudiciales para la salud de un individuo, de su descendencia o de poblaciones enteras (66).

El sistema endocrino está formado por una serie de glándulas como el tiroides, las gónadas y las glándulas suprarrenales que, mediante las hormonas que producen (tiroxina, estrógenos, testosterona, adrenalina, etc.), regulan multitud de funciones básicas como el desarrollo, el crecimiento, la reproducción y el comportamiento de personas y animales. Las hormonas, que actúan a muy bajas concentraciones (pg/L ó ng/L), son transportadas en el flujo sanguíneo hasta diferentes órganos o células diana, donde cumplen funciones específicas, o son segregadas dentro de un órgano o de un tejido y actúan localmente.

Los alteradores endocrinos pueden interferir en el funcionamiento del sistema endocrino a través de tres vías:

a) Mimetizando la acción de las hormonas naturales para producir reacciones químicas similares en el organismo.
b) Bloqueando los receptores hormonales de las células diana lo que imposibilita la acción de las hormonas naturales.
c) Interfiriendo en la síntesis, el transporte, el metabolismo y la secreción de hormonas naturales con la consiguiente alteración de sus concentraciones.

3.2. Sustancias alteradoras del sistema endocrino

Los compuestos químicos con actividad hormonal se clasifican en cuatro grupos (67):

• *Hormonas naturales* como los estrógenos, la progesterona y la testosterona, que se encuentran de forma natural en el organismo de seres humanos y animales, se liberan de forma no intencionada en el ambiente (ej. en las aguas residuales municipales) y pueden afectar a la fauna de los cauces en los que se vierten los efluentes.

(66) Comunicación de la Comisión de las Comunidades Europeas al Consejo y Parlamento Europeo. Estrategia Comunitaria en materia de alteradores endocrinos- sustancias de las que se sospecha interfieren en los sistemas hormonales de seres humanos y animales- COM (1999) 706.

(67) (http://ec.europa.eu/environment/endocrine/definitions/endodis_en.htm)

● *Sustancias naturales* que incluyen las toxinas producidas por partes concretas de las plantas (los también llamados fitoestrógenos) y ciertos hongos. Fitoestrógenos, como los producidos en brotes de alfalfa o semillas de soja tienen algunos efectos beneficiosos para la salud humana (ej. previenen las enfermedades cardiovasculares, osteoporosis y algunos tipos de cáncer) y además se cree que estas sustancias naturales se excretan rápidamente y no experimentan bioacumulación. Los riesgos en la actualidad pueden derivar de los cambios en los hábitos alimenticios que conlleven la ingesta de mayores cantidades de estos alimentos (66).

● *Hormonas sintéticas*. Su estructura es idéntica a la de las hormonas naturales (por ej. los anticonceptivos orales, los tratamientos de sustitución hormonal, algunos aditivos de los alimentos para animales, etc.) y se han diseñado con la intención de alterar y regular el sistema endocrino.

● *Compuestos químicos.* Sintetizados para ser usados en la industria (p. ej., como agentes de limpieza), la agricultura (p. ej. plaguicidas) y en bienes de consumo (p. ej., en algunos aditivos plásticos), etc. Este grupo también incluye subproductos de los procesos industriales como las dioxinas. La actividad hormonal de estos compuestos es muy débil comparada con la de las hormonas naturales.

3.3. Fuentes y efectos de la exposición

Los alteradores endocrinos se liberan al medioambiente de forma intencionada (ej. los plaguicidas) o durante la producción, uso o desecho de un compuesto químico (ej. materiales de consumo, fugas desde vertederos, etc.). Los compartimentos ambientales en los que se hallan los alteradores endocrinos, así como las vías de exposición para el ser humano y la fauna son múltiples (Figura 2). Por tanto, cualquier muestra tomada en estos compartimentos es susceptible de ser analizada para evaluar su contribución a la contaminación global y determinar su incidencia en los seres humanos y la fauna.

Los alimentos constituyen la principal vía de exposición. Diferentes estudios indican que los productos de consumo diario tales como la carne, el pescado, la leche, etc. son las fuentes de exposición más importantes para el ser humano en los países desarrollados. Los compuestos lipofílicos y persistentes a menudo se bioacumulan en los eslabones más altos de la cadena alimenticia. Así, en especies de pájaros y de mamíferos marinos que se alimentan de peces se han encontrado concentraciones de compuestos orgánicos persistentes mucho más elevadas que las halladas en los peces ingeridos o en el agua en la que habitan. En ocasiones los niveles son cientos o millones de veces más elevados (68). Es en estos animales donde más alteraciones se han observado.

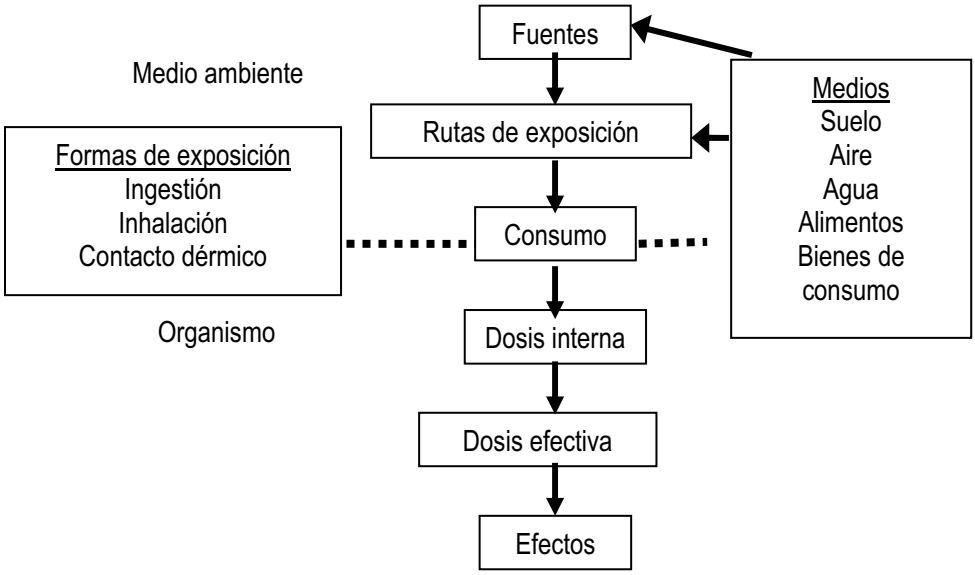

Figura 2. Principales rutas de exposición a los alteradores endocrinos

Los problemas de salud originados por los alteradores endocrinos no es un fenómeno reciente. Un ejemplo es el uso del dietilestilbestrol, iniciado a finales de la década de los 30, para prevenir los abortos en las mujeres y estimular el crecimiento del ganado. En la década de los 70 y 80 se demostró que causaba problemas graves en los aparatos reproductores masculino y femenino, incluyendo anomalías y cánceres congénitos. Era el primer ejemplo documentado de una sustancia química que, administrada a la madre, puede causar cáncer en su descendencia femenina.

El Comité Científico de la Toxicidad, Ecotoxicidad y Medio Ambiente de la Comisión Europea (SCTEE), en su dictamen de 4 de marzo de 1999 (66), revisó la bibliografía y los dictámenes científicos publicados en relación a las alteraciones endocrinas ocasionadas

(68) SEPA (2001) Miljöövervakning. Available: http://www.environ.se

por sustancias químicas. Su conclusión respecto a los efectos sobre la salud humana fue que "existe una relación entre las sustancias químicas alteradoras de los procesos endocrinos hasta ahora estudiadas y los trastornos de la salud humana como los cánceres de testículo, mama y próstata, la disminución del número de espermatozoides, las deformidades de los órganos reproductores, las disfunciones tiroideas, y los problemas neurológicos y relacionados con la inteligencia".

Respecto a los efectos sobre la fauna el Comité concluye que "existen pruebas contundentes obtenidas a partir de estudios de laboratorio que muestran el potencial de varias sustancias químicas presentes en el medio ambiente para causar alteraciones endocrinas a niveles de exposición posibles desde el punto de vista medioambiental" y que "aunque la mayoría de los efectos observados afectan a zonas gravemente contaminadas, hay posibilidades de que el problema se produzca a escala mundial".

Por otra parte, se han documentado alteraciones en la reproducción y el desarrollo en una serie de especies animales, que han afectado a poblaciones locales o regionales (66). Algunas de estas alteraciones son: la virilización de hembras de moluscos marinos a causa del tributilestaño; el adelgazamiento de la cáscara de los huevos de aves a causa del diclorodifeniletileno; las alteraciones de las funciones reproductora e inmunitaria en focas debidas a policlorodifenilos en la cadena alimenticia; las distorsiones en el desarrollo y las funciones de los órganos sexuales de los aligatores debido a un grave vertido de plaguicidas en una laguna.

Por lo general, la vulnerabilidad de una determinada especie depende de las propiedades intrínsecas de la sustancia química; del grado, la duración, la frecuencia y la vía de exposición, así como de la forma en que esa especie absorba, distribuya, transforme y elimine las sustancias. Por otra parte, también depende de la sensibilidad de órganos concretos en diversas etapas del desarrollo.

Las pautas de los efectos de los alteradores endocrinos varían de una especie a otra y de una sustancia a otra. Sin embargo pueden formularse 4 enunciados generales:

a) Las sustancias químicas de interés pueden tener efectos totalmente distintos sobre el embrión, el feto o el organismo perinatal y sobre el adulto.
b) Los efectos se manifiestan con mayor frecuencia en las crías, no en el progenitor expuesto.
c) El momento de la exposición en el organismo en desarrollo es decisivo para determinar su carácter y su potencial futuro.
d) Aunque la exposición crítica tiene lugar durante el desarrollo embrionario, las manifestaciones obvias pueden no producirse hasta la madurez. La vigilancia de los

alteradores endocrinos en una generación debe ser durante años e incluso décadas ya que las dosis que llegan al feto dependen no sólo de lo que ingiere la madre durante el embarazo, sino también de los contaminantes persistentes acumulados en la grasa corporal hasta ese momento de su vida. Las mujeres transfieren esa reserva química acumulada durante décadas a sus hijos durante la gestación y durante la lactancia (69, 70).

3.4. Estrategias de acción y legislativas en la Unión Europea

En la legislación vigente de la Unión Europea, la evaluación del potencial tóxico y las medidas legislativas para un compuesto químico depende del tipo de compuesto y no de su actividad endocrina. No obstante, la creciente preocupación social por el efecto de estas sustancias ha promovido el desarrollo de estrategias europeas específicas para el estudio y evaluación de los alteradores endocrinos.

Las acciones de la Unión Europea en relación a los alteradores endocrinos se iniciaron en 1996 cuando la Comisión patrocinó un encuentro en Weybridge (UK) para debatir el potencial impacto de los mismos en la salud humana y ambiental y donde se acordó un plan integrado para la futura investigación y actividades en este área (Informe Weybridge). En octubre de 1998, el Parlamento Europeo adoptó una Resolución en la que instaba a la Comisión a iniciar acciones para mejorar el marco legislativo, reforzar los esfuerzos en investigación y transmitir la información al público.

En 1999, el Comité Científico de la Toxicidad, Ecotoxicidad y Medio Ambiente de la Comisión Europea (SCTEE) publicó el estudio "Human and wildlife health effects of endocrine disrupting chemicals with emphasis on wildlife and ecotoxicology test methods," y la Comisión, para dar una respuesta rápida a las recomendaciones realizadas en este estudio, estableció la estrategia marco para el estudio de alteradores endocrinos en la Unión Europea (66). En esta estrategia se contemplan acciones a corto, medio y largo plazo.

A corto plazo, la principal actividad de la Comisión ha sido el establecimiento de una lista prioritaria de sustancias con el fin de evaluar su potencial como alteradores endocrinos y

(69) Andrade-Ribeiro A.L.F.A., Pacheco-Ferreira A., Lóbrega da Cunha C.L., Mendeskling A.S. *Revista Biomédica* 17 (2006) 14.

(70) Labropoulou D.A., Albanis T.A. *J. Chromatogr. A* 1061 (2004) 11.

determinar sus efectos. Entre los años 2000 y 2006, se han evaluado 575 sustancias en tres etapas (71-73). De estas sustancias, 320 mostraron evidencias de causar efectos por alteración del sistema endocrino, 109 se descartaron debido a insuficientes datos sobre sus efectos y 147 no se han evaluado debido a que se identificaron como dobles entradas o mezclas de dudosa relevancia. La evaluación del status legal de las sustancias consideradas como alteradores endocrinos muestra que la mayoría de ellas ya están sujetas a prohibición o restricción en la actual legislación comunitaria, aunque por razones no necesariamente relacionadas con sus efectos endocrinos.

En relación a las acciones a medio plazo, la Comisión y los Estados Miembros continúan participando en la *OECD-Endocrine Disrupter Testing and Assessment Task Force (EDTA),* la cual se estableció en 1998 con el objetivo de armonizar a nivel internacional las estrategias de evaluación del potencial endocrino de los compuestos químicos así como de coordinar y supervisar el trabajo de diferentes grupos encargados de desarrollar nuevos métodos o revisar los existentes. Existen ya diferentes métodos para evaluar los efectos de los alteradores endocrinos en la salud humana (ensayos *in vivo,* uterotróficos, etc*)* y ambiental (ensayo metamorfosis en anfibios, screening en pescado, etc.). Estos métodos están en la actualidad en periodo de validación bajo los auspicios de la OECD (Anexo III, 73).

Paralelamente, la investigación sobre alteradores endocrinos ha tenido un fuerte apoyo en el 5º y 6º Programas Marco de la Unión Europea (FP5 1999-2001 y FP6 2002-2006). Durante el FP5 se subvencionaron 23 proyectos con un coste total de 60 millones de euros. Los resultados de los mismos pueden consultarse (74). Con el objetivo de aunar esfuerzos, se creó en 2003 el cluster CREDO (Cluster of Research into Endocrine Disruption in Europe) que integraba a 63 laboratorios europeos (75) y cuyas

(71) Comunicación de la Comisión de las Comunidades Europeas al Consejo y Parlamento Europeo. *Aplicación de la Estrategia Comunitaria en materia de alteradores endocrinos -sustancias de las que se sospecha interfieren en los sistemas hormonales de seres humanos y animales- (COM (1999) 706).* COM (2001)262.

(72) Documento de trabajo de la Comisión de las Comunidades Europeas sobre *Implementación de la Estrategia Comunitaria en materia de alteradores endocrinos -sustancias de las que se sospecha interfieren en los sistemas hormonales de seres humanos y animales- (COM (1999)706)* SEC (2004) 1372

(73) Documento de trabajo de la Comisión de las Comunidades Europeas sobre *Implementación de la Estrategia Comunitaria en materia de alteradores endocrinos -sustancias de las que se sospecha interfieren en los sistemas hormonales de seres humanos y animales- (COM (1999)706, COM (2001)262, SEC (2004) 1372)* SEC (2007) 1635

(74) http://ec.europa.eu/research/quality-of-life/ka4/ka4_reports_en.html

(75) http://ec.europa.eu/research/endocrine/projects_clusters_en.html

investigaciones han contribuido de forma importante a mejorar nuestro conocimiento sobre alteraciones endocrinas. En el FP6 se ha continuado subvencionando la investigación en este área (50 millones de euros en 10 proyectos). Un proyecto relevante en este Programa es el CASCADE que se desarrollará en el periodo 2004-2010 con un presupuesto de 14.4 millones de euros y la participación de 24 grupos de investigación procedentes de 9 países europeos. CASCADE se centrará en la investigación de residuos químicos en alimentos, con especial énfasis en los alteradores endocrinos (76). Las subvenciones para la investigación sobre alteradores endocrinos continuarán en el 7º Programa Marco (2007-2013) dentro del tema 6 "Environment". Las investigaciones y actividades relacionadas con los alteradores endocrinos dentro de la comunidad europea pueden consultarse en la correspondiente página web (77).

Las acciones a largo plazo incluyen la revisión y adaptación de la legislación europea vigente con el objeto de tener regulación específica para los alteradores endocrinos. Los avances más relevantes en este contexto han sido la adopción de REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) que entró en vigor el 1 de Junio de 2007 (1), el establecimiento de estándares de calidad ambiental para sustancias prioritarias (78) dentro de la directiva marco del agua (79), y la propuesta para revisar la directiva 91/414/EC (80) relacionada con el uso de productos para la protección de los cultivos.

En REACH se regula el procedimiento de autorización para sustancias químicas de alto riesgo con el objetivo de proteger la salud humana y el medio ambiente a la vez que se protege la competitividad de la industria europea. En REACH se contemplan 1,350 sustancias químicas "de elevado riesgo" que son o probablemente son carcinógenas, mutágenas o tóxicas para el sistema reproductor; persistentes, bioacumulables y tóxicas (PBTs) o muy persistentes y muy bioacumulables (vPvBs). El uso de sustancias PBTs o vPvBs no será autorizado a menos que no haya una alternativa disponible o los beneficios socio-económicos pesen más que el riesgo.

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(76) http://www.cascadenet.org

(77) http://ec.europa.eu/research/endocrine/index_en.html

(78) Propuesta de Directiva del Parlamento Europeo y del Consejo de las Comunidades Europeas relativa a las *normas de calidad ambiental en el ámbito de la política de aguas y por la que se modifica la Directiva 2000/60/CE* COM (2006) 397.

(79) Directiva del Parlamento Europeo y del Consejo de las Comunidades Europeas de 23 de Octubre de 2000 por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas 2000/60/EC

(80) Directiva del Consejo de las Comunidades Europeas de 15 de Julio de 1991 relativa a la comercialización de productos fitosanitarios 91/414/CEE

Si un compuesto químico presenta riesgos inaceptables será prohibido. La Comisión de la UE decidirá dentro de seis años si los alteradores endocrinos (AEs) también son "de elevado riesgo" y por tanto deben estar sujetos a requerimientos similares.

Los alteradores endocrinos se incluyen ya en la directiva marco del agua de la UE (79) como el grupo 4 (Anexo VIII). Esto significa que existe obligación por parte de los estados miembros de prevenir la exposición humana a este tipo de sustancias a través del ambiente acuático y para ello deben establecer un programa de medida en las cuencas fluviales a partir del 2009 que debe estar completamente operativo en 2012. A nivel de legislación europea, y dentro del establecimiento de estándares de calidad ambiental, se han establecido recientemente límites para 33 sustancias de las que 21 muestran evidencia o potencial evidencia de efectos endocrinos (78).

En relación al uso de productos para la protección de los cultivos (80), la Comisión Europea inició en 1992 un proceso de revisión de todos los ingredientes activos utilizados en Europa para tal fin con el objeto de asegurar que éstos son inocuos para la salud humana y ambiental. El proceso se completará en 2008 y aunque el efecto endocrino de estos ingredientes activos no se ha incluido en la evaluación de riesgos, debido fundamentalmente a la falta de metodologías adecuadas, se han requerido ensayos adicionales si existe sospecha de efectos endocrinos. La consideración de la alteración endocrina de un compuesto como elemento de prohibición para su uso en el tratamiento de cultivos se ha adoptado por la Comisión y será tenida en cuenta en la revisión de la directiva 91/414/EC (78) a no ser que la exposición humana al compuesto sea despreciable en las condiciones reales de uso.

3.5 Alteradores endocrinos investigados

3.5.1 Criterios de selección

Los alteradores endocrinos que han constituido el objeto de las investigaciones que se presentan esta Memoria [Estrona (E_1), 17-β-estradiol (E_2), etinilestradiol (EE_2) y bisfenol A (BPA)] pertenecen a tres de los cuatro grupos (hormonas naturales y sintéticas y compuestos químicos) en los que se clasifican las sustancias con actividad hormonal (ver sección 3.2). En la Figura 3 se muestran las estructuras correspondientes. No se han incluido en este estudio alteradores endocrinos pertenecientes al grupo de las *sustancias naturales* ya que se excretan rápidamente y no experimentan bioacumulación. Las hormonas naturales investigadas (E_1 y E_2) son los estrógenos que se encuentran en mayor proporción en el plasma sanguíneo. Son importantes para mantener la salud de los tejidos reproductivos, pecho, piel y cerebro. Dentro del grupo de las hormonas sintéticas se ha seleccionado EE_2 por su amplio uso como anticonceptivo y porque junto

con el E_2 es la sustancia con mayor actividad estrogénica y por consiguiente con mayor capacidad alteradora del sistema endocrino. Como compuesto químico con actividad hormonal se ha seleccionado BPA debido a su elevado volumen de producción mundial y amplio uso. Este compuesto se utiliza como monómero en la fabricación de plásticos policarbonatados y resinas epoxi. Los primeros se emplean en la fabricación de discos compactos, lentes, electrodomésticos, embalaje de comida, recubrimiento de partes eléctricas y electrónicas y botes de plástico. Tienen alta resistencia al impacto, dureza, transparencia, resistencia a temperaturas entre ~ -40 ºC y ~ 145 ºC y resistencia a muchos ácidos y aceites. Las resinas epoxi se usan como recubrimientos en latas y contenedores metálicos para alimentos y bebidas, en la industria del automóvil, como adhesivos, en la industria aeroespacial, etc.

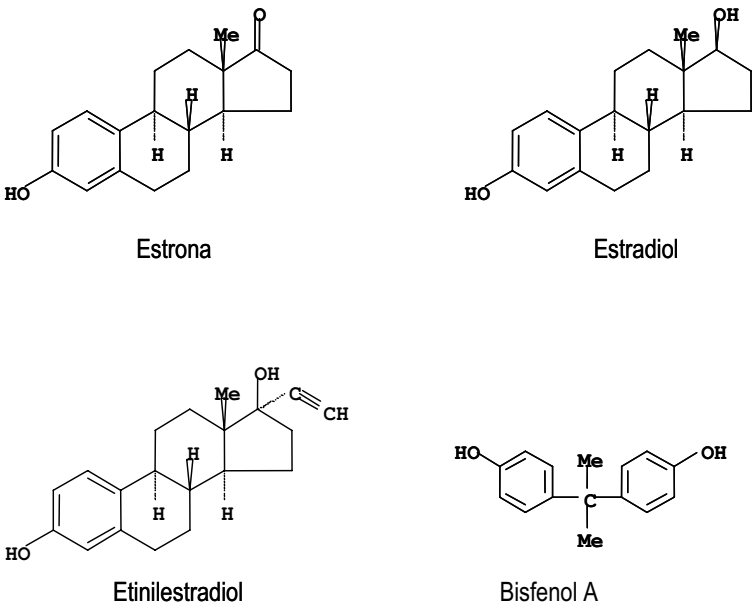

Figura 3. Estructuras de los alteradores endocrinos investigados

3.5.2 Propiedades físico-químicas

Los alteradores endocrinos seleccionados son compuestos hidrófobos (los correspondientes coeficientes de distribución octanol-agua, log K_{ow}, oscilan entre 3.25 y 4.15), no volátiles (debido a sus bajas presiones de vapor) y se encuentran en forma neutra en el medio natural (ya que los valores de pK_a son próximos a 10) (ver Tabla 2). En su estructura existen anillos aromáticos y grupos hidroxilo y/o cetónicos y por tanto, la extracción/concentración de estos analitos en las muestras de interés puede estar gobernada por interacciones hidrófobas, π-catión y/o puentes de hidrógeno, dependiendo de las características del extractante seleccionado.

Nombre	**Log $K_{O/W}$**	**Presión de vapor** (mmHg)	**pK_a**
Estrona	3.43	$2.3x10^{-10}$	10.3
17β- estradiol	3.94	$2.3x10^{-10}$	10.4
Etinilestradiol	4.15	$4.5x10^{-11}$	10.3
Bisfenol A	3.25	$4.0x10^{-8}$	9.73

Tabla 2. Propiedades físico-químicas de los alteradores endocrinos estudiados

3.5.3 Actividad estrogénica

A pesar de que cualquier glándula endocrina puede verse afectada por los alteradores endocrinos, la mayoría de los estudios realizados hasta la fecha están relacionados con su carácter estrogénico, es decir con la capacidad de los mismos de mimetizar o bloquear el efecto de los estrógenos. La potencia estrogénica de los alteradores endocrinos es muy variable y abarca desde mimetizadores tan potentes como el estrógeno natural E_2 a débiles agonistas que tan sólo tienen actividad parcial y a muy altas concentraciones. La Tabla 3 muestra la actividad estrogénica relativa de los alteradores endocrinos seleccionados en el presente estudio, considerando la potencia estrogénica de E_2=100 (81,82).

Compuesto	Potencia estrogénica relativa
17β- estradiol	100
Estrona	25
Etinilestradiol	125
Bisfenol A	0.012

Tabla 3. Potencia estrogénica relativa de los alteradores endocrinos investigados

El estrógeno sintético etinilestradiol es el alterador endocrino con mayor potencia estrogénica. BPA se ha considerado hasta hace poco tiempo como un estrógeno débil ya que tiene una potencia estrogénica aproximadamente cuatro órdenes de magnitud inferior a E_2. Sin embargo, numerosos estudios in vitro realizados recientemente han demostrado que BPA puede alterar la función normal del sistema endocrino y producir perturbaciones en las funciones celulares a concentraciones tan bajas como 0.23 ng L^{-1} (83).

3.5.4 Fuentes de contaminación

Existen varias rutas a través de las que BPA puede llegar al medio ambiente, principalmente relacionadas con su producción y fabricación de productos de consumo (84). Los niveles de BPA en aguas superficiales y residuales varían en un amplio intervalo (por ejemplo entre 5-5000 ng L^{-1} en diferentes ríos, canales y lagos alemanes; y entre 20-700 ng L^{-1} en diferentes aguas residuales alemanas (85)). En general, los niveles de BPA en ambientes acuáticos suele ser inferior a 1 µg L^{-1} (84).

(81) A WWF European Toxics Programme Report. *Bisphenol A: A Known Endocrine Disruptor* (2000).
(82) Beck I.C., Bruhn R., Gandrass J. *Chemosphere* 63 (2006) 1870.
(83) Calafat, A.M., Ye X., Wong L.Y., Reidy J.A., Needham L.L. *Environ. Health Perspect.* 116 (2008) 39
(84) Staples C.A., Dorn P.B., Klecka G.M., O`Block S.T., Hairis L.R. *Chemosphere* 36 (1998) 2149.
(85) Fromme H., Küchler T, Otto T, Pilz K, Müller J. Wenzel A. *Water Res*. 36 (2002) 1429

El consumo de alimentos embotellados y enlatados constituye la principal vía de exposición humana a BPA ya que este compuesto, como se ha comentado con anterioridad, se utiliza en la fabricación de plásticos policarbonatados y resinas epoxi y migra a los alimentos envasados (carne, pescado, verdura, bebidas, miel, leche en polvo para bebés, etc.) a concentraciones entre 0.1 y 384 ng g^{-1}) (86). Estudios recientes realizados por el National Center for Environmental Health de US han mostrado que en un 92.6% de las muestras de orina humana analizadas hay niveles detectables de BPA (entre 0.4 y 149 µg L^{-1}) (83). Considerando estos datos y teniendo en cuenta que la vida media de BPA en orina es de 12 a 48 h, puede concluirse que la exposición humana a BPA es continua.

Las hormonas naturales y sintéticas presentes en medios acuáticos proceden fundamentalmente de las aguas residuales domésticas urbanas. Este tipo de compuestos se han detectado tanto en influentes como en efluentes de estaciones depuradoras de aguas residuales de diversos países del mundo (87). Las hormonas, estradiol y estrona son excretadas de forma natural por el ser humano. En concreto las mujeres excretan 2-12 y 3-20 µg/persona/día, respectivamente. Etinilestradiol se excreta por las mujeres que están sometidas a tratamientos anticonceptivos. Otra fuente importante de estrógenos son los residuos del ganado (ovejas, cerdos, aves de corral, etc.), ya que con frecuencia se administra al ganado este tipo de compuestos para controlar el periodo de celo, para tratar desórdenes reproductivos e inducir abortos (88). Por ello es posible encontrar estrógenos en aguas superficiales y subterráneas procedentes de tierras de cultivos abonadas con estiércol animal (89-91).

(86) Thomson B.M, Grounds P.R. *Food Addit.. Contam.* 22 (2005) 65
(87) Ying G.G., Kookana R.S., Ru Y. *J. Environ. Int.* 28 (2002) 545.
(88) Refsdal A.O. *Animal Reproduction Science* 60/61 (2000) 109.
(89) Bushée E.L., Edwards D.R., Moore P. A. Trans ASAE 41 (1998) 1035.
(90) Nichols D.J., Daniel T.C., Edwards D.R., Moore Jr P.A., Pote D.H. *J. Soil Water Conserv* . 53 (1998) 74.
(91) Peterson E. W., Davis R. S., Orndorff H. A. J Environ Qual 29 (2001) 826.

3.5.5 Legislación Europea

Hasta la fecha sólo dos de los alteradores endocrinos investigados en esta Memoria están sujetos a legislación específica en la Comunidad Europea. Así, el uso de 17β-estradiol y sus derivados de tipo éster está prohibido para la cría y el tratamiento de ganado dado su carácter cancerígeno (92). Esta hormona aparece en el cuadro 3 del anexo 1 de la COM (2001): sustancias de las que se tienen pruebas que confirman su capacidad –efectiva o potencial- para causar alteraciones endocrinas (71).

En relación a BPA existen dos tipos de límites oficiales:

a) *El límite de migración*, que especifica la cantidad de compuesto que se permite migrar del envase al alimento. Las directivas Europeas 80/590, 82/711 y 90/128 (93-95) resumen las regulaciones europeas sobre polímeros que entran en contacto con alimentos. El Comité científico de la Comisión Europea ha establecido como límite de migración en alimentos 600 ng g^{-1} (96).

b) *Ingesta diaria tolerable (TDI)*, que es una estimación de la cantidad de contaminante, expresada en base al peso corporal, que puede ser ingerida diariamente durante toda la vida sin riesgo apreciable para la salud. La TDI establecida a nivel europeo para bisfenol A es de 50 µg por kilogramo de peso corporal y día.

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(92) Directiva del Consejo de las Comunidades Europeas, de 29 de abril de 1996 por la que se prohíbe utilizar determinadas sustancias de efecto hormonal y tireostático y sustancias â-agonistas en la cría de ganado y por la que se derogan las Directivas 81/602/CEE, 88/146/CEE y 88/299/C; 96/22/CEE

(93) Directiva del Consejo de las Comunidades Europeas, de 9 de junio de 1980, relativa a la determinación del símbolo que puede acompañar a los materiales y objetos destinados a entrar en contacto con productos alimenticios, 80/590/CEE

(94) Directiva del Consejo de las Comunidades Europeas, de 18 de octubre de 1982 que establece las normas de base necesarias para la verificación de la migración de los constituyentes de los materiales y objetos de materia plástica destinados a entrar en contacto con productos alimenticios 82/711/CEE

(95) Directiva del Consejo de las Comunidades Europeas, de 23 de febrero de 1990, relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios 90/128/CEE

(96) Directiva de la Comisión de las Comunidades Europeas, de 1 de Marzo de 2004, por la que se modifica la directiva 2002/72/CEE relativa a los materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios 2004/19/CEE

Parte I. Extracción coacervativa de bisfenol A en alimentos enlatados y muestras biológicas

1.- OBJETO

El efecto de la exposición humana a bajas concentraciones de bisfenol A es en la actualidad objeto de intensas investigaciones y debates, como se ha comentado en la introducción general de esta Memoria. Diferentes estudios han demostrado que la exposición humana a este contaminante es general y continua y que el consumo de alimentos enlatados constituye la principal vía de exposición, lo que ha conducido a legislación específica sobre los niveles máximos de migración permitidos. Tanto la evaluación de la exposición como el control del cumplimiento de la legislación vigente requieren el uso de métodos analíticos que, además de proporcionar datos de calidad, sean simples, robustos, de bajo costo y al alcance de cualquier laboratorio.

Las investigaciones que se presentan en la parte I de esta Memoria tienen como objeto proporcionar una estrategia general y simple para el tratamiento de muestras requerido para la determinación de bisfenol A en alimentos enlatados y orina. Para ello se propone el uso de coacervados de micelas inversas de ácido decanoico, cuyas características son idóneas para la extracción de este contaminante en muestras complejas. Entre estas características destacan las siguientes:

1) Puede solubilizar bisfenol A mediante interacciones hidrófobas y puentes de hidrógeno lo que facilitará su extracción.
2) La concentración de decanoico en el coacervado es muy elevada ($\sim$0.6 mg μL^{-1}), lo que permite el uso de bajos volúmenes de extractante. Como resultado se puede obviar la etapa de evaporación del extractante que generalmente se realiza para alcanzar los límites de detección requeridos.
3) El coacervado es compatible con cromatografía de líquidos y detección UV, fluorescente y espectrometría de masas.
4) La formación del coacervado es simple, sólo requiere mínimas cantidades de ácido decanoico (100-200 mg) y bajos volúmenes de THF (1-2 mL). Por lo tanto, su uso está al alcance de cualquier laboratorio.
5) Generalmente se usan pequeñas cantidades de muestra lo que facilita el proceso de tratamiento de las mismas.

A continuación se presentan aspectos generales de la extracción coacervativa y los resultados de las investigaciones que se han desarrollado para la extracción de bisfenol A en alimentos enlatados y orina.

2.- EXTRACCIÓN COACERVATIVA

2.1 Descripción

La coacervación es un fenómeno descrito por los químicos holandeses Bungenberg de Jong y Kruyt en 1930. Ellos acuñaron el término coacervado, del latín "co" que significa junto y "acervum" que se refiere a un montón de cosas menudas. La investigación sobre coacervados a lo largo del siglo XX ha tenido grandes discontinuidades, con décadas de relativa inactividad. En la década de los 70, el bioquímico ruso Oparin popularizó el fenómeno fuera del ámbito coloidal cuando propuso que la vida se inició en gotas de coacervados. En la actualidad, la mayoría de la información que existe sobre los mismos se encuentra dispersa en investigaciones de interés en química coloidal, química de polímeros, físico-química, y en la industria farmacéutica y alimentaria.

La IUPAC define la coacervación como un fenómeno que consiste en la separación de sistemas coloidales en dos fases líquidas (1). A la fase más concentrada en el componente coloidal se le denomina coacervado; la otra fase tiene muy baja concentración en este componente y se denomina fase en equilibrio. Un coacervado es por definición inmiscible en su propio disolvente. Las estructuras macromoleculares que experimentan coacervación son muy variadas, tal como se muestra en la Tabla 1.

PROTEINAS	POLISACÁRIDOS	POLÍMEROS SINTÉTICOS
Gelatina	Goma arábiga	Acetato de polivinilo
β-lactoglobulina	Pectina	Polivinilpirrolidona
Caseína	Quitosan	Hidroxipropilcelulosa
Albúmina	Alginato	Isobutileno-isopreno
Glicinina		Poli(diaquil)dimetilamonio

DROGAS	AGREGADOS MOLECULARES
Heparina	Tensioactivos
Gentamicina	Fosfolípidos
Morfina	Sales biliares

Tabla 1. Tipos de macromoléculas que experimentan coacervación

(1) IUPAC *Compendium of chemical terminology* 31 (1972) 611.

Los coacervados se han utilizado en múltiples aplicaciones (ej. purificación de proteínas, recuperación de acuíferos contaminados, tratamiento de aguas residuales, etc.), pero la aplicación de mayor interés a nivel industrial está relacionada con la tecnología de microencapsulamiento. Para la producción de las microcápsulas se usan macromoléculas de origen natural tales como proteínas y polisacáridos. Esta tecnología se ha empleado en gran extensión para la microencapsulación de ingredientes activos en áreas tales como farmacia, agricultura, alimentación y cosmética (2). 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.

2.1.1 Mecanismos y cinética de separación de fases

De acuerdo al mecanismo de separación de fases, se distinguen dos tipos de coacervación: simple y compleja (3,4).

En 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 que causa la coacervación puede ser un cambio en el pH o la temperatura, la adición de una determinada cantidad de electrolito, o la adición de un volumen de disolvente, miscible con el primero, pero en el que la macromolécula es poco soluble.

El mecanismo básico por el que se produce la separación de fases en coacervación simple es la desolvatación de la macromolécula (3). 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. Como resultado, el disolvente forma una capa alrededor de las macromoléculas que impide o limita la interacción entre las mismas. El agente inductor de la coacervación simple tiene como función destruir la interacción disolvente-macromolécula favoreciendo de este modo la interacción entre macromoléculas. Los agregados formados son insolubles y se separan del disolvente produciendo el coacervado.

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

(3) Wang, Y., Kimura K., Huang Q., Dubin P.L., Jaeger W. *Macromolecules* 32 (1999) 7128

(4) Mohanty B., Bohidar, H.B. *Biomacromolecules* 4 (2003) 1080

La coacervación compleja (4) se produce cuando a disoluciones acuosas de macromoléculas cargadas se le adiciona 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. Ahora bien, se deben cumplir una serie de condiciones para que se favorezca la coacervación frente a la precipitación. Así, 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. Cuando se produce la interacción, el complejo resultante retiene contraiones y un grado considerable de moléculas de disolvente. La solvatación parcial del complejo es una de las razones por la que se obtienen coacervados en lugar de productos insolubles.

La coacervación es por naturaleza un proceso cinético (5). Los modelos generales usados para describir la cinética de separación de fases son nucleación y crecimiento, y descomposición espinodal (Figura 1). En el primero se produce interacción entre macromoléculas cercanas que a continuación experimentan coalescencia hasta que

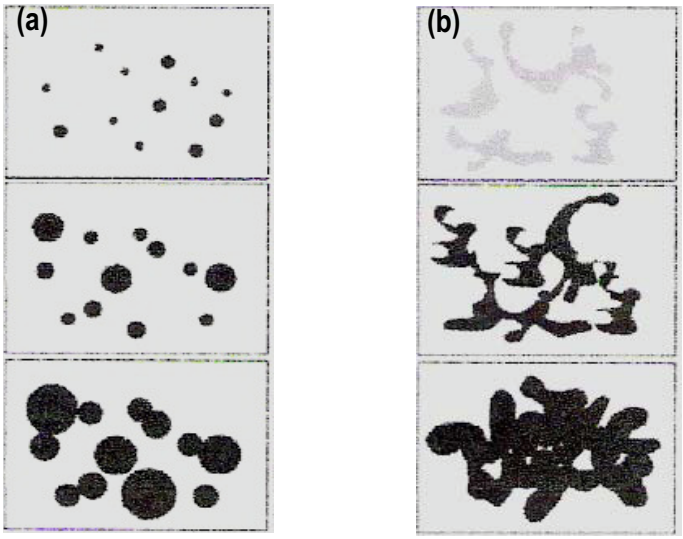

Figura 1. Cinéticas de separación de fases: (a) modelo de nucleación y crecimiento (b) modelo de descomposición espinodal.

(5) Turgeon S.L., Beaulieu M., Schmitt C., Sanchez C. Current Opinión Colloid Interface Sci. 8 (2003) 401.

alcanzan un determinado tamaño y se produce separación de fases (Figura 1a). A nivel macroscópico, el coacervado está generalmente constituido por gotas esféricas dispersas en una fase continua (Figura 2a). En el modelo de descomposición espinodal (Figura 1b) la interacción implica a muchas macromoléculas en la etapa inicial, produciendo a nivel macroscópico una red tridimensional interconectada, que se hace progresivamente más densa (Figura 2b).

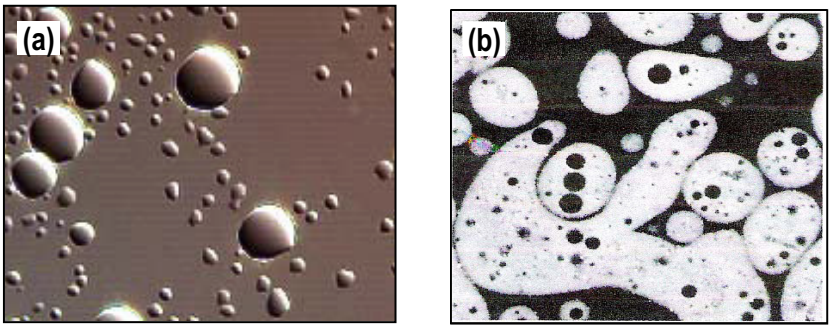

Figura 2. Coacervado compuesto por (a) ácido decanoico+ tetrahidrofurano, estructura globular; (b) β-lactoglobulina+ goma arábiga, estructura de red interconectada.

2.1.2 Diagrama de fases

Para describir el comportamiento de sistemas en equilibrio donde se generan diferentes fases se recurre a los denominados diagramas de fases, que son representaciones gráficas donde se muestra el comportamiento del sistema en función de diferentes variables. Concretamente, en el proceso de coacervación es muy importante determinar los valores relativos (concentraciones, temperatura, etc.) de los componentes del sistema a los que ocurre la separación de fases con objeto de determinar los límites en los que se obtiene el coacervado.

Como se ha comentado con anterioridad el proceso de coacervación en química analítica se produce a partir de agregados moleculares de tensioactivos en presencia de un agente deshidratante (coacervación simple), por ello son necesarios al menos dos componentes para que se genere el coacervado. En la Figura 3 se muestra el diagrama de fases de una disolución acuosa de un tensioactivo no iónico (tritón X-114) en función de la temperatura, fenómeno al que tradicionalmente se ha denominado *cloud point.* La temperatura a la que ocurre este fenómeno se denomina punto de nube. La denominada curva de coexistencia separa dos regiones; en la región L el tensioactivo forma micelas acuosas, mientras en la región L-L coexisten dos fases: el coacervado, constituido por

micelas acuosas de grandes dimensiones y la fase en equilibrio, donde el tensioactivo se encuentra en forma monomérica a la concentración micelar crítica.

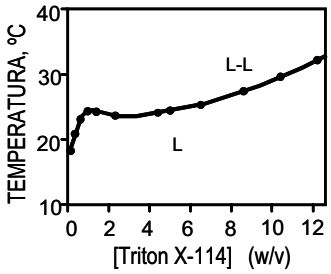

Figura 3. Diagrama de fases para el tensioactivo no iónico tritón X-114 en disolución acuosa.

El diagrama de fases de una disolución de agregados de tensioactivo que experimenta coacervación depende de la naturaleza del mismo y del agente coacervante. Así, los tensioactivos anfóteros, al contrario que los no iónicos, experimentan coacervación a bajas temperaturas. Como ejemplo, la Figura 4a muestra el diagrama de fases de un tensioactivo anfótero, C_9-$APSO_4$ (3-(nonildimetilamonio propil sulfato) en disolución acuosa (6).

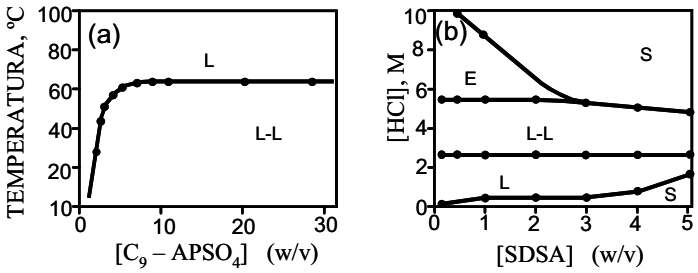

Figura 4. Diagrama de fases para los tensioactivos (a) 3-(nonildimetilamonio propil sulfato y (b) dodecano sulfonato sódico.

(6) Rubio S., Pérez-Bendito D. *Trends Anal. Chem*. 22 (2003)470.

Las disoluciones de micelas aniónicas acuosa constituidas por alquil sulfatos, alquil sulfonatos y alquil sulfosucinatos, que experimentan coacervación inducida por ácidos a temperatura ambiente, presentan diagramas de fases más complejos (7,8). En la Figura 4b se muestra el diagrama de fases de micelas acuosas de dodecano sulfonato sódico, SDSA. En este caso se definen cuatro regiones en las que se observa: una fase líquida homogénea (L), la región de coacervación (L-L), una emulsión (E) y una región constituida por dos fases una de ellas líquida y la otra sólida (S).

En general, los diagramas de fases se modifican en presencia de aditivos tales como sales, disolventes orgánicos, etc. por lo que este hecho debe tenerse en cuenta en los procesos de extracción basados en fenómenos de coacervación. Así la adición de aditivos a una disolución acuosa de Tritón X-100 puede producir un aumento o una disminución de la temperatura del punto de nube (Tabla 2).

Aditivo	Temperatura de punto de nube (ºC)
Ninguno	63.7
Urea 0.3 M	65.7
Urea 0.5 M	68.1
Azida sódica 2% (pH 7)	61.0
Cloruro sódico 0.5 M (pH 7)	26.0
Cloruro sódico 1.0 M (pH 7)	47.0

Tabla 2. Influencia de la presencia de aditivos en la temperatura de punto de nube del tensioactivo Tritón X-100

Los tensioactivos anfóteros, por lo general, exhiben el comportamiento opuesto al observado en los no iónicos, es decir la adición de una sal provoca un aumento en la temperatura del punto de nube.

Los diagramas de fases de tensioactivos iónicos también pueden modificarse por la presencia de aditivos. Como ejemplo en la Figura 5 se muestran los diagramas de fases de disoluciones acuosas ácidas de sodio dodecilsulfato (SDS), a una concentración del 5% en función de la concentración de sal y la temperatura.

(7) Casero I., Sicilia D., Rubio S., Pérez-Bendito D. *Anal. Chem*. 71 (1999) 4519.

(8) Sicilia D., Rubio S., Pérez-Bendito D. Maniasso N, Zagatto E.A.G. *Anal. Chim*. Acta 392 (1999) 29.

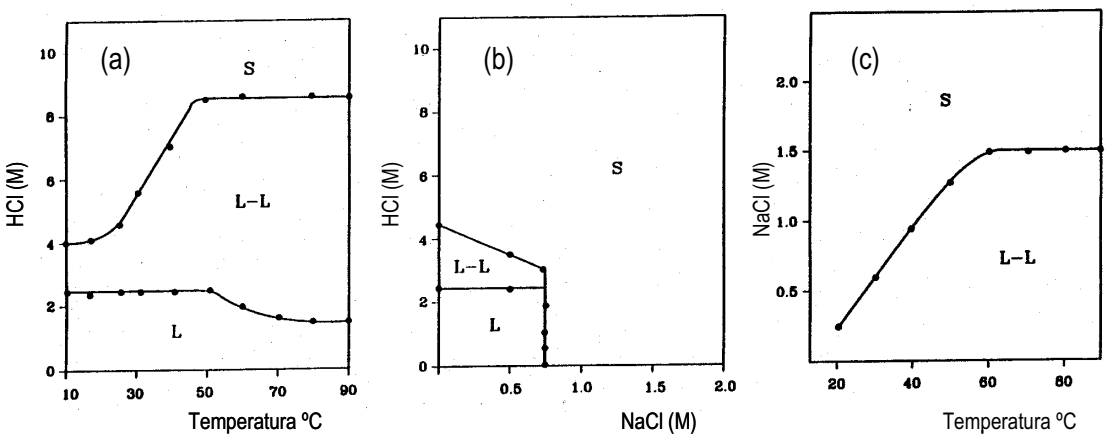

Figura 5. Diagramas de fases para SDS al 5% a) [HCl] frente a temperatura, b) [HCl] frente a [NaCl] y c) [NaCl] frente a temperatura en presencia de HCl 4 M.

2.2 Mecanismos de solubilización/extracción de los analitos

La solubilización de analitos en las macromoléculas que forman el coacervado constituye la base de su aplicación en procesos de separación. Teniendo en cuenta que el coacervado esta formado por agregados tridimensionales de sustancias anfifílicas, éstos presentan diferentes regiones donde se pueden solubilizar analitos de muy diferente polaridad y carga. Así, los analitos hidrófobos se solubilizarán en la región hidrocarbonada de las macromoléculas que conforman el coacervado debido exclusivamente a interacciones hidrófobas mientras los analitos iónicos y polares se solubilizarán en la región polar a través de diferentes interacciones iónicas, π-cation, enlaces por puentes de hidrógeno, etc. Por otro lado, los solutos con carácter anfifílico formarán agregados mixtos con el coacervado mediante interacciones con los grupos polares e hidrófobos de los tensioactivos que las constituyen.

No existe uniformidad en la bibliografía acerca de la definición del coeficiente de partición o de la constante de equilibrio que puede usarse para representar la solubilización de los componentes de una disolución en sistemas supramoleculares (9).

(9) Christian S.D., Scamehorn F.J. (Eds.). *Solubilization in Surfactant Agreggates, Surfactant Science Series 55*, Marcel Dekker (1995.) New York, USA..

Para aplicaciones analíticas, uno de los procedimientos más comunes ha sido calcular un coeficiente de distribución (D), que viene dado por la siguiente expresión matemática:

$$D = [A]_t / [A]_{aq}$$

donde $[A]_t$ y $[A]_{aq}$ son las concentraciones, en la fase rica de tensioactivo y en la fase acuosa, respectivamente.

Alternativamente, es posible relacionar la solubilización con la constante de equilibrio de la siguiente reacción:

$$\text{Tensioactivo}_{\text{coacervado}} + \text{Soluto}_{\text{agua}} \leftrightarrows \text{Tensioactivo-soluto}_{\text{coacervado}}$$

En este caso, la transferencia del soluto al coacervado pude considerarse como una reacción en la que se origina un producto (9,10) cuya constante de equilibrio viene dada por:

$$K_s = \frac{[\text{Soluto}]_{\text{coaervado}}}{[\text{Tensioactivo}]_{\text{coacervado}} \cdot [\text{Soluto}]_{\text{agua}}}$$

Los corchetes indican la concentración molar medida con respecto al volumen total de la disolución.

2.3 Modo de operación

El modo de operación cuando se trabaja en extracción líquido-líquido o sólido-líquido usando coacervados consta de dos etapas (Figura 6):

a) Formación del coacervado. Esta etapa puede llevarse a cabo en presencia o ausencia de la muestra que contiene el analito o analitos a determinar. Si la muestra es sólida puede formarse el coacervado previamente mezclando la

(10) Hinze W.L. and Armstrong D.W. (Eds.) *Ordered media in Chemical Separations, ACS Symposium Series 342,* American Chemical Society (1987) Washintong DC,USA.

disolución coloidal del tensioactivo y el agente coacervante en un tubo de vidrio especialmente diseñado para este tipo de aplicaciones; y a continuación se añade la muestra. Si la muestra es líquida, el tensioactivo generalmente se añade a ésta a concentraciones por encima de la concentración micelar crítica y se establecen las condiciones de coacervación.

b) Extracción del analito o analitos. La mezcla se agita durante un determinado tiempo con objeto de favorecer la transferencia del analito o analitos a la fase rica y a continuación se centrifuga para acelerar la separación del coacervado y la fase en equilibrio. Los analitos extraídos generalmente se determinan mediante la inyección de una alícuota de coacervado en un cromatógrafo de líquidos.

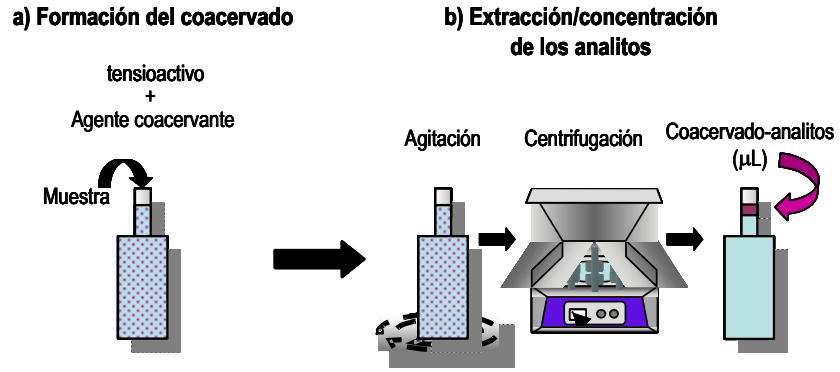

Figura 6. Modo de operación cuando se trabaja en extracción liquido-líquido con coacervados

2.4 Antecedentes

Las aplicaciones de los coacervados en procesos de extracción analítica han estado restringidas durante muchos años a la extracción de sustancias hidrófobas en muestras acuosas ambientales utilizando tensioactivos no iónicos. Esta metodología, denominada punto de nube (cloud-point extraction), presenta algunos inconvenientes asociados al tipo de tensiactivos empleados (ej. tritón X-100; la serie PONPE, eteres polietileneglicolmono-4-nonilfenol; etc) tales como la elevada temperatura necesaria para que se produzca el fenómeno y la elevada absorción en la región ultravioleta.

A pesar de estos inconvenientes, los coacervados constituidos por micelas acuosas de tensioactivos no iónicos se han usado para la extracción de una gran variedad de

compuestos tales como proteínas (11,12); iones metálicos, previa formación de complejos neutros(13); y compuestos orgánicos apolares tales como PAHs (14), PCBs (15); ácidos húmicos y fúlvicos (16); plaguicidas (17); etc .

La descripción de coacervados constituidos por micelas acuosas de tensioactivos catiónicos (18) y aniónicos (19) eliminó algunos de los inconvenientes asociados al uso de tensioactivos no iónicos. Así, fue posible la extracción de compuestos en un mayor intervalo de polaridad mediante su solubilización en los grupos polares de las micelas a través de diferentes interacciones (electrostáticas, π-catión, formación de agregados mixtos, etc.). Basada en la coacervación de tensioactivos catiónicos se ha propuesto la extracción/concentración de clorofenoles (18). En este caso las eficiencias de extracción son muy elevadas ya que además de interacciones hidróbobas entre los analitos y las micelas se producen interacciones π-catión entre el anillo aromático de los clorofenoles y el grupo amonio del tensioactivo. El inconveniente de estos coacervados es la elevada concentración de electrolito necesaria para que se produzca la coacervación (400 g/L de NaCl), lo que ha restringido su uso.

La coacervación de micelas acuosas de tensioactivos aniónicos, inducida por ácido, fue propuesta por nuestro grupo de investigación con objeto de evitar la coelución de los analitos polares y los tensioactivos en el sistema cromatográfico (7). La ausencia de anillos aromáticos en la estructura de los tensioactivos y su gran polaridad ha permitido la aplicación de estos coacervados a la extracción de un gran número de contaminantes y posterior determinación de los mismos mediante cromatografía líquida combinada con detección UV, fluorescente y espectrometría de masas. Las condiciones ácidas drásticas en las que se producen, alrededor de 3 M, han sido de gran utilidad para el tratamiento de muestras sólidas, aplicaciones en los que estos coacervados han encontrado especial relevancia.

(11) Bouvier T., Etges R.J., Bordier C.J., *J. Biol. Chem.* 260 (1985)15504.
(12) Saitoh T., Hinze W.L. *Anal. Chem.* 63 (1991) 2520.
(13) Chen J., Teo K.C., *Anal. Chim. Acta* 434 (2001)325.
(14) Ferrer R., Beltrán J.L., Guiteras J. *Anal. Chim. Acta* 330 (1996)199.
(15) Eiguren-Fernández A., Sosa-Ferrera Z. Santana-Rodriguez J.J. *Quim. Anal.* 16 (1997) 283.
(16) Revia R.L., Makharadze G.A.; *Talanta* 48 (1999) 409.
(17) García-Pinto C., Pérez-Pavón J.L., Moreno-Cordero B. *Anal. Chem.* 67 (1995) 2696.
(18) Jin X, Zhu M., Conte E.D. *Anal. Chem.* 71 (1999) 514.

Entre las aplicaciones desarrolladas destacan la extracción de PAHs en suelos, lodos y sedimentos (19) y sobre todo la extracción de sustancias anfifílicas tales como tensioactivos aniónicos (alquibenceno sulfonatos) (20), catiónicos (alquil amonio) (21) y no iónicos (alquilfenol polietoxilados y alcohol etoxilados) (22) en lodos. La formación de agregados mixtos entre los tensioactivos analitos y el tensioactivo aniónico que constituye el coacervado ha permitido la extracción de los mismos con elevada eficiencia y factores de preconcentración entre 50 y 100.

En los últimos años, una de las líneas prioritarias de investigación en nuestro grupo ha sido el desarrollo de nuevos coacervados que permitan el establecimiento de diferentes interacciones con los analitos, alcancen elevados factores de preconcentración y se produzcan en condiciones experimentales suaves. En este contexto destacan los coacervados constituidos por micelas inversas (23) y vesículas (24) de ácidos alquilcarboxílicos, inducidos por agua y tetrabutilamonio, respectivamente. Además de las interacciones que permiten establecer (hidrófobas, puentes de hidrógeno, iónicas y Π-catión), un aspecto clave de estos coacervados es la elevada concentración de ácidos alquilcarboxílicos que contienen (entre 0.7 y 1 mg μL^{-1}) lo que permite alcanzar elevados rendimientos utilizando pequeños volúmenes de los mismos. Como consecuencia, se alcanzan factores de preconcentración entre 100 y 400. Por otro lado, los coacervados de micelas inversas se producen a pH inferiores a 4 y los de vesículas en el intervalo de pH entre 5 y 10, abarcando un amplio intervalo en el que se puede seleccionar el valor más apropiado para la extracción, dependiendo de las características de los analitos.

En la Tabla 3 se comparan las recuperaciones obtenidas empleando coacervados de vesículas y micelas inversas para diferentes analitos, clasificados respecto al tipo de interacción predominante que se produce con el tensioactivo que forma el coacervado. Como puede observarse, prácticamente en todos los casos se obtienen recuperaciones cuantitativas a excepción de la extracción de alquilbenceno sulfonatos y difenzoquat cuando se utilizan coacervados de micelas inversas y vesículas, respectivamente.

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(20) Ruiz F.J., Rubio S., Pérez-Bendito D. *J.Chromatogr. A* 1030) (2004) 109.
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(23) Ruiz F.J., Rubio S., Pérez-Bendito D. *Anal. Chem.* 79 (2007) 7473.
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Tipo de interacción	Analito	[Analito](M)	Recuperaciones (%)	
			Vesículas	*M. inversas*
Hidrófoba	Naftaleno	3.9×10^{-5}	99	95
	Antraceno	2.8×10^{-5}	101	98
	Benzo(a)pireno	2.0×10^{-5}	100	97
Formación de agregados mixtos	Tritón X-100	8.3×10^{-6}	99	98
	Alquilbenceno sulfonatos	2.9×10^{-5}	100	5
Enlaces de hidrógeno	4-clorofenol	3.9×10^{-5}	95	97
	2,4-diclorofenol	3.2×10^{-5}	100	99
	2,4,6-triclorofenol	2.6×10^{-5}	98	101
	Pentaclorofenol	1.9×10^{-5}	100	101
	Bisfenol A	3.8×10^{-5}	99	95
	Bisfenol F	3.6×10^{-5}	100	97
	Bencilbutilftalato	5.1×10^{-5}	80	99
	Dibutilftalato	4.8×10^{-5}	85	100
	Paratión	4.1×10^{-5}	90	96
	Atracina		98	95
	Hidroquinona sulfonada	5.2×10^{-5}	--------	95
Iónica	Hidroquinona sulfonada	5.2×10^{-5}	95	----
	Azul brillante de cumasín	7.3×10^{-7}	100	-----
	Difenzoquat	6.9×10^{-6}	50	----

Tabla 3. Recuperaciones, expresadas en porcentajes, obtenidas en la extracción coacervativa de diferentes compuestos orgánicos usando coacervados de vesículas de ácido decanoico (C_{10}) y micelas inversas de ácido octanoico (C_8)

Hasta la fecha, estos coacervados se han aplicado a la extracción de bisfenoles A y F y los correspondientes diglicidil ésteres en aguas residuales (25) y a la extracción de contaminantes en alimentos líquidos (26).

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Capítulo 1. Decanoic acid reverse micelle-based coacervates for the microextraction of bisphenol A from canned vegetables and fruits

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Decanoic acid reverse micelle-based coacervates for the microextraction of bisphenol A from canned vegetables and fruits

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Abstract

Decanoic acid reverse micelle-based coacervates were proposed for the extraction of bisphenol A (BPA) from canned vegetables and fruits prior to its determination by liquid chromatography and fluorescence detection at λ_{exc}= 276 nm and λ_{em}=306 nm. The procedure involved the extraction of minute quantities (300-700 mg) of homogenized food sample with an aqueous solution containing 10% of THF and 0.5% of decanoic acid, conditions under which the coacervate (around 340 µL) formed *in situ* and instantaneously. The overall sample treatment, which included extraction and centrifugation, took about 25-30 min, and several samples could be simultaneously treated using conventional lab equipment. No clean-up or solvent evaporation were required. Extraction efficiencies mainly depended on the decanoic acid and THF concentration in the aqueous solution and were not affected by the pH or the temperature in the ranges studied (1-4 and 20-60ºC, respectively). Recoveries in samples ranged between about 81 and 96%. The precision of the method, expressed as relative standard

deviation, was about 3% and the quantitation limit was around 9 ng g^{-1}, which was far below the current specific migration limit (SML) set for BPA by the EU Commission (600 ng g^{-1}). The method was successfully applied to the determination of BPA in the solid content of canned fruit salad, peaches in syrup, mango slices, red peppers, sweetcorn, green beans and peas. BPA was present at concentrations in the range from 7.8 to 24.4 ng g^{-1}in canned fruits and from 55 to 103 ng g^{-1}in canned vegetables.

Introduction

Epoxy resins are widely used as internal coatings in canned food thus preventing the metal from the can corroding and migrating into the can contents. Bisphenol A (BPA) is a major component of epoxy resins [1]. The residual non-polymerized BPA easily migrates into the food during the thermal treatment required for its sterilization [2]. Because of the estrogenic character of BPA [3-5], a specific migration limit (SML) of 600 ng g^{-1} was set by the EU Commission in 2004 [6] in order to ensure consumer health protection.

The consumption of fruits and vegetables plays a vital role in providing a diversified and nutritious diet [7]. Canned vegetables are a convenient staple for evening meals and make up about 10% of total vegetable human consumption [8]. Canned fruits accounts for about 7% of total fruit intake [9]. According to the results obtained by different researchers, the migration of BPA into this type of foods causes their contamination at concentration levels around 20-100 ng g^{-1} [10-18]. Although these values are below the SML for BPA, the low-dose reproductive and developmental effects of this contaminant is currently the focus of a strong debate in the scientific community, the conclusions going from no risk to human health [11] to the need for a new risk assessment [4]. So, the determination of BPA in food is required for both the control of the compliance of current legislation and the assessment of human exposure.

Quantification of BPA in canned foods has frequently been performed by gas chromatography/mass spectrometry (GC/MS) [2,12,19] however, the need for derivatisation has fostered the use of liquid chromatography (LC) coupled to fluorimetry [15,17,20-22] or mass spectrometry (MS) [14,23]. Sample treatment is always the bottleneck of these methods since tedious, solvent-consuming and rather impracticable procedures are used.

Three main strategies have been proposed for the extraction of BPA from canned vegetables and fruits so far. The first one involves the use of repetitive extractions with high volumes of organic solvents (e.g. 270 mL of methanol [17] or 40 mL of acetonitrile [11,18]), subsequent evaporation to dryness and redissolution with a low-volume, chromatographically compatible, solvent. In the second strategy, the organic solvent

extraction, which still involves large solvent volumes (e.g. 100 mL of acetonitrile for treatment of 5 g of sample [14,15]) is followed by two clean-up steps consisting in the rinse of the acetonitrile phase with 75 mL of *n*-hexane and subsequent solid phase extraction (SPE) in Oasis HLB (hydrophilic-lipophilic balance) cartridges [14,15]. The use of MS for BPA detection simplifies this procedure (e.g. 25 mL of acetonitrile are used and the rinse with *n*-hexane becomes unnecessary [24]). Recently, sol-gel immunoaffinity columns made up of BPA antibodies immobilized in a silica matrix have been proposed for the clean-up of the acetonitrile extracts (2 mL) obtained after duplicate extraction of 1 g of canned vegetables and fruits [13,25]. Sol-gel pre-columns have to be used to prevent the clogging of the immunoaffinitty columns from small particles originating in the sample extracts. This strategy reduces drastically the volume of organic solvent required for sample treatment, but the need to prepare the columns makes it rather time consuming and complicated for routine analysis. Because of the strong demand for environmentally friendly methods that permit high sample throughput, this research assesses the use of reverse micelle-based coacervates for the extraction of BPA from canned vegetables and fruits.

Coacervates are water immiscible liquids that separate from colloidal solutions under the action of a desolvating agent, mainly changes in the temperature or the pH of the colloidal solution or the addition of electrolytes or a non-solvent for the macromolecule [26,27]. Their application to analytical extractions was proposed by Watanabe et al. a long time ago [28,29], and for many years it focused on the use of non-ionic micelle-based coacervates for the extraction of hydrophobic compounds from environmental waters [30-32]. In recent years, the development of coacervates made up of zwitterionic [33], anionic [34] and cationic [35] aqueous micelles, and reverse micelles [36] and vesicles [37] have extended significantly the scope of these extractants with regard to both the polarity range of compounds that can be extracted [36,37] and the samples that may be analysed (e.g. soil and sediment [38], sludge [39,40], etc).

Coacervates have intrinsic properties that greatly benefit extractions. Thus, because of the special structure of the supramolecular assemblies making them up, they are multifunctional solvents with the ability to establish multiple bonds with solutes, which

results in very high extraction efficiencies. Also, the high concentration of surfactant in some coacervates (e.g. around 0.7-1 mg μL^{-1} in the reverse micelle- and vesicle-based coacervates) [36,37] permits good solute recovery using minute coacervate volumes, which results in low detection limits without the need to evaporate extracts. On the other hand, the basis and procedures of coacervative extraction are similar to those of conventional solvent-based extraction, and like the latter, it uses conventional lab equipment, which makes their implantation easy. All these properties make that coacervative extraction has a great potential to simplify sample treatment in food analysis.

This paper focus on the use of decanoic acid reverse micelle-based coacervates for the extraction of BPA from canned vegetables and fruits using minute amounts of food sample and coacervate. The aim was to develop a simple, rapid and low cost method suitable for the routine control of this contaminant. The research included the study of the parameters affecting the extraction efficiency of BPA, the study of the quantitative performance of the method using LC-FL and its application to the determination of BPA in several samples of canned vegetables and fruits purchased in local supermarkets.

Experimental

Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Bisphenol A (BPA) [$(CH_3)_2C(C_6H_4OH)$] and decanoic acid [$CH_3(CH_2)_8COOH$] were obtained from Fluka (Madrid, Spain). Tetrahydrofuran, hydrochloric acid and HPLC-grade acetronitrile were purchased from Panreac (Barcelona, Spain). Stock solutions of BPA (0.5 g L^{-1}) were prepared in acetonitrile and stored under dark conditions at 4ºC not more than three months. Working solutions were made by appropriate dilution of the stock solution with acetonitrile. Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain).

Apparatus

The liquid chromatrographic system used (Spectra System SCM1000, ThermoQuest, San Jose, CA, USA) consisted of a P4000 quaternary pump, a UV6000LP diode-array detector and a FL3000 fluorescence detector. In all experiments, a Rheodyne 7125 NS injection valve, with a 20 μL sample loop, was used. The stationary-phase column was a Hypersil ODS C_{18} column (5μm, 4.6 x 150 mm) from Análisis Vinicos (Tomelloso, Spain). A Mixtasel Selecta centrifuge (Barcelona, Spain) was used for sample preparation.

Samples

Canned vegetables (red peppers, sweet corn, green beans and peas) and fruits (fruit salad, peaches in syrup and mango slices) were bought in supermarkets in Córdoba (Spain). The unopened cans were stored at room temperature. After the cans were opened, the liquid part was removed by sieving and the solid portion was homogenized using a food mixer. Every sample was analyzed in triplicate using 300 mg aliquots. Samples no immediately analyzed were stored at -20ºC in vinyl bags. The type of the internal coatings of the cans was not investigated.

Coacervative microextraction of BPA

Decanoic acid (200 mg) was dissolved in THF (4 mL) in a specially designed glass tube with a narrow neck (tube: 25 mm high, 34 mm i.d.; neck; 63 mm high, 8 mm i.d.). Then, 36 mL of 1.3 mM hydrochloric acid aqueous solution were added. Immediately, the coacervate phase separated from the bulk solution. Once the food sample (300 mg) was added to this mixture, it was stirred at 1200 rpm for 15 minutes to favor BPA extraction and then, centrifuged at 3800 rpm (2200xg) for 10 min to accelerate phase separation. Next, the volume of the coacervate, which was standing at the narrow neck of the glass tube, was measured with a digital calliper. Finally, 20 μL were withdrawn with a microsyringe and analysed.

Liquid chromatography/Fluorimetry

Quantification of BPA and separation from matrix components was carried out by liquid chromatography-fluorimetry. The mobile phase consisted of water and acetonitrile. The flow-rate was 1 mL min^{-1}. The chromatographic conditions for analysis of canned fruits were: 50:50 water:acetonitrile. The retention time for BPA was 4.0 min and the corresponding capacity factor (k '), calculated from $k' = (t_R - t_0)/t_0$ where t_0 is the retention time of acetone (hold-up time), was 1.7. The analysis of canned vegetables was carried out using the following elution program: linear gradient from 70:30 to 45:55 (water:acetonitrile) in 13 minutes and then, isocratic initial conditions (70:30) to clean and stabilize the chromatographic system. BPA eluted to a retention time of 9.3 min. The selected wavelengths were 276 nm (excitation) and 306 nm (emission). Calibration was run by injecting 20 µL of standard solutions in acetonitrile containing between 0.14 and 20 ng. Quantification was performed by measuring peak areas. Correlations between peak areas and BPA amount were in the range 0.9995-0.99990.

Results and discussion

Reverse micelle-based coacervative extraction of bisphenol A

General considerations. Decanoic acid (pK_a= 4.8±0.2) is sparingly soluble in water. It dissolves in THF by forming reverse micelles with different critical aggregation concentrations (e.g. 4.8, 7.6 and 51mM) according to a sequential-type self-association model [36]. The addition of water to THF solutions containing decanoic acid reverse micelles causes the partial desolvation of the aggregates, which makes easier micelle-micelle interaction and leads to the formation of bigger aggregates. As a result, these aggregates become insoluble in the water:THF solution and separate as an immiscible liquid. The Figure 1 depicts the region encompassed by the coacervate as a function of THF and decanoic acid concentrations. THF percentages below and above the

boundaries of this region caused precipitation and solubilization of decanoic acid respectively.

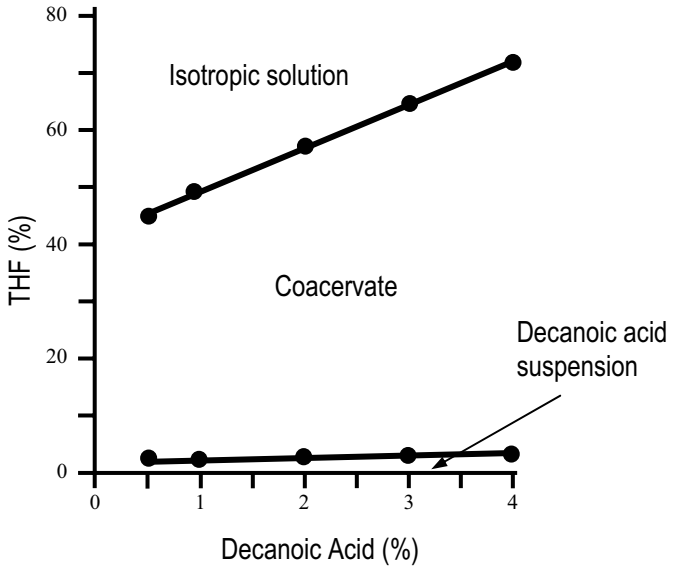

Figure 1. Binary diagram of phase boundaries for tetrahydrofuran-decanoic acid-water mixtures at room temperature

The vegetable and fruit samples to be extracted were directly added to ternary mixtures made up of decanoic acid (0.25-1% w/v), THF (5-30% v/v) and water. Three phases were always observed after the extraction and centrifugation of the food samples; namely a solid phase made up of insoluble matrix components at the bottom, a THF:water solution containing decanoic acid monomers and dissolved matrix components in the middle, and a coacervate phase containing the extracted food components at the top. At equilibrium, BPA distributed among these three phases, although the high solubility of BPA in the coacervate (~500 g L^{-1}) compared to that in THF:water (e.g. ~150 mg L^{-1} for 10% THF) greatly favoured the partition of the analyte to the coacervate phase.

Optimization. The influence of the composition of the coacervate and different operational parameters (e.g. extraction time, stirring rate, temperature, pH, etc) on both the volume of the coacervate and the recovery of BPA was investigated. The experiments were made by dissolving decanoic acid (100-400 mg) in tetrahydrofuran (5-30 %) in a specially designed centrifuge tube that had a narrow neck (7 mm i.d.). Then, water at pH about 3 was added to give a final volume of 40 mL, which caused the immediate separation of the coacervate phase from the bulk solution. Next, a food amount (100-1000 mg) fortified with 50 ng of BPA was added. The mixture was stirred in a range of conditions (600-1500 rpm, 5-30 min, room temperature-60 ºC) and then centrifuged (2200g, 15 min) to speed up the separation of the two phases. From these results and the instrumental quantification limit (IQL) obtained for BPA (injection of 0.14 ng in the chromatographic system), the corresponding method quantification limits (MQL) were estimated and used as a criterion for the selection of the optimal conditions for extraction. Conditions giving minimal MQL were selected provided that the recovery of BPA was above 70% [41], and the relative standard deviation of the method was below 10% [42].

Canned peas were used as a food sample model for optimisation studies. The selection was based on its higher protein content, compared to the other foods investigated, and its representative content in carbohydrates and fat (Table 1). The information about food composition was extracted from the can label or, as it was not supplied, from the average values provided in food composition tables [e.g. 43]. Independently on the food analysed, a whitish precipitate, which was standing at the bottom of the coacervate as a very thin layer, was extracted. This precipitate was caused by the proteins present in the samples, which were agglutinated by the reverse micelles and extracted by the coacervate, but they did not interfere in the recovery of BPA. Every optimisation experiment was carried out by analysing three non-fortified and three BPA fortified (100-200 ng g^{-1}) pea samples (300 mg) which were prepared according to the procedure specified in section 2.4.

Table 1. Protein, carbohydrate and fat composition of the canned foods selected

Food (100g)	Protein (g)	Carbohydrate (g)	Fat (g)
VEGETABLES			
Red peppers	0.6	6	0.2
Sweetcorn	2.9	11.2	0.9
Green beans[a]	1.2	4.2	0.5
Peas	5.5	10.7	0.4
FRUITS			
Fruit salad	0.4	14	0
Peaches in syrup[a]	0.5	12.3	0
Mango slices	0.4	19.2	0

[a] data obtained from reference 43

a) Influence of the coacervate composition on the extraction of BPA. Decanoic acid and THF are the major components of the coacervate, so their concentration in the colloidal solution greatly influences both the volume of extractant yielded and the extraction efficiency for BPA. Water is only a minor component of the coacervate on account of its non-solvent character for the decanoic acid reverse micelles.

The volume of coacervate increased linearly as the amount of decanoic acid did, which indicated that the composition of the surfactant rich-phase kept constant provided that the concentration of the other components remained unchanged. The corresponding equation (100-400 mg decanoic acid and 10% THF) was $y = 22 \pm 22 + 1.5 \pm 0.1x$, where y was the volume of coacervate in µL and x the amount of decanoic acid in mg. The correlation coefficient (r^2) was 0.995. The slope of the linear relationship was similar to that obtained in the absence of food sample [44], so although matrix components could be incorporated to the coacervate they did not influence its volume. Recoveries higher than 80% were obtained for BPA at decanoic acid concentrations as low as 0.5% (Table 2), which proved the high solubilization capability of the coacervate for this contaminant.

Although lower MQL could be obtained by decreasing the decanoic acid concentration used for extraction (e.g. 0.25% in Table 2) this option was not recommended because of the low recoveries obtained. So, a decanoic acid concentration of 0.5% was selected for further studies.

The relationship between the coacervate volume and the THF percentage was exponential (Table 2), which indicated that progressively more THF was incorporated to the coacervate and consequently the reverse micelles became more and more diluted. The corresponding equation (decanoic acid = 200 mg) was $y = 228 \pm 6\ e^{0.039 \pm 0.001x}$ (r^2=0.995), where *y* was the volume of the coacervate in μL and *x* the percentage of THF. Maximal extraction efficiencies were obtained in the range 10-20% of THF (Table 2). Below this range, only a fraction of the surfactant was incorporated to the coacervate [36] and as a result the recovery decreased. On the other hand, the solubility of BPA in the bulk solution increased as the THF concentration did, which resulted in decreased partition coefficients for THF percentages above 20% and consequently in lower recoveries (Table 2). We selected 10% THF, which gave the highest recoveries and MQL of around 9.3 ng g^{-1}.

b) Influence of operational parameters on the extraction of BPA. Food matrix components retain BPA through different types of interactions (e.g. Van der Waals, hydrogen bond, π-cation, etc) as it migrates from the can coating. So, the optimization of such parameters as extraction time, extraction temperature and stirring rate becomes important to break BPA-matrix interactions and consequently to make BPA extraction faster. None of the operational parameters investigated affected the volume of coacervate yielded because coacervates were formed before extraction and their formation was not influenced by the matrix components of the foods analyzed. So, MQL directly depended on the recoveries obtained for BPA under the different experimental conditions assessed. The time used for extraction was important for the recoveries obtained for BPA (Table 3). Equilibrium conditions were reached after 15 min of extraction and this time was selected as optimal for further studies. The stirring rate influenced the kinetics of the extraction and consequently more time was required to reach equilibrium conditions at the lowest stirring

rates tested (Table 3). A value of 1200 rpm was selected as optimal. The temperature scarcely influenced the extraction kinetics for BPA in the range investigated (20-60 ºC, Table 3), so the whole procedure was carried out at room temperature.

Table 2. Coacervate volumes, mean recoveries and method quantification limits obtained for BPA as a function of decanoic acid and THF concentrations

Coacervate composition	Coacervate volume (μL)	Mean recovery ± standard deviation[a] (%)	Estimated quantitation limits (ng g^{-1})
Decanoic acid[b] (%)			
0.25	161	55 ± 3	6.8
0.5	342	86 ± 2	9.3
0.75	503	90 ±3	13.0
1	620	95 ± 4	15.2
Tetrahydrofurane[c] (%)			
5	290	71 ± 3	9.5
7.5	304	77 ± 3	9.2
10	342	86 ± 2	9.3
15	392	87 ± 3	10.5
20	495	86 ± 3	13.4
25	622	78 ± 3	18.5
30	734	70 ±2	24.4

[a] n = 3; 300 mg peas spiked with 60 ng BPA
[b] THF = 10% (v/v)
[C] decanoic acid = 0.5% (w/v)

The coacervation phenomenon occurs from protonated decanoic acid (pK_a= 4.8±0.2), so extractions must be carried out below pH 4. Recoveries for BPA (pK = 9.46) were not affected by the pH in the range 1 to 4. The procedure was carried out at about pH 3.0-3.3 by the addition of hydrochloric acid.

Table 3. Mean recoveries and standard deviations obtained for BPA using different operational conditions

Extraction time (min)	$R^a \pm S^b$ (%)	Stiring rate (rpm)	$R^a \pm S^b$ (%)	Temperature (ºC)	$R^a \pm S^b$ (%)
5	44 ± 5	600	54 ± 1	20	86 ± 2
10	70 ± 4	900	81 ± 2	28	85 ± 2
15	86 ± 2	1200	86 ± 2	35	79 ± 3
25	88 ± 3	1500	87 ± 3	44	78 ± 4
30	85 ± 3			60	79 ± 4

[a] mean recoveries; 300 mg of peas spiked with 60 ng of BPA; decanoic acid = 0.5% (w/v); 10% (v/v)THF
[b] Standard deviation; n = 3

Analytical performance

Calibration curves were run by injecting 0.14–20 ng of BPA in acetonitrile. No differences in peak areas or retention times were observed for the analyte injected in organic solvent or coacervate. The slope and the intercept of the calibration curve were 5786±80 ng^{-1} and 255±680, respectively. The correlation coefficient was 0.99991. The minimum detectable amount (MDA), corresponding to the amount of BPA injected into the LC system that produced a 3:1 signal-to-noise ratio, was 0.02 ng. Taking into account the amount of sample extracted (300 mg), the volume of coacervate obtained (around 340 µL), the injection volume used (20 µL) and the recovery obtained from spiked samples (around 86%), the detection limit of BPA in canned peas was estimated to be around 1.3 ng g^{-1}. The intra-day precision was estimated by extracting six independent samples of green beans (300 mg) spiked with 60 ng of BPA. The relative standard deviation was 2.8%.

The matrix components extracted into the coacervate were chromatographically separated from BPA. In this respect, vegetable and fruit samples behaved differently; the formers presentet much more complex chromatograms that the latter ones (e.g. Figures 2

and 3). So, simpler chromatographic conditions were used to analyse BPA in canned fruits (e.g. isocratic conditions with 50:50 of water:acetonitrile, retention time for BPA 4.0 min) compared to those used for its analysis in canned vegetables (e.g. linear gradient from 70:30 to 45:55 of water:acetonitrile in 13 minutes, retention time for BPA 9.3 min).

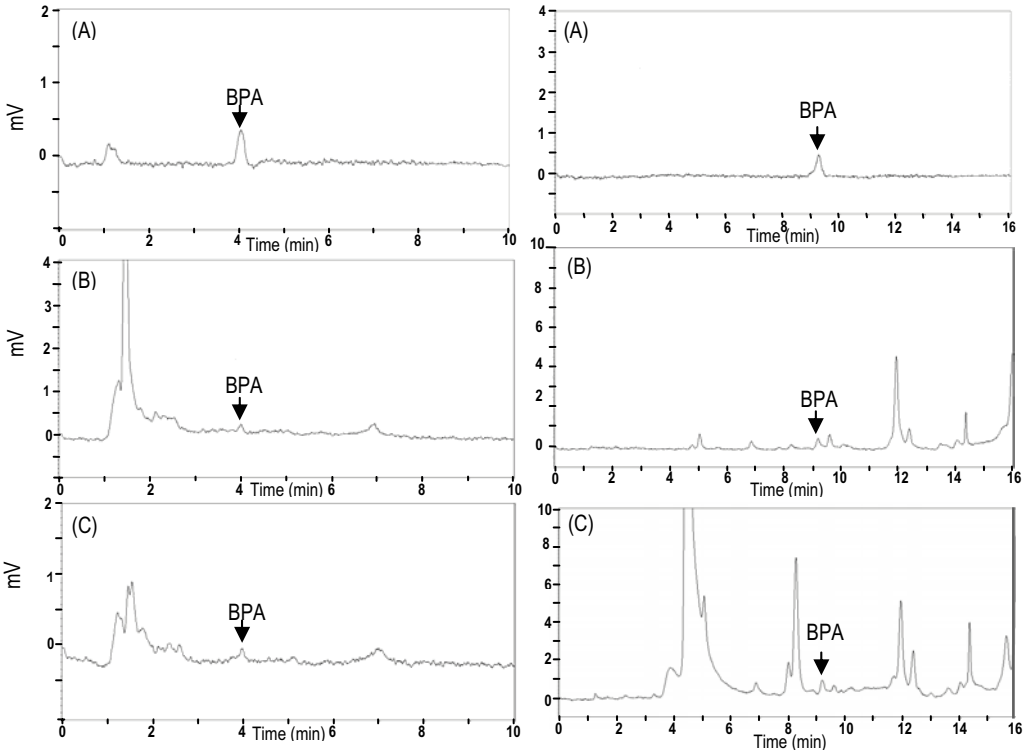

Figure 2. LC-Fluorescence chromatograms obtained from (A) a standard solution of 50 μg L^{-1} in acetonitrile; (B) 300 mg of peaches in syrup and (C) 300 mg of fruit salad.
Chromatographic conditions as specified in section 2.5 for the analysis of canned fruits

Figure 3. LC-Fluorescence chromatograms obtained from (A) a standard solution of 50 μg L^{-1} in acetonitrile; (B) 300 mg of red peppers and (C) 300 mg of sweetcorn.
Chromatographic conditions as specified in section 2.5 for the analysis of canned vegetables

The absence of interference from matrix components that could elute with BPA was checked by comparison of the slopes of the calibration curves (n=7) obtained from

standards in distilled water with those obtained from canned peas fortified with known amounts of BPA (20-300 ng) and run using the whole procedure. The difference between both slopes was found to be not statistically significant by applying the two-samples *t*-tests [45]. Therefore, matrix components were not expected to interfere in the determination of BPA.

The amount of sample analyzed did not influence the selectivity or the recoveries for BPA in the range 0.1-1g. However, the precision of the method greatly decreased for the lowest and highest sample amounts tested (e.g. the standard deviations obtained for BPA recoveries were ±21% and ±11% as 0.1 and 1g of pea samples were analysed, respectively). Very low sample amounts (e.g. 0.1g) were not representative of the bulk sample whereas the handling of sample amounts around 1g became a little more complicated for foods containing high protein levels (e.g. peas) since the protein precipitate standing at the bottom of the coacervate after centrifugation increased as the sample amount did. The precision, expressed as relative standard deviation, kept constant and around 2-4% for sample amounts between 300 and 700 mg. So, any sample amount in this range is recommended for the determination of BPA since the corresponding quantitation limits are low enough for control and risk assessment purposes.

Analysis of canned vegetable and fruit samples

Different canned vegetables and fruits were analyzed using the proposed method in order to prove its suitability for the routine control of BPA. Most of cans were products from Spain. Table 4 shows the concentrations of BPA found as well as the recoveries obtained after spiking the samples with known amounts of this contaminant. Some of the characteristics of the products analysed, as specified in the can labels, were also included. Concentrations of BPA and recoveries were expressed as the mean value of three independent determinations, besides their corresponding standard deviations. Recoveries ranged between 81 and 96% with relative standard deviations varying from 2 to 4%.

Table 4. Characteristics of the cans, mean recoveries (%) ± standard deviation obtained after spiking the samples and mean concentrations (ng g^{-1}) ± standard deviation found in the canned food analysed

Product	Origin	Lacquer area (cm^2)	Best before date	Net weight (g)	Drained weight (g)	Mean recovery[a] ± S[b] (%)	Mean concentration ± S[b] (ng g^{-1})
VEGETABLES							
Red peppers	Spain	110	July of 2010	80	60	91 ± 4	72 ± 3
Sweetcorn	Spain	220	July of 2008	150	140	81 ± 2	55 ± 1
Green beans	Spain	300	July of 2010	390	210	95 ± 3	103 ± 3
Peas	Spain	220	July 2011	200	140	88 ± 2	69 ± 1
FRUITS							
Fruit salad	Spain	300	June 2008	420	240	92 ± 3	7.8 ± 0.2
Peaches in syrup	Spain	300	April 2008	420	240	96 ± 2	10.3 ± 0.2
Mango slices	Thailand	300	May of 2007	425	230	89 ± 2	24.4± 0.7

[a] spiked sample (100 ng g^{-1}), 10% (v/v)THF, 0.5% (w/v) decanoic acid
[b] standard deviation, n = 3

BPA was quantified in all the samples analysed at concentrations in the range 7.8-103 ng g^{-1}. These concentrations were far below the current specific migration limit (SML) of 600 ng g^{-1} set by the EU Commission [6] for BPA and similar to those found by different researchers [10-18]. Figure 2 compares the chromatograms obtained from a standard solution of BPA (A) with those obtained from the analysis of canned peaches in syrup (B) and fruit salad (C). Despite the low concentration of BPA in fruits, the peak corresponding

to BPA could be clearly identified. The chromatograms corresponding to the analysis of vegetable samples were more complex (e.g. Figure 3 B and C) but using the chromatographic conditions proposed in the section 2.5, BPA was quantified with high precision (Table 4). Identification of BPA in the samples was based on retention times and the UV spectrum obtained from the diode array in line with the fluorescence detector.

Conclusions

The combination of reverse micelle-based coacervative extraction and liquid chromatography-fluorimetry constitutes a valuable strategy for the determination of BPA in canned vegetables and fruits. Coacervates surpass the current solvent-based methodologies used for the extraction of BPA in terms of simplicity (sample treatment just requires a single extraction with an aqueous solutions containing 4 mL of THF and 200 mg of decanoic acid, and no clean-up or solvent evaporation is necessary) and rapidity (the whole treatment procedure takes about 30 min and several samples can be simultaneously extracted, so sample throughput is considerable increased). There are additional assets associated to the method developed here; it involves the analysis of minute amounts of sample (300-700 mg), features low cost (the consumption of organic solvent is greatly reduced and the use of SPE columns is avoided), no special equipment is required for sample treatment and it uses fluorimetry for detection, so the method can be applied in routine analysis in labs without extra investment. The quantitation limit of the method is about 9.3 ng g^{-1}, so it can be used for the routine control of BPA in canned vegetables and fruits below the current specific migration limit (SML) of 600 ng g^{-1} set by the EU Commission [6].

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Capítulo 2. Determination of bisphenol A in canned fatty foods by coacervative microextraction, liquid chromatography and fluorimetry

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Determination of bisphenol A in canned fatty foods by coacervative microextraction, liquid chromatography and fluorimetry

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Abstract

Decanoic acid reverse micelle-based coacervates were proposed for the simple, rapid and almost solventless extraction of bisphenol A (BPA) from canned fatty foods. The procedure involved the extraction of minute quantities (200-400 mg) of homogenized food sample with an aqueous solution containing 20% THF and 200 mg of decanoic acid, conditions under which the coacervate (around 550 µl) formed *in situ* and instantaneously. The overall sample treatment took about 30 min and several samples could be simultaneously treated using conventional lab equipment. No clean-up or solvent evaporation were required before determination of BPA by liquid chromatography and fluorescence detection. Recoveries in samples were between 90 and 99%, with relative standard deviations in the range 2-7%. The quantitation limits (29-15 ng g^{-1} for 200-400 mg of sample) were far below the current specific migration limit (SML) set by the European Commission, EC (600 ng g^{-1}). The method was successfully applied to the determination of BPA in the solid content of canned fish (from 20 to 129 ng g^{-1}) and meat (from undetected to 37ng g^{-1}).

Introduction

Bisphenol A (4-4'-isopropylidenediphenol, CAS: 80-05-7; BPA) is a recognized environmental estrogen (Brotons et al. 1995; Chen et al. 2002; Vom Saal and Hughes 2005) widely used in the manufacturing of the most important internal polymeric coatings of food cans (Goodson et al. 2004). Human exposure to BPA occurs from the migration of this contaminant into the food (FSA 2001; Kang et al. 2006). The tolerable daily intake (TDI) level established by the U.S. Environmental Protection Agency, EPA (IRIS 1988), as well as that recently recommended by the European Food Safety Authority, EFSA (IRIS 1988; Vom Saal and Hughes 2005), is 50 ng g^{-1} body weight day^{-1}. The amount of BPA legally permitted to migrate from packaging into food, known as the specific migration limit (SML), is based on the TDI and it was set at 600 ng g^{-1} by the EC in 2004 (EC 2004).

Occurrence of BPA in canned foods has widely been reported (FSA 2001; Munguía-López et al. 2002; Braunrath et al. 2005; Munguía-López et al. 2005; Thomson and Grounds 2005; Sun et al. 2006; Podlipna and Cichna-Markl 2007). In 1995, Brotons and colleagues detected BPA in the liquid portion of several types of canned vegetables in the range 22-76 ng g^{-1} (Brotons et al. 1995). Later, Goodson et al. conducted a wide study in the UK that involved the analysis of 62 samples of canned foods and drinks (Goodson et al. 2002). BPA was quantified in 37 samples of canned food at levels from 7 up to 70 ng g^{-1}, with one sample of meat containing a mean level of 380 ng g^{-1}. Recently, a number of studies have confirmed the presence of BPA in canned meat (7.6- 140 ng g^{-1}), fish (0.3-110 ng g^{-1}), beverages (0.1-3.4 ng mL^{-1}), vegetables (1-212 ng g^{-1}), fruits (1.7-24 ng g^{-1}), pet food (12-206 ng g^{-1}) and so on (Brotons et al. 1995; FSA 2001; Imanaka et al. 2001; Yoshida et al. 2001; Kang and Kondo 2002; Braunrath et al. 2005; Munguía-López et al. 2005; Thomson and Grounds 2005; Sun et al. 2006; Podlipna and Cichna-Markl 2007, García-Prieto et al. 2008). The widespread human exposure to BPA from food and beverage containers has opened a strong debate in the scientific community about the low-dose reproductive and developmental effects of this contaminant, the conclusions going from no risk to human health (FSA 2001) to the need for a new risk assessment (Chen et al. 2002; Vom Saal and Hughes 2005).

Determination of BPA in food is required for both the control of the compliance of current legislation and the assessment of human exposure. Most analytical methods proposed in recent years for the determination of BPA in canned foods invariably involve the extraction of samples with organic solvents followed by liquid chromatography-fluorescence (LC-FL) or gas chromatography-mass spectrometry (GC-MS). Sample treatment is by far the most laborious and critical step.

Concerning the extraction of fatty foods, Goodson et al. have proposed the simplest BPA isolation procedure so far (Goodson et al. 2002). It involves the blending of twenty grams of fatty-sample with 20 mL of n-heptane for fat removal, duplicate extraction with 20+20 mL of acetonitrile, filtration, addition of anhydrous sodium sulphate and evaporation under nitrogen up to 5 mL. Other authors have proposed some minor modifications to this procedure, namely the treatment of ten grams of fatty sample, which reduces by half the solvent required (Podlipna and Cichna-Markl 2007), and the replacement of n-heptane by trimethylpentane, which is more effective for fat removal (Thomson and Grounds 2005). More organic solvent-consuming procedures (e.g 100 mL of methanol and 100 mL of hexane) have been proposed recently (Munguia-López et al. 2005). After isolation of BPA, the extracts are usually subjected to GC-MS, previous derivatisation of BPA, or they are analyzed by LC-FL, after a clean-up step. For this purpose, high selectivity can be achieved using sol-gel immunoaffinity columns (Braunrath et al. 2005; Braunrath and Cichna 2005; Podlipna and Cichna-Markl 2007).

The strong demand for simple, rapid, low-cost and environmentally friendly methods for food analysis has forced researchers to introduce new approaches and highly innovative technologies in food laboratories. In this context, the use of solvent-free techniques and other isolation emerging technologies is essential to increase sample throughput. The present work proposes the coacervative microextraction of BPA from fatty-foods as an innovative isolation technique to reduce time, cost and toxicity of current extraction methodologies.

Coacervates are water immiscible liquids that separate from the bulk of colloidal solutions by the action of a dehydrating agent, namely temperature, pH, electrolyte or a non-solvent for the macromolecule (IUPAC 1972; Gander et al 2002). After separation, the

coacervate, a low-volume phase, contains most of the colloid and it is in a dynamic equilibrium with the bulk solution. Coacervates are well-known in food industry where they are widely used for encapsulation of active ingredients (Gander et al. 2002). In extraction processes, applications have mainly focused on the use of surfactant aggregate-based coacervates for the extraction of pollutants from water (Saitoh and Hinze 1991; Jin et al. 1999; Casero et al. 1999; Carabias-Martínez et al. 2000; Rubio and Pérez-Bendito 2003; Merino et al. 2005; Ruíz et al. 2006; Ballesteros et al. 2007), soil, sediment (Merino et al. 2002) and sludge (Merino et al. 2003, Ruíz et al. 2003). The most used surfactant aggregates have been aqueous non-ionic (Ishii et al. 1977; Hinze and Pramauro 1993; Carabias-Martinez et al. 2000), amphoteric (Saitoh and Hinze 1991), anionic (Casero et al. 1999) and cationic (Jin et al. 1999) micelles. Recently, coacervates made up of vesicles (Ruíz et al. 2006; Ruíz et al. 2007a) and reversed micelles (Ruíz et al. 2007b; Ballesteros-Gómez et al. 2007) of alkyl carboxylic acids have been reported, which permit the extraction of organic compounds in a wide polarity range. Because of their excellent solvation and stabilizing properties, which can be varied by changing the nature of the macromolecule or the coacervating agent, coacervates have the potential to simplify extraction procedures in food analysis.

This paper focuses on the use of decanoic acid reverse micelle-based coacervates for the extraction of BPA from canned fish and meat using minute amounts of food sample and coacervate. The research includes the study of the parameters affecting the extraction efficiency of BPA, the study of the quantitative performance of the method using LC-FL and its application to the determination of BPA in several samples of canned fish and meat purchased in local supermarkets.

Materials and methods

Reagents

Bisphenol A (BPA) and decanoic acid (capric) were obtained from Fluka (Buchs, Switzerland). HPLC-grade acetronitrile, hydrochloric acid 37% and tetrahydrofuran were

purchased from Panreac (Barcelona, Spain). Stock solutions of BPA (0.5 g L^{-1}) were prepared in acetonitrile and stored under dark conditions at 4ºC not more than three months. Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain).

Apparatus

The liquid chromatrographic system used (Spectra System SCM1000, ThermoQuest, San Jose, CA, USA) consisted of a P4000 quaternary pump, a UV6000LP diode-array detector and a FL3000 fluorescence detector. In all experiments, a Rheodyne 7125 NS injection valve, with a 20 μl sample loop, was used. The stationary-phase column was a Hypersil ODS C_{18} column (5μm, 4.6 x 150 mm) from Análisis Vinicos (Tomelloso, Spain). A magnetic stirrer Basicmagmix from Ovan (Barcelona, Spain) and a digitally regulated centrifuge Mixtasel from JP-Selecta (Abrera, Spain) were used for sample preparation. Volumes of coacervate were measured using a digital calliper from Medid Precision, S.A. (Barcelona). Centrifuge tubes with narrow necks (Figure 1) were designed by authors in order to make easier the measurement and collection of the coacervate after extraction. Pobel S.A. (Madrid, Spain, web page: www.pobel.com) constructed them from commercial heavy-duty glass cylindrical centrifuge tubes with round-bottom (ref. 159050) by keeping their basic structure at the bottom (34 mm of outside diameter) but reducing the diameter from a specified height, which depended on the required tube capacity.

Determination of BPA in canned fatty foods

Sample pretreatment. Spanish manufactured cans of fish (tuna in olive oil, mackerel in vegetable oil, sardines in olive oil and mussels in pickled sauce) and meat (cooked and sterilized meatballs and lean pork cooked in its own juices) were bought in supermarkets in Córdoba (Spain). The unopened cans were stored at room temperature. For analysis, the overall content of each can was drained in a mesh strainer (holes size ~0.85 mm^2) and the liquid discarded. Then, the overall solid portion was blotted with a filter paper (base weight of 73 g/m^2, Anoia S.A., Barcelona, Spain) and homogenized using a kitchen

food mixer (~9000 rpm, Thermomix TM 21, Vorwerk, Spain). Aliquots of 200-400 mg were taken for analysis and recovery experiments, which were made in triplicate. Spiking of samples for recovery rates was carried out by adding minute volumes of a BPA standard solution in acetonitrile to the solid portion of the food before its homogenization. Samples not immediately analyzed were stored at -20 ºC until analysis. The type of the internal coatings of the cans was not investigated.

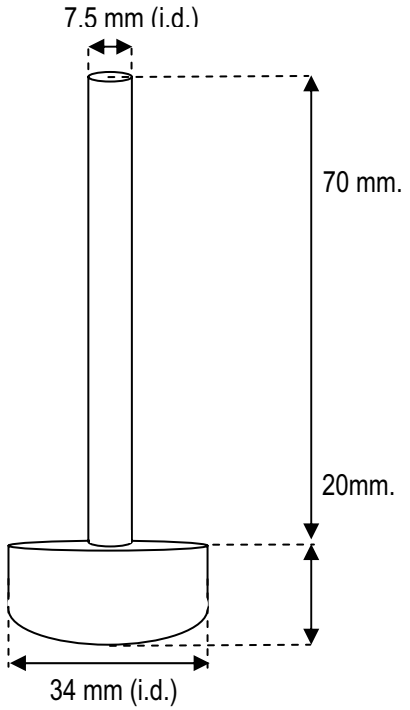

Figure 1. Schematic picture of the glass tube designed for the coacervative extraction**.**

Coacervative microextraction of BPA. Decanoic acid (200 mg) was dissolved in THF (2 mL) in a glass centrifuge tube (Figure 1). Then, 8 mL of distilled water and 140 μl of HCl 0.5 M were added. Immediately, the coacervate phase (around 550 μl) separated from the bulk solution. Next, the food (200-400 mg) was added using a small spatula (100 mm long and 4 mm width, supplied by Selecta, Barcelona, Spain). The mixture was magnetically stirred at 1200 rpm for 15 min to favour BPA extraction and then centrifuged

at 4000 rpm for 15 minutes to accelerate phase separation. Next, the volume of the coacervate, which was standing at the narrow neck of the glass tube, was measured with a digital calliper. Finally, an aliquot of 20 μl of the coacervate was withdrawn with a microsyringe and injected into the chromatograph.

Quantitation by liquid chromatography-fluorescence. Quantification of BPA and separation from the matrix components was carried out by liquid chromatography-fluorescence. The mobile phase consisted of water (A) and acetonitrile (B). The flow-rate was 1 mL min^{-1}. The gradient elution program was: linear from 70:30 to 45:55 (A:B) in 13 minutes and then isocratic initial conditions (70:30) for 7 min to clean and stabilize the chromatographic system. The selected wavelengths were 276 nm (excitation) and 306 nm (emission). BPA eluted to a retention time of 8.8 min. Calibration was run by injecting 20 μl of standard solutions in acetonitrile containing between 0.2 and 60 ng of BPA. Quantification was performed by measuring peak areas. Correlation coefficients between peak area and BPA were in the range 0.9995-0.99990.

Results and discussion

Formation and structure of decanoic acid reverse micelle-based coacervates

Decanoic acid is highly soluble in THF where it self-assembles as reverse micelles (size around 4-8 nm), according to a sequential-type self-association model (Ruiz et al. 2007b). The addition of water, in which decanoic acid is scarcely soluble, causes the partial desolvation of the reverse micelles and makes easier micelle-micelle interaction. As a result, the reverse micelles self-assemble in larger aggregates with a wide size distribution in the nano and micro scale regimes and separate from the bulk solution as an immiscible liquid phase (the coacervate). At a microscopic level, the coacervate consists of spherical droplets made up of reverse micelles which are dispersed in a THF continuous phase containing a minimal amount of water that is essential to keep the coacervate structure.

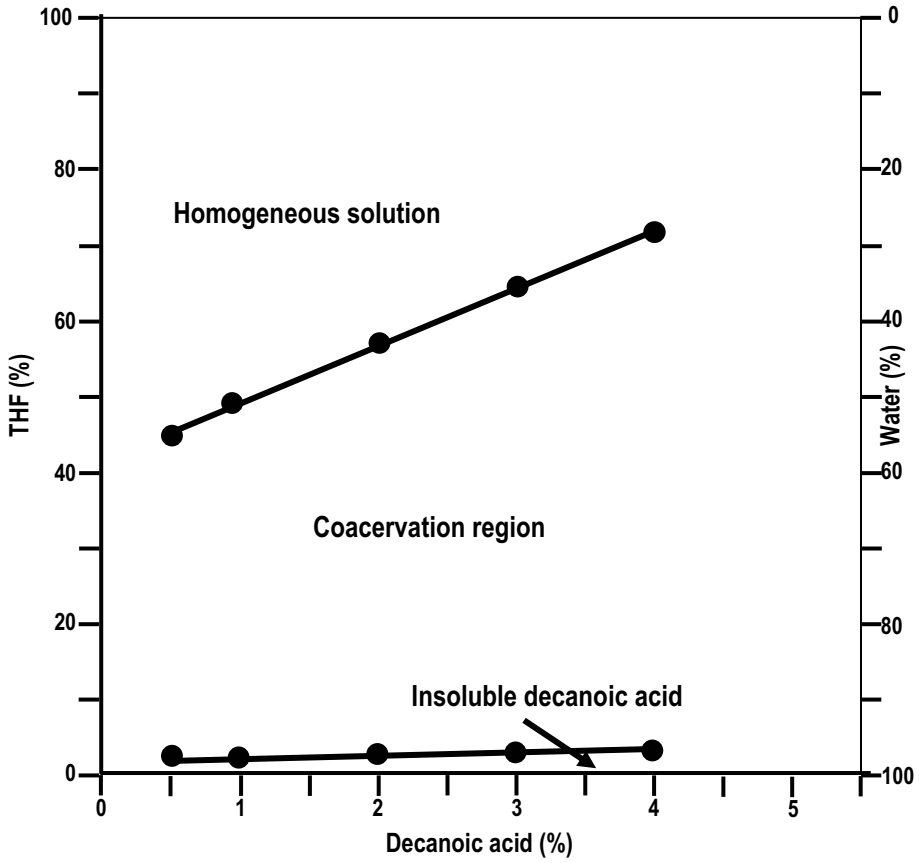

Figure 2. Phase diagram of decanoic acid in binary mixtures of THF:water. Final volume of the solution (THF+ Water) = 40 mL. Experiments made at room temperature.

Figure 2 shows the relative concentration of decanoic acid:THF:water at which the coacervation occurs. Beyond the boundaries of this region, the decanoic acid precipitates or produces a homogeneous micellar solution where the size of the aggregates is considerable smaller than those in the coacervate. Figure 3 shows a picture of the surfactant aggregates present in the different steps of the extraction process. The reversed micelles in the coacervate provide a 2-fold mechanism for BPA extraction (BPA octanol-water partition coefficient, log $K_{o/w}$, 2.91 and pK_a 9.73), namely van der Waals interactions in the decanoic acid hydrocarbon chains and hydrogen bonds in the micellar core, so it can be extracted efficiently from food samples. Since reverse micelles only

occur from protonated decanoic acid (pKa = 4.8±0.2), extractions must be carried out below pH 4.

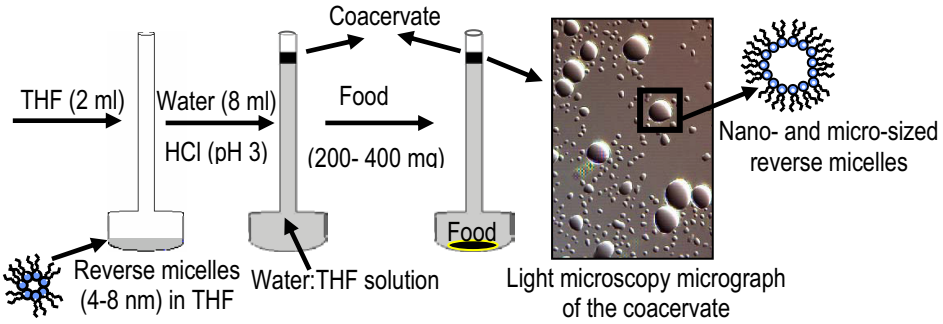

Figure 3. Illustration of the different steps and surfactant aggregates involved in the coacervative extraction of BPA.

Optimisation of the coacervative microextraction of BPA. Optimisation was carried out by extracting both fortified (n= 3; 50 ng BPA) and non fortified (n=3) tuna in olive oil samples (50-400 mg) under a variety of experimental conditions (100-600 mg decanoic acid; 5-40 % THF; pH 1-4; extraction time 10-60 min; temperature 20-60 ºC, stirring rate 600-1500 rpm). Method quantitation limits (MQL) were used as a criterion for the selection of the optimal conditions for extraction. They were estimated from the volume of the coacervate (Vc), the recovery for BPA (R) and the instrumental quantification limit (IQL) for this contaminant, which was 0.2 ng (absolute amount injected in the chromatographic system). Conditions giving minimal method quantitation limits (MQL) were selected provided that the extraction for BPA was quantitative and the relative standard deviation of the method was below 10% (Green 1996).

Influence of the coacervate amount and composition. Coacervates are mainly made up of decanoic acid and THF, so the amount of these components in the samples determines the coacervate volume and greatly influences the BPA recovery. Figure 4 shows the dependence of Vc on both the amount of decanoic acid (a) and the percentage of THF

(b). The volume of coacervate increased linearly as the amount of decanoic acid did independently of the percentage of THF investigated. This type of dependence indicated that the composition of the coacervate kept constant when the other variables remained unchanged. The corresponding equations for 5% and 20% of THF were $y = (29 \pm 28) + (1.27 \pm 0.08)\ x$ and $y = (9 \pm 15) + (2.7 \pm 0.1)\ x$, respectively, where y was the volume of coacervate in μl and x the amount of decanoic acid in mg. The correlation coefficients were 0.996 and 0.998. The slopes of the linear relationship were similar to that obtained in the absence of food sample (Ballesteros et al. 2007), so although matrix components could be incorporated to the coacervate they did not influence its volume.

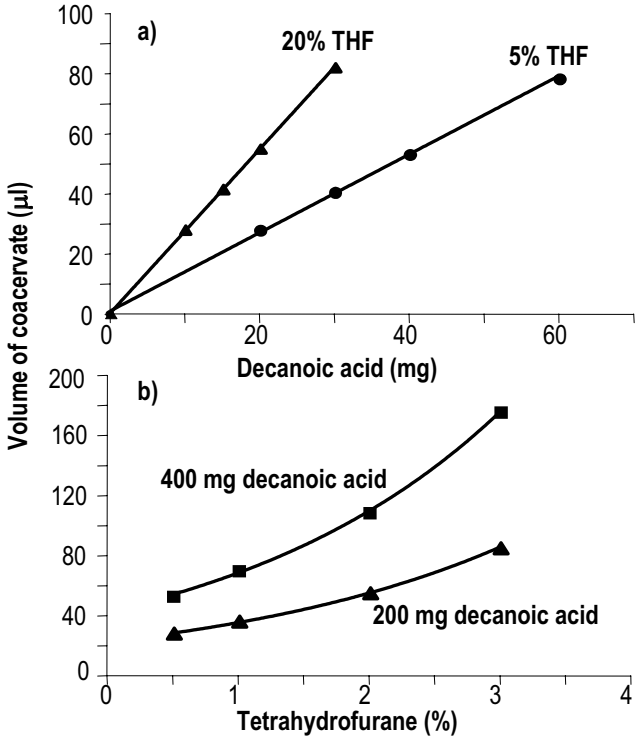

Figure 4. Volume of coacervate as a function of: a) the amount of decanoic acid at two percentages of THF, and b) the concentration of tetrahydrofuran at two amounts of decanoic acid. Extraction of 200 mg of tuna with a water:THF solution (10 mL)

The relationship between the volume of coacervate and the percentage of THF was exponential (Fig. 4 (b)) and fit to the equation $y = b_0 e^{b_1 z}$, where *y* was the volume of coacervate in μl and *z* the percentage of THF. The corresponding equations for 200 and 400 mg of decanoic acid were $y = (223 \pm 11)\ e^{(0.045 \pm 0.003)z}$ and $y = (433 \pm 17)\ e^{(0.046 \pm 0.002)z}$ respectively, with correlation coefficients of 0.997 and 0.998. This type of dependence indicated that progressively more THF was incorporated to the coacervate as the percentage of THF in the solution increased and consequently, the reverse micelles became more and more diluted. As these equations were also similar to those obtained in the absence of food sample, the general equation previously derived for the prediction of the volume of coacervate (*y*) obtained as a function of the amount of decanoic acid (x) and percentage of THF (*z*) was also applicable to tuna samples (Ballesteros et al. 2007). So, it is possible to estimate the volume of coacervate and therefore the maximum concentration factor that can be obtained under given experimental conditions through the equation $y = 1.06\ x\ e^{0.04731z}$.

Table I shows the volumes of coacervate estimated and the recoveries obtained for BPA as a function of the amount of decanoic acid and the percentage of THF. The concentration of solvent became essential to get quantitative recoveries. A THF concentration of ca. 20% was found to be necessary for efficient coacervative extraction of BPA. The recovery decreased at THF percentages above 30%, which can be explained by the increase of BPA solubility in the THF:water solution in equilibrium with the coacervate, that resulting in decreased BPA partition coefficients. From these results, the corresponding MQL values were calculated and they are included included in the Table I. Quantitative recoveries were obtained for BPA at the selected THF (20%) and decanoic acid (200 mg) values and the corresponding MQL (29 ng g^{-1}) was far below the specific migration limit (SML) set by the EC (600 ng g^{-1}, EC 2004). Under these experimental conditions, it was found that the volume of coacervate measured with a digital calliper (550.0 ± 0.4μl) coincided with that calculated by the general equation (550 μl, Table I) thus confirming the high capability of prediction of the equation used.

Table I. Coacervate volumes, mean recoveries and method quantification limits obtained for BPA as a function of THF and decanoic acid concentrations

THF (%)	Decanoic acid (mg)	Coacervate volume* (μl)	Recovery†± S‡ (%)	Method quantification limit (ng g^{-1})
5	200	269	58 ± 2	23
	300	404	48 ± 1	42
	400	538	59 ± 5	46
	600	807	48 ± 6	84
10	200	342	53 ± 3	32
	400	684	45 ± 5	76
20	100	275	76 ± 1	18
	150	413	89 ± 1	23
	200	550	95 ± 2	29
30	100	444	75 ± 5	30
40	100	714	54 ± 6	66

* Estimated from the equation $y = 1.06 \times e^{0.04731z}$, where x is the amount of decanoic acid (mg) and z the percentage of THF.
†200 mg of tuna spiked with 50 ng of BPA
‡ Standard deviation, n = 3

Influence of operational parameters. The optimization of such parameters as extraction time and temperature and stirring rate was carried out in order to select those conditions under which the extraction became faster. None of these parameters modified the volume of coacervate yielded, so MQLs directly depended on the recoveries obtained for BPA.
The stirring rate influenced the kinetics of the extraction and consequently, more time was required to reach equilibrium conditions at the lowest stirring rates tested (600-1000 rpm). A value of 1200 rpm was selected as optimal since larger stirring rates did not reduce the extraction time. Equilibrium conditions and quantitative recoveries were obtained after 15 min of extraction and this time was selected as optimal for further studies. The

temperature scarcely influenced the extraction kinetics for BPA in the range investigated (20-60 ºC), so the whole procedure was carried out at room temperature.

Recoveries for BPA were not affected by the pH of the extraction solution in the range 1 to 4, which is logical considering the type of interactions expected to be the driving forces for the extraction. So, the pH of this solution was adjusted at 3-3.5 by the addition of hydrochloric acid.

Influence of matrix components. Table II shows the concentrations of the major components in the selected foods. The data were obtained from either the can label or significant databases. Preliminary experiments indicated that independently of the food analyzed, a whitish precipitate, which was standing as a very thin layer between the bottom of the coacervate and the THF:water solution, was extracted. This precipitate decreased as the amount of sample did, so it was matrix-dependent. It was found that the precipitate was caused by the proteins present in the samples, which were agglutinated by the reverse micelles and extracted by the coacervate, but they did not interfere in the recovery of BPA.

As salt was a component of samples (see Table II), its influence on the extraction process was investigated by adding NaCl to 200 mg of tuna at the following concentrations: 1, 5, 10, 15 and 25 mg per gram of sample. Neither the recoveries for BPA nor the volume of coacervate were affected by NaCl in the range of concentrations usually present in these types of samples. The effect of larger amounts of salt: 500, 1170, 1750, 2300 and 2500 mg NaCl g^{-1} tuna sample was also investigated in order to determine if they caused a salting out effect on the extraction of BPA. Salt did not influence on the efficiency of the extraction process, so its addition to samples is not recommended. It was clearly observed in this study that the volume of coacervate increased linearly with the NaCl concentration. The slope of this linear relationship was 0.066±0.003 µl mg^{-1} NaCl g sample, so the volume of coacervate increased at a rate of 6.6 µl per 100 mg salt g^{-1} sample. Such low rate was undetectable for the low concentrations of salt present in the samples but this increase of volume should be taken into account if samples with a high salt content are analyzed.

Table II. Major components of the food samples analysed.

Sample	Component * (g 100 g^{-1} food)			
	Fat	Protein	Carbohydrate	Salt‡
Tuna in olive oil	12	24	0	0.77
Mackerel in vegetable oil	12	24	0	0.81
Mussels in pickled sauce	9	16	4	1.11
Sardines in olive oil	11†	25†	0†	0.67
Meatballs	16	8	7	1.20
Lean pork	12	16	0.3	0.99

Sources: * Content specified in the can label.
† USDA (United States Department of Agriculture) National Nutrient Database for Standard Reference.
‡ www.ocu.org/map/src/199871.htm. and http://revista.consumer.es/web/es/19990601/actualidad/analisis131221-2php

Lipids in the samples are expected to be extracted by the coacervate by the formation of mixed micelles with decanoic acid. As incorporated at enough proportion, the lipids should produce an increase in the volume of coacervate obtained. The effect of lipids on the extraction process could not be directly investigated because of both the exact proportion of lipids in the solid portion of the food is unknown (Table II gives the amount of total fat) and it is very difficult to simulate the exact composition of the lipids (i.e. free, triglyceride, phospholipids, etc) and their exact interactions with the rest of matrix components. So, we decided to asses the overall effect of matrix components, which also include lipids, on the extraction of BPA through two experiments. In the first one, different amounts (150, 200 and 400 mg) of an unfortified tuna sample were analyzed by triplicate. The concentrations of BPA found (135±6, 129±6 and 130±5, respectively) were similar in the range investigated, so it was assumed that matrix components did not influence the extraction process. In the second one, different amounts (50-400 mg) of both tuna in olive oil and mackerel in vegetable oil were spiked with 50 ng of BPA and analyzed following the procedure recommended (see the section materials and methods). The results

obtained are shown in Table III. Recoveries were quantitative and independent of the amount of sample thus confirming that matrix components, including lipids, did not affect the extraction process. On the other hand, the 95% confidence interval for the volumes of coacervate obtained in both experiments was 545±19 μL, so the content of the lipids extracted from the minute amounts of sample analyzed was not enough to produce detectable changes in the volume of coacervate.

Table III. Mean recoveries and method quantification limit as a function of the amount of sample analysed

Sample	Sample amount (mg)	Recovery* ± S† (%)	MQL‡ (ng g^{-1})
Tuna in olive oil	50	97 ±4	113
	150	104 ± 5	35
	200	95± 2	29
	400	98 ± 7	14
Mackerel in vegetable oil	50	104 ± 6	105
	75	111 ± 3	66
	120	108 ± 3	42
	200	98 ± 4	28
	400	97 ± 2	15

* Spiking level: 50 ng of BPA
† Standard deviation, n = 3
‡Method quantification limit estimated from an average volume of coacervate of 550 μl

Analytical performance

Calibration curves for BPA were run using standard solutions prepared in acetonitrile. No differences in peak areas or retention times were observed for BPA injected in acetonitrile or coacervate. The retention time for BPA was 8.8 min. Correlation coefficient between

peak areas and the amount of BPA injected every day (0.2, 1, 2, 10, 20, 30 and 60 ng) was in the range 0.9995-0.99990 indicating good fits. The slope of the calibration curve was (4.63± 0.04)∗10^3 fluorescence intensity units ng^{-1}. The instrumental detection limit (IDL) was calculated from blank determinations by using a signal-to-noise ratio of 3 and it was calculated to be ~ 0.06 ng. From this value and the average percentage of recovery of BPA in food samples (95±8%, data calculated from Table IV), an average volume of coacervate of around 550 µl and an amount of sample handled of 200 mg, the minimum detection limit that could be reached by the method was calculated to be ~ 9 ng g^{-1}. Lower detection limits can be achieved if necessary by increasing the amount of sample treated (e.g. 400 mg) or decreasing the amount of decanoic acid used for extraction (e.g. see in Table I, data for 20% THF and 100-200 mg of decanoic) since recoveries above 70% are allowed (AOAC/FAO/IAEA/IUPAC 1999, Green 1996). The relative standard deviation, calculated from the analysis of a tuna sample during five days, two complete analyses per day, was ±6%.

The accuracy of the quantitation of BPA in fatty foods using external calibration was assessed by comparison of the slopes of the calibration curves obtained from standards in acetonitrile with those obtained from 200 mg of lean pork, tuna and mackerel samples fortified with known amounts of BPA (10-300 ng) and run using the whole procedure. Six different concentrations were used for construction of each calibration curve. The slopes and correlations coefficients found for lean pork, tuna and mackerel samples were (4.6 ± 0.2)∗10^3, 0.998; (4.4 ± 0.1)∗10^3, 0.9990 and (4.26 ± 0.03)∗10^3, 0.9998; respectively. The differences found between these calibration slopes and that obtained in acetonitrile (4.63 ± 0.04)∗10^3 were exclusively due to the percentages of recovery obtained for BPA (e.g. mean recovery values of around 99, 95 and 92% for lean pork, tuna and mackerel, respectively), so calibration external is recommended for quantitation of BPA in fatty foods.

Analysis of canned fatty foods

The suitability of the proposed analytical method for the determination of BPA in canned fatty food was assessed by analyzing four samples of canned fish (tuna, mackerel, sardine and mussel) and two samples of canned meat (meatball and lean pork). Table IV shows some of the characteristics of the cans analyzed as well as the concentrations of BPA found and the recoveries obtained after spiking the samples with 50 ng of BPA.

Table IV. Characteristics of the cans analysed, mean concentrations found for BPA and recoveries obtained after spiking of the samples

Product	Lacquer area (cm^2)	Best before date	Net weight (g)	Drained weight (g)	BPA* $\pm$S‡ (ng g^{-1})	Recovery*,¶ $\pm$ S‡(%)
Fish						
Tuna in olive oil	110	12/2011	80	52	129 $\pm$ 6	95 $\pm$ 2
Mackerel in vegetable oil	120	12/2012	115	81	†20 $\pm$ 5	†92 $\pm$ 6
Sardines in olive oil	120	12/2011	125	87	119 $\pm$ 5	90 $\pm$ 5
Mussels in pickled sauce	110	12/2010	80	43	121 $\pm$ 2	95 $\pm$ 6
Meat						
Meatballs	300	12/1012	420	390	<MDL§	98 $\pm$ 2
Lean pork	243	08/2009	220	210	37 $\pm$ 5	99 $\pm$ 7

Analysis of * 200 and † 400 mg of fatty food. ‡ Standard deviation, n = 3. ¶ Food samples spiked with 50 ng of BPA. §: below the detection limit of the method.

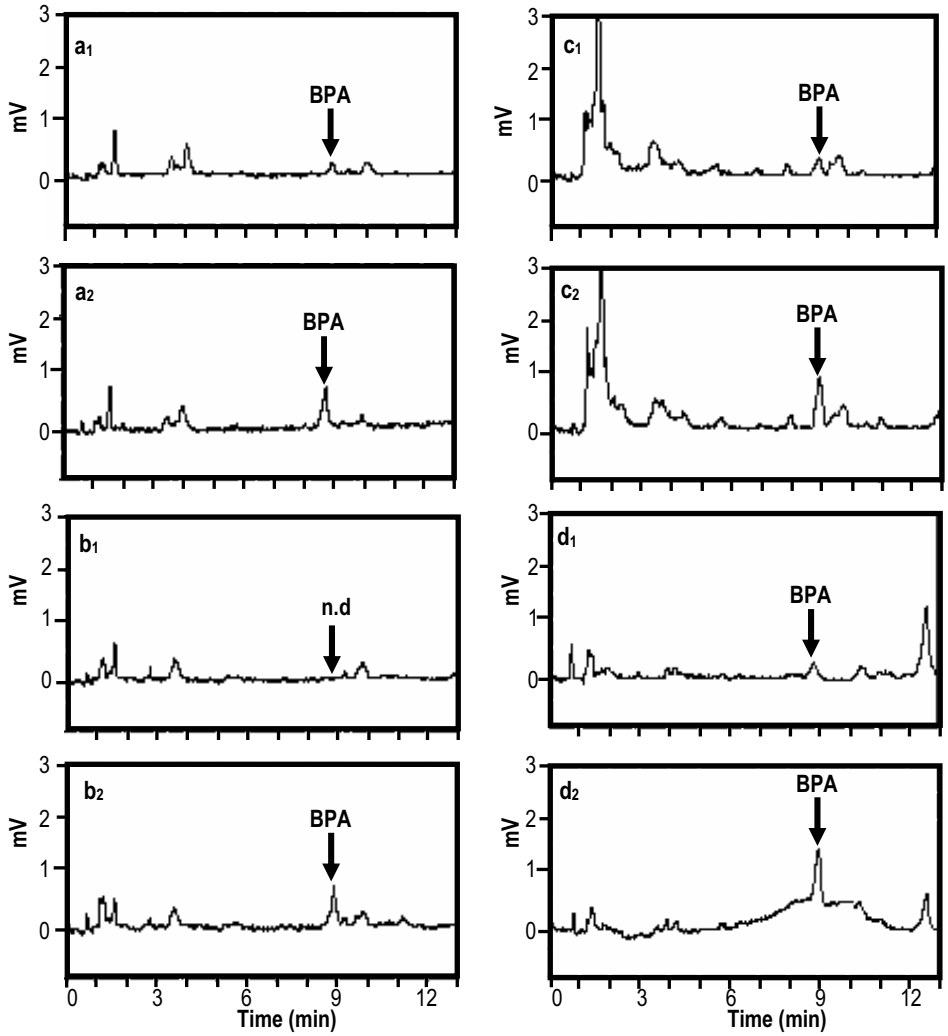

Figure 5. LC-fluorescence chromatograms obtained from 200 mg of canned sardines (a), meatballs (b), mussels (c and lean pork (d) without spiking (1) and after spiking with 50 ng of BPA (2). Experimental conditions described in the section *Materials and Methods.*

Values for concentrations and recoveries were expressed as the mean value of three independent determinations, besides their corresponding standard deviations. Recoveries of BPA were always above 90% and the standard deviations were between 2 and 7% thus indicating that the characteristic of the method were matrix-independent. BPA was present at quantifiable levels in all the canned foodstuffs analyzed except in the cooked

meatballs. The quantification of BPA in the canned mackerel involved the analysis of 400 mg of sample owing to its low content. BPA in meatballs, if any, was below the detection limit of the method (i.e. 9 ng g^{-1}). Concentrations of BPA were always below the specific migration level of 600 ng g^{-1} set by the European Commission (EC 2004).

Figure 5 shows the LC-fluorescence chromatograms obtained for the analysis of unfortified (a_1-d_1) and fortified with 50 ng de BPA (a_2-d_2) canned foods. Chromatograms were clear enough for the detection and quantitation of BPA in all the samples analyzed. Identification of the target analyte was based on the retention time and the UV spectrum, which was obtained from the diode array in line with the fluorescence detector. Analysis of the UV spectrum included both peak purity testing and spectrum matching. To calculate the purity a scan threshold of 1 mAU and peak coverage of 95% were considered. With regard to spectrum matching, the similarity threshold was set at 0.98.

Conclusions

Coacervates of decanoic acid reverse micelles constitute a valuable alternative to the current methodologies available for the extraction of BPA from canned fatty foods, which are based on the use of 30-200 mL of toxic organic solvents followed by clean-up and/or solvent evaporation. Comparatively, coacervative extraction is simpler and faster since sample treatment just requires a single extraction with an aqueous solution containing 2 mL of THF and 200 mg of decanoic acid, and no clean-up or solvent evaporation is necessary. As a result, the whole treatment procedure takes about 30 min and several samples can be simultaneously extracted, so sample throughput is considerable increased. There are additional assets associated with the proposed method; such as requires only minute amounts of sample (200-400 mg), features low cost (the consumption of organic solvent is greatly reduced and the use of SPE columns is avoided), no special equipment is required for sample treatment and it uses liquid chromatography-fluorescence for separation-detection, so the method can be applied in routine analysis in labs without extra investment. The quantitation limit of the method is

about 29 ng g^{-1}, so it can be used for the routine control of BPA in canned fish and meat below the current specific migration limit (SML) of 600 ng g^{-1}. The experimental conditions established for the coacervative extraction of BPA from fatty-food and those previously obtained by the authors for the extraction of BPA from canned vegetable and fruits (García-Prieto et al. 2008) are not essentially different, which proves the suitability of decanoic-based coacervates for the efficient and simple extraction of this contaminant from a variety of canned foods. Because of the absence of clean-up steps, chromatographic conditions are simpler for canned fruits than those for canned vegetable and fatty-foods in order to assure the exact quantitation of BPA.

Acknowledgments

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Capítulo 3. Measurement of urinary bisphenol A by coacervative microextraction and liquid chromatography-fluorescence detection

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Determination of urinary bisphenol A by coacervative microextraction and liquid chromatography-fluorescence detection

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Abstract

Total (free plus conjugated) urinary BPA is currently being used to assess human exposure to this contaminant. This work proposes the use of coacervates made up of reverse micelles of decanoic acid for the microextraction of BPA, prior to its determination by liquid chromatography and fluorescence detection (λ_{ex} = 276 nm and λ_{em} = 306 nm), with the aim of simplifying sample treatment and reducing analysis time and costs in epidemiologic studies. he procedure involves the enzymatic hydrolysis of 7 mL of urine and then the addition of decanoic acid (100 mg) and tetrahydrofurane (1 ml) to 7 ml of enzymatically hydrolyzed urine, conditions under which the coacervate (extractant phase, ~167 µl) forms *in situ* and instantaneously. The overall procedure takes about 20 min and several samples can be simultaneously treated using conventional lab equipment (i.e. stirrers and centrifuges). Extractions were independent of the pH and temperature in the ranges studied (1-4 and 25-50 ºC) rendering the method robust. Recoveries in samples ranged between 88 and 95% and the practical detection limit was 0.197µg/l, which is

below the usual concentrations of BPA in urine (ranges reported 0.4-149 µg/l). The actual concentration factor provided by the method was 38. The precision of the method, expressed as relative standard deviation, was 4.5 %. The method was successfully applied to the determination of total BPA in urine from eight healthy volunteers. BPA was detected in all the samples at concentrations ranging between 4.03 and 49 µg/l.

1. Introduction

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane) is a high-volume (> 6 billion pounds per year) production chemical mainly used in the manufacture of polycarbonate plastic and epoxy resins. Among other uses, these manufactured materials are extensively used as food containers (e.g. milk, water, and infant bottles) and food can linings [1]. Heat and either acid or basic conditions accelerate the hydrolysis of the ester bond linking BPA monomers and lead to BPA release and migration into the food. Ingestion of BPA-containing foods is thought to constitute the primary route of human exposure to this contaminant [2-5], although other potential sources are also of concern (e.g. dental fillings and sealants, leaching from landfill, indoors air, etc.[6]) .

Until recently, BPA had not been considered a chemical of “high concern” because its affinity for estrogen receptors is 10.000-100.000-fold weaker than that of estradiol. However, a large number of recent in vitro studies have shown that the effects of BPA are mediated by both genomic and non genomic estrogen-response mechanisms, with the disruption of the cell function occurring at doses as low as 1 pM (0.23 ng/l) [7]. Recent reports also indicate the potential of BPA to disrupt thyroid hormone action [8], to cause proliferation of human prostate cancer cells [9] and to block testosterone synthesis [10] at very low part-per-trillion doses. This extensive new literature concerning low-dose effects of BPA has given rise to controversy about the BPA limit values set by regulatory agencies for consumer health protection and a new risk assessment has been strongly recommended [7, 11-14]. So, extensive data are needed to clarify BPA exposures and internal dosimetry in the general population, newborns, and occupationally-exposed individuals [15].

Estimation of human BPA exposures or daily intake can be straightforwardly made by measuring the total (free plus conjugated) urinary species of this contaminant [15]. Because of extensive first-pass metabolism, most BPA is conjugated to the monoglucuronide and excreted in urine within 24 hours, whereas smaller amounts of BPA

are metabolized to and excreted as sulphate [16]. In the last decade, data on the urinary concentrations of BPA in selected populations of various countries have become available [17-24]. These data suggest that human exposure to BPA is widespread (more than 80% of the samples analysed were positive), continuous (BPA was present in most urine samples despite its estimated half-life is only of ~ 6 hr) and variable (concentrations of BPA ranged from undetected to a maximum of 3950 μg/l and were demographic and income group dependent) [15,25]. Recent studies have proved the valuable usefulness of urinary BPA measurements for different purposes. Thus, although within-person variability in urinary concentrations of BPA exists [17,26,27], concentrations based on one spot sample per person can be useful in calculating mean population concentration estimates in cross-sectional studies. On the other hand, it has been reported that a single sample is predictive of BPA exposure over weeks to months, and can provide good sensitivity to classify a person exposure in epidemiologic studies [26,27].

Most analytical methods used so far for the measurement of total urinary BPA are based on the digestion of the sample with glucuronidases, liquid-liquid (LLE) or solid phase (SPE) extraction of the sum of parent plus deconjugated BPA, and analysis by liquid chromatography (LC) with different detection techniques (mass spectrometry (MS), fluorimetry (FL) and coulometry (C)) (28-30) or gas chromatography-mass spectrometry (GC-MS), previous derivatization (31,32). Enzyme-linked immunosorbent assay (ELISA) has also been used (33), however it has poor correlation with other techniques and besides the different ELISA kits correlate poorly with each other due to lack of specificity of the antibody and effects of the biologic matrix [34]. Reliable data can be obtained with both LC and GC although the latter is more complex and prone to errors because of the need of derivatization. In order to overcome this problem, in situ derivatization has been proposed [35,36]. Most of the reported detection limits were in the range 0.1 to 1 μg/l [15], although they varied in a wider interval (e.g. from 0.01 [37] to 8.36 [38] μg/l). On the whole, sample treatment is by far the most laborious, time-consuming and critical step of urinary BPA measurement and, as a general rule, evaporation of the extract following LLE or SPE is required to achieve the required sensitivity. Stir bar sorptive extraction is simpler

[36] but the extraction time is too long (e.g. 150 min) for the large amount of measurements involved in exposure studies. So, there is still a strong requirement for more valuable sample preparation procedures that meet the demand of rapidity, simplicity and robustness required for the assessment of BPA human exposure.

In the present work, we investigate the suitability of amphiphile-based coacervative microextraction for the isolation of BPA from urine prior to LC-fluorescence detection with the aim of developing a straightforward method for assessing BPA human exposure. Amphiphile-based coacervates are water immiscible liquids that separate from the bulk of molecular aggregate solutions (e.g. aqueous or reverse micelles or vesicles) by the action of a dehydrating agent, namely temperature, pH, electrolyte or a non-solvent for the aggregates [39,40]. After separation, the coacervate, a low-volume phase, contains a high concentration of amphiphiles (typically 0.1-1 mg/µl) and therefore of binding sites. In consequence, high extraction efficiencies can be achieved using low coacervate volumes which results in high concentration factors (typically 100-500). Application of coacervates to analytical extractions was proposed by Watanabe et al. a long time ago [41], and for many years it focused on the use of non-ionic micelle-based coacervates for the extraction of hydrophobic compounds from environmental waters [42,43]. In recent years, the development of coacervates made up of zwitterionic [44], anionic [45] and cationic [46] aqueous micelles, reverse micelles [47] and vesicles [48] have extended significantly the scope of these extractants with regard to both the polarity range of compounds that can be extracted and the samples that may be analysed (e.g. soil and sediment [49], sludge [50], foods [51], etc). This paper proposes the use of coacervates made up of reverse micelles of decanoic acid for the extraction of BPA. The study includes the optimisation of the parameters affecting extraction efficiencies and concentration factors, the determination of the quantitative performance of the developed method and its successful application to the measurement of BPA in a variety of urine samples from volunteers.

2. Materials and methods

2.1 Reagents

All chemicals were of analytical reagent-grade and were used as supplied. Bisphenol A (BPA) and decanoic acid were obtained from Fluka (Madrid, Spain). Tetrahydrofuran (THF), ammonia, hydrochloric acid and HPLC-grade acetronitrile were purchased from Panreac (Barcelona, Spain). Ammonium acetate salt was purchased from Merk (Darmstadt, Germany) and β-glucuronidase (*Helix pomatia*, H1, ≥300,000 units/g solid) from Sigma Aldrich (Steinheim, Germany). A stock solution of BPA (0.5 g/l) was prepared in acetonitrile and stored under dark conditions at 4ºC. Working solutions were made by appropriate dilution of the stock solution with acetonitrile. A β-glucuronidase solution (20 mg/ml) was prepared by dissolving 0.1 g of the enzyme in 5 ml of 1 M NH_4Ac buffer (pH 5) and stored under dark conditions at 4ºC. Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain).

2.2. Apparatus

The liquid chromatrographic system used (Spectra System SCM1000, ThermoQuest, San Jose, CA, USA) consisted of a P4000 quaternary pump, a UV6000LP diode-array detector and a FL3000 fluorescence detector (LC/FL). In all experiments, a Rheodyne 7125 NS injection valve, with a 20 μl sample loop, was used. The stationary-phase column was a Hypersil ODS C_{18} column (5μm, 4.6 x 150 mm) from Análisis Vinicos (Tomelloso, Spain). A magnetic stirrer Basicmagmix from Ovan (Barcelona, Spain) and a digitally regulated centrifuge Mixtasel from JP-Selecta (Abrera, Spain) were used for sample preparation. The volume obtained of coacervate under different experimental conditions was measured using a digital calliper from Medid Precision, S.A. (Barcelona). Centrifuge tubes with narrow necks (Figure 1) were designed by authors in order to make easier the measurement and collection of the coacervate after extraction. Pobel S.A.

(Madrid, Spain, web page: www.pobel.com) constructed them from commercial heavy-duty glass cylindrical centrifuge tubes with round-bottom (ref. 159050) by keeping their basic structure at the bottom (34 mm of outside diameter) but reducing the diameter from a specified height, which depended on the required tube capacity.

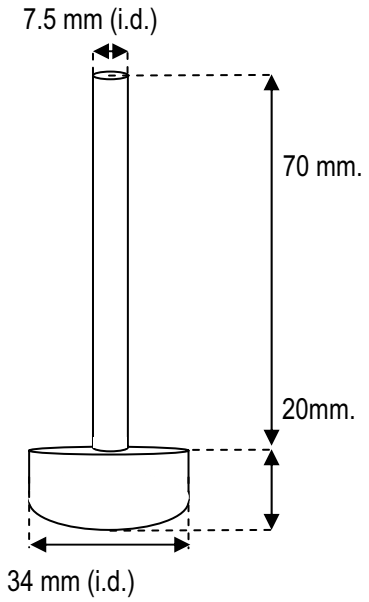

Figure 1. Schematic picture of the glass centrifuge tube designed for the coacervative extraction**.**

2.3. Determination of the total urinary BPA

2.3.1. Sample preparation

Urine was collected from eight healthy university students (23-32 years old) at an arbitrary time of the day, without considering the sex or nutritional habits. Samples, if not analyzed immediately, were stored and frozen at -20ºC. Sample preparation involved the buffering of 7 ml of urine with 1.2 ml of 1 M ammonium acetate (pH 5) and the enzymatic hydrolysis of the conjugated BPA with β-glucuronidase (H*. pomatia*) (400 U/ml) at 37ºC overnight.

After incubation, the pH of the hydrolyzed urine was adjusted at 3 with concentrated hydrochloric acid.

2.3.2. Coacervative microextraction

Decanoic acid (100 mg) was dissolved in THF (1 ml) in a glass centrifuge tube with a narrow neck (Figure 1). Then, the whole hydrolyzed urine sample (around 8.7 ml) was added and the final volume was made up to 10 ml with distilled water. Immediately, the coacervate spontaneously formed into the bulk solution. The mixture was stirred for 10 minutes at 1200 rpm to favour BPA extraction and then centifuged at 3800 rpm (2200 x g) for 10 minutes to accelerate the separation of the coacervate from the bulk solution. Finally, the exact volume of coacervate (~ 170 µl), which was standing at the narrow neck of the centrifuge tube (Figure 1), was calculated by measuring its height with a digital calliper and 20 µl were withdrawn with a microsyringe and directly injected into the LC-FL system.

2.3.3. Quantification

Quantification of total BPA (free plus conjugated) and separation from the matrix components were carried out by liquid chromatography/fluorescence detection. The mobile phase consisted of water and acetonitrile. The elution program was: linear gradient from 75 to 65% of water in 25 minutes and then isocratic conditions (100% acetonitrile) for 5 min. A re-equilibration time of 5 min was used between runs. The flow-rate was 1 ml/min. The BPA was monitored at λ_{ex} = 276 nm and λ_{em} = 306 nm and the retention time was at 26.3 min. From 0 to 14 min the excitation and emission wavelengths were fitted at 210 nm and 220 nm, respectively, in order to avoid fluorescence detector saturation owing to matrix components. Quantification was performed by measuring the peak areas corresponding to BPA. The calibration curve for BPA in acetonitrile was linear in the range 25-1000 µg/l.

3. Results and discussion

3.1 Coacervate description

Decanoic acid dissolves in THF forming reverse micelles (size around 4-8 nm) according to a sequential-type self-association model [52] with at least three critical aggregation concentrations (4.8±0.2, 7.6±0.4 and 51±2 mM) [47]. The addition of water or samples containing high water content (e.g. liquid foods [53]) to this binary system causes partial desolvation of the reverse micelles, which makes their interaction easier and promotes the formation of larger aggregates that separate from the THF:water bulk solution as an immiscible liquid phase. So, water is the agent that causes the coacervation. The resulting coacervate consists of reverse micelles with a wide size distribution in the nano and micro scale regimes, dispersed in a THF:water continuous phase. As reverse micelles are produced from the protonated decanoic acid form (pK_a 4.8±0.2), pH values below 4 are required for the formation of the coacervate. Figure 2 shows an illustration of the coacervation process including a microphotograph of the coacervate and a picture of the aggregates making it up. Figure 3 shows the relative concentration of decanoic acid:THF:water at which the coacervation occurs. Beyond the boundaries of this region, the decanoic acid precipitates or produces a homogeneous reverse micellar solution where the aggregates are considerable smaller than those in the coacervate.

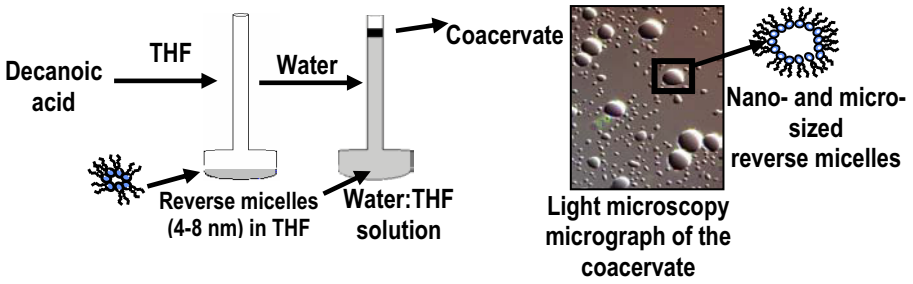

Figure 2. Illustration of the formation of the coacervate and the aggregates making it up.

The water content in urine is around 95% [54], so this biological fluid was expected to induce the coacervation of reverse micelles of decanoic acid. Production of the coacervate was investigated in ternary systems consisting of decanoic acid, THF and urine and the Figure 3 includes the phase diagram obtained. Like water, urine induced the coacervation of decanoic acid and the phase diagram showed three regions where the decanoic acid was precipitated, coacervated or solubilized. So, this coacervate is suitable to extract solutes from urine. Compared with the phase diagram obtained in distilled water, the upper boundary in the phase diagram from urine moved toward greater THF concentrations as the decanoic acid concentration was above 1% (figure 3).

In order to explain the boundary displacement, phase diagrams of ternary mixtures consisting of decanoic acid, THF and synthetic aqueous solutions containing some urine matrix components at different concentrations were constructed. We checked that urea exerted the same effect (i.e. it moved the upper boundary in the phase diagram), although the amount required (around 6%) was higher than that present in normal urine (1-3%). So, other urine constituents (e.g. ions and organic compounds) must contribute to the observed phenomenon. From an analytical point of view, it is worth noting that this effect on the phase diagram is irrelevant to the use of the coacervate for the extraction of solutes from urine since analytical applications are usually carried out near the lower phase boundary in order to use the minimal amount of THF.

The reverse micelles of decanoic acid in the coacervate provide a 2-fold mechanism for analyte solubilisation, namely van der Waals interactions at the hydrocarbon chains and hydrogen bonds at the micellar core. BPA is a relatively polar compound (log $K_{O/W}$ =3.25) that contains two protonated hydroxyl groups (pK_a = 9.73) at the pH range in which the coacervate is produced (below pH=4). So, it was expected both van der Waals interactions and hydrogen bonds to be the driving forces for BPA coacervative microextraction.

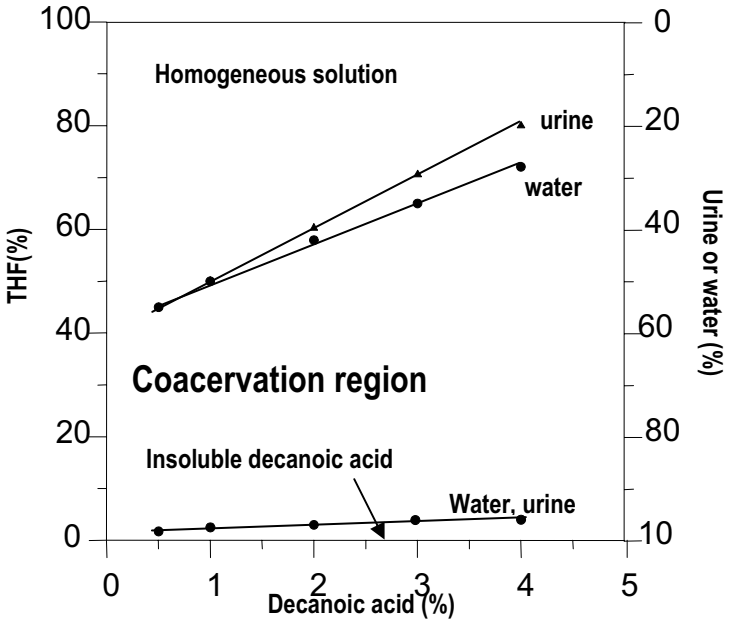

Figure 3. Phase diagram of decanoic acid in binary mixtures of THF:water and THF:urine. Final volume of the solution (THF+ Water or Urine) = 10 ml. Experiments made at room temperature.

3.2. Optimization of the coacervative microextraction

Optimal conditions for the extraction of BPA were investigated by changing each variable in turn while keeping the others constant. Optimization was carried out using samples of urine (7 ml), both unfortified and fortified with 50 µl of 1 mg/l BPA standard solution in acetonitrile, which were hydrolyzed according to the procedure specified in the section 2.3.1. In order to make hydrolysis of both fortified and unfortified samples under the same exact conditions, the latter were spiked with 50 µl of acetonitrile. Extractions were carried out under a variety of experimental conditions (50-200 mg decanoic acid; 5-20% THF; pH 1-4; temperature 25-50ºC and extraction time 5-20 min). All the optimization experiments were made in triplicate. Experimental conditions giving the minimal method detection limit (MDL) were selected as optimal provided that the extraction for BPA was above 90% and

the relative standard deviation of the method was below 10%. Method detection limits (µg/l) were estimated through the expression MDL = IDL/(*0.01∗R∗PVR*), where IDL was the instrumental detection limit for BPA (7.5 µg/l, calculated from blank determinations by using a signal-to-noise ratio of 3), *R* the recovery (%), *PVR* the phase volumes ratio (i.e. sample volume over the coacervate volume) and the product *0.01∗R∗PVR* the actual concentration factor (ACF).

3.2.1. Phase volume ratios

As decanoic acid and THF are the major components of reverse micelle-based coacervates (water hardly incorporates to the extractant phase due to its non-solvent character for the reverse micelles) their concentration in the urine sample determined the volume of extractant obtained. General equations have been previously developed for the prediction of the volume of coacervate produced in water solutions as a function of the amount of decanoic acid, the percentage of THF, and both the amount of decanoic acid and percentage of THF [55].

In order to determine whether urine matrix components influenced the volume of coacervate produced, a set of experiments was carried out using different decanoic acid amounts (50-200 mg), THF concentrations (2.5-30%) and urine samples. The volumes of coacervate obtained were measured with a digital calliper and the relationship between these volumes and the amount of decanoic acid and THF was investigated.

As expected, the volume of coacervate was linearly dependent on the amount of surfactant used, which indicated that the composition of the coacervate kept constant when the other variables remain unchanged. The slope of the linear relationship at a given THF concentration (e.g. 1.68±0.06 µl/mg for 10% THF) was similar to that obtained in water (1.67±0.04 µl/mg) [55], so although matrix components of urine could be incorporated to the coacervate they did not affect its volume.

On the other hand, similarly to water, the relationship between the volume of coacervate produced in samples of urine and the percentage of THF was exponential and fit to the equation $y = b_0\, e^{b_1 z}$, where *y* is the volume of the coacervate in µL and *z* the THF percentage (v/v). This type of dependence indicated that progressively more THF was incorporated to the coacervate as the percentage of THF in the solution increased and consequently, the reverse micelles became more and more diluted. The parameter (b_1), which describes how rapidly the volume of coacervate increases as the THF (%) does was found to be similar in all the experiments (mean value 0.046±0.001), thus indicating that it was not influenced by matrix components. The parameter b_0, which is linearly related to the amount of decanoic acid, changed similarly in urine and water (e.g. in the equation obtained for 100 mg of decanoic acid, b_0 was *108±7* µl *and 106*±3 µl in urine and water, respectively).

According to these results, the volume of coacervate produced in urine as a function of decanoic acid and THF concentration can be predicted from the general equation previously derived for water; $y = 1.035\, x\, e^{0.04731 z}$, where *y* is the volume of coacervate in µL, *x* is the amount of decanoic acid in mg, and *z* the THF percentage (v/v). The highest phase volume ratios will be obtained using low amounts of decanoic acid and THF. So, coacervate compositions near the lower boundary in the phase diagrams (Figure 3) are recommended for extraction.

3.2.2. Recoveries

Table 1 shows the results obtained for recoveries and ACFs as a function of the amount of decanoic acid and the percentage of THF. Recoveries higher than 90% were obtained for BPA at decanoic acid amounts as low as 100 mg. The recovery was quantitative as the amount of decanoic acid was about 200 mg, but the volume of coacervate, which is linearly dependent on this component, increased and consequently the corresponding MDL also did. Although lower MDL could be obtained at decanoic acid amounts below 75 mg, the recoveries did not meet the optimization criterion established above. Standard

deviations were always low enough and they were not considered for the selection of this variable. An amount of decanoic acid of 100 mg was selected for further experiments.

Table 1- Volumes of coacervate (V_c), mean recoveries (R) $\pm$ standard deviations (S), actual concentration factors (ACF) and method detection limits (MDL) as a function of the amount of decanoic acid and concentration of tetrahydrofuran.

	V_c (µl)	R $\pm$ S[c] (%)	ACF	MDL (ng/l)
mg C_{10}[a]				
50	83	54 $\pm$ 4	45	166
75	125	80 $\pm$ 1	45	166
100	167	91 $\pm$ 4	38	197
200	332	97 $\pm$ 3	20	375
% THF[b]				
5	131	77 $\pm$ 5	41	183
10	167	91 $\pm$ 4	38	197
15	210	92 $\pm$ 6	31	242
20	267	101 $\pm$ 1	26	288

[a] THF = 10%
[b] decanoic acid = 100 mg
[c] n = 3; spiked BPA concentration = 7.14 µg/l

On the other hand, recoveries progressively increased as the percentage of THF did and a percentage of at least 10% was necessary to get values above 90%. Because of the volume of coacervate increases exponentially with the concentration of this solvent, the ACFs progressively decreased and consequently the MDLs were getting worse. A 10% of THF was selected as optimal because this percentage provided the best possible MDL under the optimization criteria established. The volume of coacervate obtained under these conditions was between 165-170 µl, which allowed for at least 2-3 different chromatographic runs per sample in a reliable way (20 µl each injection).

The extraction of BPA was not affected by the pH in the range studied (1-4), which is logical considering the values of the BPA acidity constants and the type of interactions expected to be the driving forces for its solubilization into the coacervate. Because of the hydrolysis is carried out at pH 5, the pH for extraction was fixed at pH 3 by experimental convenience. The increase of the temperature of the urine sample from 25 to 50 ºC did not affect BPA extraction efficiencies or actual concentration factors. The time necessary to reach extraction equilibrium conditions using the procedure proposed was about 10 min.

3.3. Optimization of the hydrolysis of urine samples

BPA is excreted in urine mainly as water soluble conjugates with high polarity, i.e. glucuronide and sulphate, and only a minor proportion is excreted as free compound. As total urinary BPA is the parameter used to assess BPA exposure, urine samples must be hydrolysed before extraction. Enzymatic hydrolysis using β-glucuronidase (type H1), which permits the deconjugation of glucuronated- as well as sulphated-BPA, is usually preferred [e.g. 18, 21, 22, 35, 36] most authors employing between 300 and 400 U of the enzyme per ml of urine and incubation overnight. Acidic hydrolysis has been proposed as an alternative to the enzymatic one [56], the former being described as much simpler and less time consuming that the latter. So, we compared the efficiency of both procedures to hydrolyse BPA conjugates.

Different experiments were designed to investigate the effect of different factors at several levels on both types of hydrolysis. For this purpose, a non-spiked urine sample was used. Preliminary experiments were carried out in order to select an urine sample with high and low content in conjugated and free BPA, respectively. In this way, the results obtained from its hydrolysis should be useful for a wide number of samples. The concentration of conjugated and free BPA in the urine selected was 49±3 μg/l and below the quatitation limit, respectively.Table 2 shows the results obtained. The optimal conditions found for enzymatic hydrolysis were similar to those described in the literature (i.e. 400 U/ml and an incubation time of 8-12 h). The concentration of hydrochloric acid and the temperature

had notable effect on the efficiency of the acidic hydrolysis, while the influence of the time was less important. This efficiency was lower than that of the enzymatic one under all the experimental conditions investigated. At the optimal conditions (0.6 M HCl, 50 ºC and 30 min of hydrolysis) the efficiency of the acidic hydrolysis was about 65% of the enzymatic one. So, we selected the latter in our method despite the former is faster.

Table 2- Influence of different variables on the hydrolysis of BPA conjugates

Enzymatic hydrolysis					
[b]Enzyme amount (U/ml)	X ±S[a] (μg/l)	[c]Hydrolysis time (h)	X ±S[a] (μg/l)		
50	18 ± 1	1	32 ± 1		
200	30 ± 2	6	40 ± 3		
400	49 ± 3	8	48 ± 3		
500	47 ± 4	12	49 ± 3		
Acidic hydrolysis					
[d]Hydrochloric acid (M)	X ±S[a] (μg/l)	[e]Temperature (ºC)	X ±S[a] (μg/l)	[f]Hydrolysis time (min)	X ±S[a] (μg/l)
0.3	10.9 ± 0.6	30	3.61 ± 0.03	10	28 ± 1
0.6	12.3 ± 0.2	40	13.5 ± 0.5	30	32 ± 2
1.0	8.9 ± 0.2	50	17.1 ± 0.8	45	23 ± 2
2.0	------	70	12.3 ± 0.2	60	17.1 ± 0.8

[a] Mean concentration and standard deviation obtained from the analysis of three replicates of the urine samples

[b] Hydrolysis time: 12 h ; [c] β-glucuronidase: 400 U/ml; [d] temperature 70 ºC, hydrolysis time 1h; [e] [HCl]= 0.6 M hydrolysis time 1h; [f] [HCl]= 0.6 M, temperature 50ºC.

3.4. Analytical performance

Calibration curves for BPA were run using standard solutions prepared in acetonitrile. There was no difference in the peak areas or retention time of the target analyte injected in acetonitrile or coacervate. The retention time for BPA was 26.5 min. The correlation between peak areas and BPA concentration (25-1000 μg/l) was determined by linear regression and was between 0.998 and 0.9990, indicating good fit. The slope of the

calibration curve was 56.2 ± 0.4 l/μg. The instrumental detection limit (IDL) was calculated from blank determinations (i.e. bidistilled water extracted similarly to samples) considering a signal-to-noise ratio of 3 and it was 7.5 μg/l. From this value and the average percentage of recovery of BPA in urine samples (91%, calculated from Table 3), an average volume of coacervate of around 167 μl and an amount of sample handled of 7 ml, the minimum detection limit that could be reached by the method was calculated to be ~ 197 ng/l.

The precision of the method was evaluated by applying the global procedure to the extraction of BPA from 10 aliquots of a non-spiked sample taken from one of the participants in this study. The value, expressed as relative standard deviation (RSD) was about 4.5% (the average level of BPA found was 18 μg/l)

The possible interference of matrix components that could elute with BPA was assessed by comparison of the slopes of the calibration curves obtained from standards in acetonitrile (56.2±0.4 l/μg) with that obtained from different urine samples fortified with known concentrations of BPA (0-21μg/l) and run using the whole procedure (see section *Determination of the total urinary BPA*). The concentration of total BPA in urine was 43 μg/l and the concentration of free BPA used for spiking was 0.66 (the quantitation limit), 1.4, 3, 7.5, 10, 12, 15 and 21 μg/l. The slope of calibration curve in this case was 50±2 l/μg. The difference between the slopes of both calibration curves was exclusively due to the recovery obtained for BPA (~ 91%), so calibration external is recommended for quantitation of BPA in urine.

3.5. Analysis of human urine samples

The suitability of the proposed method for determining total urinary BPA was assessed by analysing the urine of a group of eight volunteers. Table 3 shows the concentrations found as well as the recoveries obtained after spiking the samples with 7.14 μg/l of BPA. Both unfortified and fortified samples were analysed in triplicate. BPA was detected in all the

samples at concentrations ranging between 4.03 and 49 μg/l. Recoveries ranged between 88 and 95 % with relative standard deviations varying from 2 to 5 %. These results are in concordance with those previously reported, in which the frequency of detection of free plus conjugated BPA was higher than 83% and the concentrations found were in the range 0.4-149 μg/l.

Table 3- Mean concentration of total BPA and recoveries after spiking the samples with 7.14 μg/l of BPA

Sample	Mean concentration ± S[a] (μg/l)	Mean recovery ± S[a] (%)
1	19.3 ± 0.4	91± 5
2	24 ± 1	90± 2
3	4.03 ± 0.08	93± 5
4	36 ± 1	88 ± 3
5	20 ± 1	89± 2
6	5.60 ± 0.04	95± 4
7	49 ± 3	89± 3
8	18.1 ± 0.9	91± 4

[a] Standard deviation from triplicate analysis of samples

Figure 4 shows the chromatograms obtained for a BPA standard solution in acetonitrile (A) and a non-spiked (B) and spiked (C) urine sample. Chromatograms were clear enough for the detection and quantitation of BPA in all the samples analysed. Identification of the target analyte was based on the retention time and the UV spectrum, which was obtained from the diode array in line with the fluorescence detector. Analysis of the UV spectrum included both peak purity testing and spectrum matching. To calculate the purity a scan threshold of 1 mAU and peak coverage of 95% were considered. With regard to spectrum matching, the similarity threshold was set at 0.98.

Additionally, unhydrolyzed aliquots of the same urine sample were analyzed. Free BPA was undetected in all the samples except in the urine from volunteers 2 and 4 (Table 3), in

which the concentrations were 4.2±0.1 and 1.80±0.05 μg/l. The concentration of free BPA in most urine samples was below the detection limit of the method, i.e. below 197 ng/l.

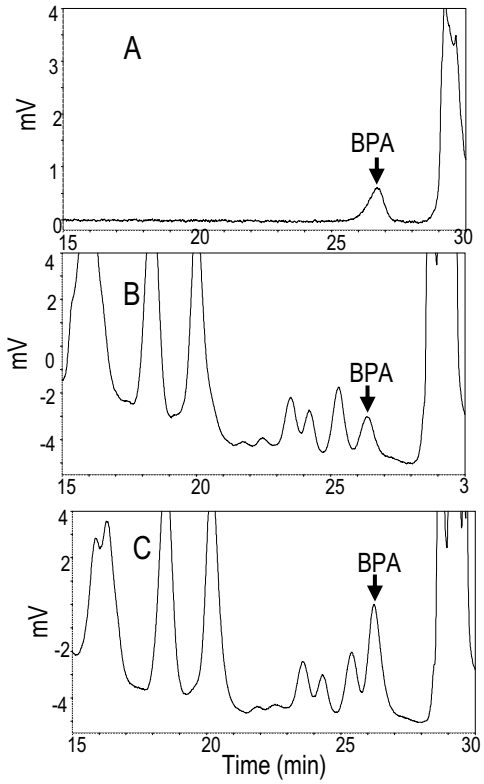

Figure 4. LC-fluorescence chromatograms obtained from a standard solution of 500 μg/l of BPA in acetonitrile (A) and an human urine sample, both unfortified (B) and fortified with 50 μg/l of BPA (C).

4. Conclusions

A method has been proposed that meets the requirements to be used in large epidemiologic studies intended to asses the relevance of human exposure to BPA. The procedure for coacervative microextraction is robust (extractions are independent of the temperature, pH or matrix components), simple (the treatment of samples just requires

the addition of 100 mg of decanoic acid and 1 ml of THF and no clean-up or solvent evaporation is needed), and rapid (the whole extraction procedure takes about 20 min and several samples can be simultaneously extracted). Another additional asset of this method is its low cost since no special equipment is required and uses fluorimetry for detection. So, the method can be used in routine analysis in labs without extra investment. The detection limit of the method (~197 ng/l) is below the usual total urinary concentration of BPA in human urine samples, which makes it widely applicable.

Acknowledgements

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PARTE II: Evaluación de la selectividad y aplicabilidad de hemimicelas y admicelas en formato SPE para la extracción de alteradores endocrinos en muestras acuosas ambientales

1.- OBJETO

Las hemimicelas y admicelas, agregados de tensioactivos sobre óxidos minerales, son nuevos tipos de adsorbentes en SPE que tienen la capacidad de proporcionar múltiples interacciones para la retención de los analitos y por tanto, presentan interés para la extracción simultánea de un gran número de compuestos en muestras ambientales. Las isotermas y cinéticas de adsorción, así como la morfología de los agregados, se han estudiado extensamente y existen excelentes revisiones en la bibliografía en el ámbito físico-químico.

Nuestro grupo de investigación ha realizado también estudios teóricos sobre hemimicelas y admicelas que han permitido establecer las pautas que deben tenerse en cuenta en el proceso de optimización y operación en SPE cuando se utilizan estos adsorbentes. Asimismo, las aplicaciones desarrolladas hasta la fecha han permitido conocer los tipos de interacciones analito-agregados que pueden establecerse lo que facilita la selección del adsorbente más apropiado para una aplicación específica.

Las investigaciones que se recogen en los dos capítulos que se presentan a continuación abordan tanto el estudio de aspectos teóricos como prácticos relacionados con el uso de adsorbentes supramoleculares para la extracción de contaminantes orgánicos en muestras acuosas ambientales. En el primer capítulo se presenta el estudio del efecto de los componentes de la matriz de aguas fluviales y residuales sobre la adsorción de dodecilsulfato sódico sobre alúmina con el objeto de establecer mecanismos de prevención y detección de interferencias que permitan el desarrollo de métodos robustos. En el segundo capítulo los agregados supramoleculares se aplican a la extracción de hormonas naturales y sintéticas en aguas residuales. Previamente, se presenta una breve introducción sobre aspectos básicos de hemimicelas y admicelas y se comentan brevemente las aplicaciones desarrolladas hasta la fecha.

2.- HEMIMICELAS Y ADMICELAS

2.1. Descripción

Los tensioactivos iónicos se adsorben sobre la superficie de óxidos minerales con carga opuesta, tales como alúmina, sílice, dióxido de titanio e hidróxidos férricos, formando agregados supramoleculares que se denominan hemimicelas y admicelas. Estos agregados tienen la capacidad de solubilizar solutos mediante un proceso denominado adsolubilización. Hemimicelas y admicelas se han propuesto como adsorbentes en SPE y se han utilizado para la extracción/preconcentración de una gran variedad de contaminantes presentes en matrices ambientales complejas (1-6). Para poder utilizar este tipo de adsorbentes en SPE es necesario conocer las isotermas de adsorción del correspondiente tensioactivo sobre el óxido adecuado en las condiciones experimentales en las que se va a llevar a cabo la extracción.

2.1.1 Isotermas de adsorción

Son numerosos los estudios que se han llevado a cabo para determinar cómo son las isotermas de adsorción de tensioactivos iónicos sobre óxidos minerales (7-12). De acuerdo a la naturaleza de la superficie del óxido se distinguen dos tipos de isotermas:

a) De carga constante. La adsorción del tensioactivo se produce sin protonación o desprotonación del óxido mineral (Figura 1a).

b) De potencial constante. La adsorción del tensioactivo induce la protonación o desprotonación de la superficie del óxido (Figura 1b). La Figura 2 muestra, a modo de ejemplo, cómo la adsorción de un tensioactivo catiónico (DPC, cloruro de dodecilpiridinio) induce la desprotonación de la superficie de la sílice incrementando

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así su densidad de carga superficial a un valor de pH dado; y en consecuencia favoreciendo la adsorción de más moléculas de tensioactivo (9).

Como puede observarse, estas isotermas se dividen en cuatro regiones bien diferenciadas cuando se representa, en una escala logarítmica, la cantidad de tensioactivo adsorbido en función de la cantidad de tensioactivo en disolución (Figura 1).

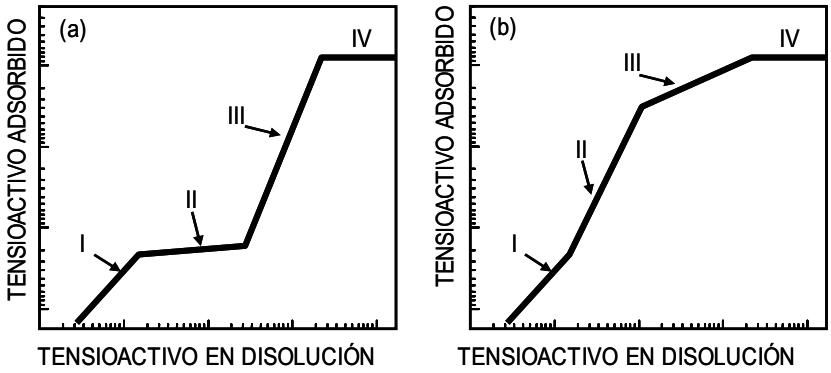

Figura 1. Isotermas de adsorción de tensioactivos iónicos sobre (a) superficies de carga constante y (b) superficies de potencial constante.

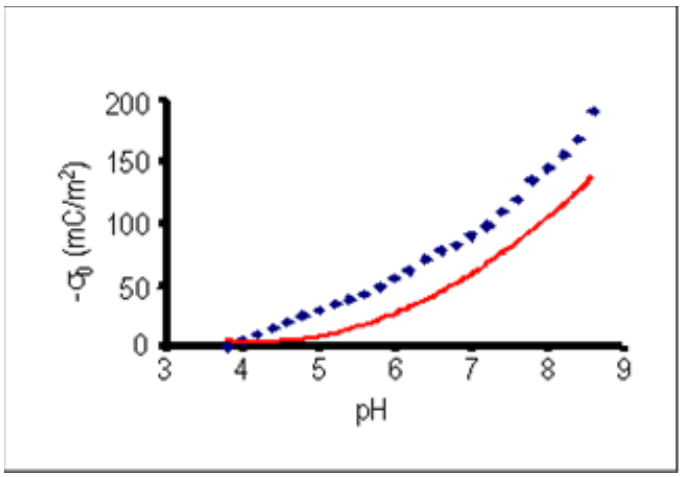

Figura 2. Curvas pH-carga de la sílice (Aerosil OX 50) en ausencia (----) y presencia (----) de 0.02 M de DPC (KCl 0.01 M)

En la región I, denominada región de Henry, los grupos cabeza de los monómeros de tensioactivo interaccionan electrostáticamente con la superficie del óxido mineral. La adsorción de las moléculas de tensioactivo ocurre de forma aislada, sin ningún tipo de interacción entre los monómeros adsorbidos (Figura 3). La formación de hemimicelas se inicia cuando se alcanza la concentración hemimicelar crítica (CHC). La adsorción de tensioactivo en la región hemimicelar se produce por interacciones electrostáticas con la superficie del óxido e interacciones laterales entre las cadenas hidrocarbonadas produciendo una monocapa con estructuras de agregación típicas (Figura 3), denominadas "teepee" en la terminología anglosajona. Si la superficie es de carga constante (Figura 1a), la pendiente de la isoterma es menor a la producida en la región I, ya que al aumentar la adsorción del tensioactivo se produce la progresiva neutralización de la carga superficial, disminuyendo la atracción electrostática. Para superficies de potencial constante (Figura 1b), la carga superficial no se compensa por la adsorción de moléculas aisladas de tensioactivo, sino que se generan nuevas cargas a medida que se produce la adsorción. Como resultado, la pendiente de la isoterma de la adsorción en la región II no disminuye, sino que aumenta, respecto a la región I.

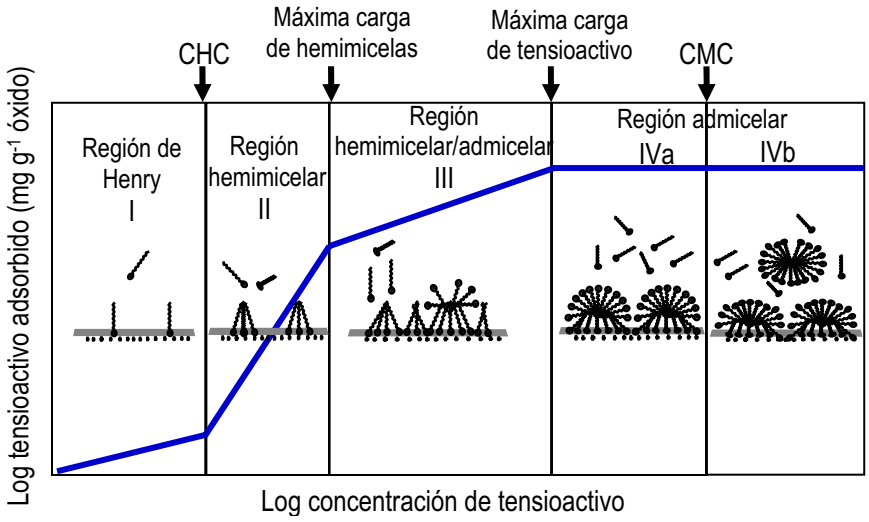

Figura 3. Representación esquemática de una isoterma de adsorción típica de cuatro regiones y estructuras propuestas para los diferentes agregados de tensioactivo formados.

La región III comienza cuando se produce la saturación de los centros activos del óxido por el tensioactivo adsorbido. En esta región, la adsorción del tensioactivo se debe exclusivamente a las interacciones hidrófobas entre las cadenas hidrocarbonadas produciéndose agregados denominados admicelas. Una vez alcanzada la carga máxima de tensioactivo sobre el óxido mineral, la adición de tensioactivo al sistema producirá el aumento de la concentración del mismo en disolución hasta alcanzar la concentración micelar crítica (CMC), a partir de la cual se forman micelas en disolución. En la tabla I se muestran algunos de los sistemas óxido mineral-tensioactivo cuyas isotermas son similares a la descrita (13).

Tabla 1. Diferentes sistemas tensioactivo-adsorbente que generan una típica isoterma de adsorción de cuatro regiones

Tensioactivo	**Adsorbente**
Dodecilsulfonato sódico	Alúmina
Alquilbenceno sulfonato	Alúmina
Cloruro de tetradecilpiridinio	Gel de sílice
Bromuro de tetradecilpiridinio	Gel de sílice
Bromuro de dodecilpiridinio	Gel de sílice
Dodecilsulfato sódico	Alúmina
Igepal Co-660	Sílice pirogénica
Cloruro de dodecilpiridinio	Rutilo
p-3 nonilbencenosulfonato sódico	Rutilo
4-C_{11} paraxilenosulfonato	Alúmina

Factores que influyen en la adsorción de tensioactivo sobre el óxido mineral

La formación de hemimicelas y admicelas sobre la superficie de un óxido mineral está influenciada por los mismos parámetros que afectan a la formación de micelas en disoluciones acuosas. Así, los parámetros que afectan a la concentración micelar crítica (CMC) tales como la presencia de electrolito, la longitud y el grado de ramificación de la cadena hidrocarbonada de la molécula de tensioactivo, la presencia de aditivos

(13) Paria S., Khilar K.C. *Adv. Colloid Interfac.* 110 (2004) 75.

de naturaleza orgánica, etc., también modifican la concentración hemimicelar crítica (CHC) y el punto de transición entre las regiones III y IV de la isoterma de adsorción (14).

El pH es uno de los parámetros más importantes ya que determina la densidad de carga superficial del óxido mineral y por tanto la cantidad de tensioactivo que puede adsorberse. Los óxidos minerales más usados, alúmina y sílice, tienen punto de carga cero (pcz) a valores de pH alrededor de 9 y 3, respectivamente. Por tanto, la alúmina se usa a valores de pH ácidos para la adsorción de tensioactivos aniónicos y la sílice a valores de pH neutros o ligeramente básicos para la adsorción de tensioactivos catiónicos. En la Figura 2 puede observarse cómo la densidad de carga de la sílice aumenta a medida que aumenta el pH, es decir a medida que el pH se aleja del pcz (9).

Con relación a la cadena hidrocarbonada, cuanto mayor es su longitud, más hidrófoba es la molécula y mayor es su facilidad para formar agregados, con la consiguiente disminución de la CMC o CHC. Un incremento de un grupo $-CH_2-$ en la cadena hidrocarbonada del tensioactivo produce una disminución de la CMC y CHC de tres veces (regla de Traube) (15).

En cuanto a la influencia de la presencia de electrolito y tipo de contraión del tensioactivo sobre la adsorción del mismo, la Tabla II muestra como ejemplo los resultados obtenidos en los estudios realizados por Velegol y Tilton (16) utilizando cetiltrimetilamonio (CTA^+) y sílice. Como puede observarse, la cantidad adsorbida de CTA^+ es mayor en presencia de electrolito. Por otro lado, además de a la adsorción, el contraión también puede afectar a la morfología del agregado, pasando de ser esférico a cilíndrico. Así, cuando disminuye la interacción del contraión con las moléculas de agua (caso del ión bromuro frente al cloruro), éste tiende a asociarse más con las moléculas del tensioactivo que constituyen el agregado, lo cuál reduce las fuerzas repulsivas entre los grupos de cabeza permitiendo la formación de agregados cilíndricos menos curvados.

La temperatura afecta de forma negativa a la cantidad máxima de tensioactivo que puede adsorberse sobre el óxido mineral. La disminución en la cantidad máxima de tensioactivo adsorbida se debe al aumento de la energía cinética de las especies y por lo tanto hay un aumento de la entropía del sistema que causa una reducción de la organización de los agregados en la superficie del adsorbente (13).

(14) Bitting D., Harwell J.H. *Langmuir* 3 (1987) 531.

(15) Fan A., Somasundaran P., Turro N.J. *Langmuir* 13 (1997) 506.

(16) Velegol S.V., Tilton R.D. *J. Colloid Interf. Sci.* 249 (2002) 282.

Tabla 2. Cantidad máxima de CTA^+ adsorbida en función del contraión del tensioactivo y la presencia de electrolito

Contraión	Cantidad máxima adsorbida (mg m^{-2})	
	En ausencia de electrolito	En presencia de KCl 10 mM
Cloruro	0.9±0.1	1.2±0.2
Bromuro	1.7±0.2	2.1±0.2
p-toluenosulfonato	2.7±0.1	2.9±0.3

2.2. Mecanismos de adsolubilización de los analitos

El fenómeno de adsolubilización de compuestos orgánicos sobre hemimicelas y admicelas presenta un gran potencial para extracción en fase sólida. Los agregados de tensioctivos poseen estructura tridimensional con diferentes microambientes donde analitos de muy diversa polaridad y carga pueden solubilizarse. Las interacciones que pueden ofrecer son muy variadas dependiendo de las características del grupo polar del tensioactivo y del tipo de agregado seleccionado.

De las cuatro regiones en las que se divide una isoterma, no todas son adecuadas para su utilización en SPE. Las regiones que pueden utilizarse son aquellas en las que hemimicelas y admicelas están en equilibrio con monómeros en disolución. Así, la región I (Figura 4), donde el mecanismo de adsorción de tensioactivo sobre el óxido mineral se debe a interacciones meramente electrostáticas, no es apta para SPE puesto que no se forma ningún tipo de agregado donde los analitos puedan solubilizarse. En la región hemimicelar (región II, Figura 4) los analitos pueden solubilizarse en el adsorbente mediante interacciones hidrófobas con la cadena hidrocarbonada del tensioactivo. Las regiones III (constituida por hemimicelas y admicelas) y IVa (admicelas) son las que ofrecen mayores posibilidades en SPE puesto que la superficie exterior es polar o iónica y el tipo de interacción puede modificarse mediante selección del tensioactivo. En estas regiones pueden adsolubilizarse analitos de muy diferente polaridad, ya que pueden producirse enlaces por puente de hidrógeno, interacciones iónicas, de Van der Waals o π-catión y formación de agregados mixtos (cuando los analitos presentan carácter anfifílico). Así, se podría lograr la retención simultánea de analitos con muy diferente polaridad, siendo esta característica muy importante en monitorización medioambiental.

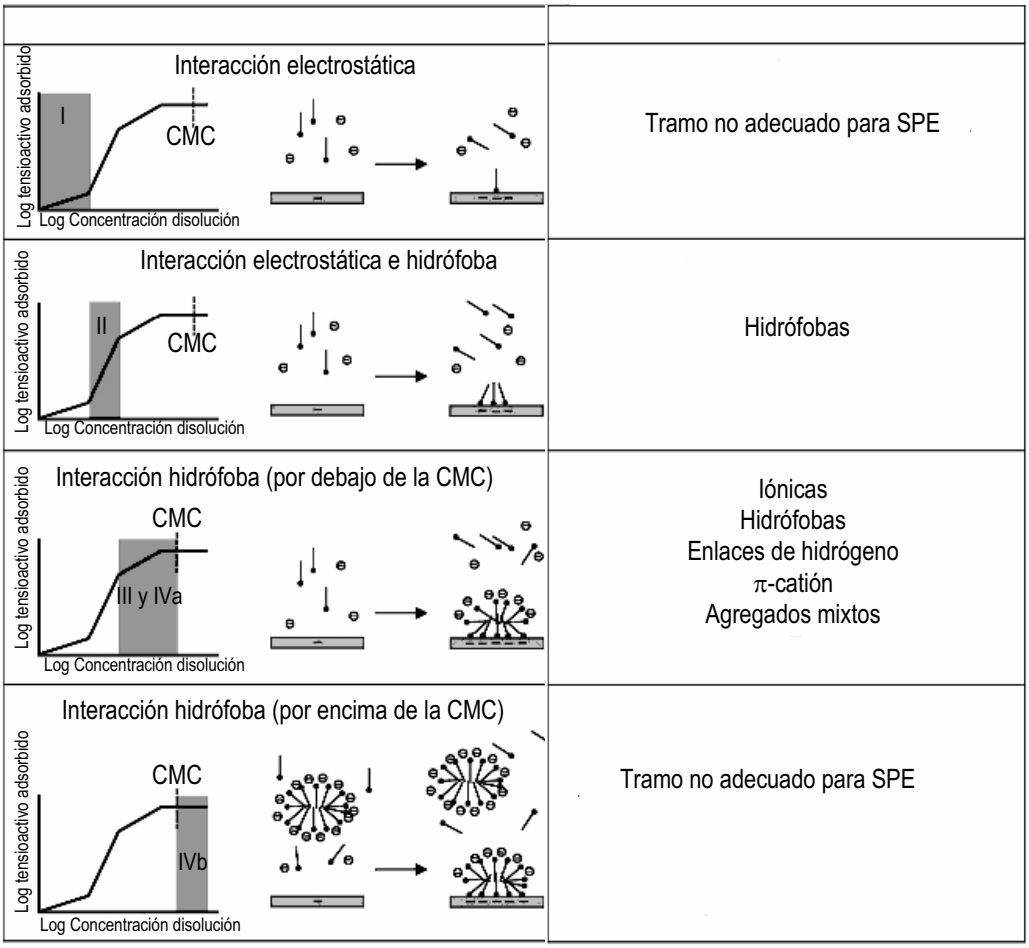

Figura 4. Mecanismo de adsorción del tensioactivo sobre el óxido mineral y de adsolubilización de solutos sobre los agregados formados en las distintas regiones de la isoterma de adsorción

Cuando la concentración de tensioactivo en disolución alcanza la CMC, región IVb, (Figura 4), comienzan a formarse micelas y en este caso la retención de los analitos sobre el adsorbente no es cuantitativa ya que éstos se distribuyen entre el adsorbente y las micelas en disolución. Por consiguiente, esta región de la isoterma no es apta para SPE.

2.3 Modo de operación

Un aspecto importante a considerar cuando se trabaja con hemimicelas y/o admicelas como adsorbente en SPE es que la adsorción del tensioactivo sobre el óxido mineral es un proceso dinámico, por lo que estos agregados se encuentran en equilibrio con moléculas de tensioactivo en disolución. Por tanto, durante la percolación de la muestra a través del adsorbente se producirá una pérdida progresiva de tensioactivo, lo cual modificará la cantidad y/o características de la fase adsorbente usada. Si se trabaja en la región hemimicelar, la concentración en disolución es del orden de $\mu g\ L^{-1}$ y la pérdida de tensioactivo durante la percolación de la muestra es despreciable, no necesitándose hacer un aporte adicional de tensioactivo a la muestra. Sin embargo, la concentración de tensioactivo en disolución en las regiones donde se producen admicelas es del orden de $mg\ L^{-1}$, y por tanto si estos agregados se usan en SPE es fundamental añadir a la muestra la concentración adecuada de tensioactivo, para mantener constante la cantidad de tensioactivo adsorbido.

El modo de operación cuando se trabaja en extracción en fase sólida con hemimicelas/admicelas es similar a la extracción en fase sólida convencional (Figura 5):

a) En primer lugar se procede a la formación de hemimicela y/o admicelas pasando una disolución del tensioactivo seleccionado, al pH al que se llevará a cabo la extracción, a través del óxido mineral.

b) A continuación se procede a la percolación de la muestra de manera que se produce la adsolubilización de los analitos (retención). Hay que tener presente el tipo de agregado con el que se trabaja, pues según se acaba de mencionar, si se trabaja en la zona admicelar hay que añadir a la muestra la concentración adecuada de tensioactivo, para mantener constante la cantidad adsorbida del mismo.

c) Finalmente, los analitos se eluyen con 1-2 mL de disolvente orgánico o disoluciones acuosas a un valor de pH adecuado para romper las interacciones tensioactivo-óxido mineral.

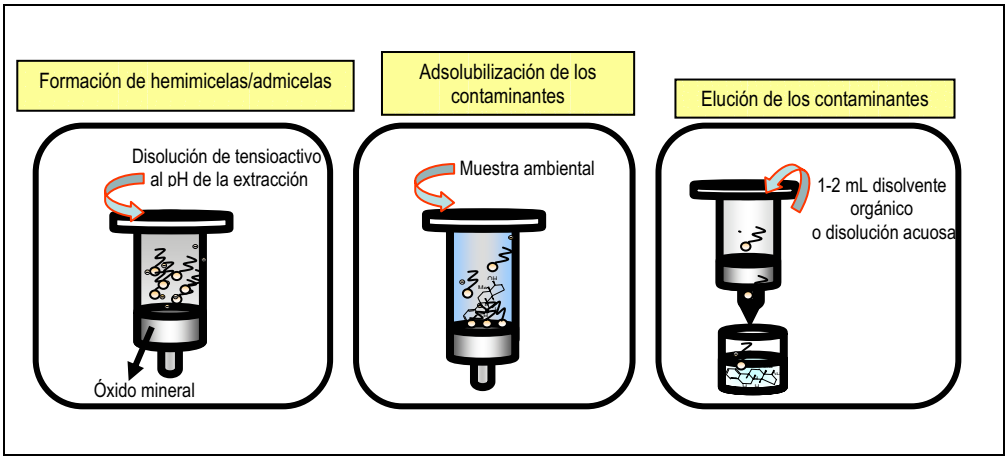

Figura 5. Modo de operación cuando se usan hemimicelas y/o admicelas como adsorbente en SPE.

2.4. Antecedentes

El fenómeno de la adsolubilización sobre hemimicelas/admicelas se ha utilizado entre otras aplicaciones para la descontaminación de suelos, tratamiento de aguas residuales, etc. (17). En Química Analítica, este fenómeno se ha utilizado para el desarrollo de la cromatografía admicelar (18), donde el principal problema hallado ha sido la progresiva elución del tensioactivo retenido en el soporte durante el desarrollo cromatográfico con la consiguiente irreproducibilidad en los tiempos de retención para los analitos cromatografiados. Asimismo, se ha aplicado a la extracción de metales pesados presentes en muestras acuosas mediante la formación de complejos con agentes quelatantes, previamente solubilizados sobre el agregado (19-22) o bien mediante formación de un quelato en disolución acuosa y posterior adsolubilización (20, 23).

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Hasta hace relativamente poco tiempo, las hemimicelas/admicelas no se habían utilizado para la extracción/preconcentración de compuestos orgánicos previa a su determinación analítica. En los últimos años, sin embargo, se han desarrollado métodos para la extracción de una gran variedad de compuestos en muestras acuosas ambientales. La presencia de regiones de diferente polaridad en estos agregados supramoleculares muestra excelente capacidad para solubilizar una gran variedad de compuestos orgánicos.

Así, se han empleado hemimicelas del tensioactivo aniónico dodecil sulfato sódico (SDS) adsorbido sobre alúmina para la extracción/preconcentración de diferentes compuestos tales como clorofenoles (24,25), alquilfenoles y alquilfenoles carboxilados (26), ftalatos (4, 25) e hidrocarburos policíclicos aromáticos (PAHs) (27) presentes en muestras acuosas ambientales. En estos ejemplos el mecanismo de solubilización de los analitos sobre el agregado se debe al establecimiento de interacciones hidrófobas. También se ha propuesto, basado en este tipo de interacciones, la determinación de PAHs en muestras de agua de río y grifo usando tensioactivos catiónicos (bromuro de cetilpiridinio, bromuro de cetiltrimetil amonio y bromuro de octodeciltrimetil amonio) adsorbidos sobre sílice (28).

Recientemente se han desarrollado métodos en los que el mecanismo de extracción se basa en la formación de agregados mixtos analito-extractante. Hemimicelas de SDS adsorbidas sobre alúmina se han empleado para extraer compuestos anfifílicos por este mecanismo, es el caso de la determinación de los homólogos del tensioactivo catiónico benzalconium (1) o de los tensioactivos no iónicos alquilfenol etoxilados y alquil etoxilados (3). Hemimicelas del tensioactivo catiónico cloruro de cetilpiridinio (CPC) adsorbidas sobre sílice se han empleado para extraer los tensioactivos aniónicos alquilbenceno sulfonatos (LAS) a partir de muestras de agua residual y de río (5).

Dado que el tipo de interacción puede variar dependiendo de la naturaleza del grupo polar del tensioactivo, se puede seleccionar a priori el adsorbente más adecuado para una aplicación concreta. Así, se ha llevado a cabo la extracción de benzimidazoles (29) y herbicidas de amonio cuaternario"quats" (2) utilizando admicelas de SDS sobre alúmina

donde los analitos se retienen mediante interacciones de tipo electrostático o por formación de pares iónicos, respectivamente.

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En la aplicación relativa a los quats, se propuso por vez primera el acoplamiento on-line de un adsorbente de SPE basado en agregados supramoleculares de tensioactivo a cromatografía de líquidos.

Las hemimicelas/admicelas mixtas de tetrabutilamonio (TBA)-SDS adsorbidas sobre alúmina ofrecen otro mecanismo de retención de analitos por el establecimiento de interacciones π-catión entre el grupo aromático de éstos y el grupo amonio del tetrabutilamonio. Con esta estrategia se han extraído bisfenoles (6) y se ha desarrollado un análisis multiresidual de plaguicidas (30).

Capítulo 4. Study of the influence of water matrix components in admicellar sorbents

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Study of the influence of water matrix components in admicellar sorbents

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Abstract

Hemimicelle and/or admicelle-based SPE have been recently proved as a fruitful strategy for the extraction and concentration of a wide variety of organic pollutants. This research focus on the effect of river and wastewater matrix components on the adsorption of sodium dodecyl sulfate (SDS) onto alumina, which is the most used sorbent in hemimicelle and/or admicelle-based SPE, and discuss the analytical consequences of the modifications observed. The effect of electrolytes (0.1 M NaCl), precipitating agents (127 and 333 mg L^{-1} $CaCl_2$) and major organic components in wastewater (19.8 mg L^{-1} of carbohydrates, proteins and fats and 10 mg L^{-1} of LAS) and river (8 mg L^{-1} humic acid) on the SDS adsorption isotherm was investigated. Also, the global effect of matrix components was assessed using a river sample. Three types of sorbents were considered (hemimicelles, mixed hemimicelles/admicelles and admicelles). Electrolytes were found to compete with surfactant molecules for charge surface sites in the early part of the hemimicellar region; precipitating agents yield insoluble salts with the aqueous

surfactant in equilibrium with admicelle-based sorbents; and organic matter did not have any influence at all. The matrix component concentrations investigated were above the usual range present in rivers and wastewater, which makes this study applicable to a wide number of environmental water samples. From the results obtained, simple rules were established to prevent and detect matrix-induced surfactant adsorption modifications, which permits to know, a priori, the suitability of these sorbents for a specific application and allows the development of more straightforward and robust methods.

Introduction

Hemimicelles and admicelles are sorbents made up of surfactant aggregates adsorbed on mineral oxides [1-8]. These systems possess unique features that convert them into very versatile materials for SPE. Thus, because of the amphiphilic character of surfactants, their aggregates have different polarity regions where analytes of a wide polarity/charge range can be solubilized. A number of analyte:surfactant aggregate interactions can be established by the appropriate selection of the surfactant polar group. To date, SPE processes based on ionic [9], π-cation [10], hydrophobic [11-13] and formation of mixed aggregates [14-16] have been developed. Pollutants such as surfactants [16,17], herbicides [9], phthalate esters [11], bisphenols [10], estrogens [13], chlorophenols [12], PAHs [18,19] and so on have been efficiently extracted by hemimicelles and/or admicelles from a variety of environmental water samples.

In addition to the investigations carried out to prove the suitability of hemimicelles/admicelles as SPE sorbents, some theoretical studies, of interest for analytical extractions, have been developed [9,20]. They have permitted us to establish some guidelines for the optimization of the parameters that affect the SPE sequence and for the way in which the SPE method must be applied. At present, we know that there are parameters which cannot be optimized in the traditional way since they modify the adsorption isotherms [9]. One example is the pH, which dramatically modifies the charge density on the oxide surface and, therefore, determines the surfactant load and/or the type of sorbent produced (hemimicelle or admicelle). On the other hand, it is now clear the consequences on the SPE operation mode arising from the fact that hemimicelles/admicelles are dynamic entities in equilibrium with aqueous surfactant monomers. This operation mode must guarantee a unique partition constant for analytes during the percolation of the sample, which requires the addition of a determined amount of surfactant to the sample in some application [9].

Up to now, the applications developed have permitted us to get an insight into the analyte:hemimicelle/admicelle interactions that can be established, and this knowledge has facilitated the selection of the appropriate surfactant/oxide system for a specific

application. However, no studies have been intended to elucidate how environmental water matrix components affect the adsorption of the surfactant on the mineral oxide, despite matrix components can modify adsorption isotherms and, as a result, the SPE of pollutants. So, a good knowledge of the interactions between matrix and sorbent is essential to prevent interferences and to get efficient and robust SPE methods.

The matrix composition of environmental water samples is highly variable, but in practical terms we can consider two kind of components; organic matter and inorganic salts. Organic matter in wastewater is mainly made up of carbohydrates, fats, proteins and surfactants [21]. In rivers, humic acids, which come from the biodegradation of animal and vegetal cells, predominate [22]. With respect to inorganic salts we must consider two possible effects on hemimicelle/admicelle-based extractions; electrolyte effect and surfactant precipitation.

A thorough review of the physical chemical literature on hemimicelles/admicelles reveals that extensive research has been conducted to elucidate the mechanism of ionic surfactant adsorption at the solid-aqueous interface, the parameters that affect adsorption isotherms (e.g. surfactant chain length, oxide surface charge, etc), and the structure of the aggregates formed [1]. A significant proportion of the available literature concerning surfactant adsorption at the solid-aqueous interface concerns amorphous silica [1,3] and comparatively few basic research has involved the study of more interesting oxides for SPE such as alumina. To the best of our knowledge, there are not antecedents about the effect of organic matter, salts or precipitating agents on the formation of hemimicelles/admicelles under the experimental conditions of interest for the SPE of pollutants.

This work was intended to investigate the effect of environmental water matrix components on the adsorption of the surfactant onto the mineral oxide in order to get an insight into the behavior of hemimicelle- and admmicelle-based sorbents when used in environmental applications. Wastewater and river water were selected as typical matrices of polluted and natural water, respectively. Dodecyl sulfate adsorbed on alumina was selected as a sorbent since it has been the most used one for the extraction of pollutants [9-17,19]. The main reason is that alumina has a high charge density at acidic pHs, and

these pH values are very convenient for the handling of environmental water samples. The effect of matrix components on the adsorption of SDS on alumina was elucidated through the study of the corresponding adsorption isotherms. Below, the main results obtained are discussed.

Experimental

Chemicals and Materials

The following reagents were of analytical reagent-grade and were used as supplied. Sodium dodecyl sulfate (SDS), sodium chloride and humic acid were obtained from Aldrich (Steinheim, Germany); formic acid from Riedel de Haën (Seelze, Germany) and calcium chloride, ammonia and HPLC-grade methanol from Panreac (Barcelona, Spain). Alumina (γ-form, for column chromatography) was purchased from Sigma (St. Louis, MO). The physical properties of this mineral oxide were as follows: surface area, 155 $m^2\ g^{-1}$; point of zero charge, pzc, 8.5; particle diameter range, 50-200 μm; mean value, 100 μm; mean pore size 58 Å; density, 3.97 g cm^{-3}.

Samples

River samples were collected in dark glass containers from the Rabanales river flowing through Córdoba city in April 2006. Samples were filtered through 7-9 μm paper filters (Anoia S.A., Spain) and then through 0.45 μm nylon membranes (Millipore, Bedford, MA, USA), both supplied by Análisis Vínicos S.L. (Tomelloso, Spain). After that, the pH was adjusted to 2 with concentrated nitric acid and the sample was stored at 4ºC under light protection conditions. To evaluate the effect of municipal wastewater organic components, a synthetic sample [23,24] containing food ingredients, as a source of carbohydrates, proteins and fatty acids, and anionic detergents (i.e. linear alkylbenzene sulfonate, LAS) was prepared weekly. Food components were obtained from a commercial chicken soup

(Gallina Blanca, Barcelona, Spain). The qualitative soup composition was as follows: chicken meat, chicken fat, celery, leek, carrot, potato, onion, boiled ham, lard, meat extract, yeast extract, egg, milk, soybean, and salt. The soup was diluted with distilled water to give final concentrations of carbohydrates, proteins and fats similar to those recommended [23,24] (i.e. 62 mg L^{-1} each). To this solution, an appropriate amount of a commercial LAS C_{10}-C_{14} mixture (Petrelab P-550) provided by Massó and Carol (Barcelona, Spain) to give a total content of anionic surfactants of 10 mg L^{-1} was added. The percentage of the different homologues in the mixture was 9.6% C_{10}, 38.1% C_{11}, 31.3% C_{12}, 19.1% C_{13} and 1% C_{14}. The final solution was filtered through a 0.45 μm nylon membrane, the pH was adjusted to 2 and the sample was stored at 4ºC.

Influence of Matrix Components on Surfactant Adsorption

Adsorption isotherms of SDS on alumina were obtained in distilled water and in the presence of different water matrix components in the batch mode. The procedure was as follows: a constant amount of alumina (0.5 g) was introduced into different 600 mL vessels. Then, 500 mL of aqueous solutions containing variable amounts of surfactant (10-3000 mg) at pH 2 (adjusted with nitric acid), and the corresponding matrix component at a fixed concentration were added. The suspensions were stirred vigorously for 5 min and then centrifuged at 3500 rpm for 15 min. The concentrations of SDS in the supernatants were determined by using a liquid chromatography/electrospray ionization/ion trap mass spectrometry, LC/(ESI-IT)-MS, system (1100 series LC/ MSD, Agilent Technologies, Waldbronn, Germany), equipped with an automatic injector. The stationary-phase used was a Hypersyl ODS column (150 x 4.6 mm, 5 μm particle diameter; Análisis Vínicos S.L., Tomelloso, Spain). The mobile phase was made of methanol/ water 67:33. Under isocratic conditions, SDS eluted at 6.7 min. Quantification was carried out in the ESI negative mode. The operational conditions of the ESI interface were: capillary voltage, 4.5 KV; capillary exit voltage, -108.3 V; skimmer, -40 V; trap drive, 37.6; source temperature, 350ºC; drying gas, 10 L min^{-1}; nebulizer gas, 80 psi; maximal accumulation time, 100 ms. Quantification was carried out under full-scan

conditions (m/z scan range, 200-300) by using the extracted molecular ion chromatogram, and the corresponding peak area was measured. Smooth chromatograms were obtained by using the Gauss function (width, 3 points; cycles, 1). Correlation between peak areas and SDS concentration was obtained in the range 0.14- 20 ng (absolute amount injected in 20 μL) with a correlation coefficient of ~0.9997. Adsorption isotherms of SDS in the presence of the following additives were obtained: a) 0.1 M sodium chloride, b) 127 and 333 mg L^{-1} calcium chloride, c) 19.8 mg L^{-1} of carbohydrates, proteins and fats and 10 mg L^{-1} of LAS, d) 8 mg L^{-1} humic acid, and e) river water. Peak areas for SDS were reduced by about a half in the presence of 0.1 M NaCl. So, calibrations in the presence of this additive required the addition of 0.1 M NaCl to the SDS standards.

Results and discussion

SDS Adsorption onto Alumina

The knowledge of the surfactant isotherms under the experimental conditions selected for SPE is essential for the correct application of these sorbents and to explain their behavior as a function of the experimental variables. Figure 1 shows the isotherm obtained for the adsorption of SDS on alumina at pH 2 and the different surfactant aggregate structures (hemimicelles and admicelles) formed in each isotherm region.

Hemimicelles consist of monolayers of surfactant monomers adsorbed head-down on the oppositely charged mineral oxide surface while the hydrocarbon chains protrude into solution with strong lateral interactions between them, which leads to teepee structures. After neutralization of the oxide surface, hydrophobic interactions are the driving force for the adsorption of surfactant molecules, which results in the formation of admicelles. These aggregates have been traditionally described as surfactant bilayers, although recent studies have brought forward evidence of the formation of discrete surface aggregates, similar to aqueous micelles [1]. Once the oxide surface is completely covered by admicelles, the amount of SDS adsorbed remains constant and the surfactant in solution increases sharply until the critical micelle concentration (cmc) is reached and aqueous

micelles are formed. The admicellar region above the cmc is not advisable for SPE since analytes partition between micelles and admicelles occurs. The maximal amount of SDS adsorbed (about 210 mg g^{-1} alumina, Figure 1) directly depends on the density of positive charges on the alumina, which in turn depends on the pH. The point of zero charge for this oxide occurs at approximately pH 8.5. The density of positive charges remains low until the solution pH reaches 5, but increases sharply between pH 5 and 2. So, pH 2 is recommended when the SDS-alumina system is used as a SPE sorbent.

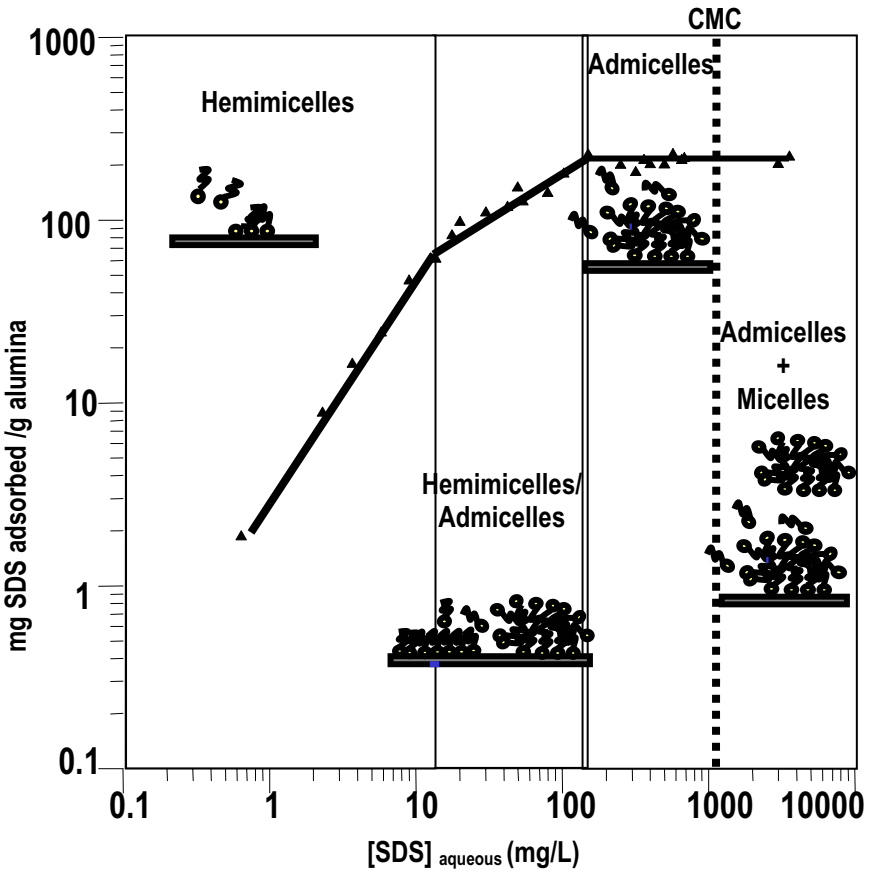

Figure 1. Experimental adsorption isotherm for sodium dodecyl sulfate (SDS) onto alumina at pH 2.

Influence of Waste and River Water Matrix Components on the Adsorption of SDS onto Alumina

The effect of three types of matrix components on the adsorption of SDS onto alumina at pH 2 was considered in this study, namely, the electrolyte effect, precipitating agents and organic matter. The concentrations of these components were selected to match real environmental conditions. In order to reproduce the experimental conditions used in SPE, the adsorption isotherms were obtained by adding each surfactant concentration to the mineral oxide directly, in one single step, and not in a step-wise manner.

Electrolyte effect

Electrolyte concentration in rivers and wastewaters. The amount of dissolved salts in environmental waters is routinely measured by the conductivity [25]. There are no official guidelines as to what is considered as a safe level for conductivity in rivers. In USA, it ranges from 50 to 1500 μS cm^{-1} [26]. Conductivity in domestic wastewaters is highly variable, but it usually ranges between 700 and 1200 μS cm^{-1} [22], while some industrial wastewater can reach conductivity values as high as 10000 μS cm^{-1} [23]. Total dissolved solids (mg L^{-1}) in a sample are estimated by multiplying conductivity by an empirical factor [25]. On the basis of these estimations, we selected 0.1 M NaCl to evaluate the electrolyte effect in the SDS adsorption onto alumina, which corresponds to 7795 μS cm^{-1} [25].

Electrolyte-induced modifications in the SDS adsorption isotherm Figure 2 compares the experimental adsorption isotherms of SDS onto alumina in the absence and presence of 0.1 M NaCl at pH 2. Similar results have been obtained for the adsorption of cationic surfactants onto rutile [5] and silica [8] (pH 7-9) and alkyl benzene sulfonates onto rutile [5] (pH 4.1) at salt concentrations ranging between 0.001 and 0.1 M. There is an intersection point between the isotherms (Figure 2) that represents the point where the orientation of adsorbing surfactant molecules changes from head-groups facing towards

the oxide to head-groups facing the solution, that it is to say, the point at which the electrostatic contribution to adsorption changes from attractive to repulsive. Below the intersection point, the addition of the electrolyte lowers adsorption by competing with monormers for charge surface sites. Thus, more bulk surfactant concentration will be needed to form hemimicelles. Above the intersection point, the addition of electrolyte lowers the mutual head-groups electrostatic repulsions, which permits tighter packing and causes an increase in the amount of surfactant adsorbed onto the oxide surface.

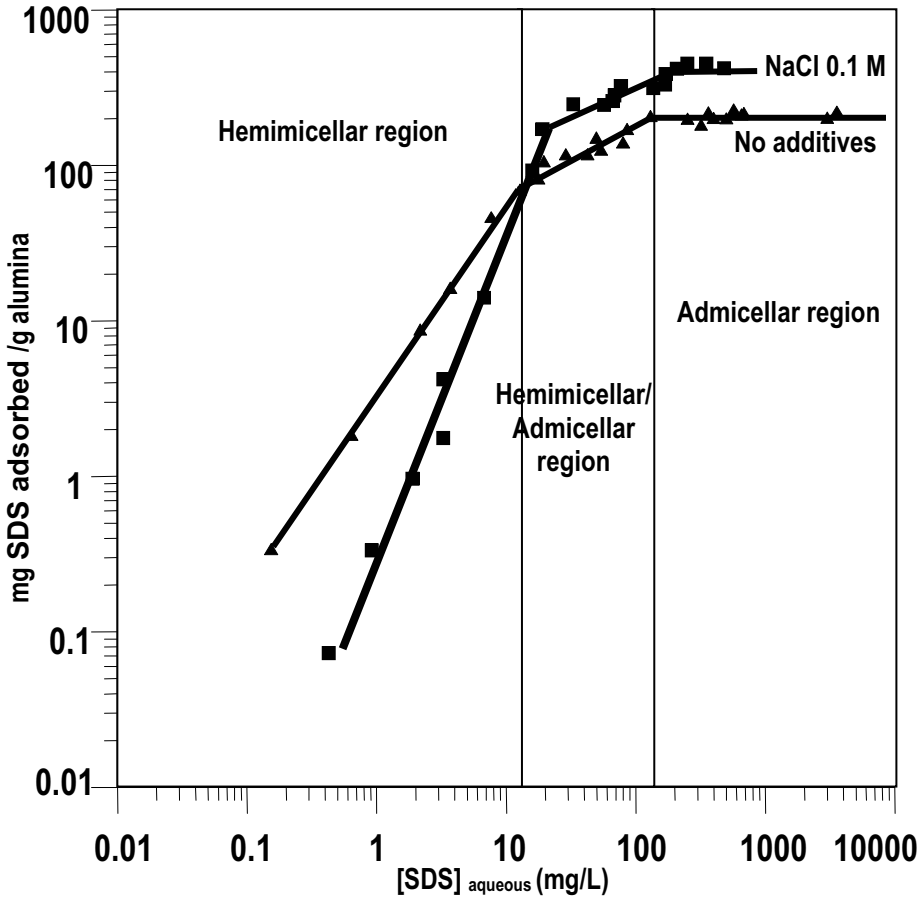

Figure 2. Experimental adsorption isotherms for SDS onto alumina at pH 2: in the absence of additives (triangles) and in the presence of 0.1 M NaCl (squares)

Analytical consequences. Isotherms in Figure 2 represent the boundaries of the adsorption of SDS onto alumina in the presence of salt in the range 0-0.1M. So, the conclusions inferred below are applicable to the SDS adsorption into this salt concentration range. Electrolytes decrease the surfactant load of hemimicelle-based sorbents in the early part of the hemimicellar region, which could have consequences on the retention of analytes and the breakthrough volume of samples. Fortunately, electrolytes do not influence the SDS load at the end of the hemimicelllar region, near the complete neutralization of the mineral oxide charge, which is just the zone usually recommended when working with hemimicelle-based sorbents for the extraction of pollutants. So, in order to prevent negative electrolyte effects and to achieve both high retention of analytes and breakthrough volumes, hemimicelle-based sorbents should be prepared at bulk surfactant concentrations at which maximal hemimicelle load is obtained.

On the other hand, electrolytes increase the amount of surfactant adsorbed in the hemimicelle/admicelle and admicelle regions by a maximum factor of around two. This electrolyte positive effect could sometimes raise the adsolubilization of analytes with very low distribution coefficients. However, the surfactant load in these regions is high enough in the absence of salts to no expect significant electrolyte effect for the extraction of the majority of pollutants, since maximal adsolubilization is generally reached before getting maximal surfactant load.

Precipitating agents

Precipitation of anionic surfactant salts. The precipitation of anionic surfactants by polyvalent cations has been largely studied [27,28] and it results in a serious problem in some surfactant applications (e.g. oil recovery). In environmental waters, calcium and magnesium salts are expected to be the main precipitating agents of SDS because of both the relatively high concentration of these ions and the insolubility of calcium and magnesium dodecyl sulfate salts ($Ca(DS)_2$, $K_{sp}= 2.14x10^{-10}$; $Mg(DS)_2$, $K_{sp}=3.09x10^{-9}$). We selected calcium as a representative ion to assess the influence of precipitating agents on the adsorption of SDS onto alumina. Figure 3a shows the precipitation boundaries for the

system $Ca(NO_3)_2$-NaDS [28]. The lower boundary is determined by the saturation, which is described by the solubility product constant. The superior boundary is explained on the basis of the formation of SDS micelles in solution which dissolve $Ca(DS)_2$ by exchange of Na and Ca ions at the micellar surface.

Precipitating agent concentration in rivers and wastewaters. Patterns of the hardness of surface waters in the United States have been investigated by monitoring 344 stations [29]. About half of the mean hardness values for the stations were in the soft to moderately hard categories (e.g. 0-120 mg L^{-1} $CaCO_3$), and about half were classified as hard to very hard (e.g. 121-more than 180 mg L^{-1} $CaCO_3$). In wastewater samples, hardness values between 90 and 250 mg L^{-1} $CaCO_3$ have been recently reported [30-32]. On the basis of these values, we investigated the influence of Ca concentrations that were representative of moderately hard (112 mg L^{-1} $CaCO_3$) and very hard (300 mg L^{-1} $CaCO_3$) waters on the SDS adsorption onto alumina.

Precipitating agent-induced modifications in the SDS adsorption isotherm . Figure 3b compares the adsorption isotherms of SDS onto alumina obtained in the absence and the presence of two calcium concentrations. No studies involving the effect of precipitating agents on the SDS-alumina system have been previously reported. The surfactant adsorption isotherms were similar in the absence and presence of calcium ions until the aqueous SDS concentration was high enough to precipitate $Ca(DS)_2$. The start of the precipitation occurred at aqueous SDS concentrations above those expected from the solubility product constant (e.g. around 100 mg L^{-1} SDS, experimental value, instead of 77 mg L^{-1} SDS, calculated value, for precipitation of 300 mg L^{-1} $CaCO_3$). The amount of $Ca(DS)_2$ increased linearly with SDS (Figure 3cI) , however calcium ions were not completely removed from the solution; only around 50-60% of the total amount of Ca precipitated. The addition of more surfactant did not have any effect on the precipitate (see the plateau in the admicellar region, figure 3cII) until its concentration was high enough to form aqueous SDS micelles (Figure 3cIII). These micelles progressively dissolved $Ca(DS)_2$. Figure 3c illustrates the most probable equilibria involved in the

$Ca(DS)_2$ precipitation region. Initially, both precipitation and admicellar solubilization of $Ca(DS)_2$ compete, which explains the incomplete precipitation of calcium.

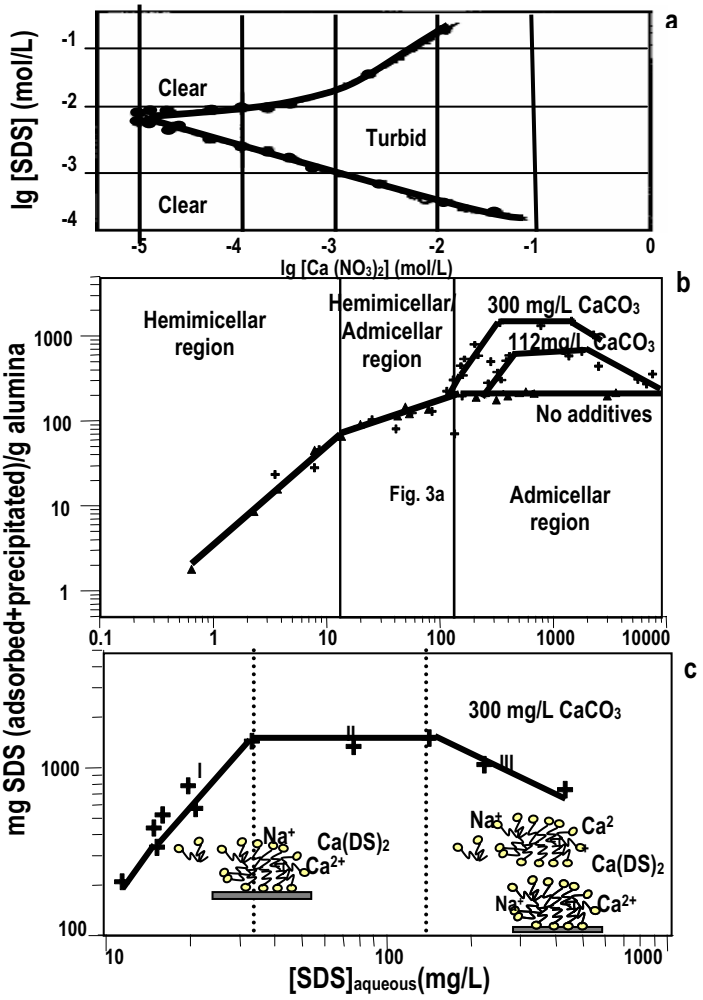

Figure 3. a Precipitation boundaries of calcium dodecyl sulfate. **b** Experimental adsorption isotherms for SDS onto alumina at pH 2 in the absence of additives (triangles) and in the presence of 112 and 300 mg/L $CaCO_3$ (crosses). **c** Chemical equilibria involved in the calcium dodecyl sulfate precipitation region. $Ca(DS)_2$ calcium dodecyl sulfate

These processes reach equilibrium conditions which are kept until aqueous micelles, that also have the capacity of dissolving $Ca(DS)_2$, are formed in solution. Admicellar and micellar solubilization of $Ca(DS)_2$ probably occurs by the exchange of Na and Ca ions at the aggregate surface.

Analytical consequences. According to the results obtained, polyvalent cations precipitate aqueous SDS but they do not precipitate adsorbed surfactant. No precipitating agent-induced modifications are expected for hemimicelle- and hemimicelle/admicelle-based sorbents from the usual polyvalent cation content of environmental water samples because of the low concentration of aqueous SDS in equilibrium with adsorbed surfactant aggregates in these isotherm regions. Most of the interferences caused by Ca and Mg ions are expected to occur in the admicellar region, where the concentration of the aqueous surfactant greatly increases. So, working with admicelle-based sorbents can require the removal of Ca and Mg before SPE of the water sample. However, the occurrence of this interference from a suspected water sample can be easily detected by adding SDS to a sample aliquot. The SDS concentration that must be tested to detect cation-induced precipitation in a specific admicelle-based sorbent can be easily inferred from the surfactant adsorption isotherm.

Organic matter

Composition and concentration of dissolved organic matter in rivers and wastewaters. Humic substances (HS) are the largest fraction (between 50-90%) of dissolved organic matter (DOM) in streams and rivers [33,34]. The rest consists of small organic molecules (carboxylic acids, amino acids, carbohydrates and so on). The content of DOM varies greatly depending on the location; but globally, dissolved organic carbon (DOC) concentrations below 10 mg L^{-1} are common [33]. Humic substances in rivers consist of humic (HA) and fulvic (FA) acids, the latter being the main fraction. For example, in several North American rivers the proportions of FA and HA were reported to be 54-68% and 13-29%, respectively, of the DOC [35]. The mean values of humic

substance concentrations reported in natural water samples have been around 6 mg L^{-1} [36,37].

Total organic matter in municipal wastewater is mainly made up of sanitary wastewater, i.e. the combination of urine and feces [38], and gray water, i.e. biodegradable surface-active agents. Main ingredients contributing to the total organic carbon (TOC) are carbohydrates (10-40 mg C L^{-1}), proteins (7-25 mg C L^{-1}), and fatty acids (18-65 mg C L^{-1}) [21]. Typical concentrations of anionic surfactants (mainly linear alkyl benzene sulfonates, LAS) are in the range 4-15 mg L^{-1} [21]. The qualitative composition of DOM in municipal wastewater is basically the same, but only the most hydrophilic organic components passing a 0.45 μm filter (i.e. molecular masses range from less than 500 to more than 5000 Da) [39] make up this organic fraction. Based on the study of acidity constants and Fourier transform infrared spectra, it is believed that the acid sites in wastewater DOM consist of carboxylic and amino functional groups [40].

The interactions occurring between anionic surfactants and predominant organic components of environmental waters influence such processes as transport, solubility, degradation and bioavailability of hydrophobic organic pollutants [41]. SDS interacts weakly with fulvic acids and strongly with the more hydrophobic humic acids forming mixed SDS-humic acid micellar aggregates [42]. Carbohydrates also favor the formation of mixed micelles [43]. Protein-surfactant interactions are commonly investigated through the binding isotherms of ionic surfactants to protein [44,45]; binding is initially specific and electrostatic, but with increasing the surfactant concentration protein unfolding is induced and surfactant binding is dominated by hydrophobic interactions.

Taking into account these data, the following experiments were carried out to evaluate the effect of environmental matrix organic components on the adsorption of SDS onto alumina. Because of the complexity of organic matter in rivers, its effect was assessed by using a real river water sample. Its DOC content (78±1 mg L^{-1}) was well above the global DOC average value (10 mg L^{-1}) in rivers. The hardness content (105 mg L^{-1} $CaCO_3$) was also calculated to evaluate their contribution to the SDS adsorption isotherm. Also, because humic acids interact strongly with surfactants and, in addition,

there is evidence of their adsorption onto alumina [46], their influence on the SDS adsorption was investigated. The HA concentration selected (8 mg L^{-1}) was well above that found in rivers. On the other hand, the effect of wastewater DOM was evaluated using a synthetic municipal wastewater sample [23,24] prepared from food ingredients, as a source of carbohydrates, proteins and fatty acids, and linear alkyl benzene sulfonates (see composition in "Samples", Experimental Section).

Organic matter-induced modifications in the SDS adsorption isotherm. Figure 4 compares the SDS adsorption isotherms obtained in the absence of additives and in the presence of (a) river matrix components, (b) humic acids and (c) synthetic wastewater organic ingredients. No organic matter-induced modifications were observed in any of the SDS isotherm regions of interest for SPE. The presence of Ca and Mg ions in the river water used as a model caused precipitation of SDS in the admicellar region below the surfactant cmc, and then complete solubilization of $Ca(DS)_2$ and $Mg(DS)_2$ in the presence of SDS micelles (Figure 4a), in accordance with the results above obtained (see Figure 3b and 3c).

Three characteristics of DOM seem to account for its lack of influence on the SDS-alumina system: 1) DOM is mainly hydrophilic; 2) at the working pH (about 2), most of DOM is mainly uncharged (e.g. humic and fulvic acids, fatty acids, carbohydrates, etc) and only proteins or LAS bear a significant net positive or negative charge, respectively; and 3) the concentration of DOM (around 6 mg L^{-1} of soluble humic substances, 4-15 mg L^{-1} of LAS, and the unknown amount of low molecular-hydrophilic fraction resulting from the filtering of the carbohydrate-fatty acid-protein mixture, 62 mg L^{-1} each component) is far lower than the SDS concentration used (20-6000 mg L^{-1}) to form the respective sorbents through the adsorption isotherm. Thus, at the hemimicellar region (SDS concentrations added between 20 and 1300 mg L^{-1}), the electrostatic binding of SDS to alumina was preferential because of the high charge density of the mineral oxide. The LAS present in wastewater also bound to alumina, but because of the low concentration of LAS, its influence on the adsorption of SDS was negligible. In isotherm regions where adsorption of SDS is driven by hydrophobic forces, the partial or complete incorporation of

DOM components to SDS admicelles probably occurred but because of the low DOM/SDS ratio, the adsolubilization of DOM did not cause detectable effects on the adsorption of SDS.

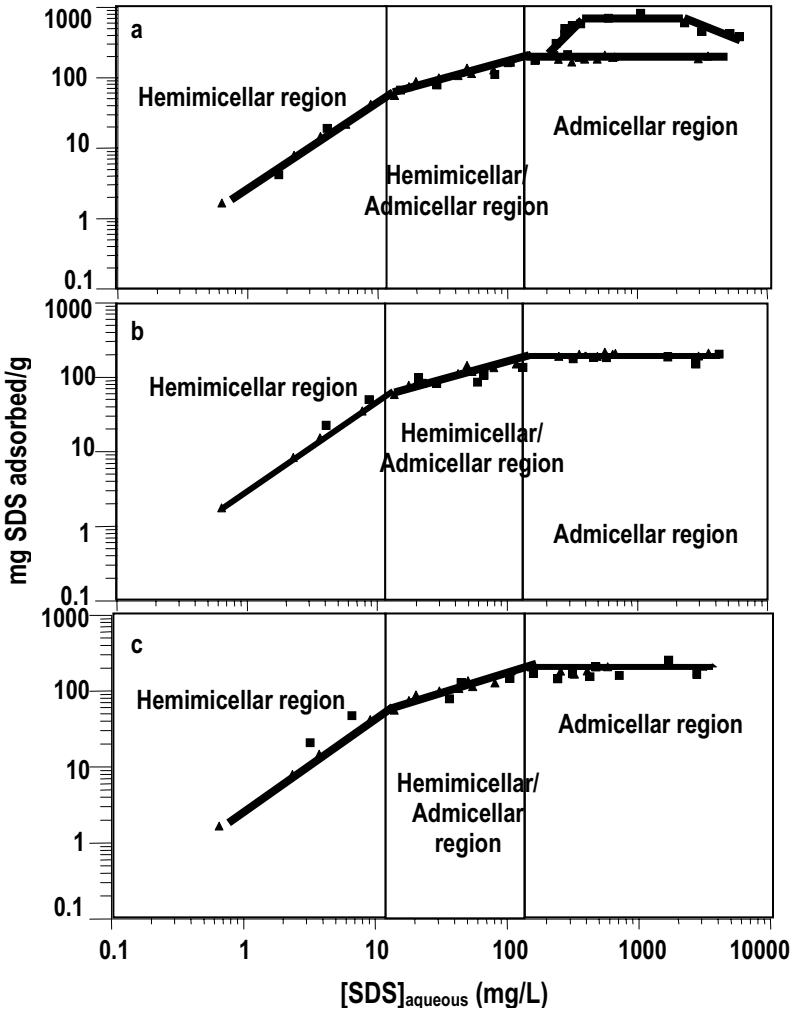

Figure 4. Experimental adsorption isotherms for SDS onto alumina at pH 2 in the absence of additives (triangles) and in the presence (squares) of **a** river matrix components, **b** humic acids and **c** synthetic wastewater organic ingredients.

Analytical consequences. No changes in the surfactant load of pure or mixed hemimicelle- or admicelle-based sorbents are expected to be caused by the common DOM present in rivers and municipal wastewaters. The high DOM concentration in the river water sample selected and the high humic acid concentration tested make the conclusions of this study relevant to a large number of river samples. On the other hand, the content in carbohydrates, proteins and fatty acids of the synthetic domestic wastewater tested was higher than that recommended for *Syntho* [23,24], which is considered standard to simulate domestic sewage.

Conclusions

The results obtained in this research have made it clear that environmental water matrix components affect the adsorption of SDS onto alumina in a predictable and easily detectable way, which allows to prevent their influence on hemimicelle- and/or admicelle-based SPE. In short, electrolytes reduce the surfactant load in the early part of the hemimicellar region and polyvalent ions can precipitate the aqueous SDS in equilibrium with the adsorbed surfactant in the admicellar one. So, mineral oxides totally covered with hemimicelles or mixed hemimicelles/admicelles will be the best selection in most of the environmental applications since no matrix component effects are expected in these isotherm spans.

Combining these results with those previously obtained [9-19] and some unpublished results, a few recommendations can be given for method development. When the SPE of hydrophobic pollutants is of concern, sorbents made up of hemimicelles covering the total oxide surface are recommended in terms of selectivity, since the adsolubilization of apolar compounds in the admicellar core can be hindered in the presence of high electrolyte concentrations (e.g. 0.1 M NaCl), because of the tighter packing of the surfactant aggregates. The SPE of amphiphilic compounds is not interfered by specific matrix-analyte interactions in any of the isotherm regions, however, the hemimicellar region is usually recommended in terms of experimental convenience [14-16], since the surfactant

load keeps constant during percolation of the sample because of the low aqueous surfactant concentration in equilibrium with the adsorbed surfactant.

The SPE of pollutants based on interactions with the surfactant polar group (e.g. ionic, π-cation, hydrogen bonds, etc) can be carried out in both the hemimicellar/admicellar or admicellar regions. The former is recommended in terms of selectivity (Ca is not expected to precipitate in this region) and experimental convenience (the surfactant load is lower which permits greater sample throughput).

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Capítulo 5. Hemimicelle-based solid-phase extraction of estrogens from environmental water samples

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Hemimicelle-based solid-phase extraction of estrogens from environmental water samples

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Abstract

Hemimicelles and admicelles of sodium dodecyl sulphate (SDS) onto alumina and cetyltrimethyl ammonium bromide (CTAB) onto silica were evaluated for the concentration and purification of the priority estrogens estrone (E_1), 17β-estradiol (E_2) and ethynylestradiol (EE_2) from sewage and river samples. Retention was based on analyte:sorbent hydrophobic and cation-π interactions. Parameters affecting the SPE of estrogens on both types of sorbents were comparatively investigated. Adsolubilization was quantitative for SDS hemimicelles/admicelles and CTAB admicelles. SDS hemimicelles-coated alumina was the sorbent selected on the basis of the lower elution volume required and the higher sample flow rate allowed. Combination of estrogens adsolubilization-based SPE with liquid chromatography/diode array/fluorescence detection permitted the quantification of the target compounds with detection limits ranging from 20 to 100 ng l^{-1}. The relative standard deviation ranged from 3 to 8%. The approach developed was applied to the determination of estrogens in raw and treated

sewage and river samples. The recovery found for estrogens in these environmental matrices was between 85 and 105%.

Introduction

Steroids hormones excreted by human and animals enter in the environment through the discharge of domestic sewage effluents and disposal of animal waste.[1] The concentrations found in sewage, surface water and ground water, although very low (ng l^{-1} level), are sufficient to induce estrogenic responses and alter the normal reproduction and development of wildlife.[2] The steroids of concern for the aquatic environment are the endogenous estrogens estrone (E_1) and 17ß-estradiol (E_2) and the synthetic estrogen most commonly used in the formulations of contraceptive pills, ethynylestradiol (EE_2). They have been included in a prioritary list of substances with endocrine-disrupting characteristics.[3] 17ß-estradiol and ethynylestradiol have the highest estrogenic activity in this list, as determined by different in vitro assays.[4-6]

Determination of natural and synthetic estrogens in environmental water samples constitutes a formidable challenge because the very low detection limits required and the complexity of sample matrices. As a result, sample preparation includes a series of complicated time consuming steps (e.g. filtration, extraction, clean-up and evaporation) in order to concentrate and purify the sample.[7]

Extraction of estrogens from environmental water samples is usually performed by off-line solid-phase extraction (SPE). C_{18}-bonded silica has been the sorbent more used,[8-11] although the use of graphitized carbon black [12,13] and styrene-divinylbenzene copolymer (SDB)[14] has been also reported. All three types of sorbents usually provide high extraction efficiencies for estrogens. However, a main drawback is the high volume of organic solvent required for elution of analytes; between 10 and 20 ml of single/various solvents added in two –three steps.[7] Purification of extracts has been carried out by liquid-liquid extraction or SPE.[16-17] Finally, reduction of volume extracts is achieved by rotatory evaporation or nitrogen evaporation. The tedious overall procedure involved in sample preparation demands the development of more simple strategies in order to reduce the time required for the analysis of estrogens.

In this research, the capability of hemimicelles and admicelles for the extraction/concentration/ purification of estrogens from environmental water samples is investigated with the aim of simplifying the sample preparation procedure. These sorbents

have been recently applied by our research group to the SPE of different pollutants from complex environmental matrices.[18-20] Some of the benefits obtained with their use have been high extraction yields, easy elution of analytes, high breakthrough volumes and high flow rate for sample loading.

Hemimicelles and admicelles are formed by the adsorption of ionic surfactants on metal oxides such as alumina, silica, titanium dioxide and ferric oxyhydroxides. [21-28] In hemimicelles, surfactant molecules are adsorbed with head-groups facing towards the surface while the hydrocarbon tail-groups protrude into solution; this creates hydrophobic patches on the surface. Admicelles are formed by hydrophobic interactions of the non-polar chain-groups of the surfactants adsorbed at the interface and the chain-groups of the molecules present in the solution bulk, so the outer surface of the admicelles is ionic. Therefore, the outer surface of the hemimicelle and admicelle provides different mechanism for retention of organics. On the other hand, the number of surfactants commercially available is very high, so both the degree of hydrophobicity and the charge of the sorbent can be easily modified according to the nature of analytes.

Sodium dodecyl sulfate(SDS)-alumina and cetyltrimethyl ammonium bromide (CTAB)-silica hemimicelles and admicelles were examined in this study for the concentration of the estrogens E_1, E_2 and EE_2 in environmental water samples. Reversed phase liquid chromatography/diode array/fluorescence detection was used for the separation and quantification of the target compounds. Predominant factors influencing the extraction efficiency were investigated. The feasibility of the method was proven by analysis of estrogens in river water samples and sewage samples from various treatment plants.

Experimental

Chemicals and materials

All reagents were of analytical reagent-grade and were used as supplied. Sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB) were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of CTAB and SDS were prepared in

distilled water and 10^{-2} M nitric acid, respectively. The estrogens estrone (E_1), 17ß-estradiol (E_2) and ethynylestradiol (EE_2) were purchased from Sigma-Aldrich, prepared at 1g l^{-1} in methanol and stored in glass containers at 4ºC. Working solutions of mixtures of these analytes were prepared by appropriate dilution of the stock solutions in water or methanol. HPLC-grade nitric acid, methanol and acetonitrile were obtained from Panreac (Barcelona, Spain) and formic acid from Merck (Darmstadt, Germany).

Silica gel (Davisil 646) and alumina (γ-form, for column chromatography) were supplied by Sigma-Aldrich. The physical properties of these mineral oxides were as follows:

- Silica gel; surface area 300 m^2 g^{-1}, point of zero charge (pzc) 2.8-3, particle diameter range 250-500 μm, mean pore size 150 Å and pore volume 1.15 cm^3 g^{-1}.
- Alumina; surface area 155m^2 g^{-1}, pzc, 8.5, particle diameter range, 50-200 μm, mean pore size 58 Å; density, 3.97 g cm^{-3} .

Cartridge columns filled with 500 mg of silica were prepared by Análisis Vínicos (Tomelloso, Spain). Bond Elut Jr. cartridge columns filled with 500 mg of alumina were purchased from Varian (Victoria, Australia).

Sample collection and preservation

Influent and effluent water samples were collected from two wastewater treatment plants (WWTPs of Bailén and Linares) in the south of Spain in September 2004. These WWTPs receive a mixture of domestic and industrial wastewater. Although the contribution of the industrial activity to the total influent is variable with time, the following inputs of industrial effluents were estimated for the two WWTPs studied from the data supplied by the personnel working in: ~20-40% for Bailén (mainly from brickworks, ceramic and olive oil industries) and ~30-50% for Linares (mainly from the car and engineering industries). River grab samples were taken from Guadalquivir and Rabanales, both flowing through Córdoba city in September 2004. Samples were collected in dark glass containers. Immediately, they were adjusted to pH 2 by the addition of concentrated nitric acid and

subsequently filtered through 7-9 μm paper filters (Anoia S.A., Spain) and 0.45 μm nylon membranes (Millipore, Bredford, MA, USA), both supplied by Análisis Vínicos S.L (Tomelloso, Spain). Finally they were stored at 4ºC until analysis.

Recommended procedure for the determination of estrogens in sewage and river water

Hemimicelles- based SPE. Bond Elut Jr. cartridge columns were conditioned with 10 ml of a 10^{-2} M nitric acid solution. Then, hemimicelles were formed on the alumina by passing a 25 ml 10^{-2} M nitric acid solution containing 42.5 mg of SDS. The samples (500 ml of wastewater or river water) were percolated through the cartridge which was subsequently dried under vacuum for 3-4 min. Then, the estrogens were eluted with 2 ml of methanol. A Visiprep SPE vacuum manifold (Supelco; Bellefonte, USA) at a pressure value of 20 kPa was used for surfactant solution and sample loading. Aliquots of the eluate were injected into the LC system under the conditions specified below.

Liquid chromatography/diode array-fluorescence detection. The different estrogens were separated and quantified using a LC system (Spectra system SCM 100, ThermoQuest , San Jose, CA, USA) consisting of a P4000 quaternary pump; a UV 6000 diode-array detector and a FL 3000 fluorescence detector. The injection volume was 20 μl. The stationary phase was a Hypersil ODS column (150x4.6 mm, 5μm; Análisis Vínicos S.L, Tomelloso, Spain). The mobile phase was made of water and acetonitrile. The elution gradient was: water:acetonitrile (77: 23) for 8 min, linear change to water:acetonitrile (54:46) for 1 min and then isocratic conditions for 11 min. The flow rate was 1 ml min^{-1}. E_2 and EE_2 were fluorimetrically monitored at λ_{ex} = 280 nm and λ_{em} = 310 nm whereas E_1 was monitored by using the diode-array detector. UV chromatograms for E_1 were recorded at 200, 210 and 280 nm. UV spectra from 190 to 600 nm were also recorded for peak purity assessment and E_1 identification. External calibration was carried out. Correlation between peak areas and estrogen concentrations (10-1000 μg l^{-1} for E_2

and EE_2 and 50-5000 μg l^{-1} for E_1) were determined by linear regression and were in the range 0.9995-0.99991.

Adsorption studies

Surfactant adsorption. The experimental isotherm corresponding to the adsorption of SDS onto γ-alumina at pH 2 was obtained as previously described[19]. The adsorption isotherm of CTAB on silica gel at pH 6.5 was determined as follows: a constant amount of silica (0.5 g) was introduced into different 50 ml test tubes. Then, 25 ml of aqueous solutions containing variable amounts of surfactant (0.018-73 mg) at pH 6.5 were added. The suspensions were vigorously stirred for 5 min and centrifuged at 3500 rpm for 10 min. The concentration of CTAB in the supernatant was determined by using liquid chromatography/electrospray ionization/ion trap mass spectrometry, equipped with an automatic injector. The stationary phase column was a 15-cm Nova-Pak C_8 with 3.9-mm i.d. and 5 μm particle diameter from Waters (Milford, MA). The mobile phase used was a mixture of 80% methanol and 20% of 50 mM ammonium formate buffer (pH, 3.5). The flow-rate was 0.6 ml/min. The surfactant analysis was carried out in the "ESI (+) mode". The set of parameters used was as follows: capillary voltage, 5 KV; capillary exit voltage, 30 V; trap drive, 30; source temperature, 350ºC; drying gas, 10 l min^{-1}; nebulizer gas pressure, 80 p.s.i. and maximal accumulation time, 50ms. Quantification was carried out under full-scan conditions (m/z scan range, 200-350) with the target mass fixed to a value of 284. CTAB was quantified from the corresponding peak areas of the extracted ion chromatograms. Smooth chromatograms were obtained by using the Gauss function (width, 3 points; cycles, 1). Correlation between peak areas and CTAB concentration was obtained in the range 3-1800 μg l^{-1} with a correlation coefficient of 0.998.

Adsolubilization of estrogens. The adsolubilization of estrogens onto SDS- or CTAB- coated mineral oxides as a function of surfactant concentration was studied by adding 25 ml of a solution containing the analytes (5 mg l^{-1} of each one) and SDS at pH 2 or CTAB at pH 6.5 to 0.5 g of alumina or silica, respectively. The amount of surfactant

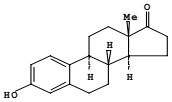
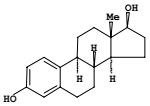
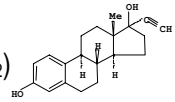
tested varied in the range 0-300 mg and 0-91 mg for SDS and CTAB, respectively. The suspensions were vigorous stirred for 5 min and then centrifuged at 3000 rpm for 10 min. Blank suspensions were prepared in the same way but without the addition of surfactant. The concentration of estrogens remaining in the supernatant was determined by LC-UV/fluorimetry as specified above.

Results and discussion

Hemimicelle/admicelle-based SPE

General considerations. The knowledge of the possible interactions between analytes and sorbent is important for setting up an efficient extraction scheme. Estrogens, which have in common a cyclopentan-o-perhydrophenanthrene ring, are hydrophobic and non-volatile compounds[1] (Table 1). Because of their pKa values, the SPE of estrogens invariably involves to the neutral form.

Table 1. Structure and physico-chemical properties of estrogens.

Analyte (acronym)	Molecular structure	Log K_{ow}	pK_a	Vapour pressure (mm Hg)
Estrone (E_1)		3.43	10.3± 0.2	2.3 x10^{-10}
17β-estradiol (E_2)		3.94	10.4± 0.2	2.3 x10^{-10}
17α-ethynylestradiol (EE_2)		4.15	10.3± 0.2	4.5 x10^{-11}

The hemimicelles and admicelles more studied in the physico-chemical literature have been made up of anionic surfactants (e.g. sulphates) adsorbed on alumina, and cationic surfactants (e.g. ammonium quaternary salts) adsorbed on silica. Figure 1 shows

the experimental isotherms obtained for SDS on alumina at pH 2 and CTAB on silica at pH 6.5.

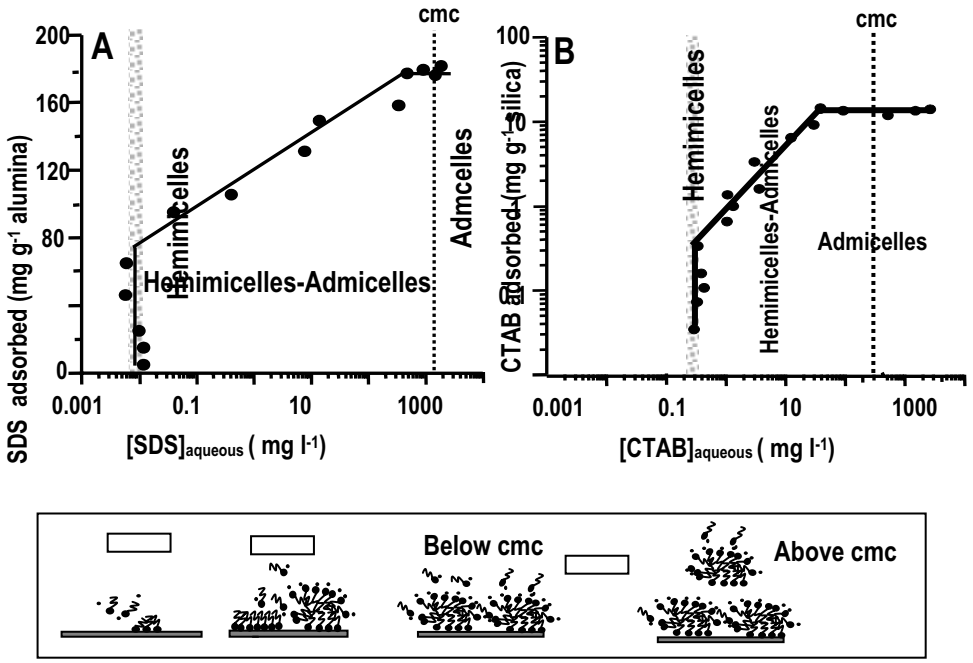


Figure 1. Experimental adsorption isotherms for (A) SDS onto alumina at pH =2 and (B) CTAB onto silica at pH = 6.5.

These isotherms are similar to those previously reported.[21,26,29-32] These surfactants were selected in this study as representative of anionics and cationics, respectively. The knowledge of the adsorption isotherms of surfactants on mineral oxides is essential to know the regions where the different aggregates form and to interpret the SPE of the target analytes. Both, SDS and CTAB isotherms showed well-differentiable regions where the distinct sorbents (hemimicelles, hemimicelles-admicelles and admicelles) exist. The concentration of surfactant monomers in solution, that was negligible in the hemimicellar region, was progressively increasing with the addition of surfactant until the critical micellar concentration (cmc) was reached. Above the cmc, aqueous surfactant micelles

were in equilibrium with admicelles which caused partition of analytes between both types of surfactant aggregates and prevented this isotherm span to be used for SPE.

The structure of the different surfactant sorbents is depicted in Figure 1. The adsorption of surfactants in the hemimicellar region is driven by both surfactant-mineral oxide electrostatic attraction and lateral hydrophobic interactions between the adsorbed surfactant molecules. At the end of this region, the overall surface charge of the oxide is neutralized and further adsorption in both the hemimicellar-admicellar and admicellar regions is only hydrophobically driven. Although admicelles have been traditionally described as surfactant bilayers, information gained in the last few years using techniques such as atomic force microscopy, fluorescence quenching and neutron reflectivity have strongly evidenced the presence of discrete surface aggregates in the admicellar region, similar to aqueous micelles.[21]

As it can be seen on Figure 1, SDS adsorbs on alumina at considerable higher amounts that CTAB on silica. This behaviour is a result of the higher charge density on alumina (point of zero charge 8.5) compared to that on silica (pcz 2.8-3) at the working pH (2 and 6.5 respectively). The charge density on silica increases exponentially with increasing pH,[22] but because the method is intended to extract estrogens from wastewater and river water, no pH values above 6.5 were considered in this study.

According to the structure of the estrogens and the surfactant sorbents above considered (Figure 1), the following interactions were expected to be the driving-force for extraction of analytes: hydrophobic for adsolubilization on SDS hemimicelles/admicelles or CTAB hemimicelles and hydrophobic/π-cation interactions for adsolubilization on CTAB admicelles.[33]Below the main results obtained in the study of the efficiency of these sorbents to extract estrogens are discussed.

Influence of the type and amount of surfactant on the adsolubilization of estrogens. Experiments to investigate the adsolubilization of the target analytes on SDS-alumina and CTAB-silica as a function of surfactant concentration were carried out in the batch mode according to the procedure specified under the Experimental section.

Figure 2 depicts the percentages of adsolubilized 17ß-estradiol (E_2) as a function of the amount of SDS (A) and CTAB (B) added to the solution containing alumina or silica, respectively. Results for estrone (E_1), and ethynylestradiol (EE_2) were similar to those obtained for E_2 (data not shown). Estrogens adsorbed at percentages between 30 and 40 % onto alumina and silica in the absence of surfactant. This behaviour has been noted before in investigations about the adsolubilization of naphthalene[34] and progesterone, testosterone and hydrocortisone[30] on CTAB-silica, and it has been related to the presence of siloxane groups, which may be considered as hydrophobic, on portions of the silica surface, which should favour the adsorption of hydrophobic solutes.

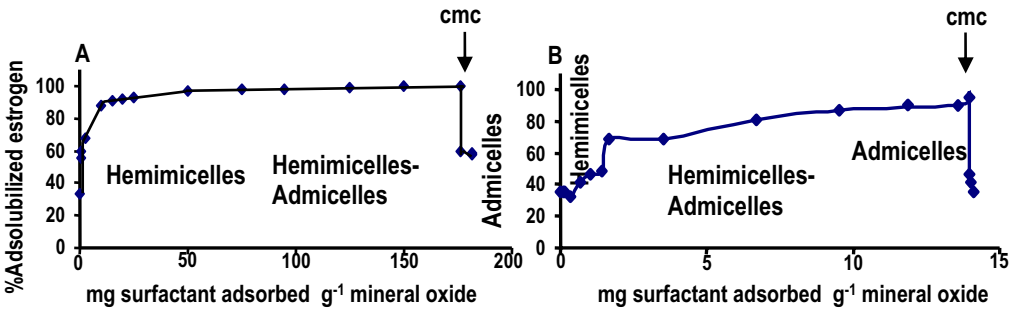


Figure 2. Effect of the amount of (A) SDS added to 0.5 g of alumina at pH 2 and (B) CTAB added to 0.5 g of silica at pH 6.5 on the adsolubilization of E_2

Estrogens were quantitatively adsolubilized by hemimicelles and admicelles of SDS (figure 2A). Hydrophobicity was the factor predominant for their adsolubilization. On the contrary, formation of admicelles of CTAB was necessary to achieve quantitative adsolubilization of estrogens on CTAB-silica (figure 2B). The low charge density on the silica surface at the working pH, which results in low surfactant loading (figure 1B), probably determined the different behaviour of CTAB- and SDS-based sorbents in the hemimicellar region. Both, the higher load of CTAB in the admicellar region and the possible π-cation interactions between the ammonium group of CTAB and the aromatic ring of analytes were considered responsible for the quantitative retention of estrogens on CTAB admicelles. Estrogens adsolubilization in the admicellar region progressively

decreased for both SDS-alumina and CTAB-silica from surfactant concentrations at which micelles were formed in the bulk aqueous solution due to partition of analytes between micelles and admicelles.

Adsorbed surfactant/estrogens molar ratios between 10 and 20 were required to achieve maximal adsolubilization of estrogens (Figure 2). These ratios were similar to those previously reported for quats on SDS admicelles[19] and toluene and acetophenone on CTAB admicelles.[32]

According to these results, both SDS hemimicelles/admicelles and CTAB admicelles were effective to retain estrogens. SDS hemimicelles were preferred on SDS admicelles on the basis of experimental convenience. In the hemimicellar region, the concentration of SDS monomers in the aqueous phase is negligible (about 8 μg l^{-1}, see Figure 1A), which permits to use these aggregates in SPE without supply of the surfactant during operation. In the admicellar region is mandatory a supply of SDS during percolation of the sample in order to keep constant the amount of SDS adsorbed, thus guaranteeing a unique partition constant for analytes.[19] The amount of SDS that should be added to the samples can be easily inferred from the corresponding adsorption isotherm (figure 1A, x-axis). A SDS amount of 85 mg g^{-1} alumina was recommended for extraction of estrogens.

On the other hand, we were interested in determining how the type of analyte:sorbent interaction can affect the SPE of estrogens. For this reason, CTAB admicelles were also selected for further studies. A CTAB amount of 36 mg g^{-1}silica was used for this purpose.

The retention capacity of SDS hemimicelles and CTAB admicelles did not change in the pH range investigated (i.e. between 2 and 6.5) despite the pH modifies the charge density on the oxide surface and therefore the amount of adsorbed surfactant, as discussed above. For example, the maximal amount of SDS adsorbed in the hemimicellar region was about 80 and 20 mg g^{-1} alumina at pH 2 and 6, respectively. Probably, estrogens adsolubilization kept constant in the pH range studied due to the excess of sorbent with respect to the target compounds. We selected pH 2 and 6.5 for working with SDS-alumina and CTAB-silica, respectively, because at these pH values the surfactant loading was maximal which should delay the breakthrough of analytes.

Desorption solution. Desorption of estrogens from SDS-coated alumina and CTAB-coated silica was investigated using acetonitrile and methanol. Organic solvents are known to disrupt surfactant aggregates, which causes elution of analytes. Table 2 lists the recoveries obtained for the target compounds. Quantitative elution of estrogens was achieved from SDS-alumina by using methanol. The lower solubility of SDS in acetonitrile could explain the lower percentage of elution of estrogens using this solvent compared to methanol. No quantitative recovery of estrogens was achieved from CTAB-silica for the volumes of organic solvents tested. The low solubility of CTAB in both solvents and the strong retention of estrogens on CTAB admicelles by hydrophobic and π-cation interactions could account for these results. Accordingly, the use of SDS-coated alumina and elution with 2 ml of methanol is recommended.

Table 2. Percentages of estrogens eluted from the SPE cartridge as a function of the eluent and its volume.

Eluent	Volume (ml)	SDS-alumina			CTAB-silica		
		E_2	EE_2	E_1	E_2	EE_2	E_1
Methanol	1	69	66	65	69	67	67
	2	95	98	97	84	81	82
	4	95	94	95	88	85	86
Acetonitrile	2	75	85	83	76	69	70
	4	73	80	80	83	88	86

Breakthrough volume. Experiments to determine the breakthrough volume were conducted by passing increasing volumes (0.025-1 l) of aqueous solutions containing 0.23 µg of each estrogen through SDS-alumina and CTAB-silica. The pH of the aqueous solutions was fixed at 2 and 6.5 when SDS hemimicelles and CTAB admicelles were respectively used as sorbents. Fortification of samples with 200 mg l^{-1} of CTAB was required for CTAB admicelles-based SPE in order to guarantee an unique partition constant of analytes during percolation of samples. Adsolubilized estrogens were eluted

with 2 ml of methanol in all experiments. Breakthrough was considered to occur when the amount eluted decreased about 5%.

Quantitative recoveries were obtained for analytes up to 0.5 l of sample using both types of sorbents. Higher volumes caused a progressive decrease in the recoveries obtained (e.g. the recovery of estrogens was about 60% when sample volumes of 1 l were percolated through SDS-hemimicelles). The SPE of estrogens using CTAB admicelles took considerable longer time than that required by SDS hemimicelles owing to the higher compactness of the surfactant on the oxide surface and the continuous supply of CTAB. Therefore, on the basis of these results, and the previous ones described above, we recommend SDS hemimicelles to extract/concentrate estrogens from environmental water samples.

Analytical performance

Calibration curves were run by injecting 0.2– 20 ng of E_2 and EE_2 and 1–100 ng of E_1, using fluorescence and UV detection, respectively. The slope of the calibration curves were $(3.46\pm0.04)x10^5$ and $(3.68\pm0.02)x10^5$ fluorescence intensity units ng^{-1} for E_2 and EE_2, respectively, and $(7.35\pm0.04)x10^5$ absorbance units ng^{-1} for E_1. The correlation coefficients ranged between 0.9995 and 0.99991. The minimum detectable amount (MDA), corresponding to the amount of chemical injected onto the LC column in a volume of 20 µl standard solution that produced a 3:1 signal-to-noise ratio, was 0.1 ng for E_2 and EE_2, and 0.5 ng for E_1. Taking in account these values, the sample volume used for analysis of wastewater and river water (0.5 l), the methanol volume containing the analytes (2 ml) and the recovery obtained from spiked samples (between 85-105%), the method detection limits were estimated to be around 0.02 µg l^{-1} for E_2 and EE_2 and 0.1 µg l^{-1} for E_1. The precision of the overall procedure, expressed as relative standard deviation (RSD), was evaluated by extracting six consecutive water samples (0.5 l) from Rabanales river spiked with 1 µg l^{-1} of each estrogen. RSD values ranged from 3 to 8% demonstrating good precision method.

Analysis of estrogens in aqueous environmental samples

Table 3. Concentrations (ng/l)±standard deviation (based on three replicates) and mean recoveries[a] (%) of target analytes found in wastewater influent and effluent samples and river water samples.

Sample location	E_2	EE_2	E_1
WWTP influent			
Linares	< LOD	< LOD	< LOD
Bailén	< LOD	< LOD	< LOD
Spiked Linares sample[a]	96	94	87
Spiked Bailén sample[a]	94	104	97
WWTP effluent			
Linares	< LOD	< LOD	< LOD
Bailén	< LOD	< LOD	223±17
Spiked Linares sample[a]	92	95	86
Spiked Bailén sample[a]	105	88	87
River sample			
Rabanales	41±1	< LOD	209±9
Guadalquivir	< LOD	< LOD	207±4
Spiked Rabanales sample[a]	95	97	88
Spiked Guadalquivir sample[a]	86	88	83

[a]Samples fortified with 0.5 µg/l of E_2 and EE_2 and 1 µg/l of E_1, n=3, range of RSD values between 1 and 7%

The performance of the method was tested by analysing sewage samples from two treatment plants and water samples from two rivers (see Experimental section). Table 3 shows the results obtained. Estrogens were not detected or their concentration was in the low ng l^{-1} range. The values found for these pollutants in wastewater and river samples were at the level previously reported by other authors.[1,35,36] Total method recoveries were assessed by analysing the same samples spiked with a standard mixture of analytes. The

values obtained are shown in table 3. Recoveries were between 83 and 105% for the samples analysed. They were similar to those found for spiked distilled water. Figure 3 shows the fluorescence and UV chromatograms from two standard solutions at different concentration levels and a river sample. The spectra for estrogens from analysed samples and standard solutions were identical.

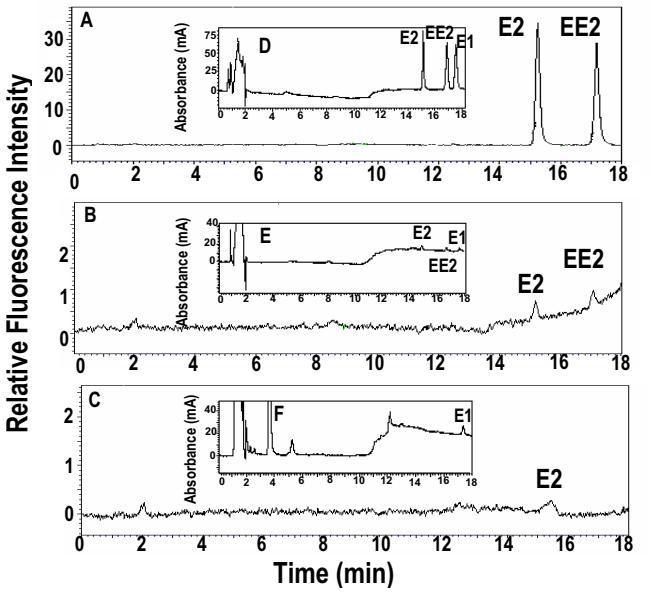


Figure 3. Fluorescence (A,B,C) and UV (D,E,F) chromatograms obtained from standard solutions (A,B,D,E,) and a river sample (Rabanales, Spain) (C,F). Concentration of standards: 1mg l^{-1} (A,D) and 10 μg l^{-1} for E_2 and EE_2 (B) and 50 μg l^{-1} for E_1 (E).

Conclusions

This work proves that SDS hemimicelles-based SPE is a valuable tool to simplify the sample preparation procedure for the analysis of the most relevant estrogens in environmental water samples. Main features of these surfactant aggregates were the

strong retention of estrogens, which permitted their quantitative adsolubilization, and the easy elution of analytes, which was the key feature to reduce the analysis time compared with previously reported methods.[7-17] No purification steps were required to the accurate determination of estrogens in the samples analysed. The detection limits achieved were similar to those found by other SPE-based LC methods.[7] Detection limits reported for GC-MS and GC-MS-MS were lower (between 0.5 and 74 ng l^{-1} and 0.1 and 2.4 ng l^{-1}, respectively) however, the derivatization step usually required for subsequent analysis with these techniques is time consuming and can be a source of inaccuracy.[37] At present, there is not regulation available for levels of estrogens in environmental aqueous samples. The concentrations reported in the literature [1,12,13,17,35-40] for the selected analytes range widely (0.1-64 ng l^{-1}; 0.1-42 ng l^{-1} and 0.012-80 μg l^{-1} for E_2, EE_2 and E_1, respectively). Taking into account the analytical features of the proposed method, it is useful to determine estrogens in those real aqueous samples whose concentration of E_2 and EE_2 is 40 ng l^{-1} or higher and the E_1 concentration is equal or superior at 200 ng l^{-1}. Both surfactant –coated mineral oxide systems investigated (SDS-alumina and CTAB-silica) were suitable for the SPE of estrogens although the former provided better analytical features on the basis of the high surfactant load achieved on the oxide surface at convenient pH values for wastewater treatment (which permitted to reach quantitative adsolubilization in the hemimicelllar region) the higher solubility of SDS compared to CTAB in organic solvents (which permitted to elute the solutes using lower volumes).

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CONCLUSIONES

Las investigaciones recogidas en esta memoria han contribuido a la consolidación de los sistemas supramoleculares como sistemas extractantes de sustancias alteradoras del sistema endocrino. Como es conocido, la característica esencial de los sistemas supramoleculares es que los componentes que lo integran son moléculas que contienen en su estructura una parte hidrófoba y otra hidrófila y/o contienen centros aceptores o donadores de protones. Esta característica hace que se puedan solubilizar en su seno una gran variedad de compuestos orgánicos e inorgánicos sobre la base de las diferentes interacciones que pueden establecerse. Los procesos de extracción desarrollados se han basado en fenómenos de coacervación y adsorción de los agregados moleculares.

Basándonos en procesos de coacervación se han puesto a punto diversos métodos para la extracción del bisfenol A presente en diferentes matrices: alimentos enlatados (frutas, verduras, pescados y carnes) y orina humana, previa a su determinación analítica. La principal vía de exposición humana a bisfenol A se encuentra en el consumo de alimentos embotellados y enlatados, ya que este compuesto se utiliza en la fabricación de plásticos policarbonatados y resinas epoxi que son empleadas como recubrimiento interior de latas y botellas. La presencia de bisfenol A en todas las muestras de orina analizadas pone de manifiesto la continua exposición humana a bisfenol A.

Por otra parte, empleando hemimicelas/admicelas como adsorbentes en SPE, se ha desarrollado un método para la determinación de sustancias con elevada actividad estrogénica (los estrógenos naturales 17-β-estradiol y estrona y el sintético etinilestradiol) presentes en muestras acuosas ambientales. Asímismo, se ha estudiado el efecto de los principales componentes presentes en matrices ambientales acuosas (carbohidratos, grasas, proteínas, tensioactivos, ácidos húmicos y sales inorgánicas)
en la extracción en fase sólida admicelar, con la finalidad de ofrecer las pautas para evitar interferencias y conseguir métodos de extracción eficientes y robustos cuando se trabaja con este tipo de muestras.

Teniendo en cuenta los resultados obtenidos en las investigaciones llevadas a cabo, se puede decir que las metodologías desarrolladas constituyen una alternativa muy ventajosa al uso de disolventes orgánicos en extracción líquido-líquido y sólido-líquido y amplía el horizonte de las fases adsorbentes utilizadas en SPE debido a:

- La reducción del consumo de disolventes orgánicos utilizados
- La simplificación de la etapa de tratamiento de la muestra, ya que no es necesaria una etapa de limpieza de la misma para obtener la selectividad adecuada en la etapa de medida de la señal analítica.

- La gran versatilidad de estos sistemas, puesto que pueden establecerse entre los agregados moleculares y los analitos una gran variedad de interacciones.

- La posibilidad de determinar los analitos mediante calibración externa debido a su separación completa en el sistema cromatográfico de las moléculas que constituyen los agregados supramoleculares empleados y de los componentes de la matriz analizada.

- La compatibilidad de los agregados moleculares con un sistema de separación-detección tan asequible para laboratorios de rutina como es un cromatógrafo de líquidos acoplado a un espectrofotómetro/fluorímetro. Los agregados micelares que constituyen los coacervados se destruyen debido a su solubilización en el disolvente orgánico de la fase móvil y las hemimicelas/admicelas se destruyen por el disolvente orgánico utilizado en la etapa de elución de analitos. La cantidad de monómeros presentes en disolución no ha presentado incompatibilidad cromatográfica en las aplicaciones desarrolladas.

Conclusiones relativas a la Parte I de la Memoria (coacervación).

• La coacervación de micelas inversas de ácido decanoico inducida por tetrahidrofurano se ha utilizado por vez primera para la extracción de bisfenol A presente en muestras de alimentos sólidos enlatados y de orina humana.

• Esta metodología aventaja a las metodologías previamente descritas para la extracción de bisfenol A presente en las matrices mencionadas en términos de:

- *Simplicidad*, ya que el tratamiento de la muestra requiere una única etapa de extracción (no es necesario clean-up ni evaporación del disolvente) con una disolución acuosa que contiene tetrahidrofurano (entre 1 y 4 ml) y una determinada cantidad de ácido decanoico (entre 100-200 mg).

- *Rapidez*, ya que el tratamiento completo de las muestras oscila entre 20 y 30 minutos y son varias las muestras que pueden extraerse simultáneamente.

- *Robustez,* ya el porcentaje de extracción del analito es prácticamente independiente de variables tales como la temperatura, pH y de la presencia de componentes de la

matriz como proteínas, sal y lípidos en el caso de los alimentos grasos enlatados, y de la urea y otros componentes propios de la orina.

• Otras ventajas adicionales asociadas a la metodología desarrollada son: a) la *baja cantidad de muestra* a tratar, especialmente en el caso de los alimentos sólidos enlatados (200-400 mg); b) el *bajo coste* requerido ya que el consumo de disolventes orgánicos se reduce considerablemente, lo que conlleva también una mayor seguridad para el analista y c) no se precisa equipamiento especial para tratar la muestra (agitador magnético, centrífuga), ni para la separación-detección de bisfenol A porque sólo es necesario un cromatógrafo de líquidos con detector fluorimétrico. En consecuencia la metodología propuesta puede llevarse a cabo en laboratorios de análisis de rutina sin inversión extra alguna.

• Los límites de cuantificación de la metodología propuesta para la determinación de bisfenol A en alimentos enlatados (9.3 ng g^{-1} para verduras y frutas y 29 ng g^{-1} para carne y pescado), están muy por debajo del límite específico de migración establecido por la Comisión de la UE de 600 ng g^{-1} por lo que este método puede proponerse para el control de rutina de bisfenol A en este tipo de muestras.

• El límite de detección del método propuesto para la determinación de bisfenol A en orina humana es 197 ng L^{-1}. Este valor está muy por debajo del nivel de concentración normalmente hallado en este tipo de muestras por lo que la metodología propuesta satisface los requisitos para ser usada en estudios epidemiológicos destinados a la evaluación de la exposición humana a bisfenol A.

Conclusiones relativas a la Parte II de la Memoria (hemimicelas/admicelas)

Las investigaciones llevadas a cabo sobre hemimicelas/admicelas se han centrado fundamentalmente en dos aspectos; por una parte se ha realizado un profundo estudio sobre el efecto de los principales componentes presentes en matrices ambientales acuosas sobre el adsorbente admicelar (hemimicelas/admicelas). Para ello se ha seleccionado el sistema constituido por el tensioactivo aniónico dodecilsulfato sódico (SDS) y el óxido mineral alúmina, que ha sido el más empleado en procesos de extracción analítica. Por otra parte, se ha desarrollado una aplicación de este tipo de adsorbentes para la extracción/concentración de estrógenos que se hallan a muy bajos niveles de concentración en muestras acuosas ambientales complejas (agua de río y residual).

Los resultados obtenidos del estudio sobre el efecto de los principales componentes presentes en matrices ambientales acuosas en el sistema SDS-alúmina han permitido establecer directrices generales para evitar interferencias en los procesos de extracción en fase sólida basados en hemimicelas/admicelas.

• Se puede predecir fácilmente el tipo de adsorbente (hemimicelas, hemimicelas-admicelas o admicelas) apropiado para la extracción de analitos de interés, respecto al tipo y composición de la muestra de agua a tratar. La influencia de cada uno de los componentes se ha evaluado mediante la construcción de la correspondiente isoterma de adsorción en presencia de cada uno de los componentes.

• La presencia de electrolito reduce la cantidad de tensioactivo adsorbida sobre la alúmina en la primera parte de la región de hemimicelas cuando aún no está neutralizada la carga superficial de la alúmina.

• Los iones divalentes (calcio y magnesio) pueden precipitar con los monómeros de SDS en disolución que se halla en equilibrio con el tensioactivo adsorbido en la región de admicelas.

• En presencia de los componentes orgánicos mayoritarios presentes en agua de río (ácidos húmicos) y agua residual (grasas, proteínas, hidratos de carbono y tensioactivos aniónicos) no se observaron modificaciones en la isoterma de adsorción de SDS en ninguna de las regiones de interés para SPE. En el estudio no se han incluido los tensioactivos no iónicos y catiónicos presentes en las aguas residuales municipales ya que es predecible que no tengan efecto a las concentraciones generalmente halladas ($<$ 0.5 mg L^{-1} los no iónicos y $<$ 0.1 mg L^{-1} los catiónicos).

• Para la extracción de compuestos presentes en muestras ambientales acuosas se recomienda utilizar como adsorbente hemimicelas que cubran completamente la superficie del óxido mineral o la mezcla de hemimicelas-admicelas, ya que no se espera influencia alguna por parte de los componentes de la matriz cuando se utilizan estos tipos de agregados.

• En función de las propiedades físico-químicas de los analitos de interés se pueden hacer algunas recomendaciones para la aplicación de esta metodología:

- Cuando los analitos a extraer tienen carácter hidrófobo, se recomienda el uso de hemimicelas en términos de selectividad ya que la adsolubilización de estos compuestos en el corazón de las admicelas puede obstaculizarse en presencia de electrolitos, que favorece el mayor empaquetamiento del agregado.

- Para la extracción en fase sólida admicelar de compuestos anfifílicos se puede utilizar cualquier tipo de adsorbente (hemimicelas, hemimicelas-admicelas o admicelas) ya que la isoterma no se afecta por la interacción analito-matriz. Sin embargo, por conveniencia experimental se recomiendan hemimicelas ya que la cantidad de tensioactivo adsorbida sobre el óxido mineral se mantiene constante durante la percolación de la muestra y no es necesario adicionar tensioactivo a la misma.

- Cuando la extracción de los analitos se basa en interacciones con el grupo polar del tensioactivo, se puede trabajar tanto en la región de hemimicelas-admicelas como de admicelas. La región de hemimicelas-admicelas es la más adecuada en términos de selectividad, porque no es probable que los iones polivalentes precipiten con SDS, y conveniencia experimental (ya que la cantidad de SDS que se ha de añadir a la muestra es menor que en región admicelar).

Para demostrar la aplicabilidad de hemimicelas/admicelas como adsorbentes en SPE, se han investigado dos sistemas SDS-alúmina y CTAB-sílice para la extracción/ preconcentración de los estrógenos estrona (E_1), 17β-estradiol (E_2) y etinilestradiol (EE_2) presentes en aguas de río y residuales. Las hormonas naturales investigadas, E_1 y E_2 son los estrógenos que se encuentran en mayor proporción en el plasma sanguíneo. Dentro del grupo de las hormonas sintéticas se ha seleccionado EE_2 por su amplio uso como anticonceptivo y porque junto con el E_2 son las sustancias con mayor actividad estrogénica y por consiguiente con mayor capacidad alteradora del sistema endocrino.

Ambos sistemas extractantes permiten la extracción cuantitativa de los analitos de interés sobre la base de interacciones hidrófobas y π-catión en la región de hemimicelas para SDS o admicelas para CTAB. Teniendo en cuenta la mayor conveniencia experimental cuando se trabaja con hemimicelas y la mayor selectividad que ofrecen éstas con respecto a las posibles interferencias de los componentes de la matriz, SDS-alúmina ha sido el sistema seleccionado para llevar a cabo la extracción admicelar de los estrógenos. Además, SDS es más soluble que CTAB en los disolventes orgánicos normalmente empleados como eluyentes de los analitos, lo que favorece la elución de los mismos. Por último, la mayor densidad de carga superficial de la alúmina al pH de trabajo permite una mayor carga de tensiactivo lo cuál es siempre ventajoso para obtener mayores volúmenes de ruptura y en consecuencia mayores factores de preconcentración.

La combinación de la extracción admicelar con cromatografía líquida con detección fluorimétrica (para estradiol y etinilestradiol) y fotométrica (para estrona) nos ha permitido

la cuantificación de los estrógenos investigados con límites de detección de 20 ng L^{-1}, para estradiol y etinilestradiol y 100 ng L^{-1}. La reproducibilidad del método expresada como desviación estándar relativa osciló entre 3 y 8%. Las concentraciones halladas de estos estrógenos en muestras de agua de río y residuales fueron del orden de ng L^{-1} y fueron similares a las encontradas por otros autores.

APÉNDICES

APÉNDICE A. PUBLICACIONES CIENTÍFICAS DERIVADAS DE LA TESIS DOCTORAL

1.- "Hemimicelle-based solid-phase extraction of estrogens from environmental water sample".
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
Analyst, **2006**,131, 407- 414.

2.- "Study of the influence of water matrix components on admicellar sorbents".
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
Analytical and Bioanalytical Chemistry, **2007**, 388, 1823-1830.

3.- "Decanoic acid reverse micelle-based coacervates for the microextraction of bisphenol A from canned vegetables and fruits".
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
Analytica Chimica Acta, **2008**, 617, 51-58.

4.- "Determination of bisphenol A in canned fatty foods by coacervative microextraction, liquid chromatography and fluorimetry".
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
Food Additives and Contaminants, **2008**, DOI: 10.1080/02652030802368740

5.- "Measurement of urinary bisphenol A by coacervative microextraction and liquid chromatography-fluorescence detection".
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito
Aceptado en Analytica Chimica Acta, 2008

APÉNDICE B. COMUNICACIONES A CONGRESOS

1.- "Use of hemimicelles and admicelles for the extraction of steroid hormones from environmetal samples". **Póster.**
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
Euroanalysis XIII; Septiembre de 2004, Salamanca (España).

2.- "Solid-phase extraction of chlorophenoxy acid herbicides from groundwater samples based on ion pairs formation with cetylpyridinium admicelles". **Póster.**
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
29TH International Symposium on High Performance Liquid Phase Separations and Related Techniques; Junio de 2005, Estocolmo (Suecia).

3.- "Environmental matrix components affecting hemimicelle/admicelle-based extractions". **Oral.**
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
12TH Symposium on Sample Handling for Environmental and Biological Analysis; Octubre de 2006, Zaragoza (España).

4.- "Determination of bisphenol A in canned vegetables and fruit samples by coacervative extraction, liquid chromatography and fluorescence detection". **Póster.**
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
3rd Internacional Symposium on Recent Advances in Food Analysis; Noviembre de 2007, Praga (República Checa).

5.- Determinación de bisfenol A en muestras de orina humana mediante extracción coacervativa y cromatografía líquida con detección fluorescente. **Póster**.
Amalia García-Prieto, Loreto Lunar, Soledad Rubio, Dolores Pérez-Bendito.
XXI Reunión Nacional de Espectroscopía V Congreso Ibérico de Espectroscopia; Septiembre de 2008, Murcia (España).

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USE OF HEMIMICELLES AND ADMICELLES FOR THE EXTRACTION OF STEROID HORMONES FROM ENVIRONMENTAL SAMPLES

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From the various groups of substances with reported endocrine-disrupting properties, the female sex hormones and the synthetic steroids are considered as the most potent estrogenic compounds. The presence of estrogenic compounds in the environment has become a concern because they may interfere with the reproduction of man, livestock and wildlife and the hormonal imbalance (feminisation). These adverse effects may occur at very low concentrations (0.1-20 ng/l). Therefore the analysis of these compounds in the environment constitutes a difficult task.

Hemimicelles and admicelles have been recently reported as excellent sorbents for the solid-phase extraction (SPE) of organic compounds of different nature such as hydrophobic, ionic and amphiphilic. These surfactant aggregates are produced by the adsorption of ionic surfactant on the surface of mineral oxides.

The objective of this contribution was to evaluate the capability of hemimicelles and admicelles for the concentration of two estrogens (estradiol and ethynylestradiol) and one progestagen (progesterone).

The aggregates studied were formed from the adsorption of the anionic surfactant sodium dodecylsulphate (SDS) on alumina and the cationic surfactant cetyltrimethylammonium bromide (CTAB) on silica. The results obtained show that the recovery in both types of aggregates, for the three analytes studied, were similar (about 90-95 %). By using hemicelles of SDS, the mechanism of interaction of analytes to the surfactant aggregates was exclusively hydrophobic. On the other hand, when the CTAB coated alumina was used as sorbent, the interaction between the admicelles and analytes can be due to hydrophobic and ionic forces. The partition of the analytes to the surfactant aggregates was independent on the pH value in the range 2-10 for SDS hemimicelles and 4-9 for CTAB admicelles.

These sorbents were successfully applied to the concentration of the analytes in various environmental water samples.

Euroanalysis 13, Salamanca 2004


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Solid-phase extraction of chlorophenoxy acid herbicides from groundwater samples based on ion pairs formation with cetylpyridinium admicelles

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Chlorophenoxy acid herbicides are of significant importance because of their wide distribution and extensive use as plant growth regulators. When applied, they are easily transferred to surface and ground waters due to their polar nature and relatively good solubility. The European Community (EC) Directive specifies a legal tolerance level of 0.1μg/l for each herbicide and 0.5 μg/l for the sum of all pesticides in water intended for human consumption. A preconcentration step is usually necessary to detect these low levels. Solid-phase extraction (SPE) is the best option for sample enrichment.

The objective of this contribution was to evaluate the capability of hemimicelles (apolar sorbent) and admicelles (ion pair and ion-exchange sorbents) for the extraction/concentration of different phenoxy acid herbicides (2,4-D, MCPA and MCCP). Because of their anionic nature, cationic surfactants (adsorbed onto silica) were selected for the formation of the aggregates. As it was expected, the adsolubilization was greater when admicelles were present on the silica owing to the ionic interaction between phenoxy acid and cationic surfactants. Among the cationic surfactant investigated, the best results were obtained using cetylpyridinium chloride (CPC). The retention for the different phenoxy acid herbicides onto CPC-coated silica was between 95-100%. No differences were found for the maximal solubilization of herbicides onto CPC admicelles at pH values between 5 and 8.The admicelles of CPC were successfully applied to the extraction/concentration of the analytes in various groundwater samples.

12th Symposium on Sample Handling for Environmental and Biological Analysis

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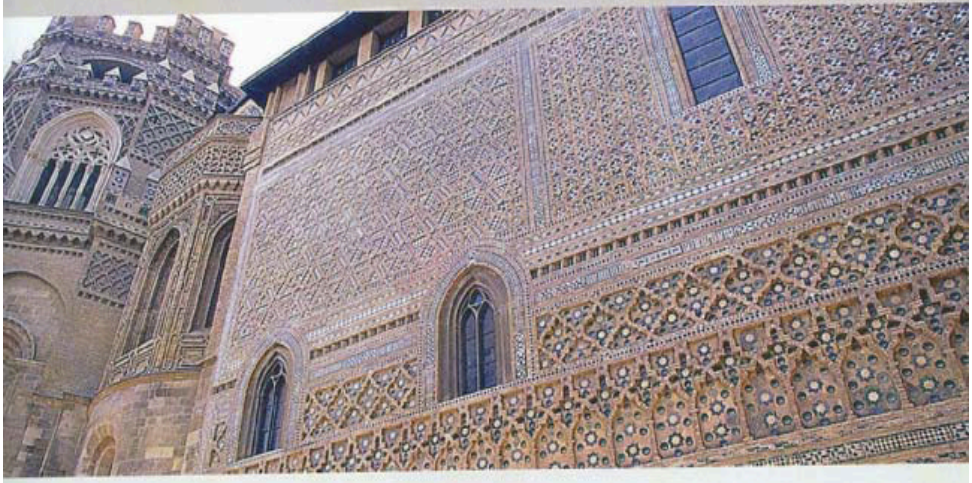

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Environmental matrix components affecting hemimicelle/admicelle-based extractions

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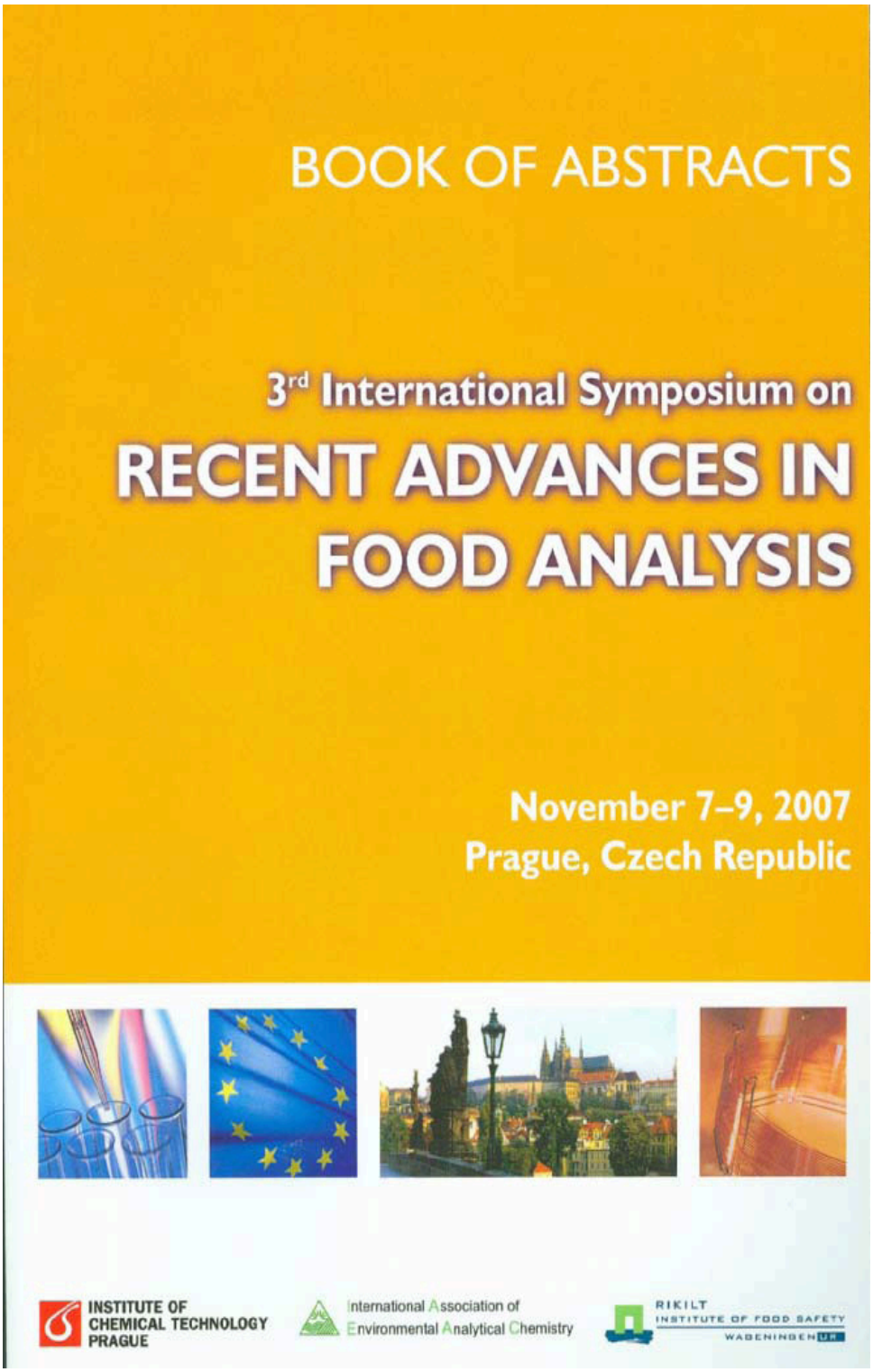
Abstract

Surfactants adsorbed on mineral oxides form aggregates termed hemimicelles and admicelles which have proved to be excellent sorbents for the solid-phase extraction (SPE) of organic pollutants from environmental samples [1,2]. Because of the amphiphilic character of the surfactants, these aggregates have regions of different polarity where pollutants of different physical chemical properties can be solubilized through the establishment of a variety of interactions such as ionic, hydrophobic, formation of mixed aggregates or hydrogen bonds.

One of the distinctive features of these sorbents is that they are dynamic entities in equilibrium with surfactant monomers in solution. The consequences of this equilibrium for monomers SPE has been investigated and different guidelines have been established for method development. On the other hand, the investigations carried out have permitted a good knowledge of the analyte:sorbent interactions which facilitates the straightforward establishment of new applications.

This contribution explores the individual and global effect of environmental matrix components on both the adsorption of the surfactant on the mineral oxide and the SPE of organic pollutants. The aim was to achieve a good knowledge of the predominant interactions between analyte:matrix and sorbent:matrix in order to prevent interferences and to get efficient and robust extraction methods.

The study was carried out using sodium dodecyl sulfate (SDS) as surfactant and alumina as mineral oxide because they have been previously proposed as sorbents for the SPE of organic pollutants by our research group. The analytes were selected on the basis of the different types of interactions that they can establish with hemimicelles and admicelles [ionic: difenzoquat methyl sulfate (FQ); hydrophobic: bisphenol A (BPA) and benzo(a)pyrene (BaPyr); and formation of mixed aggregates: benzyldimethyldodecylammonium bromide (BDDA)]. The matrix components investigated were the ionic strength (0.1 M NaCl); the water hardness (1.14 mM $CaCl_2$); and organic matter components, including those characteristics of domestic wastewater (carbohydrates, fats, proteins, detergents, etc) and natural water (humic acids). The results obtained on the influence of these matrix components on the surfactant aggregates and the adsolubilization/elution/breakthrough of analytes permit to draw different guidelines to prevent interferences in hemimicelle/admicelle-based SPE.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3rd International Symposium on
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DETERMINATION OF BISPHENOL A IN CANNED VEGETABLE AND FRUIT SAMPLES BY COACERVATIVE EXTRACTION, LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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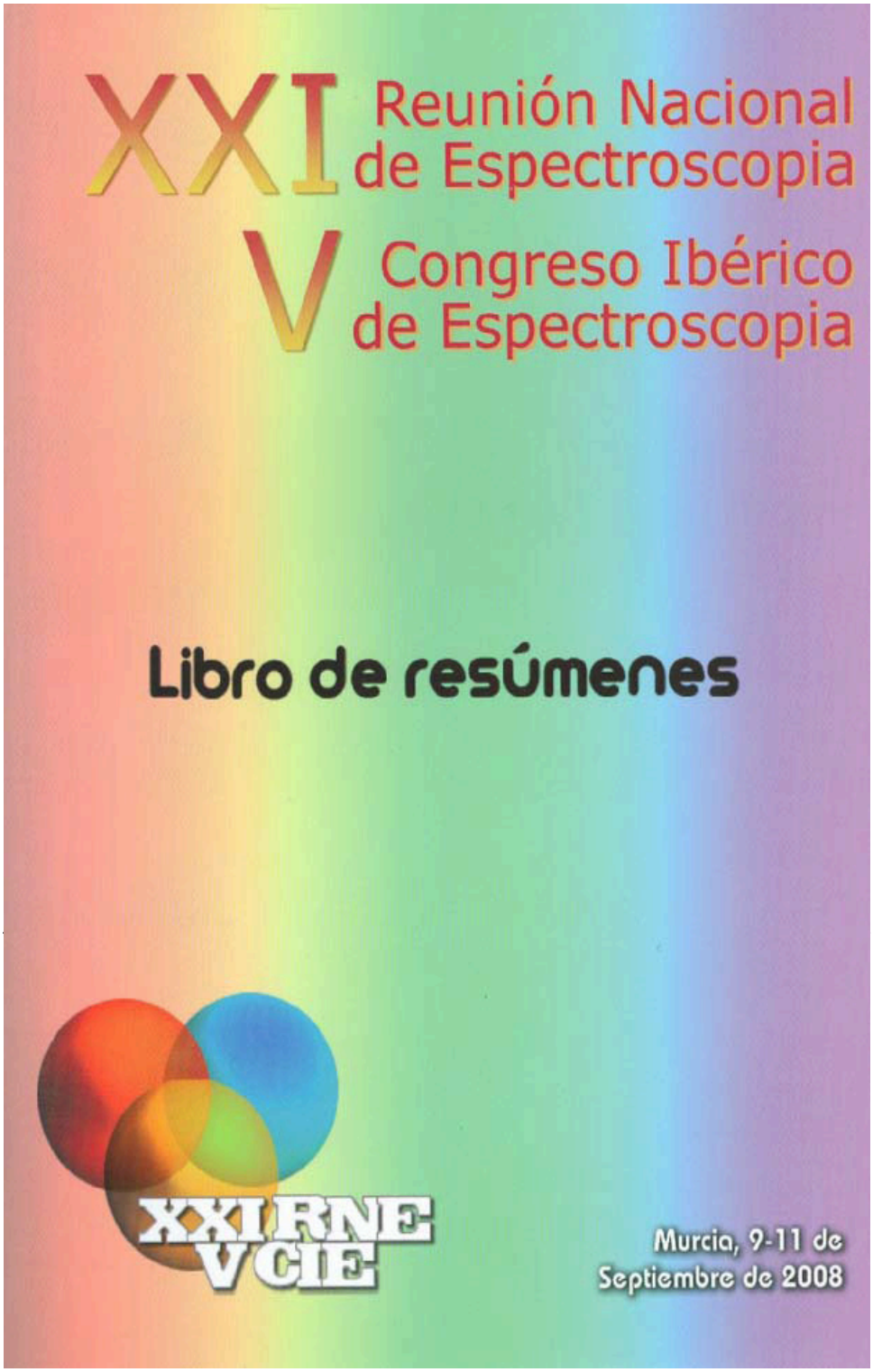
Bisphenol A (BPA) is used in the resins that coat the inside of some food cans. Human exposure to BPA occurs from the migration of this contaminant into foods. The tolerable daily intake (TDI) level established by the U.S. Environmental Protection Agency as well as that recently recommended by the European Food Safety Authority (EFSA) is 0.05 milligrams per kilogram of bodyweight. The amount of BPA legally permitted to migrate from packaging into food, known as the specific migration limit (SML), is based on the TDI and it was set at 600 ng/g by the EU Commission in 2004.

Up to now, liquid-liquid extraction and solid-phase extraction (SPE), followed by purification of extracts, evaporation with nitrogen and so on have been the most common sample preparation procedures applied to the determination of BPA in foodstuffs. The quantification is usually carried out by liquid chromatography in combination with either UV or fluorescence detection, or by gas chromatography coupled to mass spectrometry (GC-MS). The amount of sample handled usually ranges between 1 and 10 g and typical detection limits obtained vary from about 1to 20 ng BPA/g foodstuff.

The present research was intended to simplify the sample treatment required for the determination of BPA in canned food samples. For this purpose, colloid-rich liquids, named coacervates, were assessed for the first time as extractants of contaminants from solid foods. Coacervates made up of decanoic acid reverse micelles in tetrahydrofuran (THF) were proven to efficiently extract BPA on the basis of the hydrophobic and hydrogen bond interactions that were established between the analyte and the extractant. Factors affecting the extraction efficiency were optimised and the most influential ones were the decanoic acid and tetrahydrofuran concentration and the stirring time. The coacervates were formed by using 0.2 g decanoic acid and 2 mL of tetrahydrofuran. The whole procedure was developed at room temperature and the extraction time was 15 minutes. Using minute quantities of sample food (around 300 mg), the detection limit for BPA in canned vegetables and fruits was about 6 ng/g. No matrix effects were observed in any of the sample analyzed, which permitted the use of external calibration. No clean-up steps were necessary to achieve selectivity. The amount of BPA found in the solid portion of a variety of canned food samples, namely fruit salad, peach in syrup, mango slice, red pepper, sweetcorn, green bean and pea cans, ranged between 10 and 103 ng BPA/g canned food. These amounts were far below to the SML established by the EU Commission. Main assets of the use of coacervates for this application were simplicity, rapidity and low cost.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Murcia, 9-11 de Septiembre de 2008

Determinación de Bisfenol A en muestras de orina humana mediante extracción coacervativa y cromatografía líquida con detección fluorescente

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Bisfenol A (BPA), uno de los compuestos químicos con mayor producción a nivel mundial, se ha considerado tradicionalmente un estrógeno débil. Sin embargo, numerosos estudios in vitro realizados recientemente han demostrado que BPA puede alterar la función normal del sistema endocrino y producir perturbaciones en las funciones celulares a concentraciones tan bajas como 0.23 ng/l (1). El consumo de alimentos embotellados y enlatados constituye la principal vía de exposición humana a BPA ya que este compuesto se utiliza en la fabricación de policarbonato y resinas epoxi y migra a los alimentos a concentraciones entre 0.1 y 384 ng/g (2). Estudios recientes realizados por el National Center for Environmental Health de EEUU han mostrado que en un 92.6% de las muestras de orina humana analizadas hay niveles detectables de BPA (entre 0.4 y 149 µg/l) (3), por lo tanto, es de interés disponer de métodos sencillos, rápidos y económicos que permitan determinar bajos niveles de BPA en orina para evaluar la exposición humana a este contaminante.

Los métodos analíticos desarrollados hasta la fecha para la determinación de BPA en orina requieren una tediosa preparación de muestra que abarca extracción en fase sólida (SPE), purificación de extractos y evaporación con nitrógeno. La cuantificación se realiza mediante cromatografía liquida con detección ultravioleta o fluorescente o cromatografía de gases acoplada a espectrometría de masas.

En este trabajo se propone el uso de coacervados de micelas inversas de ácido decanoico para la extracción de BPA en orina con el objetivo de simplificar la etapa de tratamiento de la muestra. El procedimiento implica la adición a la orina (7 ml) de 1 ml de tetrahidrofurano (THF) y 100 mg de ácido decanoico. El coacervado se forma in situ, de forma espontánea e instantánea, es de bajo volumen (200 µl) y contiene una elevada concentración de micelas inversas (~ 0.6 mg ácido decanoico/µl), por lo tanto, posee una elevada capacidad para la solubilización de BPA mediante interacciones hidrófobas y enlaces de hidrógeno. La extracción se lleva a mediante agitación de la muestra durante 10 min y los extractos se analizan directamente mediante cromatografía líquida-fluorescencia después de centrifugación durante 10 minutos para facilitar la separación del coacervado. El método se validó mediante su aplicación a 8 muestras diferentes de orina donde los niveles hallados oscilaron entre 3.5-48.5 µg/l. El límite de cuantificación del método fue de 0.2 µg/l y la desviación estándar relativa para las muestras osciló entre el 0.7 y 6.4 %.

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