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"Profesor Fermín Capitán García"

TESIS DOCTORAL

DESARROLLO DE MÉTODOS DE ANÁLISIS PARA LA CUANTIFICACIÓN DE DISRUPTORES ENDOCRINOS QUÍMICOS EN MUESTRAS BIOLÓGICAS

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MEMORIA presentada para aspirar al Grado de Doctor en Química por la Universidad de Granada

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A todos los que lo hicieron posible

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Por último, a mi "jardinero", la persona que no ha permitido que este arbolillo se torciera, que creciera fuerte y recto, que ha sacado lo mejor de mí.

La presente Memoria de Tesis Doctoral, ha sido realizada por compilación de publicaciones. Está conformada por seis artículos publicados en revistas todas ellas pertenecientes a la Editorial Elsevier: Journal of Chromatography A, Talanta y Journal of Pharmaceutical and Biomedical Analysis.

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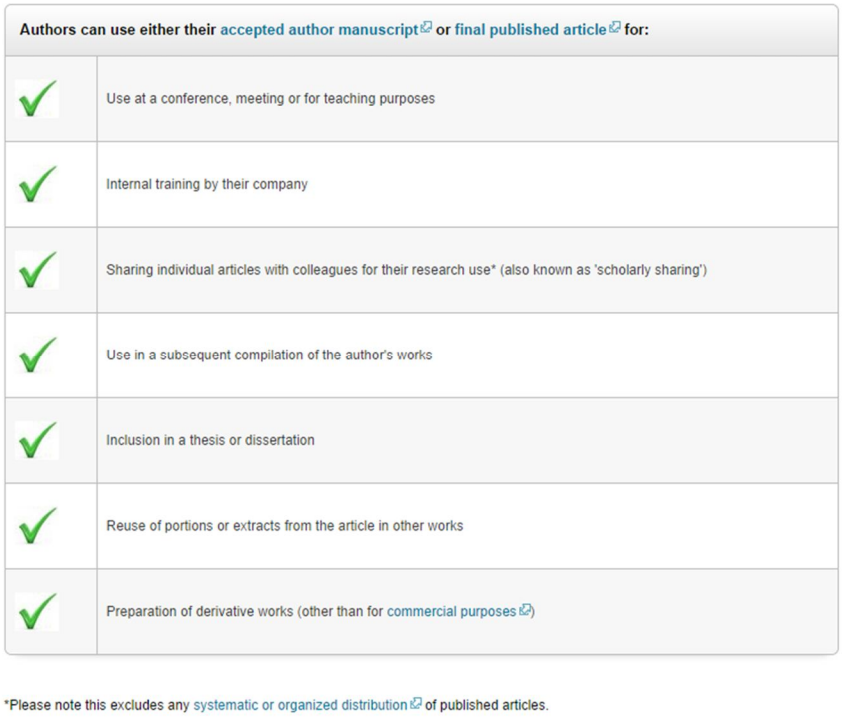

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En la presente Tesis Doctoral se proponen varias metodologías para la determinación de diferentes disruptores endocrinos químicos en leche materna humana. El estudio se ha centrado principalmente en la aplicación de diversas técnicas de tratamiento de muestra y limpieza de extractos (*clean-up*) previo al análisis mediante cromatografía de líquidos o de gases acopladas a espectrometría de masas en tándem.

En los últimos años, y debido a los cambios de hábitos experimentados por la sociedad, se han introducido en el mercado numerosas sustancias químicas de las cuales se dispone de muy poca información sobre su peligrosidad y sobre las consecuencias de su uso masivo. En muchos casos, se trata de compuestos que aparecen de forma ubicua en la actividad cotidiana del ser humano y por tanto en su entorno. Se ha demostrado que muchos de estos compuestos, además de presentar una importante actividad biológica presentan una gran capacidad de bioacumulación, debido a su bajo grado de metabolización en el cuerpo humano, por lo que se encuentran frecuentemente en fluidos y tejidos biológicos.

La selección de los compuestos objeto de estudio en la presente Tesis Doctoral (bisfenol A y sus derivados clorados, parabenos y benzofenonas), se ha realizado en base a su producción mundial actual, alta exposición humana, y principalmente a su demostrada actividad como disruptores endocrinos. Como matriz objeto de estudio, se ha seleccionado la leche humana en base a que es una de las vías de exposición más importantes en la actualidad del ser humano en las primeras etapas de la vida, donde se ha detectado una mayor vulnerabilidad de los organismos a estos compuestos químicos. Además, la leche humana es un excelente biomarcador para determinar la exposición humana, ya que es una muestra muy fácil de obtener en cantidades relativamente elevadas.

Dada la complejidad de esta matriz, y las dificultades que ofrece para su estudio desde un punto de vista analítico, la literatura científica demuestra una importante falta de metodologías para la determinación de estos compuestos

en esta matriz, existiendo escasos métodos multirresiduo publicados hasta la fecha. Para resolver este problema, la investigación se ha centrado en la optimización y aplicación de diferentes técnicas de extracción y *clean-up* con el objetivo final de proponer y validar diversos métodos multirresiduo de buenas características analíticas, que permitan la determinación de los compuestos seleccionados en muestras de leche materna. Como técnicas de análisis se han empleado tanto la cromatografía de líquidos de ultrarresolución (UHPLC) como la cromatografía de gases (GC) acopladas a espectrometría de masas en tándem.

Los métodos propuestos aportan notables mejoras y representan una importante innovación en cuanto a metodología analítica, cubriendo un amplio rango de disruptores endocrinos. Se ha llevado a cabo un estudio exhaustivo en esta matriz compleja de los parámetros más influyentes en los procedimientos de tratamiento de muestra y limpieza de extractos, empleándose en muchos casos la metodología multivariante de superficie de respuesta, y en la detección cromatográfica. Todos los métodos han sido validados siguiendo las guías internacionales de aplicación.

Como resultado de la presente investigación, se han propuesto 6 nuevos métodos con grandes prestaciones analíticas en cuanto a exactitud (veracidad y precisión), sensibilidad y selectividad:

A. Dos métodos multirresiduos para la determinación de bisfenol A y sus derivados clorados, parabenos y benzofenonas mediante el uso de la extración con barras agitadoras adsorbentes (SBSE), previa precipitación química de proteínas y grasas, y análisis mediante cromatografía de líquidos de ultrarresolución y cromatografía de gases acopladas a espectrometría de masas en tándem (UHPLC-MS/MS y GC-MS/MS).

B. Método multirresiduo para la determinación de bisfenol A y sus derivados clorados, parabenos y benzofenonas mediante la aplicación

de la extracción dispersiva con adsorbentes previa precipitación química de proteínas y grasas, y análisis mediante UHPLC-MS/MS.

C. Método multirresiduo para la determinación de bisfenol A y parabenos mediante el uso de la extracción asistida con ultrasonidos previa al análisis mediante UHPLC-MS/MS. En esta metodología se incluye además un paso de limpieza del extracto con adsorbentes dispersivos (C18).

D. Método multirresiduo para la determinación de benzofenonas mediante UHPLC-MS/MS previo tratamiento de la muestra con extracción asistida por ultrasonidos y limpieza del extracto con adsorbentes dispersivos (C18 y PSA).

E. Método multirresiduo para la determinación de parabenos mediante la aplicación de la microextracción sólido-líquido-líquido con membrana agitada (SM-SLLME) seguida de UHPLC-MS/MS.

Los métodos propuestos han sido publicados o se encuentran en fase de publicación en diferentes revistas de impacto de la especialidad de Química Analítica, dos de ellos en *Journal of Chromatography A*, otros dos en *Talanta* y el último en la revista *Journal of Pharmaceutical and Biomedical Analysis*. Todas las metodologías propuestas han sido aplicadas a la determinación de los compuestos seleccionados en cada caso a muestras reales tomadas a partir de diferentes madres lactantes en diferentes momentos del periodo de lactancia.

Como complemento al trabajo de laboratorio realizado, se ha publicado un artículo de revisión sobre la técnica *stir-bar sorptive extraction* (SBSE) en la revista científica *Talanta* y un libro a modo de monografía editado por la Editorial Académica Española, titulado *"Determinación de Disruptores Endocrinos Químicos en tejido placentario humano"*.

Finalmente, durante el periodo de desarrollo de la presente Tesis Doctoral se ha colaborado, por necesidades puntuales del Proyecto de Investigación en el que se ha desarrollado la misma, en el desarrollo de otros tres trabajos científicos publicados en revistas internacionales de alto impacto:

A. F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, M.F. Fernández, N. Olea and J.L. Vílchez. Determination of Benzophenones in Human Placental Tissue Samples A by Liquid Chromatography-Tandem Mass Spectrometry. *Talanta* 85 (2011) 1848-1855. DOI:10.1016/j.talanta.2011.07.030

B. F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, M.F. Fernández, N. Olea and A. Navalón. A Multiclass Method for Endocrine Disrupting Chemical Residue Analysis in Human Placental Tissue Samples by UHPLC-MS/MS. *Analytical Methods*, 3 (2011) 2073-2071.DOI: 10.1039/c1ay05162h

C. J. Ballesta-Claver, R. Rodríguez-Gómez, L.F. Capitán-Vallvey. Disposable Biosensor Based on Cathodic Electrochemiluminescence of Tris(2,2-bipyridine)ruthenium(II) for Uric Acid Determination. *Analytica Chimica Acta*, 770 (2013) 153-160. DOI: 10.1016/j.aca.2013.01.045

En resumen, se han publicado un total de 10 trabajos científicos a modo de trabajo científico original, monografía o revisión en publicaciones de gran notoriedad e impacto a nivel internacional.

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ACRÓNIMOS Y ABREVIATURAS

4-OH-BP	4-Hidroxibenzofenona
ANOVA	Análisis de la varianza
APCI	Atmospheric pressure chemical ionization
BP-1	Benzofenona 1
BP-2	Benzofenona 2
BP-3	Benzofenona 3
BP-6	Benzofenona 6
BP-8	Benzofenona 8
BPB	Butilparabeno
BPA	Bisfenol A
Cl-BPA	Monoclorobisfenol A
Cl_2-BPA	Diclorobisfenol A
Cl_3-BPA	Triclorobisfenol A
Cl_4-BPA	Tetraclorobisfenol A
CAS	Chemical Abstracts Service
CCD	Central composite design
CI	Chemical donization
CID	Collision induced dissociation
DBB	Diseño experimental Box-Benhken
EDCs	Disruptores endocrinos químicos
EE2	Ethynylestradiol
EI	Electron ionization
ELISA	Enzyme-linked immuno sorbent assay
EPB	Etilparabeno
ESI	Electrospray ionization
FAB	Fast atom bombardment
FDA	Food and Drugs Administration

FLD	Fluorescence detector
LC	Liquid chromatography
LDR	Linear dinamic range
LLE	Liquid-liquid extraction
LPME	Liquid-phase microextraction
LOD	Limit of detection
LOEC	Lowest observed effect concentration (Toxicology)
LOQ	Limit of quantification
MeCN	Acetonitrilo
MeOH	Metanol
MIP	Molecular Imprinted polymer
MPB	Metilparabeno
MRM	Multiple reaction monitoring
PDMS	Polydimethylsiloxane
PCBs	Polychlorinated biphenyls
PCPs	Productos del cuidado personal
POP	Persistent organic pollutant
PPB	Propilparabeno
RPLC	Cromatografía de líquidos en fase inversa
RSD	Relative standard deviation
RSM	Response surface methodology
SBSE	Stir-bar sorptive extraction
SIM	Selected ion monitoring
SM-SLLME	Stir-membrane solid-liquid-liquid microextraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring

TQD	Triple quadrupole detector
TBT	Tributiltin species
UE	Unión Europea
UHPLC	Ultra-high performance liquid chromatography
USE	Ultrasound-assisted extraction
US-EPA	United States-Environmental Protection Agency

OBJETO DE LA TESIS

El objetivo principal de la presente Tesis Doctoral es la puesta a punto y validación de diversas metodologías analíticas para la determinación de disruptores endocrinos químicos en leche materna humana. Se estudian tres de los grupos más importantes de disruptores endocrinos por su uso, presencia actual en las actividades habituales del ser humano y actividad biológica demostrada: bisfenol A y sus derivados clorados, parabenos y benzofenonas.

Los objetivos específicos de esta Memoria de Doctorado son los que se enumeran a continuación:

1. Realizar una revisión exhaustiva de la información existente hasta la fecha en relación a los contaminantes elegidos a nivel de su determinación analítica en la matriz objeto de estudio.

2. Desarrollar nueva metodología analítica para la determinación de los compuestos seleccionados en leche materna, empleando una amplia variedad de técnicas de extracción, limpieza de extractos y técnicas cromatográficas, que por sus características permitan a cualquier laboratorio realizar los análisis propuestos. Para ello se han estudiado en profundidad las variables que pueden afectar a cada etapa del proceso analítico empleando en muchos casos metodología multivariante de superficie de respuesta.

3. Validar los métodos propuestos en términos de exactitud (veracidad y precisión), sensibilidad y selectividad, siguiendo las guías internacionales de validación aplicables a la puesta a punto de métodos analíticos.

4. Estudiar la presencia de estos compuestos en muestras reales, para lo cual se han seleccionado una serie de madres voluntarias en diferentes periodos del proceso de lactancia.

Las técnicas y metodologías analíticas que se han empleado para la consecución de los objetivos de la presente Tesis Doctoral han sido:

- ✓ Cromatografía de líquidos de ultrarresolución acoplada a espectrometría de masas en tándem (UHPLC-MS/MS).

- ✓ Cromatografía de gases acoplada a espectrometría de masas en tándem (GC-MS/MS).
- ✓ Extracción asistida por ultrasonidos (USE).
- ✓ Técnicas de precipitación química de proteínas y grasas y aplicación de la extracción con adsorbentes dispersivos (C18 y PSA).
- ✓ Microextracción mediante barras agitadoras adsorbentes (SBSE).
- ✓ Microextracción sólido-líquido-líquido con membrana agitada (SM-SLLME).
- ✓ Herramientas estadísticas para llevar a cabo el tratamiento de los datos procedentes de los diseños de experimentos multivariantes empleados en la optimización de los diferentes métodos analíticos y para la validación de los métodos propuestos.

INTRODUCCIÓN

1. DISRUPTORES ENDOCRINOS QUÍMICOS

La búsqueda continua de una mejora en la calidad de vida de la sociedad actual, ha provocado un aumento en el consumo y la aparición de nuevos productos de "primera necesidad" por parte de la población. El ser humano emplea, en las diversas actividades que realiza en su vida cotidiana, numerosos productos manufacturados. Como consecuencia de estos nuevos hábitos, se han generado gran cantidad de residuos que en su mayor parte van a parar al medio ambiente. En este sentido, en los últimos años, se han realizado gran cantidad de estudios que demuestran la presencia de nuevas sustancias químicas en los diferentes compartimentos medioambientales. Son los denominados *contaminantes emergentes*. Se trata sustancias químicas que no poseen una regulación clara y específica, y cuyo impacto sobre el medio ambiente y la salud humana no están aun claramente descritos[1,2].

Muchas de estas sustancias han sido clasificadas como altamente persistentes y peligrosas para el medio ambiente, siendo potencialmente carcinogénicas, tóxicas y con propiedades como disruptores endocrinos. La situación es más preocupante aun si se considera que, debido al desconocimiento de su potencial efecto adverso sobre la salud humana, en algunos casos, aparte de masivo, su uso se ha convertido en incontrolado.

Entre estos nuevos compuestos de síntesis, existe un grupo de sustancias químicas, consumidas por la población mundial en muy elevadas cantidades (miles de toneladas por año), denominadas *productos de cuidado personal (PCPs)*. Se trata de diferentes familias de compuestos orgánicos empleados como aditivos en numerosos productos de uso cotidiano[3]. En el mundo se consumen elevadas cantidades de productos para el cuidado dental, de la piel o el cabello como jabones, champús o detergentes, así como agentes

1 Rosal R., Rodríguez A., Perdigón-Melón J.A., Petre A., García-Calvo E., Gómez M.J., Agüera A., Fernández-Alba, A.R. *Water Research,* 2010, 44:578–588.

2 Vogelsang C., Grung M., Jantsch T.G., Tollefsen K.E., Liltved H. *Water Research,* 2006, 40:3559–3570.

3 Jiménez-Díaz I., Zafra-Gómez A., Ballesteros O., Navalón A. *Talanta,* 2014, 129:448–458.

protectores solares, entre muchos otros. Los surfactantes (detergentes), las fragancias (almizcles nitrados y policíclicos), los filtros para la radiación UV (benzofenonas y canfores), los conservantes (parabenos y derivados de isotiazolina) o los antisépticos (triclosán y triclocarbán), se encuentran habitualmente como componentes de estos productos manufacturados[4].

En los últimos años, se ha demostrado que muchos de estos compuestos químicos denominados *Endocrine Disrupting Chemicals* (EDCs) son capaces tanto de mimetizar como de inhibir, la acción natural de las hormonas, provocando efectos adversos principalmente en el desarrollo de los individuos y en su capacidad reproductiva y presentando por tanto actividad biológica como disruptores endocrinos tanto en animales como en humanos[5]. En machos, se ha relacionado la exposición a los EDCs con problemas en la capacidad reproductiva y con el cáncer de próstata y testicular[6]. En hembras, las anomalías se asocian al incremento del riesgo de endometriosis, problemas reproductivos, aumento en la incidencia de cánceres relacionados con el sistema endocrino, entre otros efectos[7]. Además, es un hecho demostrado que, la incidencia de enfermedades relacionadas con el sistema endocrino de los individuos se ha incrementando vertiginosamente en los países desarrollados, habiendo claros signos de cambios en la salud reproductiva de los seres vivos, incluyendo una disminución en la fertilidad masculina, defectos de nacimiento e incidencia de cánceres.

Los disruptores endocrinos incluyen familias de sustancias de origen tanto natural como artificial tan diferentes como los estrógenos sintéticos, usados como anticonceptivos (*e.g.,* EE2 y 17α-etinilestradiol); algunos fármacos (*e.g.,* tamoxifeno o dietilestilbestrol); los fitoestrógenos (*e.g.,* isoflavonoides); los hidrocarburos aromáticos policíclicos (benzopireno, fenantreno…); los fenoles halogenados (clorofenoles y bromofenoles); el bisfenol A, sus derivados y los ésteres del ácido ftálico (ftalatos), utilizados en la fabricación o como aditivos

4 Ternes T.A., Knacker T., Oehlmann J. *Umweltchemie und Ökotoxikologie*, 2003, 15:169–180.
5 US EPA, Office of Research and Development, 1197, EPA/630/R-36/012.
6 Pflieger-Bruss S., Schuppe H.C., Schill W.B. *Andrologia, 2004,* 36:337–345.
7 Nicolopoulou-Stamati P., Pitsos M.A. *Human Reproduction Update,* 2001, 7:323–330.

en resinas, lacas, plásticos y materiales poliméricos; los pesticidas (lindano o DDT); los policlorobifenilos (PCBs); los alquilfenoles (nonilfenol y octilfenol); los filtros UV-Vis (benzofenonas, canfores o salicilatos) o los conservantes (parabenos). Se trata de una lista interminable, lo que hace pensar que la exposición humana es masiva y universal[8-10].

El ser humano se encuentra por tanto, de forma inevitable, expuesto a los EDCs, ya que vivimos rodeados de los mismos[11], por lo que la preocupación creciente que causa este problema está completamente justificada. Esta exposición se convierte en más inquietante cuando se produce durante los inicios de la vida de un individuo, ya que, aunque aún permanece poco claro, podría relacionarse con anomalías en la diferenciación sexual, problemas en el desarrollo neurológico y reproductivo y con el riesgo de desarrollo de problemas reproductivos y cáncer en el futuro[12].

En este contexto, la comunidad científica ha comenzado a plantear una posible relación entre la presencia de estas sustancias y enfermedades de diversa índole. Resulta por tanto muy interesante desde un punto de vista científico conocer la presencia y abundancia de estas sustancias en nuestro propio organismo con el fin de anticiparnos y así prever posibles consecuencias negativas sobre la salud.

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8 Stopper H., Schmitt E., Kobras K. *Mutation Research*, 2005, 574:139–155.

9 Rubin B.S. *The Journal of Steroid Biochemistry and Molecular Biology*, 2011, 127:27–34.

10 Ying G.G., Williams B., Kookana R.S. *Environment International*, 2002, 28:215–226.

11 Frye C.A., Bo E., Calamandrei G., Calzà L., Dessì-Fulgheri F., Fernández M., Fusani L., Kah O., Kajta M., Le Page Y., Patisaul H.B., Venerosi A., Wojtowicz A.K., Panzica G.C. *Journal of Neuroendocrinology*, 2012, 24:144–159.

12 Damgaard I.N., Main K.M., Toppari J., Skakkebaek N.E. *Best Practice and Research: Clinical Endocrinology and Metabolism*, 2002, 16:289–309.

1.1. Definición del problema. Antecedentes históricos

Los disruptores endocrinos químicos (EDCs) son compuestos capaces de afectar al balance normal de las funciones hormonales en animales y en su progenie. Son numerosos los contaminantes que habitualmente son liberados por la actividad humana, de los que se ha demostrado actividad estrogénica[13], existiendo evidencia de que la mayoría se encuentran presentes de forma ubicua en el medioambiente. En los años 80, la aparición de una serie de efectos adversos sobre todas las clases de organismos vivos, dirigieron la atención hacia lo que hoy conocemos como EDCs. En estos años, la zoóloga estadounidense de la *Word Wildlife Fund* (WWF), Theo Colborn, realizó estudios sobre los vertebrados que habitaban en los Grandes Lagos[14]. Colborn, realizó mediciones y analizó los efectos negativos que iba observando sobre la biota del lago, llegando a la conclusión de que los animales se estaban viendo afectados en varios sentidos. Observó una tasa anormalmente reducida de reproducción, problemas tiroidales, comportamientos alterados y cambios en el metabolismo de los animales. Todos ellos conducían al deterioro serio de la salud. Cada síntoma sugería que las alteraciones eran producidas a nivel del sistema endocrino, y por lo tanto comenzó a sospechar en la existencia del fenómeno de la disrupción endocrina. La observación más importante fue que estos problemas también eran observados en la progenie.

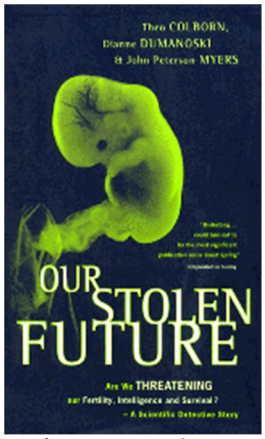

Figura 1. *"Nuestro futuro robado" Theo Colborn. Primer tratado que describe los efectos de los disruptores endocrinos.*

A partir de estas investigaciones y tras dar la voz de alerta, han sido muchos los ejemplos que se puede citar de estudios realizados en relación a la presencia de compuestos con actividad disruptora del sistema endocrino en medios naturales y las consecuencias observadas en las poblaciones.

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[13] Keith L.H. *Pure and Applied Chemistry,* 1998, 70:2319–2326.
[14] Colborn T., Dumanoski D., Myers J.P. Penguin Books, 1996, New York, USA, 336.

Así, por ejemplo, en Taiwan los elevados niveles de PCBs ingeridos por la población a través de aceites contaminados dieron como resultado importantes desórdenes en los hijos de las madres expuestas[15]. En Florida, se encontraron importantes anomalías de índole reproductiva en los caimanes (*Alligator Mississippiensis)* del lago Apopka, posiblemente producidas por el efecto de la descarga masiva de EDCs en el lago[16]. En la población de gaviotas del sur de California se observó una desviación importante en la ratio sexual, incrementándose en gran medida el número de hembras frente al de machos y observándose además conductas anómalas en la etapa de nidificación[17]. En general, en diferentes partes del mundo, se han observado numerosas anomalías en peces, como consecuencia del vertido residual de las plantas de tratamiento de papel. Se han registrado desajustes en cuanto a la ratio sexual y una importante disminución del contenido en testosterona de los individuos macho[18,19]. En España, se ha documentado, en hembras de gasterópodos, un fenómeno denominado *imposex*, que consiste en la superposición de caracteres sexuales masculinos y femeninos[20]. Se trata de uno de los pocos ejemplos de relación causa-efecto, dosis-dependiente, que se conocen en toxicología y resulta altamente específico ante la contaminación por tributiltin (TBT, compuesto organoestañoso lixiviado de las pinturas antialgas). Durante el verano de 1996 se desarrolló una campaña de muestreo a lo largo de la costa de Galicia, eligiéndose al prosobranquio marino *Nucella lapillus*como especie bioindicadora*,* por ser una especie altamente sensible. En todas las poblaciones estudiadas se registró la presencia de *imposex*[21-23].

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15 Rogan W.J., Gladen B.C., Hung K.L., Shih L. Y., Taylor J.S., Wu Y.C., Yang D., Ragan N.B., Hsu C.C. *Science,* 1988, 241:334–336.

16 Clark D.R. Technical Report W.D.C.U.S.FaX Service, Editor. 1990. USA

17 Fox G.A., 1992, 23, USA, pp 147-158.

18 Munkittrick K.R., Port C.B., Van der Kraak G.J., Smith I.R., Rokosh D.A. *Canadian Journal of Fisheries and Aquatic Sciences,*1991, 48:1371–1380.

19 Jobling S., Sumpter J.P. *Aquatic Toxicol*ogy, 1992, 27:361–372.

20 Gibbs P.E., Bryan G.W., Pascoe P.L., Burt G.R. *Journal of the Marine Biological Association,* 1987,67:507–523.

21 Barreiro R., Ruiz J.M., Quintela, M. *Cuadernos de Investigación Biológica,*1998, 20:279–282.

22 Barreiro R., Quintela M., Ruiz J.M. *Marine Ecology Progress Series,* 1999, 185:229–238.

23 Quintela M., Barreiro R., Ruiz J.M. *Marine Environmental Research,* 2002,54:657–660.

Los EDCs no sólo afectan a los animales. Las plantas también pueden sufrir sus efectos, tal como ocurre con las leguminosas. Las plantas leguminosas viven en simbiosis con las bacterias del suelo del género *Rhizobium*. La comunicación funciona vía fitoestrógenos, donde el "blanco" de estos fitoestrógenos es la proteína activadora transcripcional *nodulation D* (NodD), que puede ser considerada como el homólogo del receptor de los estrógenos de los vertebrados[24]. Se ha demostrado que los EDCs interfieren en la comunicación bacteria-planta por su interacción con los fitoestrógenos y por lo tanto afectan a la simbiosis. Este hecho ha sido probado en ensayos que han estudiado la comunicación simbiótica entre la alfalfa (*Medicago sativa*) y la bacteria del suelo *Sinorhizobium meliloti*, que es responsable de la fijación de nitrógeno, en presencia de bisfenol A[25-27].

A pesar de todas estas evidencias, a día de hoy, existen opiniones contradictorias respecto a la relación causa-efecto de estas sustancias químicas sobre los seres vivos. Algunos científicos consideran que no hay evidencia suficiente para asegurarla[28], mientras que otros establecen que esta evidencia es considerable y está claramente demostrada[29].

En cualquier caso, a raíz de las evidencias presentadas a lo largo de estos primeros años de investigación, en el Workshop europeo sobre el impacto de los EDCs en la salud humana y animal, de Weybridge (Reino Unido) celebrado en 1996, se consensuó una definición general según la cual, ***"un disruptor endocrino es una sustancia exógena que causa efectos adversos en la salud de un organismo intacto, o en su progenie, consecuencia de cambios en las funciones endocrinas"***. Ese mismo año el *Informe Weybridge* publicado tras el ***"European Workshop: On the Impact of Endocrine Disruptors on Human Health and Wildlife"***, concluye que no sólo es necesario saber qué sustancias pueden

[24] Gyorgypal Z., Kondorosi A. *Molecular & General Genetics*, 1991, 226:337–340.
[25] Fox J.E. *Integrative and Comparative Biology*, 2005, 45:179–188.
[26] Fox J.E. *Environmental Health Perspectives*, 2004, 112:648–653.
[27] Fox J.E, Starcevic M., Jones P.E., Burow M.E., McLachlan J.A. *Environmental Health Perspectives*, 2004, 112:672–677.
[28] Mathiessen P. *Ecotoxicology*, 2000, 9:21–24.
[29] Dickerson S.M., Gore A.C. *Reviews in Endocrine & Metabolic Disorders*, 2007, 8:143–159.

actuar como disruptores endocrinos o qué otros efectos pueden tener, sino qué hay que establecer las precauciones que se deben tomar para disminuir o eliminar completamente la exposición[30].

En ese momento aún se desconocía que muchos de los compuestos químicos que usamos habitualmente en nuestra vida cotidiana son potenciales EDCs. En 1999, la Comisión del Consejo y el Parlamento Europeo acordaron que había una necesidad de tener mayor conocimiento acerca de estos compuestos y dos años después, tras numerosas reuniones técnicas, se creó el *Research Cluster into Endocrine Disruption* (CREDO), publicándose una lista de sustancias prioritarias para su posterior evaluación en relación a su rol como disruptores endocrinos[31].

A partir de finales de los 90, se comenzaron a desarrollar sistemas *in vitro* para determinar qué sustancias podrían presentar esta actividad disruptora, y métodos de análisis para la determinación de estas sustancias en matrices de diferente naturaleza, principalmente medioambientales, alimentarias y biológicas. En 2001 la lista de compuestos con capacidad disruptora endocrina alcanzó los 553[32,33]. Actualmente, se ha comprobado la actividad como EDCs *in vitro* e *in vivo* en más de 900 sustancias químicas[34] y se sospecha de más de 40.000, incluyendo algunos metales pesados. Además, de acuerdo a la US-EPA, 87.000 compuestos químicos necesitan ser evaluados para descartar o confirmar esta actividad.

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30 European Commission. European workshop on the impact of endocrine disruptors on human health and wildlife. Report Eur 17549, Environment and Climate Research Programme, DG XII. Weybridge, 1996.

31 COM. 706 final, Communication from the Commission to the Council and the European Parliament, Community Strategy for Endocrine Disrupters. A range of substances suspected of interfering with the hormone systems of humans and wildlife, 1999.

32 COM. 262 final, Communication from the Commission to the Council and the European Parliament, Community Strategy for Endocrine Disrupters. A range of substances suspected of interfering with the hormone systems of humans and wildlife. Bruxelles, 2001, 55 pp.

33 BKH. Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption - preparation of a candidate list of substances as a basis for priority setting. European commission DG ENV. Delft, 2000, 29 pp.

34 TEDX List of Potential Endocrine Disruptors. Disponible en: http://endocrinedisruption.org/endocrine-disruption/tedx-list-of-potential-endocrine-disruptors/chemicalsearch?action=search&sall=1 (Visitada 29/09/2014)

1.2. Determinación de la actividad estrogénica

Es difícil deducir la actividad estrogénica en base únicamente a la estructura de un compuesto químico. Los EDCs pueden tener propiedades fisicoquímicas muy diferentes y pertenecer a familias químicas muy variadas. Sin embargo, la estructura del anillo aromático, parece ser una subestructura común en todos los compuestos con actividad estrogénica. En cualquier caso, se ha demostrado que lo contrario no es necesariamente cierto, es decir, la presencia de grupos famélicos no implica necesariamente que la sustancia tenga propiedades estrogénica[35].

Desde un punto de vista biológico, la influencia de los EDCs sobre el sistema endocrino forma parte de un proceso complejo. Los EDCs pueden utilizar rutas variadas para provocar los efectos adversos. Son capaces de interaccionar con los receptores hormonales perturbando la subsiguiente expresión génica. Al unirse al receptor, estos compuestos estimulan e inhiben la transcripción genética de forma similar a como lo hacen las hormonas naturales, o incluso pueden inactivar dicho proceso formando complejos receptor-ligando con conformaciones que anularían la transcripción. Así pues, el impacto de los EDCs en el metabolismo es de una gran importancia ya que la interacción con los receptores genera interferencias en el transporte de proteínas, alteraciones en la síntesis y biotransformación de las hormonas y efectos tóxicos en gónadas, hipotálamo, pituitaria y glándulas endocrinas.

Basados en estos datos, son varios los test que históricamente se han propuesto para la determinación de la actividad disruptora de las sustancias químicas. A pesar de esto, son pocos los bioensayos que se reconocen en la actualidad como instrumentos útiles y fiables[36].

A continuación se muestran los tests de identificación de actividad estrogénica más empleados hoy en día:

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[35] Fang H., Tong, W.D., Shi L.M., Blair R., Perkins R., Branham W., Hass B.S., Xie Q., Dial S.L., Moland C.L., Sheehan D.M. *Chemical Research in Toxicology*, 2001, 14:280–294.

[36] Korach K.S. MacLachlan J.A. *Environmental Health Perspectives,* 1995,103:5–8.

- ✓ Test de cornificación vaginal[37].
- ✓ Test de incremento del peso uterino[38].
- ✓ Test del enlace competitivo con el receptor estrogénico[39].
- ✓ Test de expresión génica e inducción de proteínas.
  - o Test de la vitelogenina[40].
  - o Test de lactoferrina [41].
  - o Ensayo CAT[36].
- ✓ Ensayos estrógeno dependiente. Ensayo de E-Screen[42].

1.3. Tipos de disruptores endocrinos químicos

Anteriormente se ha mencionado que los EDCs representan un grupo de sustancias que no están definidas principalmente por su estructura química, ya que muchos no presentan similitudes estructurales o propiedades químicas comunes, sino más bien se distinguen o reconocen a través de sus efectos biológicos.

Los EDCs, se pueden clasificar como naturales o antropogénicos de acuerdo a su origen. Entre los segundos se incluyen fármacos y productos de cuidado personal, así como compuestos químicos industriales sintetizados con diversos fines, pero que accidentalmente tienen propiedades como disruptores endocrinos[43]. La ***Figura 2*** muestra un esquema general de la clasificación de estos compuestos químicos y algunos ejemplos.

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37 Allen E., Doisy E.A. *JAMA, 1923,* 81:819–821.
38 Astwood E.B. *Endocrinology,* 1938, 23, 25–31.
39 Jensen E.V. *Recent Programme on Hormones,* 1962, 18, 387–414.
40 Heppell S.A., Denslow N.D., Folmar L.C., Sullivan C.V. *Environmental Health Perspectives*, 1995, 103, 9–15.
41 Teng C.T. *Environmental Health Perspectives,* 1995, 103, 17–20.
42 Soto A.M., Sonnenschein C. *Journal of Steriod Biochemistry,* 1985, 23:87–94.
43 Anca Caliman F., Gavrilescu M.P. *Clean*, 2009, 37:277–303.

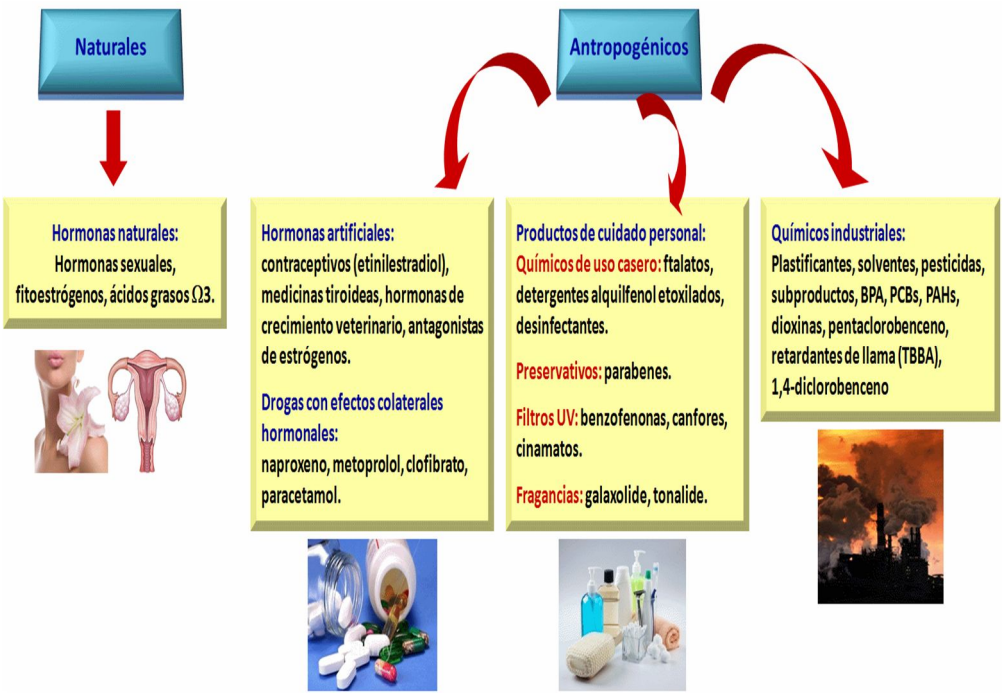

Figura 2. *Clasificación de EDCs de acuerdo a su origen*

1.4. Mecanismo de acción de los disruptores endocrinos químicos

Las hormonas son un conjunto de moléculas que, junto al sistema nervioso, coordinan diversas funciones en el organismo[44-46]. Estos compuestos se producen en células u órganos endocrinos y ejercen su acción en zonas del organismo, tras ser transportados por el torrente sanguíneo. Del conjunto de sistemas hormonales que controlan el funcionamiento del organismo se deben destacar tres por su vital importancia en el desarrollo sexual y reproductivo de los individuos:

- ✓ Hormonas **tiroideas**. Están implicadas en el crecimiento y en la diferenciación sexual (el hipotiroidismo genera, entre otros efectos, una importante merma del desarrollo físico y mental).

[44] Jacobson J.L., Jacobson S.W., Humphrey H.E. *Journal of Pediatrics*, 1990, 116:38–45.
[45] Sharpe N.E. *Lancet*, 1993, 341:1392–1395.
[46] Adami H., Bergström R., Möhner M., Teppo L. *International Journal of Cancer*, 1994, 59:33–38.

- ✓ Hormonas **retinoideas.** Básicas en el proceso de diferenciación sexual embrionaria y cuyo mal funcionamiento acarrea deficiencias en el desarrollo sexual.

- ✓ Hormonas **esteroideas.** Regulan el fenómeno reproductivo y el desarrollo de características sexuales secundarias.

Los EDCs tienen la capacidad de mimetizar a estas moléculas y realizar las funciones características de cada una de ellas, siendo su efecto mucho más potente cuando hay más de un compuesto presente de forma simultánea, aunque su acción individual pueda ser insignificante (efecto sinérgico).

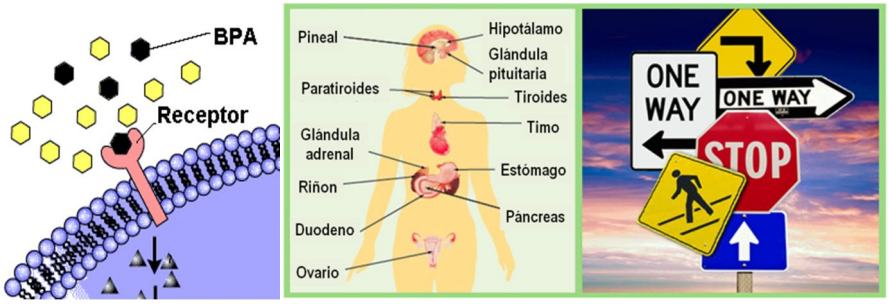

Figura 3. *Los EDCs imitan a las hormonas naturales interactuando con los receptores hormonales en el sistema endocrino, alterando la expresión genética. Los EDCs envían un mensaje equivocado en el lugar equivocado en el momento equivocado*

Un buen número de EDCs se caracterizan por enfocar su mecanismo de acción sobre las hormonas estrogénicas. Los EDCs estrogénicos pueden ser divididos en dos grupos generales: unos que imitan las hormonas esteroideas endógenas y otros que actúan como antagonistas. La estrogenicidad mediada por receptores clásicos o estrógenos endógenos ocurre cuando se unen directamente al receptor estrogénico α o β, activándolo[47]. Un antagonista clásico, por ejemplo, fulvestrant, inactiva por bloqueo el receptor al que se une el esteroide[48]. Sin embargo, existen otras maneras por las cuales los EDCs alteran los mecanismos de acción de las hormonas.

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[47] Welshons W.V., Thayer K.A., Judy B.M., Taylor J.A., Curran E.M., Vom Saal F.S. *Environmental Health Perspectives*, 2003, 111:994–1006.

[48] Howell A. *Endocrine-Related Cancer*, 2006, 13:689–706.

- ✓ **Alteración de la biosíntesis de hormonas esteroideas endógenas**. Un ejemplo clásico es el mitotano (o,p'-DDD), que afecta la biosíntesis de las hormonas esteroideas incluso en una etapa temprana. Reduce la producción de corticoesteroides por inhibición del esteroide mitocondrial 11β-hidrolasa (CYP11B1) y la actividad en el lado de ruptura del colesterol (CYP11A1)[49].

- ✓ **Alteración de la biotransformación de hormonas**. Algunos clorofenoles inhiben a las enzimas sulfotransferasas, responsables de inactivar los estrógenos, mediante la formación de sulfato de estrógeno en la sangre[48], causando un desbalance en la sulfatación y desulfatación y un incremento de los niveles intercelulares de estrógenos.

- ✓ **Interferencia con el anabolismo de las hormonas**. Por inhibición de la aromatasa (CYP19), responsable de convertir los andrógenos en estrógenos, el balance fisiológico de andrógenos y estrógenos sufre un desorden[50,51]. Un ejemplo son los fungicidas azoles (porchloraz)[50].

- ✓ **Alteración de los receptores esteroideos de membrana, localizados sobre la superficie celular**. Un ejemplo es el receptor de membrana 20β-S, responsable de la inducción de la maduración de los oocitos. La presencia de xenoestrógenos puede inhibir esta inducción[52].

- ✓ **Alteración del receptor arilhidrocarburo (AhR) o su dupla (SHP).** Ambos ejercen influencia sobre el receptor de los estrógenos[53,54].

La pregunta que realmente se plantea es cuáles son las consecuencias que causan estos compuestos en la salud en los seres humanos. En este sentido en los últimos años, se han realizado numerosos estudios que han demostrado

[49] Sanderson J.T. *Toxicological Sciences*, 2006, 94:3–21.
[50] Zarn J.A., Bruschweiler B.J., Schlatter J.R. *Environmental Health Perspectives*, 2003, 111:255–261.
[51] Sanderson J.T. *Toxicological Sciences*, 2006, 94:3–21.
[52] Thomas P. *Fish Physiology and Biochemistry*, 2003, 28:3–12.
[53] Seol W.G., Hanstein B., Brown M., Moore D.D. *Molecular Endocrinology*, 1998, 12:1551–1557.
[54] Navas J.M., Segner H. *Aquatic Toxicology*, 2000, 51:79–92.

que la continua exposición a este tipo de compuestos, podría ser la causa de numerosos efectos perjudiciales observados como son:

- ✓ Anomalías neurocerebrales y de conducta.
- ✓ Aumento en la incidencia de cánceres testicular, de próstata, de útero o de mama.
- ✓ Aumento en el número de casos de hipospadia y criptorquidia.
- ✓ Merma en la calidad seminal de los varones.
- ✓ Feminización de la ratio sexual de la población.

La exposición a los compuestos químicos con actividad hormonal no tiene por qué tener la misma repercusión sobre todos y cada uno de los individuos expuestos. Destacan, como un momento crítico, las etapas embrionaria, fetal y la primera infancia. Se cree que la exposición uterina, tiene consecuencias de tal magnitud que difícilmente se sospecharían en estudios realizados sobre individuos adultos. Esta asociación confiere a la exposición materna unas peculiaridades muy particulares y coloca a la mujer en edad fértil en el centro de atención de la mayor parte de los estudios en disrupción endocrina[55-57].

A continuación, se resumen algunas evidencias acerca de los trastornos mencionados.

- ✓ **Anomalías neurocerebrales y de conducta.** Existen varios estudios epidemiológicos que correlacionan la exposición humana a PCBs con déficit en el desarrollo de las funciones cerebrales. Así, en Taiwán y en Japón se han observado desórdenes neurológicos en los niños nacidos de madres que habían ingerido altos niveles de PCBs a través de aceite de

[55] Olsson P., Borg, B., Brunström B. *S.E.P. Agency*, Editor. 1998.
[56] Safe S. *TRENDS in Endocrinology and Metabolism*, 2005, 16:139–144.
[57] Amaral-Mendes J.J. *Food and Chemical Toxicology*, 2002, 40:781–788.

arroz. En la zona del lago Michigan se registraron casos similares. El nivel de PCBs ingerido por las mujeres a través del pescado dio como fruto una generación de niños con serios problemas de memoria a corto plazo. En general, los PCBs parecen provocar mermas en las capacidades cognitivas, lo que se asocia a una mala formación cerebral en las etapas de gestación del individuo. Otros EDCs como los ftalatos, los alquilfenoles y el bisfenol A y derivados muestran indicios de provocar anomalías de conducta en la progenie. Así, y aunque aún no hay evidencias suficientes para realizar afirmaciones categóricas, algunos grupos de investigación apuntan que existe correlación entre la exposición materna a estos xenobióticos y déficit de comportamiento en los neonatos, o incluso con desórdenes de hiperactividad en niños de edad escolar.

- ✓ **Cáncer testicular, de próstata, de útero y de mama.** La incidencia de estos tipos de cánceres se ha disparado en los últimos 40 años. La gran dependencia androgénica y estrogénica de las células tumorales de este tipo de cánceres hacen pensar que los EDCs están favoreciendo su aparición. El cáncer de mama por ejemplo, se ha convertido en países como Suecia, Noruega o Finlandia en el más común entre la población femenina. El incremento tan notorio de su incidencia no puede explicarse por los factores de riesgo ya conocidos, a saber: menopausia muy tardía, menarquía a edad muy temprana, embarazos a edades avanzadas y factores dietéticos. Por tanto, se ha sugerido que los EDCs a los que nos vemos expuestos están acelerando la aparición de este tipo de cáncer. De hecho existen estudios analíticos que ponen de manifiesto la presencia significativa de organoclorados, parabenos y algunas otros disruptores en tejido tumoral y en sangre de estos pacientes. Los casos de cáncer endometrial también han aumentado notablemente en las últimas décadas, y se ha comprobado que algunos fármacos como el tamoxifeno, usado en el tratamiento del cáncer de mama, favorecen su proliferación, por lo que se buscan nuevas vías de tratamiento.

- ✓ **Hipospadia y criptorquidia.** La hipospadia es una malformación en el tracto reproductor masculino y la criptorquidia es el no descenso testicular. La incidencia de estas anomalías ha crecido significativamente en los países altamente industrializados (Suecia, Noruega, Inglaterra, Gales…), y aunque a día de hoy no puede establecerse una correlación entre incidencia y exposición a EDCs, se ha comprobado que los tratamientos hormonales que se aplican a algunas mujeres embarazadas (fundamentalmente con dietilestilbestrol) parecen aumentar el número de hijos varones con hipospadia o criptorquidia. Así este fenómeno sugiere que la exposición podría estar relacionada con el incremento de casos observados de dicha malformación.

- ✓ **Calidad del esperma.** La infertilidad masculina es uno de los problemas reproductivos más comunes en la actualidad. Aunque existe cierta controversia, parece demostrado que los EDCs juegan un papel más que plausible en este fenómeno. De hecho, existen estudios analíticos donde se han encontrado concentraciones significativas de disruptores como el lindano o los PCBs en individuos con problemas de infertilidad.

- ✓ **Feminización de la ratio sexual de la población.** Aunque a día de hoy no puede correlacionarse este fenómeno con la exposición a los EDCs, es cierto que el número de mujeres nacidas está creciendo respecto al de varones, fenómeno que se manifiesta más claramente en países altamente industrializados. Así se sospecha que los disruptores pueden jugar un papel importante en esta feminización de la población.

En la ***Tabla 1*** se muestran, a modo de resumen, los principales efectos sospechados que provoca la exposición a los EDCs en hombres y mujeres y en su descendencia.

Tabla 1. *Posibles efectos de los EDCs en humanos.*

MUJERES	HIJAS	HIJOS	HOMBRES
Cáncer de mama	Pubertad precoz	Criptorquidia o no descenso testicular	Cáncer de testículos
Endometriosis	Cáncer vaginal	Hipospadias	Cáncer de próstata
Muerte embrionaria y fetal	Mayor incidencia de cánceres	Reducción del recuento espermático	Reducción del recuento espermático
Malformaciones en la descendencia	Deformaciones en órganos reproductores	Disminución del nivel de testosterona	Reducción de la calidad del esperma
	Problemas en el desarrollo del sistema nervioso central	Problemas en el desarrollo del sistema nervioso central	Disminución del nivel de testosterona
	Bajo peso de nacimiento	Bajo peso de nacimiento	Modificaciones de concentraciones de hormonas tiroideas
	Hiperactividad	Hiperactividad	
	Problemas de aprendizaje	Problemas de aprendizaje	
	Disminución del coeficiente de inteligencia y de la comprensión lectora	Disminución del coeficiente de inteligencia y de la comprensión lectora	

1.5. Disruptores endocrinos químicos en estudio. Evidencias experimentales de su actividad disruptora y daños producidos en la biota no humana.

1.5.1. Bisfenol A y sus derivados clorados

Los bisfenoles son compuestos aromáticos constituidos por dos anillos fenólicos que se unen a través de un grupo puente. El más común y el de uso más extendido es el bisfenol A (BPA), en el que el grupo puente es un isopropilideno. Además, en presencia de cloro y a valores de pH determinados, en torno a 8.1, este compuesto tiende a incorporar átomos de cloro en su molécula dando lugar a una serie de derivados con 1 a 4 átomos. La ***Figura 4*** muestra la estructura química de esta familia de compuestos.

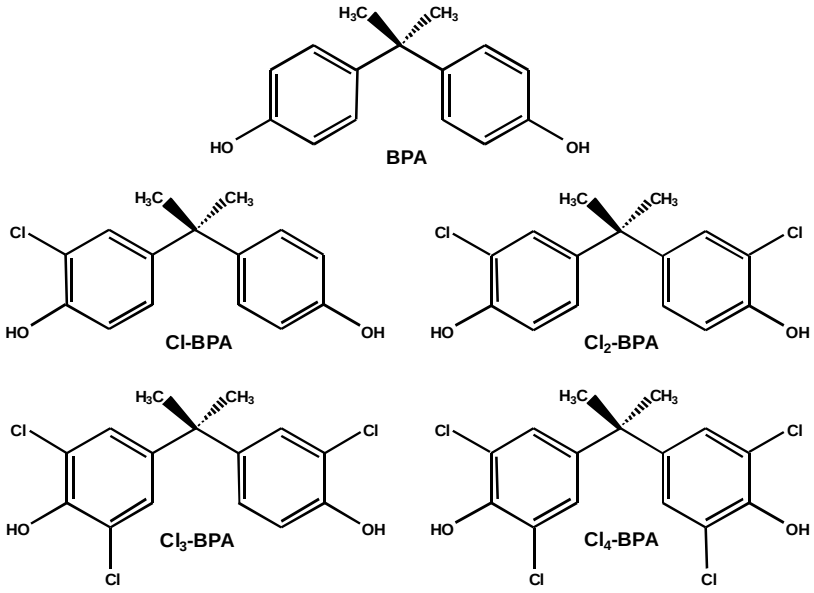

Figura 4. *Estructura química del BPA y sus derivados clorados*

Desde un punto de vista físico-químico, es un sólido blanco cristalino con un punto de fusión de 150–155 ºC[58]. Es poco soluble en agua (120–300 mg L^{-1} en función del pH del medio) y presenta propiedades como ácido debido a los grupos fenólicos que forman parte de su estructura, con valores de pK_a de 9.6 y

[58] Staples C.A, Dom P.B., Klecka G.M., O'Block S.T., Harris L.R. *Chemosphere*, 1998, 36:2149–2173.

10.2. Cuando la molécula incorpora átomos de cloro, estos le confieren una mayor acidez, disminuyendo los valores de pK_a. Su coeficiente de partición octanol-agua (log K_{ow}) se encuentra entre 2.2–3.8, valor que varía con el pH debido a sus propiedades ácido-base. Este parámetro confirma sus características hidrofóbicas, así como su elevada tendencia a adsorberse a matrices sólidas. Su baja presión de vapor ($8.7 \cdot 10^{-10}$ a $3.96 \cdot 10^{-7}$ mmHg), así como su baja constante de Henry ($1.0 \cdot 10^{-10}$ atm m^3 mol^{-1}) establecen que es un compuesto no volátil. El BPA presenta fluorescencia nativa en disolventes orgánicos, no así sus derivados clorados, con $\lambda_{excitación}$ entre 274 y 282 nm y $\lambda_{emisión}$ entre 297 y 309 nm para distintos disolventes orgánicos. Esta propiedad es empleada para su determinación analítica, como método de detección[59].

Actualmente, el BPA es uno de los compuestos de síntesis de mayor producción a nivel mundial, con una producción anual de más de 4 millones de toneladas en la última década, cifra que continúa creciendo en la actualidad. Sus aplicaciones son múltiples y su uso está muy extendido siendo el monómero base para la fabricación de las resinas epoxi, polímeros de excelentes propiedades químicas, térmicas y mecánicas; y para la obtención de los plásticos policarbonato, que poseen gran estabilidad mecánica y térmica, así como muy buena transparencia. Estos plásticos están presentes en infinidad de productos y de actividades como la industria del automóvil y aeronáutica, en instalaciones de alumbrado, en construcción, en sistemas eléctricos, tuberías de agua potable, telecomunicaciones, equipos fotográficos, lentes ópticas y discos compactos, envasado de alimentos y aguas, en productos manufacturados como los biberones e incluso en materiales relacionados con la salud humana como prótesis y lentes intraoculares; también puede destacarse el uso de BPA en fungicidas, retardantes de llama y colorantes, entre otras muchas aplicaciones[60]. La ***Figura 5*** esquematiza alguna de las más destacadas.

[59] Del Olmo M., Zafra A., Jurado A.B., Vílchez J.L. *Talanta*, 2000, 50:1141–1148.
[60] Yang M., Seon Park M., Sun Lee H. *Journal of Environmental Science and Health, Part C*, 2006, 24:183–224.

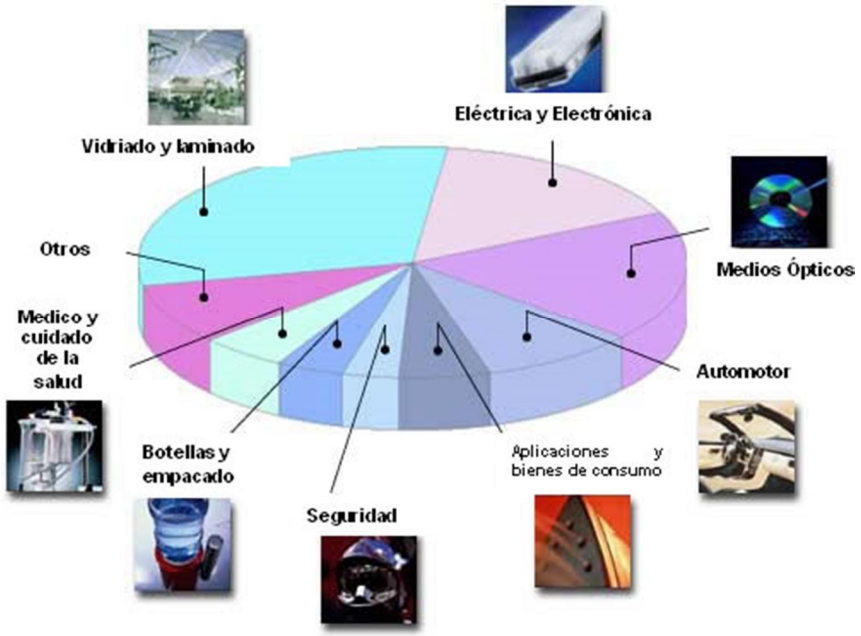

Figura 5.Campos de aplicación del BPA

La **Figura 6** muestra la distribución del BPA de acuerdo a sus usos en Europa.

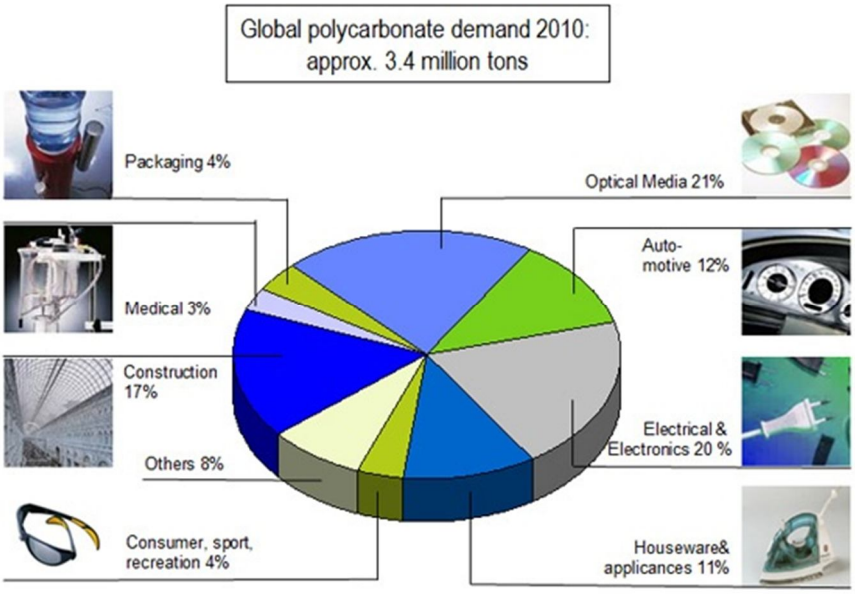

Figura 6. *Distribución de los usos del bisfenol A en Europa por sectores*

Actualmente, debido a su comportamiento bioquímico, el BPA es el disruptor endocrino que más preocupación está causando a la comunidad científico-médica ya que los estudios realizados demuestran que los efectos que produce son más perniciosos que los de otros compuestos químicos. No obstante, existe cierta controversia en torno a este compuesto ya que, aunque la industria de los plásticos establece que las concentraciones de BPA a las que está expuesta una persona no son tóxicas; existe la llamada hipótesis de la "dosis baja", aplicable a este compuesto, según la cual, dosis pequeñas pueden ser más dañinas que a dosis altas[61]. Numerosos estudios publicados en los últimos 20-30 años han demostrado que el BPA puede causar efectos adversos por debajo de la dosis "de seguridad" de 50 μg kg^{-1}/d, por lo que estos niveles deberán ser reevaluados por la US-EPA y FDA.

A continuación se resumen las diferentes actividades biológicas ya demostradas del BPA.

- ✓ **Capacidad para unirse a los receptores hormonales estrogénicos y competir con los estrógenos naturales.** La forma en la cual el organismo regula la expresión hormonal de tipo estrogénico es a grandes rasgos como sigue: el organismo segrega estrona, está viaja a través del torrente sanguíneo (en forma sulfatada), se transforma en estradiol y éste se une a los receptores específicos celulares desencadenando la respuesta conveniente. Así, si una sustancia exógena es capaz de "mimetizar" el comportamiento del estradiol y competir con él por la unión con los receptores alterará el metabolismo estrogénico.

  Se ha descrito en estudios *in vitro*, la capacidad del BPA para competir eficazmente con el estradiol por unirse a los receptores estrogénicos en humanos y animales[62-64], además demuestran que su afinidad por estos

[61] Vandenberg L., Maffini M.V., Sonnenschein C., Rubin B.S., Soto A.M. *Endocrine Reviews*, 2009, 30:75–95.
[62] Gould J.C., Leonard L.S., Maness S.C., Wagner B.L., Conner K., Zacharewski T., Safe S., McDonell D.P., Gaido K.W. *Mollecular and Cellular Endocrinology*, 1998, 142:203–214.
[63] Aloisi A.M., Della Seta D., Ceccarelli I., Farabollini F. *Neuroscience Letters*, 2001, 310:49–52.

receptores es superior a la de la mayoría de los xenobióticos conocidos. Un importante estudio ha demostrado que el BPA presenta una gran afinidad por los receptores estrogénicos de tipo γ[65], íntimamente relacionados con el desarrollo cerebral de los mamíferos. Esta afinidad es tal que se están desarrollando dispositivos que permiten la eliminación de éste y otros EDCs de efluentes acuosos destinados al consumo humano, mediante la interacción xenobiótico-receptor[66].

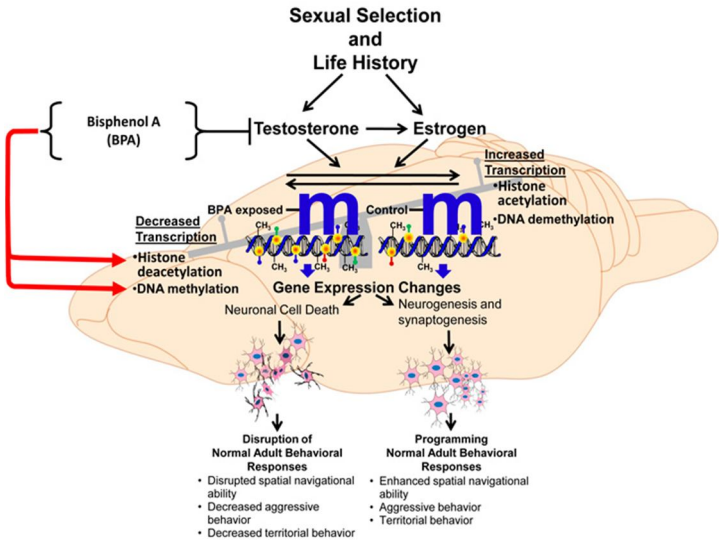

Figura 7. *Afinidad del BPA por los receptores estrogénicos*

- ✓ **Interacción con sistemas enzimáticos reguladores del balance hormonal**. En 2006 se demostró que el BPA puede sulfatarse y desulfatarse de forma muy similar a como lo hace el estradiol[67], de modo que la acción de las sulfatasas permite transportar no sólo a los estrógenos naturales, sino también al compuesto. La consecuencia de este fenómeno es que el organismo no es capaz de ofrecer una resistencia tan eficaz como debiera

[64] Kitamura S., Suzuki T., Sanoh S., Kohta R., Jinno N., Sugihara K., Yoshihara S., Fujimoto N., Watanabe H., Ohta S. *Toxicological Sciences*, 2005, 84:249–259.

[65] Takayanagi S., Tokunaga T., Liu X., Okada H., Matsushima A., Shimohigashi Y.*Toxicological Letters*, 2006, 167:95–105.

[66] Liu Z., Ito M., Kanjo Y., Yamamoto A. *Journal of Environmental Sciences*, 2009, 21:900–906.

[67] Stowell C.L., Barvan K.K., Young P.C.M., Bigseby R.M., Verdugo D.E., Bertozzi C.R., Wdlanski T. *Chemistry and Biology*, 2006, 13:891–897.

a la acción de este contaminante. Mientras que otros disruptores al sulfatarse se transportan hasta llegar a la orina y se excretan, el BPA no se elimina de forma eficaz permaneciendo en el organismo. Es decir, consigue obviar parcialmente la defensa metabólica del organismo ante la mayoría de los xenobióticos.

- ✓ **Efectos sobre la proliferación de células tumorales.** Este efecto se ha demostrado mediante la realización de los test citados con anterioridad en esta Memoria. Los test emplean células de tejidos estrógeno-sensibles, en muchas ocasiones de origen humano, establecidas en cultivo a largo plazo y que pueden ser fácilmente tratadas con 17ß-estradiol o cualquier compuesto químico sospechoso de ser un estrógeno. Estos ensayos finalizan con la cuantificación de la proliferación celular tras varios días de subcultivo, o midiendo la expresión de algún gen que codifica una proteína específica, en ese modelo, de la acción hormonal. Se establecen, además, los controles mediante el mantenimiento de grupos celulares en medio libre de estrógenos.

El test más utilizado actualmente es el ensayo ***E-SCREEN***[68]. En 1992 Soto y col. patentaron este test de estrogenicidad. Se basa en el empleo de las células MCF-7 (línea celular del cáncer de mama) en cultivo en monocapa. El test pone de manifiesto la actividad estrogénica de un compuesto químico basándose exclusivamente en la observación de su capacidad proliferativa. Estas células responden con un incremento significativo del índice proliferativo a la acción de los estrógenos naturales en concentraciones picomolares[69]. Se ha señalado que es primordial la elección del clon de células MCF-7 para que la respuesta en el E-SCREEN sea óptima. El desarrollo metodológico del test ha venido a facilitar el *screening* rápido y fiable de gran número de compuestos en los que se quiere demostrar una actividad hormonal de carácter estrogénico. Se ha

[68] Soto A.M., Lin T.M., Justicia H., Silvia R.M., Sonnenschein C. Patente USA 4, 859, 585, 1992.
[69] Soto A.M., Sonnenschein C. *Journal of Steroid Biochemistry,*1985, 23:87–94.

recomendado el uso de las células MCF-7 sobre otras células mamarias debido al conocimiento previo de su dependencia hormonal.

Son numerosos los estudios donde se ha demostrado que tanto el BPA como sus derivados mono y disulfatados provocan la proliferación de células MCF-7, y que este fenómeno está correlacionado con la concentración de disruptor a la que se somete el cultivo celular.

- ✓ **Actividad antiandrogénica.** El BPA y sus derivados son capaces de inhibir la respuesta bioluminiscente de células N1H3T3, especialmente diseñadas para expresar la enzima luciferasa tras la unión de la dihidrotestosterona con sus receptores específicos[64].

Toda esta actividad biológica demostrada ha hecho que se haya relacionado al BPA con numerosas anomalías y problemas en la salud de los individuos expuestos. Además de los daños ya descritos sobre la capacidad y el desarrollo reproductivos de las especies[70,71], en los últimos años se ha demostrado que el BPA está relacionado con numerosas anomalías neuroendocrinas[72]; alteraciones fisiológicas y estructurales del cerebro[73]; alteraciones en la conducta como agresividad, estrés; conductas sexuales anómalas; mayor vulnerabilidad del individuo ante la ingesta de drogas como la morfina; peor capacidad cognitiva[72]; y parece estar relacionado con la inhibición de los fenotipos masculinos y de la espermatogénesis, es decir, feminización de la población de ensayo[74].

Además, como ya se ha citado al inicio de este apartado, el BPA tiene la capacidad de reaccionar con el cloro libre, usado como agente blanqueante en las fábricas de papel y en la desinfección del agua potable, produciendo

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[70] Patisaul H.B., Bateman H.L. *Hormones and Behavior*, 2008, 53:580–588.
[71] Ramakrishnan S., Wayne N.L. *Reproductive Toxicology*, 2008, 25:177–183.
[72] Richter C.A., Birnbaum L.S., Farabollini F., Newbold R.R., Rubin B.S., Talsness C.E., Vandenbergh J.G., Walser-Kuntz D.R., Vom Saal F.S. *Reproductive Toxicology*, 2007, 24:199–224.
[73] Tando S., Itoh K., Yaoi T., Ikeda J., Fujiwara Y., Fushiki S. *Brain Development*, 2007, 29:352–356.
[74] Furuya M., Adachi K., Kuwahara S., Ogawa K., Tsukamoto Y. *Life Sciences*, 2006, 78:1767–1776.

determinados derivados clorados[75], algunos de los cuales tienen aplicaciones industriales, como el tetraclorobisfenol A (Cl_4-BPA), usado como retardante de llama. Los cloroderivados, especialmente el tricloro y tetraclorobisfenol A están comenzando a causar cierta preocupación, porque se ha demostrado en estudios *in vitro* e *in vivo*, que pueden ser incluso más activos que el BPA en la competencia con el 17β-estradiol para unirse a los receptores estrogénicos humanos (ER-α y ER-β)[76], incluso a concentraciones muy bajas[75,77]. Además, a través de estudios con células humanas la línea MCF-7, se ha demostrado que los cloroderivados del BPA tiene una potencia estimuladora mayor sobre su proliferación que el BPA[78].

El BPA y sus derivados clorados son capaces de producir un incremento notable en la masa del tejido uterino y el endometrio, fases previas en el desarrollo de cánceres de útero y endometrio. Además, se ha demostrado que la exposición crónica a los derivados clorados a dosis muy pequeñas es mucho más potente y peligrosa que la del propio BPA[78]. También se ha publicado que los cloroderivados poseen un mayor potencial de inhibición de la 3,3',5-triyodotironina (T3), la cual se une a la proteína tiroidea plasmática, transtirretina (TTR)[79]. Estos resultados indican que los cloroderivados también son competidores más potentes del TTR.

Además, también se ha comprobado que la citotoxicidad aguda de los cloroderivados se incrementa por radiación UVB y UVC[80]. Esto significa que cuando los cloroderivados ingresan al medio ambiente, estarán expuestos a la luz solar que contiene radiación UV, incrementando su citotoxicidad por generación de subproductos de fotodegradación.

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[75] Fukazawa H., Watanabe W., Shiraishi F., Shiraishi H., Shiozawa T., Matsushita H., Terao Y. *Journal of Health Science*, 2002, 48:242–249.

[76] Hu J., Aizawa T., Ookubo S. *Environmental Science and Technology*, 2002, 36:1980–1987.

[77] Kuruto-Niwa R., Terao Y., Nosawara R. *Environmental Toxicology and Pharmacogy*, 2002, 12:27–35.

[78] Takemura H., Ma J., Sayama K., Terao Y., Zhu B.T., Shimoi K. *Toxicology*, 2005, 207:215–221.

[79] Murata T., Yamauchi K. *Toxicology and Applied Pharmacology*, 2003, 187:110–117.

[80] Mutou Y., Ibuki Y., Terao Y., Kojima S., Goto R. *Biological and Pharmaceutical Bulletin*, 2006, 29:2116–2119.

Por otro lado, se conoce que el BPA es metabolizado y transformado en glucorónidos a nivel hepático[81], y sus metabolitos son rápidamente eliminados con las heces y la orina; sin embargo, en el caso de los cloroderivados, son degradados lentamente, acumulándose en el cuerpo humano y otros seres vivos.

El problema de salud general surge cuando se tiene en cuenta la gran exposición a la que los seres vivos están expuestos a este compuesto en la actualidad. Además del factor ambiental, debido a su presencia ubicua, existe un foco de exposición de extrema importancia a través de la alimentación. El BPA, está presente en los plásticos de los recipientes para bebidas y comidas yen los revestimientos de la comida enlatada consumida en ingentes cantidades alrededor del mundo[82]. La ***Tabla 2*** presenta algunas estimaciones de la exposición a BPA a través de la comida, basadas en estudios realizados a lo largo de los últimos años[83-87].

[81] Pottenger L.H., Domoradzki J.Y., Markham D.A., Hansen S.C., Cagen S.Z., Waechter JM. *Toxicological Sciences*, 2000, 54:3–18.
[82] Von Goetz N., Wormuth M., Scheringer M., Hungerbuhler K. *Risk Analysis*, 2010, 30:473–487.
[83] Geens T., Apelbaum T.Z., Goeyens L., Neels H., Covaci A. *Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure and Risk Assessment*, 2010, 27:1627–1637.
[84] Higuchi M., Miyata D., Kawamura S., Ueda E., Imanaka M., Tonogai Y. *Journal of the Food Hygienic Society of Japan*, 2004, 45:339–343.
[85] Lim D.S., Kwack S.J., Kim K.B., Kim H.S., Lee B.M. *Journal of Toxicology and Environmental Health-Part A-Current Issues*, 2009, 72:1327–1335.
[86] Mariscal-Arcas M., Rivas A., Granada A., Monteagudo C., Murcia M.A., Olea-Serrano F. *Food and Chemical Toxicology*, 2009, 47:506–510.
[87] Thomson B.M., Grounds P.R. *Food Additives and Contaminants*, 2005, 22:65–72.

Tabla 2. *Medición de la ingesta de BPA basado en el análisis de alimentos enlatados combinado por estudios de consumo.*

País	Tipo de comida	Ingesta promedio ($ng/kg_{masa\ corporal}$/día)	Ingesta elevada ($ng/kg_{masa\ corporal}$/día)
Bélgica	Comida y bebidas enlatadas	15	86 (p.95)
Nueva Zelanda	Comida y bebidas enlatadas	8	290 (max)
Corea	Comida enlatada	25	---
España[a]	Comida enlatada y recipientes para microondas	18	78 (max)
Japón	Comidas preparadas de hospitales	8	---

[a] *Basado en estudios de migración*

Un factor de exposición muy importante en adultos es a través de las botellas de policarbonato[88], mientras que en el caso de los niños, la exposición de BPA por los biberones de policarbonato, actualmente ya prohibidos en numerosos países, se considera la más importante[82].

Las condiciones más importantes que favorecen la migración de BPA hacia los alimentos son la temperatura, el pH, el tiempo de contacto, la cantidad de revestimiento y las condiciones de fabricación y procesamiento[89,90]. Por otro lado, no hay consenso acerca de la influencia del envejecimiento del recipiente y la liberación de BPA, sin embargo, se ha demostrado que los residuos de detergente en la superficie del recipiente después del lavado incrementa la liberación del compuesto[89]. En ningún caso se han publicado valores que excedan el límite de migración legal establecido, incluso en condiciones

[88] Carwile J.L., Luu H.T., Bassett L.S., Driscoll D.A., Yuan C., Chang J.Y., Ye X.Y., Calafat A.M., Michels K.B. *Environmental Health Perspectives*, 2009, 117:1368–1372.

[89] Aschberger K., Castello P., Hoekstra E., Karakitsios S., Munn S., Pakalin S., Sarigiannis D. European Commission–Joint Research Centre–Institute for Health and Consumer Protection, 2010.

[90] Goodson A., Robin H., Summerfield W., Cooper I. *Food Additives and Contaminants*, 2002, 21:1015–1026.

extremas[91,92]. Se ha publicado que la concentración de BPA en orina aumenta en un 66% después del consumo de bebidas a través de botellas de policarbonato durante una semana[93].

1.5.2. Parabenos

Los parabenos (PBs) Son un grupo de compuestos químicos de síntesis derivados del ácido *p*-hidroxibenzoico obtenidos mediante esterificación en la posición C-4. La ***Figura 8*** muestra su estructura general.

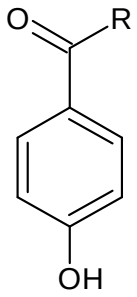


Figura 8. *Estructura química de los parabenos*

Se trata de sólidos inodoros, de color blanco, relativamente solubles en agua e hidrolíticamente estables, que presentan mayor carácter hidrofóbico al aumentar el tamaño de su cadena hidrocarbonada[94]. Son termoestables y resistentes a la hidrólisis en medios ácidos, siendo su hidrólisis apreciable a un pH superior a 7. Los puntos de fusión están comprendidos entre 68–70ºC para el butilparabeno y 131 ºC para el metilparabeno. La solubilidad en agua medida a 25 ºC oscila entre los 500 mg L^{-1} del butilparabeno y los 5.5 g L^{-1} del metilparabeno. Debido a que tienen un grupo fenólico en su estructura, presentan propiedades ácidas, con valores de pK_a comprendidos entre 8.2 y

[91] Kubwabo C., Kosarac I., Stewart B., Gauthier B.R., Lalonde K., Lalonde P.J. *Food Additives and Contaminants Part A. Chemistry Analysis Control Exposure and Risk Assessment*, 2009, 26:928–937.

[92] Le H.H., Carlson E.M., Chua J.P., Belcher S.M. *Toxicology Letters*, 2008, 176:149–156.

[93] Geens T., Goeyens L., Covaci A. *International Journal of Hygiene and Environmental Health*, 2011, 214:339–347.

[94] Núñez L., Tadeo J.L., García-Valcárcel A.I., Turiel E. *Journal of Chromatography A, 2008,* 1214:178–182.

8.3. Los PBs presentan coeficientes de partición octanol-agua (log K_{ow}) entre 1.96 y 3.47. La presión de vapor de estos compuestos es de $0.4 \cdot 10^{-3}$ mmHg para el butil y $5.5 \cdot 10^{-3}$ mmHg para el metilparabeno. Los PBs pueden determinarse mediante una detección UV a λ 257 nm o mediante fluorescencia con $\lambda_{excitación}$ de 257 nm y $\lambda_{emisión}$ de 320nm.

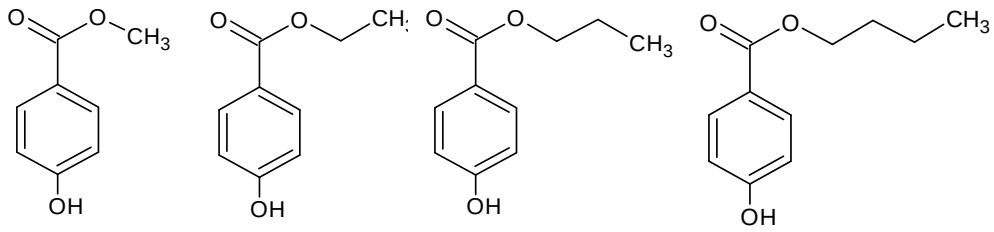
Dado su alto poder antimicrobiano, la principal utilidad de los PBs es como conservante. Son capaces de inhibir procesos mitocondriales y de membrana básicos para el desarrollo de los microorganismos. La capacidad antimicrobiana de los PBs es mayor cuanto mayor es su cadena alquílica, pero generalmente los procesos implicados en la proliferación de microorganismos suelen ser en medio acuoso, por lo que tendrá una gran influencia la cantidad de parabeno disuelto en agua. Por este motivo, los más usados a nivel industrial son los de cadena más corta (1 a 4 átomos de carbono), mas solubles en agua.

Son activos frente a hongos, levaduras, y algunos microorganismos Gram (+) y Gram (-), provocando disrupción en los procesos de transporte de membrana, inhibición de la síntesis de ADN y ARN o uniéndose a sus enzimas. Debido a la gran actividad antimicrobiana que presentan, su baja toxicidad, carácter no irritante además de su baja tendencia a hidrolizarse son muy usados como estabilizantes y conservantes en cosméticos y jabones, e incluso como antimicrobianos en alimentos[95]. También se incorporan en fármacos, anestésicos, colirios, píldoras, jarabes e inyectables en concentraciones inferiores al 1 %. En la ***Figura 9*** se muestran alguna de estas aplicaciones.

Figura 9. *Aplicaciones y usos comunes de los parabenos*

[95] Nieto A., Borrull F., Pocurull E., Marce R.M.*Trends in Analytical Chemistry,*2010, 29:752–764.

Dado que son los compuestos más empleados a nivel industrial, en esta Tesis Doctoral, se han estudiado los *p*-hidroxibenzoatos de metilo (metilparabeno), de etilo (etilparabeno), de propilo (propilparabeno) y de butilo (butilparabeno). La ***Figura 10*** muestra sus estructuras.



Metilparabeno	**Etilparabeno**	**Propilparabeno**	**Butilparabeno**
MPB	**EPB**	**PPB**	**BPB**

Figura 10. *Estructura de los parabenos estudiados*

Hasta los años 90, dada la estabilidad y las excelentes propiedades físico-químicas de estos compuestos, se pensó que eran compuestos completamente inocuos y su uso estaba aceptado sin condiciones. Sin embargo, es a finales de esta década cuando surgen los primeros estudios, tanto *in vitro* como *in vivo,* que comienzan a mostrar indicios del carácter disruptor de los PBs[96]. Hoy en día numerosos estudios han obtenido importantes conclusiones que avalan la inclusión de los PBs dentro del grupo de EDCs:

- ✓ **Capacidad para unirse a los receptores hormonales estrogénicos. Competencia con los estrógenos naturales.** Byfford y col.[97]pusieron de manifiesto la habilidad de los PBs para unirse a los receptores Erα obtenidos a partir de células cancerosas tipo MCF-7. Estos autores observaron como la unión entre el estradiol y el receptor Erα disminuye a

[96] Soni M.G., Carabin I.G., Burdock G.A. *Food Chemisty and Toxicol*ogy, 2005, 43:985–1015.
[97] Byford, J.R., Shaw L.E., Drew M.G., Pope G.S., Sauer M.J., Darbre P.D. *Journal of Steroid Biochemistry and Molecular Biology*, 2002, 80:49–60.

partir de ratios molares comprendidos entre 10^5 y 10^6M. Concluyendo además que la unión entre el estradiol y el receptor Erα se inhibe más cuanto mayor es la cadena alquílica del parabeno en cuestión.

- ✓ **Efectos de los parabenos en la proliferación de células tumorales.** Byfford y col.[97] llegaron a la conclusión de que la actividad disruptora de los PBs va más allá de una simple competición con el estradiol por los sitios diana celulares, de tal manera que estos compuestos provocan la proliferación de células MCF-7. Así, a partir de una concentración de parabeno igual o superior a 10^{-6} M, y por supuesto antes de alcanzar el nivel de citotoxicidad de cada uno de ellos, la población celular del cultivo ensayado crece notablemente, de forma similar a como lo provoca el estradiol natural.

- ✓ **Anomalías en las funciones enzimáticas que regulan el transporte de los estrógenos naturales.** Otra de las formas por la cual los PBs ejercen un efecto disruptor es la inhibición de la expresión de ciertas enzimas esenciales para el correcto balance de los niveles de estradiol natural. Prusakiewicz y col.[98] demostraron que la aplicación cutánea de los compuestos provoca una disfunción de las enzimas sulfotransferasas de la piel, por lo cual el estradiol, una vez que se une a los receptores y desencadena la repuesta endocrina, no puede transportarse y salir de estos tejidos. Así se produce una acumulación de estrógenos en la piel que finalmente podría desencadenar en la aparición de cáncer. Este fenómeno se manifiesta tanto en ensayos de cinética química como en ensayos con células vivas.

- ✓ **Actividad antiandrogénica.** Se ha demostrado que los PBs no sólo pueden interaccionar con receptores típicamente estrogénicos, sino que además merman la producción de testosterona[99]. Así, el MPB, PPB y BPB son capaces de disminuir la producción bioquímica de testosterona hasta llegar

[98] Prusakiewicz J.J., Harville H.M., Zhang Y., Ackermann C., Voorman R.L. *Toxicology,* 2007, 232:248–256.

[99] Chen J., Ahn K.C., Gee N.A., Gee S.J., Hammock B.D., Lasley B.L. *Toxicology and Applied Pharmacology,* 2007, 221:278–284.

a porcentajes del 40, 33 y 19%, respectivamente, sobre la generación normal de esta hormona.

La actividad biológica demostrada de los PBs se traduce en numerosos efectos demostrados en la salud de los animales y su progenie. Actualmente son ya numerosos los estudios en los que se concluye la importante relación existente entre la exposición a estos compuestos de los organismos vivos y las diversas anomalías detectadas en éstos[96]. Estas investigaciones se refieren tanto a organismos acuáticos (peces y gasterópodos) como a mamíferos de pequeño tamaño (roedores). Así Alslev y col.[100] demostraron que la exposición oral y ambiental de la trucha arco iris (*Oncorhynchus mykiss*) al BPB afecta directamente a la producción vitelogénica de este organismo. Oishi[101] demostró que el PPB afecta directamente al sistema reproductivo de ratas macho. Así, tras estudios con grupos de animales distintos, se puso de manifiesto la correlación negativa entre la exposición a este compuesto, la reserva espermática y niveles de testosterona de los animales. Sin embargo, el metil y el etilparabeno no parecen provocar estos efectos[102]. También se ha demostrado que los PBs inducen respuestas estrogénicas en roedores hembra que previamente habían sido sometidos a una ooforectomía (extirpación de sus ovarios). Así, por ejemplo, el peso uterino aumenta notablemente cuando se ven expuestos a distintas dosis de PBs. En otros estudios se manifiesta que la exposición materna a estos xenobióticos provoca déficit de desarrollo en las crías nacidas posteriormente: menos peso corporal, mal desarrollo de las gónadas, etc., con las subsiguientes distrofias reproductivas[103].

[100] Alslev B., Korsgaard B., Bjerregaard P. *Aquatic Toxicology*, 2005, 72:295–304.
[101] Oishi S. *Food Chemistry and Toxicolgy,* 2002, 40:1807–1813.
[102] Oishi S. *Food Chemistry and Toxicology*, 2004, 42:1845–1849.
[103] Kang K.S., Che J.H., RYu D.Y., Kim T.W., Li G.X., Lee Y.S. *Journal of Veterinary and Medical Sciences*, 2002, 64:227–235.

1.5.3. Benzofenonas

Las benzofenonas (BPs) son una familia de compuestos habitualmente empleados en la industria como filtros de la radiación ultravioleta. Los más utilizados son los denominados desde benzofenona 1 (BP-1) a benzofenona 12 (BP-12), aunque existen otros menos habituales como la 2-hidroxibenzofenona (2-OH-BP), la 3-hidroxibenzofenona (3-OH-BP) y la 4-hidroxibenzofenona (4-OH-BP) con una menor aplicación industrial. Su estructura química general, es la que se muestra en la ***Figura 13***.

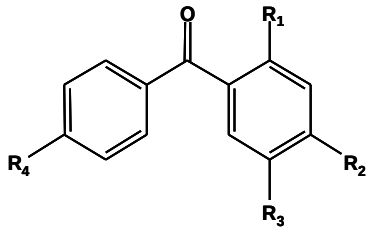


Figura 13. *Estructura química general de las benzofenonas*

Desde un punto de vista físico-químico, se trata de cetonas aromáticas con olor a rosa. Son sólidos blancos de aspecto cristalino con puntos de fusión comprendidos entre 62–65ºC para la BP-3 y 198–203 ºC para el BP-2. La solubilidad en agua medida a 25 ºC oscila entre los 70 mg L^{-1} para la BP-6 y los 1.2 g L^{-1}de la BP-2, son compuestos en general poco solubles en agua. Presentan propiedades ácidas, variando los valores de pK_a entre 6.8 para la BP-6 y 8.1 para 4-OH-BP. Las BPs presentan coeficientes de partición octanol-agua (log K_{ow}) entre 2.8–3.9. La presión de vapor de estos compuestos varía entre $3.0 \cdot 10^{-8}$ mmHg y $6.5 \cdot 10^{-6}$ mmHg. Las BPs emiten fluorescencia con $\lambda_{excitación}$ de 230 nm y $\lambda_{emisión}$ de 340nm. Por otro lado, su naturaleza química las hace especialmente eficaces en la absorción de luz ultravioleta (λ: 280 - 400 nm), ya que son capaces de absorberla (promoviendo sus electrones a un estado excitado) y disiparla en forma de calor. Esto es posible debido a que la benzofenona posee sus estados de singlete y triplete energéticamente muy próximos entre sí. De esta propiedad deriva su aplicación industrial más importante, ya que las BPs se usan ampliamente como filtros solares en cremas

protectoras y en la formulación de polímeros para evitar la degradación del color y la pérdida de características mecánicas.

Figura 14.*Uso habitual de las benzofenonas*

En la ***Figura 15***, se muestra la estructura de las BPs más importantes y utilizadas actualmente a nivel comercial, y estudiadas a lo largo de la presente Tesis Doctoral.

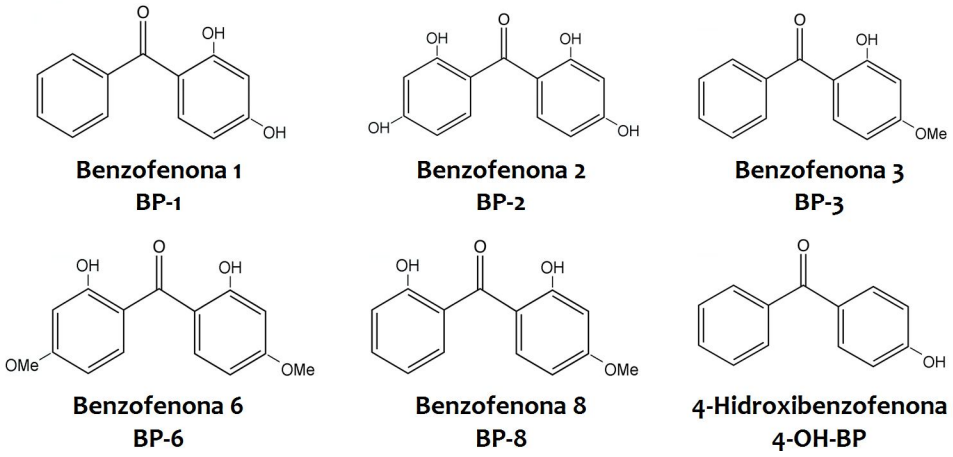


Figura 15. *Estructura de las BPs estudiadas*

Estos compuestos, que rara vez producen reacciones alérgicas o irritantes y aparentemente son carentes de toxicidad, son capaces de bioacumularse en los organismos vivos. Desde mediados de los años 90 diferentes estudios han demostrado que estos compuestos, que entran en los organismos

principalmente por vía cutánea[104-107], poseen un importante carácter como disruptores endocrinos. Así, las BPs han demostrado:

- ✓ **Capacidad para unirse a los receptores hormonales estrogénicos.** Molina-Molina y col.[108]realizaron en 2008 un estudio pormenorizado en el cual se usaron distintos tipos de células, provenientes de peces y de tejido humano, que habían sido modificadas genéticamente para que apareciera una respuesta bioluminiscente ante la unión estrógeno-receptor (a partir de la expresión de la enzima luciferasa). Cada una de estas cepas contenía un tipo determinado de receptor estrogénico, de tal manera que los autores pudieron evaluar la estrogenicidad de las BPs para distintos receptores (animales y humanos). Así, cuando se sometía uno de estos cultivos a concentraciones crecientes de benzofenona, la bioluminiscencia aumentaba, fenómeno que también provoca el estradiol natural: si el 100% de luminiscencia se alcanzaba cuando el cultivo se sometía a una concentración de estradiol de 10 nM, la señal relativa permitía evaluar el carácter estrogénico de cada benzofenona ensayada. El estudio realizado conBP-1, BP-2, BP-3 y trihidroxibenzofenona (THB) sobre las células MELN demostró que, a bajas concentraciones, el comportamiento de los compuestos sobre la actividad de la luciferasa era el mismo, permaneciendo constante hasta una concentración próxima a 10^{-6} M. A partir de este valor, cada compuesto actuaba de forma diferente sobre la actividad de la luciferasa apareciendo efectos notables. En este estudio también se ensayó la capacidad de las BPs para desplazar al estradiol de los receptores de las células una vez que éste ya está unido a los mismos. Las diferentes cepas se sometieron a estradiol marcado isotópicamente durante el tiempo suficiente para que la unión estradiol-receptor se diera por completo, añadiéndose posteriormente concentraciones crecientes de

[104] Felix T., Hall B.J., Brodbelt J.S. *Analytica Chimica Acta*, 1998, 371:195–203.
[105] Hagedorn-Leweke U., Lippold B.C. *Pharmacological Research,*1995, 12:1354–1360.
[106] Hayden C.G.J., Roberts M.S., Benson H.A.E. *Lancet*, 1997, 350:863–864.
[107] Jiang R., Roberts M.S., Collins D.M., Benson H.A.E. *British Journal of Clinical Pharmacology,* 1999, 48:635–637.
[108] Molina-Molina J.M., Escande A., Pillon A., Gomez E., Pakdel F., Cavailles V., Olea N., Ait-Aissa S., Balaguer P. *Toxicology and Applied Pharmacology,* 2008, 232:384–395.

cada compuesto (BP-1, BP-2 y THB). Se midió el porcentaje de estradiol que quedaba unido al receptor tras la competencia del xenobiótico. Hasta concentraciones de 10^{-7} M la unión estradiol-receptor era prácticamente del 100%, a partir de ese punto, el porcentaje disminuía progresivamente hasta que a de 10^{-5} M de xenobiótico el porcentaje de la unión estradiol-receptor era prácticamente cero.

- ✓ **Efectos de las benzofenonas en la proliferación de células tumorales.** Al igual que otros EDCs, las BPs favorecen el desarrollo de colonias celulares cancerosas como la MCF-7[109]. Así, concentraciones crecientes de las distintas benzofenonas provocan un mayor número de individuos en el cultivo, siempre y cuando no se alcancen los niveles de citotoxicidad.

- ✓ **Actividad antiandrogénica.** Existen diversos trabajos de investigación que ponen de manifiesto la capacidad de las BPs para inhibir la actividad propia de la dihidrotestosterona. Suzuki y col.[109]demostraron que pueden inhibir la respuesta bioluminiscente de células tipo N1H3T3, cepa que está provista de un plásmido que le permite expresar la enzima luciferasa cuando se produce la unión de la dihidrotestosterona con su receptor.

- ✓ **Efectos en animales.** También se han llevado a cabo estudios en modelos animales que han demostrado que estos compuestos tienen suficiente carácter disruptor como para provocar daños diversos en los organismos vivos. Weisbrod y col.[110] demostraron que la BP-2 es capaz de provocar efectos más que significativos en la capacidad reproductiva de los peces, inhibiendo la gametogénesis (tanto en hembras como en machos), mermando el desove y la producción vitelogénica de estos animales. La vitelogenina es una proteína que se genera en el hígado y que desencadena la maduración de los oocitos para dar lugar a óvulos plenamente formados. Así pues, los estrógenos naturales provocan la producción de vitelogenina, con el subsiguiente correcto desarrollo de las

[109] Suzuki T., Kitamura S. *Toxicology and Applied Pharmacology,* 2005, 203:9–17.
[110] Weisbrod C.J., Kunz P.Y., Zenker A.K., Fent K. *Toxicology and Applied Pharmacology,* 2007, 225:255–266.

funciones sexuales del individuo. Este proceso tiene lugar periódicamente en los peces, concretamente en la época de desove. Con la disminución de la producción vitelogénica el ciclo sexual de la especie se ve afectado, por lo que el número de óvulos maduros se hace menor y la viabilidad embrionaria del ciclo reproductivo disminuye ostensiblemente. Por lo tanto, la concentración de vitelogenina en el animal pone de manifiesto una posible acción disruptora del xenobiótico. Además se han observado alteraciones histológicas en las gónadas, muestras de la atrofia reproductiva de la población de ensayo, y una feminización de los individuos; los caracteres sexuales secundarios se manifiestan en menor medida de lo habitual, y la ratio hembras/machos crece significativamente.

Respecto a estudios con roedores, se han desarrollado estudios cuyos resultados relacionan directamente la exposición a BPs con la carcinogénesis. Sirva como ejemplo el estudio realizado por Rhodes y col.[111], donde se muestra que la BP-1 aplicada a ratas y ratones de laboratorio a través de la dieta provoca en estos animales adenoma renal, leucemia de células mononucleares y sarcoma histiocítico.

[111] Rhodes M.C., Bucher J.R., Peckham J.C., Kissling G.E., Hejtmancik M.R., Chhabra R.S. *Food and Chemical Toxicology,* 2007, 45:843–851.

2. LECHE MATERNA HUMANA

Uno de los aspectos más preocupantes de los EDCs, es la vulnerabilidad de los niños, especialmente la de los recién nacidos, a la exposición a este tipo de compuestos durante las primeras etapas del desarrollo. La exposición a los EDCs es particularmente importante en esta fase de la vida debido a la gran susceptibilidad del cerebro y otros órganos a los estrógenos[61]. La consecuencia de esta exposición puede llegar a provocar efectos irreversibles que sólo se harán evidentes a lo largo de su vida.

Como ya se ha citado con anterioridad en la presente Memoria, la exposición a los EDCs puede causar efectos adversos incluso a bajas dosis, habiéndose demostrado que los grupos más susceptibles a los efectos de los disruptores endocrinos son las madres gestantes, los fetos, los bebés lactantes y los niños menores de 5 años[112]. Estos grupos presentan una capacidad disminuida de metabolizar a estos compuestos a nivel hepático, mientras que por otro lado, los EDCs presentan una gran afinidad para almacenarse en el tejido fetal, así como en la placenta[61].

Figura 16. *Grupos susceptibles a los EDCs*

Se ha publicado que los EDCs se acumulan en determinados tejidos humanos y podrían pasar a la descendencia a través de la placenta o leche materna[113-117]. Las madres lactantes expuestas a disruptores endocrinos pueden,

[112] Diamanti-Kandarakis E., Bourguignon J.P., Giudice L.C., Hauser R., Prins G.S., Soto A.M., Zoeller R.T., Gore A.C. Endocrine Reviews, 2013, In press DOI:http://dx.doi.org/10.1210/er.2009–0002.

[113] Jiménez-Díaz I., Zafra-Gómez A., Ballesteros O., Navea N., Navalón A., Olea N., Vílchez J.L. *Journal of Chromatography B*, 2010, 878:3363–3369.

sin saberlo, exponer a sus hijos a niveles peligrosos de estos compuestos. Puesto que la leche materna es la principal vía de exposición para los bebés lactante, su análisis es de especial interés científico.

La leche materna además de ser una importante ruta de exposición para los bebés y un valioso fluido biológico que permite la evaluación de la exposición, puede servir como biomarcador de la exposición materna o prenatal[118] y es útil para estudios de biomonitorización, ya que el muestreo es no invasivo y los volúmenes disponibles de muestra son relativamente grandes[119].

Es un fluido complejo compuesto de "moléculas" bioactivas y nutrientes esenciales para el óptimo desarrollo del bebé. Los componentes de la leche materna participan en procesos tan importantes como el crecimiento, modulación y maduración del sistema inmunológico, protección ante toxinas y agentes patógenos, en el desarrollo congnitivo del bebé y en el establecimiento de la microbiota intestinal.

La composición de la leche materna la podemos resumir en tres grandes bloques: *oligosacáridos, proteínas y grasas*.

2.1. Oligosacáridos

Los oligosacáridos son el tercer componente más abundante de la leche materna. El interés por la investigación de este tipo de compuestos ha tomado

[114] Jiménez-Díaz I., Vela-Soria F., Zafra-Gómez A., Navalón A., Ballesteros O., Navea N., Olea N., Vílchez J.L. *Talanta*, 2011, 84:702–709.
[115] Vela-Soria F., Jiménez-Díaz I., Rodríguez-Gómez R., Zafra-Gómez A., Ballesteros O., Navalón A., Vílchez J.L., Fernández M.F., Olea N. *Talanta,* 2011, 85:1848–1855.
[116] Vela-Soria F., Jiménez-Díaz I., Rodríguez-Gómez R., Zafra-Gómez A., Ballesteros O., Olea N., Navalón A. *Analytical Methods*, 2011, 3:2073–2081.
[117] Stefanidou M., Maravelias C., Spiliopoulou. *Endocrine, Metabolic and Immune Disorders - Drug Targets*, 2009, 3:269–276.
[118] Hernik A., Góralczyk K., Struciński P., Czaja K., Korcz W., Minorczyk M., Łyczewska M., Ludwicki J.K. *Chemosphere*, 2014, 94:158–163.
[119] Smolders R., Schramm K.W., Nickmilder M., Schoeters G., *Environmental Health Perspectives*, 2009,8:8–18.

desde los orígenes dos vías bien diferenciadas. Por un lado, los pediatras y microbiólogos se centraban en la búsqueda de los beneficios asociados a la lactancia y por otro los químicos trataban de caracterizar los carbohidratos más abundantes encontrados exclusivamente en la leche materna. A finales del siglo XIX, la tasa de mortalidad infantil en el primer año de vida era de un 30%, y se observó que los bebés amamantados tenían una probabilidad mucho mayor de supervivencia que los neonatos alimentados con biberón. Eschbach en 1888, observó que la leche materna contenía un tipo de lactosa diferente a la bovina, poco después Deniges, encontró que no existían diferencias, y que "ambas lactosas" eran la misma, sin embargo determinó la existencia de de una fracción de carbohidratos no descrita hasta el momento. Polonowski y Lespagnol, en los años 30, caracterizaron dicha fracción de hidratos de carbono denominándola "gynolactose"[120,121].

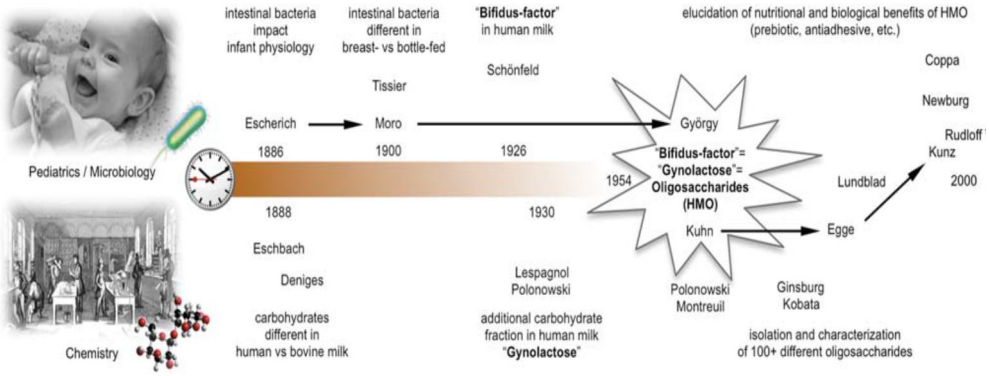


Figura17. *Principios de la investigación de los oligosacáridos en la leche materna*

Los oligosacáridos de la leche materna son una familia estructuralmente muy diversa. Originalmente, fueron descubiertos como un prebiótico "*factor bifidus*" que actúa como sustrato metabólico para las bacterias deseadas y forma una composición de la microbiota intestinal que benefician la salud del bebé lactante. Algunos estudios certifican que la presencia de oligosacáridos en la leche materna sirve para prevenir el acceso de patógenos a las superficies mucosas del bebé y disminuyen el riesgo de infecciones virales, bacterianas y parásitas. Además, los oligosacáridos pueden modular las respuestas celulares

[120] Bode L. *Glycobiology*, 2012, 22:1147–1162.
[121] Montreuil J. *New Perspectives in Infant Nutrition*, 1992, Georg Thieme Verlag. p. 3–11.

epiteliales e inmunes, reducir la activación y la infiltración de leucocitos, disminuir el riesgo de enterocolitis necrosante y aportar ácido siálico que es un nutriente esencial para el desarrollo cognitivo y del cerebro del niño.

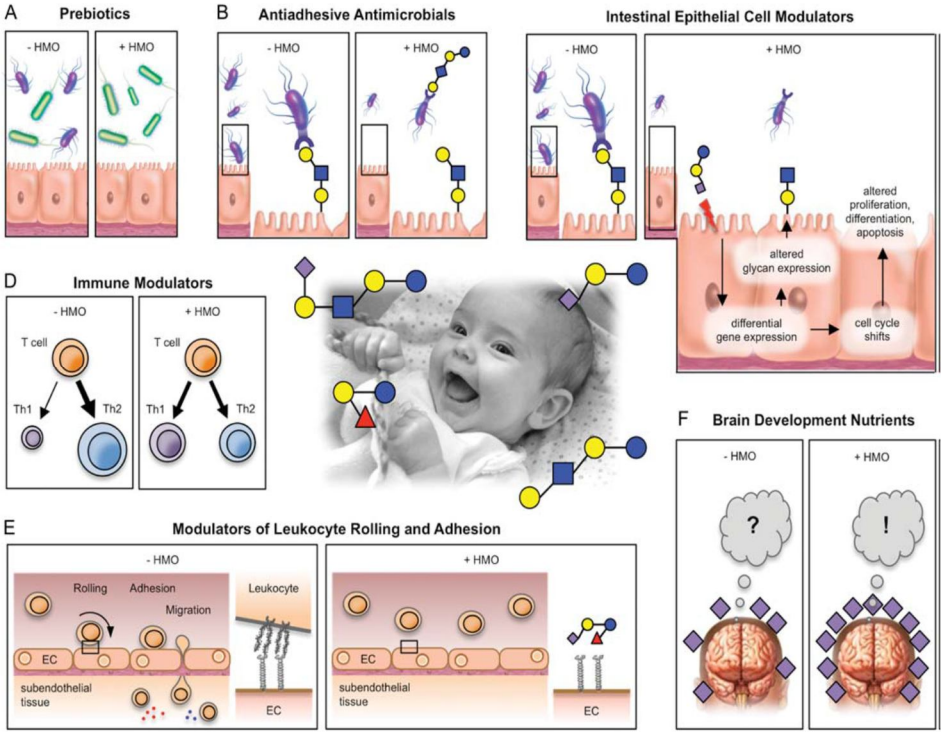


Figura 18. *Beneficios de los oligosacáridos*

La composición y cantidad de oligosacáridos en la leche materna varía entre mujeres y a lo largo del periodo de lactancia[122,123]. El calostro, que es el primer fluido secretado por la glándula mamaria unos días antes y después del parto, contiene 20-25 gL^{-1} de oligosacáridos. A medida que avanza el periodo de lactancia, la leche madura y se produce una disminución del contenido en oligosacáridos a 5-20 g L^{-1}. Los oligosacáridos encontrados en la leche materna derivan generalmente de cinco monosacáridos, concretamente glucosa, galactosa, fucosa, ácido siálico y N-acetilglucosamina.

[122] Kunz C., Rudloff S., Baier W., Klein N., Strobel S. *Annual Review of Nutrition*, 2000, 20:699–722.
[123] Gabrielli O., Zampini L., Galeazzi T., Padella L., Santoro L., Peila C., Giuliani F., Bertino E., Fabris C., Coppa G.V. *Pediatrics*, 2011, 128:E1520–E1531.

2.2. Aminoácidos y proteínas

Los aminoácidos son los componentes principales de la proteína, por lo tanto, son importantes en la leche materna para el desarrollo y el crecimiento infantil[124]. Las funciones de los aminoácidos son variadas, participan en la digestión aumentando la absorción de nutrientes e incrementan la función del sistema inmune del bebé contra bacterias patógenas, virus y levaduras[125]. El contenido proteico de la leche materna varía dependiendo del estado de lactancia de la madre. Así, durante la lactancia temprana, oscila entre 1.4-1.6g/100mL, 0.8-1.0g/100mL durante los 3-4 primeros meses de lactancia y 0.7-0.8g/100mL después de 6 meses de lactancia[126,127].

La cantidad y calidad de las proteínas son aspectos clave del valor nutricional de la alimentación infantil. El perfil de aminoácidos es un dato importante a la hora de evaluar la calidad del contenido total de proteína y por ello la composición proteica de la leche materna se suele determinar a través de la composición en aminoácidos[128].

Es importante la caracterización y la cuantificación del nitrógeno total en la leche materna ya que sirve como guía nutricional y para entender y definir los requerimientos del bebé en términos de proteína y aminoácidos. En la mayoría de los estudios sobre la composición en aminoácidos de la leche materna, se exponen valores en cantidad de proteína total o nitrógeno total, sin diferenciar cuál se debe a la proteína y cual es nitrógeno "libre" o no proteico. El contenido total de aminoácidos, está compuesto por los aminoácidos que constituyen las proteínas y por lo tanto cuantifican valor del nitrógeno proteico y el contenido de aminoácidos libres que participan en el valor del contenido del nitrógeno no proteico[129]. Alrededor del 20-25% del nitrógeno total en la leche humana corresponde al nitrógeno que no procede de la proteína. El

[124] Zhang Z., Adelman A.S., Rai D., Boettcher J., Lőnnerdal B. *Nutrients*, 2013, 5:4800-4821.
[125] Leonnerdal B. *American Journal of Clinical Nutrition,*2003, 77:1537S–1543S.
[126] Michaelson K.F. *American Journal of Clinical Nutrition,* 1994, 59:600–611.
[127] Jensen R.G. *Academic Press, Inc.*, 1995, San Diego, USA.
[128] Raiten D.J., Talbot J.M., Waters J.H. *Journal of Nutrition,* 1998, *128*:2116S–2118S.
[129] Lemons J.A., Reyman D., Moye L. *Early Human Development*, 1983, 8:323–329.

conjunto de aminoácidos libres, constituyen entre el 8%-22% del contenido en nitrógeno no proteico y entre el 5-10% del total de aminoácidos[130-133].

El contenido de aminoácidos libres suele ser pasado por alto en la literatura científica, normalmente los estudios se centran en la composición proteica. Los escasos estudios que se han centrado en el contenido de aminoácidos libres[134], confirman que la mayor parte de la variación en la composición de aminoácidos de la leche materna se produce en la etapa de lactancia, ocurriendo la mayor disminución durante los primeros meses. Sin embargo, algunos aminoácidos libres tales como alanina, glicina, serina, glutamina y glutamato, aumentan con el paso del tiempo de lactancia.

La taurina, el ácido glutámico y la glutamina son los aminoácidos libres más abundantes en la leche materna, comprenden casi el 50% del total de los aminoácidos libres[135-138]. A diferencia de los datos obtenidos para el contenido total de aminoácidos, los valores para el contenido de aminoácidos libres, presenta una gran variabilidad. Existe una distribución anormal para estos aminoácidos, por lo que podría considerarse como una característica diferencial o huella dactilar de cada leche materna.

2.3. Lípidos

Los lípidos constituyen la segunda fracción en abundancia de la leche materna. Su principal función es proporcionar energía para el crecimiento y

[130] Agostoni C., Carratù B., Boniglia C., Lammardo A.M., Riva E., Sanzini E. *Journal of* Pediatric Gastroenterology *and* Nutrition*,* 2000, *31*:508–512.
[131] Atkinson S.A., Schnurr C., Donovan S.M., Lönnerdal B. Atkinson, S.A., Lönnerdal, B., Eds.; CRC Press Boca Raton, 1989, FL, USA, 117–133.
[132] Carratù B., Boniglia C., Scalise F., Ambruzzi A., Sanzini E.*Food Chemistry*, 2003, 81:357–362.
[133] Svanberg U., Gebre-Medhin M., Ljungqvist B., Olsson M. *American Journal of Clinical Nutrition*, 1977, 30:499–507.
[134] Zhang Z., Adelma A.S, Rai D., Boettcher J.**,** Lőnnerdal B. *Nutrients,*2013, *5*:4800-4821.
[135] Agostoni, C., Carratù, B., Boniglia, C., Riva, E., Sanzini, E. *Journal of the American College Nutrition*, 2000, 19:434–438.
[136] Chuang C.K., Lin S.P., Lee H.C., Wang T.J., Shih Y.S., Huang F.Y., Yeung C.Y. *Journal of Pediatrics Gastroenterolgy and Nutrition*, 2005, 40:496–500.
[137] Elmastas M., Keha E.E., Keles M.S., Aboul-Enein H.Y. *Analytical Letters*, 2008, 41:725–736.
[138] Sarwar G. *Journal of the American College Nutrition,* 2001, 20:92–93.

desarrollo del bebé[139]. Los lípidos esenciales son estructuras complejas que varían en contenido y composición. Los triglicéridos representan aproximadamente el 98% del contenido total de lípidos en la leche materna, por lo tanto se puede hablar del contenido total de triglicéridos como el contenido total de grasas[140,141]. Curiosamente, la etapa del calostro es la que tiene menor contenido (2.0 g L^{-1}), aumentando durante los 30 primeros días de lactancia hasta los 4.0 g L^{-1}. Sin embargo, según avanza el periodo de lactancia el contenido en triglicéridos varía considerablemente entre 2.5 y 4.5 g L^{-1}. Debido a que la grasa es la principal fuente de energía, es la responsable del contenido calórico de la leche materna[141,142].

La alta actividad biosintética de los triglicéridos en la glándula mamaria humana, implica la secreción de 20–30 g/día de triglicéridos. Para ello se requieren grandes cantidades de ácidos grasos. Estos ácidos grasos proceden de tres fuentes principales[142,143]:

- ✓ Directamente de la dieta de la madre.
- ✓ Previamente almacenados o sintetizados en los tejidos maternos.
- ✓ Sintetizados en la glándula mamaria.

Estas tres fuentes deben ser equiilibradas para así poder mantener constante el suministro de ácidos grasos y aportar la energía al bebé de manera adecuada. Se han identificado más de 150 ácidos grasos diferentes en la leche materna, la mayoría provienen de la dieta de la madre, ya sea directamente o por el almacenaje en los tejidos[142]. Los más comunes son el ácido oleico, que se

[139] Innis S. M. *Current Nutrition Reports*, 2013 2:151–158
[140] Jensen R.G., Bitman J., Carlson S.E., Couch S.C., Hamosh M., Newburg D.S. Academic Press 1995, San Diego, USA, 495–542.
[141] Jensen R.G. *Lipids*, 1999, 34:1243–1271.
[142] Innis S.M. *Advances in Experimental Medicine and Biology*, 2004, 554:27–43.
[143] Rudolph M.C., McManaman J.L., Phang T., Russell t., Kominsky D.J., Stein T., Anderson S.M. Neville M. *Physiologycal Genomics*, 2007, 8:323–336.

puede encontrar entre 27–35 g/100 g de leche materna, el ácido palmítico, 22-27 g/100 g de leche materna y el ácido linoléico, 8-14 g/100 g de leche materna[144].

En la siguiente tabla se recogen los porcentajes de los ácidos grasos más abundantes en leche materna.

***Tabla 3**. Ácidos grasos saturados, monoinsaturados y poliinsaturados omega-6 y omega-3 (moles %) en leche materna madura*

Ácido graso	
8:0 Ácido Caprílico	0.28 ± 0.091
10:0 Ácido Cáprico	0.98 ± 0.11
12:0 Ácido Laurico	5.10 ± 0.43
14:0 Ácido Mirístico	6.07 ± 0.31
15:0 Ácido Pentadecílico	0.43 ± 0.04
16:0 Ácido Palmítico	21.23 ± 0.39
18:0 Ácido Esteárico	10.55 ± 0.71
20:0 Ácido Arquídico	0.28 ± 0.05
≤14-carbonos saturados	12.48 ± 0.74
Total saturados	45.12 ± 1.32
16:1 n-7 Ácido Palmitoléico	2.78 ± 0.19
18:1 n-9 Ácido Eláidico	31.10 ± 1.54
18:1 n-7 Ácido Vacénico	0.32 ± 0.04
20:1 n-9 Ácido Eicosenoico	0.11 ± 0.04
Total monoinsaturados	34.41 ± 0.66
18:2 n-6 Ácido Linoléico	17.56 ± 1.121
18:3 n-6 Ácido γ- Linoléico	0.57 ± 0.05
20:2 n-6 Ácido Eicosadienoico	0.36 ± 0.04
20:3 n-6 Ácido dihomo-γ-Linoléico	0.42 ± 0.07
20:4 n-6 Ácido Arquidónico	0.48 ± 0.04
22:4 n-6 Ácido Adrénico	0.09 ± 0.02
22:5 n-6 Ácido Docosapentaenoico	0.13 ± 0.02
18:3 n-3 Ácido α-Linoléico	0.48 ± 0.07
20:5 n-3 Ácido Eicosanpentenoico (EPA)	0.11 ± 0.04
22:5 n-3 Ácido Docosapentaenoico (DPA)	0.03 ± 0.01
22:6 n-3 Ácido Docosahexaenoico (DHA)	0.14 ± 0.01
Total poliinsaturados	20.41 ± 1.51

[144] Ilyasoglu H. *Food Science and Tecnology*, 2013, 54:179–185.

2.4. Resumen de la composición genérica de una leche materna

En las ***Tablas 4*** y ***5***[145] se presenta un resumen de la composición general de una leche materna madura.

Tabla 4. *Resumen de la composición media de una leche materna madura*

Componente	Concentración (gL^{-1})
Proteína[146]	8
Grasa[124]	41
Lactosa[124]	70
Oligosacáridos[123,147]	5-15
Número oligosacáridos identificados[123,125]	+100

Tabla 5. *Componentes identificados en un extracto de leche materna*

N°	Nombre	Grupo
1	2-oxoglutarato	
2	*2'fucosillactosa*	Fuc (α1-2) Gal (β1-4)
3	*3'fucosillactosa*	Fuc(α1-3)αGlc Fuc(α1-3)βGlc
4	*3'sialil-lactosa*	Neu5Ac(α2-3)
5	4-hidroxifenilactato	
6	*6'sialil-lactosa*	Neu5Ac(α2-6)
7	Ácido acético	
8	Alanina	
9	Carnitina	
10	Colina	
11	Ácido cítrico	
12	Fucosa (1)	Fuc(α1-3)GlcNAc
13	Fucosa (2)	Fuc(α1-3)GlcNAc
14	Galactosa (1)	Gal(β1-4)GlcNAc
15	Galactosa (2)	Gal(β1-4)GlcNAc
16	Galactosa (3)	Gal(β1-4)GlcNAc
17	Glucosa	βGlc

[145] Pratico G., Capuani G., Tomassini A., Baldassarre M., Delfini M., Miccheli A. *Natural Product Research*, 2014, 28:95–101.
[146] Hale T.W., Hartmann P.E. Hale Publishing, L.P.2007, MA, USA.
[147] Bao Y., Zhu L., Newburg D.S. *Analytycal Biochemistry*, 2007, 370:206–214.

Tabla 5 cont. *Resumen de los componentes identificados en un extracto de leche materna*

N°	Nombre	Grupo
18	Ácido glutámico	
19	Histidina	
20	Isoleucina	
21	Ácido láctico	
22	Fracción Lactosil	Gal(β1-4) αGlc βGlc
23	Lactodifucotetraosa	Fuc(α1-2) Fuc(α1-3)αGlc Fuc(α1-3)βGlc
24	Lacto-N-difucohesaosa I	Fuc(α1-2) Fuc(α1-4) GlcNAc(β1-6)
25	Lacto-N-difucohesaosa II	Fuc(α1-4) Fuc(α1-3)αGlc Fuc(α1-3)βGlc Gal(β1-4)
26	Lacto-N-fucopentaosa I	Fuc(α1-2) GlcNAc
27	Lacto-N-fucopentaosa II	Fuc(α1-4)
28	Lacto-N-fucopentaosa III	Fuc(α1-3)GlcNAc Fuc(α1-3)GlcNAc
29	Lacto-N-fucopentaosa V	Fuc(α1-3)αGlc Fuc(α1-3)βGlc
30	Lisina	
31	Me-Histidina	
32	Me-Histidina	
33	Inositol	
34	N-acetilcarnitina	
35	N-Acetil (1)	
36	N-Acetyl (2)	
37	Fenilalanina	
38	Ácido succínico	
39	Treonina	
40	Tirosina	
41	Ácido valérico	
42	Valina	

La ***Figura 19*** muestra una imagen del aspecto general de la leche materna, correspondiente a distintas etapas del periodo de lactancia y a distintas madres lactantes.

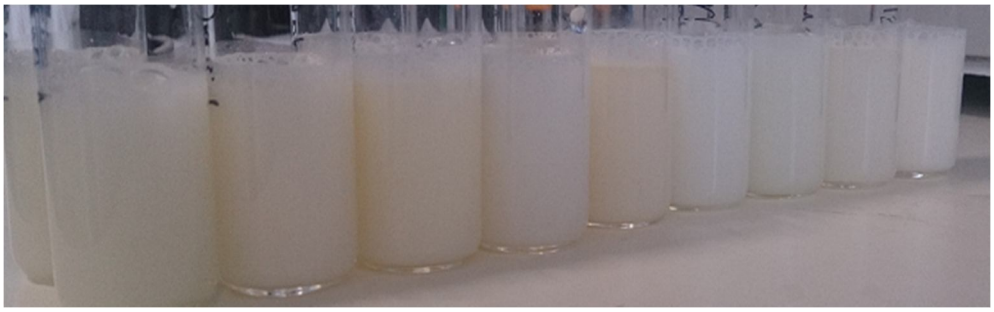


Figura 19. *Aspecto de muestras de leche materna en diferentes estadíos del periodo de lactancia*

3. METODOLOGÍA ANALÍTICA PARA LA DETERMINACIÓN DE LOS CONTAMINANTES SELECCIONADOS EN LECHE MATERNA

La importancia de la detección de estos compuestos en muestras biológicas ya ha quedado bien definida a lo largo de la introducción de la presente Tesis Doctoral. Los EDCs objeto de estudio se encuentran en las muestras biológicas en concentraciones a nivel de traza ($\mu g\ L^{-1}$– $ng\ L^{-1}$). El primer problema que surge por tanto, a la hora de desarrollar un método de análisis a nivel de trazas, es el efecto matriz de la muestra.

La leche materna es una matriz biológica de elevada complejidad. Como anteriormente hemos definido, se trata de una matriz compuesta principalmente por proteínas y lípidos que son los principales interferentes que dificultan el análisis. Por lo que no hay publicados un gran número de métodos para el análisis de los analitos objeto de estudio en la literatura científica. Por esta razón y debido a que un número muy limitado desarrolla métodos multirresiduo, es importante el desarrollo de nuevas metodologías que mejoren la calidad de los ya existentes.

En la ***Tabla 6***, se muestra un resumen de los trabajos más relevantes publicados para la determinación de los compuestos objeto de estudio en leche materna en las distintas fases del periodo de lactancia.

Tabla 6. *Métodos analíticos para la determinación de EDCs en leche materna*

Analitos	Matriz	Técnica de extracción	Extractante	Técnica instrumental	LOD (ng mL^{-1})
PBs[148]	Leche materna madura	On-line SPE	LiChrosphere RP-18	LC-MS/MS	<1
BPA y derivados[149]	Leche materna madura	On-line SPE	X-Bridge C8	UHPLC-MS/MS	0.01-0.09
BPA y derivados[150]	Calostro	On-line SPE	X-Bridge C8	UHPLC-MS/MS	0.01-0.09
BPA[151]	Calostro	LLE	MeCN	ELISA	----
PBs, BP-2, BP-3[152]	Leche materna madura	LLE	Hexano	LC-MS GC-MS	----
BPA/BP-3[153]	Leche materna madura	On-line SPE	LiChrosphere RP-18	LC-MS/MS	0.28-0.51
PBs[154]	Leche materna madura	MISPE	MIP	LC-UV	----
BPA[155]	Leche materna madura	LLE	2-propanol	UHPLC-FLDLC-MS/MS	0.6 y 0.39
BPA[156]	Leche materna madura	LLE	Hexano	LC-FLD	0.11
BPA[157]	Leche materna madura	SPE	Glass Oasis™ HLB	UHPLC-MS/MS	0.22

Los métodos propuestos en la literatura, se basan principalmente en el uso de la cromatografía de líquidos/gases acopladas a espectrometría de masas y ELISA como técnicas de análisis. El tratamiento de muestra, para la

[148] Ye X., Calafat A.M., Bishop L.L. *Analytica Chimica Acta*, 2008, 622:150–156.
[149] Cariot A., Dupuis A., Albouy-Llaty M., Legube B., Rabouana S., Migeot V. *Talanta*, 2012, 100:175–182.
[150] Migeot V., Dupuis A., Cariot A., Albouy-Llaty M., Pierre F., Rabouan S. *Enviromental Science and Technology*, 2013, 47:13791–13797.
[151] Kuruto-Niwa R., Tateoka Y., Usuki Y., Nozawa R. *Chemosphere*, 2007, 66:1160–1164.
[152] Schlumpf M., Kypke K., Wittassek M., Angerer J., Mascherd H., Mascher D., Vökt C., Birchler M. Lichtensteiger W. *Chemosphere*, 2010, 81:1171–1183.
[153] Ye X., Kuklenyik Z., Needham L.L., Calafat A.M. *Journal of Chromatography B*, 2006, 831:110–115.
[154] Melo L.P., Queiroz M.E.C. *Analytical Methods*, 2013, 5:3538–3545.
[155] Yia B., Kimb C., Yanga M. *Journal of Chromatography B*, 2010, 878:2606–2610.
[156] Sun Y., Irie M., Kishikawa N., Wada M., Kuroda N., Nakashima K. *Biomedical Chromatography*, 2004, 18:501–507.
[157] Zimmersa S.M, Browne E.P., O'Keefe P.W., Anderton D.L., Kramer L., Reckhow D.A, Arcaro K.F. *Chemosphere,* 2014, 104:*237–243.*

preconcentración y aislamiento de los analitos, se lleva a cabo empleando técnicas de extracción tanto clásicas como la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE), como técnicas de reciente desarrollo como la extracción en fase sólida con el empleo de polímeros impresos molecularmente (MISPE).

A continuación se comentan brevemente las técnicas empleadas para la determinación de EDCs en leche materna.

A. Extracción en fase sólida (SPE). SPE es una de las técnicas de tratamiento de muestra más utilizadas en la actualidad para la determinación de EDCs en muestras biológicas ya que permite tanto la limpieza de la muestra como la concentración de los analitos[158]. El proceso completo consiste en las 4 etapas mostradas en la ***Figura 20***:*(i)* acondicionamiento del adsorbente, *(ii)* carga de la muestra, *(iii)* lavado y *(iv)* elución y recuperación de los analitos[159].

[158] Hennion M.C. *Journal of Chromatography A*, 1999, 856:3–54.
[159] Pichon V. *Journal of Chromatography A*, 2000, 885:195–215.

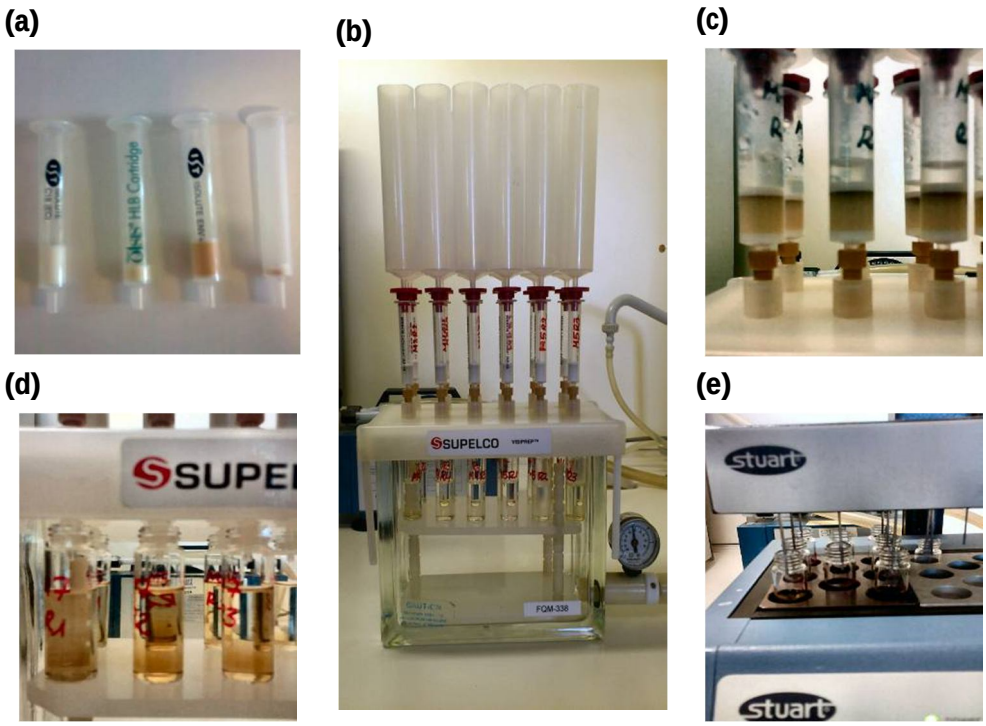


Figura 20. *Proceso de SPE.* ***(a)*** *cartuchos, de izquierda a derecha: Isolute® C18, Oasis® HLB, Isolute®ENV+, Empore MPC.* ***(b)*** *Montaje del sistema completo de SPE.* ***(c)*** *Detalle de los cartuchos durante la carga de la muestra.* ***(d)*** *Detalle de la etapa de elución.* ***(e)*** *Detalle de la etapa de evaporación a sequedad de los extractos.*

La SPE se puede llevar a cabo *off-line*, cuando el proceso se realiza de manera separada al análisis cromatográfico, o puede ser *on-line*, cuando el proceso está integrado con el sistema de análisis[160].Los adsorbentes convencionales están fabricados a base de sílice porosa a la que se le enlazan grupos apolares, siendo C18 el más usado. Sin embargo, estos adsorbentes no ofrecen altas recuperaciones para compuestos de elevada polaridad. Con la introducción de nuevos adsorbentes de tipo polimérico, donde existe un balance hidrofílico-lipofílico, se puede aplicar la SPE a este tipo de analitos[161,162].

[160] Rossi D.T., Zhang N. *Journal of Chromatography A*, 2000, 885:97–113.
[161] Gros M., Petrović M., Barceló D. *Analytical Chemistry*, 2009, 81:898–912.
[162] Fatta D., Nikolaou A., Achilleos A., Meriç S. *TrAC Trends in Analytical Chemistry*, 2007, 26:515–533.

En el caso de los compuestos y la matriz objeto de estudio, la SPE se ha usado para la determinación de BPA y sus derivados en leche madura y calostro empleando la modalidad *on-line*, y usando cartuchos X-Bridge C18, Glass Oasis™ HLB y LiChrosphere RP-18, con límites de detección excelentes en el orden de los ng L^{-1} en todos los casos, siendo más favorables en el caso del primer tipo de cartuchos. Por otro lado, Ye y col.[148] proponen el uso de rellenos LiChrosphere RP-18 para la determinación, mediante SPE-*on-line*, de PBs en leche madura obteniendo límites de detección inferiores a 1 ng mL^{-1}. En todos los casos la técnica analítica empleada fue la cromatografía de líquidos acoplada a la espectrometría de masas.

Una variante de la SPE, introducida en los últimos años, emplea como adsorbentes los llamados polímeros impresos molecularmente (MIPs). La técnica MISPE se basa en el uso de polímeros de reconocimiento molecular específico para el analito en función de la forma y posición de los grupos funcionales. Esta selectividad se debe a que para la síntesis del MIP se emplea una molécula a modo de "molde" denominada *template*, de naturaleza muy similar al analito que se pretende determinar, que se une al polímero mediante enlaces a través de monómeros que están unidos al polímero por *linkers*. Una vez formado el polímero, la molécula molde se elimina generando unos espacios moleculares que serán ocupados por el analito de interés durante la extracción.

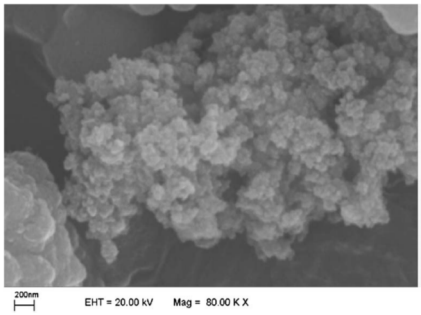


Figura 21. *Microscopía de barrido electrónico de un MIP*[154]

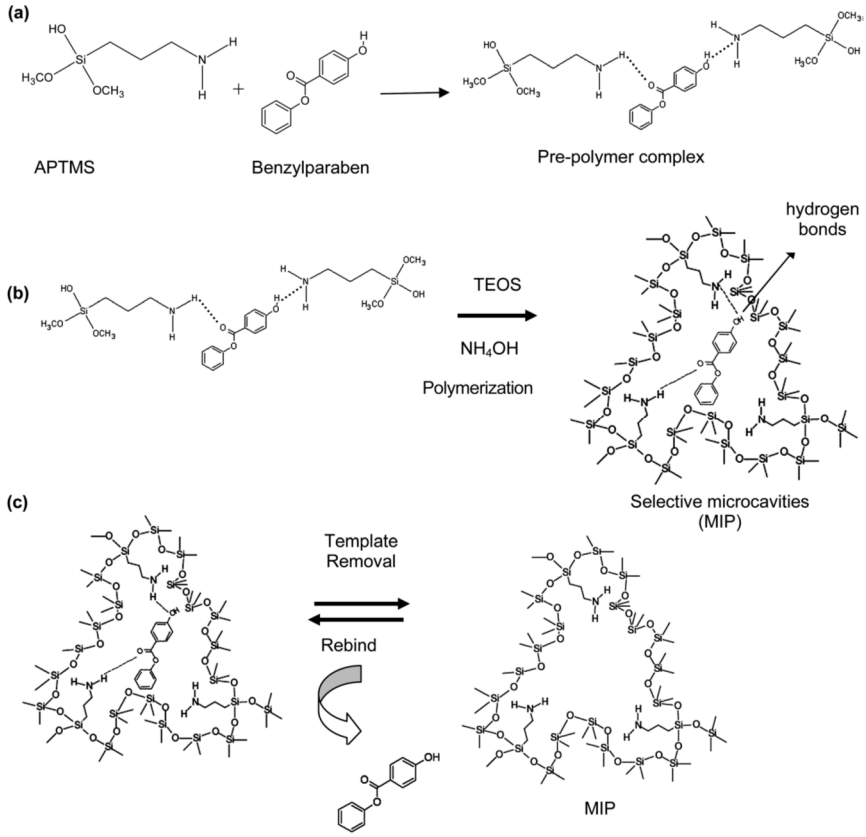


Figura 22. *Esquema de la síntesis de MIPs para la determinación de PBs en leche materna*[154]

Una vez construido el polímero, este se pone en presencia de los analitos que se pretenden extraer, los cuales pueden unirse a la cavidad de manera selectiva, presentando una mayor afinidad que otros compuestos presentes en la muestra. Los analitos pueden ser liberados cuando se establecen unas condiciones adecuadas, consiguiendo así su aislamiento.

Esta técnica ha sido aplicada por Melo y Queiroz[154] para la determinación de PBs en leche madura empleando cromatografía de líquidos con detección ultravioleta. En este caso, los autores no suministran datos sobre los límites de detección obtenidos.

B. Extracción Líquido-Líquido (LLE). Esta técnica ha sido ampliamente empleada para la determinación de EDCs en matrices biológicas. Se puede definir como la transferencia de una sustancia "x" de un disolvente A a un

disolvente B inmiscible con el primero. Los parámetros que se deben controlar en este caso son la selectividad o relación entre los coeficientes de reparto de dos sustancias ($\alpha=K_x/K_y$); la recuperabilidad, la volatilidad relativa del disolvente de extracción respecto de los componentes del extracto debe ser muy distinta; el coeficiente de partición. La afinidad del compuesto "x" por el disolvente A debe ser mayor que en B es decir, un alto coeficiente de partición para así utilizar el mínimo volumen de disolvente; la capacidad o carga de analito por el peso de disolvente de extracción; la densidad, cuanto mayor sea la diferencia mejor se realizará su separación; la tensión interfacial, se requiere alta ya que promueve una rápida coalescencia de las gotas de la emulsión, favoreciendo la separación; la reactividad química del disolvente, que debe ser estable e inerte químicamente respecto al analito y al material de trabajo; la toxicidad del disolvente, que debe ser baja; y el costo.

Esta técnica ha sido empleada para la determinación BPA en leche madura[155], empleando 2-propanol y hexano como extractantes. Como técnicas analíticas se ha empleado cromatografía de líquidos con detección fluorescente y espectrometría de masas en tándem. Los límites de detección obtenidos son inferiores a 1 ng mL^{-1}. Esta técnica ha sido también empleada para la determinación de BPA en calostro[151] empleando MeCN como extractante y Enzyme-Linked Immuno Sorbent Assay (ELISA) como técnica de detección.

La técnica ELISA se basa en el uso de antígenos o anticuerpos marcados con una enzima, de forma que los conjugados resultantes tengan actividad tanto inmunológica como enzimática. Al estar uno de los componentes (antígeno o anticuerpo) marcado con una enzima e insolubilizado sobre un soporte (inmunoadsorbente) la reacción antígeno-anticuerpo quedará inmovilizada y, por tanto, será fácilmente revelada mediante la adición de un sustrato especifico que al actuar la enzima generará un color cuantificable mediante el uso de un espectrofotómetro o un colorímetro.

Existen diversas modalidades de ELISA. En la ***Figura 24*** se muestra un resumen de las mismas.

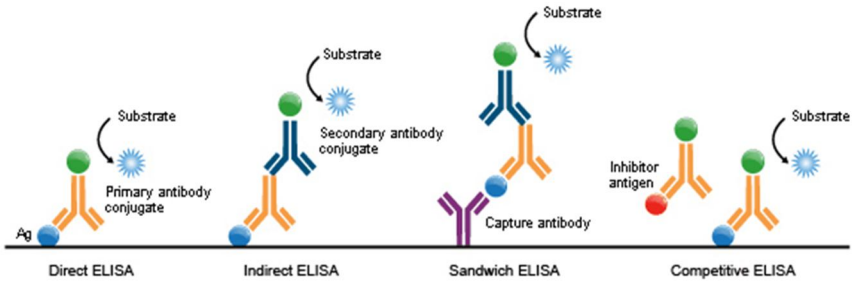


Figura 24. *Tipos de Métodos ELISA*[163]

Finalmente, uno de los escasos métodos propuestos en la literatura científica que analizan varias familias de EDCs es el de Schlumpf y col.[152] que aplican la LLE con hexano como extractante a la determinación de PBs y BPs en leche madura, utilizando tanto cromatografía de gases como de líquidos acoplada a espectrometría de masas como técnica de análisis.

[163] http://www.abnova.com/support/resources.asp?switchfunctionid=%7B70196CA1-59B1-40D0-8394-19F533EB108F%7D.

CAPÍTULO I

Materiales y métodos

En este capítulo se describen las disoluciones, reactivos, material de laboratorio, instrumentación y programas informáticos utilizados para la realización de la presente investigación. También, se presentan los tratamientos estadísticos empleados para la calibración y validación de los métodos analíticos desarrollados.

1. REACTIVOS Y DISOLVENTES

- ✓ **Patrones.** En la ***Tabla I.1*** se muestran los patrones empleados a lo largo del desarrollo de la fase experimental de la presente Tesis Doctoral.

Tabla I.1. *Analitos, abreviaturas, marcas comerciales y números CAS*

Nombre	Abreviatura	Número CAS	Marca Comercial
Metilparabeno	MPB	99-76-3	Alfa Aesar
Etilparabeno	EPB	120-47-8	Alfa Aesar
Propilparabeno	PPB	94-13-3	Alfa Aesar
Butilparabeno	BPB	94-26-8	Alfa Aesar
Benzofenona 1	BP-1	131-56-6	Sigma-Aldrich
Benzofenona 2	BP-2	131-55-5	Sigma-Aldrich
Benzofenona 3	BP-3	131-57-7	Sigma-Aldrich
4-Hidroxibenzofenona	4-OH-BP	1137-42-4	Sigma-Aldrich
Benzofenona 6	BP-6	131-54-4	Alfa Aesar
Benzofenona 8	BP-8	131-53-3	Sigma-Aldrich
Bisfenol A	BPA	80-05-7	Sigma-Aldrich
Monoclorobisfenol A	Cl-BPA	---	---
Diclorobisfenol A	Cl_2-BPA	---	---
Triclorobisfenol A	Cl_3-BPA	---	---
Tetraclorobisfenol A	Cl_4-BPA	79-95-8	Sigma-Aldrich
Bisfenol A deuterado	BPA-d_{16}	96210-87-6	Isotec
Etilparabeno-ring-$^{13}C_6$	EPB-$^{13}C_6$	EC200-662-2	Sigma-Aldrich
Etilparabeno-d_5	EPB-d_5	126070-21-1	Toronto Research Chemicals Inc
Benzofenona-d_{10}	BP-d_{10}	22583-75-1	Sigma-Aldrich

- ✓ Metanol grado gradiente (Hipersolv) y LC-MS (Fluka).
- ✓ Agua mili-Q y agua grado LC-MS (Fluka).
- ✓ Acetonitrilo LC-MS (Fluka).
- ✓ Amoniaco, aditivo para LC-MS (Fluka).
- ✓ Acetato amónico (J.T. Baker).
- ✓ Etanol, grado gradiente (Fluka).
- ✓ PSA (amina primaria/secundaria) (Scharlab).
- ✓ C18 granel (SCHARLAB).
- ✓ Cloruro de sodio (Panreac).
- ✓ Sulfato de sodio (J.T. Baker).
- ✓ Ácido fórmico (Sigma-Aldrich).
- ✓ Ácido clorhídrico (Panreac).
- ✓ Disolución de precipitación de proteínas y grasas: compuesta por 9.10 g de acetato de cinc hidratado (Sigma-Aldrich), 5.46 g de ácido fosfotúngstico hidratado (Sigma-Aldrich) y 5.8 mL de ácido acético glacial (Panreac) y hasta 100 mL de Agua Milli-Q.

2. MATERIAL DE LABORATORIO

Para el desarrollo de todo el trabajo experimental, se utilizaron los siguientes materiales de laboratorio:

- ✓ Matraces aforados clase A, de diferentes volúmenes.
- ✓ Pipetas graduadas y aforadas clase A de diferentes capacidades.
- ✓ Viales de vidrio para inyección cromatografía de 2 y 1.5 mL.
- ✓ Tapones de rosca y capsula para viales cromatográficos.
- ✓ Tubos de centrifuga de 100 mL de capacidad.
- ✓ Tubos de Falcon de 45 mL de capacidad.
- ✓ Tubos de cónicos de 10 mL de capacidad.
- ✓ Tubos Eppendorf de 1.5 y 2 mL de capacidad.
- ✓ Twister ^{TM}0.5 mm x 20 mm
- ✓ Encapsulador y descapsulador para viales cromatográficos.
- ✓ Micro pipetas de 250 y 1000 µL.
- ✓ Botellas de vidrio graduadas de 1 L Pírex®.
- ✓ Botellas de vidrio ámbar de 100mL de capacidad.
- ✓ Vasos de precipitados de diferentes capacidades.

- ✓ Cinta de teflón de 75 µm x 0.5 µm de dimensiones

- ✓ Nevera capaz de alcanzar y mantener una temperatura de 4.0 ± 1.0 ^{o}C.

- ✓ Congelador capaz de alcanzar y mantener una temperatura de -80.0 ± 1.0 ºC.

La limpieza y mantenimiento de todo material empleado a lo largo del trabajo experimental se realizó con mezcla crómica o HNO_3 al 50% (v/v) y enjuagando posteriormente con abundante agua desionizada antes de su utilización.

3. INSTRUMENTACIÓN

Los instrumentos y aparatos empleados para el desarrollo de los experimentos realizados en la presente Tesis Doctoral fueron:

- ✓ Cromatógrafo Waters Acquity UPLC™ acoplado a espectrómetro de masas triple cuadrupolo Waters H-Class-Xevo TQS™. Provisto de:
  - o Bomba: Quaternary Solvent Manager. Acquity UPLC H Class. Waters.
  - o Inyector: Sample Manager-FTN. Acquity UPLC. Waters.
  - o Detector: Xevo TQ-S. Waters.

- ✓ Cromatógrafo de gases Agilent 7890 GC acoplado a un espectrómetro de masas triple cuadrupolo, provisto de:
  - o CIS-4 programmable temperature vaporization (PTV) inlet.
  - o Inyector: muestreador automático (MPS).
  - o Detector: Agilent 7000B.

- ✓ Sonda de ultrasonidos Digital Sonifier S450D (BRANSON) provista de:
  - o Convertidor tipo 102.
  - o Resonador estándar de 12.7 mm de diámetro.
  - o Punta enroscable de 12.7 mm de diámetro.
  - o Punta enroscable de 12.7 mm de diámetro.
  - o Sonda de temperatura.
  - o Micropunta enroscable de diámetro final 3 mm.

- ✓ Liofilizador SCANVAC CoolSafe™.

- ✓ Concentrador a vacío SCANVAC CoolSafe™ ScanSpeed MaxiVac.

- ✓ Balanzas analíticas Mettler PJ 360 Delta Range y Mettler AE 163 y AND GX-400.

- ✓ pH-metro digital CRISON. Modelo Micro-pH 2000 y pH-metro digital CRISON, modelo pH/mV-meter digit 501, provistos de electrodo combinado de vidrio y plata/cloruro de plata.

- ✓ Estufa de secado HEAREUS de 50-300^{o}C.

- ✓ Baño de ultrasonido SELECTA de 1000 mL de capacidad.

- ✓ Centrífuga Hettich Zentrifugen, Universal 32.

- ✓ Placa agitadora–calefactora SELECTA AGIMATIC-N.

- ✓ Placa agitadora, calefactora de 9 posiciones OVAN.

- ✓ Agitador tipo vortex y agitador de brazos multipuesto SELECTA.

4. PROGRAMAS INFORMÁTICOS

- ✓ Software MassLynx V.4.1 SCN.803 Programa de gestión y tratamiento de los datos obtenidos con el cromatógrafo Waters Acquity UPLC™ H-Class-Xevo TQS™.

- ✓ Paquete Microsoft® Office: Word®, Excel® y PowerPoint® 2007.

- ✓ Software de tratamiento estadístico Statgraphic Plus, versión 5.0.

- ✓ MassHunter B.03.02 Programa de gestión y tratamiento de los datos obtenidos con el cromatógrafo de gases Agilent 7890.

5. CROMATOGRAFÍA DE GASES Y DE LÍQUIDOS ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM

De la bibliografía consultada se desprende que, tanto la cromatografía de líquidos (LC) como la de gases (GC) con diferentes modalidades de detección, son las técnicas más empleadas para separar y detectar los compuestos seleccionados en la presente Tesis Doctoral en matrices de interés biológico a nivel traza.

Los EDCs seleccionados generalmente son compuestos poco volátiles que contienen grupos funcionales ionizables, con coeficientes de partición octanol/agua moderados. En consecuencia, la técnica seleccionada suele ser la cromatografía de líquidos de alta resolución (LC o UHPLC) aunque también en algunos casos se ha optado por la cromatografía de gases previa derivatización de los analitos para aumentar su volatilidad.

La evolución de la cromatografía de líquidos acoplada a espectrometría de masas, la ha convertido en la técnica de elección habitual, evitando así las desventajas inherentes al tratamiento de muestra en cromatografía de gases y los procesos de derivatización previos a la inyección cromatográfica[164,165]. Habitualmente se ha empleado la cromatografía de fase inversa (RPLC) con columnas estándar C8 ó C18, de < 3 μm de tamaño de partícula, con 2.1 mm de diámetro interno (i.d.) y 100 mm de largo, las cuales toleran flujos menores (200–250 μL min^{-1}), hasta un máximo de 500 μL min^{-1}, especialmente si se usan interfases de tipo ESI en el espectrómetro de masas.

En la ***Tabla I.2*** se muestran las fases estacionarias empleadas durante el desarrollo de los métodos contemplados en esta Memoria.

[164] Ballesteros O., Zafra A., Navalón A., Vílchez J.L. *Journal of Chromatography A*, 2006, 1121:154–162.

[165] Hernando M., Mezcua M., Gomez M., Malato O., Aguera A., Fernandez Alba A. *Journal of Chromatography A*, 2004, 1047:129–135.

Tabla I.2.*Características de las columnas cromatográficas usadas en esta Tesis Doctoral*

Nombre	Relleno	Dimensiones
Acquity UPLC BEH™	columnaC_{18}	100 mm × 2.1 mm i.d., 1.7 μm de tamaño de partícula
Acquity UPLC CORTEX™	columnaC18	50 mm × 2.1 mm i.d., 1.6μm de tamaño de partícula
HP-5MS capillary™	5% diphenyl 95% dimethylpolysiloxane	30 m × 0.25 mm i.d.; 0.25 μm

En lo que respecta a la fase móvil, en el caso de la cromatografía de líquidos, los disolventes de uso común en la separación los compuestos de interés son agua, metanol y acetonitrilo. Para la detección por espectrometría de masas, se utilizan como aditivos para incrementar la eficiencia en la ionización, electrolitos que no dejan residuo como acetato de amonio, amoníaco, ácido acético, ácido fórmico o trialquilaminas, especialmente en la interfase ESI. Para cromatografía de gases se ha usado helio (99.9999%) como gas portador y como gas de colisión (99.999%).

Para el desarrollo de los métodos analíticos desarrollados en esta memoria, se emplearon los equipos de cromatografía de líquidos y gases acoplados a espectrometría de masas que se muestran en las ***Figuras I.1***. y ***I.2.***

Figura I.1. *Equipos de cromatografía de líquidos acoplado a espectrometría de masas empleado. Cromatógrafo Waters Acquity UPLC™ H-Class–Xevo TQS™*

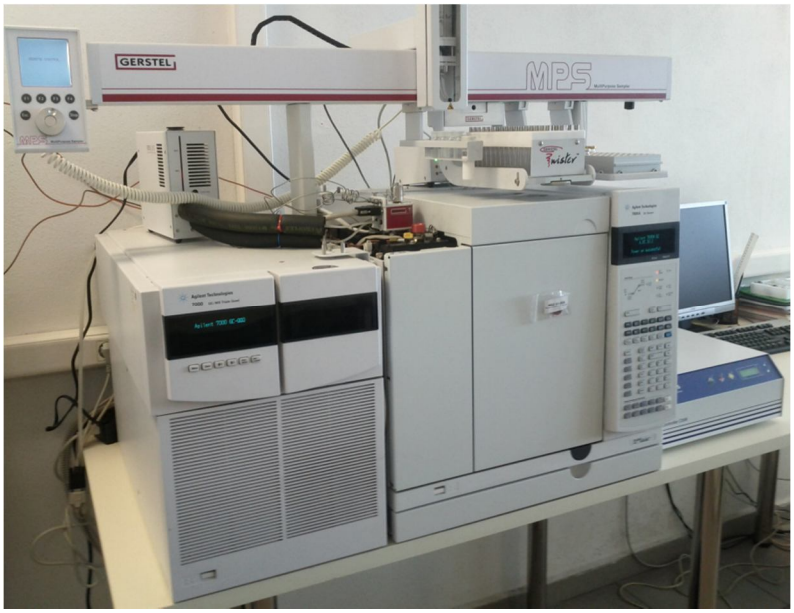


Figura I.2. *Equipos de cromatografía de gases acoplado a espectrometría de masas empleado. Cromatógrafo Agilent 7890 Triple Quad*

Dado que tanto la cromatografía de líquidos como la espectrometría de masas de simple cuadrupolo son técnicas muy extendidas y ampliamente utilizadas, sus fundamentos teóricos son de sobra conocidos por cualquier usuario de la técnica o personal formado en química y no serán objeto de explicación. Sin embargo, si se realizará un breve recorrido por los fundamentos básicos de la espectrometría de masas en tándem.

La forma más simple de entender un Espectrómetro de Masas en tándem sería visualizarlo como la combinación en línea de dos espectrómetros simples. El proceso consistiría en emplear dos sistema de generación de iones y dos sistemas de filtrado de iones (cuadrupolos), o dos procesos de aislamiento (trampa de iones) dentro de un solo instrumento. El primer espectrómetro (o sistema de filtro) es empleado para realizar la fragmentación de la molécula neutra del analito de interés y seleccionar, en función de su relación masa/carga (*m/z*), un único fragmento (ion precursor), que debe ser característico del propio analito que pretendemos analizar. El ion seleccionado pasa a una segunda zona (cámara de colisión) en la que se induce una nueva fragmentación, generalmente producida por la colisión con un gas neutro (gas de colisión en los equipos de triple cuadrupolo) o con el propio gas portador (trampa de iones), este proceso se denomina disociación inducida por colisión (collision-induced dissociation, CID). El segundo espectrómetro separa y analiza los fragmentos (iones producto) generados por la disociación del ion precursor en función de su relación *m/z*. El espectro de MS/MS resultante consiste en los iones productos formados a partir del ion precursor seleccionado. Este proceso puede ser continuado de manera que nuevamente se puede aislar uno de los iones producto y nuevamente ser fragmentado, de modo que se generen nuevos iones producto, dando lugar a la Espectrometría de Masas multietapa (MS^n).

En la ***Figura I.3*** se muestra un esquema del funcionamiento de un espectrómetro de masas de triple cuadrupolo.

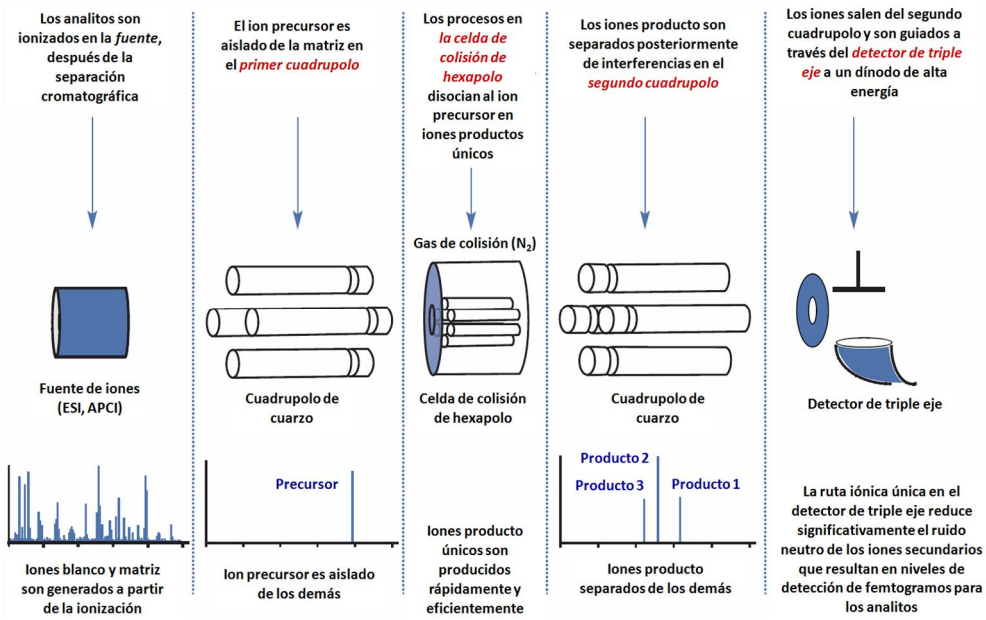


Figura I.3. *Esquema del funcionamiento de un espectrómetro de masas de triple cuadrupolo*

A. Los analitos son ionizados en la fuente de iones después de la separación cromatográfica.

B. Los iones precursores seleccionados que han sido generados en la fuente son aislados de la matriz y del resto de iones en el primer cuadrupolo.

C. Los procesos de colisión en la celda de colisión disocian el ion precursor en iones productos únicos.

D. Los iones producto generados son separados y aislados en función de su relación *m/z*.

E. Los iones que salen del tercer cuadrupolo son detectados por el electrón-multiplicador y registrados por el software.

La principal diferencia entre los componentes de un espectrómetro de masas de simple cuadrupolo y de triple cuadrupolo es la inclusión de la celda de colisión que actúa como una nueva fuente de ionización produciendo la fragmentación del ion precursor mediante CID. En dicho proceso el ion precursor entra en la celda de colisión (o en el caso de las trampas de iones el ion precursor es confinado en la trampa) que contiene una alta presión de un gas inerte (argón, helio, nitrógeno, etc.). En el interior de la celda, el ion precursor sufre repetidos impactos con el gas de colisión, aumentando la energía potencial del ion, hasta que se alcanza la energía umbral de fragmentación y se generan los iones productos. Los tipos de fragmentación que se pueden producir pueden variar considerablemente con el tipo de ion precursor y la cantidad de energía aplicada. A bajas energías (cercana a la energía umbral), las reacciones de fragmentación se limitan a pérdidas neutras (H_2O, MeOH, CO, CO_2, MeCN), que no se consideran estructuralmente significativas, a menos que puedan aportar información sobre los grupos funcionales en la molécula. A altas energías, se pueden obtener reacciones más complejas, llegando a producirse ruptura de enlaces C-C, produciéndose la fragmentación de la molécula y generándose los iones producto.

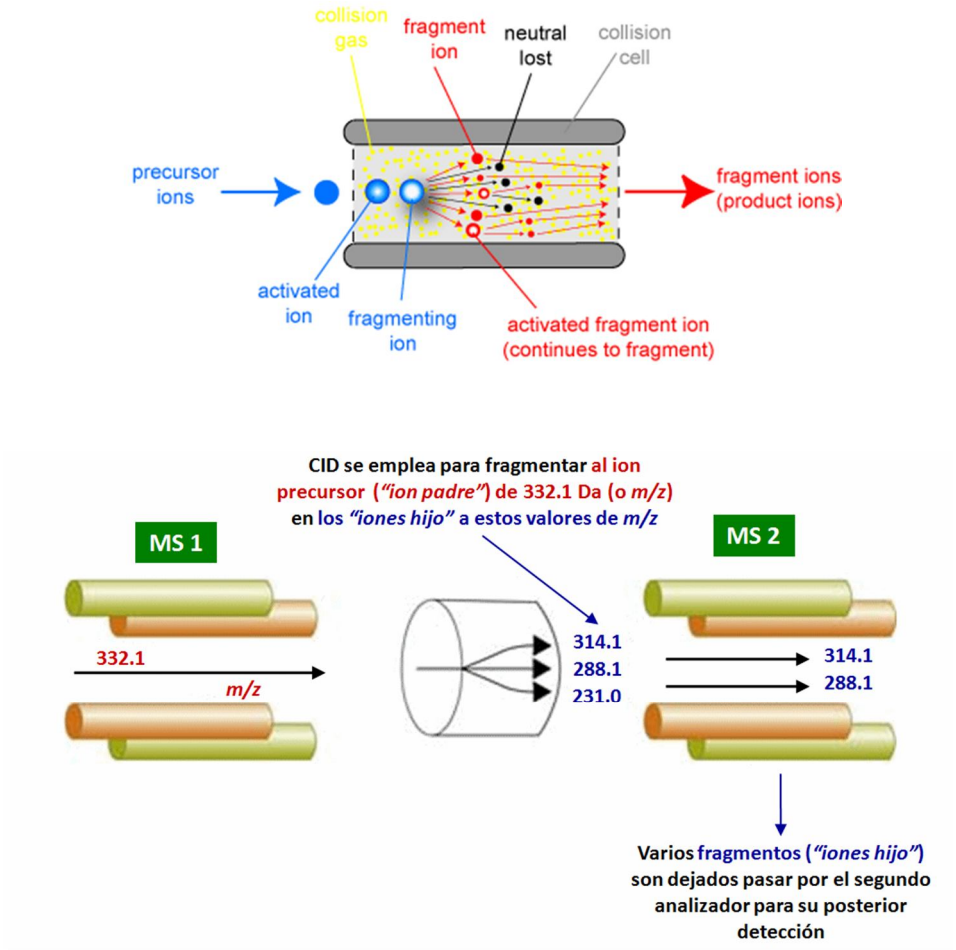


Figura I.4. *Visión esquemática de una celda de colisión de un espectrómetro de triple cuadrupolo*

Los modos de trabajo en Espectrometría de Masas de triple cuadrupolo son los que se enumeran a continuación:

- ✓ ***Product ion scan.*** En este modo, el primer cuadrupolo (Q1) selecciona un ion que es introducido en la celda de colisión (q2) donde se induce la disociación mediante la colisión con el gas inerte que hay en su interior y se generan los iones productos. Posteriormente, todos los fragmentos son analizados por el tercer cuadrupolo (Q3) mediante la realización de un barrido o scan. Como resultado se obtiene un espectro MS/MS completo,

similar a los obtenidos en modo *full-scan* en los espectrómetros de masas de simple cuadrupolo.

- ✓ ***Precursor ion scan.*** En este modo, el Q3 está fijado para únicamente medir una relación *m/z* seleccionada, mientras el Q1 realiza un barrido de todas las masas. Solo se obtiene una respuesta cuando el Q1 selecciona una masa que genere la masa seleccionada en el Q3. En el espectro obtenido aparecen únicamente los iones precursores que generan dicho fragmento.

- ✓ ***Neutral loss scan*.** En este caso mientras el Q1 realiza un barrido de masas, el Q3 lo realiza también pero con una determinada diferencia de masas con respecto al Q1, de modo que únicamente se obtiene respuesta para aquellos iones en el Q1 que sufran una pérdida de masa igual a la diferencia establecida entre los cuadrupolos.

- ✓ ***Selected reaction monitoring (SRM).*** Este modo de trabajo es similar al SIM (*selected ion monitoring*) empleado en los sistemas de simple cuadrupolo. El Q1 y el Q3 están fijados para analizar un único ion precursor e ion producto que son característicos de la sustancia que pretendemos analizar, mejorándose la selectividad y la sensibilidad de los análisis. Este modo de trabajo sólo se emplea para el análisis de sustancias previamente pre-seleccionadas (*target*), puesto que requiere que los iones precursores y producto sean conocidos. Este modo es el más adecuado para análisis cuantitativos. A cada análisis SRM se le conoce como transición, el análisis simultáneo de varias transiciones en un mismo análisis se denomina *Multiple Reaction Monitoring* (MRM). En este modo, la confirmación de los positivos obtenidos se realiza midiendo una segunda transición, en la cual el ion producto seleccionado es diferente al primero. La relación entre las intensidades de la transición de cuantificación y la transición de confirmación es comparado frente a la relación de las intensidades de dichas transiciones de un patrón de control, si es igual (con una determinada tolerancia) la identidad de la sustancia puede ser inequívocamente confirmada.

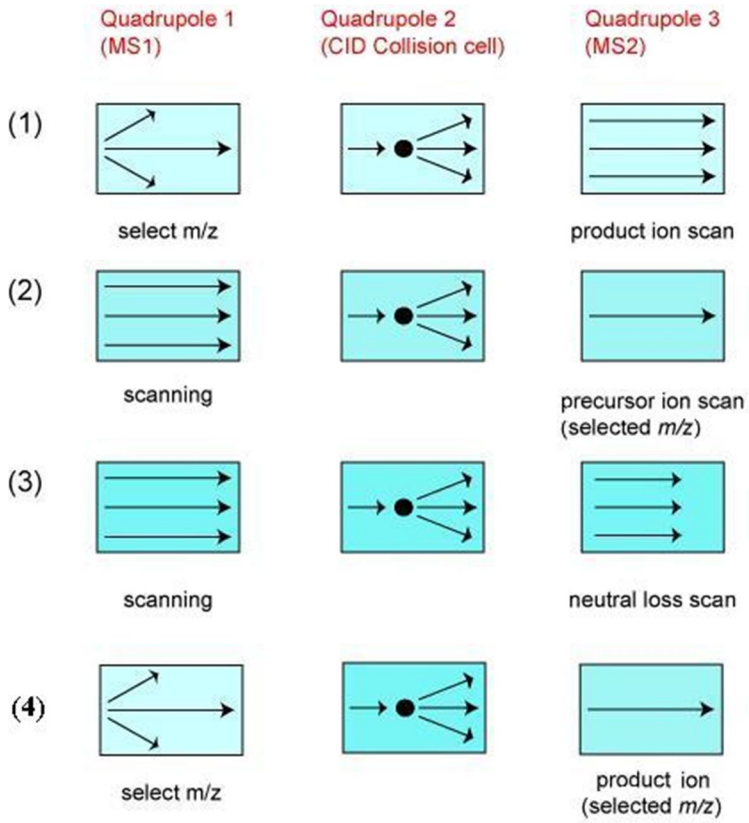


Figura I.5. *Modos de adquisición de un espectrómetro de masas de triple cuadrupolo*

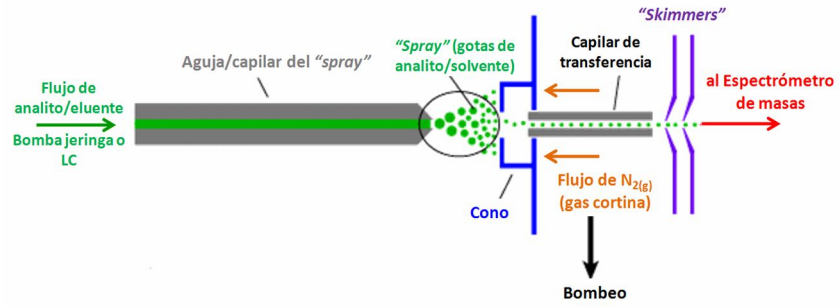
El paso previo a la entrada en el primer cuadrupolo de los compuestos es la ionización de los analitos. Los métodos de ionización más habituales son por impacto electrónico (EI), ionización química (CI), bombardeo rápido de átomos (FAB), ionización por *termospray* (TSP), ionización por *electrospray* (ESI) e ionización química a presión atmosférica (APCI).

A lo largo de esta Memoria se ha trabajado con los modos de ESI en el caso de la cromatografía de líquidos y EI en el caso de cromatografía de gases.

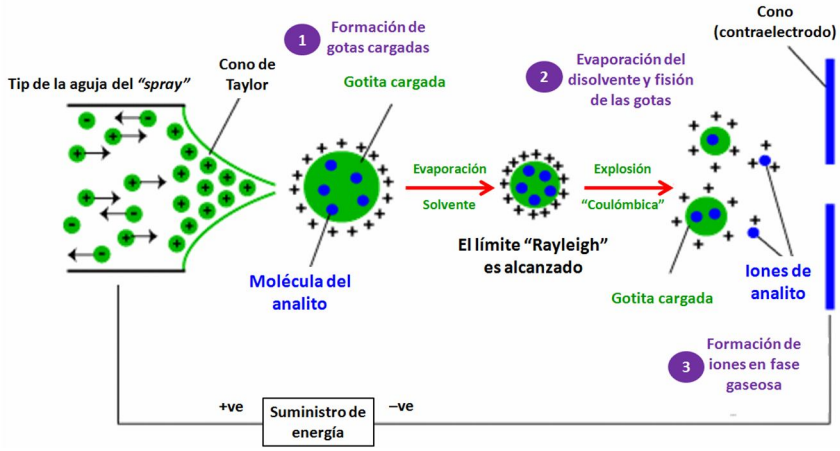
El método de ionización ESI es muy suave con muy poca energía residual retenida por el analito después de la ionización. Habitualmente no se produce fragmentación dentro de la fuente. Los compuestos de bajo peso molecular forman generalmente iones monocargados por pérdida o ganancia de un

protón y las biomoléculas de alto peso molecular y polímeros forman iones multicargados. Se pueden formar aductos o multímeros con el disolvente. Los aductos de NH_4^+ (M+18), Na^+ (M+23), y K^+ (M+39) son muy comunes. La ***Figura I.6*** muestra un esquema de la interfase ESI.

(a)



(b)



(c)

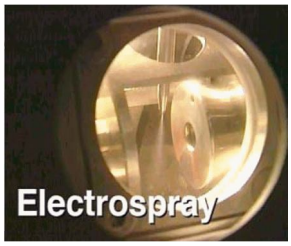


Figura I.6. *Método de ionización "electrospray" (ESI).* ***(a)*** *Estructura de la interfase.* ***(b)*** *Mecanismo de ionización.* ***(c)*** *Imagen de la formación del spray".*
(Tomado de http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html)

El espectrómetro de masas Xevo TQS™ que está acoplado al cromatógrafo UPLC™ H–Class de Waters, se caracteriza por tener una interfase *ESI ortogonal Z–Spray*™. El *spray* es generado de la forma tradicional, pero con la diferencia de que no todo este *spray* es arrastrado a través del orificio del cono de muestra, ya que debido a la diferencia de presión y voltaje, sólo la porción cargada (que contiene al analito) ingresa al analizador de masas, que se encuentra en posición perpendicular a la trayectoria del *spray*. El resto, que debe contener a componentes de la matriz no cargados, va al desecho (escape de gases). Este sistema permite disminuir/eliminar en gran extensión la cantidad de componentes de la matriz que han sido coextraidos, y que son responsables del efecto matriz.

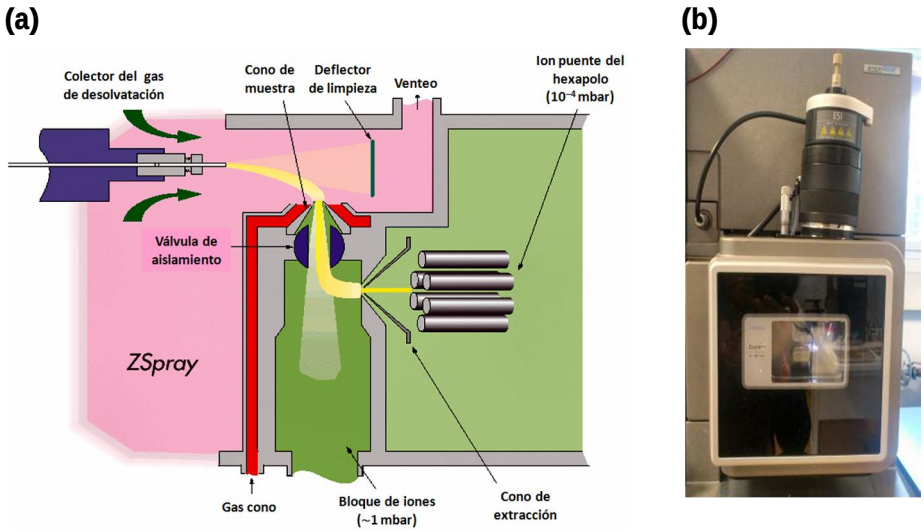


Figura I.7.(a) *Esquema;* ***(b)*** *imágenes, de la interfase ESI ortogonal Z-spray™. (Tomado del Curso "Introduction to LC/MS" de Waters®)*

En general, la interfase ESI presenta una gran sensibilidad y selectividad para el análisis de los compuestos seleccionados en muestras complejas. El bisfenol A, derivados y los parabenos se suelen analizar usando el modo de ionización negativo, mientras que la mayoría de las benzofenonas en el modo positivo, empleando aditivos apropiados en las fases móviles para mejorar la eficiencia de la ionización.

En cuanto al acoplamiento y la ionización en cromatografía de gases con espectrometría de masas, por su concepción elemental, se trata de algo mucho más sencillo que el caso de la cromatografía de líquidos, ya que no es necesario interponer una interfase específica para cambiar el estado físico de la muestra. La muestra procedente del proceso cromatográfico entra en el espectrómetro ya en estado gaseoso. El espectrómetro de masas empleado en la presente Tesis Doctoral ha sido el de triple cuadrupolo con ionización por impacto electrónico (EI). Este modo de ionización es el de uso más común en los equipos de espectrometría de masas acoplados a cromatografía de gases. En este tipo de ionización, la energía con la que un electrón es acelerado puede, al chocar con el analito, provocar la formación de iones negativos, positivos o la fragmentación con producción de especies neutras e iónicas. La ***Figura I.8*** muestra el esquema básico de este tipo de ionización.

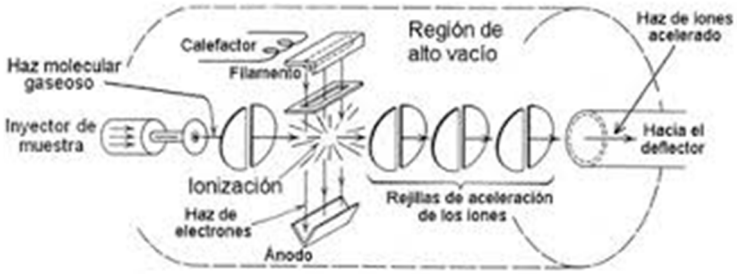


Figura I.8. *Ionización por impacto electrónico*

La GC-MS/MS (QqQ), debido a su alta selectividad y sensibilidad, es una importante herramienta de análisis ampliamente utilizada en la determinación de EDCs en matrices de diversa naturaleza (alimentarias, biológicas y medioambientales). Sin embargo, una de las principales limitaciones que tradicionalmente ha presentado esta técnica es el requerimiento de que los analitos sean térmicamente estables y suficientemente volátiles, teniendo que recurrirse a tratamientos de muestra complejos y a etapas de derivatización. Por este motivo, en los últimos años se ha impuesto el uso de la cromatografía de líquidos.

6. TRATAMIENTO ESTADÍSTICO DE LOS DATOS ANALÍTICOS

6.1 Optimización de variables. Diseño de experimentos

El empleo de técnicas de extracción de microcontaminantes en matrices de naturaleza compleja requiere de una optimización adecuada de todas las variables implicadas en cada caso. Del entendimiento de la naturaleza y las características del proceso de extracción resultará el establecimiento de las variables relevantes más influyentes que serán estudiadas en la optimización del método.

Si los factores involucrados en el análisis son independientes (lo cual es una situación poco habitual), la práctica más común es el *diseño univariante*. Sin embargo, este método clásico de optimización es largo, tedioso y presenta numerosos inconvenientes.

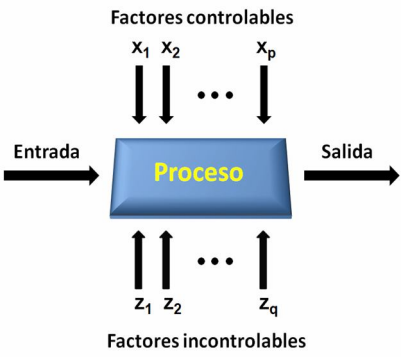


Figura I.9. *Esquema general de un proceso o sistema*[166].

Las interacciones entre variables suelen ser muy frecuentes y a veces son los efectos más importantes sobre la respuesta de un sistema, por lo que conocerlas es imprescindible para comprender su comportamiento. La solución, por lo tanto, puede consistir en variar más de un factor simultáneamente al realizar un nuevo experimento *(diseño multivariante)*, que de forma general se aplica a sistemas como el mostrado esquemáticamente en la ***Figura I.9***. En todos ellos se observa una o más variables experimentales dependientes (respuestas *(y)*) cuyo valor depende de una o más variables independientes controlables (factores *(x)*). Las respuestas también pueden estar influidas por otras variables que no son controladas por el experimentador *(z)*.

El diseño estadístico de experimentos es el proceso de planificar un experimento para obtener datos apropiados que puedan ser analizados mediante métodos estadísticos con el objeto de obtener conclusiones válidas y objetivas[166]. El diseño experimental ayuda a seleccionar la estrategia óptima para obtener la información buscada con el mínimo coste y evaluar los datos experimentales obtenidos, garantizando la máxima fiabilidad en las conclusiones que se obtengan.

El diseño experimental multivariante conduce a una planificación con menos experimentos proporcionando un conocimiento mayor del sistema o proceso en estudio[167]. Las herramientas quimiométricas que se aplican permiten el desarrollo de modelos matemáticos para describir el comportamiento global del sistema, determinar la relevancia estadística de los efectos independientes que están siendo investigados, así como establecer las interacciones. Si existen interacciones significativas entre variables, las condiciones óptimas indicadas por estudios univariantes serán muy diferentes de los resultados correctos de la optimización multivariante. A mayores interacciones, mayor será la diferencia.

En la optimización multivariante se siguen una serie de etapas ordenadas:

- ✓ Definición del problema y selección de las variables apropiadas y respuesta(s) a través de estudios de diagnóstico.
- ✓ Elección del diseño de experimentos.
- ✓ Selección de los niveles de variables y codificación.
- ✓ Ajuste matemático del modelo.

[166] Montgomery D.C.. Diseño y Análisis de Experimentos, 2da ed. Limusa Wiley, México, 2002, 686.
[167] Ferreira S.L.C., Bruns R.E., Ferreira H.S., Matos G.D., David J.M., Brandão G.C., Da Silva E.G.P., Portugal L.A., Dos Reis P.S., Souza A.S., Dos Santos W.N.L. *Analytica Chimica Acta*, 2007, 597:179–186.

- ✓ Revisión de la idoneidad del modelo.
- ✓ Análisis del modelo y estimación del efecto.
- ✓ Determinación de los óptimos.
- ✓ Revisión de la robustez.

A. Definición del problema y selección de las variables apropiadas y respuesta(s) a través de estudios de diagnóstico.

En general, las variables pueden ser de dos tipos:

- ✓ ***Variables continuas.*** Representan mediciones en una escala continua, como la presión, temperatura y tiempo de extracción.
- ✓ ***Variables categóricas o cualitativas.*** Representan información sobre características discretas, como el número de ciclos de extracción.

La distinción entre ambos tipos de variable es importante cuando se quieren añadir puntos centrales al diseño, para aplicar posteriormente, pruebas para la presencia de curvatura, y para establecer la validez de un modelo simple de efectos lineales. En relación a la elección del parámetro de respuesta (s), habitualmente se consideran la recuperación, la eficiencia de la extracción o el factor de enriquecimiento. Para la optimización de un método analítico, puede seleccionarse una o más respuestas si se observa que la variación de la respuesta en función de las variables es muy pequeña. Si se debe considerar más de una respuesta, se pueden determinar prioridades entre ellas. En el caso de los análisis realizados en esta Tesis Doctoral, se observó que la evaluación del porcentaje de recuperación del analito es suficiente para cumplir los objetivos fijados.

Cuando el proceso de extracción involucra un gran número de variables, es necesario identificar primero aquellas que presentan los efectos más importantes que puedan afectar significativamente la respuesta. Con este objeto se utilizan los *diseños de diagnóstico*, también llamados de cribado o *screening*. Estos diseños suelen aplicarse en las primeras fases de un proceso de optimización, cuando es probable que muchos de los factores inicialmente considerados tengan poco o ningún efecto sobre la respuesta. Los factores que se identifican como importantes en esta primera etapa son investigados entonces con mayor detalle en diseños posteriores. Entre los diseños de diagnóstico destacan los diseños factoriales fraccionados[168], dentro de los cuales, el más utilizado es el *diseño Plackett-Burman* (diseño fraccionado de dos niveles).

B. Aplicación de métodos de superficie de respuesta en química analítica

La metodología de superficie de repuesta (RSM) se refiere a un grupo de técnicas estadísticas y matemáticas empleadas durante la optimización de una respuesta que es función de una serie de variables significativas. El modelado de estas superficies se realiza empleando un modelo empírico. Existe una gran versatilidad a la hora de describir la gran variedad de superficies de respuesta que se pueden ajustar dentro del dominio impuesto por los factores, ya que no sólo explican el efecto de cada factor sobre la respuesta del sistema, sino que además revelan la existencia de interacciones entre factores cuando éstas están presentes en un sistema.

En la mayoría de los casos inicialmente se desconoce la forma de la relación entre la respuesta y las variables independientes estudiadas. Por ello, en primer lugar se debe establecer una aproximación adecuada a la relación funcional real entre la ellas. En general, se emplea un polinomio de orden bajo en alguna región de las variables independientes. Si la respuesta se describe adecuadamente por una función lineal de las variables, se podrá emplear como

[168] Box G.E.P., Hunter J.S. *Technometrics*, 1961, 3:311–351.

función de aproximación un modelo de primer orden, mientras que si existe curvatura en el modelo, deberá emplearse un modelo de segundo orden.

Una vez ajustada la superficie de repuesta al modelo, y tras comprobarse que no existe falta de ajuste, se localiza mediante análisis canónico el punto estacionario (máximo, mínimo o punto de silla). Los modelos más empleados son los de segundo orden, con al menos tres niveles por factor y, si es posible, cumpliendo las premisas de ortogonalidad y rotabilidad. Estas técnicas se emplean cada vez más en química analítica, sobre todo en procedimientos de optimización de variables experimentales en la puesta a punto de las metodologías analíticas.

Esta metodología fue desarrollada por Box y colaboradores en los años 50[169,170]. Este término se originó en base a una perspectiva gráfica generada después del ajuste a un modelo matemático. Su utilidad es ya reconocida por lo que su aplicación en distintas áreas de la ciencia se ha extendido ampliamente a lo largo de los años. La RSM consiste en un grupo de técnicas matemáticas y estadísticas que se basan en el ajuste de modelos empíricos a datos obtenidos a partir de un diseño experimental. Para cumplir este objetivo, se emplean funciones lineales o polinomiales para describir el sistema estudiado, y adicionalmente, para explorar (modelar y desplazar) las condiciones experimentales hasta su optimización.

El modelo más simple que se puede emplear en la RSM se basa en una función lineal. Pero en este caso, la respuesta no presentará ninguna curvatura. Para evaluar la curvatura, se debe usar un modelo de segundo orden. Los *diseños factoriales de dos niveles* se emplean para estimar los efectos de primer orden, pero fallan en la evaluación de efectos adicionales, como los efectos de segundo orden. Si adicionalmente se desean estudiar las *interacciones entre las variables*, será necesario seleccionar modelos más complejos, donde hay que asegurarse que en el diseño experimental, todas las variables se estudien por lo

[169] Gilmour S.G. *Biometrics*, 200662:323–331.
[170] Bruns R.E., Scarminio I.S., Neto B.B. Elsevier, Amsterdam, Holanda, 2006.

menos a tres niveles. Con este objeto, se requieren de diseños de segundo orden simétricos, dentro de los que destacan: el *diseño factorial completo a tres niveles*, *diseño Box-Benhken*, *diseño compuesto central* y *diseño Doehlert*. Estos diseños difieren uno de otro en relación a la selección de los puntos experimentales, número de niveles por variables y número de experimentos y bloques. En esta Tesis doctoral se han empleado diseños factorial completo y Box-Benhken.

B.1. Diseño factorial completo a tres niveles. Es una matriz de experimentos que tiene una aplicación limitada en RSM cuando el número de factores es superior a 2, porque el número de experimentos que se requiere para este diseño (que se calcula por el expresión N = 3*k*, donde N es el número de experimentos y *k* es el número de factores) sería demasiado grande, con lo que se perdería la eficiencia en la modelación de funciones cuadráticas. La ***Figura I.10*** muestra la representación de este diseño para dos variables, que fue el que se empleó en la presente Memoria para la optimización del método de *clean-up* de un extracto de leche materna mediante una mezcla de dos adsorbentes usados comúnmente en SPE dispersiva. Asimismo, la ***Tabla I.3*** muestra la matriz experimental para la optimización de dos variables usando este diseño.

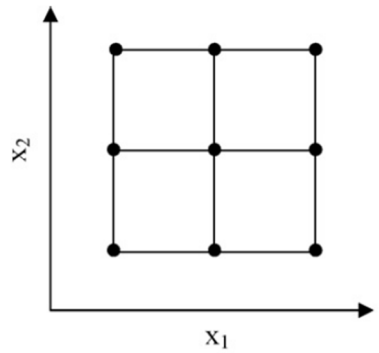


Figura I.10. *Diseño factorial completo de tres niveles para la optimización de dos variables (indicadas como x_1 y x_2)*[171]

[171] Almeida Bezerra M., Erthal Santelli R., Padua Oliveira E., Silveira Villar L., Escaleira L.A. *Talanta*, 200876:965–977.

Tabla I.3. Matriz de experimentos para el diseño factorial completo de tres niveles para dos variables (indicadas como x_1 y x_2).

Experimento	x_1	x_2	Experimento	x_1	x_2
1	–1	–1	6	0	1
2	–1	0	7	1	–1
3	–1	1	8	1	0
4	0	–1	9	1	1
5*	0	0			

* *Las réplicas del punto central no están incluidos en esta matriz*

Debido a que un diseño factorial de tres niveles para más de dos variables requiere de un mayor número de experimentos que difícilmente son manejables a nivel práctico, existen diseños que presentan un número menor de puntos experimentales, como por ejemplo el *diseño Box-Behnken*, que se basa en un diseño factorial a tres niveles para tres factores que está incompleto.

B.2. Diseño Box-Benhken (DBB). Este diseño constituye una alternativa al diseño central compuesto, muy ampliamente usado en química analítica. Es un tipo de diseño de segundo orden rotable o casi rotable que se basa en un diseño factorial de tres niveles incompleto[172]. La ***Figura I.11*** muestra el DBB para tres factores, que corresponden a los valores codificados en la ***Tabla I.4.*** En general, el número de experimentos (N) requerido para el desarrollo de un DBB es definido como $N = 2k\ (k - 1) + C_0$ (donde k es el número de factores y C_0 es el número de puntos centrales). Este diseño fue empleado en la presente Memoria para la optimización de la extracción mediante la técnica de ultrasonidos de los EDCs seleccionados en leche materna una vez liofilizada.

Este diseño consiste en tres partes de cuatro experimentos cada una. Dentro de cada parte, dos factores se arreglan como un diseño completo de dos niveles, mientras que el nivel del tercer factor es fijado a cero. La representación gráfica del DBB para tres factores puede ser interpretada

[172] Box G.E.P., Behnken D.W. *Technometrics*, 1960 2:455–475.

de dos maneras: primero, como un cubo que consiste de un punto central y el resto de puntos, cada uno en el medio de todas las aristas del cubo, como se observa en la ***Figura I.11a***; y segundo, un sólido geométrico que se forma a partir de tres diseños factoriales 2^2 unidos y un punto central, como se indica en la ***Figura I.11b***. Los puntos caen en la superficie de una esfera centrada en el origen del sistema coordinado.

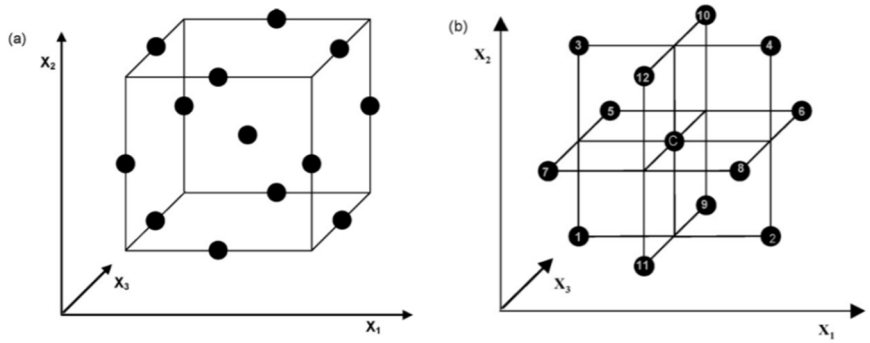


Figura I.11. *Diseño Box-Behnken para la optimización de tres variables (indicadas como x_1, x_2, yx_3)*[173]

Tabla I.4. *Matriz de experimentos para el diseño Box-Benhken para tres variables (indicadas como x_1, x_2, y x_3).*

Experimento	x_1	x_2	x_3	Experimento	x_1	x_2	x_3
1	–1	–1	0	8	1	0	1
2	1	–1	0	9	0	–1	–1
3	–1	1	0	10	0	1	–1
4	1	1	0	11	0	–1	1
5	–1	0	–1	12	0	1	1
6	1	0	–1	13	0	0	0
7	–1	0	1				

* *Las réplicas del punto central no están incluidos en esta matriz*

La comparación del diseño Box-Behnken con otros diseños de superficie de respuesta (compuesto central, Doehlert y diseño factorial

[173] Ferreira S.L.C., Bruns R.E., Ferreira H.S., Matos G.D., David J.M., Brandão G.C., da Silva E.G.P., Portugal L.A., do Reis P.S., Souza A.S., dos Santos W.N.L. *Analytica Chimica Acta*, 2007597:179–186.

completo de tres niveles) demuestra que el DBB es más eficiente que el diseño compuesto central, pero menos que el factorial de tres niveles, si se define la eficiencia de un diseño experimental como el número de coeficientes en el modelo estimado dividido por el número de experimentos.

Algunos autores han propuesto el *diseño Box-Behnken* como idóneo para estimar una superficie de respuesta adecuada para la optimización de métodos espectrométricos, electroanalíticos y cromatográficos. Se trata de uno de los diseños de segundo orden más usados en la actualidad. La ***tabla I.5*** presenta la estructura y la eficiencia de este diseño hasta un máximo de 8 variables.

Tabla I.5.*Diseño Box-Behnken para hasta 8 variables*

Variables *(k)*	Número de coeficientes *(p)*	Número de experimentos *(f)*	Eficiencia *(p/f)*
2	6	---	---
3	10	13	0.77
4	15	25	0.60
5	21	41	0.61
6	28	61	0.46
7	36	85	0.42
8	45	113	0.40

El diseño debe luego ajustarse a un modelo matemático. Por ejemplo, el diseño Box-Behnken para dos y tres variables puede describirse por los siguientes modelos:

Para dos variables: $Y = b_o + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2$

Para tres variables: $Y = b_o + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3$

donde b_o, b_1, b_2 … b_{23} son los coeficientes y X_1, X_2 y X_3 son las variables codificadas.

A continuación se debe examinar el modelo ajustado para asegurar que se adecua al comportamiento del sistema real. El modelo queda validado si muestra una buena capacidad de predicción. El método más potente para la validación del modelo es la aplicación del *análisis de varianza (ANOVA)*. ANOVA comprueba la adecuación del modelo de regresión en términos de la prueba de falta de ajuste (*lack-of-fit test*) y si los coeficientes estimados son influyentes. ANOVA puede comparar la variación debido al tratamiento (cambio en la combinación de los niveles de las variables) con la variación debido a errores aleatorios inherentes a las mediciones de las respuestas. A partir de esta comparación, es posible evaluar la significancia de la regresión empleada para prever las respuestas considerando las fuentes de varianza experimental[174]. Esta comparación es realizada a través del valor F, el cual es la proporción entre el cuadrado medio del modelo y el error residual. Si el modelo es un buen predictor de los resultados experimentales, el valor F debe ser mayor que el valor tabulado en un cierto número de grados de libertad y viceversa. En el trabajo realizado en esta Tesis Doctoral se empleó un 95% de nivel de confianza.

También se debe evaluar el coeficiente de determinación *(R^2)*, que mide cuantitativamente la correlación entre los datos experimentales y las respuestas predichas. Un valor de R^2 igual a 1.0 indica que la regresión se ajusta perfectamente a los datos.

Finalmente, también se puede validar el modelo de forma gráfica. El método gráfico más conocido es el *diagrama de Pareto* (***Figura I.12***). En este diagrama, las variables y las interacciones posibles aparecen en el eje *y*, mientras el valor del efecto estimado estandarizado es representado en el eje *x*. Estos efectos, para cada variable, aparecen como una barra horizontal, cuya longitud es proporcional al valor absoluto del efecto estimado, mientras que también se incluye una línea vertical de referencia que corresponde al intervalo de confianza especificado (95% habitualmente). El efecto se considera significativo si su valor sobrepasa esta línea de referencia mientras que los signos positivo y negativo revelan los casos cuando la respuesta es disminuida

[174] Stalikas C., Fiamegos Y., Sakkas V., Albanis T. *Journal of Chromatography A*, 2009, 1216:175–189.

o incrementada, respectivamente, cuando se pasa del nivel más bajo al más alto de una variable específica.

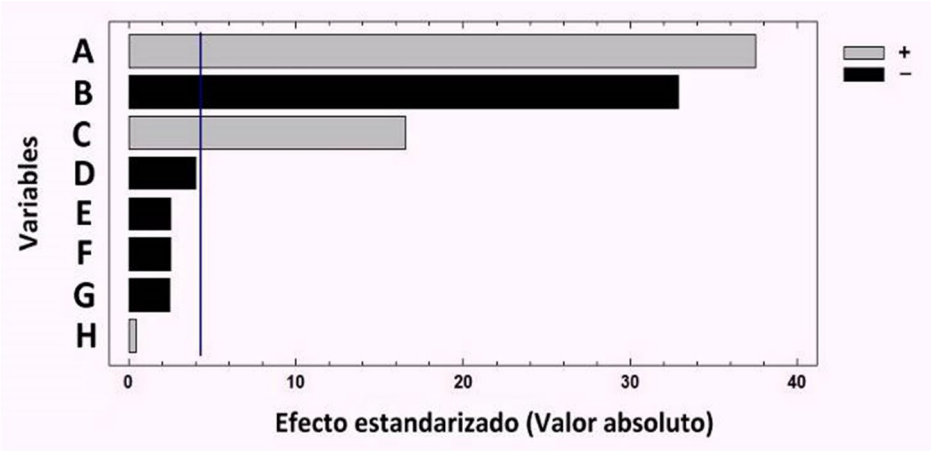


Figura I.12. *Diagrama de Pareto. La línea vertical en el diagrama define el 95% del nivel de confianza, y permite determinar que en este caso, solamente las variables A, B, C ejercen una influencia estadísticamente significativa en la respuesta*

6.2. Calibración analítica

La calibración, como etapa integrante del proceso analítico, es de gran importancia y sólo se puede obtener una buena exactitud en los resultados si se aplican buenos métodos de calibración. El proceso de calibración consta de dos etapas:

- ✓ **Etapa de calibración**. En ella se establece el modelo que relaciona la variable dependiente (señal analítica) con la variable independiente (concentración): ***Señal analítica = f (concentración)***

- ✓ **Etapa de predicción.** Consiste en obtener las variables independientes, es decir, las concentraciones, de una o más muestras problema a partir del valor obtenido para la variable dependiente. El valor de la variable independiente correspondiente a muestras patrón junto con las sensibilidades, permiten predecir los valores de estas variables en las muestras problema.

El modelo de calibración seleccionado en la presente Tesis Doctoral fue el lineal de regresión univariante por mínimos cuadrados, donde la variable independiente (concentración) se relaciona con la respuesta (señal instrumental) mediante una relación lineal de tipo $\boldsymbol{y = a + b \cdot x}$, donde ***y***es la variable dependiente, ***x*** la independiente, y ***a*** y ***b*** dos parámetros estimados a partir de los datos experimentales.

En todos los casos se ha estudiado además la homogeneidad de varianzas, es decir, la condición de homocedasticidad, a partir ***del análisis gráfico de los residuos***[175].

6.2 Validación del método analítico. Cálculo de los parámetros de calidad

La validación de cualquier método analítico debe realizarse a través de diferentes pruebas de laboratorio: *"La validación de un proceso analítico es el procedimiento por el cual éste es establecido a través de estudios de laboratorio, de modo que la información que de él se derive sea útil para cumplir los requerimientos necesarios de los problemas analíticos en los que será aplicado"*[176].

Son numerosas las razones que justifican la necesidad de validar un método analítico. Entre ellas, caben destacar requerimientos regulatorios, buenas prácticas de laboratorio y control de calidad. El *Código de Regulaciones Federales* (CFR) 311.165c establece explícitamente que *"la exactitud, sensibilidad, especificidad, y reproducibilidad de los métodos analíticos empleados deberán ser establecidos y documentados"*.

La validación implica en primer lugar la verificación de la exactitud de los resultados que genera un método analítico. La exactitud se define por la Norma ISO 5725 como *"el grado de concordancia entre el resultado de un ensayo y un*

[175] Boqué R., Rius F.X., Avances en Quimiometría Práctica, Servicio de publicaciones e intercambio científico de la Universidad de Santiago de Compostela. *Santiago de Compostela, España*, 1994 150–157.

[176] USP 32. General Chapter 1225. Validation of Compendial Methods. United States Pharmacopeia XXXII. Rockville: United Pharmacopoeial Convention, Inc., 2009.

valor de referencia aceptado"[177]. La exactitud combina dos propiedades complementarias: la veracidad y la precisión de los resultados. La veracidad se logra al eliminar los errores sistemáticos, mientras que la precisión se obtiene al minimizar los errores aleatorios. Para completar la validación de un método de análisis es necesario establecer otros parámetros de calidad del mismo, dentro de los cuales podemos incluir: rango lineal, linealidad, sensibilidad, selectividad, límites de detección y de cuantificación.

A. Evaluación de la exactitud

La exactitud de cualquier método analítico debe ser evaluada en términos tanto de veracidad como de precisión.

Veracidad. Se define como *"el grado de concordancia entre la media aritmética de un conjunto de resultados y el valor aceptado o de referencia"*[177]. Su evaluación consiste en estimar la concentración media del analito en una muestra y compararla con el valor verdadero empleando el protocolo estadístico apropiado. Ya que es imposible conocer el valor verdadero de una especie química en una muestra, es necesario obtener un valor verdadero convencional o aceptado para emplearlo como valor de referencia. Este último puede ser obtenido mediante dos estrategias: empleando un estudio interlaboratorio o un estudio intralaboratorio. Dentro de un laboratorio están disponibles dos alternativas para demostrar la veracidad de un método analítico: el uso de materiales certificados de referencia y la comparación con métodos de referencia. Antes de realizar las experiencias necesarias para demostrar la veracidad del método de análisis desarrollado, es necesario evaluar si los constituyentes de las muestras sobre las que se aplicará el método generan errores sistemáticos constantes o proporcionales sobre la respuesta analítica.

[177] Norma ISO 5725. Partes 1 y 2. Exactitud (Veracidad y Precisión). International Organization for Standarization, Ginebra, 1994.

El *"efecto matriz"* puede causar un error sistemático proporcional, manifestándose con un cambio en las pendientes de las rectas de calibrado con patrones y en presencia de matriz. Para detectar este efecto, se deben comparar las pendientes de ambas rectas de calibrado[178] mediante un test de la *t de Student*. Las soluciones posibles para reducir/compensar el *"efecto matriz"* consisten en el uso de patrones internos y la preparación de rectas de calibrado de acuerdo al método de adición de patrón o la calibración en matriz, *matrix-matched calibration*, los cuales se aplican para matrices complejas que no pueden ser imitadas artificialmente. Ninguna de estas soluciones ofrece garantías totales de que el error sistemático por efecto matriz se haya eliminado por completo, lo que obliga a validar siempre la veracidad de los métodos analíticos.

Para la estimación de la veracidad en el trabajo desarrollado a lo largo de la presente Tesis Doctoral, se han empleado los *ensayos de recuperación* sobre muestras libres de los analitos, dopadas con los compuestos objeto de estudio a un mínimo de tres niveles de concentración. La incertidumbre asociada al valor asignado está limitada exclusivamente al error que pueda cometerse en la adición de la cantidad de analito al material. El porcentaje de recuperación *(%R)*, se calcula dividiendo la cantidad de analito encontrada mediante la interpolación en la función de calibración entre la concentración añadida al blanco de muestra. Esta forma de validación de la veracidad es la aceptada en la Decisión 2002/657/EC[179], cuando no existen materiales de referencia certificados.

Precisión. Mide el grado de concordancia entre resultados independientes de un ensayo obtenidos bajo condiciones estipuladas[177]. Desde un punto de vista práctico, la precisión viene determinada por la desviación estándar relativa o coeficiente de variación de la serie de resultados. La desviación estándar

[178] Massart D.L., Vandeginste B.G.M., Buydens L.M.C., De Jong S., Lewi P.J., Smeyers–Verbeke J. Handbook of Chemometrics and Qualimetrics: Part A, Elsevier Science B.V., Amsterdam, 1997, 887 pp.

[179] Commission Decision 2002/657/EEC. Official Journal of the European Communities, L 221, 2002.

relativa de la concentración, RSD (*relative estándar deviation*), se puede calcular a partir de la expresión:

$$RSD = 100 \cdot s_c / c$$

donde s_c es la desviación estándar obtenida a partir de replicados[180], y c es el valor de la concentración media.

La precisión se puede calcular en condiciones de *repetibilidad* o de *reproducibilidad*. Puede estimarse también la *precisión intermedia*, a partir de las variaciones dentro de un mismo laboratorio, por ejemplo, con medidas realizadas en diferentes días.

B. Otros parámetros de calidad

Los parámetros evaluados a lo largo de esta Tesis Doctoral han sido[181]:

- ✓ Rango dinámico lineal y linealidad.
- ✓ Sensibilidad. Límite de detección y de cuantificación.
- ✓ Selectividad.

Estos parámetros se han evaluado a partir de las correspondientes rectas de calibrado. Para ello, se ha establecido un número de tres réplicas para cada nivel de calibración, empleando en todos los casos al menos seis valores de concentración igualmente espaciados sobre el intervalo de aplicación del método.

Rango dinámico lineal y Linealidad. El rango dinámico lineal viene definido por el límite superior del intervalo de concentraciones en el que ha aplicado el método analítico y el límite de cuantificación de dicho método. Por otra parte,

[180] Cuadros-Rodríguez L., García-Campaña A.M., Jiménez-Linares C., Román-Ceba M. *Analytical Letters*, 1993, 26:1243–1258.
[181] Shabir G.A. *Journal of Chromatography A*, 2003, 987:57–66.

la linealidad indica el grado de ajuste de los valores de la señal medidos sobre la recta de calibrado[182]. En la presente Memoria se han evaluado dos tipos de linealidad:

- ✓ La linealidad *in-line* (en línea) estimada con el valor *P* del test de fallo de ajuste. Estableciéndose como condición de linealidad un valor P ≥ 5%.

- ✓ La linealidad *on-line* (*sobre la línea)*, estimada con el coeficiente de correlación (*r*), o como el coeficiente de determinación (R^2), sin embargo valores de (*r*) no indican necesariamente una alta calidad de los datos.

Sensibilidad. Límites de detección (LOD) y de cuantificación (LOQ). La capacidad de detección, límite de detección o concentración mínima detectable se define por la Norma ISO 11843[183] para una probabilidad de falso positivo, α, como *"la verdadera concentración neta de analito en el material a analizar, la cual permite, con una probabilidad 1-β la correcta conclusión de que la concentración en el material analizado es diferente de la del blanco"*. Se asocia con la menor concentración de analito en una muestra que puede ser separado del ruido de fondo, siendo posible su detección. El límite de cuantificación o de determinación (LOQ) es una medida de la capacidad de cuantificación inherente de un proceso de medida químico, es decir, la concentración de analito mínima cuantificable en una muestra. En esta Memoria el límite de detección y de cuantificación se han calculado utilizando dos formas diferentes:

- ✓ Según la recomendación de la IUPAC[184], el LOD corresponde a tres veces la desviación estándar del blanco (s_0). El límite de cuantificación o determinación (LOQ) corresponde a 10 veces la desviación estándar del blanco.

[182] Analytical Methods Committee. *Analyst*, 1994, 119:2363–2366.
[183] Norma ISO 11843. Capability of detection, Ginebra, Part 1, 1997 y Part 2, 2000.
[184] Guidelines for Single–Laboratory Validation of Analytical Methods for Trace-Level Concentrations of Organic Chemical, AOAC/FAO/IAEA/IUPAC, 2002.

En la bibliografía se proponen diversas formas de calcular esta desviación estándar. En el caso de la presente Tesis Doctoral, se ha calculado a partir de la función de calibración. En primer lugar se calcula la desviación estándar de la regresión ($s_{y/x}$), a partir de las respectivas desviaciones estándar de la ordenada en el origen (s_a) y la pendiente (s_b). El valor de la desviación estándar para el blanco vendrá dado por la expresión:

$$S_0 = \frac{S_{y/x}}{b}\sqrt{\frac{1}{n}+\frac{1}{m}+\frac{c_m^2}{\sum_{i=1}^{m}(c_i-c_m)^2}}$$

donde n el número total de niveles de calibración; m es el número de repeticiones del análisis realizadas; c_i es cada uno de los valores individuales de concentración y c_m es la media aritmética de todos los valores de concentración.

- ✓ A partir de la relación señal-ruido, obtenida directamente a partir del cromatograma. El LOD correspondería a aquella concentración en la que dicha relación es igual a 3 y el LOQ es igual a 10. Esta modalidad de cálculo cumple con los requerimientos de la Comisión Europea que estable los criterios relativos a la validación de un método y la interpretación de los resultados analíticos[185].

Selectividad (especificidad). La *International Conference on Harmonization* (ICH) define a la "especificidad" como *"la habilidad de distinguir inequívocamente la presencia del analito en medio de otros componentes que pueden encontrarse presentes. Típicamente se pueden incluir a impurezas, componentes de la matriz, etc."*.

[185] European Commission. COMMISION DECISION 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. L 221 17/8/2002.

Aunque es un parámetro de los más importantes a establecer en el proceso de aseguramiento de la calidad de un método de análisis, se trata de un concepto difícil de definir desde un punto de vista cuantitativo. En la práctica se suele representar la selectividad a través de la cuantificación de la interferencia producida por una especie química concreta en la determinación de un analito en un procedimiento dado, de modo que se establece un nivel máximo de interferente que no produce interferencia. En esta Tesis Doctoral se ha evaluado este parámetro mediante la comparación del cromatograma correspondiente al blanco en cada caso y el mismo cromatograma correspondiente a dicho blanco dopado con diferentes concentraciones de cada uno de los analitos objeto de estudio.

CAPÍTULO II

Aplicación de la *stir-bar sorptive extraction* previa precipitación de grasas y proteínas

1. OBJETIVOS

Los objetivos principales de los trabajos realizados y presentados en este capítulo son:

- ✓ Proponer metodología de buenas características analíticas para la determinación de bisfenol A, parabenos y benzofenonas mediante cromatografía de líquidos y cromatografía de gases acoplados a la espectrometría de masas en tándem (LC-MS/MS y GC-MS/MS) en muestras de leche materna.

- ✓ Aplicar la técnica de extracción con *stir-bar* seguida de un proceso de desorción química de los analitos.

- ✓ Validar los métodos analíticos propuestos en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación aplicables.

- ✓ Aplicar las metodologías desarrolladas en el control y evaluación del contenido de los EDCs seleccionados en muestras reales de leche materna humana.

2. STIR-BAR SORPTIVE EXTRACTION

La técnica que se propone en el presente capítulo es un sistema de extracción libre de disolventes introducido por Baltusen y col.[186], ampliamente empleada en la preparación, extracción y preconcentración de compuestos orgánicos en matrices acuosas. Se basa en los principios de la microextracción en fase sólida (SPME), aunque el volumen de fase extractante empleada en la *stir-bar sorptive extraction* (SBSE) es entre 50 y 250 veces mayor que el empleado en SPME, lo que provoca que la sensibilidad de esta técnica se multiplique por un factor de entre 100 y 1000 veces. Los analitos son extraídos por la fase estacionaria en función de sus coeficientes de partición octanol-agua ($K_{o/w}$) y del ratio de volúmenes muestra-adsorbente. Esta técnica ha sido aplicada con buenos resultados para el análisis de trazas en análisis medioambiental, biomédico, y análisis de alimentos[187-189].

Se trata de unas barras magnéticas agitadoras recubiertas de una película de un material polimérico, generalmente polidimetilsiloxano (PDMS) que se introducen en la muestra acuosa y se emplean para agitarla a la vez que se produce la extracción y pre-concentración de los analitos sobre el PDMS. Es una técnica de extracción basada en equilibrios, en la que la cantidad de analito extraída de la muestra está controlada por el coeficiente de partición de los analitos entre la muestra y el material de recubrimiento.

Las barras agitadoras están constituidas por tres componentes. El primero de ellos de naturaleza metálica, está situado en la parte más interna y consiste en una barra magnética agitadora, la cual es necesaria para provocar la rotación y, por tanto, la agitación de la muestra. El segundo componente es una fina

[186] Baltussen E., Sandra, P., David F., Cramers C. *Journal of Microcolumn Separations*, 1999, 11:737–747.
[187] Vercauteren J., Peres C., Cevos C., Sandra P., Vanhaecke F., Moens L. *Analytical Chemistry*, 2001, 73:1509–1514.
[188] Bicchi C., Iori C., Rubiolo P., Sandra P. *Journal of Agricultural and Food Chemistry*, 2002, 50:449–459.
[189] Kawaguchi N., Ito R., Saito K., Nakazawa H. *Journal of Pharmaceutical and Biomedical Analysis*, 2006, 40:500–508.

capa de vidrio que recubre el núcleo magnético. Esta capa actúa como barrera entre el PDMS y el metal para prevenir la descomposición del recubrimiento. La tercera capa, la más externa, es el recubrimiento de PDMS donde los analitos son adsorbidos.

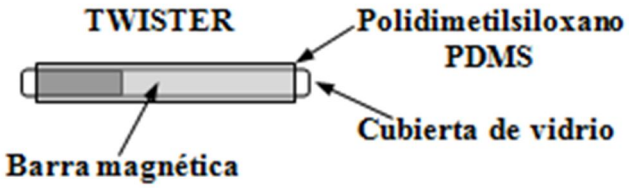


Figura II.1. *Representación esquemática de un Twister*

Las barras de PDMS están disponibles comercialmente por la empresa alemana Gerstel, y su nombre comercial son *Twister*. Pueden adquirirse en cuatro tamaños dependiendo de la longitud de la barra y el espesor del recubrimiento: 20 mm de largo x 1.0 mm de espesor, 20 x 0.5 mm, 10 x 1.0 mm y 10 x 0.5 mm. Generalmente los de 10 mm son empleados para volúmenes de muestra comprendidos entre 1 y 50 mL, y los de 20 mm para volúmenes de muestra de 50-250 mL.

La extracción de la muestra se realiza introduciendo la muestra en un vial o matraz. Posteriormente, se añade el *Twister* y se agita durante un determinado periodo de tiempo. El tiempo de extracción, que debe ser optimizado para cada aplicación, está controlado cinéticamente y viene determinado por el volumen de muestra, la velocidad de agitación, el volumen de fase estacionaria, y el tipo de analito. Al igual que en SPME, la optimización se realiza representando el tiempo de extracción frente a la tasa de recuperación, para determinar en qué tiempo la tasa de recuperación se mantiene constante. Una vez se ha alcanzado el tiempo de equilibrio, el *Twister* se retira de la muestra y se seca.

Los analitos extraídos pueden ser analizados mediante cromatografía de líquidos o de gases. En GC, los analitos pueden ser desorbidos térmicamente empleando un equipo de desorción térmica acoplado en línea con el

cromatógrafo. Un segundo modo de desorción, aplicable a LC y GC, es la desorción química empleando un disolvente apropiado. El proceso generalmente consiste en introducir el *Twister* en un vial con un volumen determinado de disolvente y aplicar ultrasonidos.

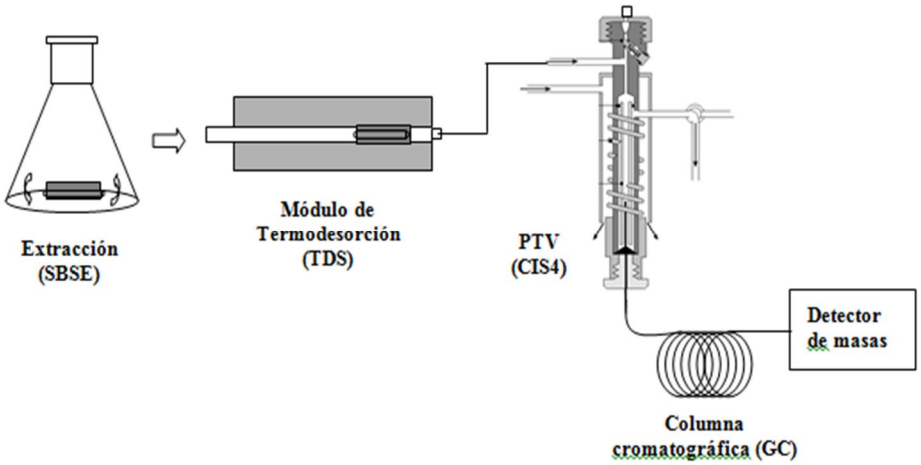


Figura II.2. *Esquema del proceso de extracción, desorción y análisis empleando cromatografía de gases.*

La teoría de la SBSE está estrechamente relacionada y es similar a la de SPME. En esta Tesis Doctoral, se presenta un trabajo de revisión publicado en la revista internacional de impacto *Talanta* en el que se describen los principios de esta técnica.

3. PUBLICACIONES

3.1. Publicación I

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Gas chromatography and ultra high performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction

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Abstract

In the present work, two specific, accurate and sensitive methods for the determination of endocrine disrupting chemicals (EDCs) in human breast milk are developed and validated. Bisphenol A and its main chlorinated derivatives, five benzophenone-UV filters and four parabens were selected as target analytes. The method involves a stir-bar sorptive extraction (SBSE) procedure followed by a solvent desorption prior to GC–MS/MS or UHPLC–MS/MS analysis. A derivatization step is also necessary when GC analysis is performed. The GC column used was a capillary HP-5MS with a run time of 26 min. For UHPLC analysis, the stationary phase was a non-polar Acquity UPLC® BEH C18 column and the run time was 10 min. In both cases, the analytes were detected and quantified using a triple quadrupole mass spectrometer (QqQ). Quality parameters such as linearity, accuracy (trueness and precision), sensitivity and selectivity were examined and yielded good results. The limits of quantification (LOQs) ranged from 0.3 to 5.0 ng mL^{-1} for GC and from 0.2 to 1.0 ng mL^{-1} for LC. The relative standard deviation (RSD) was lower than 15% and the recoveries ranged from 92 to 114% in all cases, being slightly unfavorable the results obtained with LC. The methods were satisfactorily applied for the

determination of target compounds in human milk samples from 10 randomly selected women.

Keywords: Stir-bar sorptive extraction; GC–MS/MS; UHPLC–MS/MS; Endocrine disrupting chemicals; Human breast milk

Highlights:

- ✓ Analysis of endocrine disrupting chemicals: bisphenol A, parabens and benzophenones.
- ✓ Stir-bar sorptive extraction procedure for isolation of EDCs from breast milk samples.
- ✓ Validation of GC-MS/MS and UHPLC-MS/MS methods to measure target compounds.
- ✓ The methods were applied in real samples collected from randomly selected mothers.

1. Introduction

It is well known that many industrial chemicals show estrogenic activity in addition to their desired chemical properties. Many of these compounds are found in the environment and in biological fluids or tissues. Bisphenol A (BPA) and its chlorinated derivatives, parabens (PBs) and benzophenone-UV filters (BPs) belong to this group of compounds [1-4].

BPA, with an annual production of 2-3 million tons, is a fundamental building block in the synthesis of polycarbonate plastics and epoxy resins used in the production of a large variety of manufactured products [5]. BPA has been associated with a large list of adverse reproductive and developmental effects [6-9]. The European Food Safety Authority (EFSA), in agreement with the US-EPA, has established the maximum acceptable exposure to BPA to be 50 μg kg^{-1} body-weight/day [10]. Although humans are frequently exposed through multiple sources, diet is considered the major source of exposure [11].

In addition, BPA in water can be transformed to its chlorinated derivatives due to chlorine treatments [12].

PBs are a family of compounds with bactericide and antimicrobial properties. Due to their low toxicity, cost, inert nature and worldwide regulatory acceptance, PBs are widely used as preservatives in personal care products (PCPs), pharmaceuticals, food and beverages. These compounds are found, individually or in combination, in more than 13,200 formulations in nearly all types of cosmetics [13-15]. Several studies have demonstrated the ability of PBs to disrupt physiological functions in both in vitro [16] and in vivo [14, 17, 18]. Exposure to PBs may occur through inhalation, dermal contact or ingestion [19].

The family of BPs is one of the most frequently used groups of UV-filters. There are 12 main compounds, called benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less known as 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP) and 4-hydroxybenzophenone (4-OH-BP). Despite some toxic effects of BPs, such as hepatotoxicity, they are safe for topical application in humans. However, there is increasing evidence that BPs are able to interfere with the endocrine system [20, 21]. These compounds are reported to enter the human body through the food chain [22] or by absorption through the skin [23].

Children are particularly vulnerable to the effects of exposure to EDCs because they are at the most sensitive stages of human development and therefore most susceptible to endocrine disruption. Human breast milk is a major route of exposure for breastfed infants and a valuable biological marker for exposure assessment (both maternal an prenatal exposure) [24]. In addition, breast milk is very useful in biomonitoring studies since it enables non-invasive sampling and relatively large volumes for large-scale biomonitoring programs [25].

To date, very few methods have been proposed for the determination of the selected EDCs in human milk. Most of these methods only determine BPA in human milk or colostrum [26-33] and in some cases their chlorinated derivatives [30, 31]. However, there is a marked lack of analytical methods in the literature regarding the determination of PBs and BPs, and they only focus on the determination of very few PBs [34-36] or BPs [26, 36]. Due to the complexity of human milk matrix, the sample treatment step involves a multi-step process that generally consists of an extraction procedure followed by clean-up and preconcentration steps prior to the analysis. In recent years, stir-bar sorptive extraction (SBSE) has been employed as an efficient sample preparation technique. This technique, developed by Baltussen et al. [37], is based on the same principles of solid-phase micro-extraction (SPME), but because of the larger volume and surface area of the adsorbent, SBSE possesses much higher extraction capacity [38-40].

The main purpose of the present work is to develop two accurate and sensitive methods for the determination of different families of EDCs in human breast milk. The introduction of a SBSE procedure and subsequent solvent desorption with an organic solvent prior to GC–MS/MS or UHPLC–MS/MS analysis is proposed. The use of tandem MS allows the identification and quantification of the analytes. The method was satisfactorily applied for the determination of target compounds in human milk from 10 randomly selected women.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB) and butylparaben (BPB) were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), tetrachlorobisphenol A (Cl_4-BPA), deuterium labelled

bisphenol A (BPA-d_{16}), benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP), labelled deuterium benzophenone (BP-d_{10}) and ethylparaben $^{13}C_6$-ring labelled (EPB-$^{13}C_6$) were supplied by Sigma-Aldrich (Madrid, Spain). Monochloro-, dichloro- and trichlorobisphenol A (Cl-BPA, Cl_2-BPA, Cl_3-BPA) were synthesized in our laboratory (purity > 99 %) by direct chlorination of BPA [41]. The chemical structures of target analytes are shown as supplementary material (Figure S1).

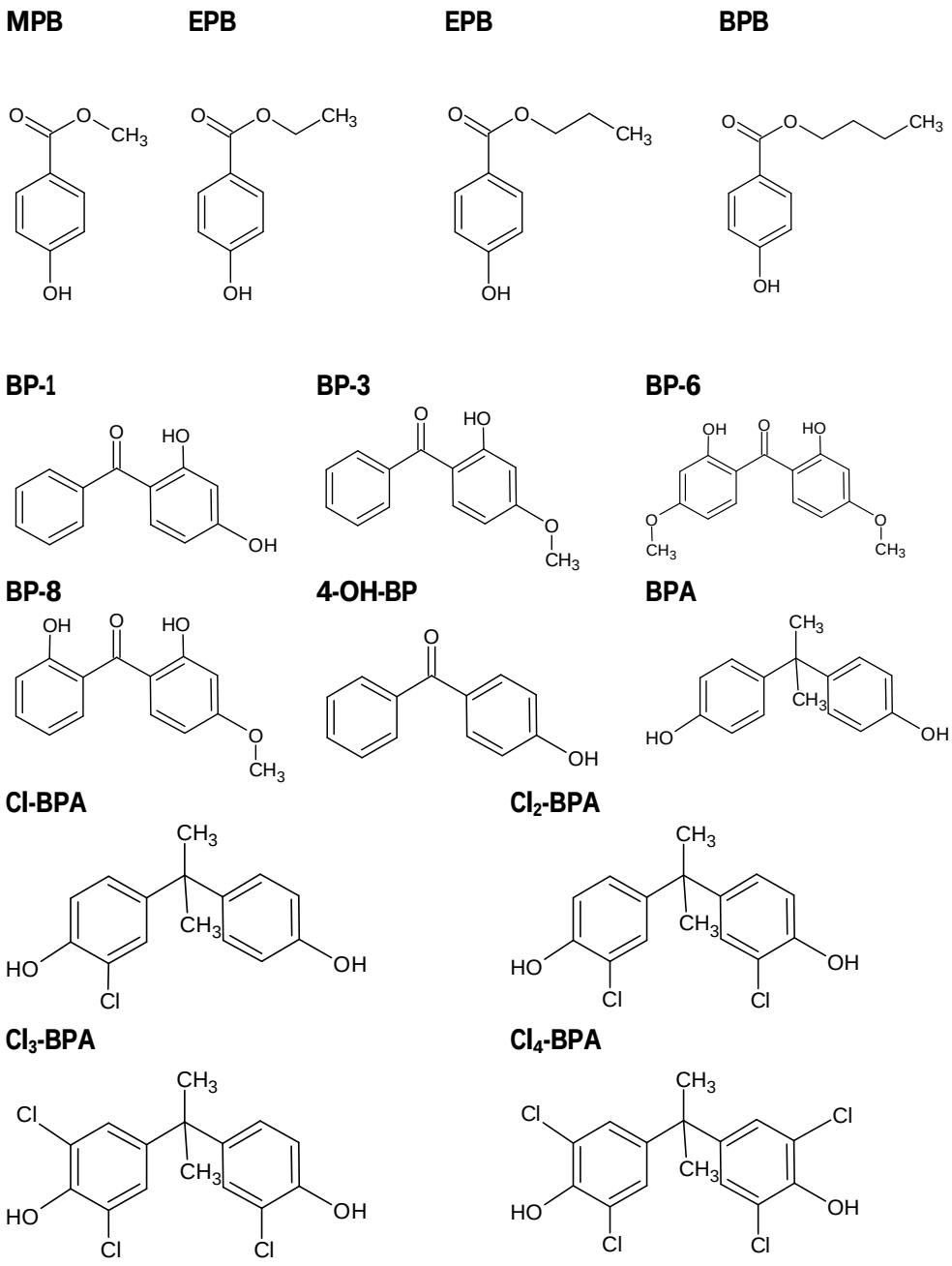

Figure S1. *Chemical structure of target compounds*

Stock standard solutions (100 µg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile up to the final volume was added. The solution remained stable for at least four months at 4 ºC in the darkness. Three intermediate solutions, No. 1, 2 and 3 (2.5, 5 and 10 µg mL^{-1}) were prepared by diluting 0.25, 0.5 and 1 mL respectively of the stock solution to 10 mL in acetonitrile. Subsequently, two new intermediate solutions No. 4 and 5 (0.5 and 1 µg mL^{-1}) were prepared by diluting 0.5 and 1 mL respectively of solution No. 3 to a final volume of 10 mL in acetonitrile. Finally, intermediate solutions No. 6 and 7 (0.05 and 0.1 µg mL^{-1}) were prepared by diluting 1 and 2 mL respectively of solution No. 5 to a final volume of 10 mL in acetonitrile. Working standards for calibration purposes were prepared by diluting 100 µL of the intermediate solutions No. 1 to 7 to a final volume of 10 mL in human breast milk. Working standards were prepared fresh from the acetonitrile solutions prior to the experiments.

Methanol, ethyl acetate, acetone and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). LC-MS grade methanol, water, formic acid, ammonia (25% w/v), hydrated zinc acetate, polyhydrated phosphotungstic acid, sodium hydroxide and N,O-bis(trimethylsilyl)trifluoro-acetamide with trimethylchlorosilane (BSTFA/1% TMCS) were purchased from Sigma-Aldrich(Madrid, Spain). Sodium chloride, glacial acetic acid (99%) and o-phosphoric acid (85%) were obtained from Panreac (Barcelona, Spain). The fat/proteins precipitation solution was prepared at time of use by dissolving 9.10 g of zinc acetate hydrated, 5.46 g of polyhydrated phosphotungstic acid and 5.8 mL of glacial acetic acid in 100 mL final volume of deionized water.

2.2. Instrumentation and software

The gas chromatograph was an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a CIS-4 programmable temperature vaporization (PTV) inlet, a multipurpose (MPS) autosampler. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert electron-impact ion source. The mass spectrometer worked in selected reaction

monitoring (SRM) mode and electron impact (EI) mode at 70 eV. For control and data analysis, Agilent Mass Hunter B.03.02 was used. Helium with a purity of 99.9999% was used as carrier gas and quenching gas, and nitrogen with a purity of 99.999% as collision gas, both them supplied by PRAXAIR España S.L. (Madrid, Spain). An HP–5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm) from Agilent was used.

UHPLC–MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class (Waters, Manchester, UK), consisting of Acquity UPLC™ binary solvent manager and Acquity UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for EDCs detection. An Acquity UPLC® BEH C_{18} (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (UK) was used as chromatographic column.

Sample agitation was carried out in a nine-position digital stirrer purchased from Ovan (Badalona, Spain). Polydimethylsiloxane (PDMS) stir bars (Twisters™) were provided by Gerstel (Mülheiman der Ruhr, Germany). The size of the stir bar was 0.5 mm (PDMS thickness) × 20 mm (length). A vacuum centrifugal evaporator was used to concentrate samples (LaboGene, Lynge, Denmark). Statgraphics Plus version 5.0 software (Manugistics Inc., Rockville, USA, 2000) was used for statistical and regression analyses (linear mode).

2.3. Sample collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. Samples were anonymized, frozen at -20 ºC and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form.

2.4. Preparation of spiked samples

Due to the absence of certified reference materials, for recovery studies, blank samples were spiked at different concentrations (from 0.1 ng mL^{-1} to 100 ng mL^{-1}) by adding 100 µL of different spiking standard solutions to 9.9 mL of human breast milk. In order to attain equilibrium, the mixtures were vortexed for 2 min and then left to stand for 24 h at 4 ºC in the dark before analysis. This allows the analytes to come into full contact with the sample. The blank samples were previously analyzed in order to ensure the absence of analytes or that these were below the LODs of the method.

2.5. Extraction procedure

An aliquot of 9.9 mL of human breast milk sample was placed into a 45 mL centrifuge tube and 100 µL of a 10 µg mL^{-1} acetonitrile solution of the surrogates (BPA-d_{16}, BP-d_{10} and EPB-$^{13}C_6$) was added. The final concentration in sample of surrogates was 100 ng mL^{-1}. The sample was vigorously shaken in a vortex-mixer for 1 min and then, 7.5 mL of acetonitrile and 7.5 mL of the fat/proteins precipitation solution were added. The mixture was stirred on a vortex-mixer for 1 min, and centrifuged for 10 min at 5000 rpm (4050 × *g*). The underlying liquid layer was filtered through a 0.22 µm nylon filter and transferred to a Falcon tube for evaporation to near dryness in a vacuum centrifugal evaporator at 2000 rpm (760 × *g*) and 60 ºC. The residue was re-dissolved in a final volume of 20 mL of deionized water and shaken vigorously for 4 min. The extract was transferred to a 100 mL bottle containing 17.5 g of NaCl in 20 mL of deionised water and the PDMS twister. The operation is repeated with 10 mL of water to properly clean the Falcon tube containing the original sample. The final solution (about 50 mL) was stirred for 24 h at 600 rpm. Subsequently, the twister was removed and washed with deionised water to remove any remaining salts, dried on a lint-free tissue and introduced into an Eppendorf tube. In order to carry out the chemical desorption of the analytes from the stir-bar, 1 mL of acetonitrile was added and sonicated for 5 min in an ultrasound bath (1 cycle is enough). Next, the solvent was evaporated to

dryness at room temperature under a nitrogen stream. For GC analysis, analytes must be previously derivatized in order to increase their volatility. For this purpose, the residue was dissolved into50 µL of a mixture of ethyl acetate and BSTFA/1% TMCS (60:40, v/v), and heated at 60 ºC for 20 min [42]. For UHPLC analysis, sample was simply dissolved in 50 µL of the initial mobile phase.

3. Results and discussion

3.1. Optimization of the chromatographic procedure

3.1.1. GC–MS/MS analysis

Analytes were separated on a HP-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 µm). The injection port of the GC was set at 250 ºC. Samples were automatically injected in splitless-injection mode. The injection volume was 1 µL. The helium carrier gas flow was maintained at a constant pressure of 20 psi. The initial oven temperature was set at 70 ºC (held for 2.0 min). Then, three linear ramps were established in order to reach 120 ºC at 25 ºC min^{-1}, 260 ºC at 10 ºC min^{-1} hold for 2 min, and finally 280 ºC at 20 ºC min^{-1}. This final temperature was maintained for 5 min. Total time of analysis was 26 min. In the inlet a temperature ramp of 12 ºC s^{-1} was established in order to reach a final temperature of 325 ºC to transfer the analytes into the GC column.

A selected reaction monitoring (SRM) method was created in the triple quadrupole mass spectrometer. Two transitions were monitored for each analyte, the first for quantification and the second one for confirmation. Table 1 shows the selected mass spectrometer conditions. The method was divided into 3 segments to obtain enough sampling points for each chromatographic peak and adequate dwell times to obtain an optimal sensitivity and signal-noise relationship (SNR). Resolution was adjusted to 1.0 Da for quadrupole 1 and 3. Temperatures of the transfer line, ion source and quadrupole 1 and 2 were 290 ºC, 290 ºC and 180 ºC, respectively. The mass spectrometer was auto-tuned on a weekly basis.

Table 1. *Transitions and optimized potentials for GC–MS/MS and UHPLC-MS/MS*

GC–MS/MS					
	Transitions	CE		Transitions	CE
BPA	372.0 →191.2[a]	20	BP-1	343.2 → 271.1[a]	20
	357.0 → 191.2[b]	30		343.2 → 105.1[b]	30
Cl-BPA	406.0 → 391.1[a]	10	BP-3	285.1 → 242.1[a]	20
	391.1 → 73.0[b]	30		285.1 → 212.0[b]	30
Cl_2-BPA	442.0 → 427.1[a]	10	BP-6	403.0 → 360.1[a]	20
	425.0 → 225.1[b]	20		403.0 → 73.0[b]	30
Cl_3-BPA	476.0 → 461.0[a]	10	BP-8	373.2 → 73.0[a]	30
	459.0 → 73.0[b]	30		299.1 → 73.0[b]	20
Cl_4-BPA	495.0 → 73.0[a]	30	4-OH-BP	270.1 → 193.1[a]	10
	495.0 → 277.1[b]	10		270.1 → 255.1[b]	10
MPB	224.0 → 209.0[a]	5	BPA-d_{16}	386.0 → 368.3[a]	10
	224.0 → 131.0[b]	30		386.0 → 73.0[b]	30
EPB	238.2 → 223.2[a]	5	EPB-$^{13}C_6$	244.0 → 229.2[a]	10
	238.2 → 195.1[b]	10		244.0 → 201.1[b]	10
PPB	252.0 → 195.1[a]	15	BP-d_{10}	192.0 → 82.0[a]	20
	252.0 → 210.1[b]	5		192.0 → 110.0[b]	30
BPB	210.0 → 195.1[a]	5			
	210.0 → 151.1[b]	5			

UHPLC–MS/MS							
	Transitions	CV	CE		Transitions	CV	CE
BPA	227.2→ 211.9[a]	-50	-22	BP-1	214.9→136.8[a]	2	18
	227.2→ 132.9[b]	-50	-26		214.9→105.1[b]	2	32
Cl-BPA	261.1→182.0[a]	-56	-30	BP-3	229.0→150.8[a]	4	20
	261.1→210.0[b]	-56	-22		229.0→104.9[b]	4	18
Cl_2-BPA	295.0→215.9[a]	-74	-30	BP-6	275.0→150.9[a]	14	18
	295.0→243.9[b]	-74	-24		275.0→94.9[b]	14	34
Cl_3-BPA	329.0→249.8[a]	-2	-32	BP-8	245.0→120.9[a]	14	20
	329.0→277.9[b]	-2	-24		245.0→150.9[b]	14	20
Cl_4-BPA	365.0→313.9[a]	-50	-28	4-OH-BP	199.0→120.8[a]	36	20
	365.0→285.9[b]	-50	-32		199.0 →104.8[b]	36	18
MPB	151.1→91.8[a]	-38	-22	BPA-d_{16}	241.2→223.0[a]	-46	-22
	151.1→135.8[b]	-38	-14		241.2→141.9[b]	-46	-32
EPB	165.1→91.9[a]	-38	-24	EPB-$^{13}C_6$	171.1→98.0[a]	-36	-24
	165.1→136.6[b]	-38	-16		171.1→142.7[b]	-36	-14
PPB	179.1→91.8[a]	-42	-24	BP-d_{10}	193.1→109.8[a]	18	16
	179.1→136.1[b]	-42	-16		193.1→81.8[b]	18	30
BPB	193.1 →91.4[a]	-42	-24				
	193.1 →136.1[b]	-42	-16				

[a] *SRM transition used for quantification;* [b] *SRM transition used for confirmation*
CV: Cone voltage (V); CE: Collision energy (eV)

3.1.2. UHPLC–MS/MS method

Chromatographic separation of compounds was performed using an Acquity UPLC® BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The compounds were separated using a gradient mobile phase consisting of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 0.1% (v/v) ammonia in methanol (solvent B). Gradient conditions were: 0.0-2.0 min, 30% B; 2.0-5.0 min, 30-90% B; 5.0-5.1 min, 90-100% B; 5.1-7.0 min, 100% B and back to 30% in 0.1 min. Flow rate was 0.25 mL min^{-1}. The injection volume was 10 μL. The column temperature was maintained at 40 °C. Total run time was 10.0 min.

ESI was simultaneously performed in both negative and positive ion modes. The tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuous infusion of standard solutions (1 mg L^{-1}). The ion source temperature was maintained at 150 ºC. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1}, and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell time was set at 25 ms. Optimized parameters for each compound are also listed together with the mass transitions in Table 1.

3.2. Optimization of the SBSE procedure

A study of the major variables affecting the process of SBSE was performed. The effect of the amount of matrix modifiers (methanol and sodium chloride), sample volume, extraction time, stirring speed, desorption solvent and desorption time were optimized.

3.2.1. Effect of matrix modifiers. Methanol and NaCl

To study the influence of these variables, spiked samples with a final concentration of 100 ng mL^{-1} of each analyte were extracted following the previously described protocol (section 2.5.1). Five spiked samples with methanol percentages of 0, 5, 15, 25 and 35% (v/v) were extracted in triplicate. The presence of methanol resulted in a drastic decrease (90%) in the response of all analytes. The experiment was repeated using acetonitrile and tetrahydrofuran as solvents but the results did not improve. Regarding the salt content, seven samples with NaCl contents of 0, 5, 15, 25, 35, 40 and 50% (w/v) were also extracted in triplicate and the maximum signals were obtained when the percentage of NaCl was 35%, close to saturation. It was also observed that at concentrations above 35% (supersaturated solutions), the analytical response decreased (see supplementary material, Figure S2). Therefore 35% of salt and the absence of methanol were selected as optimum values for those variables.

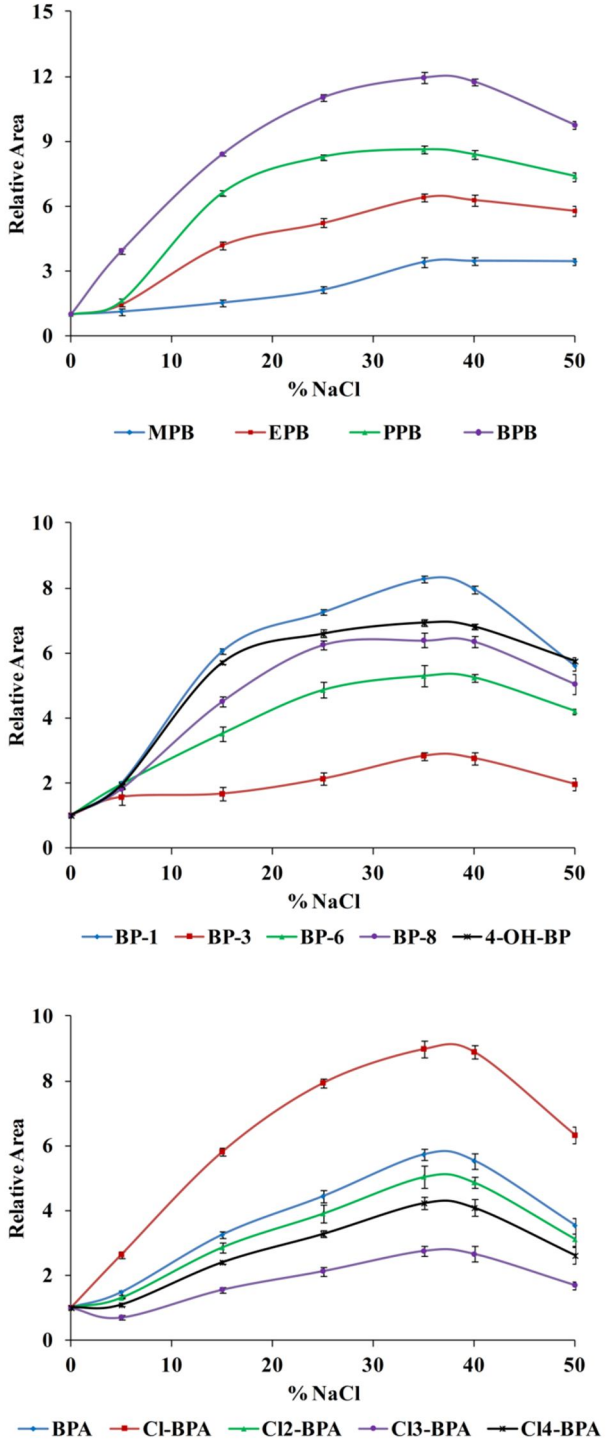

Figure S2. *Influence of ionic strength*

3.2.2. Optimization of milk amount (sample dilution)

The influence of milk amount and sample dilution was studied. Due to the high matrix effect observed in previous experiments, it was necessary to dilute the sample in water in order to perform the SBSE process. A final volume of 50 mL was set and different volumes of human breast milk (2, 5, 10 and 15 mL) were assayed. Spiked samples (100 ng mL^{-1} of final concentration) were analyzed in triplicate and extracted for 24 h in order to ensure that the equilibrium was reached. It was observed that the analytical signal decreases when the amount of milk increases, probably because of the huge increase in matrix effect due to the complexity of human milk samples (see supplementary material, Figure S3). Since this effect is much more marked for volumes higher than 10 mL, this volume was selected as a compromise to get good analytical signals.

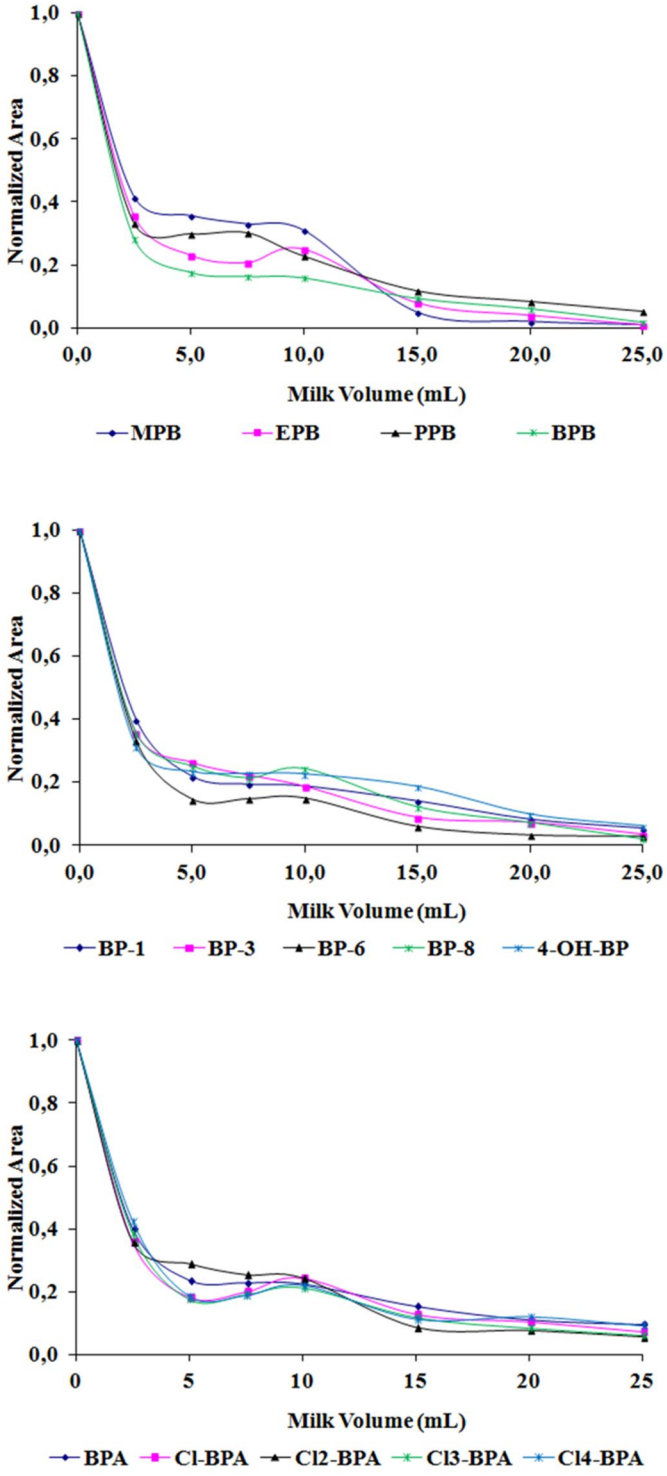

Figure S3. *Influence of human milk amount*

3.2.3. Optimization of the extraction time

To study the effect of extraction time, nine spiked samples (100 ng mL^{-1}) were extracted and analyzed. The experiments were performed in triplicate. The twister was removed from the sample at different times, stopping the extraction at 0.5, 1, 2, 5, 15, 24, 30 and 48 h. It was observed that the equilibrium was reached at 24 h, and this value was the selected time for further experiments. The results are shown as supplementary material (Figure S4).

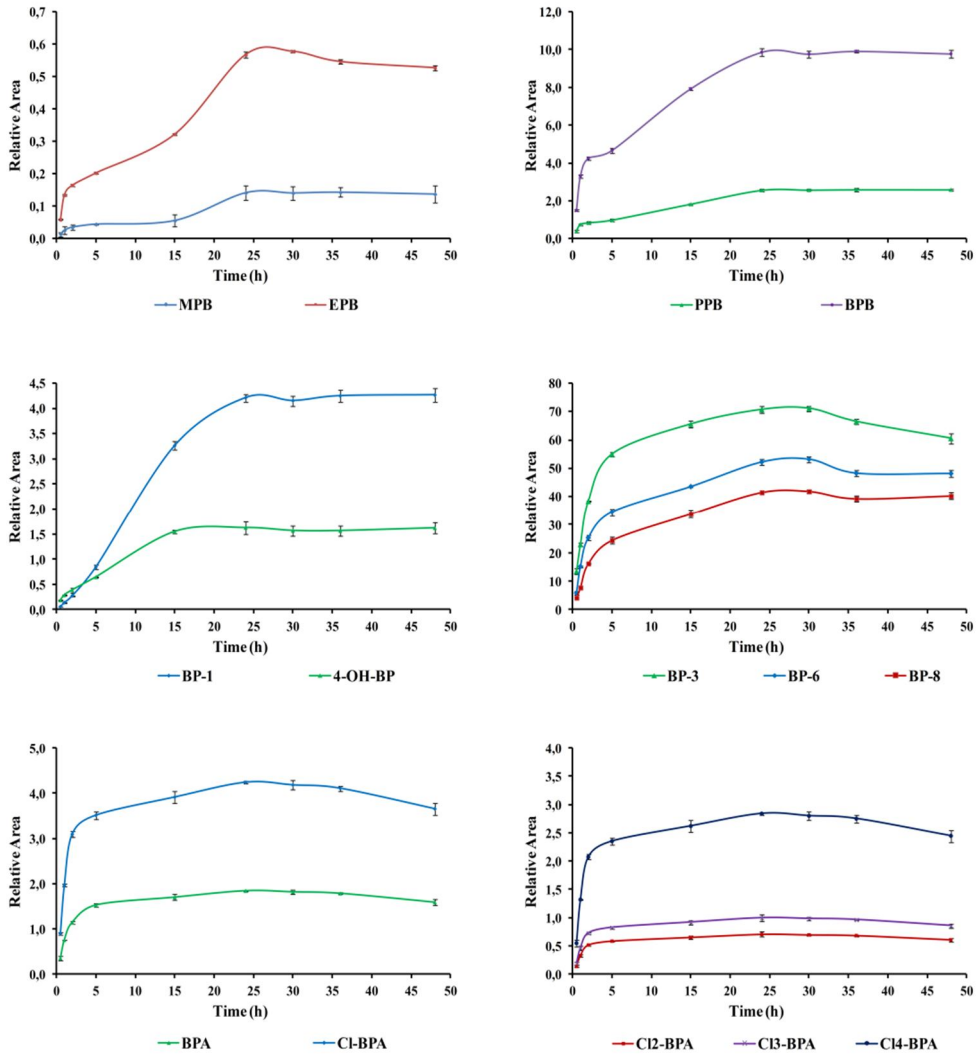

Figure S4. *Influence of the extraction time*

3.2.4. Optimization of stirring speed

Stirring rate was studied between 200 and 1200 rpm, minimum and maximum for the instrument used. The extraction yield improves when stirring speed increases from 200 to 600 rpm. Twisters showed unstable motion for speeds higher than 600 rpm. Thus, this was the selected stirring rate.

3.2.5. Optimization of desorption solvent

The effect of different solvents and the effect of solvent acidification with formic acid in order to protonate the ionizable groups of all compounds were evaluated. Methanol, acetonitrile, ethyl acetate, ethyl acetate/acetone (1:1, v/v), methanol/formic acid (0.1%, v/v), acetonitrile/formic acid (0.1%, v/v), ethyl acetate/formic acid (0.1%, v/v) and ethyl acetate/acetone (1:1, v/v)/formic acid (0.1%, v/v) were assayed. Each experiment was carried out in triplicate. As shown in Figure 1, the optimal results for all compounds were obtained with pure acetonitrile. The acidification of solvents did not improve the extraction yields.

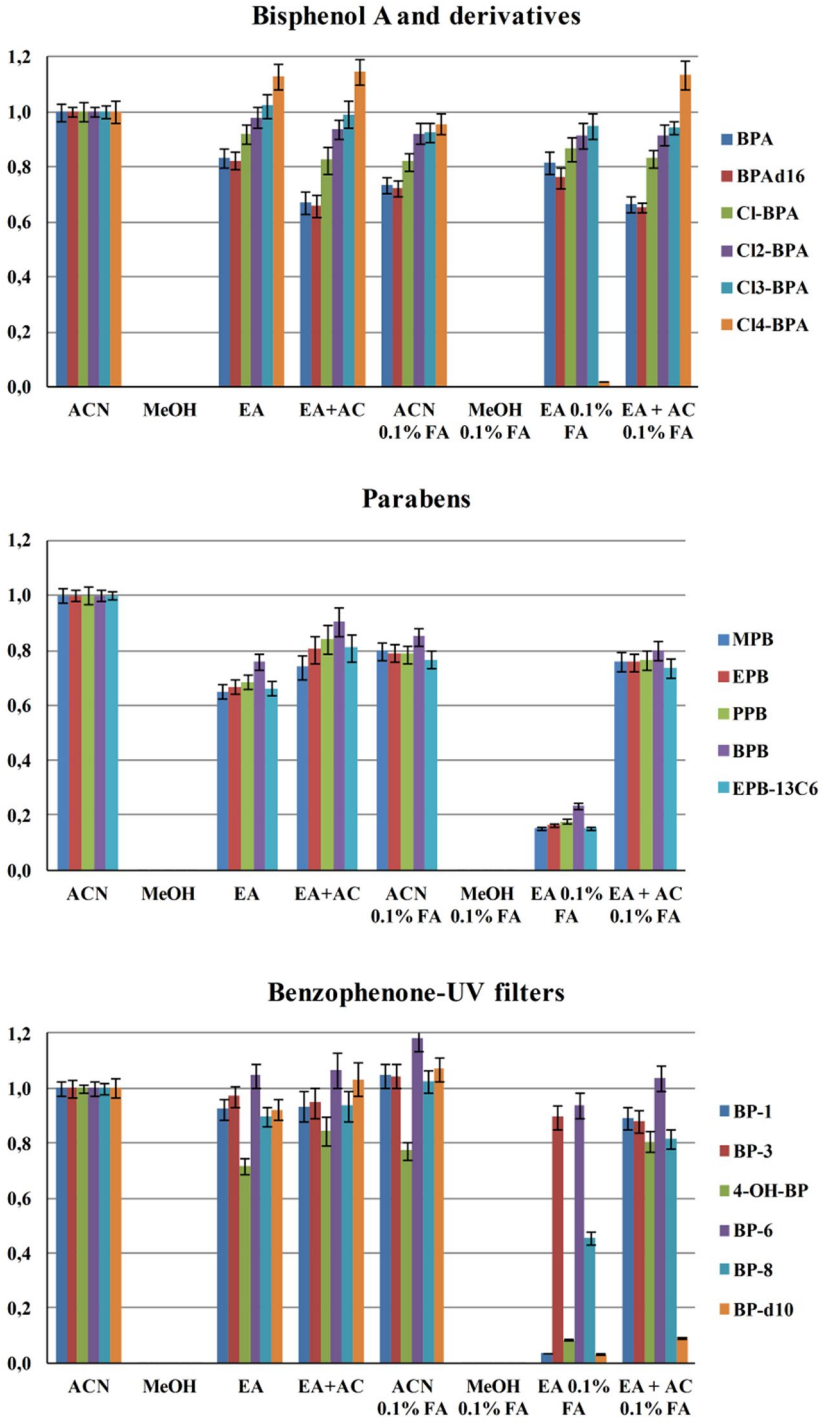

Figure 1. *Optimization of desorption solvent. ACN: acetonitrile; MeOH: methanol; EA: ethyl acetate; AC: acetone; FA: formic acid. Relative areas with respect to ACN (100 %).*

3.2.6. Optimization of desorption time

Seven spiked samples were extracted in triplicate and the twister desorbed for 1, 5, 10, 15, 20, 25, and 30 min respectively. Since a constant response was observed from 5 min for all analytes, this was the selected desorption time.

3.3. Calibration curves

A six-concentration level calibration curve for GC–MS/MS and a seven-concentration calibration curve for UHPLC–MS/MS were built for each compound. Each level of concentration was made in triplicate. Calibration graphs were constructed using analyte/surrogate peak area ratio versus concentration of analyte. Calibration graphs were made using SRM mode. BPA-d_{16}, EPB-$^{13}C_6$ and BP-d_{10} (100 ng mL^{-1}, final concentration in milk samples) were used as surrogates for BPA and derivatives, PBs and BPs, respectively.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in distilled water and the other in blank human milk. The *Student's t*-test was applied in order to compare the calibration curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a *Snedecor's F*-test. The *Student's t*-test showed statistical differences among slope values for the calibration curves of chlorinated derivatives of BPA, BPB and BPs 3, 6 and 8 and consequently, a significant matrix effect was observed and the use of matrix-matched calibration was necessary. A possible explanation for this behaviour could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are relatively variable within the same family of compounds, especially in the case of BPs. Therefore, although the three compounds selected have a similar basic structure compared to the analytes of the same family, and the use of these compounds as internal standards or as surrogates is accepted in scientific literature, they differ slightly due to the presence of different substituents in the molecule. Since it is impossible to have the corresponding isotopically

labeled standard for each one of the studied analytes (many of them do not exist in the market), we decided to work with matrix-matched calibration in all cases. Table 2 shows the statistical and the analytical parameters obtained for each analyzed compound using both GC and LC analytical techniques.

Table 2. *Analytical and statistical parameters*

	GC–MS/MS						
	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BPA	$2.29 \cdot 10^{-2}$	$2.21 \cdot 10^{-4}$	99.7	86.5	0.2	0.5	0.5 - 100
Cl-BPA	$5.26 \cdot 10^{-2}$	$6.01 \cdot 10^{-4}$	99.6	92.8	0.1	0.5	0.5 - 100
Cl_2-BPA	$2.01 \cdot 10^{-2}$	$3.25 \cdot 10^{-4}$	99.3	89.0	0.3	1.0	1.0 - 100
Cl_3-BPA	$1.10 \cdot 10^{-2}$	$1.16 \cdot 10^{-4}$	99.7	18.2	1.0	3.0	3.0 - 100
Cl_4-BPA	$1.70 \cdot 10^{-3}$	$2.48 \cdot 10^{-5}$	99.5	48.9	1.5	5.0	5.0 - 100
MPB	$1.15 \cdot 10^{-2}$	$9.53 \cdot 10^{-4}$	99.8	6.0	0.1	0.4	0.4 - 100
EPB	$2.04 \cdot 10^{-2}$	$1.42 \cdot 10^{-4}$	99.8	45.7	0.1	0.4	0.4 - 100
PPB	$8.02 \cdot 10^{-2}$	$3.91 \cdot 10^{-4}$	99.9	32.6	0.1	0.3	0.3 - 100
BPB	$1.65 \cdot 10^{-1}$	$1.25 \cdot 10^{-3}$	99.1	10.5	0.1	0.3	0.3 - 100
BP-1	$4.33 \cdot 10^{-2}$	$5.06 \cdot 10^{-4}$	99.5	11.3	0.2	0.6	0.6 - 100
BP-3	$8.31 \cdot 10^{-2}$	$6.94 \cdot 10^{-4}$	99.8	17.0	0.1	0.4	0.4 - 100
BP-6	$3.53 \cdot 10^{-2}$	$3.77 \cdot 10^{-4}$	99.6	7.8	0.2	0.6	0.6 - 100
BP-8	$7.19 \cdot 10^{-2}$	$6.74 \cdot 10^{-4}$	99.7	78.0	0.1	0.5	0.5 - 100
4-OH-BP	$8.13 \cdot 10^{-2}$	$6.70 \cdot 10^{-4}$	99.8	49.7	0.1	0.4	0.4 - 100
	UHPLC–MS/MS						
	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BPA	$1.90 \cdot 10^{-2}$	$3.23 \cdot 10^{-4}$	99.4	5.8	0.1	0.3	0.3 - 100
Cl-BPA	$6.65 \cdot 10^{-2}$	$8.47 \cdot 10^{-4}$	99.6	98.8	0.1	0.2	0.2 - 100
Cl_2-BPA	$4.95 \cdot 10^{-2}$	$7.97 \cdot 10^{-4}$	99.4	62.7	0.1	0.3	0.3 - 100
Cl_3-BPA	$6.75 \cdot 10^{-2}$	$7.85 \cdot 10^{-4}$	99.7	9.0	0.2	0.5	0.5 - 100
Cl_4-BPA	$1.67 \cdot 10^{-3}$	$3.20 \cdot 10^{-4}$	99.5	5.6	0.3	1.0	1.0 - 100
MPB	$2.22 \cdot 10^{-2}$	$2.61 \cdot 10^{-4}$	99.7	28.7	0.1	0.2	0.2 - 100
EPB	$7.61 \cdot 10^{-2}$	$6.75 \cdot 10^{-4}$	99.8	73.0	0.1	0.3	0.3 - 100
PPB	$2.84 \cdot 10^{-1}$	$5.25 \cdot 10^{-3}$	99.7	12.1	0.2	0.7	0.7 - 100
BPB	$5.62 \cdot 10^{-1}$	$2.29 \cdot 10^{-3}$	99.6	59.8	0.1	0.2	0.2 - 100
BP-1	$4.04 \cdot 10^{-2}$	$5.25 \cdot 10^{-4}$	99.6	91.6	0.2	0.6	0.6 - 100
BP-3	$8.31 \cdot 10^{-2}$	$6.94 \cdot 10^{-4}$	99.8	17.0	0.1	0.4	0.4 - 100
BP-6	$5.30 \cdot 10^{-2}$	$6.78 \cdot 10^{-5}$	99.6	46.7	0.3	1.0	1.0 - 100
BP-8	$7.24 \cdot 10^{-3}$	$8.46 \cdot 10^{-5}$	99.7	80.1	0.1	0.2	0.2 - 100
4-OH-BP	$7.92 \cdot 10^{-2}$	$1.04 \cdot 10^{-3}$	99.6	18.6	0.1	0.2	0.2 - 100

b: slope; s_b: slope standard deviation; R^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification; LDR: linear dynamic range

3.4. Methods validation

The validation of the methods in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity was performed according to the protocols described in the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [43]. The results obtained were:

3.4.1. Linearity

A concentration range from the minimal quantified amount (LOQ) to 100 ng mL^{-1} was selected. Linearity of the calibration graphs was tested using the determination coefficients (% R^2) and the *P*-values (% P_{lof}) of the *lack-of-fit* test [43]. The values obtained for R^2 ranged from 99.1 to 99.9 % and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges.

3.4.2. Limits of detection and quantification

In the present work, limit of detection (LOD) and limit of quantitation (LOQ) were calculated by taking into consideration the standard deviation of residuals, $S_{y/x}$, the slope, b, of the calibration graphs and an estimate s_0 obtained by extrapolation of the standard deviation of the blank [44]. The LOD was $3 \cdot s_0$ and the LOQ was $10 \cdot s_0$. Found limits of quantification ranged from 0.3 to 5 ng mL^{-1} for GC–MS/MS analysis, and from 0.2 to 1.0 for UHPLC–MS/MS analysis. These results are also summarized in Table 2.

3.4.3. Selectivity

The specificity of the method was determined by comparing the chromatograms of a blank sample with the corresponding to a spiked breast milk sample. No interferences from endogenous substances were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensure the high selectivity of the methods. Figure 2

shows the SRM chromatograms obtained a sample spiked with the analytes (5 x LOD ng mL^{-1}) analyzed with GC-MS/MS and Figure 3 shows the chromatograms obtained when UHPLC–MS/MS was used as analytical technique.

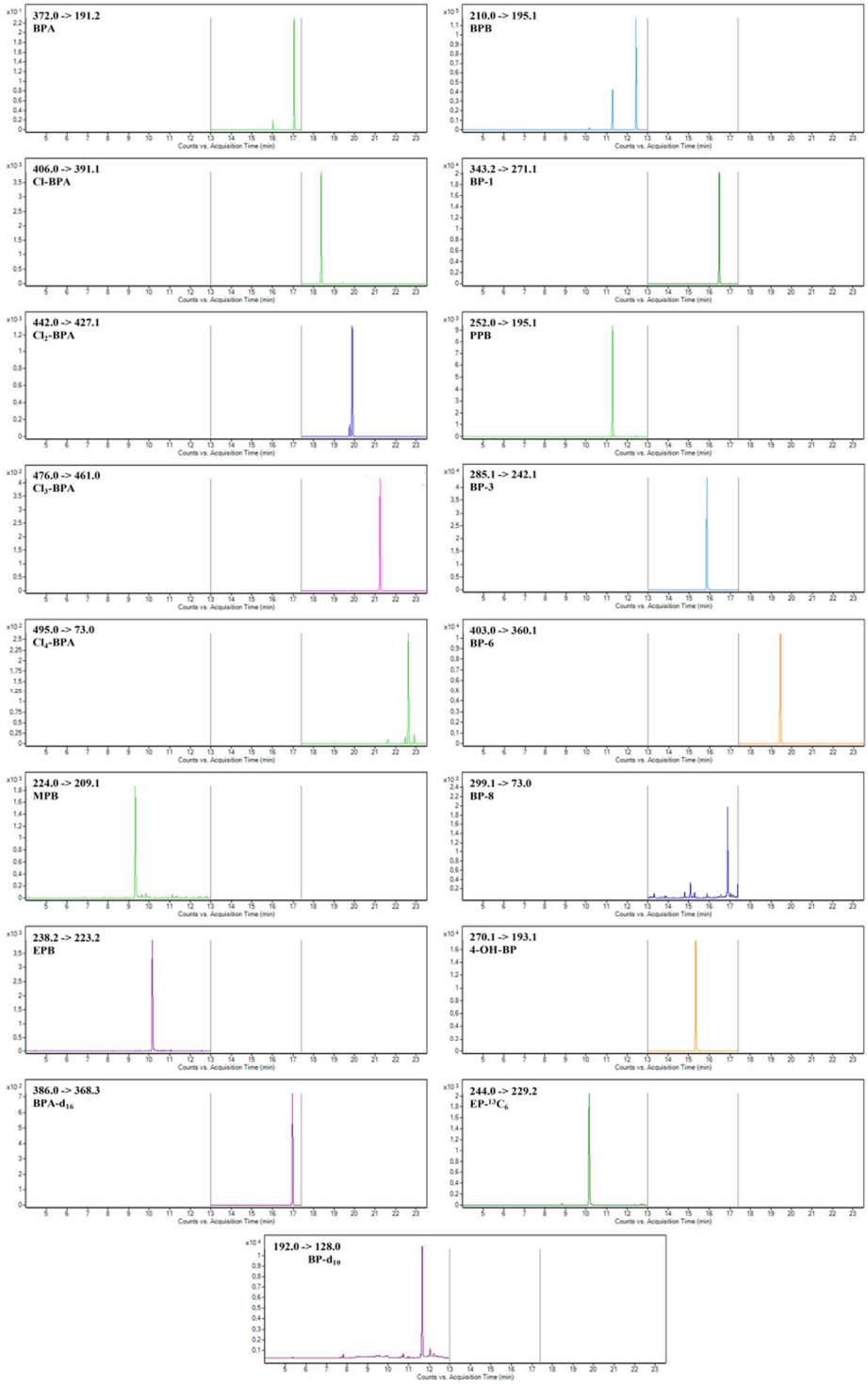

Figure 2. *SRM-GC-MS/MS chromatogram of a spiked blank of human milk sample (5 x LOQ).*

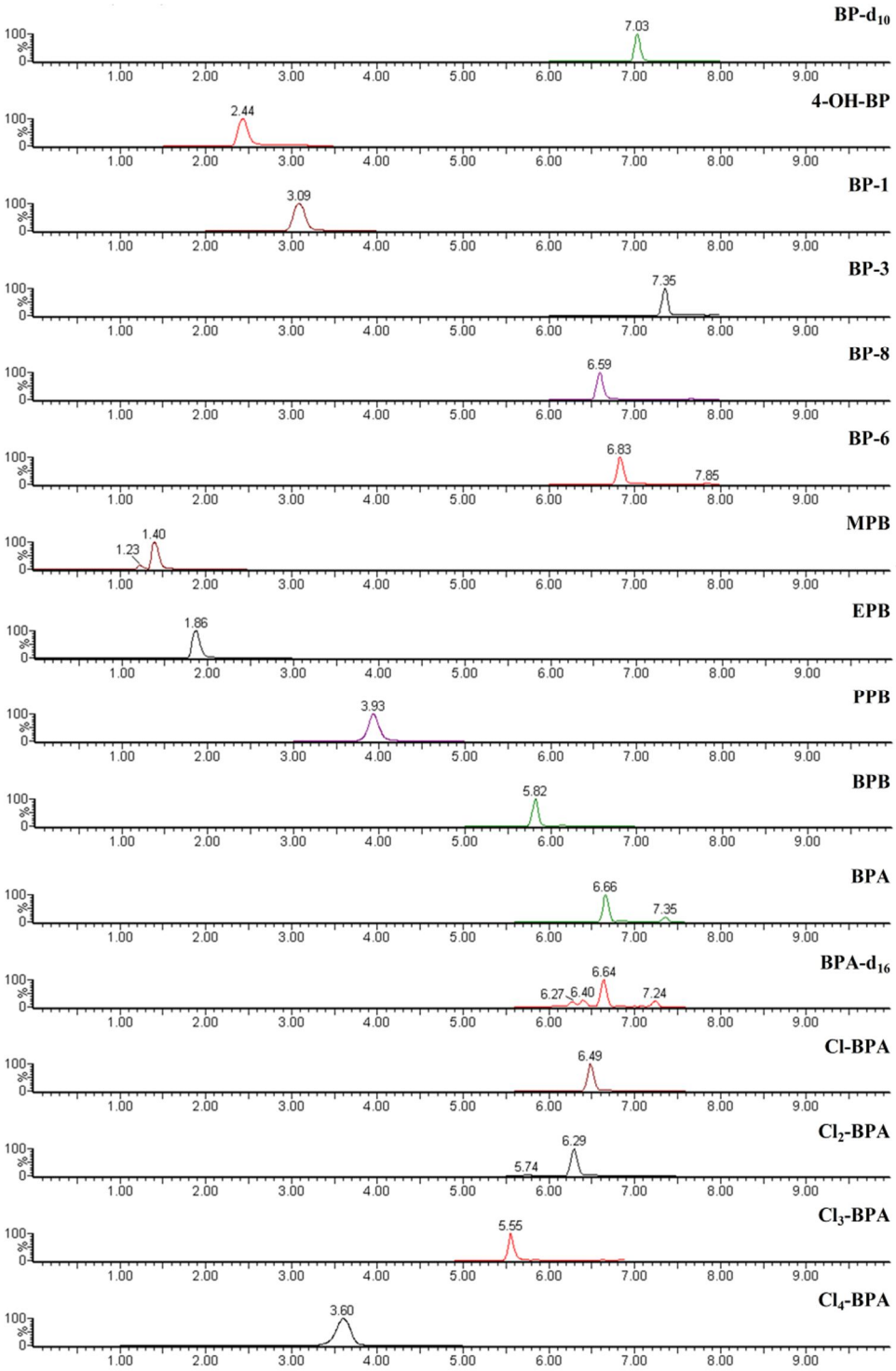

Figure 3. *UHPLC-MS/MS chromatogram of a spiked blank of human milk sample (5 x LOQ).*

3.4.4. Accuracy

Due to the absence of certified materials, in order to evaluate the trueness and the reproducibility of the method, a study with spiked human breast milk samples, at three concentrations levels for each compound was performed on 6 consecutive days. Spiked samples were analyzed in triplicate each day in repeatability conditions. A total of 18 measurements for each level were carried out. The concentrations studied were 1, 50 and 100 ng mL^{-1}, except for the determination of Cl_2-BPA and Cl_3-BPA by GC-MS/MS, where the lower level was 5 ng mL^{-1} and for Cl_4-BPA which was 10 ng mL^{-1}, respectively. The precision was expressed as relative standard deviation, RSD, and the trueness was evaluated by a recovery assay. The precision and the trueness of the proposed analytical method are shown in Table 3.

Table 3. *Recovery assay, precision and trueness of compounds in human breast milk*

GC–MS/MS									
	Spiked (ng mL^{-1})	Found[a] (SD) (ng mL^{-1})	RSD (%)	R (%)		Spiked (ng mL^{-1})	Found[a] (SD) (ng mL^{-1})	RSD (%)	R (%)
	1	1.1 (0.1)	8.4	110		1	1.1 (0.1)	6.7	114
BPA	50	50.2 (2.1)	4.2	101	PPB	50	51.0 (1.6)	3.1	102
	100	99.7 (3.9)	3.9	100		100	98.8 (1.6)	1.7	99
	1	0.9 (0.1)	7.3	95		1	1.1 (0.1)	5.0	106
Cl-BPA	50	49.2 (3.6)	7.3	98	BPB	50	51.3 (2.5)	4.9	103
	100	100.2 (3.4)	3.4	100		100	99.5 (3.5)	3.5	99
	5	4.6 (0.3)	5.8	92		1	1.0 (0.1)	10.0	102
Cl_2-BPA	50	50.9 (3.2)	6.4	102	BP-1	50	48.2 (2.4)	5.0	96
	100	99.4 (5.5)	5.5	99		100	101.5 (4.8)	4.8	102
	5	5.3 (0.3)	5.7	106		1	1.1 (0.1)	8.9	105
Cl_3-BPA	50	52.8 (2.1)	3.9	106	BP-3	50	47.8 (1.9)	4.0	96
	100	104.8 (3.6)	3.5	105		100	100.8 (3.7)	3.7	101
	10	9.4 (0.8)	8.6	94		1	1.0 (0.1)	10.0	103
Cl_4-BPA	50	48.2 (2.5)	5.1	96	BP-6	50	47.9 (2.7)	5.7	96
	100	95.8 (3.1)	3.3	96		100	98.7 (3.2)	3.2	99
	1	1.1 (0.1)	5.3	111		1	0.9 (0.1)	7.9	93
MPB	50	48.6 (1.7)	3.6	97	BP-8	50	52.4 (2.5)	4.7	105
	100	100.9 (2.4)	2.4	101		100	106.2 (4.3)	4.1	106
	1	1.1 (0.1)	5.3	109		1	1.1 (0.1)	7.1	107
EPB	50	48.5 (0.9)	1.9	97	4-OH-BP	50	48.9 (2.5)	5.0	98
	100	100.9 (2.4)	2.8	101		100	100.3 (2.9)	2.9	100
UHPLC–MS/MS									
	Spiked (ng mL^{-1})	Found[a] (SD) (ng mL^{-1})	RSD (%)	R (%)		Spiked (ng mL^{-1})	Found[a] (SD) (ng mL^{-1})	RSD (%)	R (%)
	1	1.1 (0.1)	8.0	106		1	1.1 (0.2)	15.0	107
BPA	50	54.3 (3.5)	6.5	109	PPB	50	51.1 (2.8)	5.5	102
	100	98.6 (2.8)	2.8	99		100	99.9 (3.3)	3.4	100
	1	1.0 (0.1)	7.3	92		1	0.9 (0.1)	13.0	92
Cl-BPA	50	50.0 (3.2)	6.3	100	BPB	50	48.5 (2.9)	6.0	97
	100	99.9 (3.4)	3.4	100		100	100.8 (4.0)	3.9	101
	1	1.1 (0.1)	8.1	109		1	1.1 (0.1)	5.7	111
Cl_2-BPA	50	49.5 (3.8)	7.7	99	BP-1	50	49.4 (4.0)	8.2	99
	100	100 (5.3)	5.3	100		100	100.4 (4.5)	4.5	100
	5	0.9 (0.1)	5.5	108		1	1.0 (0.1)	10.4	105
Cl_3-BPA	50	51.5 (1.6)	3.0	103	BP-3	50	52.6 (2.7)	5.1	105
	100	99.7 (3.1)	3.1	98		100	99.4 (3.3)	3.3	99
	10	1.1 (0.1)	9.6	106		1	1.1 (0.1)	8.0	105
Cl_4-BPA	50	53.6 (3.4)	6.3	107	BP-6	50	51.8 (2.4)	4.7	104
	100	98.2 (3.9)	4.0	98		100	99.5 (4.6)	4.6	99.5
	1	0.9 (0.1)	9.7	95		1	0.9 (0.1)	7.2	94
MPB	50	47.8 (3.1)	6.5	96	BP-8	50	50.8 (3.3)	6.5	102
	100	101.1 (3.9)	3.9	101		100	98.9 (3.2	3.3	99
	1	1.1 (0.1)	6.0	111		1	1.0 (0.1)	5.4	105
EPB	50	50.5 (2.6)	5.1	101	4-OH-BP	50	52.3 (2.4)	4.5	106
	100	99.0 (3.1)	3.1	99		100	98.9 (3.7)	3.7	99

[a] *Mean of 18 determinations; SD: standard deviation; RSD: relative standard deviation; R: Recovery*

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in human breast milk samples. Recoveries were determined analyzing the spiked samples and the concentration of each compound was determined by interpolation in the standard calibration curve within the linear dynamic range and compared with the amount of analytes previously added to the samples. A recovery test was carried out. The results are also shown in Table 3. The recoveries were close to 100% in all cases.

Inter-day precision (expressed as RSD) was lower than 15%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data (shown in Table 3) demonstrated that the proposed method is reproducible.

Precision and trueness data indicate that the methodology to determine the target compounds in human milk samples is accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

3.5. Application of the methods

The validated methods were applied to the determination of the amounts of EDCs in 10 human breast milk samples. The results obtained as mean of six determinations are summarized in Table 4.

Table 4. *Application to human breast milk samples*

	GC–MS/MS				
Sample	Found amount (ng mL^{-1}) [a,b]				
	BPA	MPB	EPB	PPB	BPB
01	D	ND	0.7 (0.1)	1.3 (0.2)	ND
02	3.4 (0.3)	ND	2.5 (0.2)	14.9 (1.4)	D
03	5.7 (0.4)	7.2 (0.6)	13.8 (1.1)	43.5 (3.4)	14.5 (1.3)
04	D	ND	0.7 (0.1)	0.8 (0.1)	ND
05	3.2 (0.3)	0.7 (0.1)	0.9 (0.1)	1.4 (0.1)	ND
06	10.8 (0.8)	ND	1.0 (0.1)	0.8 (0.1)	0.6 (0.1)
07	ND	1.2 (0.1)	D	D	ND
08	5.2 (0.4)	1.4 (0.2)	ND	0.8 (0.1)	0.8 (0.1)
09	ND	ND	ND	ND	ND
10	6.2 (0.4)	3.9 (0.3)	8.6 (0.7)	38.7 (2.3)	9.0 (0.8)
Sample	BP-1	BP-3	BP-6	BP-8	4-OH-BP
01	1.0 (0.1)	8.8 (0.8)	1.9 (0.1)	D	D
02	D	2.9 (0.2)	D	2.4 (0.2)	D
03	1.3 (0.1)	0.9 (0.1)	ND	ND	9.7 (0.8)
04	ND	1.3 (0.1)	ND	ND	D
05	ND	D	ND	ND	ND
06	D	0.7 (0.1)	ND	ND	9.9 (0.8)
07	D	D	ND	ND	6.5 (0.5)
08	ND	1.2 (0.1)	ND	ND	ND
09	ND	ND	ND	ND	ND
10	0.8 (0.1)	1.5 (0.1)	ND	ND	8.4 (0.8)

[a] *Mean of 6 determinations; ND: not detected (<LOD); D: detected (>LOD and <LOQ)*
[b] *Standard deviations are in parentheses*

Table 4 cont. *Application to human breast milk samples*

Sample	UHPLC–MS/MS				
	Found amount (ng mL^{-1}) [a,b]				
	BPA	MPB	EPB	PPB	BPB
01	D	ND	0.7 (0.1)	1.1 (0.2)	ND
02	3.2 (0.3)	ND	2.7 (0.2)	14.1 (1.2)	D
03	6.1 (0.4)	7.8 (0.6)	15.0 (1.2)	41.2 (2.9)	13.4 (1.2)
04	D	ND	0.7 (0.1)	0.7 (0.1)	ND
05	3.5 (0.3)	0.8 (0.1)	0.8 (0.1)	1.5 (0.1)	ND
06	11.5 (1.1)	ND	0.9 (0.1)	0.7 (0.1)	0.7 (0.1)
07	ND	1.3 (0.1)	D	D	ND
08	5.4 (0.5)	1.5 (0.1)	ND	0.7 (0.1)	0.8 (0.1)
09	ND	ND	ND	ND	ND
10	6.8 (0.5)	4.3 (0.4)	9.3 (0.8)	36.1 (3.1)	8.0 (0.8)
Sample	BP-1	BP-3	BP-6	BP-8	4-OH-BP
01	0.8 (0.1)	9.6 (0.8)	1.7 (0.2)	ND	D
02	D	2.7 (0.3)	D	2.6 (0.2)	D
03	1.5 (0.1)	0.9 (0.1)	ND	ND	10.2 (0.7)
04	ND	1.4 (0.1)	ND	ND	D
05	ND	D	ND	ND	ND
06	D	0.8 (0.1)	ND	ND	9.2 (0.8)
07	D	D	ND	ND	7.1 (0.7)
08	ND	1.06 (0.1)	ND	ND	ND
09	ND	ND	ND	ND	ND
10	0.9 (0.1)	1.4 (0.1)	ND	ND	7.7 (0.8)

[a] *Mean of 6 determinations; ND: not detected (<LOD); D: detected (>LOD and <LOQ)*
[b] *Standard deviations are in parentheses*

As it is shown in the table, BPA, PBs and BPs were detected and quantified in almost one of the analyzed samples. However, none of the chlorinated derivatives of BPA were detected. BPA was detected in 8 samples and was quantified in 6 of them at concentrations ranging from 3.15 to 10.8 ng mL^{-1}. Among the four parabens analyzed, EPB and PPB were the major compounds found in most milk samples, with concentrations ranging from 0.8 to 43.5 ng mL^{-1} for PPB and from 0.8 to 15.0 ng mL^{-1} for EPB. This indicates concurrent exposure to these two compounds. MPB also was detected frequently (n=5/10), although their concentrations were lower than those of EPB and PPB.

On the other hand, detection rate of BPB were lower than those of the others PBs. Regarding to BPs, BP-3 was detected in almost all samples (n = 9/10) and was quantified in seven samples at concentrations ranging from 0.7 to 9.6 ng mL^{-1}. BP-1 was detected in six of the samples and quantified in only three of them and at low levels. 4-OH-BP was detected in seven samples and quantified in four of them, at concentrations ranging from 6.5 to 10.2 ng mL^{-1}. Finally, BP-6 and BP-8 were only detected in two samples and quantified in one of them in each case.

It is important to emphasize that the concentrations of total BPs and PBs in two of the samples were significantly higher than those in the rest of the samples. Possible causes could be high exposure of those mothers to compounds due to abusive use of sun blockers or other cosmetic products [36].

3.6. Comparison of the methods

Finally, in order to compare the applicability of both proposed methods, a statistical comparison between EDC concentrations found in human milk samples was carried out. A *Student's t*-test was used to compare two means. The values of the statistical *t-calculated* ranged between 0.994 and 2.105. Since the tabulated for α = 5% and 10 degrees of freedom is 2.228, in all cases, t_{calc} were lower than t_{tab}, in consequence, it can be concluded that the two methods can be used with no statistically significant differences between them ($p < 0.05$).

4. Conclusions

In the present work, two analytical multi-residue methods (SBSE in combination with GC–MS/MS or UHPLC–MS/MS) have been proposed for the determination of EDCs in human breast milk. BPA and its chlorinated derivatives, four PBs and five BPs were selected as target analytes. The

introduction of a SBSE procedure and subsequent solvent desorption with an organic solvent prior to the chromatographic analysis is proposed. SBSE, among other advantages, has a high pre-concentration capacity being a very simple and efficient extraction technique that provides enough sensitivity to detect very low quantities of compounds in complex matrices. Although the technique has a broad spectrum of applications, SBSE has never been used for de determination of EDCs in human milk. Due to these advantages SBSE is becoming one of the most studied sample extraction techniques for the analysis of organic compounds. On the other hand, the use of tandem MS, in both GC and LC, allows the unequivocal identification and quantification of these compounds. The main parameters affecting the SBSE (ionic strength, stirring speed and extraction time) have been completely characterized studying in depth the influence of all variables on the analytical signal. The nature of the organic solvent used in the solvent desorption procedure and the optimal instrumental conditions for GC–MS/MS and UHPLC–MS/MS analysis were also evaluated and optimized. Both developed methods show better analytical performance in terms of accuracy (precision and trueness) and sensitivity than previously published methods for the determination of EDCs in human breast milk. However, the most remarkable improvement of the proposed methods is the simultaneous detection of 14 compounds from three different families, while previous methods only involve analysis of one family of compounds, and even in some cases, only a number of compounds of the same family. The methods were satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women.

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3.2. Publicación II

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Stir-barsorptive extraction. Recent applications, limitations and future trends

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Abstract

Stir bar sorptive extraction (SBSE) has generated growing interest due to its high effectiveness for the extraction of non-polar and medium-polarity compounds from liquid samples or liquid extracts. In particular, in recent years, a large amount of new analytical applications of SBSE have been proposed for the extraction of natural compounds, pollutants and other organic compounds in foods, biological samples, environmental matrices and pharmaceutical products. The present review summarizes and discusses the theory behind SBSE and the most recent developments concerning its effectiveness. In addition, the main results of recent analytical approaches and their applications, published in the last three years, are described. The advantages, limitations and disadvantages of SBSE are described and an overview of future trends and novel extraction sorbents and supports is given.

Keywords: Stir bar sorptive extraction; Solid phase extraction; Sample preparation; Analytical applications; Environmental samples; Food samples; Clinical samples

1. Introduction

Sample preparation is perhaps one of the most important stages of the analytical process. This step becomes more important as the complexity of the samples increases and when the concentration levels to be detected are minimal. In fact, selecting the detection technique for an analysis is currently considered easier than choosing the sample treatment technique.

Sample preparation involves clean-up and pre-concentration procedures aimed to improve the sensitivity, specificity and selectivity of the analytical methods. Current trends in analytical chemistry focus on miniaturization of these steps and of the amount of toxic reagents in order to reduce wastes [1, 2]. Solid phase microextraction (SPME), micro liquid-liquid extraction (MLLE), dispersive liquid-liquid extraction (DLLE) or stir bar sorptive extraction (SBSE) are the most popular among all the techniques proposed in recent years for reducing wastes. In the last ten years, these techniques have been widely applied in hundreds of families of compounds, in all analytical fields. During the last few years, research has focused on miniaturization of the entire sample preparation workflow, including the collection of smaller sample sizes that leads to complete automation of almost all these procedures that are tailored to this small sample size.

SPME was developed in the early 90s by Arthur et al. [3] and was the first modern solventless extraction technique for organic compounds. The technique soon became very popular due to its broad application field, simplicity, and low cost, among other reasons. However, at the beginning, the extraction procedure was completely manual with the consequent loss in reproducibility and sample throughput capacity. This limitation was overcome with the advent of commercial solutions that coupled the extraction fibers to generic autosamplers, allowing a completely automated and unattended analysis in both immersed and head space fiber extraction modes. This new extraction technique was successfully applied by modifying previously well-defined methods [4] and it was also used in novel applications [5]. Different

fibers that would allow the extraction of compounds with very different polarities and molecular weights were developed to broaden the applications of SPME. However, because of its limitations, SPME is not the preferred technique for the analysis of organic compounds. Due to the low fiber volume, the mass of analyte extracted was limited by the kinetics of the extraction process, and it was mainly affected by sample volume [6, 7]. Certainly, SPME can be applied to very small to extremely large volumes (i.e., an entire lake), but if quantitative recoveries are needed, only small sample volumes could be analyzed, affecting consequently to the sensitivity of the methods. Other limitations include that the precise control of the extraction time, since the extraction is developed out of the equilibrium state; the premature contamination and degradation of the fiber; the displacement effects due to the matrix compounds; and its relatively low specificity that requires the use of several fibers for multi-residue analysis. SBSE has overcome some of these limitations by allowing larger solid phase volumes.

SBSE was introduced in 1999 by Baltussen et al. [8] who proposed a novel application involving polydimethylsiloxane (PDMS) polymer as sorbent for solid phase extraction. PDMS is coated onto a glass-coated magnetic bar. Sampling is done by directly introducing the SBSE device into the aqueous sample. While stirring, the bar adsorbs the organic compounds to be extracted. The bar is removed from the sample, rinsed with deionized water and dried. After sorption, the compounds are chemically desorbed in a liquid or gas chromatography inlet, but capillary electrophoresis (CE) [9] or even inductively coupled plasma (ICP) [10,11] can also be used. SBSE was developed by Gerstel GmbH & Co. KG (Mülheim an der Ruhr, Germany) and is commercialized under the trade name Twister®. Although the first applications of SBSE were published in 2001 [12] and it cannot be considered a novel technique, nowadays a large amount of new applications are being continuously developed. This technique has been successfully applied to all analytical fields, including environmental, clinical and food analysis, and to a large variety of matrices including soils, environmental water and wastewater, solid and liquid foods, gaseous samples, and biological fluids. Due to the high pre-concentration

capacity, broad spectrum of applications and simplicity, SBSE is becoming one of the most studied sample extraction techniques for the analysis of organic compounds. However, it has some disadvantages such as the limited spectrum of analyte polarities for the available stationary phases, the presence of strong matrix effects and the need of high control of extraction conditions.

There are few reviews on SBSE in the literature, and they mainly focus on the general theoretical principles of this technique [13] or the recently developed applications [14-16]. In contrast, the present review offers a different point of view. The text is divided into three sections: 1) a review of current procedures and approaches used in SBSE and the correlation between the results obtained and the theoretical data; 2) a review of the most recent trends in SBSE applications published in the last four years; and 3) discussion of the main disadvantages and limitations of SBSE that must be overcome in the future in order to improve this technique. The information presented is intended to be useful for the development of future applications and solutions to overcome the limitations of the technique.

2. Theoretical data and actual data obtained

The theory behind SBSE is the same as that of SPME. Baltussen et al. made an extensive study of the theory and thermodynamic principles of SBSE in 1999 [8]. Previously, they had published other approaches related to this technique that finally led to the development of SBSE [17]. Although, the objective of the present work is not to discuss these principles, some concepts must be explained.

It is well known that the extraction efficiency of SBSE and SPME —in PDMS stationary phases— is correlated to the octanol-water partitioning coefficient ($K_{o/w}$) and to the phase ratio (β). The equations that guide the partition between the liquid and the stationary phases are:

$$\frac{m_{SBSE}}{m_0} = \frac{\left(\frac{K_{o/w}}{\beta}\right)}{1+\left(\frac{K_{o/w}}{\beta}\right)} \quad (1)$$

$$\beta = \frac{Volume of sample}{Volume of stationary phase} \quad (2)$$

Where m_{SBSE} is the mass of analyte in the sorbent and m_0 is the mass of the analyte in solution. Both equations are equally valid regardless of the stationary phase or the nature of the sample, but if the stationary phase is not PDMS, the $K_{o/w}$ constants cannot be applied and other appropriated partitioning constants must be used.

The phase ratio is responsible for the better extraction efficiency of SBSE over SPME because the volume of stationary phase used in SBSE is about hundreds to thousands times higher than the one used in SPME. According to this theory, for a sample volume of 10 mL, a quantitative extraction using SPME is only possible for compounds with a Log $K_{o/w}$> 5, while for SBSE, a quantitative extraction can be obtained for compounds with Log $K_{o/w}$> 2.7 using a common PDMS stir bar.

However, it is possible to obtain quantitative extraction (100%) using the SBSE technique? Certainly, there is a huge number of substances with Log $K_{o/w}$ values higher than 2.7. Quantitative extraction was obtained for a volume of 10 mL, which is not a very large volume. In order to obtain lower limits of quantification (LOQs), the sample volume must be increased and recoveries of 100% will only be obtained for compounds with increasing values of $K_{o/w}$. Table 1 shows the theoretical recovery values and the amount of analyte extracted for two substances with a Log $K_{o/w}$ = 2.7 and 4.0, respectively, and the estimated Log $K_{o/w}$ for what quantitative recovery (>80%) is obtained if sample volume is increased.

Table 1
Theoretical recoveries and amount of analyte extracted as a function of the sample volume for two analytes with different $K_{o/w}$, and progression of the $K_{o/w}$ with the sample volume in order to obtain a quantitative recovery

Sample volume	Phase ratio[a]	Log $K_{o/w}$[b]	Log $K_{o/w}$ = 2.7		Log $K_{o/w}$ = 4.0	
			Recovery (%)	Extracted amount (µg)[c]	Recovery (%)	Extracted amount (µg)[c]
5 mL	50	2.30	90.9	0.045	99.5	0.049
10 mL	100	2.60	83.3	0.083	99.0	0.099
20 mL	200	2.90	71.4	0.143	98.0	0.196
50 mL	500	3.30	50.0	0.250	95.2	0.476
100 mL	1000	3.60	33.3	0.333	90.9	0.909
200 mL	2000	3.90	20.0	0.400	83.3	1.667
500 mL	5000	4.30	9.1	0.455	66.7	3.333
1000 mL	10000	4.60	4.8	0.476	50.0	5.000

a: phase ratio calculated using a PDMS volume of 100 µL; b: Log $K_{o/w}$ for a theoretical recovery of 80%; c: extracted amount calculated for a concentration of analyte of 10 µg L^{-1}.

These results demonstrate that the increase in sensitivity of a method based on SBSE, and the increase in the number of substances that can be quantitatively extracted are inversely correlated. These data also show that for the substance with Log $K_{o/w}$ = 2.7 there is no significant increase in the mass of analyte extracted for sample volumes over 200 mL (12.5% when the sample volume increases to 500 mL). For the substance with a Log $K_{o/w}$ = 4.0, the loss in extraction efficiency is not as pronounced and an increase of up to 52.9% is obtained when the sample volume increases from 500 to 1000 mL, resulting in a significant improvement. However, a recovery < 80% implies an unacceptable systematic error in the analysis and requires that the calibration standards must be extracted in the same way as the samples. It could be argued that the only parameter that improves the LOQ in SBSE is the $K_{o/w}$ of the analyte, but this does not seem to be true. The reasons why the experimental data deviate from theoretical data are described below.

2.1. Sample volume and equilibrium time

As previously indicated, the easiest way to improve the recoveries in SBSE is increasing the sample volume. This could be extended until the loss of extraction efficiency according equation 1 overcomes the gain of mass analyte obtained by the increase of sample volume. However, increasing the sample volume involves other issues.

SBSE is a particular type of mass transfer process, considered a multiphase system where a substance is transferred from a liquid to a solid phase. As many other physicochemical processes, the SBSE process depends on the corresponding equilibrium constant (K_c), defined as the ratio between the concentration of the substance in the absorbent and in the sample.

If the solid support is PDMS, K_C is closely correlated with $K_{o/w}$ [8,18]. Nonetheless, equation 1 is only valid when the equilibrium has been reached, this equation also considers that the absorbent is a liquid, but this is only an approximation. Since the sample transfer process implies two or more insoluble phases, the phases must remain in intimate contact in order to reach a complete equilibrium between them. SBSE uses magnetic stirring for this purpose, but due to the capability of disaggregate in liquid media, much longer equilibrium times are needed in SBSE when PDMS is used as absorbent, in comparison to LLE with octanol. Despite all this, the theory behind Equation 1 provides a good practical approach to predict recovery data in SBSE.

This is the first example of deviations from the theory. The equations that define the SPME process are valid only when the equilibrium has been reached and because volumes of sample and stationary phase are higher than those used in SPME, much longer equilibrium times are required. As a result, if out-of-equilibrium conditions are selected (e.g., shorter extraction times because the application requires maximum throughput), Equation 1 would not be applicable. Camino-Sánchez et al. presented data about analyte response as a function of extraction time [19]. Their results showed that compounds with

equal $K_{o/w}$ had different curves and equilibrium times. A trend in the experimental data according to $K_{o/w}$ and equilibrium time can be observed; in general, for some medium-polarity compounds, the experimental data show the following tendency: small molecules with high $K_{o/w}$ have short equilibrium times, while substances with low $K_{o/w}$ show longer times (e.g., the equilibrium between water and PDMS for terbuthylazine and atrazine pesticides is not reached before 48 h). Liu et al. reported identical conclusions for the determination of organophosphorus pesticides (POP) in cucumber and potato [20]. They use compromise conditions due to the long extraction times required to reach the equilibrium (5 h). High precision and reproducibility were obtained when extraction time was strictly set at 30 min.

Therefore, extractions under equilibrium conditions are not always possible and compromise conditions must be set. However, if pre-equilibrium conditions are used, small deviations in the selected experimental variables can lead to a significant loss of reproducibility, and in these cases the use of appropriated internal standard to correct these deviations is mandatory.

2.2. Temperature

Unlike SPME, the effect of temperature on SBSE is not usually evaluated and the extractions are usually conducted at room temperature. Only a few authors have analyzed the effect of temperature [21,22]. The reason for this is that SBSE is considered an "in equilibrium" extraction process; nonetheless, as previously stated, this is not always true. Temperature has two opposite effects on SBSE— the equilibrium state is reached faster at higher temperatures, while the amount of extracted analyte must remain constant, and in contrast, according to Henry's Law, the solubility of the analytes in water increases with temperature and therefore the amount of extracted analyte will be lower ($K_{o/w}$ decrease). The possibility of increasing the temperature will depend on the aim of the method— for maximum sensitivity, the extraction should be performed at room temperature, but for maximum throughput (and minimum extraction time), the temperature should be increased.

Ochiai et al. published one of the first SBSE applications in food analysis [22]. They proposed a method for the determination of different preservatives in diverse aqueous matrices and evaluated the extraction efficiency of SBSE at 25, 45 and 70 ºC. For all compounds, increasing the extraction temperature modified the kinetics of the mass transfer process, which resulted in shorter equilibrium times. However, a decrease in analyte recovery was observed at 45 and 70 ºC. Responses up to four times higher were obtained for some of the studied analytes at the minimum temperature. Liu et al. also studied the effect of temperature on the extraction process, drawing similar conclusions [20]. They observed that the extraction phase began to degrade at 40 ºC, finally setting the temperature at 30 ºC.

On the other hand, analyte stability should also be considered when the temperature extraction increases, since many organic compounds are thermally unstable. Balbao et al. reported recoveries of 76.6 and 56.8% for rifampicin in plasma at 38 and 50 ºC, respectively, in comparison with the ones obtained at 24ºC [21].

2.3. Matrix effect

Matrix effect is one of the major limitations of SBSE. Quantitative recovery strongly depends on sample volume and $K_{o/w}$ coefficients. Samples with high organic matter or suspended solid component, such as environmental samples, biological fluids or foods, are very difficult to extract with SBSE. Adsorption of the analytes onto the organic matter surface competes with the stir bar in the sorption. In addition, partitioning of analytes between water phase and organic matter ($K_{c/w}$) is strongly correlated with $K_{o/w}$, so these compounds with high affinity for PDMS will also exhibit high adsorption to the matrix components. Therefore, lower recoveries are expected for compounds with higher $K_{o/w}$ values. To our knowledge, few authors have developed an exhaustive study of how organic matter influences the recovery of analytes depending on their $K_{o/w}$ or $K_{c/w}$. Further comparison between SBSE and other extraction techniques in terms of recoveries of organic compounds would be useful because otherwise,

false negatives could be obtained with SBSE. This matrix effect is not only limited to organic matter or suspended soils, but it also affects any substance present in the sample that can give rise to a three-phase partitioning (solvent, sorbent and competitor) of the analyte.

The simplest way to solve these issues is to dilute the sample until matrix effects are not significant, but higher LOQs and LODs will be obtained. The second way is to perform matrix-matched calibration, which is probably the best approach, where LOQ and LOD are not affected. However, matrix-matched calibration does not overcome other type of matrix effects: uncontrolled differences in the physicochemical properties between samples and the matrix used for calibration standard matrix-matching. Both matrix effects described imply a decrease in the recovery, but this third type of sample matrix can result in an increase of recoveries over 100%. SBSE recoveries highly depend on the ionic strength of the sample, and therefore, on the salt content. Differences in salt content of samples and calibration standards can lead to unacceptable biases in analyte recovery. Camino-Sanchez et al.[23] developed an SBSE-based method to determine tributyltin species in seawater. For the calibration standards, they prepared water with identical salt content to that of seawater and compared the slope of the calibration graph with the slope from seawater, used as blank for calibration standard. The results demonstrated that there was a significant difference between slopes. Environmental samples have a huge variability between them in their physical and chemical properties, so matrix-matched calibration is not always the best solution for these samples. For wastewater analysis, this effect is even more pronounced.

Actually, the only efficient way to overcome, or at least minimize, matrix effects is using the adequate internal standards. These compounds must be added at the beginning of the extraction process. Although the most expensive option, the use of isotope-labeled compounds is the optimal choice. However, this option is not always feasible when multi-residue methods (MRMs) are used because there are not labeled compounds available for all the substances or because the number compounds included in the method would imply a high

number of costly internal standards, making the method unfeasible. In SBSE, internal standards are subjected to the overall analytical process in both the samples and calibration standards. As a result, high deviation in their responses and more random errors in samples and standards are obtained in SBSE-based techniques [15].

But how much surrogate correction can be done? If the recovery of the internal standard (IS) falls to 20%, do the recoveries of all the analytes included in the method also fall in the same percentage? Authors never set the maximum admissible IS correction when they validate a method. The behavior of IS cannot be expected for all the substances when the recoveries are very far from 100%, although all analytes were from the same family. Isotope-labeled internal standards might not show similar behavior to that of the analyte; effects such as matrix bonding are not equal for the analyte and the IS. Robustness should be studied in depth in SBSE applications and setting the maximum admissible IS correction and variability should be compulsory for each matrix in order to avoid false negatives and false positives. These recommendations are particularly important in MRMs.

2.4. *Matrix modifiers*

In SBSE, like in other extraction techniques, MeOH and NaCl are widely used as matrix modifiers. NaCl is mainly used to cause a salting-out effect, improving the recoveries of polar analytes, and MeOH is added to water samples to increase water solubility of hydrophobic compounds, such as polycyclic aromatic hydrocarbons (PAH) and polychlorobiphenyls (PCBs). The addition of MeOH also prevents the quick adsorption of these compounds over the glass walls of the flask. Both matrix modifiers have a major drawback and in non-polar compounds a decrease in recovery is observed when NaCl is added, which results in increased water density and subsequent lower mass-transfer rate, and in polar compounds an increase in recovery is observed when MeOH is added, which results in decreased water solubility. Therefore, the matrix modifier and its amount must be carefully selected particularly in multi-target

methods.

Another matrix modifier commonly used is pH adjustment. The analyte comes from acid to basic form depending on their *pKa*, making the analyte available to be extracted by SBSE based on the presence of neutral or ionic species. In addition to this well-known procedure, pH adjustment can be used in complexation reactions in order to obtain the neutral specie of an ionic compound. Villaverde-de-Sáa et al. used an ion-pair agent in order to extract several very polar compounds including seven perfluorinated carboxylic acids (PFCs) and perfluorooctane sulfonate (PFOS) in aqueous samples [24]. They added tetrabutylammonium (TBA) as ion-pairing agent to the water samples to obtain the neutral species or an ion-pair with a neutral net charge. General procedures of ion-pair extraction or other methods that lead to the formation of neutral species by decreasing the polarity of the compounds can be applied to SBSE.

Hyamine has also been used as modifier by several authors. This is a cationic surfactant that decreases the surface tension and the adsorption of non-polar compounds onto the glass wall. Kolahgar et al. reported an increase of sensitivity in PAH analysis when a concentration of 10 µg L^{-1} of hyamine was used [25]. Although hyamine was one of the first matrix modifier studied in SBSE, it is not very used and its effect on newly developed applications is no longer studied.

2.5. Deviations from theoretical data

Organic compounds do not always show the predicted behavior according to the theoretical data. An example of a group of compounds that deviates from the predicted data is the four isomers of hexachlorocyclohexane (BHC). These compounds are non-polar and have Log $K_{o/w}$ values ranging from 3.7 to 4.3. Therefore, they should be successfully extracted by SBSE and their behavior could be predicted. However, they have been experimentally proved to show an opposite response to matrix modifiers from that expected. The

recovery of those compounds increases when NaCl is added, conversely, the recovery decreases when an organic solvent such as MeOH is added [19,26]. Similar conclusions have also been reported by Margoum et al. and Ochiai et al. [27,28]. Figures 1 and 2 show the extraction recoveries of the organochlorinated pesticides (OCP) in relation to NaCl and MeOH concentrations, respectively. An opposite behavior is observed between BHC isomers and other OCPs when the content of NaCl or MeOH increases, although they have similar $K_{o/w}$ values.

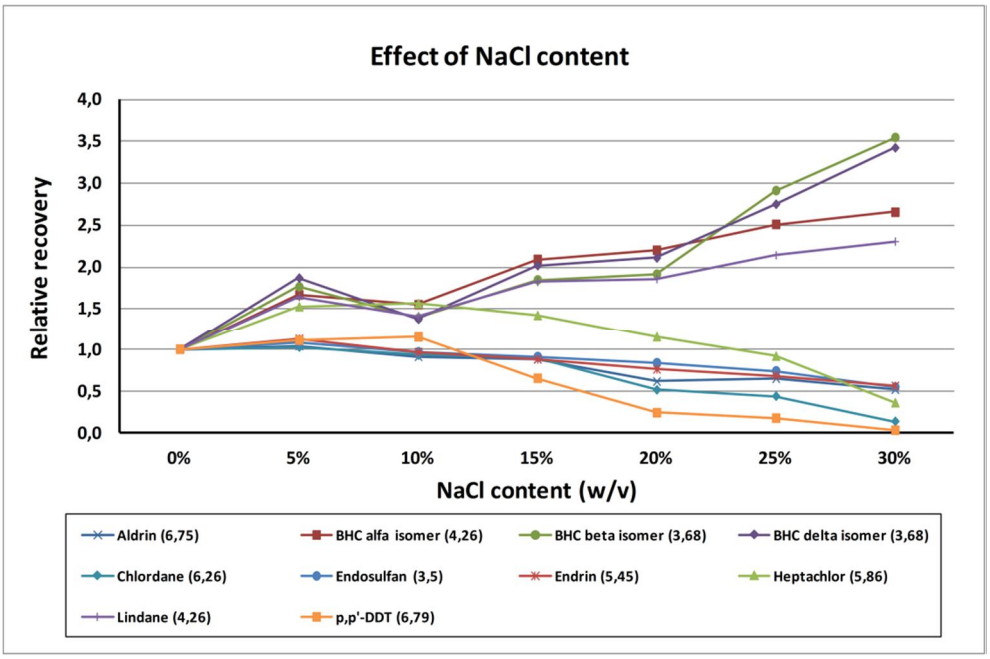

Figure 1. *Effect of matrix modifier NaCl on the recovery of organochlorinate pesticides. Into parentheses is included the Log $K_{o/w}$ of each substance*

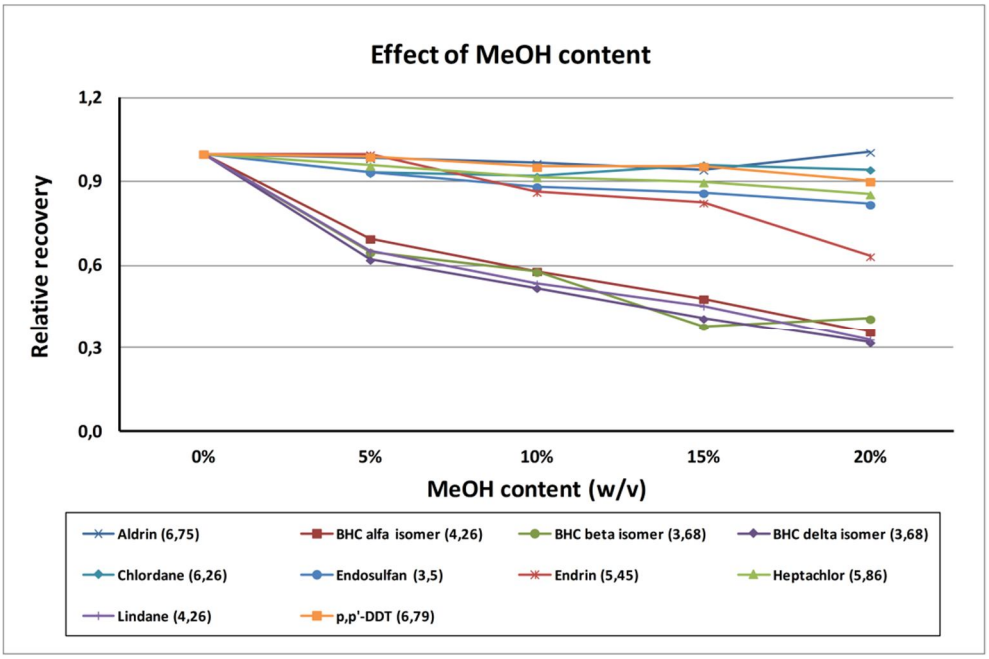

Figure 2.*Effect of matrix modifier MeOH on the recovery of organochlorinated pesticides. Into parentheses is included the Log $K_{o/w}$ of each substance*

Another common deviation from theoretical data is shown by compounds with very high Log $K_{o/w}$. This issue has been previously reported by many authors and discussed above. This effect is due to the adsorption of very hydrophobic compounds onto the glass walls of the extraction flask, which makes them unavailable to the extraction sorbent. Since this deviation has been well described by other authors, it will not be discussed here.

Methods of analysis that include compounds whose behavior is different the theoretically expected should be carefully optimized and validated. The selection of the appropriate internal standard is essential, especially in multi-compound applications.

3. Latest applications and trends

Since the number of SBSE-based methods published has experienced a linear increase in the last ten years, this paper reviews the literature published in the last four years. Originally, SBSE was intended for environmental analysis, but over time, hundreds of applications for almost every field of analytical chemistry have been developed. In addition, new materials and coatings are being developed in order to overcome the limitations of PDMS and to expand the technical applicability of SBSE. In the present review, the application fields of SBSE are divided into the following groups for further discussion: environmental analysis, food analysis, clinical and pharmaceutical analysis, and development of new coatings.

3.1. Environmental analysis

For years, SBSE has been widely applied to the analysis of a large number of compounds known as Persistent Organic Pollutants (POPs), most of them included in the Stockholm Convention. These families of compounds include PAH, organochlorinated pesticides (OCPs), organophosphorus pesticides (OPPs), organonitrogenated pesticides (ONPs), PCBs and polybrominated diphenylethers (PBDE). Water and soil were the most commonly studied

matrices using SBSE, but air, sewage sludge and other complex matrices are left out of the scope of SBSE. It has been demonstrated that SBSE can be applied for the analysis of almost all of these pollutants. Accurate results, good recoveries and very low LOQs have been reported for these applications [12,29]. Recently, the application of SBSE has extended to a new group of pollutants generically known as emerging contaminants. Since the awareness of the risk of these pollutants is recent, the development of new analytical methods is mandatory. The most recently developed methods are summarized in Table 2.

Table 2
Recent SBSE-based methods for environmental analysis

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Ref
Fungicides / preservatives	Wastewater	TCC	Liquid	LC-MS/MS	[30]
Organic pollutants	Sea water	OCPs, PAHs, PCBs, PBDEs, NP	Thermal	GC-MS	[31]
Odors/synthetic compounds/ detergents	Natural water and wastewater	18 synthetic musk fragrances	Thermal	GC-MS	[32]
PAHs	Wastewater	24 PAHs	Thermal	GC-MS/MS	[33]
Organic pollutants	Marine sediments	84 compounds: OCPs, OPPs, ONPs, ureic pesticides, PAHs, PCBs, PBDEs	Thermal	GC-MS/MS	[26]
EDCs	Environmental water samples	20 Compounds: APs, BPA, estrogens and sterols	Thermal	GC-MS	[34]
PAHs	Eluates of contaminated soils	15 PAHs	Liquid	HPLC-FLD	[35]
UV-filters	Sea water	6 UV-filters	Liquid	LC-(APCI)-MS/MS	[37]
Drugs residues	Wastewater and river water	6 statin drugs	Liquid	HPLC/Q-TOF-MS	[36]
Pesticides	River water	16 OCPs	Thermal	GCxGC-HRTOF-MS	[38]
Odors/synthetic compounds/det ergents	Natural water and wastewater	9 synthetic musk fragrances	Thermal	GC-MS	[39]
Organic pollutants	Environmental water samples	45 compounds EU list of priority substances and EPA method 625	Thermal	GC-MS	[40]
Organic pollutants	River water	77 compounds: OCPs, OPPs, ONPs, PAHs, PCBs, PBDEs	Thermal	GC-MS/MS	[19]

Table 2 cont
Recent SBSE-based methods for environmental analysis

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Ref
Organotin compounds (anti-fouling)	Sea water	TBTs	Liquid	LC-MS/MS	[23]
PAHs	Environmental water samples	6 PAHs	Liquid	LC-FLD	[41]
Pesticides	Surface water	15 pesticides and metabolites	Liquid	LC-MS/MS	[27]

APs: alkylphenols; BPA: bisphenol A; DI: direct immersion; EDCs: endocrine disrupting chemicals; NP: nonylphenol; OCPs: organochlorinated pesticides; ONPs: organonitrogenated pesticides; OPPs: organophosphorous pesticides; PAHs: polycyclic aromatic hydrocarbons; PBDEs: polybrominated diphenyl ethers; PCBs: polychlorinated biphenyls; PLE: pressurized liquid extraction; TBTs: tributiltin species; TCC: triclocarban

3.2. Food analysis

In this field, SBSE has been mainly used for the analysis of pollutants and toxics. Very few SBSE-based methods have been validated for the analysis of nutrients or major constituents, with some exceptions, such as the method published by Horák et al. (2008) for the determination of free medium-chain fatty acids in beer [42].

Recently, Ha et al. (2014) have reported an SBSE-based method for the determination of E,E-farnesol and other related volatile compounds in rice wine [43]. They compared the efficiency of SBSE and the dynamic headspace sampling (DHS) obtaining sensitive and accurate results, and they conclude that SBSE is a good option for volatile compounds analysis. Jin et al. (2013) [44] developed a method for the simultaneous determination of six commonly used preservatives with low $K_{o/w}$in beverages. Since PDMS cannot extract these compounds efficiently, dual coated bars were tested. The performance of the proposed coatings was evaluated against PDMS, resulting in a dramatic improvement of the extraction efficiency. The SBSE-based methods more recently published for food analysis are summarized in Table 3.

Table 3.
Recent SBSE-based methods for food analysis

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Ref
Strobilurin pesticides	Fruits	Metominostrobin, azoxystrobin, dimoxystrobin, kresoxim-methyl, picoxystrobin, pyraclostrobin, trifloxystrobin	Liquid	LC-DAD	[45]
Volatile compounds (aroma)	Grapes	Glycosidic aroma compounds	Thermal	GC-MS	[46]
Furan	Coffee and jarred baby food	Furan	Thermal	GC-MS	[47]
Haloanisoles	Cork stoppers	2,4,6-TCA	Thermal	GC-MS	[48]
Pesticides	Fruit-based soft drinks	7 OCPs, 6 OPPs	Thermal	GC-MS	[49]
EDCs (bisphenols)	Canned beverages and filling liquids of canned vegetables	BPA, BPF, BPZ, BP	Thermal	GC-MS	[50]
Pyrethroid pesticides	Tea	Fenson, allethrin, ovex, tetramethrin, fenpropathrin, permethrin, t-cypermethrin, c-cypermethrin, deltamethrin, fenvalerato, bifenthrin, cyfluthrin	Thermal	GC-MS	[51]
Volatile compounds	Vinegars	Short-chain esters, acids, acetates and alcohols, phenols, lactones and benzenic and furanic compounds	Thermal	GC-MS	[52]
Volatile compounds (aroma)	Rice wine	E,E-farnesol and other volatile compounds: volatile alcohols, 1-butanol-3-methyl acetate, stearol, and phytane	Thermal	GC-MS	[43]

BP: bisphenol; BPA: bisphenol A; BPF: bisphenol F; BPZ: bisphenol Z; DHS: dynamic headspace sampling; EDCs: endocrine disrupting chemicals; MAE: microwave extraction; OCPs: organochlorinated pesticides; OPPs: organophosphorous pesticides; PCA: principal components analysis; TCA: trichloroanisole; VOCs: volatile organic compounds

3.3. Pharmaceutical and clinical applications

Table 4 summarizes the SBSE-based methods most recently developed in the pharmaceutical and clinical fields and in related fields. The number of applications is lower than in the fields of environmental and food analysis. The pharmaceutical and medical field is perhaps the area where SBSE has been less applied because of the following reasons: (i) in pharmaceutical production and quality assurance (QA) analysis, the concentration of the target analytes is usually in a range in which pre-concentration is not necessary; a dilution of the initial sample is usually performed, therefore, SBSE is not useful and simpler extraction procedures are used. (ii) Biological matrices are generally very complex samples and SBSE may be not the best choice for the elimination of matrix interferences because this is not a highly selective or specific extraction technique. SPE has been used instead during the last decades for treatment of biological samples with better results than SBSE or SPME. (iii) In medical and pharmaceutical research, the sample volume is often limited to few milliliters or few microliters due to the nature of the sample (plasma, serum, tissues, etc.) and the difficulty in sample collection. Although SBSE requires very low sample volumes, a minimal volume is required to cover adequately the stationary phase and to obtain reliable extractions. (iv) The two most important reasons are related to the polarity of the analytes. First, these substances are polar compounds and they have a poor extraction efficiency when PDMS is used as stationary phase. Second, the compounds are commonly analyzed by liquid chromatography using liquid desorption. The procedure cannot be easily automated and it is less sensitive than thermal desorption.

Table 4
Recent SBSE-based methods for medical and pharmaceutical analysis

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Ref
Drugs	Plasma	Rifampicin	Liquid	LC-UV	[21]
SRI	Brain tissue, plasma and urine	Fluoxetine, citalopram, venlafaxine, norfluoxetine, desmethylcitalopram didesmethylcitalopran and o-desmethylvenlafaxine.	Liquid	LC-FLD	[53]
CCS	Smoke	17 VOCs	Thermal	GC-MS	[54]
Drugs	Urine	DIC	Liquid	LC-UV	[55]
Drugs	Pharmaceutical liquid formulations	DIC	Liquid	LC-UV	[56]
Phenols	Solid drugs	2,4,6-TBA, 2,4,6-TBP, 2,4,6-TCA and 2,4,6-TCP	Thermal	GC-MS/MS	[57]
Antioxidants	HMWP implantable medical devices	2-Tert-butyl-6-(prop-1-en-2-yl)phenol, 2,6-Di-tert-butylphenol, BHT-quinone, BHT, BHT-aldehyde, Metilox®	Thermal	GC-MS/MS	[58]

BHT: butylated hydroxytoluene; CCS: components of cigarette smoke; DIC: diclofenac; HMWPE: high molecular weight polyethylene; SRI: serotonin reuptake inhibitors; TBA: tribromoanisole; TBP: tribromophenol; TCA: trichloroanisole; TCP: trichlorophenol; VOCs: volatile organic compounds

3.4. *New coating materials*

In recent years, researchers have focused on the development of new coatings [59] but despite this fact, there are only three commercially available coatings for SBSE: PDMS, Polyacrylate (PA) and ethylene glycol/silicone (EG/silicone). The last two coatings were introduced by Gerstel GMbH (Mülheim an der Ruhr, Germany) in 2011 for the extraction of polar and medium-polarity compounds. Since EG/silicone is a silicone-based compound, it will also extract non-polar compounds. Few papers on the use of PA and EG/silicone have been published [60-62], and in all cases, authors have reported

higher extraction efficiency of these new sorbents for polar compounds than PDMS. However, Ochiai et al. [61] have reported some mechanical instability of EG-silicone coatings. This coating degraded quickly and each stir-bar could be used only about 20 times but to solve this issue, they modified the stirring of the sample. The EG-silicone bar is placed on the wall of the extraction jacket while the sample is stirred by a magnetic stir bar or a conventional PDMS-coated sorptive bar.

The development of new coatings is, in fact, the most relevant improvement to expand the applicability of SBSE, allowing the analysis of polar compounds. Nonetheless, these compounds generally cannot be analyzed using gas chromatography, and thermal desorption cannot be used. Table 5 shows a total of 21 methods that use liquid desorption, while only four that use thermal desorption.

Table 5.
Recent SBSE-based methods that use new coating materials

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Stir bar coating material	Ref
Triazine herbicides	Rice, apple, lettuce and soil	Cyanazine, simazine, simetryne, atrazine, ametryn, propazine, terbuthylazine, prometryn, terbutryn	Liquid	HPLC-UV	MIP	[66]
Inorganic anions	Purified water	Br^-, NO_3, PO_4^{3-} and SO_4^{2-}	Liquid	IC	Monolithic material poly(2-(methacryloyloxy) ethyltrimethylammonium chloride-co-divinylbenzene)	[67]
Drugs	Urine	AMP, mAMP, 3,4-methylenedioxy-AMP, 3,4-methylenedioxy-mAMP and ketamine	Liquid	HPLC-UV	Titania-OH-TSO	[68]
β_2-agonists	Pork, liver and feed	Ractopamine, isoxsuprine, clenbuterol and fenoterol	Liquid	HPLC-UV HPLC-FLD	MIP with ractopamine	[69]
Herbicides (sulfonylurea herbicides)	Environmental water	Nicosulfuron	Liquid	HPLC-UV	MIP	[70]
Emerging pollutants (polar pharmaceuticals and personal care products)	River water, effluent and influent waste water	Paracetamol, caffeine, antipyrine, propranolol, carbamazepine, ibuprofen, diclofenac, methylparaben, ethylparaben, propylparaben, triclocarban, DHB, DHMB and BP3	Liquid	LC-MS/MS	Hydrophilic polymer based on poly(N-vinylpyrrolidone-co-divinylbenzene)	[71]
Industrial residues	Wastewater	Benzothiazole	Thermal	GC-MS	PA	[60]
Drugs	Pork, liver and chicken samples	Sulfamethazine, sulfachloropyridazine, sulfamethizole, sulfathiazole, sulfameter and sulfamethoxazole	Liquid	HPLC-UV	MIP	[73]

Table 5 cont.
Recent SBSE-based methods that use new coating materials

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Stir bar coating material	Ref
Phenyl arsenic compounds and their possible transformation products	Chicken tissues	cMMA,DMA,p-ASA,4-OH,3-NHPAA,PA,4-NPAA	Liquid	LC-ICP-MS	TiO_2-PPHF	[72]
Polar pharmaceuticals	Environmental water	Paracetamol, caffeine, antipyrine, propranolol, carbamazepine, naproxen and diclofenac	Liquid	LC-MS/MS	poly(MAA-co-DVB)	[74]
Chemical warfare agents and degradation products	Environmental water	EMPA, PMPA and MPA	Liquid	CE	ZrO_2-PDMS	[75]
Seleno-amino acids and seleno-oligopeptides	Biological samples	SeCys2, MeSeCys, SeMet, SeEt, γ-GluMeSeCys, GS-Se-SG	Liquid	LC-ICP-MS	PSP-TiO_2	[76]
Drugs	Human serum	Carvedilol	Liquid	LC-UV	Poly(methyl-PA-EG) and PDMS	[77]
Hormones	Water samples	Estriol, estradiol, ethynylestradiol, estrone, progesterone, medroxyprogesterone, levonorgestrel, northindrone	Liquid	LDTD-APCI-GC-MS/MS	PDMS/PTS/β-cyclodextrin	[78]
Antibacterials synergist and sulfonamides	Urine, plasma and milk	Trimethoprim, sulfamether, sulfamethazine and sulfamerazina	Liquid	LC-UV	MIP	[80]
Drugs	Milk and milk powder	SDZ, SMR, SMZ, SMT, SMX and SDM	Liquid	LC-MS/MS	C18-PDMS	[81]
Bisphenols	Personal care products	BPA, BPF and BPZ	Thermal	GC-MS	EG-Silicone	[82]

Table 5 cont.
Recent SBSE-based methods that use new coating materials

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Stir bar coating material	Ref
Drugs	Pork meat	Ractopamine	Liquid	ECL	MIP	[79]
Organophosphorus pesticides	Environmental waters	Phorate, fenitrothion, malathion, parathion and quinalphos	Liquid	GC-FPD	PDMS/PTH	[83]
Metals	Drinking water	Copper	Liquid	FAAS	MIP, Cu-morin based	[84]
Estrogens	Environmental water	17β-estradiol, dienestrol, diethylstilbestrol, estrone, 4-t-OP, BPA and 17α-ethynylestradiol	Liquid	LC-UV	Metal-organic frameworks (OF) combined with PDMS (PDMS/MOF-5, PDMS/MOF-199 and PDMS/IRMOF-3)	[85]
Volatile compounds	Green Tea	32 VOCs present in beverages	Thermal	GC-MS	EG-Silicone	[60]
Alkyl phenols and metals	Water samples	APs, Cu(II), Cr(III) and Ni(III)	Liquid	GC/MS and ICP-OES	Silica gel modified with ketamine groups	[11]
Drugs	Urine	(+)-(S)-citalopram	Liquid	LC-MS/MS	Chiral-MIP	[86]
Drugs	Food samples	Vardenafil, tadalafil and sildenafil	Liquid	HPLC-UV	Endrimer-based MIP	[87]
Preservatives	Beverages (cola, orange juice and herbal tea)	BA, SA, MP, EP, PP and, BP	Liquid	HPLC-UV	APTES/OH-TSO and C_{18}-PDMS	[44]

Table 5 cont.
Recent SBSE-based methods that use new coating materials

Family of analyzed compounds	Sample type	Analytes	Desorption	Technique	Stir bar coating material	Ref
Drugs	Pork meat	Ractopamine	Liquid	ECL	MIP	[79]
Wine taint compounds	Wine	CPs and CAs	Thermal	GC-MS	EG–Silicone and PA	[62]

AMP: amphetamine; APCI: atmospheric chemical ionization; APs: alkyl phenols; APTES: 3-aminopropyltriethoxysilane; BA: benzoic acid; BP: butyl p-hydroxybenzoate; BP3: benzophenone-3; BPA: bisphenol A; BPF: bisphenol F; BPZ: bisphenol Z; CAs: chloroanisoles; CPs: chlorophenols; DHB: 2,4-dihydroxybenzophenone; DHMB: 2,2-dihydroxy-4-methoxy benzophenone; ECL: electrochemiluminescence; EG: ethyleneglycol; EMPA: ethyl methylphosphonic acid; EP: ethyl p-hydroxybenzoate; γ-GluMeSeCys: γ-glutamyl-Se-methyl-selenocysteine; GS-Se-SG: selenodiglutathione; LDTD: laser diode thermal desorption; mAMP: methamphetamine; MIP: molecularly imprinted polymer; MP: methyl p-hydroxybenzoate; MPA: methylphosphonic acid; MeSeCys: methylseleno-cysteine; OF: organic frameworks; OH-TSO: hydroxy-terminated silicone oil; PA: polyacrilate; PDMS: polydimethylsiloxane; PMPA: pinacolyl methylphosphonate; poly(MAA-co-DVB): copolymer of methacrylic acid and divinylbenzene; PP: propyl p-hydroxybenzoate; PSP-TiO_2: partially sulfonated polystyrene-titania; PTH: polythiophene; PTS: phenyltrimethylsiloxane; SA: sorbic acid; SDM: sulfadimethoxine; SDZ: Sulfadiazine; SeCys2: Selenocystine; SeEt: selenoethionine; SeMet: selenomethionine; SMR: sulfamerazine; SMT: sulfamethizole; SMX: sulfamethoxazole; SMZ: sulfamethazine; TiO_2-PPHF: high polar extraction phase of titania immobilized polypropylene hollow fiber; titania-OH-TSO: titania-hydroxy-terminated silicone oil; 4-t-OP: 4-t-octylphenol; VOCs: volatile organic compounds; ZrO_2: zirconia

The new coatings are typically manufactured using two technologies: Molecular Imprinting Technology (MIT) and sol-gel processes. MIT is a technique to create artificial receptors or ligands with a predetermined selectivity and specificity for a given analyte. These materials are known as Molecularly Imprinted Polymers (MIPs). MIPs are robust molecular recognition elements, such as antibodies or biological receptors, and they are useful to separate and analyze complex samples such as biological fluids and environmental samples [63]. MIPs applied to SBSE allow the selective extraction of the analyte based on its tridimensional structure or functional groups. On the other hand, the sol-gel process is a method for producing advanced solid materials from small molecules. The method is used for the creating organic-inorganic hybrids materials, mainly metal oxides of silicon, titanium and zirconium. In general, the sol-gel process involves the transition of a solution system from a liquid "sol" (mostly colloidal) into a solid "gel" phase. Using the sol-gel process, it is possible to manufacture advanced materials in a wide variety of forms: ultrafine or spherical shaped powders, thin film coatings, fibers, porous or dense materials. These materials can be applied onto a

magnetic rod for the production of new sorptive bars with new physical and chemical properties [64,65].

4. Some outstanding issues

4.1. Stir bar status control

One of the most common issues that researchers are faced with when they start working with SBSE is the maximum number of analysis that can be made with one sorptive bar before degradation. Obviously, this number depends on several factors including the matrix properties, extraction conditions, but particularly on the desorption conditions. High desorption temperature, long desorption times, extreme sample pH, oxidation of the adsorbent and irreversible adsorption of matrix components are some of the most common factors which decrease the useful life of the adsorbent.

In this regard, it is important to know how the degradation of the adsorbent affects the analytical results. The degradation of the coating bar generally is progressive and results in gradual decrease of the stationary phase volume. As previously discussed, the extraction efficiency strongly depends on the volume of adsorbent, and this effect is different for each analyte depending on the physicochemical properties. Therefore, the effect of the degradation of the sorptive bars will not be equal for all analytes, even to the extent that bars that show an extraction efficiency similar to new bars for some analytes will show total inefficiency for the extraction of others. The selection of the IS will play an essential role in the accuracy of the results. The effects of the aging of the adsorbent on the recovery of analytes must be studied in the validation step of any SBSE-based method. The maximum number of extractions that can be made with each sorptive bar and the maximum correction of the internal standard for each analyte must be established. A control of each batch of sorptive bars or an individual control of each one must be made in order to obtain adequate results in a long period of time. This procedure can be made by setting a minimum recovery of the

internal standards or, in a simpler way, by recording the number of extractions made with each bar or batch.

4.2. Stir bar conditioning

An important factor that must be taken into account during SBSE is the "memory effect" of the adsorbent after sample desorption. Stir bars are reusable and as any other laboratory material, they must be cleaned and properly conditioned before the next use. Compounds with high boiling points, such as PAHs, PCBs and PBDEs, are the most critical when thermal desorption is used. Typical thermal desorption instruments do not reach more than 300 – 350 ºC and PDMS cannot be heated to temperatures higher than 300 ºC because it is degraded. Although desorption is performed under an intense flow of inert gas (50 – 200 mL min^{-1}) and for long desorption times (5 – 15 min), the total desorption of compounds with high boiling point is no easy to obtain. In addition, incomplete desorption can be more marked when liquid desorption is used because a thermodynamic equilibrium is established between the coating and the extraction solvent, but since the coating is selected to have a high affinity for the analyte, the equilibrium seldom is completely displaced to the solvent, and a portion of the total amount in the sorbent could not be extracted. This fact has many implications including false positives, blank issues, loss in reproducibility, as well as a reduction of the concentration range of the method.

Therefore, extreme care should be taken in the conditioning of bars and adequate procedures should be established when a method is developed in order to avoid cross contamination between uses. Stir-bar conditioning can be performed in two ways, thermal desorption or a solvent conditioning procedure. The former can be carried out in the same thermal desorption instrument that is coupled to the gas chromatograph or using a specific equipment. This procedure has several disadvantages, the specific equipment is expensive and the use of the gas chromatograph is highly time-consuming and the instrument can be contaminated by previous processes. Residues of

coating or non-volatile matter from the matrix can enter into the thermal desorption unit or into the gas chromatograph. The shelf life of the coating can be dramatically reduced if very high temperatures are applied. Solvent conditioning could be the best approach, but the solvent must be carefully selected in order to avoid coating damages. Acetonitrile and mixtures of this solvent with dichloromethane are the most common solvents used for this purpose. Methanol is not recommended since it is a protic solvent.

4.3. Stability of new coatings

Although new coatings have been developed in recent years and their suitability for the extraction of a wide variety of compounds has been proved, their quality must be further assessed beyond extraction efficiency. New coatings must be physically stable and have good mechanical properties that ensure they do no break or degrade after their use. Bonding of the coating to the cover of the magnetic rod, generally glass, is one of the difficulties. Bars are subjected to several processes during the extraction and analysis that can damage the coating. During the stirring, the surface of the coating is continuously rubbing the bottom surface of the glass flask at a very high stirring rate (usually up to 1000 to 2000 rpm) and these frictions can break the union between the coating and the supporting surface where is attached, generally glass, and then the coating will break away. The bonding must be strong enough to allow an intense friction between the coating and the surface of the sample recipient without material losses. Some authors have employed a metal-organic framework in order to increase the mechanical resistance of the bar [85]. These frameworks consist of metal ions or clusters coordinated to increase the rigid properties of organic molecules. In addition, the coating must also be thermally stable in order to tolerate the high temperature needed for desorption.

The coatings must be also chemically inert. The coating material will be in constant contact with oxygen, both atmospheric and dissolved in the water samples. Consequently, it could be quickly and easily oxidized and will lead to a

modification of the coating structure and properties which can result in loss of extraction capabilities. Furthermore, coatings could be damaged by a large number of compounds including matrix modifiers such as acids, bases or organic solvents, major components of the sample (proteins or lipids) that may obturate the surface of the coating, or any substance that could permanently bind to the coating.

The most critical step in which the coating can be damaged is desorption (liquid and thermal). New developed materials must be able to tolerate the high temperatures used in thermal desorption without degrading or losing extraction efficiency. When liquid desorption is used, the material should not be damaged or dissolved by the organic solvent. The coating must be chemically stable and it should not dissolve into the extraction solvent. All these challenges must be solved and the performance parameters presented before claiming that a new coating is valid for SBSE. An assessment of the robustness, chemical, physical and thermal resistance would be mandatory every time a new coating is proposed.

5. Conclusions

It has been ten years since SBSE was first applied. Since then, there has been a steady increase in the number of published methods that propose the use of SBSE. Although environmental have been the most studied matrices, SBSE has been successfully applied to almost all types of matrices, covering all the fields of analytical chemistry research. The main limitation of SBSE in the early stages of development was that there was only one available coating material, which allowed only the extraction of compounds with high $K_{o/w}$. This limitation was partially overcome by the use of matrix modifies. However, compounds with low $K_{o/w}$ values, or relative low values, could not be successfully extracted with PDMS stationary phases. SBSE was developed as an improvement to SPME and it was therefore designed for thermal desorption coupled to a gas chromatograph only. Later, liquid desorption of the stir-bars was proposed in order to use liquid or gas chromatography without using

expensive thermal extraction units.

Current trends in research related to SBSE are focused toward the development of new coating materials for the stir-bars, which extend the versatility and applicability of the technique. These new materials are divided in two main groups: coating for the extraction of polar compounds (i.e., PA and EG-based coatings), and coating for selective extractions (i.e., MIPs). But besides the extraction performance, these proposed new coatings still have to prove their robustness and quality in order to claim that they are valid for their intended purpose. It is worth noting that most of the new proposed materials are desorbed by liquid desorption, and this could be due to the fact that polar compounds are not susceptible to thermal desorption, or the coating cannot tolerate high temperatures. The number of extractions that can be made with a new material, before its performance begins to decrease, could be a good reference to evaluate its robustness.

Despite the continuous development and evaluation of new coatings, there are others limitations that must be overcome, like the automation of liquid desorption, the impossibility of re-analysis after thermal desorption, the control of coating status after several uses and the mixing up of used twisters with new ones. Taking into account that stir-bars are expensive and must be reused for several extractions as long as the coating is in appropriate conditions, SBSE has not the best precisions (RSD) when compared to other extraction techniques such as SPE. Therefore, SBSE may be the best choice for ultra-trace analysis, but probably not the best technique for small sample volumes or when high precision is required.

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CAPÍTULO III

Aplicación de adsorbentes dispersivos previa precipitación de grasas y proteínas

1. OBJETIVOS

Los principales objetivos de los trabajos realizados y presentados en este capítulo son:

- ✓ Proponer metodología de buenas características para la determinación de bisfenol A, parabenos y benzofenonas mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS) en leche materna humana.

- ✓ Optimizar un tratamiento de muestra simple basado en una precipitación de proteínas y grasas seguido de una técnica de limpieza del extracto con adsorbentes dispersivos (PSA, C18) previo al análisis cromatográfico.

- ✓ Validar el método analíticos propuesto en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación aplicables.

- ✓ Aplicar la metodología desarrollada al control y evaluación del contenido de los disruptores seleccionados en muestras reales de leche materna humana.

2. INTRODUCCIÓN

En los últimos años, se encuentran disponibles una amplia variedad de técnicas de extracción, que incluyen desde las más tradicionales como la extracción sólido-líquido, en fase sólida y líquido-líquido, hasta otras más modernas, como la extracción por ultrasonidos, microondas y por líquidos presurizados; además de las "sofisticadas" como la extracción dispersiva líquido-líquido (DLLME), la extracción sobre barras magnéticas (*stir bar sorptive extraction*) y la extracción dispersiva en fase sólida (MSPD).

En general, en todas estas técnicas se hace uso de algún disolvente orgánico en alguna de sus etapas, siendo acetonitrilo, metanol, acetona y acetato de etilo, los más comúnmente usados. Estos disolventes, además de su función extractiva, en el caso de matrices biológicas y alimentos, también producen precipitación de proteínas y de algunos lípidos, lo cual constituye una función importante, ya que de esta manera se eliminan simultáneamente componentes de la matriz que pueden afectar la eficiencia de la extracción de los compuestos, así como los parámetros de calidad del método analítico, como por ejemplo, la sensibilidad.

Por este motivo, independientemente del método de extracción utilizado, el tratamiento de muestra incluye casi siempre un paso de limpieza de los extractos o *clean-up*, que tiene por objeto reducir los efectos matriz, y de esta manera, superar las limitaciones prácticas que tienen las diversas técnicas de extracción, especialmente cuando se trata de matrices complejas, como son las matrices biológicas.

Fue Anastassiades[190], quien al introducir la técnica QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) como tratamiento de muestras para la extracción de residuos de contaminantes, incluyó un paso de limpieza del extracto mediante el uso de adsorbentes para la SPE dispersiva, que consistía

[190] Anastassiades M., Lehotay S.J., Stajnbaher D., Schenck F.J. *Journal of AOAC International*, 2003, 86:412-431.

en agregar una pequeña cantidad del adsorbente a una alícuota del extracto, agitarlo por un corto período de tiempo, con la consiguiente recuperación del extracto "limpio" luego de la centrifugación para la separación del adsorbente. Este procedimiento demostró ser además de muy simple, también muy eficiente como método de *clean-up*, por lo que su uso se ha extendido con los años a una amplia variedad de técnicas de extracción y de matrices. La elección adecuada del tipo de adsorbente garantizará la eliminación selectiva de diversos componentes dependiendo de la naturaleza de la matriz, y debido a esto, en la actualidad, se ha incrementado en el mercado, el número de adsorbentes disponibles para una amplia variedad de matrices. Es importante indicar, que a diferencia de la MSPD, donde un factor limitante de la técnica es el costo de estos adsorbentes dispersivos, en el caso de este método de *clean-up*, la cantidad de adsorbente que se requiere es comparativamente mucho menor, ya que usualmente es aplicado en alícuotas de los extractos o extractos reconstituidos con un volumen pequeño de disolvente, lo cual incrementa sus prestaciones en relación al balance positivo eficiencia *vs* precio.

Además, la SPE dispersiva tiene importantes ventajas sobre la SPE tradicional, como el uso de una menor cantidad de disolventes, lo que la ratifica como una técnica de bajo costo y "amigable" con el medio ambiente, ya que tampoco requiere el uso de cartuchos, evitándose en consecuencia, las etapas de acondicionamiento, lavados, etc. Por otro lado, es una técnica versátil ya que permite utilizar mezclas de diversos tipos de adsorbentes, de acuerdo a las necesidades del analista (tipo de matriz y de analitos), y no limitarse solamente a los cartuchos que existen en el mercado, con lo cual se pueden implementar métodos nuevos de tratamiento de muestra.

A continuación, se hará una breve descripción de dos de los adsorbentes más comúnmente usados, y que se han aplicado en las técnicas de *clean-up* incluidas en esta Memoria: PSA y C18.

A. Amina primaria secundaria (PSA). Este adsorbente actúa como un intercambiador aniónico que puede interactuar con los compuestos a

través de enlaces de hidrógeno y/o fuerzas dipolo-dipolo. Debido a que establece interacciones fuertes con los componentes de la matriz, es ampliamente usado para la eliminación de varios tipos de interferentes[190]. La estructura bidentada del PSA (Figura 1) tiene un elevado efecto quelante, debido a la presencia de los grupos amino primario y secundario en su estructura[191]. En consecuencia, este adsorbente retiene típicamente ácidos grasos libres, azúcares y otros compuestos polares que usualmente interfieren durante el análisis cromatográfico. Sin embargo, no es eficiente en la eliminación de grasas y lípidos, por lo que usualmente es usado de forma conjunta con otros adsorbentes, por ejemplo C18 o carbono grafitizado.

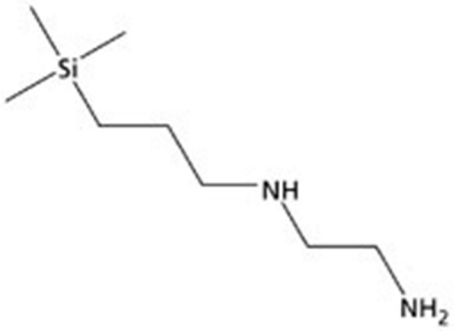

Figura III.1. *Estructura química de PSA*

B. ***Octadecilsilano (C18)***. El uso de este adsorbente es muy importante y reconocido en la SPE dispersiva, ya que permite una eliminación efectiva de interferentes apolares, como grasas y lípidos de la matriz. Su uso se recomienda cuando la composición de grasas de la matriz es superior al 2%.

En el caso del uso de esta técnica de *clean-up* en extractos de leche, se ha demostrado que la mezcla PSA-C18 resulta ser idónea para la eliminación de los principales componentes de la leche que usualmente interfieren en su análisis, como ácidos grasos, triglicéridos, fosfolípidos, esteroles (colesterol) y azúcares. Este procedimiento de limpieza permite complementar la eliminación de proteínas, que usualmente ocurre a través

[191] Prestes O.D., Adaime M.B., Zanella R. *Scientia Chromatographica*, 2011, 3:51–64.

de protocolos conocidos de precipitación que utilizan disolventes orgánicos, dentro de los cuales, el acetonitrilo es el más reconocido. También se ha demostrado que la proporción que se use de ambos adsorbentes depende fundamentalmente del tipo de analito, especialmente para el caso del PSA, ya que al ser un intercambiador aniónico, puede asociarse a los compuestos dependiendo de su grado de acidez o polaridad, como ocurre en el caso de los disruptores endocrinos que se contemplan en esta Memoria, por lo que fue importante optimizar cuidadosamente la cantidad de PSA que fue empleada, para no reducir la eficiencia en la recuperación de los analitos de interés durante el proceso de extracción.

En el presente capítulo se propone el uso de estos dispersantes para llevar a cabo la limpieza de los extractos previamente obtenidos mediante la precipitación de proteínas y grasas.

3. PUBLICACIONES

3.1. Publicación III

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A multiresidue method for the determination of selected endocrine disrupting chemicals in human breast milk based on a simple extraction procedure

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Abstract

In recent decades, in parallel to industrial development, a large amount of new chemicals have emerged that are able to produce disorders in human endocrine system. These groups of substances, so-called endocrine disrupting chemicals (EDCs), include many families of compounds, such as parabens, benzophenone-UV filters and bisphenols. Given the demonstrated biological activity of those compounds, it is necessary to develop new analytical procedures to evaluate the exposure with the final objective of establishing, in an accurate way, relationships between EDCs concentrations and the harmful health effects observed in population. In the present work, a method based on a simplified sample treatment involving steps of precipitation, evaporation and clean-up of the extracts with C18 followed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis for the determination of bisphenol A and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachlorobisphenol A), parabens (methyl-, ethyl-, propyl- and butylparaben) and benzophenone-UV filters (benzophenone-1, -2, -3, -6, -8 and 4-hydroxybenzophenone) in human breast milk samples is proposed and validated. The limits of detections found ranged from 0.02 to 0.05 ng mL^{-1}. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery rates

ranged from 91% to 110% and the precision (evaluated as relative standard deviation) was lower than 15% for all compounds, being within the acceptable limits for the selected bioanalytical method validation guide. The method was satisfactorily applied for the determination of these compounds in human breast milk samples collected from 10 randomly selected women.

Keywords: Endocrine disrupting chemicals; Bisphenol A; Parabens; Benzophenone-UV filters; Human milk samples; UHPLC-MS/MS

Highlights:

- ✓ The determination of three families of EDCs in human milk samples is developed
- ✓ A simple treatment of human milk for isolation of EDCs from samples is proposed
- ✓ UHPLC-MS/MS is used as analytical technique to measure the target compounds
- ✓ The method was satisfactorily validated and applied to human breast milk samples

1. Introduction

The overall development occurred in the last century has led man to have available a lot of manufactured products with a wide applicability that have significantly eased the life. Nevertheless, this massive development has brought an important inconvenient to the population: the exposure to a high variety of xenobiotics that could cause negative health effects. Among these compounds, endocrine disrupting chemicals (EDCs) have become in a special concern in the last years.

EDCs cover an important range of synthetic and natural substances able to alter the normal hormone function of wildlife and humans. The endocrine and reproductive effects of those compounds are believed to be due to their ability to mimic or antagonize the effects of endogenous hormones, such as

estrogens and androgens, or to disrupt synthesis and metabolism of endogenous hormones and hormone receptors [1]. Beside some naturally occurring compounds (lignans, coumestans, isoflavones, mycotoxins), numerous synthetic chemicals such as are bisphenol A (BPA) and its chlorinated derivatives, benzophenone-UV filters (BPs) and parabens (PBs) have been implicated in endocrine disruption.

Since its effects, even at very low concentrations, are more detrimental and pernicious than other EDCs, BPA has received a tremendous attention from the scientific-medical community and governments [2, 3]. It is the raw material used in the manufacturing of epoxy resin and polysulfones. It is also applied as antioxidant or stabilizer. However, the most important use of BPA is the production of polycarbonate plastics for a great variety of applications such as digital media (e.g., CDs, DVDs), electrical and electronic equipment, automobiles, sports safety equipment, reusable food and drink containers, medical devices and many other products [4]. Moreover, when BPA is present in treated waters, it may react with residual chlorine originally used as disinfectant, producing chlorinated BPA derivatives depending on the pH of the medium [5]. Regarding to BPs, those compounds are used as UV filters in sunscreens to protect the skin and hair from UV irradiation as they are able to absorb UV light that is harmful to the human body in the form of UVA (320 to 400 nm) and UVB (290 to 320 nm). Finally, PBs (alkyl esters of *p*-hydroxybenzoic acid) are widely used as antimicrobial preservatives, especially against mold and yeast, in cosmetic products and pharmaceuticals, and in food and beverage processing [7].

The widespread use of BPA, PBs and BPs and their potential risk to human health have prompted interest in assessing human exposure to them. It may occur through inhalation, dermal contact or ingestion [7-10] and their metabolism may differ depending upon the exposure route [7, 11]. These compounds may conjugate to β-D-glucuronide and sulfate, thus reducing their bioactivity and facilitating their urinary excretion. Although free and conjugate forms can be measured in humans, only the free forms are biologically active.

Developmental exposure to EDCs is particularly important in the first stages of life because of the increased susceptibility of the brain and other organs to estrogens during this period [12]. It has been postulated that EDCs accumulate in certain human tissues and their effects might pass to the offspring via the placenta and/or breast milk [13-17]. Breastfeeding mothers exposed to EDCs may be unknowingly exposing their children to harmful levels of these compounds. In this context, it is particularly important to develop strategies for the study of this exposure through the mother after childbirth and therefore, to develop sensitive analytical methods to monitor EDCs in human milk.

Sample preparation is a critical step in complex biological matrices analysis, such as human milk. An extraction technique is usually required to purify and isolate the target compounds. Moreover, because of the low levels of EDCs in human milk, these extraction techniques must be able to concentrate the analytes. To date, BPA and its chlorine derivatives, PBs and BPs have been extracted from human milk using liquid-liquid extraction (LLE) [18-20], ultrasound assisted extraction (UAE) [21], off-line solid-phase extraction (SPE) [22-25] and on-line SPE [21, 26-28]. In the present work, a simple and cost effective sample treatment based on a precipitation of fat and proteins followed by a clean-up using a simplification of the Quick, Easy, Cheap, Effective, Rugged &Safe (QuEChERS) methodology is proposed. QuEChERS was developed by Anastassiades et al. in 2003 for the analysis of pesticides in fruits and vegetables [29]. Since then, it has become an important and widely used technique in the analysis of multiple chemical residues, including EDCs, in a great variety of matrices. Thus, it has been used for the analysis BPA and bisphenol S in canned vegetables and fruits [30], pesticides and mycotoxins in commercial milk [31] and steroid hormones or BPA and the active metabolites of methoxychlor and vinclozolin in rat testis [32]. Recently, the efficacy of this methodology has been also proven for the extraction of organochlorine pesticides in human milk [33]. However, to our knowledge, QuEChERS has not been applied for the EDCs selected in the present work in human milk samples.

The aim of the present work was to develop a sensitive multiresidue method based on a precipitation of fat and proteins followed by a clean-up step for the simultaneous determination of free amounts of BPA and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachloro-); four PBs (methyl-, ethyl-, propyl- and butylparaben) and six BPs (benzophenone-1, 2, 3, 6, 8 and 4-hidroxybenzophenone) in human milk samples. UHPLC-ESI-MS/MS has been used as detection technique. The proposed method was satisfactorily validated and applied for the determination of the free content of the above mentioned compounds in 10 human milk samples from volunteers lactating mothers who live in the province of Granada (Spain).

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. PBs standards were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), tetrachlorobisphenol A (Cl_4-BPA), deuterium-labelled bisphenol A-d_{16} (BPA-d_{16}), benzophenone-UV filter standards (BPs) and deuterium-labelled benzophenone-d10 (BPA-d_{10}) were supplied by Sigma-Aldrich (Madrid, Spain). Monochloro-, dichloro- and trichlorobisphenol A (Cl-BPA, Cl_2-BPA, Cl_3-BPA) were synthesized in our laboratory (purity > 99%) by direct chlorination of BPA [34]. Deuterium-labelled ethylparaben-d5 (EPB-d_5) was purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). Stock standard solutions (100 µg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile up to the final volume was added. The solution remained stable for at least four months at 4 ºC in the darkness. For calibration and validation purposes, two intermediate solutions, No. 1 and 2 (10 and 2.5 µg mL^{-1}) were prepared by diluting 1.0 and 0.25 mL respectively of the stock solution to 10 mL in acetonitrile (MeCN). Subsequently, two new intermediate solutions No. 3 and 4 (1.0 and 0.5 µg mL^{-1}) were prepared by diluting 1.0 and 0.5 mL respectively of solution No. 1 to a final volume of 10 mL

in MeCN. Then, two new intermediate solutions No. 5 and 6 (0.1 and 0.05 μg mL^{-1}) were prepared by diluting 1 and 0.5 mL respectively of solution No. 3 to a final volume of 10 mL in MeCN. Finally, intermediate solution No. 7 (0.01 μg mL^{-1} was prepared by diluting 1 mL of solution No. 5 to a final volume of 10 mL in MeCN. Working standards for calibration and validation purposes were prepared by diluting 100 μL of the intermediate solutions No. 2 to 7 to a final volume of 10 mL in human breast milk. Working standards were prepared fresh from the MeCN solutions prior to the experiments.

Methanol (MeOH) and MeCN gradient grade were obtained from Merck (Darmstadt, Germany). LC-MS grade methanol and water, formic acid, ammonia (25%), zinc acetate dihydrate, phosphotungstic acid hydrate and primary-secondary amine (PSA), were also purchased from Sigma-Aldrich (Madrid, Spain). Octadecyl (C18) solid sorbent (40 μm) was supplied by J.T. Baker (Deventer, Netherlands). Glacial acetic acid (99%) was obtained from Panreac (Barcelona, Spain). The fat/proteins precipitation solution was prepared at time of use by dissolving 9.10 g of zinc acetate hydrated, 5.46 g of hydrated phosphotungstic acid and 5.8 mL of glacial acetic acid in 100 mL final volume of deionised water.

2.2. Instrumentation and software

UHPLC-MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class from Waters (Manchester, UK). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for EDCs detection. An Acquity UPLC® BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (UK) was used as chromatographic column. A vacuum centrifugal evaporator was used to concentrate samples (LaboGene, Lynge, Denmark). MassLynx 4.1 software was used for instrument control, peak detection and integration. Statgraphics Plus version 5.0 (Manugistics Inc., Rockville, USA, 2000) was used for statistical and regression analyses.

2.3. Sample collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. Samples were anonymized, frozen at -20 ºC and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form.

2.4. Preparation of fortified milk samples (calibration and validation standards)

For calibration and recovery studies, blank samples were spiked at different concentrations (from 0.1 ng mL^{-1} to 25 ng mL^{-1}) by adding 100 µL of the different spiking standard solutions (No. 2-7) to 9.9 mL of human breast milk. In order to attain equilibrium, the mixtures were vortexed for 2 min and then left to stand for 24 h at 4 ºC in the dark before analysis. This allows the analytes to come into full contact with the sample. The blank samples were obtained from two different mothers and in different days. Although the exposure to these compounds through numerous pathways is very usual, the metabolism of these substances varies from one individual to another; in this sense, the fact of being exposed does not necessarily mean bioaccumulation of these compounds in every biological fluid. Thus, taking into account the results obtained in previous analyses of different tissues and biological fluids (placenta or serum of more than 50 parturient women), they were selected six mothers that had very low or even null concentrations of the analytes under study in those samples. Finally, the breast milk of each mother was analyzed for several days and the samples from two of the mothers, in different days, was found to be "free of the analytes" (under LOD of proposed method), and therefore these samples were selected as blank for further experiments.

2.5. Sample treatment

An aliquot of 9.9 mL of human breast milk sample was placed into a 45 mL centrifuge tube and 100 µL of a 0.5 µg mL^{-1} acetonitrile solution of the surrogates (BPA-d_{16}, BP-d_{10} and EPB-d_5) was added. The final concentration of surrogates in sample was 5 ng mL^{-1}. The sample was vigorously shaken in a vortex-mixer for 1 min and then, 7.5 mL of MeCN and 7.5 mL of the fat/proteins precipitation solution were added. The mixture was stirred on a vortex-mixer for 1 min and centrifuged for 10 min at 4050 × *g*. The underlying liquid layer was filtered through a 0.22 µm nylon filter and transferred to a Falcon tube for evaporation to dryness in a vacuum centrifugal evaporator at 760 × *g* and 60 ºC.

After evaporation was complete, a clean-up step was carried out. For this purpose, the dry residue obtained was dissolved in 7.5 mL of MeCN and 150 mg of C18 were added as clean-up sorbent. $MgSO_4$ (about 0.1 g) was also added to the mixture in order to remove any traces of moisture. The mixture was stirred for 3 min at room temperature and after centrifugation at 4050 × *g*, the supernatant was transferred to a 8 mL glass vial and evaporated to dryness in the vacuum centrifugal evaporator at 760 × *g* and 60 ºC. The residue was dissolved with 300 µL of MeOH (twice) and transferred to a 1.5 mL Eppendorf tube for evaporation to dryness at room temperature. Finally, the residue was dissolved in 100 µL of initial mobile phase and after stirring for 60 s in vortex, it was filtered through a 4 mm and 0.22 µm nylon filter. The sample was ready to be injected into the chromatographic system.

2.6. Liquid chromatography-mass spectrometry analysis

The chromatographic separation of targets analytes was performed using an Acquity UPLC® BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size). The compounds were separated using a gradient mobile phase consisting of 0.1% (v/v) aqueous ammonium formate solution (pH = 9) as solvent A and 0.1% (v/v) ammonia in MeOH as solvent B. Gradient conditions were as follow: 0.0-4.0 min, 40% B; 4.0-6.0 min, 40-90% B; 6.0-6.1 min, 90-100% B; 6.1-7.5 min, 100% B

and back to 40% in 0.5 min. Then, 5 min for conditioning of column were added. Total run time was 13.0 min. Flow rate was 300 µL min^{-1} and the injection volume was 10 µL. The column temperature was maintained at 40 ºC.

ESI was performed in both negative and positive ion modes. The tandem mass spectrometer was operated in the multiple reactions monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each analyte by continuous infusion of concentrate standard solutions (1 µg mL^{-1}). The ion source temperature was maintained at 150 ºC. Other instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1} and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell times were set at 25 ms. Collision energies (CE) and cone voltages (CV) were optimized for each analyte. Optimized parameters for each compound are listed together with the mass transitions in Table 1.

Table 1
Transitions and optimized potentials for UHPLC–MS/MS analysis

	Transitions	CV	CE		Transitions	CV	CE
BPA	227.2 → 211.9[a]	-50	-22	BP-1	214.9 → 136.8[a]	2	18
	227.2 → 132.9[b]	-50	-26		214.9 → 105.1[b]	2	32
Cl-BPA	261.1 → 182.0[a]	-56	-30	BP-2	245.1 →134.8[a]	-40	-16
	261.1 → 210.0[b]	-56	-22		245.1 →108.9[b]	-40	-22
Cl_2-BPA	295.0 → 215.9[a]	-74	-30	BP-3	229.0 → 150.8[a]	4	20
	295.0 → 243.9[b]	-74	-24		229.0 → 104.9[b]	4	18
Cl_3-BPA	329.0 → 249.8[a]	-2	-32	BP-6	275.0 → 150.9[a]	14	18
	329.0 → 277.9[b]	-2	-24		275.0 → 94.9[b]	14	34
Cl_4-BPA	365.0 → 313.9[a]	-50	-28	BP-8	245.0 → 120.9[a]	14	20
	365.0 → 285.9[b]	-50	-32		245.0 → 150.9[b]	14	20
MPB	151.1 → 91.8[a]	-38	-22	4-OH-BP	199.0 → 120.8[a]	36	20
	151.1 → 135.8[b]	-38	-14		199.0 → 104.8[b]	36	18
EPB	165.1 → 91.9[a]	-38	-24	BPA-d_{16}	241.2 → 223.0[a]	-46	-22
	165.1 → 136.6[b]	-38	-16		241.2 → 141.9[b]	-46	-32
PPB	179.1 → 91.8[a]	-42	-24	EPB-d_5	170.1 → 92.1[a]	-38	-24
	179.1 → 136.1[b]	-42	-16		170.1 → 136.0[b]	-38	-16
BPB	193.1 → 91.4[a]	-42	-24	BP-d_{10}	193.1 → 109.8[a]	18	16
	193.1 → 136.1[b]	-42	-16		193.1 → 81.8[b]	18	30

[a] SRM transition used for quantification; [b] SRM transition used for confirmation
CV: Cone voltage (V); CE: Collision energy (eV)

3. Results and discussion

3.1. Sample treatment

3.1.1. Protein and fat precipitation

The sample treatment techniques for isolation of EDCs from biological samples published in the scientific literature are usually laborious and time-consuming and particularly, in the case of human breast milk, there is an important lack of information. After a carefully study of possible sample treatments for cow milk-related products a simple precipitation procedure with a solution containing zinc and tungsten salts in an acidic media was selected.

This solution has been currently used for milk treatment in the analysis of lactulose as described in the method by the International Dairy Federation [35]. It was decided to evaluate a similar procedure to remove fat and proteins from human milk by precipitation. The results were excellent in terms of sensitivity and sample handling and on the one hand a white solid fraction corresponding to protein, fat and other precipitated salts was obtained, and it was also separated a clear solution containing the analytes that after filtering was completely transparent.

Since heavy metals are present in the precipitation solution, in order to minimize the amounts of reagents and to decrease final residues on the analysis, the ratio of precipitation solution/acetonitrile and human milk sample was optimized for maximum recovery. For 10 mL of milk sample, volumes ranging from 2.5 to 10 mL of precipitation solution and MeCN were assayed; being the optimum value obtained 7.5 mL for both them.

3.1.2. Optimization of the clean-up sorbent

After the precipitation of proteins and fat, the extract was completely evaporated to dryness in a vacuum centrifugal evaporator at 760 × *g* and 60 ºC. A final extract with an oily aspect was obtained. In consequence, a clean-up step was necessary in order to minimize matrix effects and to avoid instrumental problems in the UHPLC system. Based on our previous experience with this type of analytes in other biological and environmental matrices, and in the principles of extraction technique QuEChERs, acetonitrile was chosen as solvent to dissolve the extract. In order to minimize the solvent amounts, the volume of acetonitrile was studied in the range from 1 to 10 mL and the minimal amount of solvent necessary to dissolve adequately the oily extract was 7.5 mL.

Then, a mixture of PSA and C18 as clean-up sorbents was studied. Since the optimal amount of both sorbents in the mixture could be related each other, a simple factorial 3^2 response surface design (three central points) was performed in order to optimize the mass of both sorbents. The design allows

the simultaneous optimization of two variables at three levels [36, 37]. The pairs selected for the assay and the optimum values for each variable are shown as supplementary material (Table S01). Figure 1 shows the response surfaces obtained for each compound. The following model was determined for each response: $y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X^2_1 + b_{22}X^2_2$ [38] where, y is the measured response (relative area) for each compound, b_0 the intercept, b_i the regression coefficients and X_i the values of variables (X_1 = mass of PSA; X_2 = mass of C18).

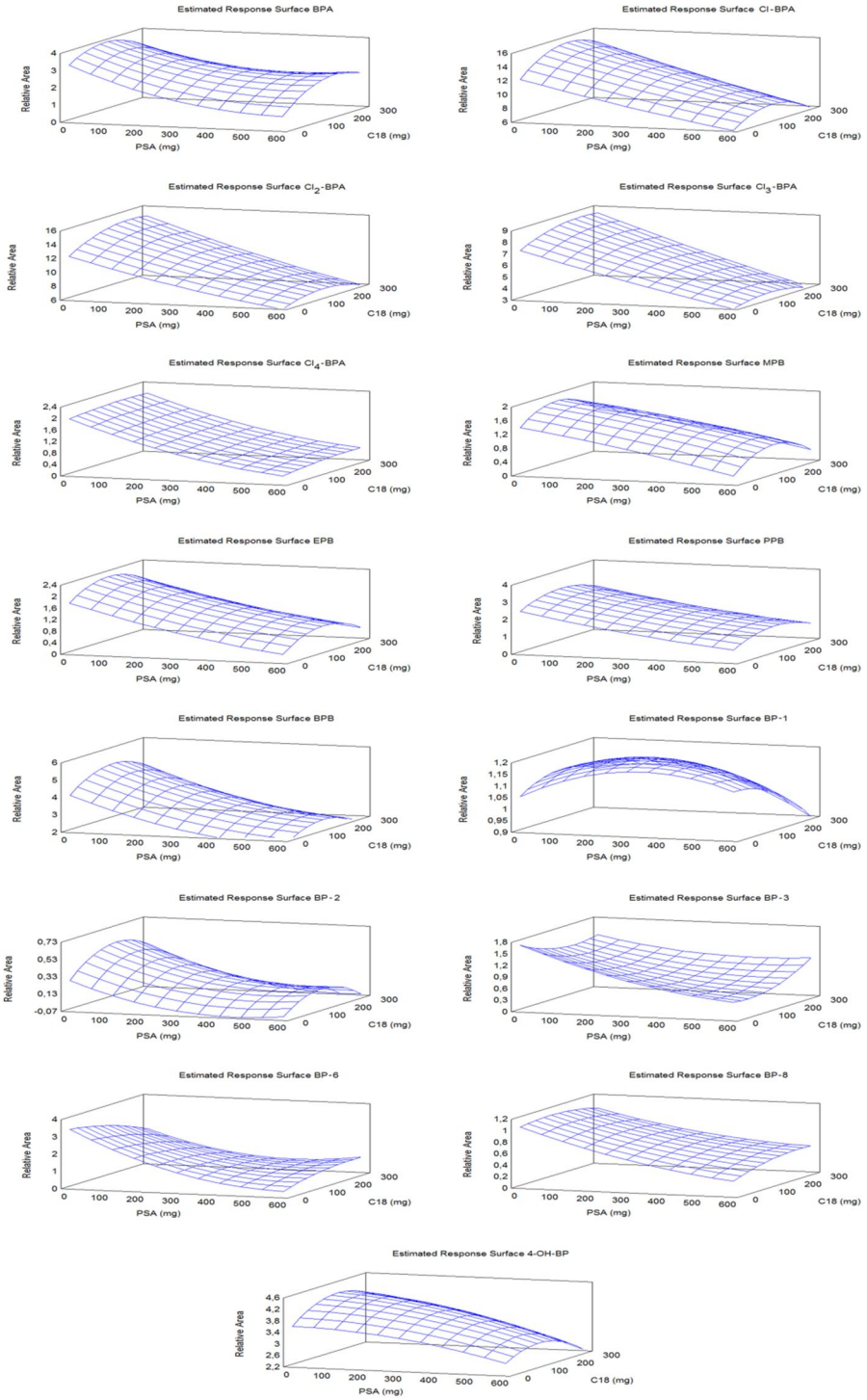

Fig. 1. *Optimization of the amount of clean-up sorbent. Response surfaces obtained for the factorial experimental design.*

As the figure evidence, the presence of PSA does not affect the response and therefore, its presence in the cleaning up process is not necessary. In the case of C18 sorbent, some differences in the optimal values obtained were observed depending on the compound. The combination of the optimized experimental values obtained for each compound for the two variables allowed the determination of the best overall extraction efficiency, which was calculated with the desirability function. The plot of this function versus sorbent amounts is shown in Figure 2.

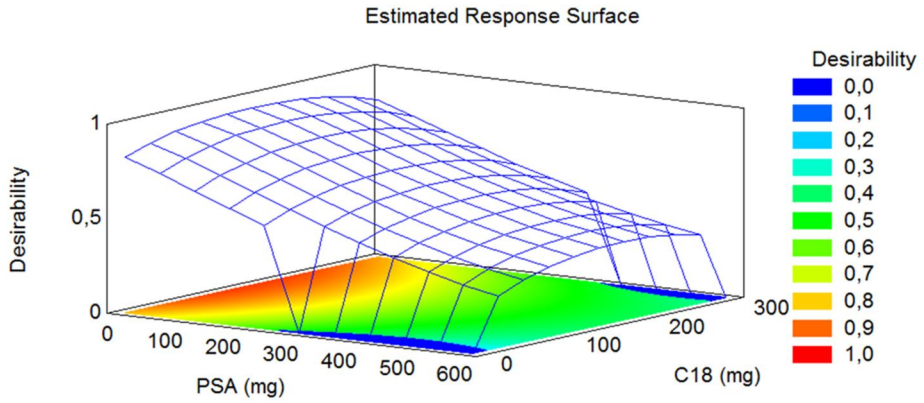

Fig. 2. *Representation of the global desirability function vs PSA and C18 amounts. Results were evaluated using a 95% confidence interval.*

Responses for each compound in the different experiments of the design were first normalized between 0 and 1, and then the global desirability function, was defined as their geometric mean. As Figure 2 shows, the optimal quantities were 0 mg for PSA and approximately 150 mg for C18, and these values were then used for further experiments.

3.2. Analytical performance

A calibration curve for each compound, with six concentration levels (six fold) was built. The curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using SRM

mode. Surrogates (BPA-d_{16}, EPB-d_5 and BP-d_{10}) were added at a concentration of 5 ng mL^{-1}.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in the initial mobile phase and the other in blank human milk. The Student's *t*-test was applied in order to compare the calibration curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a Snedecor's *F*-test. The Student's *t*-test showed statistical differences among slope values for the calibration curves of some of the target analytes and consequently, a significant matrix effect was observed in those cases. A possible explanation for this not correction of the matrix effects by the surrogates employed, could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are relatively variable within the same family of compounds, especially in the case of BPs. Therefore, although the compounds selected have a similar basic structure compared to the analytes of the same family, and the use of these compounds as internal standards or as surrogates is accepted in scientific literature, they differ slightly due to the presence of different substituents in the molecule. Since it is impossible to have the corresponding isotopically labelled standard for each one of the studied analytes (many of them do not exist in the market), it was decided to work with matrix-matched calibration in all cases. Table 2 shows the statistical and the analytical parameters obtained for each compound.

Table 2
Analytical and statistical parameters

Compound	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BPA	0.190	$1.457 \cdot 10^{-3}$	99.8	64.7	0.05	0.15	LOQ - 25.0
Cl-BPA	1.160	$6.426 \cdot 10^{-3}$	99.9	30.0	0.04	0.12	LOQ - 25.0
Cl_2-BPA	0.870	$5.961 \cdot 10^{-3}$	99.8	40.2	0.04	0.12	LOQ - 25.0
Cl_3-BPA	0.472	$6.334 \cdot 10^{-3}$	99.4	29.6	0.04	0.14	LOQ - 25.0
Cl_4-BPA	0.198	$1.015 \cdot 10^{-3}$	99.9	31.0	0.04	0.13	LOQ - 25.0
MPB	0.468	$2.553 \cdot 10^{-3}$	99.9	13.6	0.03	0.09	LOQ - 25.0
EPB	0.631	$3.906 \cdot 10^{-3}$	99.9	5.1	0.03	0.09	LOQ - 25.0
PPB	0.846	$4.381 \cdot 10^{-3}$	99.9	92.6	0.03	0.09	LOQ - 25.0
BPB	0.961	$7.095 \cdot 10^{-3}$	99.8	32.0	0.03	0.10	LOQ - 25.0
BP-1	0.220	$1.361 \cdot 10^{-3}$	99.9	36.9	0.03	0.09	LOQ - 25.0
BP-2	0.408	$2.672 \cdot 10^{-3}$	99.8	27.8	0.03	0.09	LOQ - 25.0
BP-3	0.109	$7.398 \cdot 10^{-4}$	99.9	7.8	0.03	0.09	LOQ - 25.0
BP-6	0.319	$3.805 \cdot 10^{-3}$	99.5	48.1	0.03	0.10	LOQ - 25.0
BP-8	0.131	$7.339 \cdot 10^{-4}$	99.9	40.0	0.02	0.08	LOQ - 25.0
4-OH-BP	0.862	$6.414 \cdot 10^{-3}$	99.8	99.2	0.03	0.09	LOQ - 25.0

b, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [39].

Linearity. A concentration range from the minimal quantified amount, limit of quantification (LOQ) (see Table 2) to 25 ng mL^{-1} was selected. Linearity of the calibration graphs was assessed with the determination coefficients (% R^2) and the P-values (% P_{lof}) of the *lack-of-fit* test [40]. The values obtained for R^2 ranged from 99.4% for Cl_3-BPA to 99.9% for Cl-BPA, Cl_4-BPA, MPB, EPB, PPB, BP-1, BP-3 and BP-8, and P_{lof} values were higher than 5% in all cases. This indicates a good linearity within the stated ranges.

Limits of detection and quantification. Limits of detection (LOD) and quantification (LOQ) are two fundamental parameters that need to be calculated in the validation of any analytical method in order to determine if an analyte is present in the sample. In the present work, these parameters were established as the minimum concentration of analyte that the method can detect and with a signal-to-noise ratio of 3 for LOD and 10 for LOQ, using the quantification transition. The values obtained for the LOQ ranged from 0.08 ng mL^{-1} for BP-8 to 0.15 ng mL^{-1} for BPA. The results are also summarized in Table 2.

Accuracy (precision and trueness). Due to the absence of certified materials and in order to evaluate the trueness and the precision of the method, a recovery study with spiked human breast milk samples, at three concentrations levels for each compound (0.5, 5.0 and 25 ng g^{-1}), was performed on six different days. The precision was expressed as relative standard deviation (RSD) and the trueness was evaluated with the percentage of recovery. The precision and the trueness of the proposed analytical method are shown in Table 3.

Table 3
Recovery assay, precision and trueness of the method

	Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	RSD (%)	Recovery (%)		Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	RSD (%)	Recovery (%)
	0.50	0.55	4.6	109.8		0.50	0.47	7.5	96.0
BPA	5.00	4.77	8.0	95.5	BPB	5.00	4.72	4.1	94.4
	25.0	25.0	3.5	100.2		25.0	24.9	3.6	99.9
	0.50	0.51	5.4	102.6		0.50	0.54	7.7	108.2
Cl-BPA	5.00	5.19	4.6	103.8	BP-1	5.00	5.09	6.1	101.8
	25.0	25.1	1.5	100.4		25.0	25.1	1.9	100.3
	0.50	0.54	6.9	108.0		0.50	0.55	3.6	109.3
Cl_2-BPA	5.00	5.17	5.3	103.5	BP-2	5.00	4.98	5.0	99.5
	25.0	25.1	2.9	100.5		25.0	25.2	2.3	100.6
	0.50	0.47	4.2	93.1		0.50	0.49	3.1	99.0
Cl_3-BPA	5.00	4.93	14.6	98.6	BP-3	5.00	4.86	5.0	97.2
	25.0	25.2	5.2	100.8		25.0	24.9	2.0	99.7
	0.50	0.55	3.2	110.0		0.50	0.55	7.1	109.8
Cl_4-BPA	5.00	4.89	3.0	97.8	BP-6	5.00	5.13	5.4	102.7
	25.0	25.1	2.0	100.3		25.0	25.1	5.3	100.6
	0.50	0.46	4.6	93.0		0.50	0.53	7.1	106.6
MPB	5.00	4.76	2.0	95.1	BP-8	5.00	4.82	5.4	96.4
	25.0	25.0	2.5	100.0		25.0	25.1	1.9	100.2
	0.50	0.46	12.4	91.6		0.50	0.53	5.8	106.0
EPB	5.00	4.73	4.4	94.6	4-OH-BP	5.00	5.02	6.2	100.4
	25.0	24.9	2.4	99.8		25.0	25.0	2.4	100.1
	0.50	0.45	6.3	91.0					
PPB	5.00	4.98	3.0	99.6					
	25.0	24.9	2.0	99.9					

[a] *Mean of 18 determinations; RSD: relative standard deviation*

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in spiked human breast milk samples. Samples were analyzed using the proposed method and the concentration of each compound was compared with the amount of analyte previously added to the samples. In all cases, the recoveries were close to 100%. Precision (expressed as relative standard deviation, RSD) was lower than 15% for all compounds. Therefore, it was within the acceptable limits for bioanalytical method validation, which are

considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data, also shown in Table 3, demonstrated that the proposed method is reproducible. Therefore, precision and trueness data indicate that the method is highly accurate.

Selectivity. The specificity of the method was demonstrated by analyzing the chromatograms of a human milk sample spiked with the analytes and the corresponding blank. No interferences from endogenous substances were observed at the retention time of the compounds. These findings suggest that the spectrometric conditions ensured high selectivity of the UHPLC-MS/MS method. Figure 3 shows a chromatogram of a spiked milk sample.

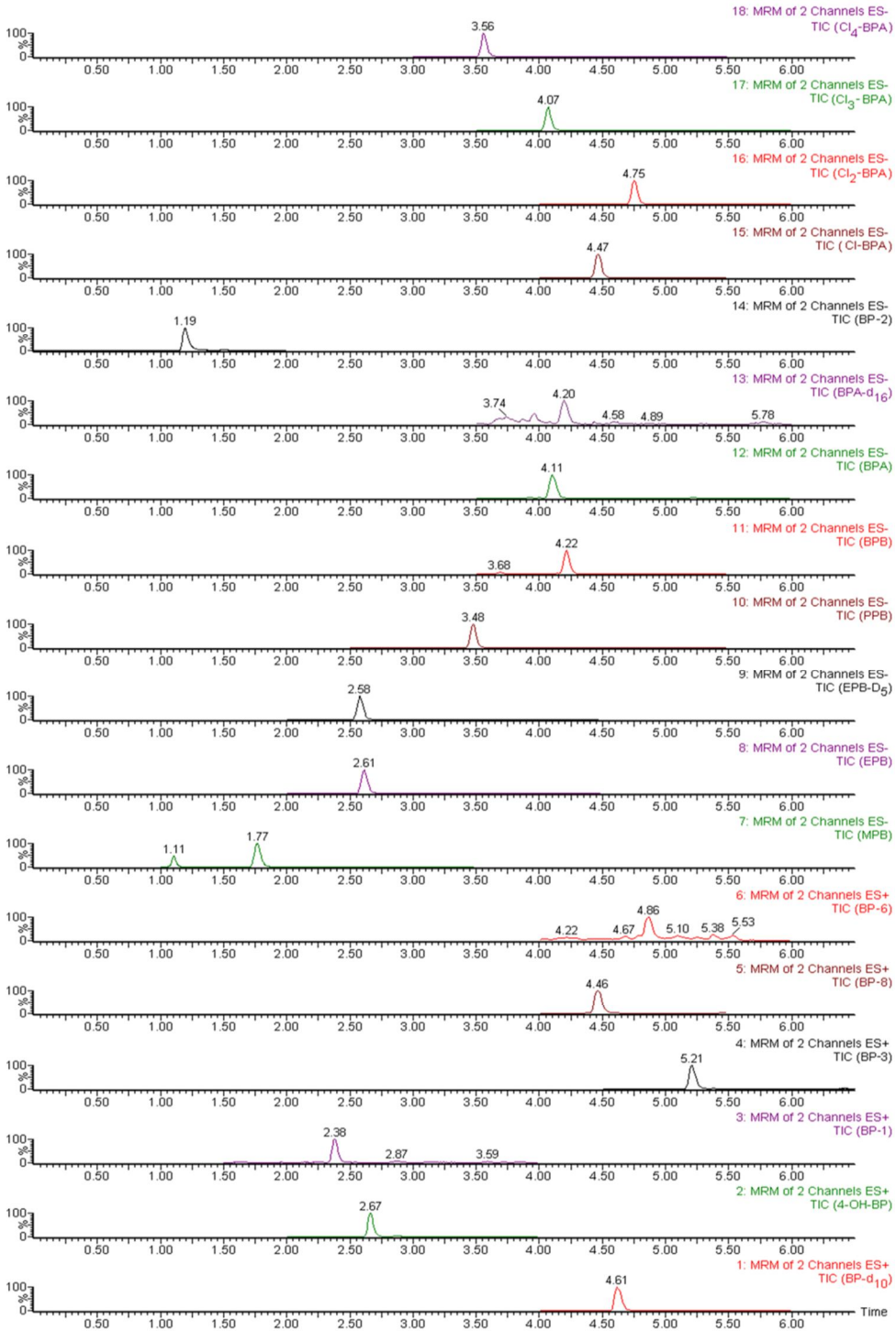

Fig. 3.*Chromatograms of a standard mixture in human milk (5 ng mL^{-1} of each studied analyte)*

3.4. Application of the proposed method

The validated method was applied to the determination of the selected EDCs in 10 samples of human breast milk. The results obtained as mean of six determinations are summarized in Table 4. Figure 4 shows the chromatograms obtained for a natural sample (M01).

Table 4
Application to human breast milk samples

	Found amount (ng mL^{-1})* (RSD, %)				
	MPB	EPB	PPB	BPB	BPA
M01	3.5 (4.3)	3.4 (2.9)	7.5 (5.8)	0.3 (5.8)	0.8 (4.8)
M02	0.8 (2.5)	0.3 (2.2)	1.3 (3.2)	0.4 (8.2)	ND
M03	2.9 (5.4)	0.2 (2.5)	0.8 (3.6)	0.2 (3.9)	ND
M04	0.9 (3.0)	1.5 (3.0)	6.5 (7.3)	1.3 (3.3)	0.7 (10.9)
M05	1.1 (6.1)	0.2 (2.2)	0.3 (5.1)	D	0.6 (6.5)
M06	0.4 (8.1)	0.2 (3.5)	0.1 (6.8)	0.2 (4.3)	ND
M07	3.3 (2.3)	0.8 (2.2)	0.8 (2.3)	0.3 (1.2)	2.3 (4.4)
M08	0.6 (3.4)	0.8 (4.9)	0.6 (4.2)	0.5 (4.9)	9.3 (3.1)
M09	1.4 (1.8)	0.3 (4.3)	2.4 (2.3)	1.3 (1.3)	13.8 (1.1)
M10	ND	ND	ND	ND	ND
	Cl_2-BPA	BP-1	BP-2	BP-3	4-OH-BP
M01	0.2 (6.2)	ND	0.4 (8.1)	8.1 (7.0)	2.3 (4.3)
M02	ND	D	ND	0.9 (4.4)	0.4 (2.7)
M03	ND	D	ND	7.1 (5.9)	4.1 (5.2)
M04	ND	D	ND	17.4 (2.1)	3.4 (3.8)
M05	0.4 (4.2)	D	0.5 (8.5)	6.8 (3.6)	5.8 (4.4)
M06	ND	0.2 (4.8)	D	7.4 (4.1)	2.7 (3.5)
M07	ND	D	ND	2.4 (2.2)	1.3 (4.6)
M08	ND	0.5 (3.9)	ND	D	D
M09	ND	1.3 (5.1)	2.1 (5.4)	11.8 (0.8)	3.3 (2.2)
M10	ND	ND	ND	ND	ND

** Mean of 6 determinations; ND, not detected (< LOD); D: detected (> LOD and < LOQ); RSD, Relative standard deviation*

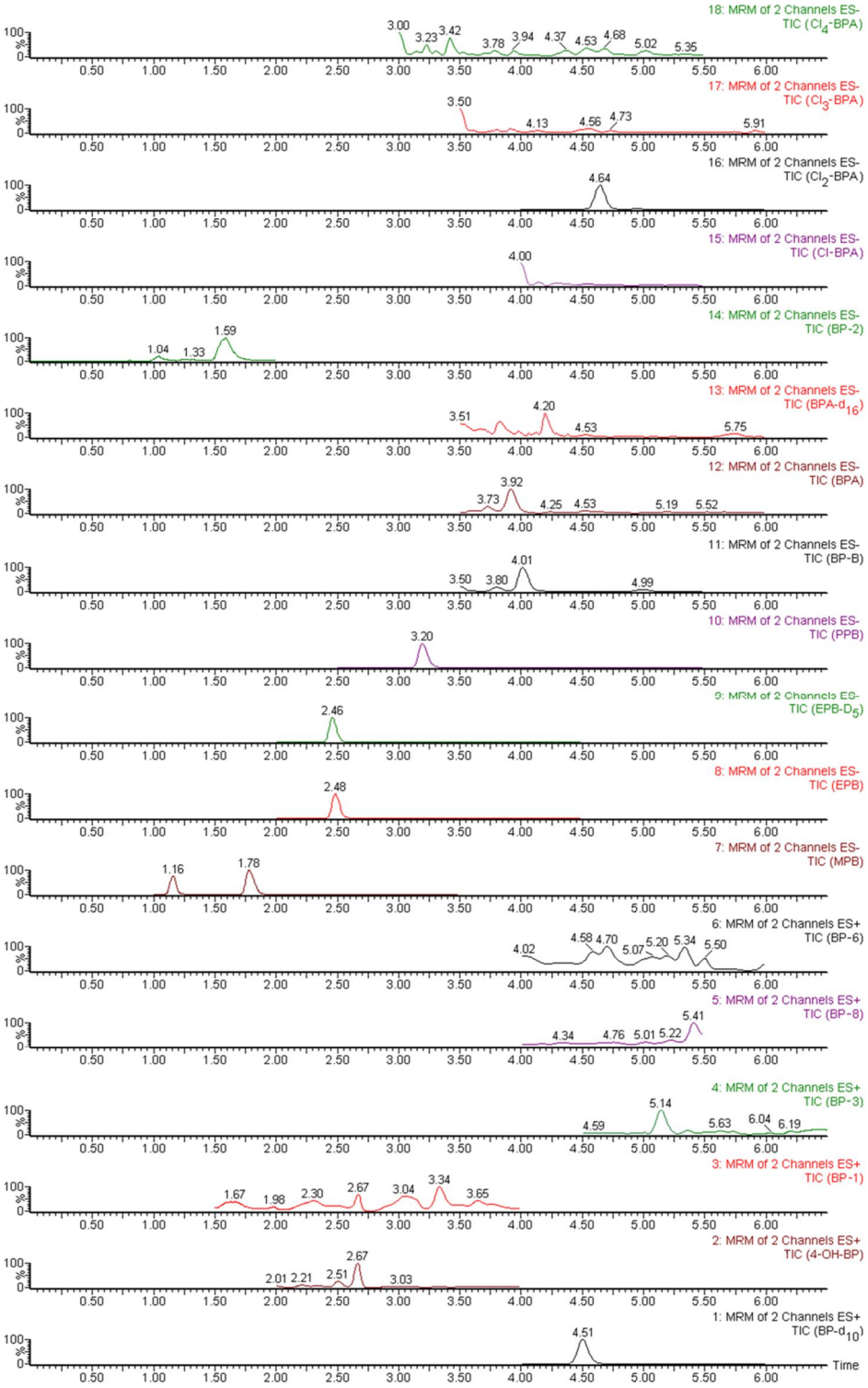

Fig. 4. *Chromatograms of a real human milk sample (M01)*

As it is shown in Table 4, ten of the fifteen analyzed compounds were detected and quantified in almost one of the samples. Regarding BPA, the compound was quantified in six of the ten samples in a concentration range from 0.6 to 13.8 ng mL^{-1} (mean: 4.6 ng mL^{-1}). However, in the case of chlorinated derivatives, only Cl_2-BPA was detected in two of the samples at concentration levels lower than 0.5 ng mL^{-1}. PBs were detected in almost samples, being MPB and PPB clearly the predominant. MPB was detected and quantified in 9 of the analyzed samples, in a concentration range from 0.4 to 3.5 ng mL^{-1} (mean: 1.7 ng mL^{-1}). PPB was also detected in nine samples in concentrations ranging from 0.1 to 7.5 ng mL^{-1} (mean: 2.3 ng mL^{-1}). EPB was quantified in nine of the samples but at lower concentrations than MPB and PPB (mean: 0.9 ng mL^{-1}). Finally, BPB was quantified in 8 samples and detected in one more of the analyzed samples (mean: 0.6 ng mL^{-1}). On the other hand, only four of the six BPs were detected and/or quantified in at least one sample. BP-3 and 4-OH-BP were the most frequently detected compounds being detected in nine of the ten samples and quantified in eight of them in a concentration ranges from 0.9 ng g^{-1} to 17.4 ng mL^{-1} (mean: 7.7 ng mL^{-1}) and from 0.4 to 5.8 ng mL^{-1} (mean: 2.9 ng mL^{-1}) respectively. BP-1 was detected in eight of the samples but quantified in only three of them (mean: 0.7 ng mL^{-1}) while BP-2 was detected in four samples and quantified in three of them (mean: 1.0 ng mL^{-1}). BP-6 and BP-8 were not detected in any of the analyzed samples.

Not many papers on the determination of these compounds in breast milk been published in the literature. When the results obtained in the present study are compared with those found by other authors, it can be concluded that the data are consistent but slightly different. Some authors [18, 19, 21-23, 27] have found BPA in 100% of analyzed samples (n = 23, 100, 3 and 4 respectively), while other authors such as Zimmers et al. [24], Ye et al. [26] and in our case, have found BPA in 60% of the analyzed samples (n = 21, 20 and 10, respectively). Regarding PBs, Schlumpf et al. [20] established the presence of MPB, EPB and PPB in 26%, 15% and 11% of samples (n = 54) respectively, while Ye et al. [27] determined PPB at MPB in 50% and 25% of the samples (n = 4). In the present work, four PBs (MPB, EPB, PPB and BPB) were detected in most of the samples

(10 samples, 90% of positives). Finally, in relation to BPs, Schlumpf [20] analyze only BP-2 and BP-3 in 54 samples and he only found BP-3 in 13% of them. Ye et al. analyzed BP-3 and BP-4 in 20 breast milk samples, with 25% [27] and 15% [26] of positive samples, compared to 90% of positive samples found in our study. Furthermore, we have found trace amounts of BP-1, BP-2 and 4-OH-BP in 80%, 40% and 90% of the samples, respectively.

4. Conclusions

The identification and quantification of free concentrations of BPA and chlorinated derivatives, four PBs and six BPs in human breast milk samples was successfully performed using a simple precipitation procedure followed by a clean-up with C18 and UHPLC-MS/MS analysis. The isolation of analytes from samples was accurately optimized and the procedure was validated. The methods were satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women. The analytical method can be applied can be very useful for the design of further studies for the determination of human exposure to EDCs. To our knowledge and to date, the proposed method presents the lower detection limits published in the scientific literature for the multiresidue determination of these fifteen compounds. This is a potent analytical tool that can be used in further studies for the determination of human exposure to those EDCs through human milk in the first stages of the life. As evidenced in this study and in studies by other authors [18-28], EDCs transfer from mother to child is not limited to the pregnancy period across the placenta, but it extends to the period of lactation.

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CAPÍTULO IV

Aplicación de la extracción asistida con ultrasonidos

1. OBJETIVOS

Los objetivos de los trabajos realizados y presentados en este capítulo son:

- ✓ Proponer metodología de buenas características para la determinación de bisfenol A, parabenos y benzofenonas mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS) en leche materna humana.

- ✓ Aplicar la técnica de extracción asistida por ultrasonidos seguida de un proceso de limpieza de los extractos con adsorbentes dispersivos, para el aislamiento de los analitos a partir de la matriz inicial.

- ✓ Validar los métodos analíticos propuestos en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación aplicables.

- ✓ Aplicar las metodologías desarrolladas en el control y evaluación del contenido de los EDCs seleccionados en muestras reales de leche materna humana.

2. EXTRACCIÓN ASISTIDA POR ULTRASONIDOS

Se define como ultrasonido a aquella onda acústica o sonora cuya frecuencia es superior a los 20,000 Hz, estando por encima del rango audible por los humanos (10–16,000 Hz) (***figura II.1***).

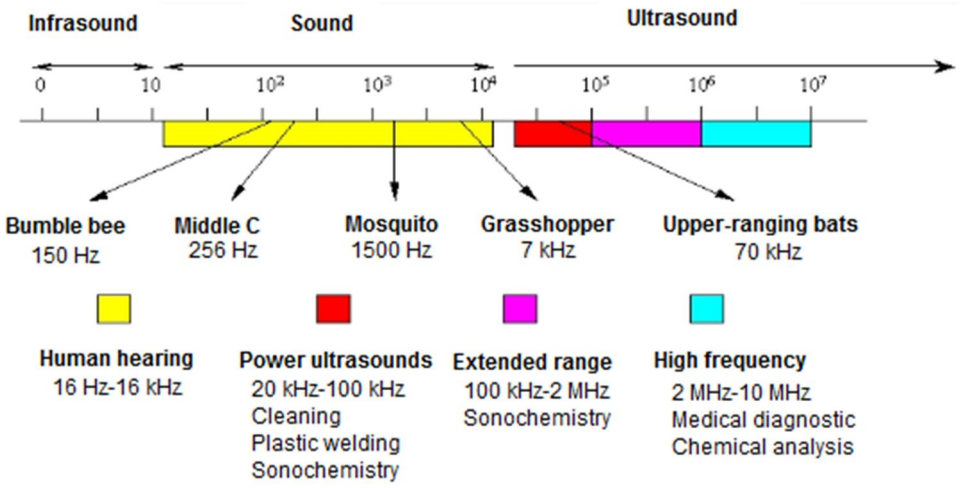

Figura II.1. *Región de frecuencia del sonido*[192].

El ultrasonido es una herramienta de gran potencial desde un punto de vista de la Química Analítica, y no sólo en los procesos de extracción de compuestos químicos, sino también en otros procedimientos como son la dispersión de suspensiones acuosas, la homogeneización, la nebulización, el lavado o la derivatización[193,194].

En un líquido, cuando se irradia con ultrasonidos, el ciclo de expansión produce presión negativa. Si la onda de ultrasonido es de una potencia suficiente, este ciclo crea burbujas o cavidades en el seno líquido. La presión negativa excede la fuerza de tensión local creando burbujas de vapor (***Figura II.2***). El proceso por el cual se forman dichas burbujas, crecen y sufren el

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colapso implosivo recibe el nombre de *"cavitación"*, el cual ocurre en aproximadamente 400 ms (***Figura II.3***).

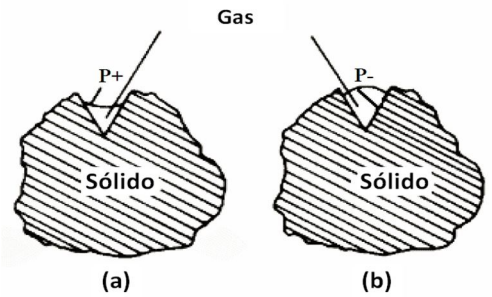

Figura II.2. *Efecto del gas aprisionado en el poro de una partícula:* ***(a)*** *presión positiva,* ***(b)*** *presión negativa*[195].

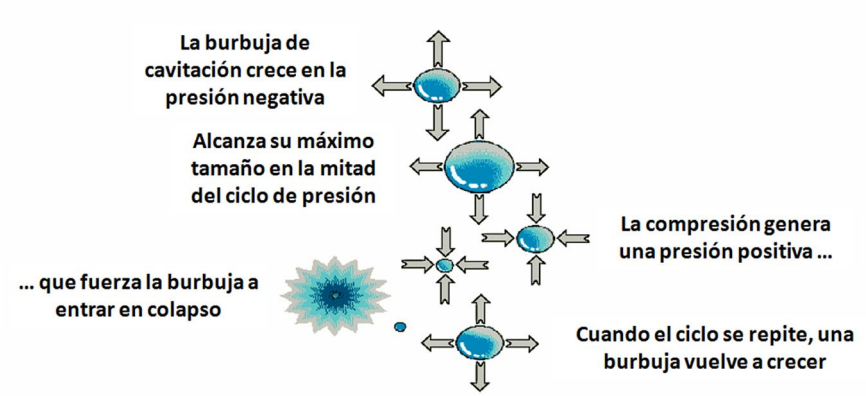

Figura II.3. *Esquema de la implosión de una burbuja*[195].

El fenómeno de cavitación genera una importante interacción entre la materia y la energía. Cuando ésta ocurre, la energía potencial de la burbuja expandida se transforma en cinética y se propaga a una velocidad considerable, alcanzando el recorrido de las partículas de la matriz. Estas colisiones provocan la ruptura de la muestra, generando partículas más pequeñas, y aumentando por tanto la superficie de exposición al disolvente[196]. Este hecho, junto a las altas temperaturas a las que trascurre el proceso provoca un aumento de la

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solubilidad y la difusividad, además las altas presiones favorecen la penetración y el transporte de materia[197].

Para generar la radiación ultrasónica, en la mayoría de los laboratorios se suelen emplear baños de ultrasonido, sin embargo, dada las desventajas que presenta este sistema, como la falta de uniformidad en la distribución de la energía y la disminución de la potencia con el tiempo, lo que repercute en pérdida de reproducibilidad experimental, en la presente Tesis Doctoral se ha optado por el empleo de un sistema de sonda (***Figura II.4***) capaz de enfocar toda la energía en una región localizada. Este sistema proporciona una cavitación muy eficiente y en consecuencia una gran reproducibilidad.

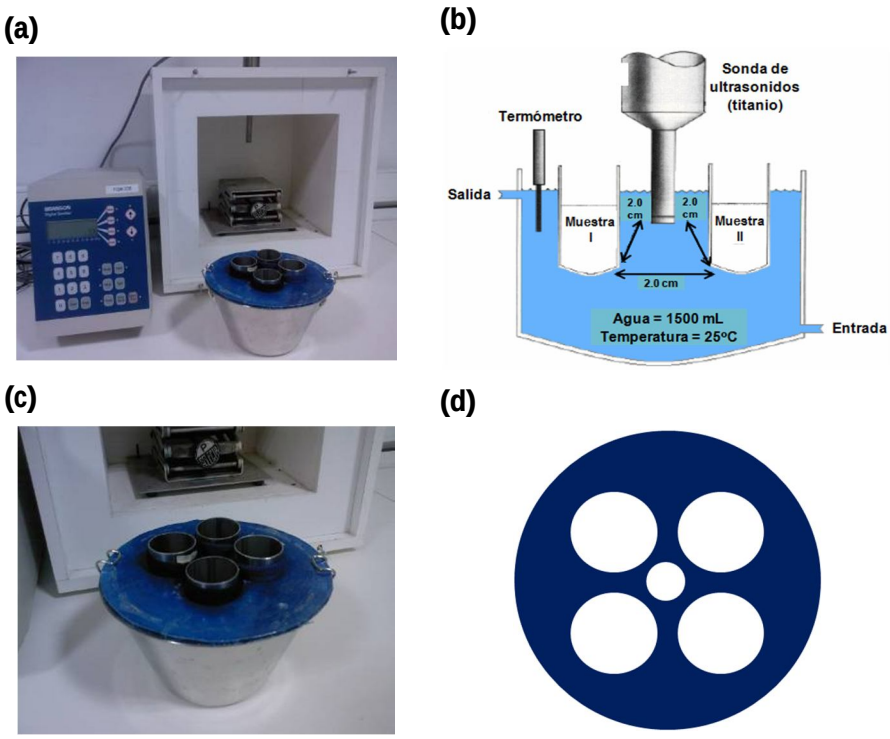

Figura II.4.(a) *Equipo de ultrasonidos.* ***(b)*** *Esquema del diseño.* ***(c)*** *Detalle del portaceldas y celdas de acero inoxidable.* ***(d)*** *Detalle de la tapa del portaceldas. Los cuatro agujeros grandes dan cabida a las celdas, mientras que el agujero central es para la sonda*

[197]Bossio J.P., Harry J., Kinney C.A. *Chemosphere*, 2008, 70:858–864.

La extracción se lleva a cabo introduciendo la sonda de ultrasonido en un baño con agua donde se encuentran las celdas que contienen a la muestra. Aunque en teoría la introducción directa de la sonda en la muestra favorecería el proceso de cavitación, incrementando la eficiencia de la extracción[193], en este trabajo se ha comprobado que este modo de trabajo no mejora notablemente el rendimiento de la extracción, afectando de forma drástica a la vida útil de la sonda. El modo de trabajo seleccionado permite además el procesado de un mayor número de muestras simultáneamente (4 por ciclo).

Son numerosas las variables del sistema de extracción que deben ser tenidas en cuenta[198]. Entre otras, las más importantes son la naturaleza del líquido empleado para la propagación de las ondas, la cantidad de líquido en el baño, la temperatura del líquido, la forma geométrica del baño y de la cápsula de extracción, la composición material de la cápsula, el número de cápsulas y por último, la distancia entre la sonda y la cápsula de extracción. Todas estas variables se encuentran fijadas por los trabajos previos realizados en nuestro grupo de investigación, siendo fijas en todos los análisis. Por tanto, con estas condiciones preestablecidas la optimización de variables se ha centrado exclusivamente en aquellas que afectan al proceso de extracción particular de los analitos seleccionados. Los parámetros fueron los siguientes:

- ✓ Disolvente de extracción.
- ✓ Volumen del disolvente de extracción.
- ✓ Tiempo de extracción.
- ✓ Número de ciclos de extracción.

Para no reducir el tiempo de vida de la sonda, se emplearon valores de amplitud menores del 70%, y para evitar el sobrecalentamiento, el tiempo de

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extracción máximo predeterminado fue de 20 min. De acuerdo a cada situación particular, estos parámetros fueron optimizados empleando métodos univariantes o mediante diseño de experimentos, especialmente por las importantes interacciones que existen entre parámetros como el tiempo de extracción y la amplitud del ultrasonido.

3. PUBLICACIONES

3.1. Publicación IV

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Enviado (Septiembre 2014)

Ultrasound-assisted extraction and clean-up with dispersive sorbents followed by UHPLC–MS/MS for the determination of parabens and bisphenol A in human milk samples

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Abstract

A sensitive and accurate analytical method for the determination of four parabens (methyl-, ethyl-, propyl- and butylparaben) and bisphenol A in human milk samples is optimized and validated. The combination of a commonly used extraction technique, such as ultrasound-assisted extraction (USE) followed by a simplified and fast clean-up technique with sorbent materials was successfully applied for sample preparation prior to ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis. The analytes were first extracted from freeze-dried human milk samples with acetonitrile by applying ultrasonic irradiation (3 cycles of 15 min and 70% amplitude) and further cleaned-up using C18 as sorbent. Different extraction solvents, sorbent types and amounts, and the most influential parameters affecting USE were optimized using design of experiments. For MS detection, negative electrospray ionization (ESI) in the selected reaction monitoring (SRM) mode was used. The use of two reactions for each compound allowed simultaneous quantification and identification in one run. The analytes were separated in less than 10 min. Deuterium-labelled ethylparaben-d_5 (EPB-d_5) and deuterium-labelled bisphenol A-d_{16} (BPA-d_{16}) were used as surrogates. The limits of quantification found ranged from 0.4 to 0.7 ng mL^{-1}, while inter- and intra-day variability was under 11.1% in all cases. Due to the absence of certified

reference materials, the method was validated by recovery assays with spiked samples using matrix-matched calibration. Recovery rates ranged from 93.8% to 112.2%. The proposed method was satisfactorily applied for the determination of selected parabens and bisphenol A in human milk samples of nursing mothers from the province of Granada (Spain).

Keywords: Human milk; Endocrine disrupting chemicals; Ultrasound-assisted extraction; Sorbent materials; UHPLC-MS/MS

Highlights:

- ✓ The determination of parabens and bisphenol A in human milk samples is proposed
- ✓ An ultrasound assisted extraction procedure followed by a cleanup step are optimized
- ✓ Box–Behnken experimental design is used for the optimization of the different variables
- ✓ UHPLC-MS/MS is used as analytical technique to measure the target compounds
- ✓ The method was satisfactorily validated and applied to real human milk samples

1. Introduction

The preservation of health and well-being of children have been cause of concern for generations. Therefore, it is of special interest to assess the exposure of infants to a many different contaminants, because a central issue in this context is the age of exposure. Early life stages are particularly vulnerable and disturbance of developmental processes can lead to persisting alterations in structure and function that sometimes becomes manifest only later in life in terms of children's cognition, chronic diseases pubertal development or adult obesity. Infant growth retardation may be due to various reasons among which includes disruption of hormone systems, potentially caused by environmental pollutants [1, 2].

In the last decades, industrial development has experienced a substantial growth to be consistent with the needs of today's society, which results in the use of a large number of chemical compounds to improve the quality of life. However, this vertiginous industrial growth has also produced an increase in human exposure to xenobiotics which are able to cause adverse effects on human health. This is particularly important in the case of endocrine disrupting chemicals (EDCs), which belong to a large range of different chemicals. EDCs are ubiquitous, especially in food and consumer products. These compounds belong to an extensive group of both synthetic and natural substances that are able to interfere with the normal hormone function and homoeostatic control of wildlife and humans [3].

Industrial synthetic chemicals such as bisphenol A (BPA) or parabens (PBs) are among the most recognized EDCs. BPA is one of the high-volume compounds produced, with an annual production around of 2-3 million tons, and it is widely used in the manufacture of polycarbonate plastics and epoxy resins [4]. As a result, it may be found in many common consumer products and is abundantly retrieved in the environment leading to widespread exposure to BPA among the general population. On the other hand, PBs are alkyl esters of *p*-hydroxybenzoic acid, these compounds are widely used as bactericide and antimicrobial preservatives, especially against mold and yeast in cosmetic products, pharmaceuticals and food and beverages [5, 6]. Individually or in combination, PBs are used in over 13,200 formulations in nearly all types of cosmetics. In 2011, PBs have been permitted to be used in the European Union in cosmetic products with a maximum concentration of each one of 0.4% and a total maximum concentration of 0.8% [7]. However, a recent review has recommended the reduction of propyl and butylparaben levels in cosmetic products to a combined maximum concentration of 0.19% [8]. The ability of PBs to disrupt important physiological functions has been widely demonstrated [6, 9-11]. As well, the presence of non-metabolized PBs in breast cancer tissues [12] has focused the attention in their potential carcinogenic and toxic nature [11, 13, 14].

The routes of human exposure to these compounds are dermal contact, ingestion or inhalation [6, 15-17]. The study of exposure to EDCs is especially important in the case of breastfed infants, who are in the first stages of development and therefore are more vulnerable and susceptible to changes in the endocrine system. Human milk has been proposed in the assessment of exposure to environmental chemicals [18], particularly to EDCs [19]. It can be the main route of exposure for nursing babies, and consequently it may serve as biomarker of exposure to xenobiotics, providing important information to biomonitoring studies. For this reason, the development of analytical methods for determination of EDCs in this matrix at trace levels becomes of great importance.

The isolation of the analytes from this complex biological matrix is a critical issue; an extraction technique is usually required in order to fulfill this purpose. Only few studies have been reported on methods for the determination of these compounds in human milk, which have been mainly focused on BPA, being scarce the methods for PBs. Classical techniques such as liquid-liquid-extraction (LLE) [1, 20, 21], solid phase extraction (SPE) [22-25] including on-line SPE [26-28] have been used, being SPE, the most widely applied. However, several disadvantages are associated to both techniques, including the need of large sample volumes and toxic organic solvents, which make them costly, time-consuming and environmentally-unfriendly techniques. For these reasons, in the last decades, new extraction techniques for the determinations of EDCs in human milk samples has been developed, such as solid-liquid-liquid microextraction [29], stir-bar sorptive extraction [30], extraction with stir bar coated with molecularly imprinted polymers [31] or molecularly imprinted solid-phase microextraction [32].

In this work, the use of a simple and efficient solid-liquid extraction technique (ultrasound-assisted extraction) of freeze-drying samples is proposed. This technique is relatively simple in comparison with those recently applied techniques and requires not very high solvent consumption such as the classical ones besides being highly efficient and fast. The freeze-drying of

samples as preliminary step before extraction incorporates important advantages to sample treatment, such as the use of smaller sample volumes and water removal to favour the partitioning of the analytes into the extraction solvent [33]. Moreover, considering the complexity of biological matrices, the co-extraction of matrix components that reduce the sensitivity of the method is the major drawback of any extraction technique. Consequently, it is proposed a simplified clean-up step with sorbent materials to reduce matrix effects and improve analytical performance of the method.

In summary, the aim of the present work is to develop a sensible, selective and accurate method based on USE followed by a clean-up step for the determination of BPA and four PBs, (methyl-, ethyl-, propyl- and butylparaben) in human milk samples. Ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) has been used as detection technique. The method has been satisfactorily applied for the determination of these compounds in 10 samples collected from volunteer nursing mothers from the province of Granada (Spain).

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB) and butylparaben (BPB) were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA) and deuterium-labelled bisphenol A-d_{16} (BPA-d_{16}), were supplied by Sigma-Aldrich (Madrid, Spain). Deuterium-labelled ethylparaben-d_5 (EPB-d_5) was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). BPA-d_{16} and EPB-d_5 were used as surrogates.

Stock standard solutions (100 µg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile (ACN) up to the final

volume was added. The solution remained stable for at least four months at 4 ºC in the darkness. Three intermediate solutions, No. 1, 2 and 3 (2.5, 5 and 10 µg mL^{-1}) were prepared by diluting 0.25, 0.5 and 1 mL, respectively, of the stock solution to 10 mL with ACN. Subsequently, two new intermediate solutions No. 4 and 5 (0.5 and 1 µg mL^{-1}) were prepared by diluting 0.5 and 1 mL, respectively, of solution No. 3 to a final volume of 10 mL with ACN. Finally, intermediate solutions No. 6 and 7 (0.05 and 0.1 µg mL^{-1}) were prepared by diluting 1 and 2 mL, respectively, of solution No. 5 to a final volume of 10 mL with ACN. Working standards for calibration purposes were prepared by diluting 100 µL of the intermediate solutions No. 1 to 7 to a final volume of 10 mL with human milk. Working standards were prepared fresh from the ACN solutions prior to the experiments.

Methanol (MeOH), ethanol, ethyl acetate and ACN (HPLC grade), used for the preparation of standards and for the selection of the extraction solvent, were purchased from Merck (Darmstadt, Germany). LC–MS grade water, methanol, ammonia (≥25%) and formic acid (≥98%) —used for the preparation of mobile phases and pH adjustments— were purchased from Fluka (St. Louis, MO, USA). Anhydrous $MgSO_4$ was provided by Panreac (Barcelona, Spain). BAKERBOND® octadecyl C18 sorbent (40 µm particle size) was purchased from J.T. Baker (Deventer, The Netherlands), and PSA (40–60 µm particle size) was provided by Scharlab (Barcelona, Spain).

2.2. Instrumentation and software

The extraction of samples was performed with a Branson digital Sonifier®unit model S-450D (Danbury, CT, USA), operated with a standard 12.7 mm titanium disruptor horn, a flat and replaceable 12.7 mm titanium tip and a temperature probe.

UHPLC–MS/MS analysis was performed using a Waters Acquity UPLC™ H–Class (Waters, Manchester, UK), consisting of an Acquity UPLC™ binary solvent manager and an Acquity UPLC™ sample manager. Separation of compounds

was obtained with a CORTECS UPLC™ C18 column (1.6 μm, 2.1 mm × 50 mm) (Waters, UK). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z–spray™ electrospray ionization (ESI) source was used for analytes detection. Samples were freeze-dried using a SCANVAC CoolSafe™ freeze dryer. Extracts were evaporated with a SCANVAC CoolSafe™ ScanSpeed MaxiVac centrifuge for vaccum evaporation (Lynge, Denmark). For pH measurements, a EUTECH PCD 650 digital pH–meter with a combined glass–Ag/AgCl (KCl 3 M) electrode (EUTECH Instruments Ltd, Singapore) was used. A vortex–mixer (IKA, Staufen, Germany), a Hettich Universal 32 centrifuge (Tuttlingen, Germany) and a Spectrafuge™ 24D centrifuge from Labnet International, Inc. (New Jersey, USA) were also used. Samples agitation during the extraction procedure was carried out in an eight-position digital agitator-vibrator purchased from J.P. Selecta (Barcelona, Spain). Statgraphics Plus software version 5.1 (Statpoint Technologies Inc., Virginia, USA) was used for statistical treatment of data.

2.3. Sample collection and storage

Human milk samples were obtained from healthy nursing mothers living in Granada, Spain. Samples were anonymized, frozen at –20 ºC and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form. All of the samples were obtained under strictly controlled collection.

2.4. Basic procedure

1.4.1. Preparation of spiked samples

For the preparation of the spiked samples used for the optimization of the extraction procedure, a concentrate standard solution of the analytes was added to human milk and agitated to obtain a final concentration of 20 ng mL^{-1} for each compound. Then, in order to allow the analytes to interact with the human milk, the spiked samples were left to stand for 24 h at 4 ºC in the dark before analysis. Next, milk was aliquoted in portions of 10 mL in glass vials and frozen at –8o ºC for 12 h prior being freeze-dried.

For method validation purposes (recovery assays, precision and trueness), due to the absence of certified reference materials, 9.9 mL of blank human milk samples were spiked at different concentrations by adding 100 µL of the spiking standard solutions containing the analytes and surrogates. Then the spiked samples were treated as it was previously indicated. The blank samples were previously analyzed in order to ensure the absence of analytes or that these were below the LODs of the method.

2.4.2. Extraction procedure

Figure 1 illustrates a schedule of the steps of the extraction procedure. Freeze-dried samples were placed into stainless steel capsules and 10 mL of ACN were added. The capsules were vortexed for 2 min and sonicated for 15 min at 70% amplitude. Four samples could be simultaneously extracted. Three extraction cycles were required. The extracts obtained were merged and concentrated to a volume of approximately 10 mL in Falcon tubes at 40 ºC using the SpeedVac concentrator. Then, the remaining extracts were cleaned-up with 500 mg of anhydrous $MgSO_4$ and 300 mg of C18 sorbent. The Falcon tubes were placed on the eight-position digital agitator-vibrator, stirred for 3 min at room-temperature and centrifuged for 3 min at 3634 × *g*. The supernatants were then decanted into polypropylene conical tubes and evaporated to

dryness using the SpeedVac concentrator at 40 ºC. The dried extracts were redissolved with two portions of 300 µL MeOH each one, then centrifuged using Eppendorf tubes for 20 min at 16,300 × *g*, and evaporated again to dryness. The residue was redissolved in 100 µL of the initial mobile phase, centrifuged for 30 min at 16,300 × *g* and finally injected into the LC system.

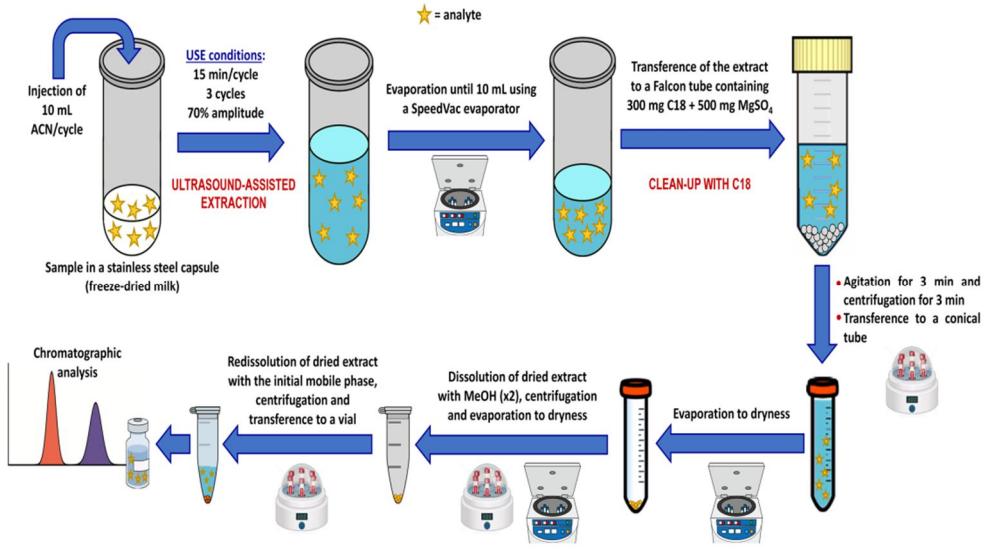

Fig. 1. *Schematic illustration of the extraction procedure.*

2.5. Ultra high performance liquid chromatography-tandem mass spectrometry

The chromatographic separation was performed using a binary gradient mobile phase consisting of 0.025% (v/v) ammoniacal aqueous solution (solvent A) and 0.025% (v/v) ammonia in MeOH (solvent B). The flow rate was 300 µL min^{-1}, the column was maintained at 40 ºC and the injection volume was 10 µL. Gradient conditions were as follows: initial mobile phase, 80% (A), maintained for 2 min, then it was linearly decreased to 10% (A) within 3.0 min, and to 0% within 0.1 min and held for 1.9 min to clean the column using 100% organic mobile phase. Finally, back to 80% (A) in 0.1 min and kept for 2.9 min to equilibrate the column. Total run time was 10 min.

The mass spectrometer (MS) was operated in negative electrospray ionization (ESI) mode. For increased sensitivity and selectivity, the analyses were performed in selected reaction monitoring mode (SRM) using $[M–H]^-$ as precursor ion. Instrument parameters were as follows [30]: capillary voltage, 0.60 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1}, and nebuliser gas flow, 7.0 bar. Nitrogen (≥99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell time for each transition was 25 ms, and inter-scan delay was set at 3 ms. In order to obtain the maximum sensitivity, the most abundant transition was used for quantification. Table 1 summarizes the transitions and potentials used for UHPLC–MS/MS analysis for the selected EDCs and the surrogates.

Table 1
Transitions and optimized potentials for UHPLC–MS/MS analysis

	Transitions	CV	CE
BPA	227.2 → 211.9[a]	-50	-22
	227.2 → 132.9[b]	-50	-26
MPB	151.1 → 91.8[a]	-38	-22
	151.1 → 135.8[b]	-38	-14
EPB	165.1 → 91.9[a]	-38	-24
	165.1 → 136.0[b]	-38	-16
PPB	179.1 → 91.8[a]	-42	-24
	179.1 → 136.1[b]	-42	-16
BPB	193.1 → 91.4[a]	-42	-24
	193.1 → 136.1[b]	-42	-16
BPA-d_{16}	241.2 → 223.0[a]	-46	-22
	241.2 → 141.9[b]	-46	-32
EPB-d_5	170.1 → 92.1[a]	-38	-24
	170.1 → 136.0[b]	-38	-16

[a] SRM transition used for quantification; [b] SRM transition used for confirmation
CV: Cone voltage (V); CE: Collision energy (eV)

3. Results and discussion

3.1. Optimization of the extraction parameters

Although MS is a selective and sensitive detection technique, lipids and other matrix interferences can deteriorate the sensitivity of the instrumental technique by increasing the signal background; therefore, an efficient clean-up step is necessary to overcome this analytical inconvenient. It is necessary to count on an extraction technique to disrupt the matrix and efficiently extract the analytes. In this sense, several variables were considered for the ultrasound-assisted extraction and during the clean-up with sorbents, which was carried out by design of experiments. Each assay was done by triplicate.

3.1.1. Selection of the extraction solvent

The extraction-solvent is a critical variable for developing an efficient USE technique. The extraction solvent must be able to solubilise the analytes of interest, minimizing the co-extraction of other matrix components. In this sense, four organic solvents were evaluated, including MeOH, ethanol, ACN and ethyl acetate. These solvents were selected because of their recognized efficiency in the extraction of several families of EDCs from biological and environmental matrices [5, 34, 35], as well as they facilitate the precipitation of proteins from milk, which can be easily separated from the obtained extract.

For solvent optimization, the following basic procedure was applied: freeze-dried milk samples were extracted by USE with 10 mL of the evaluated solvent during 15 min at 70% amplitude. Two extraction cycles were applied in all cases. The obtained extracts were evaporated to dryness using the SpeedVac concentrator at 40 ºC. The dried residues were redissolved with the initial mobile phase and injected to LC system.

MeOH and ethanol extracts were characterized by the highest extraction of matrix components, making difficult the handling of the extracts and were

not analysed further. The results of the comparison of the extraction efficiency of ACN and ethyl acetate are shown in Figure 2 (A). Mean values of relative area for each target analyte with each solvent were compared using the least significant difference (LSD) multiple range test with a 95.0% confidence level. The results showed statistically significant differences only for PPB and BPB, being acetonitrile the best option for solvent. In the case of MPB, EPB and BPA, none significant differences were observed between both solvents. Consequently, ACN was selected as optimal extraction solvent.

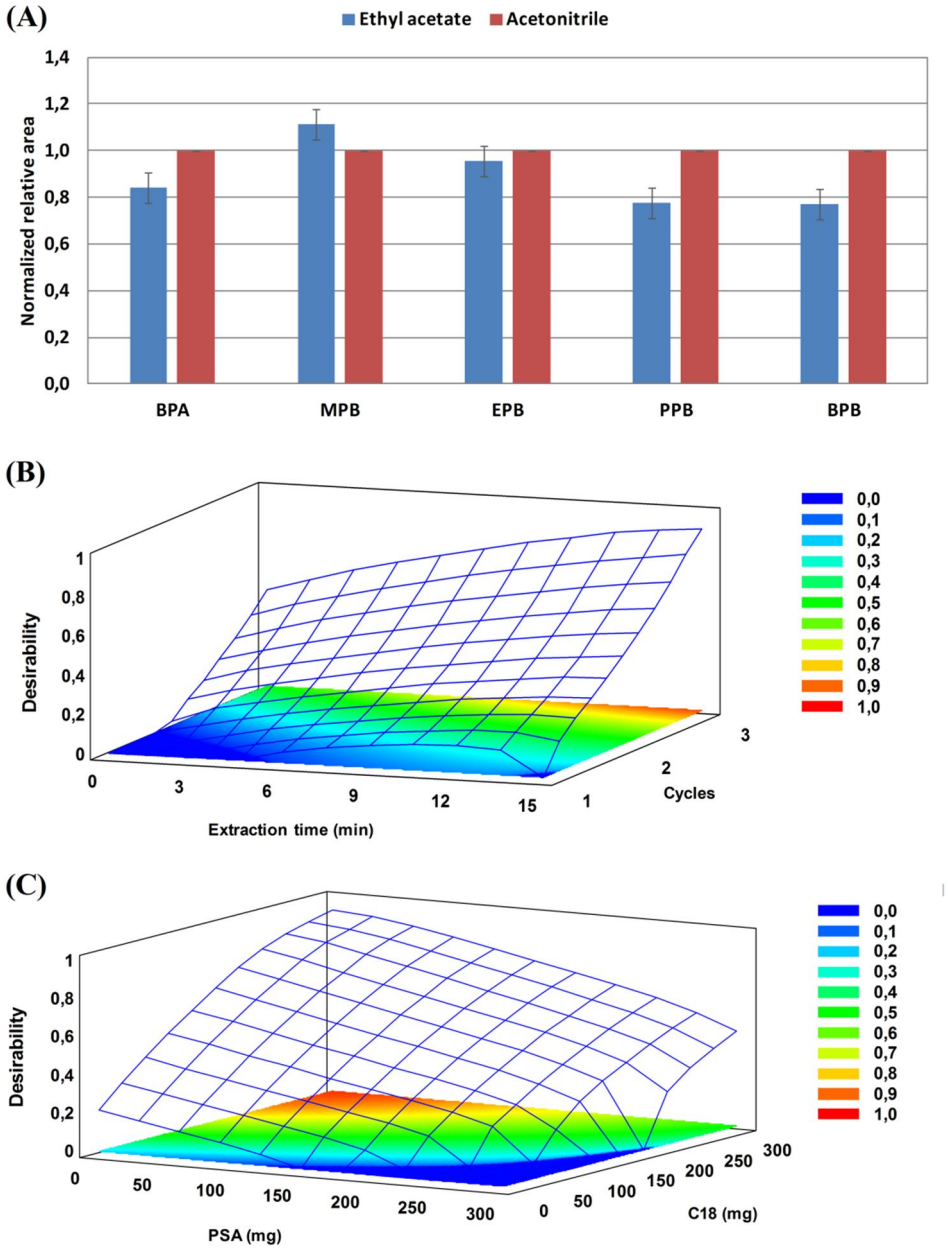

Fig. 2. (A) *Normalized relative areas for the selection between ACN and ethyl acetate as extraction solvent. BPA (LSD = 0.08); MPB (LSD = 0.10); EPB (LSD = 0.16); PPB (LSD = 0.15); BPB (LSD = 0.39). Significance level of 95% was selected for all cases. Representation of the global desirability function versus* ***(B)*** *extraction time and number of cycles for ultrasonic extraction, and* ***(C)*** *amount of PSA and C18 sorbents for the clean-up step for the determination of targets EDCs.*

3.1.2. Optimization of USE conditions

In order to optimize the USE parameters and study possible interactions between them, a 15-run Box–Behnken design including three replicates at the centre point was used for fitting a second-order response surface, with three factors and three levels for each one: extraction time (1, 8 and 15 min), number of cycles (1, 2 and 3) and extraction solvent volume per cycle (10, 15 and 20 mL). The maximum amplitude was 70% in order to not reduce the lifespan of the ultrasound probe. This value was set in all assays, since there are studies that indicate that this amplitude is required to obtain the highest extraction efficiency for EDCs [36]. The matrix applied in the Box–Behnken design is shown as supplementary material.

The data were evaluated by ANOVA. The test gave determination coefficients (R^2) between 0.813 and 0.930. Since the P values for the *lack-of-fit* test were >0.05 in all cases, the model appears to be satisfactory with the 95% of confidence level. It was observed that the number of extraction cycles was the most important parameter, followed by the extraction time, which was not significant only for BPA. The observed tendency for all the analytes was that both parameters had always a positive effect. These results proved that although the long exposure to solvent allows the matrix to swell, thus improving the penetration of solvent into the sample interstices and the contact of the solvent with the analytes, however, there is an important advantage in increasing the number of cycles instead of extraction time, since with each new cycle, the introduction of fresh solvent maintains a favourable solvent/sample equilibrium and, hence, improving partitioning into the liquid phase and increasing recoveries [35]. On the other hand, the volume of extraction solvent was not an influential variable in any case, therefore this value was set at 10 mL, that was the lowest assayed volume, being chosen in order to reduce the solvent consumption and facilitate the throughput of samples since as higher is the volume of the extraction solvent, the longer is the evaporation time.

The combination of the optimized experimental values obtained for each compound for the evaluated variables allowed the determination of the best overall extraction efficiency, which was calculated with the desirability function. Responses for each compound in the experiments of the Box-Benhken design were first normalized between 0 and 1, and the global desirability function was defined as their geometric mean. The plot of this function versus the number of cycles and the extraction time, being fixed the volume of the extraction solvent at 10 mL, is shown in Figure 2 (B). The optimum corresponded to 3 extraction cycles of 15 min each one, 10 mL of extraction solvent volume and 70% of amplitude.

3.1.3. Optimization of the clean-up procedure

When an extraction procedure is applied to a biological sample, many interferences are also co-extracted. Therefore, it was of interest to perform a clean-up step to remove matrix components, especially in this case, the lipid content of the extract. It was selected a clean-up procedure with sorbents that are frequently used in dispersive solid phase extraction.

For the development of the clean-up procedure, a mixture of two solid sorbents was checked: PSA and C18. These sorbents are commonly used in the removal of extracted interferences in biological matrices. This selection was also due to the fact that the extracted interferences in milk, such as fatty acids, sugars, triglycerides, phospholipids and cholesterol (sterols), have higher affinity for this sorbents mixture than for the other type of sorbents [37-41]. C18 is specifically used for removal of co-extracted fat and other lipophilic compounds from ACN extracts. In addition, it is worth to notice, that although bulk fats represented by triacylglycerols (TAGs) are not soluble in ACN, some TAGs and other lipophilic compounds move to the organic layer during a partitioning step in the form of micro-micelles. Under these conditions, they can cause clogging of an analytical column. To eliminate these non-polar co-extracts, the use of C18 sorbent can be also an efficient alternative [35, 42]. On the other hand, PSA could be also a good alternative for clean-up, since its

bidentate structure is responsible for its high chelating effect. As a result of its secondary and primary amino groups, the retention of free fatty acids and other polar matrix compounds is very strong. Therefore, matrix components can be more efficiently removed from the extracts leading to cleaner chromatograms. The composition of sorbent mixture for the clean-up step was determined with a Box-Benhken design, being also included as a variable the amount of $MgSO_4$ that is applied during the clean-up phase as a desiccant, in order to eliminate traces of water which complicate sample evaporation and concentration.

Box-Behnken matrix also consisted of 15 experiments, including three central points. The variables, evaluated at 3 levels, were C18 amount (0, 150 and 300 mg), PSA amount (0, 150 and 300 mg) and $MgSO_4$ amount (0, 250, 500 mg). The Box–Behnken design matrix, i.e. the extraction conditions for each run, is also shown as supplementary material.

Figure 2 (C) shows the plot of the desirability function versus the amounts of PSA and C18 sorbents, with the amount of $MgSO_4$ set at 500 mg, corresponding to the optimum value according to the Box-Benhken design. Parameters corresponding to both sorbents, particularly their quadratic terms, resulted statistically significant for all compounds. Despite the already indicated advantages in the removal of matrix interferences from milk, the use of PSA showed in this case an important but negative effect, this behaviour may be due to this sorbent is a weak anion exchanger, and therefore could be not suitable due to the acidic nature of the studied EDCs. It was also observed a significant positive interaction between C18 sorbent and $MgSO_4$, which could be explained considering $MgSO_4$ eliminates traces of water that could produce emulsions in presence of C18, making difficult the separation of the cleaned extract, reducing the recovery of the analytes. According to the results of the Box-Behnken design, a mixture of 300 mg of C18 and 500 mg of $MgSO_4$ was selected as the compromise mixture used in the clean-up step.

3.2. Method validation

First, a seven-point matrix–matched calibration curve was obtained for each studied compound in the range from the limit of quantification (LOQ) to 50 µg mL^{-1} Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. EPB-d_5 and BPA-d_{16} (50 µg mL^{-1}) were selected as surrogates for PBs and BPA, respectively. Each calibration level was made in triplicate and analyzed twice. Table 2 shows the statistical and the analytical parameters obtained for each compound.

Table 2
Analytical and statistical parameters

	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BPA	$1.278 \cdot 10^{-2}$	$1.010 \cdot 10^{-4}$	99.8	47.4	0.2	0.5	0.5 - 50
MPB	$1.870 \cdot 10^{-2}$	$1.340 \cdot 10^{-4}$	99.8	17.1	0.2	0.5	0.5 - 50
EPB	$1.952 \cdot 10^{-2}$	$1.723 \cdot 10^{-4}$	99.6	17.5	0.1	0.5	0.5 - 50
PPB	$1.590 \cdot 10^{-2}$	$1.322 \cdot 10^{-4}$	99.7	54.0	0.1	0.4	0.4 - 50
BPB	$8.057 \cdot 10^{-3}$	$5.509 \cdot 10^{-5}$	99.8	15.0	0.2	0.7	0.7 - 50

b: slope; s_b: slope standard deviation; R^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification; LDR: linear dynamic range

The analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision), according to the protocols described in the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [43].

Linearity. The determination coefficient (R^2) and the *lack-of-fit* test (P_{lof}) were evaluated. The values obtained for R^2 ranged from 99.6 to 99.8% and P_{lof} values were >5% in all cases [44], and then good linearity was observed within the concentration range (LOQ–50 ng mL^{-1}).

Sensitivity. The limits of detection (LODs) and quantification (LOQs) were calculated by taking into consideration the minimum concentration of analyte that the method can detect and with a signal-to-noise ratio of 3 for LODs and 10

for LOQs, using the quantification transition. Found LODs ranged from 0.1 to 0.2 ng mL^{-1} and found LOQs ranged from 0.4 to 0.7 ng mL^{-1}. These results are also summarized in Table 2.

Accuracy (precision and trueness). The precision of the method in terms of intra– and inter–day variability was evaluated using spiked human milk samples at three concentration levels (1, 10 and 50 ng mL^{-1}) for each compound. Precision, expressed as relative standard deviation (%, RSD) was determined from triplicate spiked samples during the same day and in 6 different days. The values obtained are summarized in Table 3. RSD values fell between 1.3% and 11.1% being lower than 15%. Therefore, all compounds were within the acceptable limits for method validation guide, which are considered ≤15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data indicate that the methods are reproducible.

Table 3
Recovery assay, precision and trueness of target compounds in human breast milk

	Spiked (ng mL^{-1})	[a]Found ± CI (ng mL^{-1})	RSD (%)	Recovery (%)
	1.0	1.02 ± 0.03	5.8	102.7
BPA	10	9.9 ± 0.2	3.1	99.6
	50	49.6 ± 0.5	1.3	100.2
	1.0	0.93 ± 0.04	6.5	94.0
MPB	10	9.94 ± 0.3	5.0	100.0
	50	49.6 ± 1.2	3.2	100.3
	1.0	0.93 ± 0.03	5.1	93.8
EPB	10	10.6 ± 0.5	7.3	107.0
	50	50.0 ± 1.6	4.5	101.1
	1.0	1.09 ± 0.07	9.3	109.6
PPB	10	10.1 ± 0.8	11.1	101.7
	50	49.8 ± 1.2	3.7	100.7
	1.0	1.12 ± 0.03	3.3	112.2
BPB	10	9.68 ± 0.5	6.8	97.4
	50	49.7 ± 0.8	2.4	100.5

[a] *Mean of 18 determinations; CI: confidence interval (P=95% and 17 freedom degrees); RSD: relative standard deviation*

For validation of the method in terms of trueness, a recovery assays were carried out. The recovery of known amounts of tested compounds in milk samples was evaluated by comparing the spiked amount with the concentration of each compound determined by using the proposed method. As shown in Table 3, the recoveries were close to 100% (93.8% to 112.2%) in all cases.

Precision and trueness data indicate that the method is accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Selectivity. This parameter was demonstrated by LC–MS/MS analysis of blanks. A blank sample and a spiked blank sample with the analytes were extracted and their chromatograms were compared. No interferences from endogenous substances were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensure the high selectivity of the methods. Figure 3 (A) shows the SRM chromatograms obtained from a spiked milk sample with the analytes.

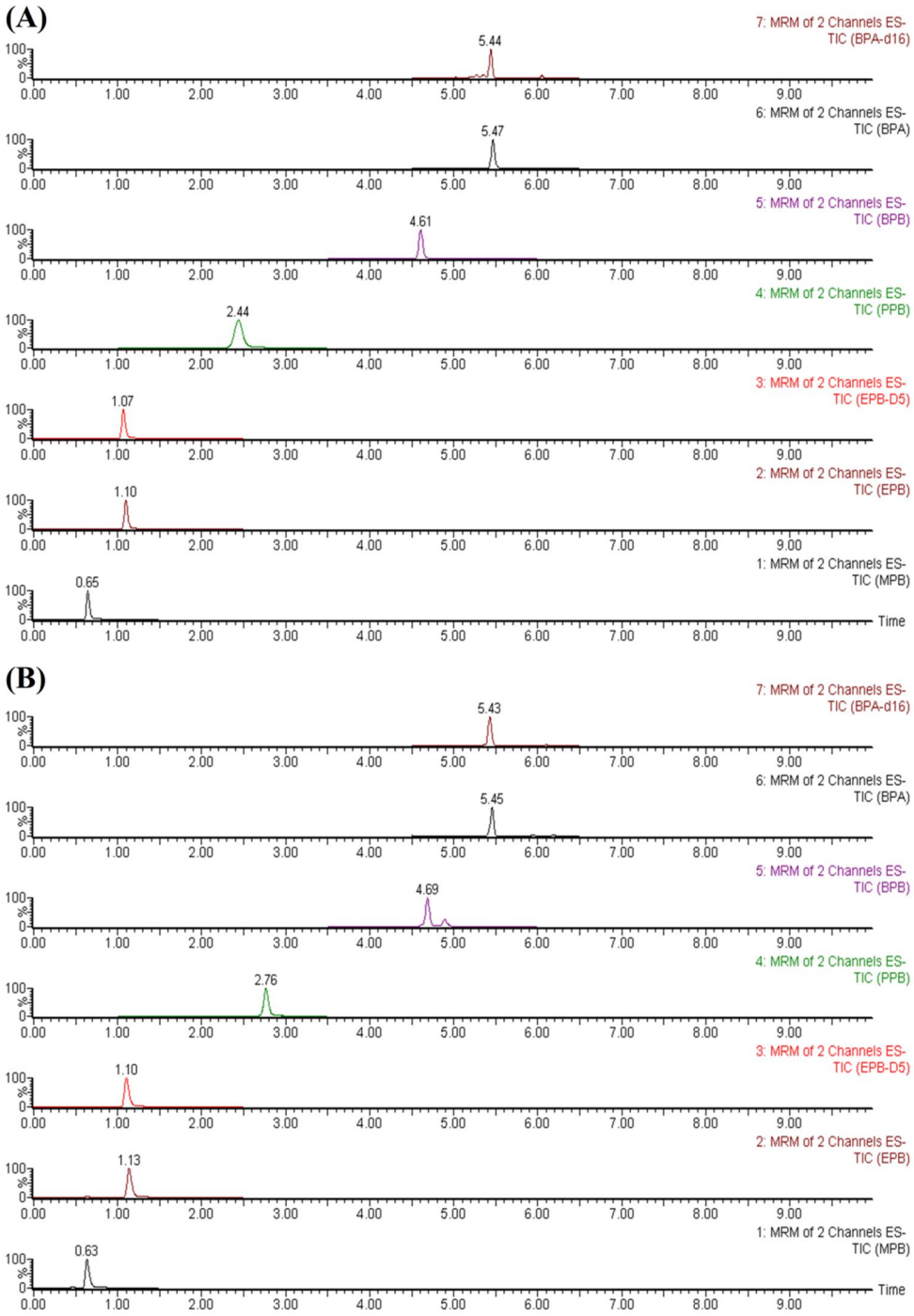

***Fig. 3**. UHPLC–MS/MS chromatograms of: **(A)** a spiked blank; **(B)** a positive human breast milk sample with the target analytes, containing the corresponding surrogates (BPA-d_{16} and EPB-d_5).*

3.3. Application of the method

The proposed method was applied to determine free concentrations of selected PBs and BPA in human milk samples collected from 10 anonymous women with no known occupational exposure to these compounds. The results obtained as a mean of six determinations are summarized in Table 4.

Table 4
Application to human breast milk samples

Sample	[a,b]Found amount (ng mL^{-1})				
	BPA	MPB	EPB	PPB	BPB
01	1.31 (0.04)	2.80 (0.09)	2.00 (0.06)	4.0 (0.4)	5.4 (0.4)
02	ND	1.82 (0.06)	1.62 (0.06)	1.3 (0.2)	D
03	D	16.3 (0.5)	18.1 (1.2)	12.6 (1.5)	12.1 (1.0)
04	D	4.65 (0.25)	D	1.98 (0.07)	1.19 (0.09)
05	0.60 (0.04)	1.74 (0.09)	0.97 (0.03)	1.39 (0.06)	D
06	1.76 (0.07)	1.26 (0.07)	D	1.02 (0.08)	D
07	2.10 (0.09)	1.99 (0.07)	1.15 (0.02)	1.13 (0.09)	1.06 (0.05)
08	D	1.81 (0.06)	1.13 (0.03)	D	1.17 (0.03)
09	D	D	D	ND	D
10	ND	ND	ND	ND	ND

[a] *Mean of 6 determinations; ND: not detected (<LOD); D: detected (>LOD and <LOQ)*
[b] *Standard deviations are in parentheses*

BPA was found in 8 samples but quantified in 5 of them at concentrations ranging from 0.6 to 2.1 ng mL^{-1}. These concentration levels of free BPA are similar to those found in other three published studies in Caucasian women, one from France (0.80–3.29 ng mL^{-1}) [45] and the other two from the United States (0.22–10.8 ng mL^{-1}) [24, 27], considering there was a significant association between BPA concentration and race. Caucasian women had significantly higher levels of free BPA in their milk than non-Caucasian women (mean value 0.52 ng mL^{-1}) [24]. Concentrations levels of PBs were alike in most of the samples (between 1–2 ng mL^{-1}), except for two of them, sample 1 showed concentrations slightly higher than the others, and sample 3 contained about 10 times higher concentrations of PBs than the others. Only one sample did not contain any of the analyzed compounds. Except for sample 3, all the other results are also agreed with a previous work (0.32–3.04 ng mL^{-1}) [27]. In general, these results confirm the statement that these substances are

ubiquitous, which could be due to the daily and massive use of personal care products by people, especially women. Figure 3 (B) shows the SRM chromatograms obtained from one of the positive analyzed samples.

4. Conclusions

A relatively simple, efficient and robust extraction technique by USE and a clean-up step using C18 sorbent material has been proposed as a convenient alternative for sample preparation for determining some selected EDCs (PBs and BPA) at trace levels in previously freeze-dried human milk samples by UHPLC–MS/MS. Due to the known complexity of biological matrices, the obtained extracts shown significant matrix effects. However, the thorough optimization by design of experiments of both, the most influential parameters of USE and the type and amount of sorbents for clean-up step, together with the sensitive detection and quantification by UHPLC–MS/MS, allowed the validation of the analytical performance of the method, which showed good analytical parameters in relation to sensitivity, selectivity, accuracy and precision in the determination of the selected compounds, achieving low LOQs (between 0.4 and 0.7 ng mL^{-1}), high recoveries and precision. The proposed sample preparation procedure was considered a good choice, because of its higher extraction yields and easy operation, especially when it is compared with the SPE technique that is traditionally used for human milk analysis, and then this alternative saves time and requires lower volumes of solvents than SPE, reducing costs and waste.

This method is useful for the determination of the levels of PBs and BPA in human milk samples, and it may be used to perform biomonitoring studies, since this matrix is a valuable biological fluid that may serve as a biomarker of both maternal and prenatal exposure to many different environmental chemicals, particularly to EDCs. Human milk could at once be a major route of exposure for breastfed infants to exogenous contaminants, so that the development of analytical methods for the determination of these substances will allow developing more studies about the incidence and onset of diseases

and other adverse effects that are being related with EDCs, such as different types of cancer, changes in brain structure and function, behaviour, male and female reproductive tracts, metabolism, hormone signalling, and the immune system.

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3.2. Publicación V

Talanta, Enviado
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Analysis of benzophenone-UV filters in human milk samples using ultrasound-assisted extraction and clean-up with dispersive sorbents followed by UHPLC–MS/MS

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Abstract

A new sample preparation method for the determination of five benzophenone UV-filters in human breast milk has been developed. The procedure involves the lyophilization of the sample, and its subsequent extraction by ultrasound sonication using acetonitrile. In order to reduce matrix effects produced by milk components that are coextracted, mainly proteins, sugars and lipids, a further clean-up step with a mixture of dispersive-SPE sorbents, C18 and PSA was applied. Extraction parameters were optimized using experimental design based optimization techniques, and the compounds were detected and quantified by ultrahigh performance liquid–chromatography tandem mass spectrometry (LC–MS/MS) in positive ESI mode. Analytes were separated in 10 min. BP-d_{10} was used as internal standard. The limits of detection (LODs) were between 0.1 and 0.2 ng mL^{-1}, and the limits of quantification (LOQs) were between 0.3 to 0.6 ng mL^{-1} for the target analytes. The inter– and intra–day variability was < 12%. The method was validated using matrix–matched calibration and recovery assays with spiked samples. Recovery rates were between 90.9% and 109.5%. The method was successfully applied for the determination of these compounds in human milk samples collected from volunteers lactating mothers with no known occupational exposure to these compounds who live in the province of Granada (Spain). The analytical method developed here may be useful for the development of more in–depth studies on the prenatal exposure and biomonitoring of these commonly used UV-filters.

Keywords: Benzophenone-UV filters; Human breast milk analysis; Ultrasound-assisted extraction; Sorbent materials; UHPLC–MS/MS

Highlights:

- ✓ The analysis of benzophenone UV-filters in human milk samples is validated
- ✓ An ultrasound assisted extraction procedure followed by a cleanup step is proposed
- ✓ Box–Behnken experimental design is used for the optimization of the different variables
- ✓ UHPLC-MS/MS is used as analytical technique to measure the target compounds
- ✓ After validation, the method has been applied in real human milk samples

1. Introduction

Endocrine disrupting chemicals (EDCs) are a group of natural and synthetic chemicals that may interfere with the normal function of the endocrine system in animals and humans [1]. EDCs can either mimic or inhibit the action of natural hormones, leading to adverse reproductive and developmental effects. In men, exposure to EDCs is associated with problems in reproductive capacity and testicular or prostate cancer [2]. In women, abnormal endocrine function may be associated with increased risk for endometriosis, reproductive and endocrine-related cancers, or impaired oocyte competence, ovarian function or menstrual cycling [3]. Although, the effects of early life exposure to EDCs remain still unclear, it has been suggested that fetal or childhood exposure may lead to abnormal sex differentiation, abnormal neurological and reproductive development, and to risk of reproductive problems or cancer later in life [4].

A wide variety of chemicals have been shown to have estrogenic activity [5], being the majority of them synthetic compounds. Many are components of personal care products (e.g., UV-filters) [6], which comprise different groups of

compounds that are currently used as additives in different common products such as cosmetic, household, food or pharmaceutical products, among others. Considerable amounts of PCPs are used in everyday human activities, so they are produced in large quantities (thousands of tons per year). Although these compounds are used in some products intended for direct ingestion, the main route of exposure to PCPs is the absorption through the skin, being further metabolized and eventually bioaccumulated and/or excreted [7-13].

Organic UV-filters are often used to protect skin against UV radiation damage. They are constituents of many daily products such as skin creams, body lotions, hair sprays, hair dyes, shampoos and sunscreen. The European Union (EU) Regulation 1223/2009 –Cosmetics Regulation– provides a robust, internationally recognized regime, which reinforces product safety. It stipulates the compounds that are allowed to be used as UV-filters in cosmetics and their maximum concentrations [14]. The family of benzophenones (BPs) is one of the most frequently used groups of UV-filters as they are able to absorb UV light that is harmful to the human body in the form of UVA (320 to 400 nm) and UVB (290 to 320 nm). BPs consists of 12 main compounds, called from benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less known as 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP) and 4-hydroxybenzophenone (4-OH-BP) [15, 16].

There is increasing evidence that BPs are able to interfere with the endocrine system. *In vitro* studies have shown that BPs stimulates the proliferation of the breast cancer cell line MCF-7 due to their estrogenic activity and that these compounds have also antiandrogenic activity [17, 18]. Carcinogenesis and reproductive organ malformations have been also reported in rodents after exposure to BP-UV filters [19, 20]. During the biotransformation of BPs, they can suffer yet a "cross-transformation" to other types of BPs which often show more dangerous disrupting activities than the original forms [21]. It has been reported that BP-3 is metabolized to BP-1 and BP-8 in animals [21, 22], and there are some evidence that BP-1 possesses higher

estrogenic activity than BP-3 [17, 22, 23]. Others BPs such as 4-OH-BP are also metabolites of BP-3.

Since human milk is the main route of exposure to chemical compounds for infants, its analysis is of special interest. The early life stages are very important due to vulnerability in developmental processes and any disturbance can lead to persistent alterations in structure and function that sometimes becomes manifest later in life. Information on early exposure to BPs, for example, can be obtained from analyses of human milk, which reflect exposure of the infant and, also provides information on prenatal exposure. Although BPs are widely present in aquatic ecosystems and exhibit bioaccumulation in invertebrates and fish, their presence in human tissues appears to correlate with consumer habits rather than with environmental exposure. This leads to a high inter-individual variability of human exposure patterns to UV filters. It has been proved that there is a positive correlation between the use of cosmetics containing BPs and their presence in the milk of individual mothers [24].

To date, very few studies have been focused in the development of analytical methods for the determination BPs in human milk, which are mainly focused on BP-3, since it is the most widely used and therefore the most important member of this family of compounds. Due to the complexity and composition of this biological matrix, especially in relation to its significant fat and protein content, the isolation of the target analytes becomes critical for the development of any analytical method. The selection of an adequate sample treatment and the extraction technique is very important to obtain a selective and efficient recovery of the analytes from samples, reducing matrix effects and improving the sensitivity of the method, considering the great importance of detecting trace levels of these substances, since it is proved their adverse effects even at very low concentrations. For determination of BPs in human milk samples classical techniques such as liquid-liquid-extraction (LLE) [24, 25] or solid phase extraction (SPE) including on-line SPE [26, 27] have been used, however very few works have developed a multiresidue method [15, 28].

In this work, a sensible, selective and accurate multiresidue method for determination of BP-1, BP-3, 4-OH-BP, BP-6 and BP-8 in human milk samples is proposed. This method is based on the use of ultrasound assisted extraction, followed by a clean-up step with PSA and C18, commonly used as dispersive-SPE sorbents. UHPLC–ESI–MS/MS has been used as analysis technique. Previous lyophilization of milk samples is an important innovation, since this treatment clearly facilitates the extraction procedure, which will be completed through a further clean-up step, improving the quality parameters of the method. The method was satisfactorily validated and applied for the determination of the concentrations of free forms of the above mentioned compounds in 10 human milk samples from volunteers lactating mothers who live in the province of Granada (Spain).

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP) and labelled-deuterium benzophenone (BP-d_{10}) were supplied by Sigma-Aldrich (Madrid, Spain). BP-d_{10} was used as internal standard.

Stock standard solutions (100 µg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile (MeCN) up to the final volume was added. The solution remained stable for at least four months at 4 ºC in the darkness. For calibration and validation purposes, two intermediate solutions, No. 1 and 2 (5.0 and 2.5 µg mL^{-1}) were prepared by diluting 0.5 and 0.25 mL, respectively, of the stock solution to 10 mL with MeCN. Subsequently, two new solutions No. 3 and 4 (1.0 and 0.5 µg mL^{-1}) were prepared by diluting 2 and 1 mL, respectively, of solution No. 1 to a final volume of 10 mL with MeCN. Finally, solutions No. 5 and 6 (0.1 and 0.05 µg mL^{-1}) were prepared by diluting 2

and 1 mL, respectively, of solution No. 4 to a final volume of 10 mL with MeCN. Working standards were prepared fresh from the MeCN solutions prior to the experiments.

Methanol (MeOH), ethanol, ethyl acetate and MeCN (HPLC grade), used for the preparation of standards and for the selection of the extraction solvent, were purchased from Merck (Darmstadt, Germany). LC–MS grade water, methanol, ammonia (≥25%) and formic acid (≥98%), used for the preparation of mobile phases and pH adjustments, were purchased from Fluka (St. Louis, MO, USA). Anhydrous $MgSO_4$ was provided by Panreac (Barcelona, Spain). BAKERBOND® octadecyl C18 sorbent (40 µm particle size) was purchased from J.T. Baker (Deventer, The Netherlands), and primary-secondary amine (PSA) (40–60 µm particle size) was provided by Scharlab (Barcelona, Spain).

2.2. Instrumentation and software

The extraction of samples was performed with a Branson digital Sonifier®unit model S-450D (Danbury, CT, USA), operated with a standard 12.7 mm titanium disruptor horn, a flat and replaceable 12.7 mm titanium tip and a temperature probe. UHPLC–MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class from Waters (Manchester, UK). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for BPs detection. Separation of compounds was obtained with a CORTECS UPLC™ C18 column (50 mm × 2.1 mm; 1.6 µm particle size) (Waters, UK). MassLynx 4.1 software was used for instrument control, peak detection and integration. Samples were lyophilized using a SCANVAC CoolSafe™ freeze dryer and extracts were evaporated with a SCANVAC CoolSafe™ ScanSpeed MaxiVac centrifuge for vaccum evaporation (Lynge, Denmark). For pH measurements, a EUTECH PCD 650 digital pH-meter with a combined glass-Ag/AgCl (KCl 3 M) electrode (EUTECH Instruments Ltd, Singapore) was used. A vortex–mixer (IKA, Staufen, Germany), a Hettich Universal 32 centrifuge (Tuttlingen, Germany) and a Spectrafuge™ 24D centrifuge from Labnet International, Inc. (New Jersey,

USA) were also used. Samples agitation during the extraction procedure was carried out in an eight-position digital agitator-vibrator purchased from J.P. Selecta (Barcelona, Spain). Statgraphics Plus software version 5.1 (Statpoint Technologies Inc., Virginia, USA) was used for statistical treatment of data.

2.3. Sample collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. Samples were anonymized, frozen at -20 ºC and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form.

2.4. Preparation of spiked milk samples

For the optimization of experimental variables, an aliquot of 9.9 mL of human milk sample was placed into a glass vial of 20 mL and 100 µL of a concentrate standard solution was added to obtain a final concentration of 20 ng mL^{-1} of all analytes. Then, the spiked samples were left to stand for 24 h at 4 ºC in the dark before analysis to allow the analytes to come into contact with the whole human milk sample. Next, the samples were frozen at -80 ºC for 12 h prior to lyophilization.

For validation purposes (recovery assays, precision and trueness) blank samples were spiked at different concentrations (from 0.5 ng mL^{-1} to 50 ng mL^{-1}) by adding 100 µL of the different spiking standard solutions (No. 1-6) to 9.9 mL of human milk, left at 4 ºC for 24 h and frozen at -80 ºC. The blank samples were previously analyzed in order to ensure the absence of analytes or that these were below the limits of detection (LODs) of the method.

2.5. Extraction procedure

Lyophilized human milk samples were placed into stainless steel capsules and 10 mL of MeCN were added. In order the sample comes into contact with the solvent, these capsules were vortexed for 2 min, and then sonicated for 15 min at 70% amplitude. Three cycles were required. The obtained extracts were merged and concentrated to a volume around 10 mL using the SpeedVac concentrator at 760 × *g* and 40 ºC. Then, the extracts were cleaned-up with a mixture of 500 mg anhydrous $MgSO_4$, 250 mg PSA and 300 mg C18 sorbents. The extract was stirred for 3 min at room temperature at an eight-position digital agitator-vibrator and centrifuged for 3 min at 3634 **×** *g*. The supernatant was transferred to a 10 mL polypropylene conical tubes and evaporated to dryness in the SpeedVac concentrator at 760 × *g* and 40 ºC. The residue was dissolved with two portions of 300 µL of MeOH and transferred to a 1.5 mL Eppendorf tube for evaporation to dryness at room temperature. Finally, the residue was dissolved in 100 µL of initial mobile phase and after stirring for 60 s in vortex, then it was filtered through a 4 mm and 0.22 µm nylon filter. The sample was ready to be injected into the chromatographic system.

2.6. Liquid chromatography-mass spectrometry analysis

The chromatographic separation of target analytes was performed using a CORTECS UPLC™ C18 (Waters) column (50 mm **×** 2.1 mm; 1.6 µm particle size). The compounds were separated using a gradient mobile phase consisting of 4 mM aqueous ammonium formate solution (pH = 9) as solvent A and 0.025% (v/v) ammonia in MeOH as solvent B. Gradient conditions were as follow: initial mobile phase, 80% (A), maintained for 2 min, then it was linearly decreased to 10% (A) within 3.0 min, and to 0% within 0.1 min and held for 1.9 min to clean the column using 100% organic mobile phase. Finally, back to 80% (A) in 0.1 min and kept for 2.9 min to equilibrate the column. Total run time was 10 min. Flow rate was 300 µL min^{-1}, injection volume 10 µL and column temperature 40 ºC.

For MS analysis, ESI was performed in positive ion mode. The tandem mass spectrometer was operated in the multiple reactions monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Instrument parameters were as follows [15]: capillary voltage, 0.60 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1} and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell times were set at 25 ms. Collision energies (CE) and cone voltages (CV) were optimized for each analyte. Optimized parameters for each compound are listed together with the mass transitions in Table 1.

Table 1
Transitions and optimized potentials for UHPLC–MS/MS analysis

	Transitions	CV	CE
BP-1	214.9 → 136.8[a]	2	18
	214.9 → 105.1[b]	2	32
BP-3	229.0 → 150.8[a]	4	20
	229.0 → 104.9[b]	4	18
BP-6	275.0 → 150.9[a]	14	18
	275.0 → 94.9[b]	14	34
BP-8	245.0 → 120.9[a]	14	20
	245.0 → 150.9[b]	14	20
4-OH-BP	199.0 → 120.8[a]	36	20
	199.0 → 104.8[b]	36	18
BP-d_{10}	193.1 → 109.8[a]	18	16
	193.1 → 81.8[b]	18	30

[a] SRM transition used for quantification; [b] SRM transition used for confirmation
CV: Cone voltage (V); CE: Collision energy (eV)

3. Results and discussion

3.1. Sample treatment

Although MS is a selective and sensitive detection technique, human milk is a complex matrix that contains lipids, proteins, carbohydrates, minerals and

vitamins that can be also co-extracted and interfere with the detection of the target analytes. The previous lyophilization of the sample minimizes the extraction of many of these matrix components, which remain precipitated during the extraction step (*i.e.,* proteins). In this sense, ultrasound-assisted extraction and a clean-up step with sorbents were selected. Several variables were considered for the optimization of both procedures and design of experiments was applied. Each experiment was done in triplicate.

3.1.1. Selection of the extraction solvent

The solvent is an important factor to be considered when biological samples are processed. The selected solvent must have certain characteristics, mainly to extract the analytes of interest minimizing the extraction of matrix components. In this case, the following solvents were evaluated, since they have been widely used for the extraction of different families of EDCs from biological samples: methanol, ethanol, acetonitrile and ethyl acetate [29, 30].

For solvent optimization, the following basic procedure was applied: spiked lyophilized milk samples were extracted with 10 mL of each solvent by USE. Two extraction cycles of 15 min at 70% amplitude were applied in all cases. The obtained extracts were evaporated to dryness in the SpeedVac concentrator at 760 × *g* and 40 ºC. In the case of methanol and ethanol, the use of both solvents was directly discarded because their extracts contained high amounts of matrix components. The dried residues from acetonitrile and ethyl acetate extracts were redissolved with 100 µL of the initial mobile phase containing the internal standard (50 ng mL^{-1}) and directly injected into the LC system. The Figure 1 shows the comparison of the extraction efficiency between acetonitrile and ethyl acetate. Mean values of normalized areas for each target analyte with each solvent were compared using the least significant difference (LSD) multiple range test with a 95.0% confidence level. The results showed statistically significant differences for all analysed BPs, being acetonitrile the best option for solvent, which was selected for further experiments.

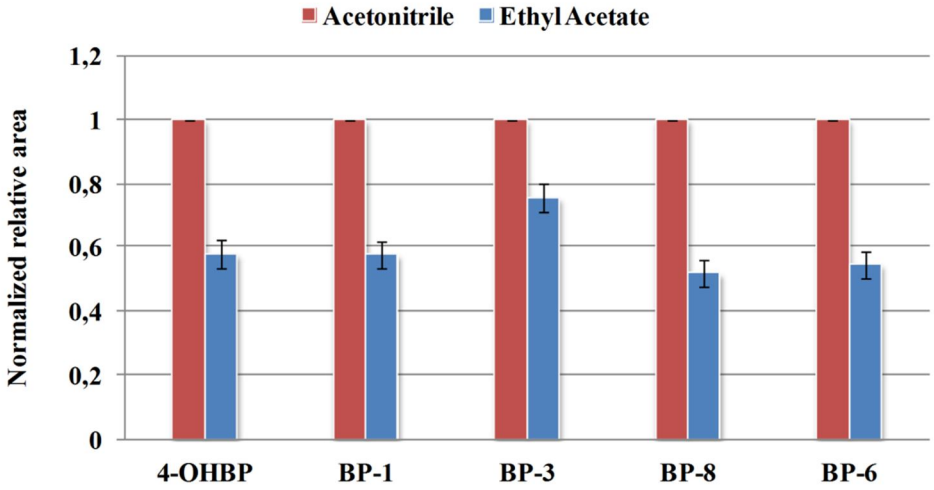

Fig. 1. (A) *Normalized relative areas for the selection between ACN and ethyl acetate as extraction solvent. BP-1 (LSD = 1.47); BP-3 (LSD = 1.17); 4-OH-BP (LSD = 0.11); BP-6 (LSD = 0.12); BP-8 (LSD = 0.23). Significance level of 95% was selected for all cases.*

3.1.2. Optimization of USE conditions

In order to optimize of the variables involved in the USE extraction procedure and to study possible interactions between them, a 15-run Box–Behnken design with three replicates of the central point was used for fitting a second-order response surface. Three factors and three levels for each one were checked: extraction time (1, 8 and 15 min), number of extraction cycles (1, 2 and 3) and volume of solvent per cycle (10, 15 and 20 mL). Amplitude was set at the maximum recommended value of 70% in all the experiments. The data were evaluated by ANOVA and the test gave determination coefficients (R^2) between 0.833 and 0.897. Since the P-values for the *lack-of-fit* test were >5% in all cases, the model appears to be satisfactory with the 95% of confidence level. It was observed that the number of extraction cycles was the most influential parameter, unlike extraction time and volume, which were not significant. However, a significant negative interaction between the number of cycles and volume was observed. For this reason, 10 mL was selected as optimum for the volume of solvent, which was the lowest assayed volume.

The combination of the optimized experimental values obtained for each compound allowed the determination of the best overall extraction efficiency, which was calculated with the desirability function. Responses for each compound in the experiments of the Box-Behnken design were first normalized between 0 and 1, and the global desirability function was defined as their geometric mean. The plot of this function versus the number of cycles and the extraction time, with the extraction solvent volume set at 10 mL, is shown in Figure 2A. Although there were not significant differences in desirability along the evaluated extraction time interval at 3 cycles, it was selected 15 min in order to assure a more complete extraction of the analytes in samples. Consequently, the optimum values corresponded to 3 extraction cycles of 15 min each one, 10 mL of extraction solvent volume and 70% of amplitude.

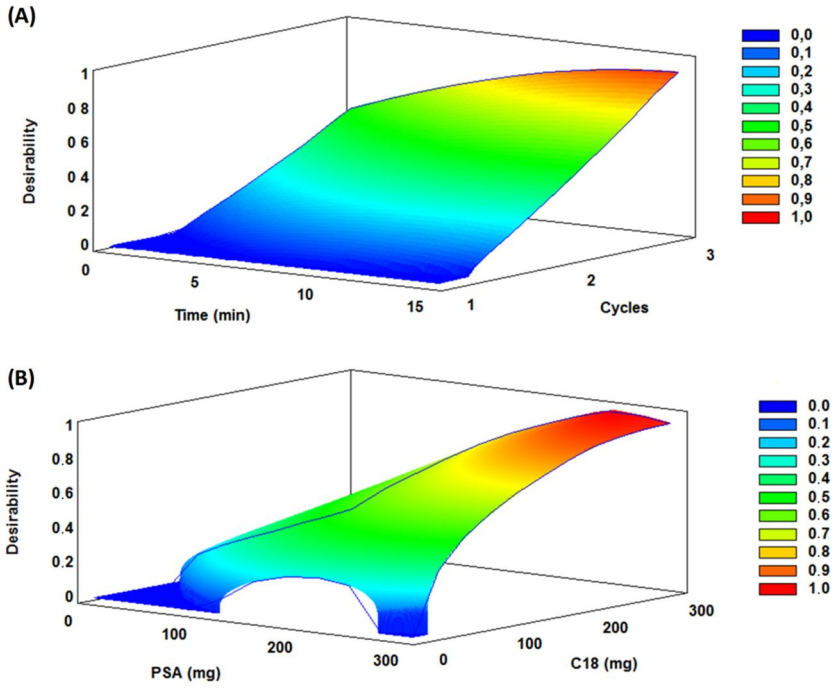

Fig. 2. *Representation of the global desirability functions.* ***(A)*** *extraction time and number of cycles for ultrasonic extraction, and* ***(B)*** *amount of PSA and C18 sorbents for the clean-up step for the determination of BPs.*

3.1.4. Optimization of the clean-up procedure

A clean-up step to obtained extracts from USE was necessary, almost mandatory, especially for removal of remaining lipids from samples. It was selected a clean-up procedure using different sorbents that are frequently used in dispersive solid phase extraction. Since they are commonly used in the removal of extracted interferences in biological matrices [24-28], a mixture of two solid sorbents was evaluated: PSA and C18. C18 is specifically used for removal of co-extracted fat and other lipophilic compounds from acetonitrile extracts [29, 30]. On the other hand, PSA could be also a good alternative for clean-up, since its bidentate structure is responsible for its high chelating effect. The composition of sorbent mixture for the clean-up step was also determined with a Box-Benhken design, being also included as a variable the amount of $MgSO_4$ that is applied during the clean-up phase as a desiccant, in order to eliminate traces of water which complicate sample evaporation and concentration [15].

Box-Behnken matrix also consisted of 15 experiments, including three central points. The variables studied (at 3 levels), were C18 (0, 150 and 300 mg), PSA (0, 150 and 300 mg) and $MgSO_4$ amounts (0, 250, 500 mg). Figure 2B shows the plot of the desirability function versus the amounts of PSA and C18 sorbents, with the amount of $MgSO_4$ set at 500 mg, corresponding to the optimum value according to the Box-Benhken design. The three variables resulted influential, $MgSO_4$ and C18 amounts showed a positive effect for all analytes, while PSA amount had a positive influence for BP-3, BP-6 and BP-8, but negative for 4-OH-BP and BP-1. Moreover, significant positive interactions among all variables were observed: PSA and C18, as well as $MgSO_4$ with each one of the sorbents. According to the observed positive influence of the variables, the optimum values were the maximum for C18 and $MgSO_4$, but slightly lower for PSA, due to the negative effect showed for some compounds. Consequently, a mixture of 250 mg of PSA, 300 mg of C18 and 500 mg of $MgSO_4$ was selected as optimum for the clean-up step.

3.2. Analytical performance

A calibration curve for each compound, at six concentration levels (six fold) was built, in the range from the limit of quantification (LOQ) to 50 µg mL^{-1}. The curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using SRM mode. Internal standard (BP-d_{10},) were added at a concentration of 50 ng mL^{-1}. Each calibration level was made in triplicate and analyzed twice. Table 2 shows the statistical and the analytical parameters obtained for each compound.

Table 2
Analytical and statistical parameters

	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BP-1	$1.334 \cdot 10^{-1}$	$1.191 \cdot 10^{-3}$	99.6	12.1	0.1	0.5	0.5 - 50
BP-3	$4.402 \cdot 10^{-2}$	$4.910 \cdot 10^{-4}$	99.5	6.5	0.2	0.6	0.6 - 50
BP-6	$6.021 \cdot 10^{-2}$	$5.417 \cdot 10^{-4}$	99.7	89.4	0.1	0.5	0.5 - 50
BP-8	$4.992 \cdot 10^{-2}$	$3.363 \cdot 10^{-4}$	99.8	91.5	0.1	0.4	0.4 - 50
4-OH-BP	$5.898 \cdot 10^{-1}$	$5.650 \cdot 10^{-3}$	99.6	7.3	0.1	0.3	0.3 - 25

b: slope; s_b: slope standard deviation; R^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification; LDR: linear dynamic range

The presence/absence of matrix effect was evaluated with two calibration curves for each compound, one in the initial mobile phase and the other in blank human milk. A Student's t-test was applied in order to compare the calibration curves, showing high statistical differences among slope values for the calibration curves in all cases and consequently, a significant matrix effect was observed. A possible explanation for this not correction of the matrix effects by the internal standard, could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are relatively variable. Therefore, although the compounds studied have a similar basic structure, and the use of this internal standard is accepted in scientific literature, they differ due to the presence of different substituents in the molecule. Then, it was decided to work with matrix-matched calibration in all cases.

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [38].

Linearity. A concentration range from the minimal quantified amount, limit of quantification (LOQ) (see Table 2) to 50 ng mL^{-1} was selected for BP-1, BP-3, BP-6 and BP-8, and to 25 ng mL^{-1} for 4-OH-BP. The determination coefficient (R^2) and the *lack-of-fit* test (P_{lof}) were evaluated. The values obtained for R^2 ranged from 99.5 to 99.8% and P_{lof} values were >5% in all cases [39]. This indicates a good linearity within the stated ranges (LOQ–50 ng mL^{-1}).

Sensitivity. The limits of detection (LODs) and quantification (LOQs) were calculated by taking into consideration the minimum concentration of analyte that the method can detect and with a signal-to-noise ratio of 3 for LODs and 10 for LOQs, using the quantification transition. Found LODs ranged from 0.3 for 0.6 ng mL^{-1}. The results are also summarized in Table 2.

Accuracy (precision and trueness). The precision of the method in terms of intra– and inter–day variability was evaluated using spiked human milk samples at three concentration levels, 1, 10 and 50 ng mL^{-1} for BP-1, BP-3, BP-6 and BP-8; and 1, 10 and 25 ng mL^{-1} for 4-OH-BP. Precision, expressed as relative standard deviation (%, RSD) was determined from triplicate spiked samples during the same day and in 6 different days. The values obtained are summarized in Table 3. RSD values fell between 2.0% and 12.3% being in lower than 15% for all compounds. Therefore, it was within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data indicate that the method is reproducible.

Table 3
Recovery assay, precision and trueness of target compounds in human milk

	Spiked (ng mL^{-1})	[a]Found ± CI (ng mL^{-1})	RSD (%)	Recovery (%)
	1.0	1.09 ± 0.05	8.4	108.7
BP-1	10	9.21 ± 0.2	4.0	92.1
	50	50.6 ± 0.9	3.5	101.1
	1.0	1.02 ± 0.03	5.1	102.1
BP-3	10	9.6 ± 0.2	5.0	95.9
	50	50.7 ± 0.9	3.4	101.3
	1.0	1.12 ± 0.05	9.4	109.5
BP-6	10	9.7 ± 0.4	7.6	97.1
	50	50.0 ± 0.9	3.8	100.1
	1.0	1.12 ± 0.07	12.3	108.6
BP-8	10	9.9 ± 0.1	2.0	98.6
	50	50.1 ± 0.9	3.5	100.3
	1.0	1.05 ± 0.03	5.0	104.9
4-OH-BP	10	9.1 ± 0.2	3.3	90.9
	25	25.3 ± 0.3	2.6	101.2

[a] *Mean of 18 determinations; CI: confidence interval (P=95% and 17 freedom degrees); RSD: relative standard deviation.*

Meanwhile, the trueness was evaluated by a recovery assay. The recovery of known amounts of tested compounds in milk samples was evaluated by comparing the spiked amount with the concentration of each compound determined by using the proposed method. In all cases, the recoveries were close to 100% in all cases (between 90.9% and 109.5%).

Selectivity. The specificity of the method was demonstrated by analyzing the chromatograms of the blank and the corresponding spiked human milk sample. No interferences from endogenous substances were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensured high selectivity of the UHPLC–MS/MS method. Figure 3A shows the SRM chromatograms obtained from the spiked blank sample (sample 5).

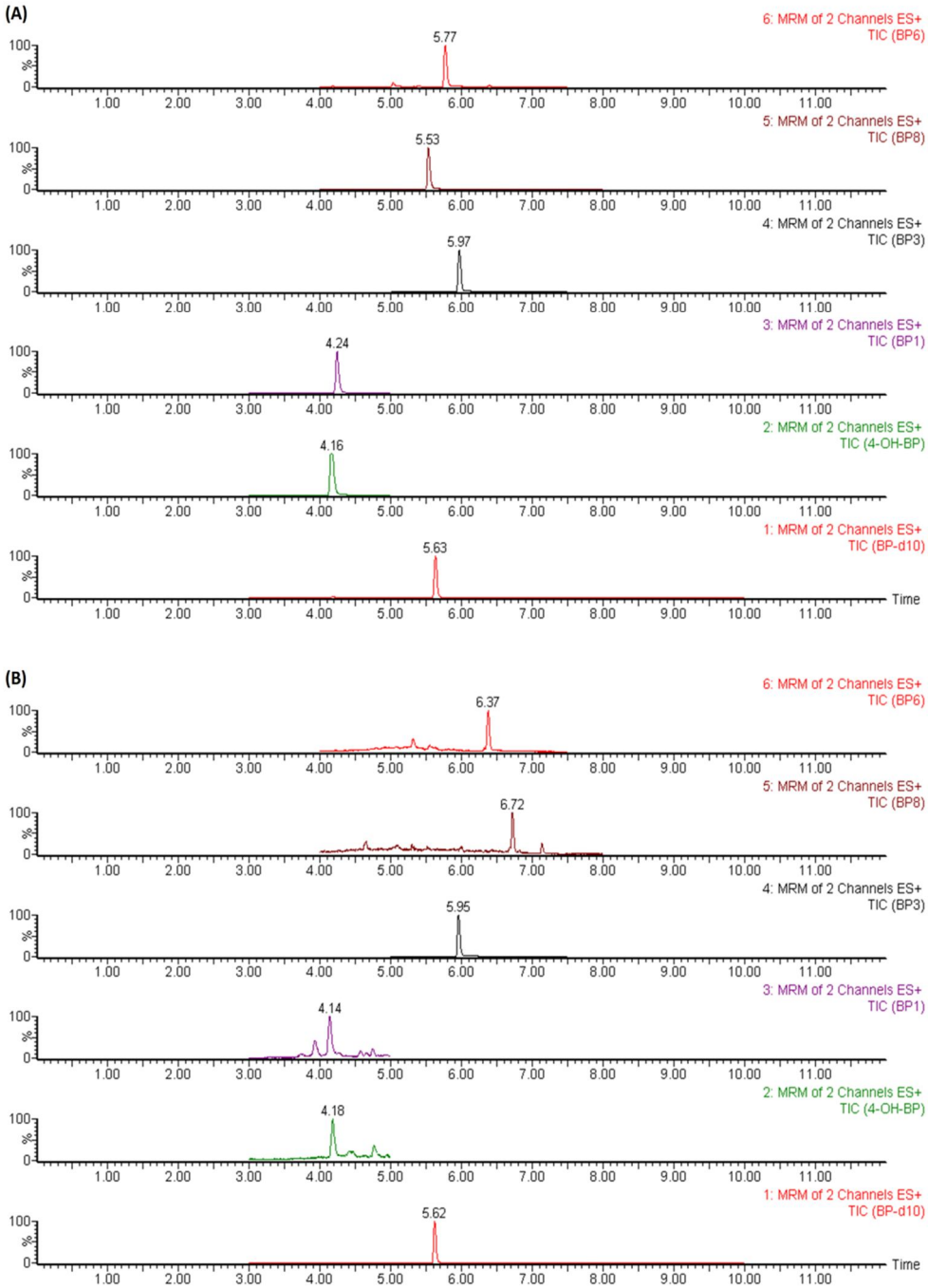

Fig. 3.*UHPLC–MS/MS chromatograms of:* ***(A)*** *a spiked blank sample;* ***(B)*** *a positive human milk sample with the target analytes, containing the corresponding internal standard (BP-d_{10}).*

3.4. Application of the method

The proposed method was applied to the determination of the content of the free forms of the selected BPs in human milk samples collected from 10 anonymous women with no known occupational exposure to these compounds. The results obtained as a mean of six determinations are summarized in Table 4.

Table 4
Application to human breast milk samples

Sample	[a,b]Found amount (ng mL^{-1})				
	BP-1	BP-3	BP-6	BP-8	4-OH-BP
01	D	5.8 (0.5)	D	ND	0.31 (0.02)
02	D	D	ND	D	ND
03	D	5.9 (0.4)	D	0.73 (0.02)	D
04	D	4.7 (0.2)	D	ND	ND
05	D	6.7 (0.2)	D	D	0.48 (0.05)
06	0.61(0.03)	8.5 (0.8)	ND	ND	1.92 (0.04)
07	D	4.7 (0.3)	ND	ND	ND
08	D	15.7 (0.5)	ND	D	3.9 (0.3)
09	ND	ND	ND	ND	ND
10	D	4.5 (0.1)	ND	ND	D

[a] *Mean of 6 determinations; ND: not detected (<LOD); D: detected (>LOD and <LOQ)*
[b] *Standard deviations are in parentheses*

Although the number of analyzed samples is not very high, it is possible to formulate some interesting conclusions. The results demonstrate the widespread occurrence of BP-3 in human samples. It was detected in nine of the analyzed samples, and quantified in eight of them at concentrations ranging from 4.5 to 15.7 ng mL^{-1} (mean: 7.1 ng mL^{-1}). The presence of 4-OH-BP was also important, since it was detected in the 60% of analysed samples, and quantified in the 40% of them, but in lower concentrations than BP-3 (0.31 to 1.92 ng mL^{-1}, mean: 1.6 ng mL^{-1}). It is important to remark that both compounds are the most used in commercial applications. BP-1 was detected in 9 of the samples but quantified in only one of them. This compound is described as one of the most important metabolites of BP-3. Finally, BP-6 and BP-8 were the least detected. Both were detected in four samples, but BP-8 was quantified in

one of them. Figure 3B shows the SRM chromatograms obtained from a human milk sample (sample 5).

4. Conclusions

There are very few analytical works published in the scientific literature for the determination of the selected compounds in human milk samples. Most of them have been focused on the determination of BP-3 using classical extraction techniques for compound isolation such as SPE (off- and on-line) or LLE. Other papers analyze BP-2, BP-3 and BP-4 and very few publications have been developed for multiresidue analysis.

In this context, the application of ultrasound-assisted extraction followed by a clean-up step using C18 and PSA sorbents is proposed for the identification and quantification of free forms of five BPs in human milk samples previously lyophilized. UHPLC–MS/MS is proposed as analytical technique. The isolation of analytes from samples was properly optimized by means of two Box-Behnken type experimental designs. The proposed sample preparation procedure was considered a good choice, because of its higher extraction yields and easy operation, especially when it is compared with the SPE technique that is traditionally used for human milk analysis, so that this alternative saves time and requires lower volumes of solvents than SPE, reducing costs and waste.

The procedure was accurately validated, being useful for the determination of trace levels of BPs in human milk, since limits of quantification ranged between 0.3 to 0.6 ng mL^{-1}, therefore it may be used to perform biomonitoring studies, since this matrix is a valuable biological fluid that may serve as a biomarker of both maternal and prenatal exposure to many different environmental chemicals, particularly to EDCs.

The method was satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women.

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CAPÍTULO V

Aplicación de la microextracción sólido-líquido-Líquido con membrana agitada

1. OBJETIVOS

Los objetivos de los trabajos realizados y presentados en este capítulo son:

- ✓ Proponer metodología analítica de buenas características para la determinación de parabenos mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS) en leche materna humana.

- ✓ Aplicar de una novedosa técnica de extracción solido-líquido-líquido simultánea con membrana agitada, para el aislamiento de los analitos a partir de la matriz inicial.

- ✓ Validar los métodos analíticos propuestos en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación aplicables.

- ✓ Aplicar la metodología desarrollada en el control y evaluación del contenido de los disruptores seleccionados en muestras reales de leche materna humana.

2. MICROEXTRACCIÓN SÓLIDO-LÍQUIDO-LÍQUIDO CON MEMBRANA AGITADA

En las últimas décadas, se ha extendido en el campo de la ciencia aplicada el uso de membranas en diferentes procesos de separación. Así, se han usado en desalinización, diálisis, ultrafiltración, separación de gases, ósmosis inversa, deshumidificación o electrodiálisis. Por tanto, es concebible que puedan utilizarse desde un punto de vista químico analítico para la separación y preconcentración en aplicaciones concretas.

Las membranas pueden ser selectivas a una determinada especie, pero principalmente funcionan como separador de fases, controlando la transferencia de masa entre las mismas, permitiendo la preconcentración de las especies de interés y la eliminación de la matriz de la muestra. El uso de membranas en el pretratamiento demuestra, en muchos casos, se ha convertido en una opción de elección dado que facilitan la extracción sin la mezcla de fases, permitiendo el contacto continuo entre muestra y extractante, evitando problemas como la formación de emulsiones. Existe una gran variedad de membranas comerciales que pueden clasificarse en función de su geometría, morfología, estructura, capacidad o composición. La extracción con membrana agitada (SME) fue desarrollada en 2009 por el grupo de investigación liderado el profesor Varcárcel en la Universidad de Córdoba[199].

Se diseñó una novedosa unidad de extracción que incorporaba tanto la agitación magnética como una membrana polimérica en el mismo dispositivo. La agitación incrementa la velocidad de extracción, permitiendo alcanzar eficiencias mayores a tiempos menores. La gran variedad de membranas poliméricas disponibles en el mercado hacen de la microextracción con membrana agitada una técnica muy versátil. Además, se puede acoplar tanto a

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técnicas cromatográficas como espectroscópicas. Recientemente, el grupo de investigación ha avanzado en la mejora de esta modalidad de extracción con vistas a incrementar la versatilidad de la misma desarrollándose la llamada microextracción líquida con membrana agitada (SM-LLME),que incluye las ventajas de la *Liquid-Phase Microextraction (*LPME) y la agitación en la misma unidad, permitiendo la extracción de los analitos de un modo simple y eficiente. La SM-LLME puede operar en dos formatos diferentes según el número de fases implicadas: dos o tres fases. La elección del formato se realiza teniendo en cuenta las características químicas de los analitos a determinar.

La pieza clave de esta metodología es la unidad de extracción (***Figura V.1***), que está constituida por 5 elementos básicos: (i) una barrita de hierro, (ii) un tapón comercial de politetrafluoroetileno (PTFE) para los cartuchos de SPE de 3 mL, (iii) la sección superior de uno de estos cartuchos, (iv) una membrana de PTFE, y (v) una sección de una punta de pipeta de 5 mL. El empleo de elementos comerciales en su diseño, garantiza la reproducibilidad en su construcción, aunque, por otro lado, la optimización de estos sistemas de extracción está limitada únicamente al volumen de la fase aceptora.

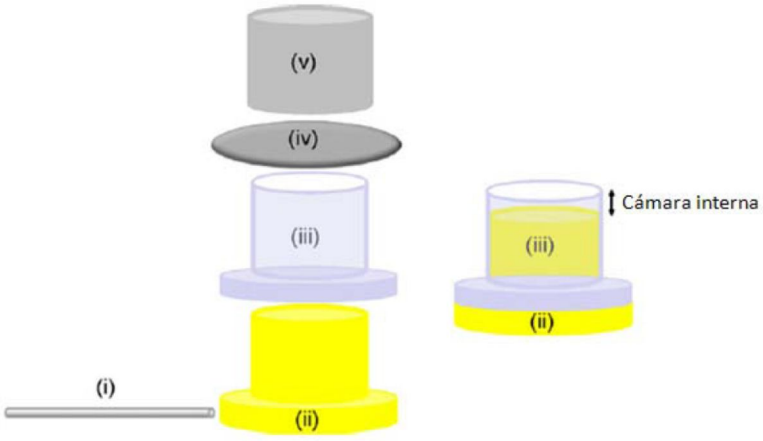

Figura V.I. *Elementos básicos del dispositivo original de extracción*

El proceso de ensamblaje es bastante sencillo, y puede realizarse en un par de minutos. En primer lugar, el elemento interno (iii) se sella a presión con el

tapón (ii), previamente perforado por la barrita de hierro (i) que es la que permite la agitación magnética de la unidad de extracción. El ensamblaje de los elementos (ii) y (iii) es crucial, ya que definen la cámara interna donde más tarde se situará el extractante. Una vez añadido éste, se deposita la membrana (iv) sobre la unidad, fijándola por desplazamiento del elemento externo (v), siendo el volumen interno de la cámara de 50 μL.

Durante el desarrollo del presente trabajo de investigación y con objeto de aumentar la reproducibilidad del sistema, se realizaron una serie de modificaciones en el dispositivo, que proporcionaron unos resultados excelentes. En la ***Figura V.2***, se muestra el dispositivo final empleado. La innovación principal consistió en sustituir la cámara que contiene a la muestra y la fase orgánica por un tubo Eppendorf de fondo curvo, permitiendo una mayor reproducibilidad en la geometría del dispositivo.

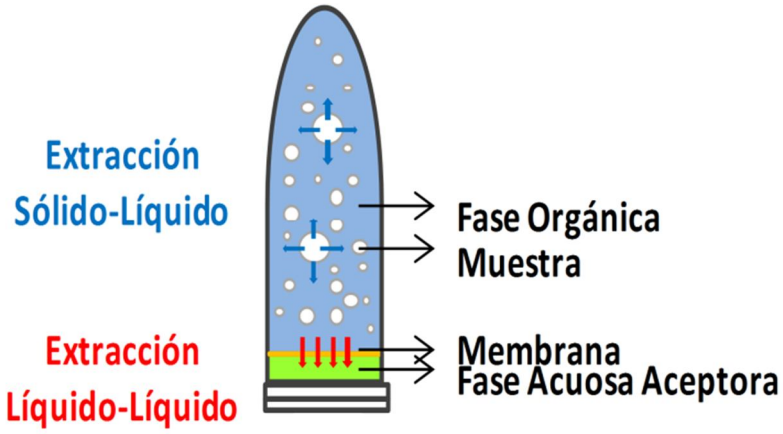

Figura V.II. *Elementos básicos del dispositivo modificado de extracción*

Como se ha indicado anteriormente la SM-LLME puede operar con dos o tres fases, de tal manera que los analitos migran desde la muestra a la fase aceptora confinada en el interior de la unidad, a través de la membrana líquida soportada. Esta fase aceptora puede ser un disolvente orgánico (el mismo que se ha usado para constituir la membrana líquida soportada), dando lugar a la

modalidad de dos fases; o bien una disolución acuosa ácida o básica, dando lugar a un sistema de extracción en tres fases.

En la investigación desarrollada en la presente Tesis Doctoral, aunque se ensayó la posibilidad de usar tres fases, no se obtuvieron buenos resultados y se optó por el trabajo en dos fases. El disolvente orgánico empleado como extractante juega un papel importante en el procedimiento de extracción líquido-líquido; debiendo cumplir una serie de requisitos. En primer lugar, este debe presentar una elevada afinidad por los analitos de interés, para poder extraerlos de la matriz de la muestra, debe ser químicamente compatible con la membrana empleada y debe presentar una baja solubilidad en agua para evitar su pérdida durante la extracción. Por último, debe tenerse en cuenta su compatibilidad con la técnica instrumental empleada para el análisis. También es necesario optimizar variables del proceso de extracción como la velocidad y el tiempo de agitación, el volumen de muestra y el de extractante.

Las técnicas que integran la extracción y la agitación han seguido una evolución continua en los últimos años[200]. Entre ellas, la extracción con membrana agitada ha sido especialmente empleada principalmente en aplicaciones medioambientales. Las características inherentes de los nuevos desarrollos, hacen de esta técnica un instrumento prometedor para el aislamiento y la preconcentración de compuestos químicos en muestras biológicas, incluyendo aquellas de disponibilidad limitada, se debe trabajar en el diseño de nuevos dispositivos de extracción construidos a partir de materiales inertes y en su potencial automatización.

[200] Lucena R. *Analytical and Bioanalytcal Chemistry*, 2012, 403:2213–2223.

3. PUBLICACIÓN

3.1. Publicación VI

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Stir-membrane solid-liquid-liquid microextraction for the determination of parabens in human breast milk samples by ultra high performance liquid chromatography-tandem mass spectrometry

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Abstract

In this article, stir-membrane solid-liquid-liquid microextraction (SM-SLLME) is tailored for the analysis of solid matrices and it has been evaluated for the determination of parabens in human breast milk samples. A three-phase microextraction mode was used for the extraction of the target compounds taking advantage of their acid-base properties. The unit allows the simultaneous extraction of the target compounds from the solid sample to an organic media and the subsequent transference of the analytes to an aqueous acceptor phase. The method includes the identification and quantification of the analytes by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). All the variables involved in the extraction procedure have been accurately studied and optimized. The analytes were detected and quantified using a triple quadrupole mass spectrometer (QqQ). The selection of two specific fragmentation transitions for each compound allowed simultaneous quantification and identification. The method has been analytically characterized on the basis of its linearity, sensitivity and

precision. Limits of detection ranged from 0.1 to 0.2 ng $mL^{-1}$with precision better than 8%, (expressed as relative standard deviation). Relative recoveries were in the range from 91 to 106% which demonstrated the applicability of the stir-membrane solid-liquid-liquid microextraction for the proposed analytical problem. Moreover, the method has been satisfactorily applied for the determination of parabens in human breast milk samples from 10 randomly selected individuals.

Keywords: Stir-membrane solid-liquid-liquid microextraction; Human milk samples; Paraben; UHPLC-MS/MS.

Highlights:

- ✓ Stir-membrane device is adapted to solid-liquid-liquid extraction
- ✓ This combination is used to the isolation of parabens from lyophilized human milk
- ✓ The extracted analytes are finally determined by UHPLC-MS/MS
- ✓ Limits of detection were in the low ng mL^{-1} range with precision better than 8%
- ✓ Recoveries varied between 91 and 106% indicating the applicability of the proposal

1. Introduction

The alkyl esters of *p*-hydroxybenzoic acid (parabens, PBs) are a group of compounds widely used as bactericide and antimicrobial preservatives, especially against mold and yeast in cosmetic products, pharmaceuticals, and in food and beverage processing [1]. The biological activity of PBs is based on their inhibitory effects on membrane transport and mitochondrial function processes. These compounds are present, individually or in combination, in a large amount of commercial formulations. Although PBs have been considered for years to be relatively safe compounds with a low bioaccumulation potential [2], some studies suggest that they present a moderate endocrine disrupting activity and therefore they can cause adverse effects on humans and wildlife. In

fact, the ability of PBs to disrupt physiologically important functions in both *in vitro* systems [3] and *in vivo* models [4-6] has been demonstrated. As well, the presence of non-metabolized PBs in breast cancer tissues [7] has focused the attention in their potential carcinogenic and toxic nature [2, 6, 8].

The human exposure to PBs may occur through ingestion, inhalation or dermal absorption. This exposure, estimated in 76 mg per day, involves different sources such as cosmetics and personal care products (50 mg/day), drugs (25 mg/day) or food (1 mg/day) [1]. After intake, PBs are metabolized by hydrolysis of the ester bond and by glucuronidation [9]. However, the parent compounds (free forms) can still be detected in biological samples such as urine [10], serum and seminal plasma [11] and human milk [12].

Since breast milk is the main route of exposure for breastfed infants, its analysis is of special scientific interest. The isolation of the target analytes from this complex biological sample is a critical aspect even when a chromatographic technique is employed due to selectivity and sensitivity issues. Selectivity aspects, including ion suppression, are also critical when mass spectrometry is employed as detection technique. Current sample preparation of breast milk typically involves a previous acid treatment and a centrifugation step [13] to release the target analytes from the sample matrix. Classic liquid-liquid extraction [14] and solid phase extraction [12] with intense cleanup procedures have been proposed for the extraction of these compounds from such samples. These classic techniques, which are usually tedious and require high volume of sample and organic solvents, are gradually being replaced by microextraction techniques, which are characterized by their simplicity, green nature and efficiency. Microextraction techniques, especially solid phase microextraction, have been proposed for the isolation of pesticides [15], polychlorinated biphenyl congeners [16] and monocyclic aromatic amines [17] from human milk samples.

Stir membrane extraction (SME) [18, 19], which use a polymeric membrane as extracting phase, has been proposed as a novel technique that integrates

the extraction and stirring element in the same device. The main shortcoming of SME is the limited adsorption capacity of conventional membranes which can be enhanced changing the nature of the extracting phase. The use of liquid extracting phases has brought the development of the stir-membrane liquid-liquid microextraction (SM-SLLME), both in the two-phase [20] and three-phase modes [21, 22]. However, the design of the unit does not allow the processing of sample volumes lower than 10 mL which is an obvious restriction in bioanalysis. The adaptation of stir-membrane liquid phase microextraction (SM-LPME) for the extraction of volume limited biological samples, such as saliva, has also been recently published [23].

In the present work, SM-SLLME is tailored to the analysis of solid samples and it has been evaluated for the determination of PBs in human milk samples. The unit allows the extraction of the target compounds from the solid sample to an organic media and the subsequent transference of the analytes to an aqueous acceptor phase which is compatible with ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The new proposal gives a selectivity and sensitivity enhancement which is critical when biological samples are studied, even if a UHPLC–MS/MS system is employed. In addition, the extraction technique seems to be versatile enough to face up the isolation and preconcentration of hydrophobic ionizable compounds from different solid matrices. The method was validated and satisfactorily applied to determine the free PBs content in samples collected from 10 volunteers.

2. Experimental

2.1. Chemicals and reagents

All reagents were of analytical grade or better. Methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), butylparaben (BPB) and ethylparaben $^{13}C_6$-ring labelled (EPB-$^{13}C_6$) were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions of each compound were prepared in

acetonitrile (Panreac, Barcelona, Spain) at a concentration of 100 mg L^{-1} and stored at 4 ºC in the dark. Working solutions were prepared by a rigorous dilution of the stocks in human milk samples prior to their lyophilization. Potassium hydroxide, hydrochloric acid, acetonitrile, hexane and dichloromethane, employed in the sample treatment, were purchased from Panreac (Barcelona, Spain). LC–MS grade acetonitrile and water used as components of the chromatographic mobile phase were also purchased from Sigma-Aldrich. Polytetrafluoroethylene (PTFE) tape (75 µm, 0.5 µm of pore size) from Miarco (Valencia, Spain) and Eppendorf tube (2 mL in volume) were employed in the construction of the extraction device. Sample agitation for the extraction procedure was carried out in an eight-position digital agitator-vibrator purchased from J.P. Selecta (Barcelona, Spain).

2.2. Chromatographic analysis

Two chromatographic systems, including different detectors, were employed in the development of the present research. The optimization of the extraction procedure was carried out on a Waters-Acquity™ Ultra Performance LC system (Waters, Manchester, UK) using an Acquity UPLC® BEH C_{18} column (1.7 µm particle size, 2.1 mm × 100 mm) maintained at 45 ºC. The mobile phase consisted of 60 % of Milli-Q ultrapure (Millipore Corp, Madrid, Spain) water and 40 % acetonitrile under isocratic conditions. The flow rate was maintained at 0.5 mL min^{-1}. The injection volume was 1 µL with partial loop with needle overfill mode. The separated analytes were detected using a PDA eλ (extended wavelength) Detector (Waters) at 254 nm. System control was achieved with Empower software.

The extraction performance is calculated in relative terms (extraction recovery and enrichment factors) and the obtained values are independent of the instrument employed. For this reason, UHPLC–MS/MS was finally used as instrumental technique in order to improve the sensitivity and selectivity of the UV detection. In this sense, method validation and sample analysis were performed on a Waters Acquity UPLC™ H-Class (Waters, Manchester, UK),

consisting of an ACQUITY UPLC™ binary solvent manager and an ACQUITY UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z–spray™ electrospray ionization (ESI) source was used for PBs detection. An Acquity UPLC® BEH C_{18} column (1.7 μm particle size, 2.1 mm × 100 mm) was used. The compounds were separated using a gradient mobile phase consisting of LC–MS grade water (solvent A) and LC–MS grade acetonitrile (solvent B). Gradient conditions were: 0.0-2.0 min, 40% B; 2.0-5.0 min, 40-90% B; 5.0-5.1 min, 90-100% B; 5.1-8.0 min, 100% B and back to 40% in 0.1 min. Flow rate was 0.5 mL min^{-1}. Total run time was 10.0 min. The injection volume was 10 μL and the column temperature was maintained at 40 ºC.

For mass spectrometric analysis, the tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. ESI was performed in the negative ion mode. The mass spectrometric conditions were optimized for each compound by continuous infusion of standard solutions (1 mg L^{-1}). The ion source temperature was maintained at 150 ºC. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1}, and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell time was set at 25 ms. Optimized parameters for each compound are also listed together with the mass transitions in Table 1.

Table 1. *Transitions and optimized potentials for UHPLC–MS/MS analysis*

Compound	Transitions	CV	CE
MPB	151.1→91.8[a]	-38	-22
	151.1→135.8[b]	-38	-14
EPB	165.1→91.9[a]	-38	-24
	165.1→136.6[b]	-38	-16
PPB	179.1→91.8[a]	-42	-24
	179.1→136.1[b]	-42	-16
BPB	193.1 →91.4[a]	-42	-24
	193.1 →136.1[b]	-42	-16
EPB-$^{13}C_6$	171.1→98.0[a]	-36	-24
	171.1→142.7[b]	-36	-14

[a] *SRM transition used for quantification;* [b] *SRM transition used for confirmation*
CV, Cone voltage (V); CE, Collision energy (eV)

2.3. Samples collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. It is important to point out that none of them follow a medical treatment. Samples were collected using a breast pump in a 100 mL PTFE flask and immediately cold storage at -20 ºC. All volunteers signed their informed consent to participate in the study.

2.4. Preparation of spiked samples

The blank samples were previously analyzed in order to ensure the absence of the analytes or that these were below the limits of detection of the method (LODs). Due to the absence of certified reference materials, for recovery studies, blank samples were spiked at different concentrations by adding 100 µL of spiking standard solutions to 3 mL of human breast milk. The mixtures were vortexed for 2 min and then, left to stand for 24 h at 4 ºC in the dark before analysis. This allows the analytes to interact with the human milk sample. Then, aliquots of 3 mL of spiked samples were placed in an 8 mL glass vial and frozen at -80 ºC for 12 h prior being lyophilized.

2.5. Extraction unit

The design of the extraction device is the consequence of a deep research in stir membrane extraction and related techniques during the last years. The unit is based on our previous adaptation of stir-membrane liquid phase microextraction to the analysis of volume limited biological samples [23]. In this case, the unit has been simplified and only two commercial elements are needed for its construction: an Eppendorf tube of 2 mL and a PTFE tape (75 µm in thickness, 0.5 µm of pore size).

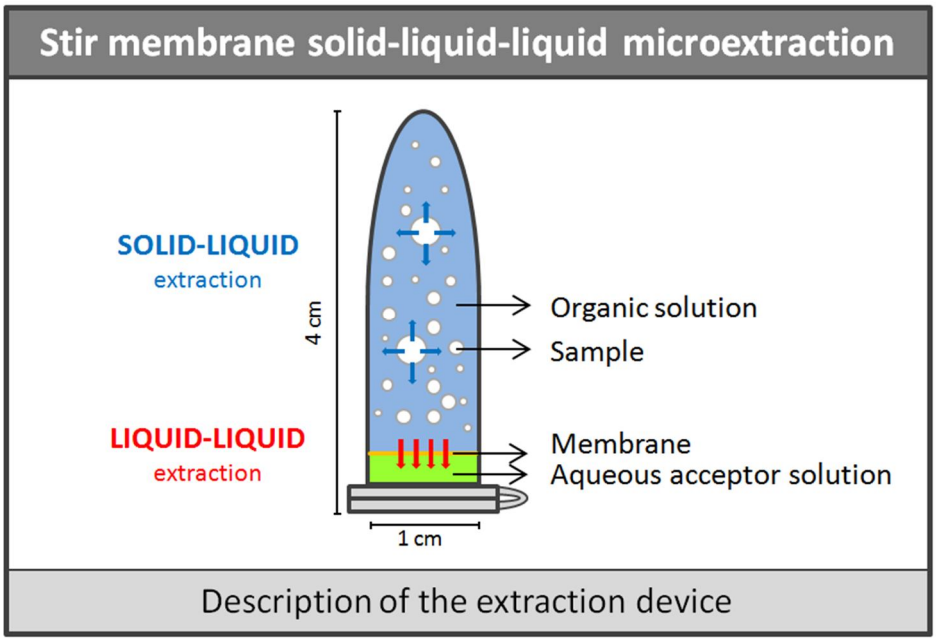

Figure 1. *Description of the extraction device*

The assembly process generates two different chambers (lower and upper) separated by a polymeric membrane which avoids direct contact and mixing. The upper section is filled with an appropriate organic solvent where the solid sample is dispersed while the lower chamber (ca. 100 µL) is filled with the acceptor alkaline phase. The extraction device can be simply agitated in an eight-position digital agitator-vibrator allowing the solid-liquid-liquid extraction of the analytes. In addition, the sample throughput is enhanced since various

units can be built in a reproducible way, thus allowing that eight samples can be extracted at the same time. Moreover, each unit can be re-used several times just replacing the membrane and cleaning the Eppendorf tube with Milli-Q water and methanol.

2.6. Extraction procedure

First, the cap of the Eppendorf unit is filled with 100 µL of a 10^{-2} M potassium hydroxide solution, which acts as acceptor solution. Then, the polymeric membrane is conveniently placed over the cap. Later on, 0.1 g of freeze-dried human milk containing the analytes (corresponding to 1 mL of milk) and 1.5 mL of a mixture of hexane:dichloromethane 80:20 (v/v), were added inside the Eppendorf tube. Once ready, the extraction units are placed on the eight-position digital agitator-vibrator and stirred for 45 min at room temperature (25 ºC). After this time, the sample and the solvent are withdrawn from the unit and the acceptor phase is collected using a 100 µL microsyringe and transferred to a chromatographic vial. Finally, 40 µL of a mixture of hydrochloric acid:acetonitrile 1:1 (v/v) are added and 10 µL of the final solution are injected into the chromatographic system.

3. Results and discussion

Different variables, which are summarized in Table 2, may affect the efficiency of the extraction procedure and therefore their effects on the analyte extraction were considered in the optimization of the extraction procedure. Table 2 also reflects their initial values, the studied interval and the final optimum values. The optimization was performed under a univariate approach although two couples of variables were considered jointly since they are directly related. Lyophilized human milk containing the four PBs studied, at a concentration of 100 µg L^{-1}, was used in these studies.

Table 2. *List of the variables involved in the SM-SLLME extraction process*

Variable	Initial value	Optimization range	Optimum
Solvent (HEX:DCM)	0:100 (v/v)	0:100-100:0 (v/v)	80:20 (v/v)
Acceptor phase pH	9	9-12	12
Sample amount (g)	0.05	0.05-0.30	0.10
Organic solvent volume (mL)	0.5	0.5-2.0	1.5
Extraction time (min)	5	5-60	45

HEX: hexane; DCM: dichloromethane

3.1. Selection of the extraction solvent and the final acceptor phase

The proposed extraction procedure allows the solid-liquid microextraction of PBs from lyophilized samples and the subsequent liquid-liquid microextraction from the organic solvent to an aqueous phase compatible with UHPLC–MS/MS. Therefore, the appropriate selection of the organic solvent is a critical step as it plays a double role. On the one hand, the organic solvent should be able to extract the analytes from the freeze-dried breast milk and the analytes should have a higher solubility in the final aqueous solution.

As target compounds present a marked interaction with the fatty compounds of the sample, we think in the possibility of using hexane as solvent. Nevertheless, the extraction with pure hexane did not provide good results. In fact, the signals obtained for MPB and EPB were too low while no peaks were obtained for the other compounds. In order to enhance the extraction of the compounds, dichloromethane was added as modifier. The hexane:dichloromethane volume ratio was evaluated in the range from 100:0 to 0:100 (v/v). The results showed that the efficiency of the extraction for all analytes increase up to the 80:20 ratio and an intensive decrease being observed for higher values. The use of the 80:20 ratio produces a 3-fold increase of the signals of MPB and EPB compared to that obtained with pure hexane and it also allows the extraction of PPB and BPB. In addition, this ratio gives an average signal improvement of 20% compared with that obtained with pure dichloromethane.

This behavior can be ascribed to two different aspects. On the one hand, the analytes present a high solubility in dichloromethane but in real samples the analytes are interacting with the fatty compounds of the sample matrix which are soluble in hexane. A mixture of both solvents provides a balanced media to break the analyte-matrix interaction and to solubilize the analytes. Moreover, the optimum ratio of solvents also influences the final liquid-liquid partition since the solubility of the target in the aqueous phase should be higher than that in the organic donor phase.

Considering the acid–base properties of PBs, with pKa in the range of 8.1-8.5, their water solubility can be modulated by an effective pH adjustment. This fact has been exploited to recover the target compounds from the organic solvents mixture. The pH of the acceptor phase was evaluated in the range from 9 to 12 by using different potassium hydroxide concentrations. In the pH range studied the results showed an analytical signal increase with the pH. According to the results, pH 12 was selected as the optimum value since it provides a 2,9-fold (average value for all the analytes) signals improvement compared to that obtained at pH 9. This fact can be ascribed to the complete ionization of the target compounds at these pH values which increases their solubility in the aqueous medium.

3.2. Optimization of sample amount and organic solvent volume

Sample amount and solvent volume are crucial variables in this extraction procedure. Moreover, both variables are related to each other and when higher sample amounts are processed, higher volumes of solvent are required to effectively leach the analytes. Sample amount and organic solvent volumes were optimized together considering four different sample amounts (0.05, 0.10, 0.20 and 0.30 g) and four different solvent volumes (0.5, 1.0, 1.5, and 2.0 mL). The maximum value of both variables was selected according to practical considerations. Amounts higher than 0.30 g produce the clogging of the membrane while extractant volumes larger than 2.0 mL cannot be used due to the limited device dimensions.

Figure 2 shows the bivariant effect on the extraction factors (EFs) for all analytes by means of a contour surface graph. In the light of the results, when an amount of 0.05 g was employed, the EFs were very low regardless the organic solvent volume tested. When 0.10 g of sample was extracted, the EFs increased with the organic solvent volume up to 1.5 mL, decreasing for higher volumes. This behaviour was observed for all analytes except for MPB, the most polar analyte, which was preferably extracted with sample amount of 0.20 g and 1.0 mL.

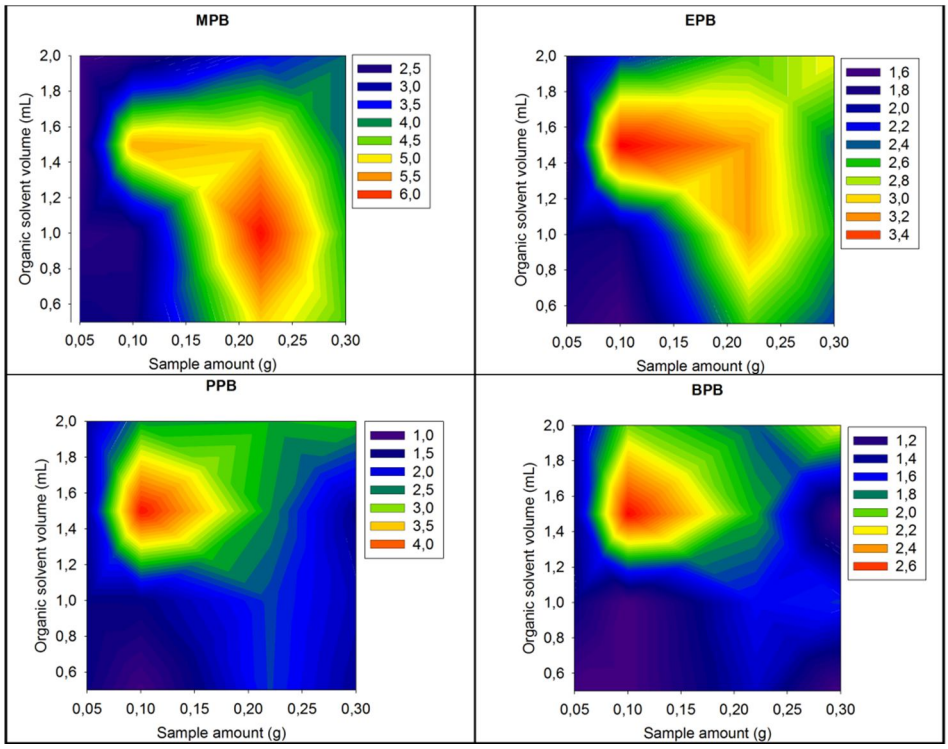

Figure 2. *Bivariant effect of sample amount and organic phase volume on the enrichment factors of the analytes.*

It might be expected that when 0.3 g of sample was extracted, the EFs increase. However, the experimental results showed a notable decrease on the extraction efficiency. This could be ascribed to the low organic solvent volume which is insufficient to leach the analytes from the sample. According to the obtained results, a sample amount of 0.10 g and a volume of 1.5 mL of organic

solvent were selected as the optimal values to carry out the extraction procedure.

3.3. Optimization of the extraction time

Extraction time was investigated in the range from 5 to 60 min and the results, which are depicted in Figure 3, showed two different behaviors depending on the polarity of the analyte. The extraction of the most polar analytes (MPB and EPB) increases markedly and almost linearly with the time up to 45 min, while from 45 to 60 min the increase was less pronounced. On the other hand, the extraction of the most hydrophobic analytes slightly increases up to 15 min and the extraction equilibrium is achieved, remaining almost constant for higher times. Thus, 45 min was selected as the optimum value as a compromise between the sample throughput and sensitivity.

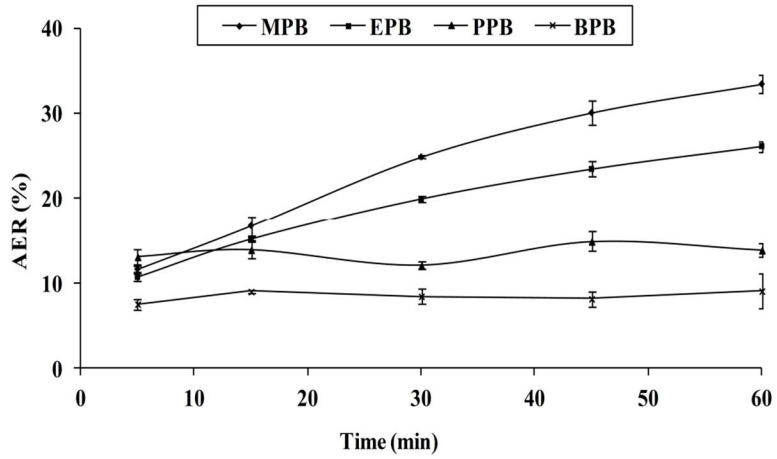

Figure 3. *Effect of the extraction time on the absolute recovery of the analytes.*

3.4. Analytical characterization of the method

3.4.1. Calibration curves

A seven-concentration calibration curve for UHPLC–MS/MS was built for each compound. Each level of concentration was made in triplicate. Calibration graphs were constructed using analyte/surrogate peak area ratio versus concentration of analyte. Calibration graphs were made using SRM mode. EPB-$^{13}C_6$ (100 ng mL^{-1}, final concentration in milk samples) was used as surrogate. Table 3 shows the analytical parameters obtained.

Table 3. *Analytical and statistical parameters*

Matrix Matched Calibration (human breast milk)							
Compound	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	% P_{Lof}	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
MPB	$1.367 \cdot 10^{-2}$	$1.292 \cdot 10^{-4}$	99.8	19.0	0.2	0.5	0.5-100
EPB	$5.281 \cdot 10^{-3}$	$5.825 \cdot 10^{-5}$	99.7	33.9	0.1	0.5	0.5-100
PPB	$3.080 \cdot 10^{-3}$	$2.980 \cdot 10^{-5}$	99.7	91.6	0.1	0.4	0.4-100
BPB	$1.020 \cdot 10^{-3}$	$1.068 \cdot 10^{-5}$	99.7	28.0	0.2	0.5	0.5-100
Calibration in solvent							
Compound	b (mL ng^{-1})	s_b (mL ng^{-1})	% R^2	t_{calc}	Slopes	Matrix effect corrected by the surrogate?	
MPB	$1.479 \cdot 10^{-2}$	$1.709 \cdot 10^{-4}$	99.9	1.944	Equal	Yes	
EPB	$5.035 \cdot 10^{-3}$	$4.588 \cdot 10^{-5}$	99.6	0.961	Equal	Yes	
PPB	$3.398 \cdot 10^{-3}$	$3.746 \cdot 10^{-5}$	99.8	2.399	Different	No	
BPB	$1.320 \cdot 10^{-3}$	$9.005 \cdot 10^{-6}$	99.5	6.385	Different	No	

b, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; t_{tab} (α=0.05 and 38 f.d) = 2.03

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in the initial mobile phase and the other in blank human milk. The Student's *t*-test was applied in order to compare the calibration curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a Snedecor's *F*-test. As is shown in Table 3, the

Student's *t*-test showed statistical differences among slope values for the calibration curves of PPB and BPB and consequently, a significant matrix effect was observed being of a great magnitude in the case of BPB, and the use of matrix-matched calibration was necessary. A possible explanation for this subject could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are slightly differents within the same family of compounds. Therefore, although the compound selected as surrogate has a similar basic structure compared to the analytes, and the use of this compound as internal standard or as surrogate is accepted in scientific literature, it differ slightly due to the presence of different substituents in the molecule. Since it is impossible to have the corresponding isotopically labeled standard for each one of the studied analytes, it was decided to work with matrix-matched calibration in all cases.

3.4.2. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [24].

Linearity. A concentration range for the minimal quantified amount (LOQ) to 100 ng mL^{-1} PBs was established. Linearity of the calibration graphs was tested using the determination coefficients (% R^2) and the *P*-values (% P_{lof}) of the *lack-of-fit* test [25]. The values obtained for R^2 ranged from 99.7 for EPB, PPB and BPB and 99.8% for MPB, and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges.

Limits of detection (LODs) and quantification (LOQs). Those are two fundamental parameters that need to be examined in the validation of any analytical method to determine if an analyte is present in a sample. In the present work, these parameters were calculated by taking into consideration the standard deviation of residuals, $S_{y/x}$, the slope, b, of the calibration graphs and an estimate s_0 obtained by extrapolation of the standard deviation of the

blank [26]. The LOD was $3 \cdot s_0$ and the LOQ was $10 \cdot s_0$. Limits of quantification ranged from 0.4 to 0.5 ng mL^{-1}. These values, which are referred to the original breast milk samples before their lyophilization, are also summarized in Table 3.

Accuracy (precision and trueness). Due to the absence of certified materials, in order to evaluate the trueness and the reproducibility of the method, a study with spiked human milk samples, at three concentrations levels for each compound was performed on 6 consecutive days. Spiked samples were analyzed in triplicate each day in repeatability conditions. A total of 18 measurements for each level were carried out. The concentrations studied were 1, 50 and 100 ng mL^{-1}. The precision was expressed as relative standard deviation (RSD) and the trueness was evaluated by a recovery assay. The precision and the trueness of the proposed analytical method are shown in Table 4.

Table 4. *Recovery assay, precision and trueness of the method*

Compound	Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	RSD (%)	Recovery (%)
MPB	1.0	0.91	7.7	91
	50.0	51.3	3.1	103
	100.0	96.4	1.1	96
EPB	1.0	0.93	8.0	93
	50.0	53.0	5.0	106
	100.0	98.1	3.6	98
PPB	1.0	0.95	7.5	95
	50.0	49.2	3.9	98
	100.0	103.0	4.1	103
BPB	1.0	0.96	6.0	96
	50.0	51.8	1.8	104
	100.0	102.8	3.1	103

[a] *Mean of 18 determinations; RSD, relative standard deviation*

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in human breast milk samples. Recoveries were determined analyzing the spiked samples and the concentration of each compound was determined by interpolation in the standard calibration curve within the linear dynamic range and compared with the amount of analytes

previously added to the samples. The results, which are also summarized in Table 4, were between 91 and 106%. Therefore, it can be concluded that the extraction process fulfills the 70-130% recovery criterion [27]. The results show the potential of the proposed stir membrane solid-liquid-liquid microextraction for the extraction of the four PBs from lyophilized human milk samples.

Inter-day precision (expressed as RSD) was lower than 8%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate more than 20%. The data obtained demonstrated that the proposed method is highly reproducible. Precision and trueness data indicate the potential of the proposed stir-membrane solid-liquid-liquid microextraction for the extraction of the PBs from human milk samples.

3.5. Application of the method

The validated method was applied to the determination of the amounts of EDCs in 10 human breast milk samples. The results obtained as mean of six determinations are summarized in Table 5. Figure 4 shows a chromatogram of a contaminated natural sample.

Table 5. *Application to human breast milk samples*

[a]Found amount (%RSD)				
	MPB	EPB	PPB	BPB
01	11.3 (6.8)	13.2 (3.1)	37.0 (6.4)	11.3 (4.7)
02	6.0 (5.5)	D	5.9 (6.8)	2.2 (4.5)
03	ND	ND	D	D
04	0.9 (4.4)	0.7 (5.1)	D	ND
05	1.5 (5.1)	0.5 (4.9)	5.9 (4.8)	3.2 (4.7)
06	1.1 (6.9)	ND	0.5 (6.0)	0.6 (4.7)
07	3.3 (4.9)	8.6 (4.8)	27.2 (2.2)	6.5 (5.7)
08	1.1 (6.8)	D	2.6 (6.1)	1.7 (3.1)
09	ND	ND	ND	ND
10	1.8 (5.4)	ND	0.6 (5.1)	1.5 (5.3)

[a] *Mean of 6 determinations (ng mL^{-1}); ND, not detected (<LOD); D, detected (>LOD and <LOQ); %RSD, Relative standard deviation (%)*

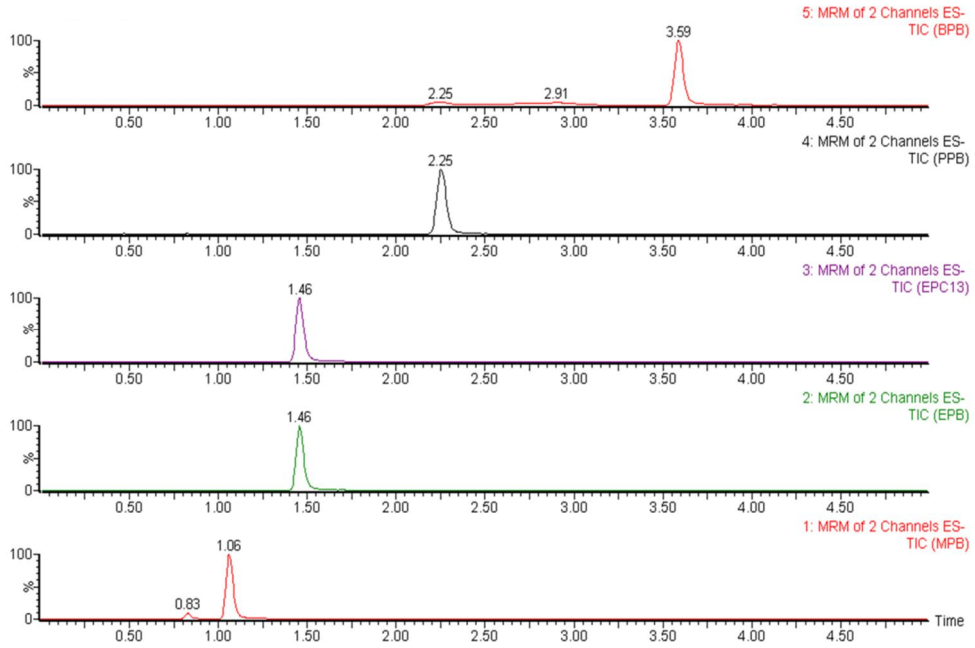

Figure 4.*Chromatogram of a contaminated human milk sample (Sample 01).*

As it is shown in the table, PBs were detected and quantified in many of the analyzed samples. MPB was quantified in 80% of samples (8/10) at concentrations ranging from 0.9 to 11.3 ng mL^{-1}. EPB was detected in 6 samples and quantified in 4 of them, with concentrations ranging from 0.5 to 13.2 ng mL^{-1}. Among the four PBs analyzed, PPB was the major compound found in most milk samples (9/10), with the higher concentrations (from 0.5 to 37.0 ng mL^{-1}). This indicates concurrent exposure to this compound. Regarding to BPB, this compound was also frequently detected (8/10) although the concentrations (0.6–11.3 ng mL^{-1}) were lower than those of PPB. It is important to remark that the concentrations of total PBs in two of the samples (mothers 01 and 07) were significantly higher than those in the other samples. This fact suggests higher exposures of those mothers to PBs, which can be attributed to the abusive usage of products such as personal hygiene products and cosmetics containing these compounds, recognized by two of the mothers in the initial survey.

4. Conclusions

In the present work, a new extraction procedure evolved from stir membrane extraction technique is proposed for the isolation and preconcentration of four PBs from human breast milk before their final chromatographic analysis. The extraction procedure allows the simultaneous solid-liquid extraction of the PBs from the lyophilized samples and the subsequent liquid-liquid extraction from the organic extract to an aqueous phase.

The integration of both extractions allows not only the improvement of basic analytical properties but also the simplification and miniaturization of the sample treatment. In addition, the sample throughput is enhanced since several samples can be extracted at the same time. The new proposal, which potential has been proved, is highly versatile and it can be easily adapted to face up different analytical problems.

The use of tandem MS, allows the unequivocal identification and quantification of these compounds in samples. The main parameters affecting the SM-SLLME procedure (pH and extraction time), have been completely characterized studying in depth the influence of these variables on the analytical signal. The nature of the organic solvent employed for the phase extractants; and the optimal instrumental conditions for UHPLC–MS/MS analysis were also evaluated and optimized. As a result, a selective, sensitive and accurate method for determination of four PBs in human breast milk samples has been developed and validated. The method was satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women.

The comparison of the proposed method with other counterparts [12, 28-34] for the determination of parabens in biological fluids is presented in Table 6. Compared with those method focused on human milk analysis [12, 32] the new proposal provides the better sensitivity, the narrower interval of relative

recovery values and a good precision. However, our method uses a higher sample volume and requires a larger extraction time.

The comparison with those methods focused on the analysis of urine [28-30, 33, 34] shows a similar sensitivity and precision (reference 30 excepted), but some of the counterparts require larger extraction times. Finally, our proposal provides better LODs that those obtained for the analysis of plasma samples [31].

Table 6. *Comparison of the SM-SLLME method with other counterparts for the determination of parabens in biological samples*

Extraction technique[a]	Matrix	Sample amount	Extractant[b]	Extraction time (min)
On-line SPE	Human milk	0.1 mL	LiChrosphere RP-18	15
SPE	Urine	1 mL	Strata X	>120
SPE	Urine	3 mL	Bond Elut Certify LRC	---
SPE	Urine Serum Seminal plasma	0.5 mL	Strata XL (200 mg)	---
SPE	Plasma	0.5 g	Oasis HLB (540 mg)	---
MISPE	Human milk	0.2 mL	MIP	---
LLE	Urine	0.5 mL	EA (9 mL)	>70
DLLME	Urine	5 mL	TCM	>20
SM-SLLME	Human milk	1 mL	HEX:DCM (4:1) / aqueous phase pH 12	45

Instrumental technique[c]	LOD[d] (ng mL^{-1})	Relative recovery (%)	Precision (%)	Reference
HPLC-MS/MS	<1	84-119	3.5-5.6	[12]
LC-MS/MS	0.08-0.3	97.4-100.5	4.7-6.8	[28]
UPLC- MS/MS	0.09-0.3	89.3-101.5	2.6-16.0	[29]
LC-MS/MS	0.07-0.4 0.02-0.36 0.03-0.41	82.8-115.9 75.6-135.6 78.0-120.4	4.2-18.3 8.0-29.2 2.8-26.9	[30]
UHPLC-TOF	2-7 (MDLs[f])	45-67	2.0-11.9	[31]
FTIRLC-UV	10-20 (LOQ[e])	86-117	3.0-12	[32]
LC-MS/MS	0.5-1 (LOQ[e])	67-126	5.8-18.6	[33]
UHPLC-MS/MS	0.1	94-103	2.0-9.9	[30]
LC-MS/MS	0.1-0.2	91-106	1.1-7.7	This method

[a] *SPE, solid phase extraction; LLE, liquid liquid extraction; DLLME dispersive liquid liquid microextraction; MISPE, molecularly imprinted solid phase extraction; SM-SLLME, stir membrane solid liquid liquid microextraction.*

[b] *HLB, hydrophilic-lipophilic balance; MIP, molecularly imprinted polymer; EA: ethyl acetate; TCM, trichloromethane; HEX, hexane; DCM, dichloromethane.*

[c] *HPLC, high performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; UHPLC, ultra high performance liquid chromatography; UPLC, ultra performance liquid chromatography; TOF, time of flight; FTIR, fourier transform-infrared spectroscopy; UV, ultraviolet.*

[d] *LOD, limit of detection;* [e] *LOQ, limit of quantification;* [f] *MDL, method detection limit.*

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CAPÍTULO VI

Conclusiones

Como resultado de la investigación realizada en la presente Tesis Doctoral, se han obtenido las siguientes conclusiones:

I. Se han desarrollado y validado varios métodos analíticos para la determinación de diferentes disruptores endocrinos químicos (bisfenol A y sus derivados clorados, parabenos y benzofenonas) en muestras de leche materna humana.

II. Los métodos propuestos ofrecen mejoras importantes e innovaciones en relación a métodos ya existentes, ya que solo algunos estudios han considerado la determinación de estos compuestos en este tipo de matriz, y no existen estudios de determinación simultánea de varios compuestos de una misma familia y menos aun de distintas familias de estos compuestos simultáneamente en un rango tan amplio.

III. La validez de la metodología desarrollada se ha demostrado aplicándola al análisis de muestras reales de leche materna obtenida a partir de diferentes madres lactantes voluntarias. De esta forma, los métodos podrán ser aplicables a la realización de investigación exhaustiva sobre la presencia y exposición a estos contaminantes.

IV. Se han desarrollado especialmente los procedimientos de tratamiento de la muestra. Las matriz estudiada es compleja, de modo que el análisis de contaminantes orgánicos en general genera numerosos inconvenientes experimentales, por lo que la etapa de preparación de muestra representa un reto real debido a las bajas recuperaciones de los analitos durante la extracción y los fuertes efectos de supresión de la señal. La determinación de los parámetros más influyentes en la extracción selectiva de los analitos, evitando la co-extracción de los componentes de la matriz ha sido determinante para lograr tratamientos de muestra efectivo y extracciones cuantitativas.

V. El empleo de la espectrometría de masas-masas con dos transiciones SRM por cada analito, ha permitido la cuantificación y confirmación de los resultados positivos en un mismo análisis, mejorando y optimizando aún más la productividad del método

VI. La resolución de este problema analítico ha permitido obtener métodos con buenas características analíticas, presentando elevada exactitud, sensibilidad, selectividad, amplios rangos dinámicos lineales, y tiempos de análisis más cortos con la consiguiente reducción de costes.

ANEXO I

Publicaciones de la Tesis

ANEXO II

Otras Publicaciones