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Nuevas aportaciones al análisis de estrógenos y derivados mediante el uso de técnicas analíticas miniaturizadas

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Nuevas Aportaciones al análisis de estrógenos y derivados mediante el uso de técnicas analíticas miniaturizadas

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2016

D. MIGUEL ÁNGEL RODRÍGUEZ DELGADO, CATEDRÁTICO DE UNIVERSIDAD, Y D. JAVIER HERNÁNDEZ BORGES, PROFESOR CONTRATADO DOCTOR, DEL DEPARTAMENTO DE QUÍMICA, UNIDAD DEPARTAMENTAL DE QUIMICA ANALÍTICA, FACULTAD DE CIENCIAS, DE LA UNIVERSIDAD DE LA LAGUNA, Y D. SALVATORE FANALI, PROFESOR DE INVESTIGACIÓN, DEL CONSIGLIO NAZIONALE DELLE RICERCHE DE ROMA (ITALIA)

INFORMAN:

Que D. Giovanni D'Orazio, Licenciado en Química por la Università degli Studi di Roma "La Sapienza", ha realizado bajo nuestra dirección los trabajos conducentes a la elaboración de su Tesis Doctoral titulada *Nuevas aportaciones al análisis de estrógenos y derivados mediante el uso de técnicas analíticas miniaturizadas*.

Revisado el trabajo, autorizamos su presentación bajo la modalidad de Compendio de Publicaciones, para que se pueda proceder a su lectura y defensa pública, y optar al grado de Doctor en Química con Mención Internacional por esta Universidad.

Y para que así conste, firmamos el presente en San Cristóbal de La Laguna, a quince de julio de dos mil dieciséis.

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DEPARTAMENTO DE QUÍMICA
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NUEVAS APORTACIONES AL ANÁLISIS DE ESTRÓGENOS Y DERIVADOS MEDIANTE EL USO DE TÉCNICAS ANALÍTICAS MINIATURIZADAS

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE
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GIOVANNI D'ORAZIO
LA LAGUNA, TENERIFE
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NEW CONTRIBUTIONS TO THE ANALYSIS OF ESTROGENS AND THEIR DERIVATIVES USING MINIATURIZED ANALYTICAL TECHNIQUES

DISSERTATION PRESENTED TO OBTAIN THE Ph.D. DEGREE IN CHEMISTRY AT THE UNIVERSITY OF LA LAGUNA

GIOVANNI D'ORAZIO
LA LAGUNA, TENERIFE
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La Laguna, 11 settembre 2016

Giovanni

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ai miei genitori, Rino e Giovanna

a mia moglie, María

"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza."

Dante, canto XXVI, Inferno

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PRÓLOGO

El final del siglo XX ha definido una nueva era marcada por grandes cambios, no solamente sociales y económicos, sino sobre todo medioambientales. Se trata de una época caracterizada por la escasez de materias primas, de energía, e incluso por la dificultad de muchos países de tener acceso al agua potable.

Todo lo anterior ha impulsado la creación del programa "*Horizonte 2020*", Programa Marco de Investigación e Innovación de la Unión Europea (UE) que es, sin duda alguna, el programa de investigación e innovación más importante realizado por la UE y que está haciendo especial hincapié en la transferencia de ideas de investigación básica y grandes resultados al mercado y a la sociedad en general. Se trata de una inversión financiera de aproximadamente 80 millones de euros para un período de 7 años (2014-2020) de los cuales aproximadamente el 35% ha sido, es y será destinado a hacer frente al cambio climático (Horizon, 2020a).

La quinta prioridad temática de los Retos Sociales planteados en el programa "*Horizonte 2020*" (Horizon, 2020b) es precisamente la dedicada al clima, al medio ambiente, a la eficiencia de los recursos y a las materias primas. Entre sus principales objetivos destacan el aumento de la competitividad europea, la seguridad de las materias primas y la mejora del bienestar humano, garantizando al mismo tiempo la integridad del medio ambiente, la resiliencia y la sostenibilidad, para poder así limitar el aumento de la temperatura global en menos de 2 ºC respecto a los niveles preindustriales.

Dentro de este programa, las líneas prioritarias de acción también se han definido como una transición hacia una economía "verde" y una sociedad sostenible, a través de la innovación ecológica y el desarrollo de un sistema de información y vigilancia del medio ambiente, promoviendo así la denominada *Química Verde* y, por consiguiente, la *Química Analítica Verde*.

En 1991, a través de un programa especial puesto en marcha por la Agencia de Protección Ambiental de Estados Unidos (EPA) con el fin de promover el desarrollo sostenible de las tecnologías químicas en la industria, la universidad y el gobierno, Anastas definió la *Química Verde* como: "*El diseño de productos y procesos químicos que reducen o eliminan el uso o generación de sustancias peligrosas. La Química Verde se aplica en todo el ciclo de vida de un producto químico, incluyendo su diseño,*

fabricación, uso y eliminación final. De ahí que también se la identifique como Química Sostenible" (EPA-US Environmental Protection Agency).

Dicha definición abarca la idea de sustituir las sustancias peligrosas por otras menos contaminantes o, si fuera posible, por productos inocuos, así como de establecer una previsión de los desechos junto con el uso limitado de las materias primas y la energía.

En 1998 el propio Anastas propuso, conjuntamente con Warner (Anastas y Warner, 1998), lo que hoy en día se conoce como "*los doce principios de la química sostenible*", que se podrían resumir de la siguiente forma: "*1. Evitar la formación de residuos; 2. Maximizar la economía atómica; 3. Diseñar síntesis químicas menos peligrosas; 4. Diseñar productos químicos y compuestos más seguros; 5. Utilizar disolventes y condiciones de reacción más seguros; 6. Aumentar la eficiencia energética; 7. Utilizar materias primas renovables; 8. Evitar derivados químicos innecesarios; 9. Potenciar la catálisis; 10. Generar productos biodegradables; 11. Desarrollar metodologías químicas para la monitorización en tiempo real de la contaminación; 12. Reducir al mínimo los posibles accidentes químicos*".

En general, estos principios encierran una filosofía de prevención de la contaminación y los efectos de los productos químicos, proporcionando indicaciones para mejorar los métodos de síntesis, a través del uso de materias primas renovables, la optimización de los productos finales y la reducción del consumo de energía. En última instancia, se recomienda reducir la toxicidad química de los productos involucrados y la utilización de catalizadores. Por otra parte, y haciendo referencia directa a las metodologías analíticas, se indica la necesidad de llevar a cabo un seguimiento en tiempo real de los procesos realizados y, sobretodo, un control preventivo de la formación de sustancias peligrosas.

En lo que respecta a la Química Analítica, y con el objetivo de superar la paradoja de que frecuentemente los productos químicos utilizados en los análisis eran más tóxicos que las especies analizadas, nació una nueva filosofía como resultado directo de la *Química Verde*: la *Química Analítica Verde*, término acuñado por Namiestnik a finales de los años 90 (Namiesnik, 1999; Namiesnik, 2001), aunque la definición más actual y completa la propuso Lawrence posteriormente como (Sandra y cols., 2010): "*El uso de técnicas y metodologías analíticas que reducen o eliminan los disolventes, reactivos, conservantes y otros productos químicos que son peligrosos para la salud humana o el medio ambiente, y que también pueden permitir un análisis más rápido y más eficiente desde un punto de vista energético, sin comprometer los criterios de funcionamiento de dichas técnicas o métodos".*

En esta definición se identifican varios aspectos clave para conseguir que una metodología sea "verde". En particular, se debe promover el uso de metodologías analíticas basadas en: i) el análisis de muestras tratadas pero no sujetas a manipulación con disolventes y reactivos; ii) la rapidez en la obtención de los resultados mediante la utilización de instrumentos portátiles y multi-paramétricos para obtener más información en un número limitado de medidas y iii) la reducción de la cantidad y la toxicidad de los disolventes y reactivos utilizados en la fase de medida, sobre todo promoviendo la utilización de la automatización y la miniaturización. En este sentido, cabe destacar que la reducción de escala dimensional, del volumen y de la cantidad de muestra del procedimiento analítico, tanto en la fase de pre-tratamiento de muestra como en la determinación analítica, es un factor clave en la reducción del consumo y el aprovechamiento de la energía utilizada.

Esta nueva filosofía queda patente cuando se realiza un análisis retrospectivo del papel de la Química Analítica durante los últimos 40 años (*Figura 0.1*) ya que, durante todo ese tiempo, su objetivo principal ha sido la miniaturización del sistema analítico, para reducir costes y tiempos de análisis manteniendo, al mismo tiempo, parámetros como la exactitud, sensibilidad, selectividad y precisión a niveles aceptables. En este contexto de avances tecnológicos, la *Química Analítica Verde* no se presenta como una alternativa, sino más bien como un nuevo impulso, añadiendo a sus criterios un mayor respeto por el medio ambiente.

Considerando la miniaturización como un aspecto clave de una estrategia verde, la reducción del consumo de reactivos, de los volúmenes de residuos tóxicos, de la energía y el tiempo de análisis son también buenas oportunidades para minimizar los costes en los laboratorios. De hecho, el concepto "verde" también implica pensar en invertir en nuevos equipos miniaturizados, más rápidos en la respuesta analítica, y también en el uso de técnicas de detección multiparamétricas.

Con esta filosofía se desea recuperar el equilibrio entre el hombre y la biosfera, después de años de desarrollo tecnológico desordenado que no ha tenido en cuenta el impacto ambiental de las actividades humanas y los riesgos de este tipo de actividades a largo plazo. Por esta razón, la Química ha sido percibida tradicionalmente como un concepto inherente a las malas prácticas. Sin embargo, la responsabilidad actual de la ciencia es transmitir a la sociedad que otra química es posible y que ella en sí misma es la única solución para la prevención de la contaminación y la restauración y conservación del medio ambiente, para asegurar así la continuidad del ecosistema, de la vida y del futuro de las generaciones venideras.

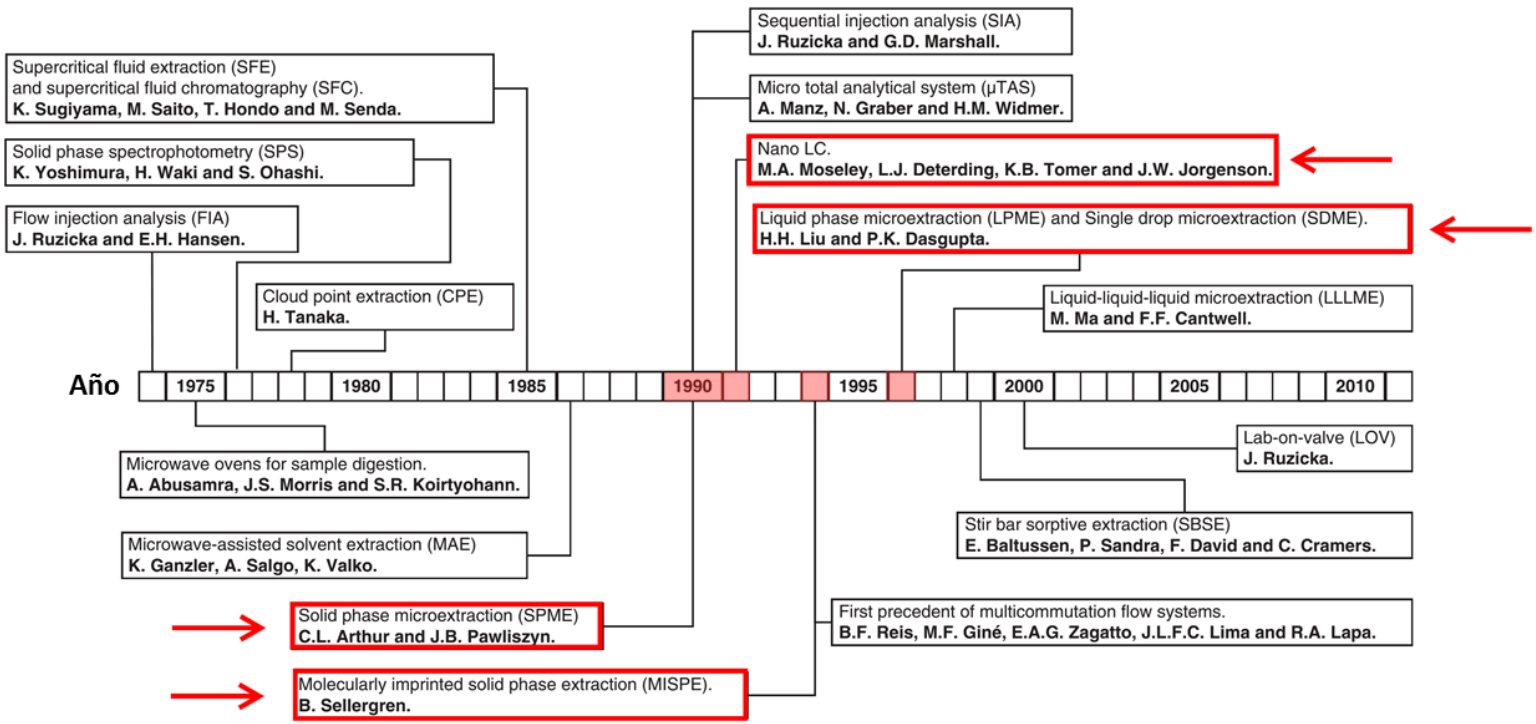


Figura 0.1. Hitos más importantes en el desarrollo de metodologías para una Química Analítica Sostenible. Adaptada de De La Guardia y Garrigues (2012).

I. INTRODUCCIÓN

I. INTRODUCCIÓN

I.1. Las hormonas

I.1.1. El origen del concepto de la comunicación celular

La célula, como unidad formológica funcional de un ser vivo, es el elemento de menor tamaño que puede considerarse que está vivo. De hecho, existen organismos unicelulares como las bacterias o los protozoos, pero también existen organismos pluricelulares, como el caso del ser humano, en el que una célula no existe de manera aislada, de forma que una compleja red de comunicación las coordina para que puedan trabajar en conjunto. Todas ellas llevan a cabo procesos corporales necesarios para la vida, tales como el crecimiento, la diferenciación y el metabolismo.

Las responsables de la comunicación celular son generalmente moléculas extracelulares secretadas por una célula específica que pueden actuar sobre ella misma o sobre otra/s célula/s, a cortas o largas distancias. Tales mensajeros químicos, denominados *hormonas*, son capturados por receptores específicos, que son a menudo proteínas. La comunicación intercelular puede ser *autocrina* (cuando la acción de la hormona se ejerce sobre la misma célula que la sintetiza), *paracrina* (cuando la acción se ejerce en zonas cercanas a su liberación), *endocrina* (cuando existe un retardo importante, de minutos u horas, ya que la acción se ejerce en una zona lejana y es necesario un transporte de la hormona a través de la sangre), *sináptica* o incluso *de contacto* (Nelson y Cox, 2013).

En 1905, Starling, profesor de Fisiología en el *University College of London*, utilizó por primera vez la palabra "*hormona*" en su famoso discurso "*La correlación química de las funciones del organismo*" que envió al *Royal College of Physicians* de Londres para describir la acción de la secretina, una molécula secretada por el duodeno que estimula el flujo del jugo pancreático (Starling, 1905). La etimología de la palabra deriva del griego antiguo ὁρμῶν (hormôn) que significa *"que impulsa, que pone en movimiento*" (Tata, 2005).

Sin embargo, la naturaleza y el papel de estos mensajeros químicos en el organismo ya había atraído el interés de científicos anteriores a Starling. A mediados del siglo XIX, y atendiendo a descripciones puramente anatómicas, se tomó conciencia de la existencia de ciertas glándulas que formaban parte de los vasos sanguíneos, a las que se calificaba como "glándulas de la sangre", como la tiroides o los ganglios linfáticos. En aquella época, científicos de todo el mundo realizaron múltiples estudios al respecto, obteniendo importantes resultados. Éste fue el caso de Berthold en 1849 (Berthold, 1849;

Quiring, 1944), Carpenter en 1852 (Carpenter, 1852) o Addison en 1855 (Addison, 1868).

Prácticamente finalizando el siglo XIX (1889), Brown-Séquard formuló la hipótesis de la *"organoterapia"* (administración de secreciones endógenas) en una reunión de la Société de Biologie en París (Brown-Séquard, 1889; Brown-Séquard, 1893). Según una práctica que data de la época de los antiguos egipcios, griegos (Hipócrates, 450-400 a.C.) y romanos, basada en el principio de "*similia similibus curantur*" (lo semejante se cura con lo semejante), el mal funcionamiento de un órgano podía ser curado con un derivado de un órgano similar. Brown-Séquard planteó utilizar inyecciones hipodérmicas de un fluido preparado a partir de las secreciones testiculares de perros y conejillos de Indias, sugiriendo un efecto terapéutico. Estos estudios inspiraron los trabajos del premio Nobel Fritz Pregl, que centró su investigación en la realización de experimentos para verificar la mejora del rendimiento físico mediante la administración de sustancias testiculares (Hobermann y Yesalis, 1995).

Ya en los años 20 del siglo pasado, el químico y bioquímico Kendall (Premio Nobel compartido con Reichstein y Showalter Hench en 1950) aisló la cortisona y la tirosina y determinó sus estructuras (Kendall, 1950). En 1923, Banting y Macleod descubrieron y aislaron la insulina (Banting, 1925), mientras que, durante los años siguientes, Butenandt (premio Nobel en 1939 compartido con Ruzicka y Doisy por el descrubimieneto de la estrona en 1929) descubrió y caracterizó varias hormonas sexuales como la progesterona, la androsterona y diferentes estrógenos (Butenandt, 1931). En 1950, Hench obtuvo el premio Nobel por el descubrimiento de la cortisona y su aplicación en el tratamiento de la artritis reumatoide.

I.1.2. Las glándulas endocrinas

Las glándulas endocrinas (del griego *endon*=dentro y *krinien*=liberar), a diferencia de las glándulas exocrinas, que secretan sustancias que son liberadas sobre la superficie del cuerpo humano (glándulas sebáceas, sudoríparas, lacrimales, etc.), son capaces de secretar diferentes sustancias entre las que se encuentran las hormonas. Éstas son producidas por unos grupos de células epiteliales localizadas en diferentes partes del cuerpo humano y que juntas definen el sistema endocrino.

Las principales glándulas endocrinas incluyen el hipotálamo, la glándula pineal o epífisis cerebral, la pituitaria o hipófisis, la tiroides, las paratiroides, las suprarrenales, el páncreas, los ovarios y los testículos. La Figura I.1 muestra la ubicación de las principales glándulas endocrinas en el ser humano.

Las hormonas secretadas por las glándulas endocrinas actúan en muchos procesos de los seres vivos, como el crecimiento y el desarrollo, el metabolismo de los alimentos, la función sexual, la reproducción, el comportamiento y el estado de ánimo.

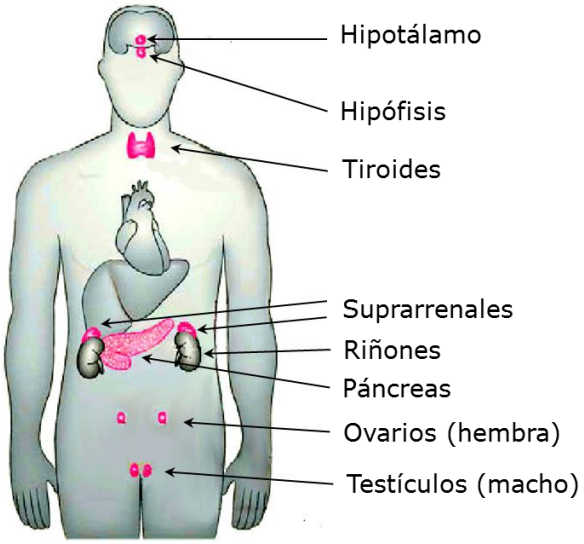


Figura I.1. Las principales glándulas endocrinas.
Adaptada de Nelson y Cox (2013).

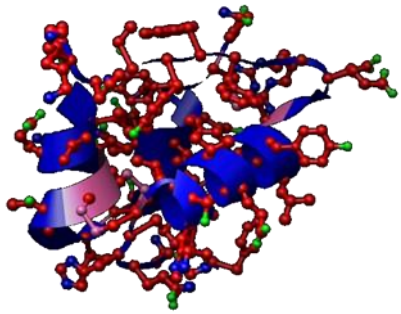
I.1.3. Clasificación de las hormonas

La clasificación de las hormonas se puede realizar teniendo en cuenta diferentes criterios (Nelson y Cox, 2013). Por un lado, se pueden clasificar según su solubilidad y la localización de los receptores en:

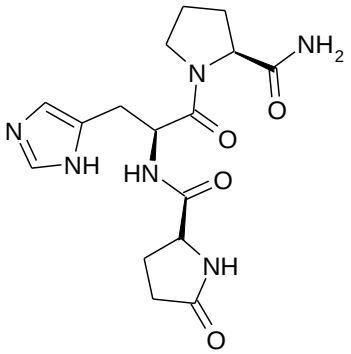
1) *Hormonas hidrófilas.* No permean fácilmente a través de la bicapa lipídica de la membrana celular, actuando en el exterior de la célula, donde transmiten su señal a los receptores situados en la membrana celular.
2) *Hormonas hidrofóbicas.* Pueden traspasar la membrana celular y se unen a un receptor que se encuentra en el interior de la célula, en ocasiones en la propia membrana nuclear.

Por otra parte, también se pueden clasificar según su estructura bioquímica, siendo ésta la clasificación más utilizada en la actualidad (Herrera, 1991). Atendiendo a este criterio las hormonas se pueden dividir en:

I) *Hormonas peptídicas.* Se trata de cadenas de aminoácidos, desde pequeñas moléculas formadas por tan solo tres aminoácidos a grandes moléculas formadas por grandes proteínas y glicoproteínas. Se sintetizan en forma de pre-hormonas, y sólo después de una modificación posterior se activan. Ejemplos de ellas son la insulina (Figura I.2.a), producida por las células β del páncreas y cuya función es el control de los niveles de glucosa en la sangre; o la tirotropina (Figura I.2.b), denominada también hormona estimulante de la tiroides, y que regula la producción de hormonas tiroideas.



a) Insulina[(1)]



b) Tirotropina

Figura I.2. Estructura de algunas hormonas amínicas, [(1)] © Wikipedia.

II) *Hormonas esteroideas.* Son de naturaleza lipídica y tienen un precursor común, que es el colesterol. Actúan sobre el metabolismo del crecimiento y la reproducción. Son transportadas a través de la sangre por las denominadas proteínas transportadoras que las protegen de la degradación enzimática. Ejemplos de ellas son la aldosterona (Figura I.3.a), que favorece la eliminación de potasio en el riñón y provoca la retención de sodio; o el cortisol (Figura I.3.b), cuyas funciones son aumentar el nivel de glucosa en la sangre, suprimir el sistema inmunológico y ayudar al metabolismo de las grasas, proteínas e hidratos de carbono.

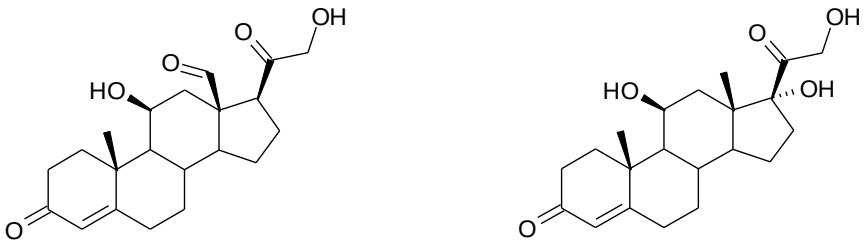


Figura I.3. Estructura de algunas hormonas esteroideas.

III) *Hormonas amínicas*. Son pequeñas moléculas sintetizadas a partir del triptófano, como la melatonina, o de la tirosina, como las catecolaminas y las hormonas tiroideas. Ejemplos de este tipo de hormonas son la adrenalina (Figura I.4.a), una catecolamina que incrementa la frecuencia cardíaca y participa en la *reacción de lucha o de huida* (respuesta fisiológica ante la percepción de daño o ataque) del sistema nervioso simpático; o la tiroxina (Figura I.4.b), que regula el metabolismo celular.

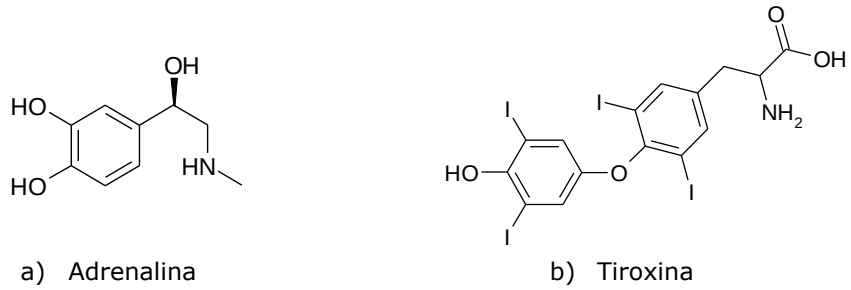


Figura I.4. Estructura de algunas hormonas amínicas.

I.1.4. Producción y mecanismos de actuación de las hormonas

El sistema nervioso no sólo es el medio de comunicación de todo el organismo, sino que también se encarga de la coordinación muscular a través de los neurotransmisores y de la secreción hormonal de las glándulas endocrinas (Figura I.5). Desde la recepción de la información interna o externa, como por ejemplo, una situación peligrosa o la sensación de hambre, el sistema nervioso responde mediante la administración controlada de hormonas. En este sentido, el sistema endocrino está construido de acuerdo con un modelo de cadena de control, donde la señal hormonal está sujeta a una estricta jerarquía.

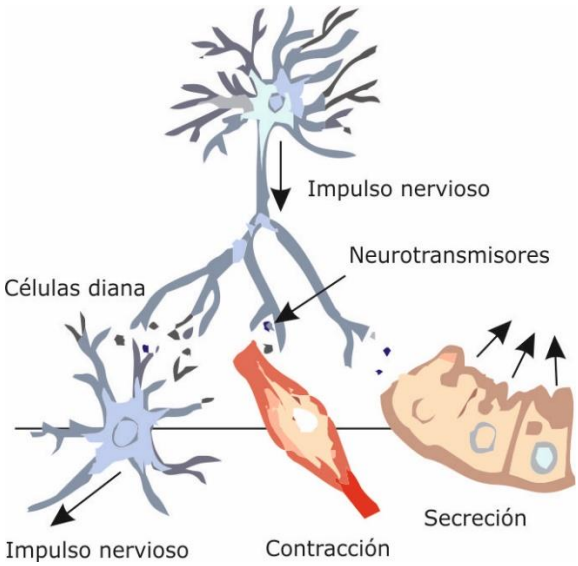


Figura I.5. La generación de señales en el sistema neuro-endocrino. Adaptada de Nelson y Cox (2013).

El hipotálamo es a su vez el centro de coordinación del sistema endocrino que recibe e integra los mensajes provenientes del sistema nervioso central. En respuesta a estos mensajes, el hipotálamo produce hormonas reguladoras que se dirigen directamente a la hipófisis. La hipófisis posterior contiene las terminaciones de los axones de muchas neuronas que se originan en el hipotálamo, a partir de los que se producen dos pequeños péptidos, la oxitocina y la vasopresina. Éstas, moviéndose a través de los axones, llegan a las terminaciones nerviosas de la hipófisis, para ser almacenadas en gránulos de secreción, a la espera de la señal para su liberación.

La hipófisis anterior responde a las hormonas hipotalámicas transportadas a través de la sangre mediante la producción de hormonas trópicas que activan las glándulas endocrinas al siguiente nivel, como la corteza suprarrenal, la tiroides, los ovarios y los testículos. Estas glándulas secretan a su vez sus hormonas específicas, que son transportadas por el torrente sanguíneo a los tejidos diana.

En este sistema en cascada, una pequeña señal provoca una respuesta más grande, generando una amplificación de un millón de veces. Todo el proceso está regulado por un mecanismo de retroalimentación del nivel superior que permite un control de la concentración requerida de la hormona específica en tiempo real.

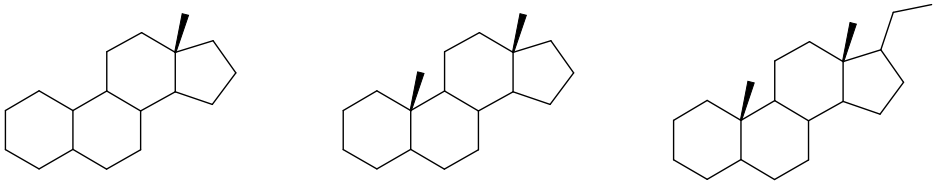
I.1.5. Hormonas esteroideas

Las hormonas esteroideas incluyen aquellas secretadas por la corteza adrenal (adenocorticales), las formadas a partir de la vitamina D, las hormonas sexuales masculinas y femeninas (andrógenos y estrógenos) y los progestágenos. Este tipo de hormonas circulan en el torrente sanguíneo unidas a proteínas transportadoras específicas debido a su elevado carácter lipofílico.

Las hormonas adenocorticales se diferencian a su vez en tres tipos: los *corticosteroides* (como el cortisol o la aldosterona), los *glucocorticoides*, que afectan al metabolismo de los glúcidos, y los *mineralocorticoides*, que regulan la concentración de electrolitos en sangre. Por su parte, las producidas por la vitamina D controlan el metabolismo del calcio y el fosfato, incluyendo la formación y movilización de $Ca_3(PO_4)_2$ en los huesos, mientras que los andrógenos y estrógenos se sintetizan en los testículos y en los ovarios, respectivamente, y afectan al desarrollo sexual y reproductor, entre otras funciones. Por último, los progestágenos se sintetizan en los ovarios o en la propia placenta, y su función es mantener el embarazo (pre-gestacional).

Esta distribución no es estricta, ya que la corteza suprarrenal también sintetiza una pequeña cantidad de esteroides gonadales. Por otra parte, los testículos también pueden producir estrógenos y los ovarios andrógenos. Además, todas las glándulas esteroidogénicas son capaces de producir progesterona, precursora de todas las hormonas esteroideas.

Desde un punto de vista químico, estas hormonas se sintetizan a partir del colesterol y pueden diferenciarse en función de su estructura química, compuesta de un esqueleto básico (Figura I.6) de 18 átomos de carbono (estrano), como los estrógenos; 19 átomos de carbono (androstano), como los andrógenos; o de 21 átomos de carbono (pregnano), como los progestágenos, los mineralocorticoides y los glucocorticoides.



Estrano Androstano Pregnano

Figura I.6. Hidrocarburos cíclicos relacionados con las hormonas esteroideas.

La Tabla I.1 recoge, a modo de resumen, las características más representativas de las hormonas esteroideas, algunos ejemplos, sus principales funciones y su glándula secretora mayoritaria.

En las rutas biosintéticas de esta clase de hormonas se producen varias reacciones características que están catalizadas por diferentes enzimas como las hidroxilasas, desmolasas, deshidrogenasas e isomerasas. En todos los casos, la biosíntesis comienza por la escisión de la cadena lateral del colesterol para dar lugar a pregnenolona, tal y como muestra la Figura I.7.

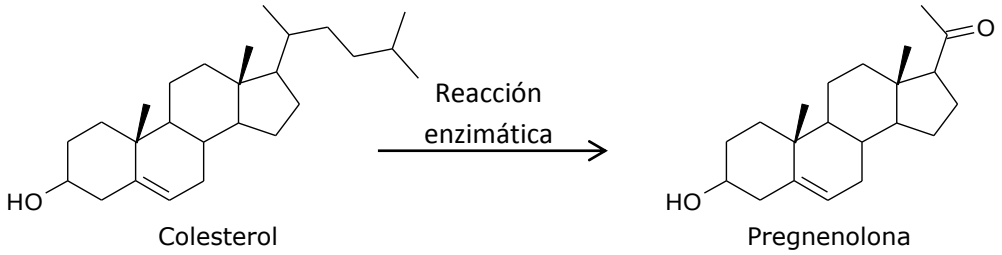


Figura I.7. Ejemplo de reacción enzimática característica de la biosíntesis de las hormonas esteroideas.

I.2. Compuestos con actividad estrogénica: endo y exoestrógenos

Con frecuencia, el grupo de estrógenos naturales anteriormente descrito y sus metabolitos reciben el nombre de *endoestrógenos* (Noppe y cols., 2008), para diferenciarlos de todo un conjunto de compuestos que también posee actividad endocrina pero que, sin embargo, tiene otro origen.

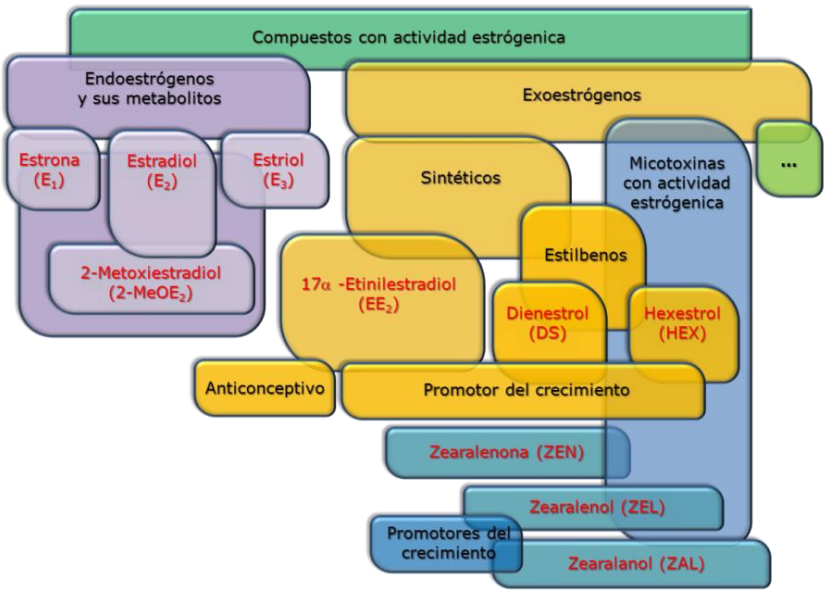


Figura I.8. Clasificación de compuestos con actividad estrogénica atendiendo a su origen.

Tabla I.1. Características más importantes de las hormonas esteroides (Herrera, 1991).

Categoría	Estructura química	Nº de átomos de C	Función principal	Glándula secretora mayoritaria	Ejemplos
Estrogénos	Estrano	18	Desarrollo de los caracteres sexuales secundarios femeninos	Ovarios, placenta	17-β-estradiol, estrona, estriol
Andrógenos	Androstano	19	Desarrollo de los caracteres sexuales secundarios masculinos	Testículos, glándulas suprarrenales	Testosterona, androstenediona, dehidroepiandrosterona, androsterona
Progestágenos	Pregnano	21	Regulación del desarrollo y mantenimiento del embarazo	Ovarios, placenta	Progesterona, pregnenolona, 17-hidroxiprogesterona, 17-hidroxipregnolona
Mineralcorticoides	Pregnano	21	Regulación de las concentraciones de electrolitos (K^+, Na^+, Ca^{2+}, Cl^-) en la sangre	Células glomerulares de la corteza suprarrenal	Peterserona, aldosterona
Glucocorticoides	Pregnano	21	Regulación del metabolismo de los hidratos de carbono	Glándulas suprarrenales, células fasciculadas y reticulares	Cortisol, corticosterona, cortisona

Estos últimos reciben el nombre de *exoestrógenos*, frecuentemente denominados también como *disruptores endocrinos*, ya que tienen un origen ajeno al cuerpo humano, aunque éste puede ser tanto natural como sintético (Figura I.8).

Dentro de los endoestrógenos destacan la estrona, el estradiol y el estriol, frecuentemente abreviados como E_1, E_2 y E_3, respectivamente, en función del número de grupos hidroxilo que poseen en su estructura. Se caracterizan por poseer una estructura de estrano (C18) con la variabilidad en los sustituyentes del ciclopentano (Figura I.9).

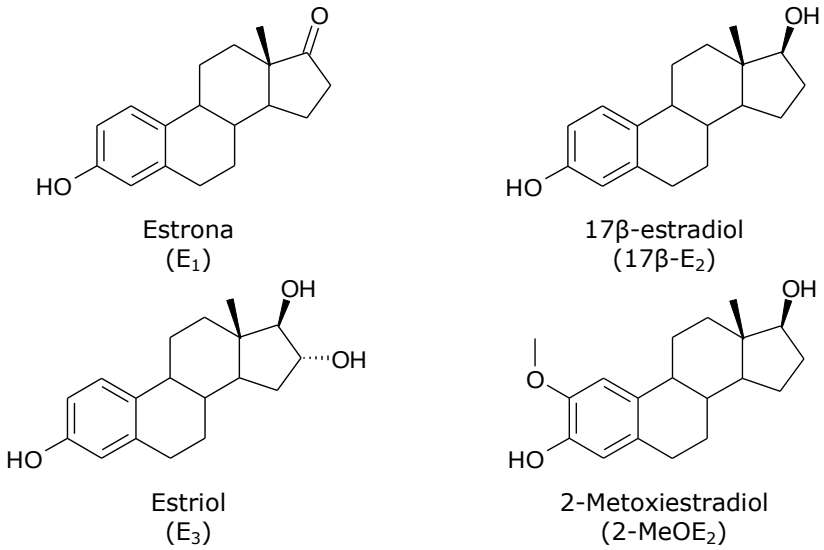


Figura I.9. Estructura de los estrógenos naturales.

La E_1, secretada por el ovario y el tejido adiposo, es la menos abundante de las tres hormonas naturales, pero después de la menopausia se convierte en el estrógeno predominante. Se considera una forma poco activa y, en caso de necesidad, puede convertirse en E_2 (Varney y cols., 2004). Por su parte, el E_2 es secretado durante los años reproductivos por las células granulares de los ovarios mediante la conversión de E_1 en E_2. Una pequeña cantidad de E_2 también es producida por la corteza suprarrenal y por los testículos. Es el estrógeno más abundante y también el más importante por su efecto estrogénico, siendo aproximadamente 80 veces más potente que el E_3 y 12 veces más que la E_1 (Serrano y cols., 2001). La forma isomérica que tiene propiedades estrogénicas es el 17β-E_2 aunque el 17α-E_2 se ha encontrado a bajas conentraciones en la sangre y la orina de diversas especies animales y en la leche de vaca (Jouan y cols., 2006), poseyendo una

actividad hormonal débil (Hobe y cols., 2002). El E_3 es secretado mayoritariamente en los ovarios durante el embarazo, aunque también constituye un producto de oxidación de la E_1 y del 17β-E_2, conversión que tiene lugar en el hígado y en el intestino delgado (Serrano y cols., 2001). El 2-metoxiestradiol (2-MeOE_2) es un metabolito endógeno del 17β-E_2, que posee una potente actividad apoptótica contra las células tumorales de crecimiento rápido (Lakhani y cols., 2003).

En lo que respecta a los exoestrógenos, éstos pueden imitar la actividad de los endoestrógenos, debido a que presentan geometrías estructurales similares (mecanismo de suplantación), pero también pueden bloquearla o incluso modular o alterar los niveles hormonales (Hallegue y cols., 2003). De hecho, pueden interferir negativamente en el sistema reproductor, neurológico e inmunológico. Se les atribuye incluso la proliferación de determinados tipos de cáncer (Ganmaa y cols., 2001; Serrano y cols. 2001). Un exoestrógeno de gran importancia es el 17β-etinilestradiol (EE_2) (Figura I.10), de origen semisintético, que se ha utilizado comúnmente con fines terapéuticos, como anticonceptivo, como terapia hormonal sustitutiva durante la menopausia y como tratamiento complementario de algunos tipos de cáncer (Díaz-Cruz y cols., 2003; Dörner y cols., 1985; Iwase y cols., 2013). También se ha utilizado en ganadería como promotor del crecimiento (Shao y cols., 2005).

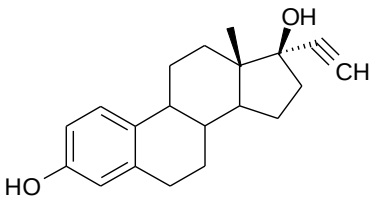
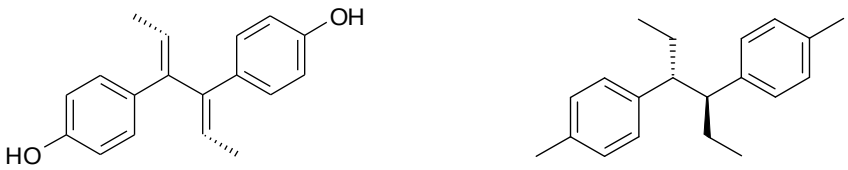


Figura I.10. Estructura del 17α-etinilestradiol (EE_2).

Los estilbenos sintéticos como el dienestrol (DS) y el hexestrol (HEX) también se incluyen en este grupo. Son moléculas estructuralmente parecidas al E_2, debido tanto a su geometría como a la distancia de separación entre los grupos hidroxilo (Figura I.11). En general, y debido a sus propiedades anabólicas, estas moléculas se emplean como promotores del crecimiento, aunque también se utilizan como medicamentos veterinarios para tratar neoplasias malignas y controlar la lactancia y el ciclo reproductivo (Dickson y cols., 2003). También se ha utilizado como medicamento en humanos para el tratamiento de algunas distrofias vaginales (Bygdeman y Swahn, 1996).

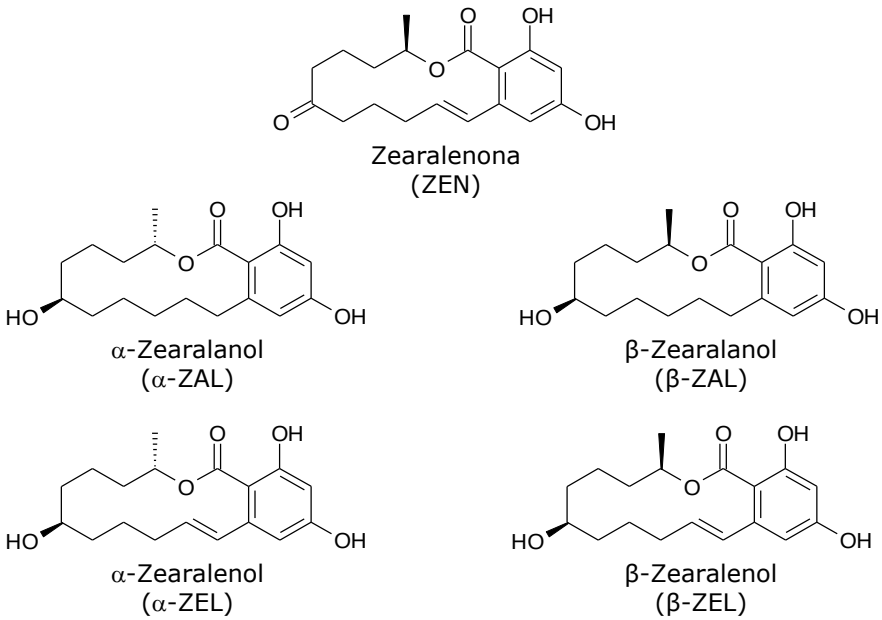


Dienestrol
(DS)

Hexestrol
(HEX)

Figura I.11. Estructura de algunos estilbenos de importancia.

Otro grupo importante de compuestos que poseen actividad estrogénica es el de las micotoxinas de la familia de la zearalenona (ZEN), tratándose del único conjunto de micotoxinas que presenta este tipo de actividad (Zain, 2011). De todas ellas, la ZEN es la más representativa y es producida por diferentes especies de hongos del género *Fusarium* (*F. graminearum, F. culmorum* y *F. equiseti*, entre otros). Se encuentra de forma común en el maíz, pero también puede estar presente en otros cereales como la cebada, el trigo, el mijo y el arroz. Su estructura química corresponde a una lactona del ácido resorcíclico y sus principales metabolitos son el zearalanol (ZAL) y el zearalenol (ZEL), en sus formas isoméricas α y β (Figura I.12) (Emídio y cols., 2015; Sørensen y Elbaek, 2005). Se sospecha que tanto la ZEN como el ZAL y el ZEL son hepatotóxicos, inmunotóxicos y genotóxicos (Zinedine y cols., 2007).



Zearalenona
(ZEN)

α-Zearalanol
(α-ZAL)

β-Zearalanol
(β-ZAL)

α-Zearalenol
(α-ZEL)

β-Zearalenol
(β-ZEL)

Figura I.12. Estructuras de las micotoxinas de la familia de la ZEN.

En lo que respecta a su actividad estrogénica, los metabolitos α-ZEL y α-ZAL tienen una actividad de 3 a 4 veces superior a la de la ZEN (Minervini y cols., 2005; Mirocha y cols., 1979;). Por otro lado, y debido a su efecto anabolizante, es importante destacar que algunos de estos compuestos han sido utilizados durante mucho tiempo como promotores del crecimiento en ganado en determinados países (Noppe y cols., 2008), aunque teniendo en cuenta el elevado grado de toxicidad que presentan tanto en el ser humano como en los mamíferos en general, dicho uso está actualmente prohibido en Europa.

I.2.1. Importancia del análisis de compuestos con actividad estrogénica en aguas y alimentos

Como es bien conocido, las actividades humanas globales están causando una profunda alteración del medio ambiente, no sólo en el paisaje, sino también en los sutiles equilibrios biológicos. Esto se debe a la introducción de productos químicos que actúan como contaminantes ambientales. En este sentido, la preocupación por las posibles consecuencias de la presencia de estos contaminantes en el medio ambiente ha aumentado, sobre todo en el caso de aquellos previamente desconocidos o no reconocidos como tales. Se trata de los denominados "contaminantes emergentes". Entre ellos, los disruptores endocrinos han suscitado un gran interés, ya que no identifican a una clase de contaminantes, sino que se trata de una amplia gama de compuestos (Ciofi y cols., 2013) que son introducidos en el organismo a través de la ingesta de agua y alimentos principalmente.

Los estudios sobre el metabolismo de los compuestos con actividad estrogénica sugieren que son absorbidos por el intestino y metabolizados en el hígado (Filannino y cols., 2011). Posteriormente, estas sustancias llegan a los riñones y se transforman en derivados más polares que se conjugan en forma de ésteres sulfato o glucurónidos hidrosolubles, pudiendo ser eliminados por medio de la orina (Pacáková y cols., 2009). Por esta razón, la presencia de este tipo de compuestos se ha detectado en el ciclo del agua (Heberer, 2002; Richardson y Bowron, 1985), habiéndose encontrado en aguas ambientales de efluentes de fábricas, de plantas de tratamiento de aguas residuales y de aguas residuales residenciales. En el segundo caso, se han detectado a niveles desde por debajo de los ng/L a decenas de ng/L (Miège y cols., 2009b).

Por otra parte, y dada su elevada estabilidad, las micotoxinas suponen una aportación adicional de contaminantes a las aguas de drenaje de campos cultivados con plantas infectadas, aguas de escorrentía de instalaciones ganaderas y campos agrícolas abonados con

estiércol de ganado (Emídio y cols., 2015), así como en sedimentos y lodos (Gabet y cols., 2007).

A pesar de que la presencia de sustancias con actividad estrogénica en la mayoría de los casos es a concentraciones tan bajas como para no suponer una preocupación medioambiental, se ha demostrado que pueden ejercer una actividad negativa significativa en organismos acuáticos, provocando, por ejemplo, trastornos endocrinos en peces a una concentración de 0,1 a 1,0 ng/L (Hansen y cols., 1998; Kramer y cols., 1998). Algunos de estos problemas han sido publicados en la bibliografía, destacando los cambios de sexo y disfunciones reproductivas en reptiles, aves, anfibios, peces y crustáceos (Jobling y cols. 1998; Miège y cols., 2009a).

Por otra parte, una fuente importante de compuestos con actividad estrogénica son los alimentos. La presencia de estrógenos de origen animal en la dieta humana es debida a una alimentación rica en carne, huevos y especialmente leche, que de acuerdo con Hartmann et al. (Hartmann y cols., 1998), supone aproximadamente el 60-70% del total de estrógenos ingeridos. Aunque para el resto de mamíferos este producto es un alimento que no se consume después del destete (Pereira, 2014), la leche y sus derivados forman parte de la dieta del ser humano incluso en la fase adulta. Los datos más recientes de la FAO muestran que la producción de leche en 2012 se estimaba en más de 150 y 90 millones de toneladas en Europa y Estados Unidos, respectivamente (FAOSTAT), siendo la ingesta de leche per cápita mayor de 150 kg al año (FAOSTAT) en ambos países. Esta estadística no hace más que ratificar que la leche es posiblemente un importante vehículo para introducir compuestos con actividad estrogénica en el organismo con un nivel de exposición continuo y diario.

Para satisfacer esta gran demanda, se han desarrollado procedimientos en zootecnia que permiten aumentar y mantener la producción de leche de vaca constante durante todo el año. Para ello, se han seleccionado razas de vaca como la *Holstein*, que es capaz de proporcionar una producción de leche durante casi todo el periodo de gestación, es decir, durante más de 300 días al año (Maruyama y cols., 2010).

La presencia de estrógenos naturales en la leche es endógena debido precisamente a la naturaleza biológica de la misma. Sin embargo, las malas prácticas ganaderas, así como la recolección durante el periodo de gestación o el uso y abuso de compuestos anabólicos ilegales como el HEX y el DS que extienden los ciclos de producción de la leche, pueden elevar el contenido de estos compuestos por encima de los niveles normales (Maruyama y cols., 2010). Además, existen hipótesis que

señalan que ni la pasteurización ni el proceso de trasformación en los productos derivados reducen los contenidos de los mismos completamente (Qin y cols., 2004).

En las últimas décadas, se ha puesto en duda la utilidad o necesidad de consumir leche. Lo cierto es que, por un lado, se trata de uno de los alimentos más completos y equilibrados, ya que aporta un elevado contenido de nutrientes como proteínas, hidratos de carbono, vitaminas liposolubles y grasas (Aluko, 2012; Rice y cols., 2013). Sin embargo, su consumo se ha relacionado con la posibilidad de un aumento del riesgo de sufrir diversas enfermedades como trastornos cardiovasculares, anomalías en el sistema reproductor (Massart y cols., 2005; Rasier y cols., 2006), diabetes e incluso cáncer testicular, de mama, próstata, ovario o cuerpo uterino (Ganmaa y cols., 2002; Ganmaa y Sato, 2005; Malekinejad y cols., 2006; Maruyama y cols., 2010; Melnik, 2009; Parodi, 2012; Pereira, 2014).

Otra fuente de disruptores endocrinos son las plantas afectadas por el hongo *Fusarium* que pueden ser consumidas por el ganado y pasar al ser humano. En particular, la carne, la leche y los huevos se han estudiado como portadores no sólo de la ZEN, sino también de su metabolitos, el ZAL y el ZEL (Kleinova y cols, 2002). Por otra parte, la presencia no natural de α-ZAL se asocia con prácticas antropogénicas en biotecnología, relacionadas con su uso como promotor del crecimiento para el aumento de la producción y como "remedio" para reducir el estrés en el ganado (Chighizola y Meroni, 2012), ambas prohibidas en Europa desde 1985 (Directiva 85/649/EEC).

Con este panorama, aparte de inculcar una buena educación alimentaria promoviendo la ingesta equilibrada de alimentos, es de vital necesidad llevar a cabo un control estricto de los alimentos en base a una serie de leyes para obtener información fidedigna que permita salvaguardar la salud de los consumidores.

I.2.2. Legislación en materia de estrógenos

La globalización de los mercados, la seguridad y la calidad de los alimentos se ha convertido en una preocupación para los consumidores, los fabricantes y los gobiernos. Los contaminantes pueden estar presentes en los productos alimenticios debido al proceso de producción del embalaje, el transporte y el almacenamiento. Además, el uso de nuevos procesos industriales y prácticas agrícolas, así como la contaminación del medio ambiente son las principales fuentes de los denominados contaminantes emergentes. Por esta razón, los centros de investigación y los gobiernos trabajan en conjunto para mejorar la seguridad alimentaria con reglamentos en los que se definen las

limitaciones, las prohibiciones y los niveles máximos de residuos (LMRs) permitidos.

En lo que concierne a los compuestos con actividad estrogénica señalados anteriormente, aun hoy en día se utiliza una amplia gama de esteroides anabólicos para el crecimiento de los animales y para aumentar la eficiencia de la conversión alimenticia, es decir, la relación entre el alimento suministrado y la ganancia de peso que experimentan. Una de estas sustancias es el α-ZAL, utilizado en Estados Unidos desde 1969, y todavía utilizado y permitido en Canadá (H. Noppe y cols., 2008). El empleo de esteroides anabólicos, incluyendo el α-ZAL, el 17β-E_2 y los estilbenos, han sido objeto de discusión en la Directiva 81/602/CEE y sucesivamente prohibidos por la UE mediante las Directivas, 85/649/EEC, 88/146/CEE, 88/299/CEE, 96/22/CE, 2003/74/EC y 2008/97/CE, que en general prohíben la administración de sustancias con efecto hormonal y tirostático y sustancias β-agonistas en la cría de ganado.

A finales de 2011, la Comisión Europea propuso la adición de 15 sustancias nuevas a la lista de los 33 contaminantes que deben de controlarse en las aguas superficiales de acuerdo con la Directiva 2008/105/CE. Esta propuesta, conocida como COM(2011)876 (Directiva 2011/876), que modifica las Directivas 2000/60/EC y 2008/105/EC, se convirtió en la Directiva 2013/39/EU y se elaboró considerando el efecto nocivo de estas sustancias basándose en las evidencias del riesgo que suponen para la salud humana. Por primera vez, se incorporaron dos compuestos con actividad estrogénica: el EE_2 y el 17β-E_2. En lo que se refiere a los alimentos, en el año 2010 se establecieron LMRs de algunas hormonas en carne, aunque no hormonas estrogénicas, a través de la Directiva 37/2010.

Por lo tanto, a pesar de la importancia que posee el consumo de la leche y de los productos lácteos, la legislación en materia de seguridad alimentaria es todavía escasa, por no decir prácticamente nula. Sin embargo, a diferencia de la baja importancia que la EU ha concedido a esta materia, la investigación científica continúa concentrando sus esfuerzos en la monitorización y el desarrollo de métodos analíticos más eficaces y sensibles.

I.3. Determinación de compuestos con actividad estrogénica: uso de técnicas miniaturizadas

Como es bien conocido, el análisis de compuestos orgánicos puede llevarse a cabo en la actualidad mediante técnicas cromatográficas, ya sea cromatografía de gases (GC) o de líquidos (LC), o bien mediante técnicas electroforéticas, dependiendo de las propiedades de los analitos. Éste es también el caso de los compuestos con actividad estrogénica

objeto de estudio en esta Tesis Doctoral: los estrógenos naturales, sintéticos y micoestrógenos. De hecho, en la bibliografía existen diversos ejemplos de su aplicación al análisis de diferentes tipos de muestras, tal y como se verá a continuación. En este sentido, es importante destacar que algunas de estas técnicas, en particular sus versiones miniaturizadas, cumplen los principios de sostenibilidad ambiental de la *Química Analítica Verde* anteriormente señalados, de ahí que sean descritas con un mayor grado de detalle.

I.3.1. Cromatografía de gases

Hoy en día, la GC es considerada en cierta medida como una técnica *verde*, dado que el uso de disolventes orgánicos es extremadamente bajo (Płotka y cols., 2013). De hecho, la muestra se disuelve en una cantidad mínima de disolvente orgánico y, a continuación, se vaporiza en el interior del inyector a partir del cual es transportada a través de la columna capilar por el gas portador.

En lo que respecta al análisis de estrógenos naturales, sintéticos y micoestrógenos, éstos se han analizado mediante GC en pocas ocasiones debido a su baja volatilidad (Hájková y cols., 2007; Soliman y cols., 2004). Sin embargo, dado que su derivatización es factible, la versatilidad de la técnica para este fin se ha visto incrementada.

Una de las reacciones de derivatización más comunes en GC y que es utilizada para el análisis de este grupo de analitos, es la la sililación, que se produce por sustitución de los hidrógenos activos de ácidos, alcoholes, tioles, aminas, amidas y cetonas y aldehídos enolizables por grupos trimetilsilil (TMS). (Azzouz y cols., 2011; Capriotti y cols., 2013). El derivado resultante se caracteriza por tener una menor polaridad, mayor volatilidad, mayor estabilidad térmica y, en última instancia, un patrón de fragmentación más claro que la molécula de origen (Pacáková y cols., 2009).

Entre los reactivos de derivatización más comunes que conducen a la formación de TMS-derivados se encuentran el N-metil-N-trimetilsililtrifluoroacetamida (MSTFA), trimetilyodosilano (TMIS) y el N-O-bis-(trimetilsilil)-trifluoroacetamida (BSTFA), que resultan particularmente eficaces en matrices agroalimentarias (Capriotti y cols., 2013). En lo que respecta a los compuestos estudiados en la presente Tesis Doctoral, se ha demostrado que el BSTFA resulta más eficaz, asegurando una mejor selectividad y una mayor sensibilidad en GC-MS (Ding y Chiang, 2003).

En la Figura I.13 se representa la reacción de sililación que tiene lugar entre un analito que contiene un grupo hidroxilo y el BSTFA. Con el objetivo de que la derivatización sea máxima, así como de evitar la

aparición de otros derivados intermedios que puedan interferir en su determinación, se añade a la mezcla de reacción trimetilclorosilano (TMCS), que actúa como catalizador (Azzouz y cols., 2011).

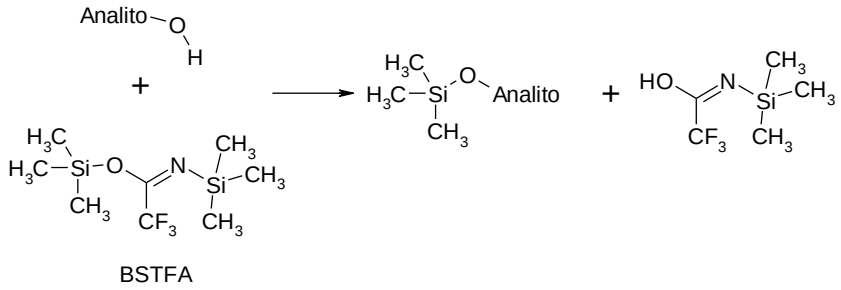


Figura I.13. Reacción de sililación de un alcohol utilizando BSTFA.

A pesar de que la derivatización de estrógenos naturales, sintéticos y micoestrógenos (y, en general, de cualquier otro analito) supone la introducción de una etapa adicional en la metodología analítica utilizada, su utilización mejora significativamente la volatilidad de dichos compuestos, permitiendo su adecuada determinación mediante GC. De hecho, la técnica se ha utilizado para su determinación en muestras biológicas (Biddle y cols., 2006; Blokland y cols., 2006; Caron y cols., 2015; Choi y cols., 2002), medioambientales (González y cols., 2015; Kelly, 2000; Nakamura y cols., 2001; Pacáková y cols., 2009) y alimentarias (Capriotti y cols., 2013; Noppe y cols., 2008; Socas-Rodríguez y cols., 2013a). En la Tabla I.2 se recogen ejemplos de artículos científicos publicados hasta la fecha para la determinación de compuestos con actividad estrogénica mediante GC en leche y derivados lácteos.

Como se puede apreciar en la tabla, para lograr la máxima selectividad en la separación de compuestos estrogénicos se han empleado columnas que contienen una fase estacionaria apolar, del tipo 5% difenil - 95% dimetil polisiloxano, ya que la presencia de grupos fenilo ofrece la posibilidad de que tengan lugar interacciones de van der Waals y de tipo π-π con los anillos aromáticos de los compuestos estrogénicos derivatizados (Dettmer-Wilde y Engewald, 2014).

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Tabla I.2. Ejemplos de artículos publicados sobre la determinación de estrógenos naturales, sintéticos y micoestrógenos mediante GC en leche y derivados lácteos.

Analitos	Muestra	Tratamiento de muestra	Columna	Técnica de análisis	Referencia
E_1, 17β-E_2, 17α-E_2, E_3	Leche de vaca, nata, mantequilla, yogur y queso	Hidrólisis enzimática, SPE, LLE y HPLC semipreparativa	DB-5MS (0,25 mm d.i. x 30 m)	GC-MS (Q)	Hartmann y cols., 1998
DES, 17β-E_2, HEX, α-ZAL, β-ZAL, α-ZEL, β-ZEL, ZEN	Leche humana	SPE	Ultra 2 - SE-54 (0,2 mm d.i. x 25 m)	GC-MS (IT)	Choi y cols., 2002
E_1, 17α-E_2, 17β-E_2	Leche de vaca	Hidrólisis enzimática, LLE y SPE	ZB-5MS (0,25 mm d.i. x 30 m)	GC-MS/MS (QqQ)	Courant y cols., 2007
E_1, 17α-E_2, 17β-E_2	Leche de vaca	Hidrólisis enzimática, SPE, LLE y HPLC semipreparativa	ZB-5MS (0,25 mm d.i. x 30 m)	GC-MS/MS (QqQ)	Courant y cols., 2008
E_1, 17β-E_2, EE_2	Leche de vaca, de cabra y humana	SPE	DB-5MS (0,25 mm d.i. x 30 m)	GC-MS (Q)	Azzouz y cols., 2011
E_1, 17α-E_2, 17β-E_2, EE_2, E_3	Mantequilla	MWCNTs-MSPD	DB-5MS (0,25 mm d.i. x 30 m)	GC-MS (Q)	Su y cols., 2011
E_1, 17α-E_2, 17β-E_2, EE_2, E_3	Leche de vaca	HF-SEBLLME asistida con microondas	DB-5MS (0,25 mm d.i. x 30 m)	GC-MS (Q)	Xu y cols., 2013

Por otro lado, también se puede observar en la Tabla I.2 que en todos los casos se ha llevado a cabo el acoplamiento de la técnica con un detector de MS, haciendo uso de la ionización por impacto electrónico (EI) y de un analizador de tipo cuadrupolo simple (Q), triple cuadrupolo triple (QqQ) o trampa de iones (IT).

I.3.2. Cromatografía de líquidos

El desarrollo de la LC también se ha visto influenciado, sobre todo durante los últimos años, por las tendencias de la Química Verde, sobre todo en lo que respecta al consumo de disolventes.

Entre estas tendencias se encuentran, entre otros: i) el empleo de fases estacionarias con grupos funcionales del tipo -CN, -NH_2, -C_8, etc. que requieren un mayor contenido de agua en la fase móvil, disminuyendo así el uso de disolventes orgánicos (De La Guardia y Garrigues, 2012); ii) la reducción del tiempo de análisis mediante el empleo de columnas cromatográficas más cortas, empaquetadas con fases estacionarias con un diámetro reducido de partícula, inferior a 3 µm (Veuthey y cols., 2006); y iii) el empleo de columnas cromatográficas con d.i. inferior a 300 µm, lo que genera una importante reducción del flujo de fase móvil de mL/min a nL/min o µL/min (Vissers, 1999).

Tabla I.3. Clasificación de los tipos de LC (Agilent, library; Noga y cols., 2007).

Denominación	D.i. de la columna	Flujo de fase móvil utilizado (valores más comunes entre paréntesis)
Nano-LC	25-100 µm	100-500 nL/min
Nano-LC/LC capilar	150 µm	300-500 nL/min
LC capilar	300-500 µm	1-20 µL/min (4-12 µL/min)
Micro-LC	1,0 mm	20-100 µL/min (50 µL/min)
***Narrow bore*-LC**	2,1 mm	0,1-0,5 mL/min (0,2 mL/min)
LC	4,6 mm	0,2-2,0 mL/min (1 mL/min)
LC semipreparativa	8-20 mm	>2 mL/min

La Tabla I.3 muestra una clasificación de los diferentes tipos de LC atendiendo al d.i. de la columna y al flujo de la fase móvil. Como se puede observar, en nano-LC tanto el d.i. de la columna, como el flujo son del orden de entre 60 y 5000 veces menores respecto a los de un sistema LC tradicional (Agilent, library; Noga y cols., 2007).

I.3.2.1. Nano-cromatografía líquida

La nano-LC se ha desarrollado sobre los mismos principios de la cromatografía convencional, aunque todos los componentes del sistema, como las bombas, el inyector, la columna y los detectores se han reducido en sus dimensiones.

En este tipo de cromatografía se utilizan columnas capilares de sílice fundida de d.i. entre 25 y 150 µm. Entre las columnas utilizadas destacan, por un lado, las denominadas "de tubo abierto" (*open tubular*), muy poco utilizadas, y, por otro, las columnas monolíticas y las empaquetadas (Pesek y Matyska, 2000).

Las columnas monolíticas se obtienen mediante la polimerización *in-situ* de un monómero orgánico adecuado (Guiochon, 2007) o bien a partir de un proceso sol-gel dentro de la columna (Li y cols., 2004). En este último caso, se obtiene una columna a base de sílice. El monolito constituye una única estructura sólida con un reticulado con poros de pequeñas y grandes dimensiones. Por este motivo, la estructura es altamente porosa y muy permeable, pudiéndose emplear elevados flujos de fase móvil con bajas contrapresiones, lo que reduce considerablemente los tiempos de análisis. A pesar de que tienen evidentes ventajas por su porosidad, robustez y versatilidad, existen muy pocas columnas de este tipo disponibles comercialmente y la polimerización en el propio laboratorio requiere una gran experiencia y un elevado control de las condiciones experimentales para asegurar una reproducibilidad adecuada.

En lo que a las columnas empaquetadas se refiere, éstas se rellenan con una fase estacionaria constituida por partículas, generalmente a base de sílice o de un material polimérico, de dimensiones entre 2-5 µm de diámetro de partícula (Angus y cols., 2000). Según el mecanismo de separación que se persiga, la fase estacionaria puede funcionalizarse en su superficie, tal y como sucede en la LC convencional. En este sentido, se puede emplear sílice modificada con una cadena hidrocarbonada como C_4, C_8, C_{18}, C_{30} o carbón grafitizado para conseguir un mecanismo de interacción de tipo hidrofóbico; sílice modificada con grupos fenilo para conseguir una interacción de tipo π-π; sílice modificada con grupos -CN para interacciones polares; o resinas de intercambio iónico, sílice modificada con grupos amino, amida, sílice o diol para conseguir interacciones hidrofílicas (HILIC).

Hoy en día existe una gran variedad de columnas de nano-LC rellenas disponibles comercialmente (Nazario y cols., 2015). Sin embargo, su coste aún sigue siendo muy elevado. Para superar este inconveniente y, sobre todo, para posibilitar el relleno de las columnas con la fase estacionaria más apropiada para cada aplicación, diversos

laboratorios han optado por fabricar sus propias columnas desarrollando una metodología de empaquetamiento específica. En este sentido, se han propuesto diferentes técnicas de empaquetamiento para la preparación de columnas capilares, que principalmente son versiones ligeramente modificadas de las ya utilizadas para preparar columnas de LC convencional. Cabe destacar que la introducción de la fase estacionaria en la columna se ha llevado a cabo tanto por gravedad (Kimberly y cols, 1998) como utilizando un gas (Crescentini y cols., 1988) o un líquido a alta presión (Broquaire, 1979; Linder, 1976), fluidos supercríticos (Malik y cols., 1993), mediante fuerza centrípeta (Fermier y Colón, 1998) o utilizando una diferencia de potencial para generar flujo electroosmótico (EOF) (Yan y cols., 1995).

Hasta la fecha, el método de empaquetamiento más utilizado, ya sea por su facilidad operativa como por la disponibilidad de la instrumentación necesaria, ha sido el denominado método de *slurry*. En la presente Tesis Doctoral, este método ha sido utilizado para el empaquetamiento de columnas capilares para nano-LC y CEC siguiendo el procedimiento descrito por Fanali et al. (Fanali y cols., 2004; 2007). La Figura I.14 muestra un esquema del sistema empleado para llevar a cabo este tipo de empaquetamiento. El capilar de sílice fundida se conecta por un lado a un frit mecánico y, por el otro, a un depósito de acero de 1 mL en el que se coloca una suspensión o *slurry* de fase estacionaria en un disolvente apropiado, que generalmente es acetona.

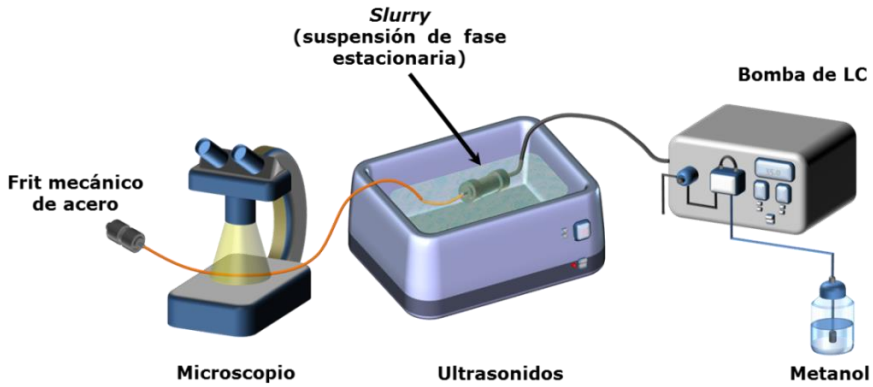

Figura I.14. Esquema del sistema utilizado para llevar a cabo el empaquetamiento de capilares mediante el método de *slurry*.

La bomba de LC conectada al depósito bombea un disolvente orgánico, que suele ser metanol (MeOH), a través del mismo a una presión de unos 300-350 bar, con lo que se consigue introducir de manera gradual la suspensión en el capilar. Para conseguir un lecho de

fase estacionaria homogéneo, el capilar se coloca en el interior de un baño de ultrasonidos durante todo el proceso. El control en tiempo real de la fase de empaquetamiento se realiza mediante un microscopio óptico, como se puede observar en la Figura I.15.

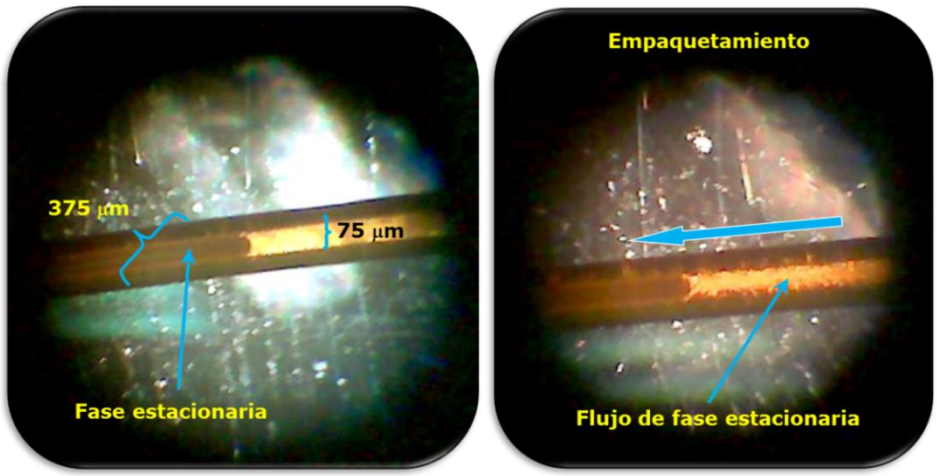

Figura I.15. Imagen del microscopio óptico del empaquetamiento de un capilar de nano-LC.

Para mantener la fase estacionaria dentro del capilar, se procede a la fabricación de los frits que deben tener una alta resistencia mecánica, elevada permeabilidad y longitud corta. Es altamente deseable que la técnica de fabricación sea sencilla, rápida y perfectamente reproducible. Para ello, se han utilizado frits mecánicos, monolíticos, frits de sílice sinterizada, etc. (Xue y cols., 2015), cada uno de los cuales presenta sus correspondientes ventajas e inconvenientes.

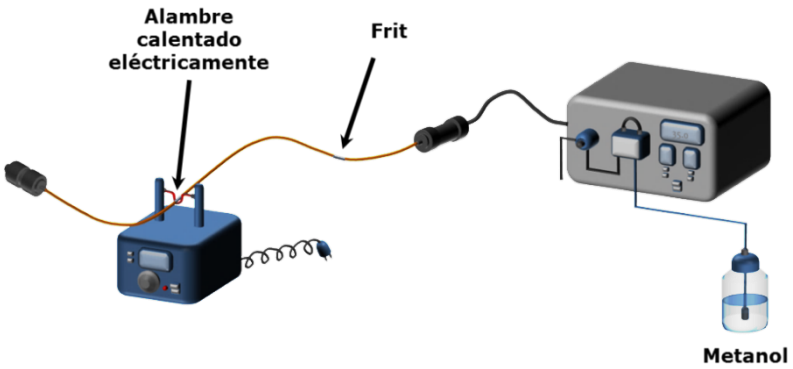

Figura I.16. Esquema del procedimiento de preparación de los frits de sílice sinterizada.

A pesar de que la preparación de los frits de sílice sinterizada tiene una reproducibilidad más baja respecto a otros métodos, la sencillez del procedimiento ha hecho que su empleo se haya extendido ampliamente. Para su preparación, una vez finalizada la etapa de empaquetamiento el capilar se lava con agua destilada a una presión de 300 bar y, sucesivamente, se coloca sobre un alambre calentado eléctricamente a 700 °C durante algunos segundos (Fanali y cols., 2004), tal como se muestra en la Figura I.16.

I.3.2.1.1. Incremento de la sensibilidad en la nano-cromatografía líquida

Aunque la nano-LC presenta grandes ventajas como la baja dilución cromatografía y la elevada eficacia, el pequeño volumen de inyección (menor de 100 nL) hace que, en general, la sensibilidad no supere a la alcanzada en la LC de alta eficacia (HPLC) convencional (Vissers, 1999). Además, en el caso de los detectores espectrofotométricos, la celda de detección está en la propia columna para minimizar la dispersión, con lo que el paso óptico coincide con el d.i. de la misma (≤ 100 μm).

Con el objetivo de mejorar la sensibilidad, se han propuesto diferentes soluciones: i) emplear celdas de detección más largas, con forma de "U" o de "Z" (Prüß y cols., 2003); ii) utilizar detectores más sensibles que el de UV-Vis como, por ejemplo, un espectrofluorímetro o un MS (Vissers, 1999) o iii) inyectar grandes volúmenes de muestra en condiciones de focalización (Héron y cols., 2000; Malcolm y cols., 1997; Vissers y cols. 1996).

Es importante destacar que dado que la duración de la etapa de inyección depende del volumen de muestra inyectado, y éste depende a su vez del flujo de la fase móvil, para inyectar un volumen de 20 μL en una columna de 4,6 mm de d.i. a una velocidad de flujo de 1 mL/min se requieren sólo unos segundos, mientras que para inyectar unos cientos de nL en una columna capilar a un flujo de 200 nL/min, se requieren desde varias decenas de segundos hasta varios minutos. En este último caso, el *plug* de inyección, que teóricamente debería ser estrecho, se extiende durante el tiempo de inyección provocando una sobrecarga de la columna (Prüß y cols., 2003). Por este motivo, la inyección de grandes volúmenes puede empeorar la separación cromatográfica en términos de selectividad y eficacia, ya que la capacidad de carga de las columnas capilares es extremadamente reducida.

Una posible solución a este último problema es hacer uso de la técnica de la focalización en línea (Héron y cols., 2000; Malcolm y cols., 1997; Vissers y cols. 1996), que consiste en disolver la muestra en un

disolvente con una fuerza elutrópica más baja con respecto a la fase móvil inicial, de modo que, cuando la muestra es inyectada, los analitos quedan retenidos en la parte inicial de la columna. Finalizada la etapa de inyección, que puede ser de pocos segundos a algunos minutos, los analitos son eluidos por la fase móvil. La elección del disolvente de focalización depende de los analitos y, sobre todo, de la interacción que estos tengan con la fase estacionaria (Buonasera y cols., 2009; D'Orazio y Fanali, 2008)

Esta técnica de focalización también se puede aplicar haciendo uso de una columna "trampa" que se coloca antes de la columna analítica, de forma similar a la denominada extracción en fase sólida (SPE) en línea (Asensio-Ramos y cols., 2011a). Sin embargo, se trata de una solución más compleja y costosa, ya que se debe disponer de un sistema de nano-LC equipado con una micro-bomba y válvulas, siendo también necesario cambiar la columna trampa periódicamente con el consecuente gasto económico.

I.3.2.1.2. Acoplamiento con la espectrometría de masas

Los primeros intentos de acoplamiento de una técnica miniaturizada de LC con la MS se realizaron a finales de los años 70 y principios de los 80, gracias a los resultados obtenidos por Takeuchi y cols. (Takeuchi y cols., 1978) y Henion y Maylin (Henion y Maylin, 1980). En este último caso, los autores describieron la introducción directa de la salida de una columna de 500 µm de d.i. en un MS mediante una interfase que denominaron "interfase de introducción directa del líquido" (*direct liquid introduction*). A mitad de la década de los 80, Tsuda y cols. (1985) utilizaron esa misma interfase con una columna de 50 µm de d.i. empaquetada. Seis años más tarde, Caprioli y cols. (1986) desarrollaron la interfase de bombardeo por átomos rápidos en flujo continuo (CF-FAB) y a finales de los años 80, Moseyley y cols. (1989) la acoplaron a una columna de 50 µm de d.i.

Sin embargo, la gran revolución en este campo fue, indudablemente, la construcción de las interfases de ionización a presión atmosférica (API). Entre ellas, las basadas en el fenómeno de ionización por electrospray (ESI) han predominado en todos los campos de aplicación por su alta compatibilidad con la nano-LC. En este sentido, basándose en los experimentos preliminares de Dole, Iribarne y Thomson (Dole y cols., 1968; Gieniec y cols., 1984; Iribarne y Thomson, 1979; Thomson y Iribarne, 1979), Fenn y sus colaboradores estudiaron la teoría del fenómeno del electrospray y desarrollaron la tecnología del mismo (Fenn y cols., 1990; Whitehouse y cols., 1985). El proceso que tiene lugar en la ESI consiste, no sólo en la nebulización de un líquido para

formar un aerosol de pequeñas gotas altamente cargadas, sino también en la ionización de los analitos. Este proceso se inicia mediante la aplicación de un potencial eléctrico de varios kV entre un capilar metálico por el que fluye el líquido y un electrodo conectado a tierra, que es el orificio de entrada al espectrómetro de masas. Cuando se aplica dicha diferencia de potencial, el menisco del líquido que sale del capilar se deforma, generando un cono que se conoce con el nombre de *"cono de Taylor"*. Posteriormente, la hipótesis teórica establece que: i) las pequeñas gotas abandonan el cono para generar sucesivos conos, hasta la formación de un gas de iones y ii) las gotas abandonan el cono y comienzan a evaporarse hasta que la carga superficial y la densidad de carga aumentan hasta un cierto límite, definido como *"límite de Reyleigh"*, tras el cual tienen lugar explosiones de Coulomb, formando finalmente un gas de iones.

Para aumentar la eficacia de la ionización y, por tanto, la sensibilidad, se puede utilizar una interfase de bajo flujo, denominada interfase nano-ESI (Wilm y Mann, 1996). Esto, conjuntamente con la aplicación de bajos potenciales y con una mejor desolvatación de los iones en fase gaseosa, mejoran la eficacia del fenómeno de ionización (Gelpí, 2002). En este sentido, es importante tener en cuenta que se ha demostrado que la eficacia de la ionización en la ESI disminuye al aumentar el flujo en la interfase (Abian y cols., 1999; Oosterkamp y cols., 1998).

Experimentalmente, la interfase nano-ESI propuesta por Abian y cols. (1999) y Davis y cols. (1995) consiste en unir la salida de la columna capilar (normalmente de 75 µm d.i. x 25 cm) a un capilar de 25 µm de d.i. en forma de punta, denominado emisor-ESI (Andren y cols., 1994), a través de una pieza de acero de volumen muerto cero. El dispositivo se dispone sobre un soporte con control de movimiento XYZ para ajustar la punta a pocos milímetros del orificio del espectrómetro. La ionización se genera por la aplicación de 2 kV a la unión de acero (Figura I.17), demostrándose que el acoplamiento de un sistema cromatográfico miniaturizado a través de una interfase nano-ESI resulta ser más sensible que un sistema LC-MS que utiliza una interfase ESI tradicional (Legido-Quigley y cols., 2002). La Figura I.17 muestra un esquema de un sistema de nano-LC fabricado en el laboratorio acoplado a la MS a través de una interfase nano-ESI (Fanali y D'Orazio, 2006). Esta interfase ha sido utilizada en la presente Tesis Doctoral.

Es importante destacar que en la bibliografía se han propuesto diferentes métodos para llevar a cabo el análisis de compuestos con actividad estrogénica mediante el acoplamiento de la MS con técnicas cromatográficas convencionales (HPLC y GC) en matrices tanto biológicas

(Choi y cols, 2002; Vanhaecke y cols., 2011), como medioambientales (Gorga y cols., 2014; Petrovic y cols., 2002; Tomšíková y cols., 2012) y alimentarias (Azzouz y cols., 2011; Socas-Rodríguez y cols., 2013a). Sin embargo, en lo que se refiere al uso de técnicas miniaturizadas, en el momento de la realización de esta Tesis Doctoral, sólo existía un trabajo en el que se separara y analizara un grupo de estrógenos (E_1, E_3, 17α-E_2, 17β-E_2, EE_2) mediante LC capilar acoplada a la MS en tándem (MS/MS) desarrollado por Kozlík y cols. (2011).

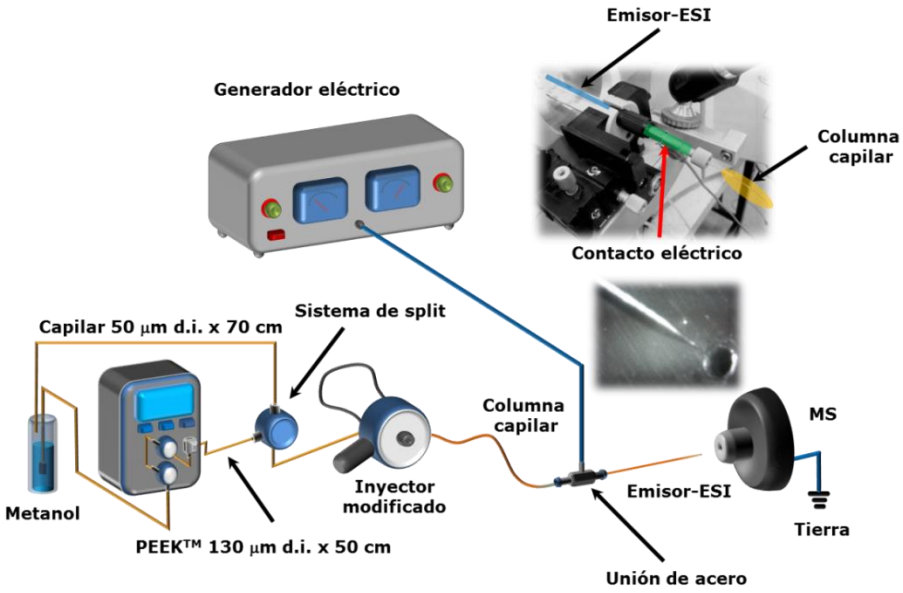

Figura I.17. Esquema del sistema nano-LC-MS utilizando una interfase nano-ESI.

I.3.3. Electroforesis capilar

La CE permite llevar a cabo separaciones rápidas y con una alta eficacia de compuestos con o sin carga, dependiendo del modo de separación, a partir de un pequeño volumen de muestra. Para ello, se aplica una diferencia de potencial de hasta 30 kV en los extremos de un capilar de sílice fundida de 10-100 µm de d.i. en el que se introduce una disolución denominada electrolito de fondo (BGE) o electrolito de separación.

La muestra se puede inyectar en el capilar por aplicación de una diferencia de potencial (inyección electrocinética), de presión o incluso mediante la aplicación de vacío en uno de los extremos del capilar (inyección hidrodinámica). Una vez inyectada la muestra, y debido a la influencia del campo eléctrico, las especies cargadas y no cargadas se mueven como resultado de la suma de su movilidad electroforética (μ_e),

de su tamaño y carga y de la movilidad del EOF (μ_{EOF}). Este último se puede modificar o incluso inhibir añadiendo determinados componentes al BGE o incluso modificando la superficie interna del capilar.

Los modos de separación empleados en CE, se pueden clasificar en dos grupos (Buszewski y cols., 2013). El primero se basa únicamente en los principios de separación electroforéticos e incluye la electroforesis capilar en zona (CZE), la electroforesis capilar en gel (CGE), el isoelectroenfoque capilar (CIEF), la isotacoforesis capilar (CITP) y la electroforesis capilar en medio no acuoso (NACE). El segundo modo combina tanto los principios de separación electroforéticos como los cromatográficos, e incluye técnicas como la electrocromatografía capilar (CEC) y la cromatografía electrocinética (EKC) (de Boer y cols., 1999). Dentro de la EKC existen a su vez diferentes modalidades en función del tipo de fase pseudo-estacionaria (PSP) empleada, entre las que destaca la cromatografía electrocinética micelar (MEKC), que se describirá a continuación, dado que ha sido empleada en esta Tesis Doctoral.

Es importante destacar que a pesar del hecho de que los compuestos con actividad estrogénica estudiados en esta Tesis Doctoral tengan un pK_a superior a 7,5 (Tabla I.4) y, por lo tanto, sean neutros en un amplio rango pH, también han sido separados y cuantificados mediante CZE (Tabla I.5) utilizando tampones muy básicos. En particular se han analizado en aguas (Li y cols., 2013) en fluidos biológicos (Kuehnbaum y Britz-McKibbin, 2011) y harina (Arribas y cols., 2009), aunque no en leche y derivados.

Tabla I.4. Algunas propiedades de los compuestos con actividad estrogénica analizados en la presente Tesis Doctoral

Analitos	**Acrónimo**	**Mw (g/mol)**	**pK_a[a)]**
Estrona	E_1	270,4	10,25
17α-Estradiol	17α-E_2	272,4	10,27
17β-Estradiol	17β-E_2	272,4	10,27
17α-Ethinilestradiol	EE_2	296,4	10,24
Estriol	E_3	288,4	10,25
2-Methoxiestradiol	2-MeOE_2	302,4	10,29
Dienestrol	DS	266,3	9,21
α-Zearalanol	α-ZAL	322,4	8,08[b)]
β-Zearalanol	β-ZAL	322,4	8,08[b)]
α-Zearalenol	α-ZEL	320,4	7,61[b)]
β-Zearalenol	β-ZEL	320,4	7,61[b)]
Zearalenone	ZEN	318,4	7,58[b)]

a) Dato extraído de la base de datos SciFinder (Scifinder); b) correspondiente al pK_{a1}.

Tabla I.5. Algunos ejemplos de trabajos publicados en la bibliografía sobre la determinación de estrógenos naturales, sintéticos y micoestrógenos mediante CE.

Analitos	Muestra	Tratamiento de muestra	BGE	Técnica de análisis	Referencia
α-ZEL, β-ZEL, ZEN	Harina de maíz	SFE	25 mM tampón borato, pH 9,2	CE-AD	Arribas y cols., 2009
E_1, E_2, , EE_2, E_3	Orina	-	50 mM NH_4HCO_3, pH 9,5	CE-TOF/MS	Kuehnbaum y Britz-McKibbin, 2011
E_1, 17β-E_2, E_3	Agua de río	LLE (dietiléter)	60 mM NaOH, pH 12,8	CE-ECD	Li y cols., 2013

El empleo de la CE ofrece, con respecto a la HPLC, numerosas ventajas entre las que destacan la reducción de los tiempos de análisis, las elevadas eficacias de las separaciones, el bajo consumo de disolventes y, sobre todo, de muestra (Koel y cols., 2006). Sin embargo, dado que la separación tiene lugar en un capilar, tal y como ocurre en nano-LC, existe una limitación clara en lo que a la sensibilidad se refiere debido al bajo volumen de inyección y al pequeño paso óptico. Para solventar este inconveniente, se han utilizado métodos de pre-concentración en línea y fuera de línea, o bien sistemas de detección más sensibles, como la detección electroquímica (ED), la detección por fluorescencia inducida por láser (LIF) o la MS.

En lo que respecta a las técnicas de pre-concentración en línea, éstas se pueden considerar como casos particulares de las técnicas de electromigración. Básicamente, se pueden distinguir dos técnicas claramente diferenciadas: el *stacking* y el *sweeping*. Ambas se basan en las diferencias de velocidad de los analitos en el seno del *plug* de inyección con respecto al BGE. En ambas técnicas, existen a su vez diferentes modos de preconcentración que a su vez dependen del tipo de inyección llevado a cabo (Breadmore, 2007).

De todas ellas, la técnica de *stacking* más utilizada hasta la fecha, y también la empleada en la presente Tesis Doctoral, es el "*stacking* en modo normal" (NSM), también denominado frecuentemente como "preconcentración de muestra por amplificación de campo" (FASS), introducida por Mikkers y colaboradores en 2007 (Breadmore, 2007; Mikkers y cols., 1979). En este caso, la muestra se encuentra en un medio con una conductividad inferior a la del BGE y es inyectada hidrodinámicamente en el capilar. Al aplicar una diferencia de potencial,

el *plug* de muestra se ve sometido a un campo eléctrico más elevado que el del BGE, lo que hace que los analitos se muevan rápidamente hacia la interfase con el BGE, donde se acumulan y pre-concentran en bandas muy estrechas.

I.3.3.2. Cromatografía electrocinética micelar

En 1984, Terabe y cols. (1984) introdujeron una nueva modalidad de CE, denominada EKC, que se podía llevar a cabo con la misma instrumentación que la utilizada en la CZE. La técnica supuso un importante punto de inflexión en el desarrollo de la CE, dado que permitió separar una amplia variedad de compuestos neutros, algo que no podía realizarse mediante otros modos de CE desarrollados hasta esa fecha.

Cuando al BGE se le adiciona un tensioactivo (aniónico, catiónico, zwitteriónico, no iónico o incluso mezclas de los anteriores) por encima de su concentración micelar crítica (CMC), se forman micelas que interaccionan con los analitos cargados y neutros. Las micelas se comportan como una especie de fase estacionaria y, de hecho se las denomina PSPs, siendo el mecanismo de reparto de los analitos entre el BGE y las propias micelas la base del principio de la separación. Fruto de todo lo anterior, la técnica recibió el nombre de EKC micelar (MEKC). Sin embargo, la EKC no sólo está limitada al uso de micelas, sino que también se pueden obtener PSPs utilizando, por ejemplo, microemulsiones (Altria, 2000) o dendrímeros (Peric y Kenndler, 2003), entre otros.

En MEKC se suelen utilizar tensioactivos iónicos para la separación de analitos neutros, siendo el dodecilsulfato sódico (SDS, con una CMC de 8 mM a 25 °C) el tensioactivo aniónico más empleado (Nishi y Terabe, 1996) para trabajar en modo normal, o el bromuro de cetiltrimetilamonio (CTAB) para trabajar en modo inverso, es decir, invirtiendo la dirección del EOF (Su y cols., 2009).

Cuando el analito se encuentra en el interior del BGE, puede interaccionar con la micela de tres formas diferentes (de Boer y cols., 1999): i) con la superficie de la misma mediante interacciones electroestáticas o fuerzas dipolo-dipolo; ii) con el centro de la micela mediante interacciones de tipo hidrofóbico o iii) comportándose como un co-tensioactivo, participando en la formación de la propia micela (Figura I.18). Estos mecanismos son dinámicos y están gobernados por el equilibrio de reparto de los analitos entre la PSP micelar y el BGE (Hancu y cols., 2013).

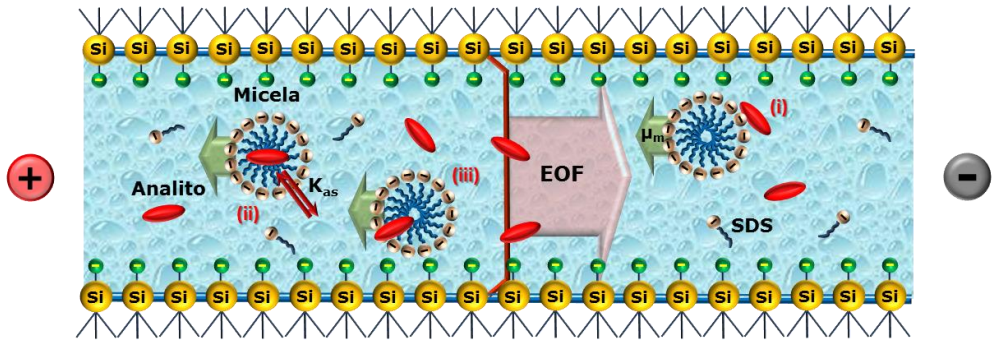

Figura I.18. Mecanismos de separación en MEKC utilizando micelas aniónicas.

En lo que respecta al sistema de detección más empleado en MEKC, éste es, sin duda alguna, el espectrofotométrico por absorción UV-Vis (Hancu y cols., 2013). Sin embargo, y a pesar de su amplía aplicabilidad a un bajo precio, la detección en el capilar y la absorción asociada al tensioactivo hacen que la sensibilidad de la técnica sea baja (Tomer, 2001).

La Tabla I.6 recoge los trabajos publicados hasta la fecha sobre la separación de estrógenos naturales, sintéticos y micoestrógenos mediante MEKC (Bykova y Holland, 2008; Fogarty y cols., 2000; Gañán y cols., 2014; Harino y cols., 2001; Katayama y cols., 2003; Ji y cols., 1995; Silva y cols, 2007; Sirén y cols., 2008; Tripodi, y cols., 2006) o mediante la cromatografía electrocinética de microemulsión (MEEKC) (Tripodi y cols, 2006).

La técnica de pre-concentración en línea más utilizada en MEKC es, sin lugar a dudas, el *sweeping*. En este caso, los analitos neutros se disuelven en un medio idéntico al BGE pero sin micelas y se inyectan electrocinéticamente. Bajo la aplicación del campo eléctrico, las micelas del BGE se desplazan a través del *plug* de muestra, generando una acumulación de los analitos en el frente de las micelas que se mueven. Después de que la zona de muestra ha sido "barrida" por las micelas, tiene lugar la separación (Quirino y Terabe, 1999). A pesar del importante incremento de sensibilidad obtenido, el NSM ha alcanzado en ocasiones mejores resultados que el *sweeping* en MEKC, como demostraron Quirino y Terabe para la separación de ciertas moléculas neutras (Quirino y Terabe, 1997a; Quirino y Terabe, 1997b).

Tabla I.6. Algunos ejemplos de trabajos publicados en la bibliografía sobre la determinación de estrógenos naturales, sintéticos y micoestrógenos mediante MEKC.

Analitos	Muestra	Tratamiento de muestra	BGE	Técnica de análisis	Referencia
E_1, E_2, E_3	Orina	Hidrólisis, LLE (diclorometano)	5 mM borato, 5 mM fosfato, 75 mM colato sódico a pH 8,86 y 20% ACN	MEKC-DAD	Ji y cols., 1995
E_1, 17β-E_2, E_3, EE_2	Agua de río	-	20 mM tampón CAPS, 25 mM SDS; 15% ACN	MEKC-DAD	Fogarty y cols., 2000
E_1, 17α-E_2, EE_2	Agua	SPE (C18)	75 mM SDS, pH 7,0	MEKC-UV	Harino y cols., 2001
E_1, E_2, E_3	Suero	LLE (cloroformo)	10 mM tampón borato-ácido fosfórico, pH 8 conteniendo 50 mM sal sódica del ácido cólico	MEKC-UV	Katayama y cols., 2003
E_1, E_2, E_3	Fármacos	Extración (MeOH)	Sistema de microemulsión AOT	MEEKC-UV	Tripodi y cols., 2006
E_1, 17β-E_2, E_3, EE_2	Orina	LLE (diclorometano)	20 mM SDS en 20 mM tampón tetraborato, pH 9,4 y 20% EtOH	MEKC-DAD	Silva y cols., 2007
E_1, 17α-E_2, EE_2	Plasma de pescado	LLE (acetato de etilo), SPE (NR_4^+)	30 mM SDS, 13 mM hidroxipropil-β-CD, 200 mM tampón fosfato, pH 2,5	MEKC-DAD	Bykova y Holland, 2008
17β-E_2	Orina, suero	Orina: hidrólisis enzimática, SPE, evaporación y disolución Suero: desproteinización	29,3 mM SDS y 1,1 mM taurocolato sódico en 20 mM acetato de amonio, pH 9,68	PF-MEKC-ESI-MS	Sirén y cols., 2008
E_1, 17β-E_2, EE_2	Leche fresca de cabra, leche de cabra preñada	MSPD-MIP(E_2)	25 mM tampón borato, pH 9,3, 10 mM SDS, 20% ACN	MEKC-DAD	Gañán y cols., 2014

I.3.3.2.1. Acoplamiento con la espectrometría de masas

El acoplamiento de la MS con las técnicas de electromigración no resulta sencillo. Debido al bajo flujo de trabajo (nL/min) y a la necesidad de mantener una elevada diferencia de potencial entre los extremos del capilar de separación y, por consiguiente, una alta corriente, el acoplamiento CE-MS se puede llevar a cabo sólo con ciertas interfases.

Desde la publicación del primer trabajo de CE-MS en 1987 por Smith y su grupo (Nguyen y cols., 1987), se han desarrollado diferentes interfases construidas en el laboratorio, cuyas prestaciones y aplicaciones han sido demostradas en una gran variedad de trabajos (Klampfl, 2009; Schmitt-Kopplin y cols., 2003).

Habitualmente el acoplamiento de la CE con la MS se consigue utilizando una ESI comercial con líquido adicional, por la posibilidad que ofrece para realizar análisis rutinarios. Esta interfase, desarrollada por Banks en 1995 (Banks Jr., 1995) es un tubo triple coaxial con toma de tierra (Bertsch y cols., 1996) (Figura I.19). El capilar es en realidad el tubo más interno que está rodeado coaxialmente por un capilar de acero por el que fluye el líquido adicional, que suele ser un disolvente orgánico o acuo-orgánico que contribuye al cierre del contacto eléctrico y favorece la ionización de los analitos. Por el tubo externo circula un gas inerte, normalmente nitrógeno, que se utiliza para asistir la nebulización en la fuente de ionización y crear así el correspondiente aerosol.

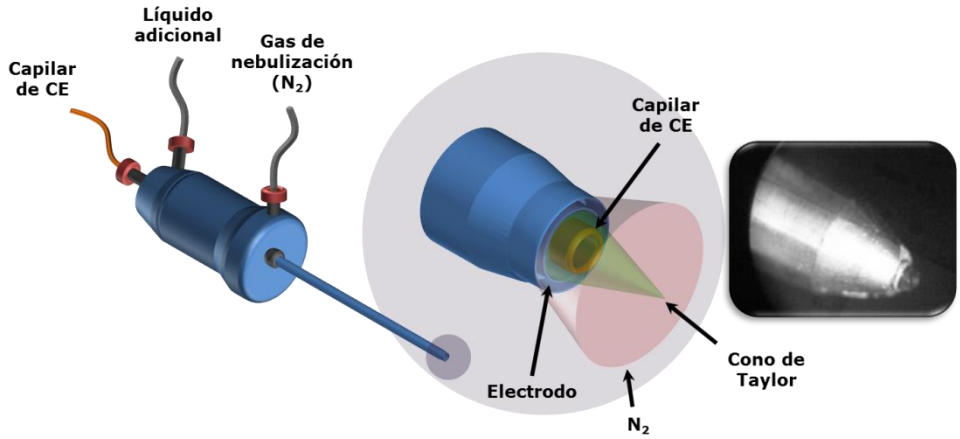

Figura I.19. Dibujo de la ESI con líquido adicional e imagen fotográfica de la misma.

Esta interfase presenta una serie de limitaciones debidas principalmente al elevado flujo de líquido adicional requerido (de 2 a 5 µL/min) que contribuye a la dilución y a la mezcla de las bandas electroforéticas, provocando así una cierta pérdida de sensibilidad y resolución. A pesar de ello, ésta constituye la mejor solución comercial,

dada su alta robustez, buena repetibilidad, reproducibilidad y sencillez (Klampfl, 2009).

En lo que respecta al acoplamiento MEKC-MS, los tensioactivos no volátiles utilizados normalmente, como el SDS, interfieren negativamente en el acoplamiento a través de una interfase ESI. Este tipo de aditivo puede provocar deposiciones y contaminaciones en la interfase y, sobre todo, una elevada supresión iónica (Yang y Lee, 1997).

La estrategia más común en MEKC-MS para impedir que la PSP entre en la interfase y también para preconcentrar parcialmente a los analitos (Quirino y Haddad, 2009), es el empleo de la denominada técnica de llenado parcial (*partial filling,* PF) (Nelson y cols., 1996), que se basa en la menor movilidad aparente que muestra la PSP hacia la salida del capilar con respecto al EOF y a los analitos. La técnica se ha aplicado con éxito para el análisis de diferentes compuestos como esteroides, lactonas, fármacos, herbicidas (Somsen y cols., 2010) y, también, aunque en una sola ocasión, para la determinación del estrógeno natural 17β-E_2 en orina y suero (Tabla I.6) (Sirén y cols, 2008).

A pesar de que estos estudios han demostrado que el PF reduce la supresión iónica y aumenta la sensibilidad, su uso también plantea ciertas desventajas. Por un lado, la limitación del tiempo efectivo de análisis, que viene dado por la migración de las micelas hacia la interfase y, por otro, la necesidad de acondicionar el capilar entre análisis, contaminando en cierta medida la interfase.

Otro modo de impedir que la PSP pueda contaminar la fuente de ionización es el empleo del método de migración inversa de micelas (*reverse-migrating micelles*, RMM) (Somsen y cols., 2010). En este caso, la pared del capilar se reviste con un polímero neutro capaz de reducir o incluso eliminar el EOF. De este modo, la PSP cargada negativamente se mantiene en el interior del capilar y sólo los analitos cargados positivamente, o aquellos neutros con una baja interacción con la micela cuando el EOF es bajo, salen del capilar y pueden ser analizados. Aunque este método resuelve el problema del tiempo de análisis, el problema de la contaminación en la etapa de acondicionamiento del capilar persiste, de ahí que sea una técnica muy poco utilizada.

La mejor solución para el acoplamiento MEKC-MS rutinario es el empleo de una PSP que sea compatible con la interfase de ionización. Éste es el caso del uso de polímeros o compuestos semivolátiles (Somsen y cols., 2010). En el primer caso, se han empleado tensioactivos poliméricos de elevado peso molecular (cuya m/z no coincide con la de los analitos) y una CMC extremadamente baja (prácticamente cero), sobre todo en el campo de las separaciones quirales (Hou y cols., 2006;

2007). En el segundo caso, se han utilizado tensioactivos fluorados como el ácido perfluorooctanoico (PFOA, Figura I.20) o el ácido perfluorooctanosulfónico (PFOS), ambos semivolátiles y capaces de formar micelas en medios acuosos.

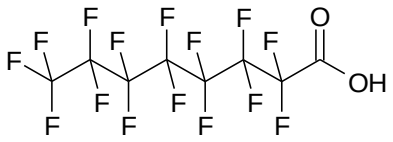

Figura I.20. Estructura del tensioactivo PFOA.

En particular, el PFOA ha proporciona resultados parecidos al SDS y garantiza una elevada compatibilidad con la MS (Ishihama y cols., 2003). Hasta la fecha de realización de esta Tesis Doctoral, su uso sólo ha sido empleado para la separación mediante MEKC-MS de fármacos (Petersson y cols., 2003), plaguicidas, (Moreno-González y cols., 2012; Van-Biesen y Bottaro, 2006) y, recientemente, aminoácidos (Moreno-González y cols., 2013).

I.3.4. Electrocromatografía capilar

La CEC es una técnica de separación híbrida entre la CE y la LC. Además, dado que se emplean columnas capilares de 50-100 µm de d.i., también es considerada una técnica de separación miniaturizada (D'Orazio y cols., 2016; Mistry y cols., 2002).

A pesar de que la miniaturización es en sí una importante ventaja, la capacidad analítica de la técnica ha suscitado un interés adicional debido, por un lado, a su capacidad para separar simultáneamente especies cargadas y no cargadas y, por otro, a su elevada eficacia cromatográfica, por encima de la que normalmente se obtiene con la HPLC (Mistry y cols., 2002).

En la CEC, el movimiento de los analitos y el transporte de la fase móvil ocurren, no sólo por el empuje neumático de una bomba como en la HPLC, sino también por el EOF generado en las paredes del capilar (si se utiliza un capilar de sílice fundida, similar a los utilizados en CE), en la superficie de las partículas de sílice en una columna empaquetada o en la red polimérica de una columna monolítica. De hecho, el EOF se genera debido, mayoritariamente, a la propia fase estacionaria produciendo un movimiento de la fase móvil del orden de varios cientos de nL/min (Keith y cols., 2001).

Tal y como sucede en la CE, las moléculas cargadas se moverán en el seno de un campo eléctrico según sus movilidades electroforéticas

que se sumarán a la del EOF. Las moléculas neutras, en cambio, migrarán con el EOF pero estarán sometidas además a un mecanismo de reparto cromatográfico con la fase estacionaria. A pesar del componente electroforético de la técnica, las ecuaciones que cuantifican los aspectos termodinámicos de la separación cromatográfica, como el coeficiente de reparto, los factores de retención y selectividad, la resolución y la eficacia, etc., siguen siendo válidas en CEC.

La instrumentación que se utiliza para llevar a cabo la CEC (Figura I.21) no es muy diferente a la de la CE, pero la primera incluye un sistema de alta presión de gas para la inyección y evitar así la formación de burbujas en la columna capilar, lo que ya está incluido en los instrumentos de CE actualmente comercializados.

Uno de los principales problemas que plantea la CEC desde un punto de vista operacional, es la generación de burbujas en el interior del capilar, que se manifiesta con un importante aumento del ruido y con la interrupción de la corriente eléctrica y, por lo tanto, del EOF. La formación de las burbujas puede ser generada por efecto Joule pero, sobre todo, por la presencia de frits de sílice sinterizada en la columna capilar. Éstos no sólo generan una cierta heterogeneidad en la permeabilidad de la columna, sino también en el campo eléctrico, en el potencial zeta y, por tanto, en el EOF, lo que provoca la aparición de las burbujas (Pyell, 2000).

Aunque desde hace ya un tiempo se están desarrollando columnas sin frits con fases estacionarias poliméricas (Guiochon, 2007) o de tipo sol-gel (Pyell, 2000), desde un punto de vista instrumental, la formación de burbujas se puede reducir significativamente controlando la temperatura de la columna mediante un sistema de termostatación, o bien aplicando una presión de un gas inerte en ambos extremos de la misma.

Es importante señalar que dado que se emplean instrumentos de CE comerciales, la CEC sólo se puede llevar a cabo en régimen isocrático o en gradiente por pasos (interrumpiendo la separación y cambiando el vial de la fase móvil). Sin embargo, existen ciertas aplicaciones en las se han desarrollado separaciones en régimen de gradiente, cuando se han utilizado instrumentos no comerciales, fabricados en el propio laboratorio (Rimmer y cols., 2000).

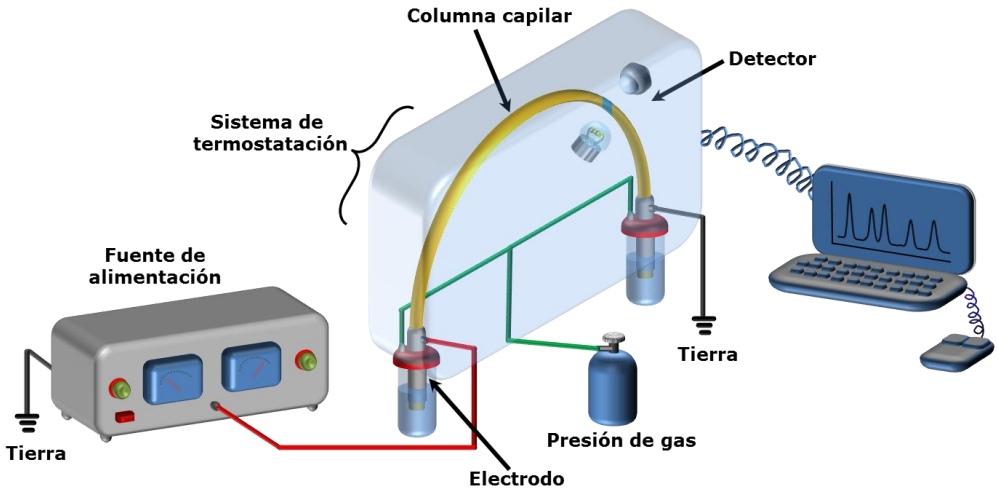

Figura I.21. Esquema básico de un equipo de CEC con detección UV.

I.3.4.3. Acoplamiento con la espectrometría de masas

La baja sensibilidad de la CEC se ha tratado de solventar de forma análoga a la ya descrita para la nano-LC y la CE, es decir, a través del incremento del volumen de inyección, el empleo de técnicas de preconcentración en línea y/o fuera de línea, así como mediante el empleo de sistemas de detección más sensibles como la MS (Apffel y cols., 2000; Klampfl, 2004).

Las interfases utilizadas para el acoplamiento CEC-MS deben garantizar el transporte de las bandas cromatográficas hasta la formación de la nebulización con la menor dispersión posible. Además, debe tenerse en cuenta la necesidad de cerrar el circuito eléctrico y, al mismo tiempo, mantener el electrospray y la corriente eléctrica constante durante el análisis, evitando la formación de burbujas. Por último, y no por ello menos importante, existe un inconveniente respecto a la distancia de separación entre los instrumentos empleados para el acoplamiento. Cualquier solución comercial requiere una distancia mínima de 50 cm entre el equipo de CE/CEC y el de MS, que para una separación en CEC, en la que las columnas más utilizadas suelen ser de 25-30 cm, se traduce en largos tiempos de análisis y en la necesidad de aplicar una diferencia de potencial demasiado elevada.

El acoplamiento CEC-MS se puede llevar a cabo mediante tres tipos diferentes de interfases: i) con líquido adicional (*sheath flow interface*); ii) sin líquido adicional o iii) por unión líquida.

En general, la interfase con flujo de líquido adicional es una solución comercial que se emplea para el acoplamiento CE-MS, como se ha descrito anteriormente. En el caso de la CEC-MS, ésta se utiliza sobre

todo cuando se emplean columnas largas y casi siempre en la modalidad presurizada (pCEC) (Desiderio y Fanali, 2000; Lord y cols., 1995; Lu y cols., 2008), aplicando presión a la entrada de la columna capilar. Sin embargo, tal y como ocurre en el acoplamiento CE-MS, el uso del líquido adicional hace que se produzca, no sólo una dilución de los analitos, sino también una mezcla parcial de las bandas cromatográficas.

La interfase sin líquido adicional es un diseño no comercial en el que el extremo de salida de la columna se diseña en forma de punta, mediante el calentamiento y alargamiento del capilar. A continuación, se reviste de un material conductor, lo que permite el cierre del circuito eléctrico. Este capilar se integra luego en un sistema coaxial de gas (generalmente hexafluoruro de azufre) que ayuda a la formación del spray y evita el efecto de descarga. Este tipo de interfase permite un óptimo transporte de los analitos a través de la columna, evita su dilución y proporciona una elevada sensibilidad. Sin embargo, la inestabilidad de la corriente, sumada a la fragilidad de la punta metálica, la han convertido en una solución poco utilizada (Zamfir, 2007).

Por su parte, la interfase de unión líquida representa una alternativa que combina los dos diseños anteriores. En este caso también se trata de una interfase no comercial que tiene su origen en la interfase nano-ESI, desarrollada para el acoplamiento CE-MS en 1996 por Wachs y cols. (1996). En este caso, el capilar de separación se une con un capilar en forma de punta (emisor-ESI) a través de una "T", a la que se le aplica el voltaje de spray. En la tercera entrada de la "T" se introduce el líquido adicional, creando así la unión líquida.

Durante el desarrollo de esta Tesis Doctoral, se ha llevado a cabo el acoplamiento CEC-MS para el análisis de los compuestos con actividad estrogénica de interés a través de una interfase de unión líquida presurizada. Ya empleada anteriormente para CE-MS por Fanali y cols., (2006), la interfase está constituida por un bloque de polisulfona en el que existe un depósito donde se introduce el líquido adicional (la presión es controlada hidrostáticamente) y que también conecta la salida de la columna capilar con el capilar emisor-ESI, separados entre sí algunas decenas de micras (Figura I.22). El depósito está conectado a un electrodo al que se le aplica una diferencia de potencial de 1,8-2,0 kV.

La interfase de unión líquida se coloca en un soporte-controlador de movimiento XYZ que permite situar el capilar emisor-ESI en la posición correcta, a pocos milímetros del orificio del espectrómetro de masas. En esta interfase, los analitos son trasportados hasta la unión líquida bajo la influencia del campo eléctrico y, sucesivamente, el líquido adicional impulsa las bandas cromatográficas al capilar emisor-ESI a un flujo parecido al del EOF.

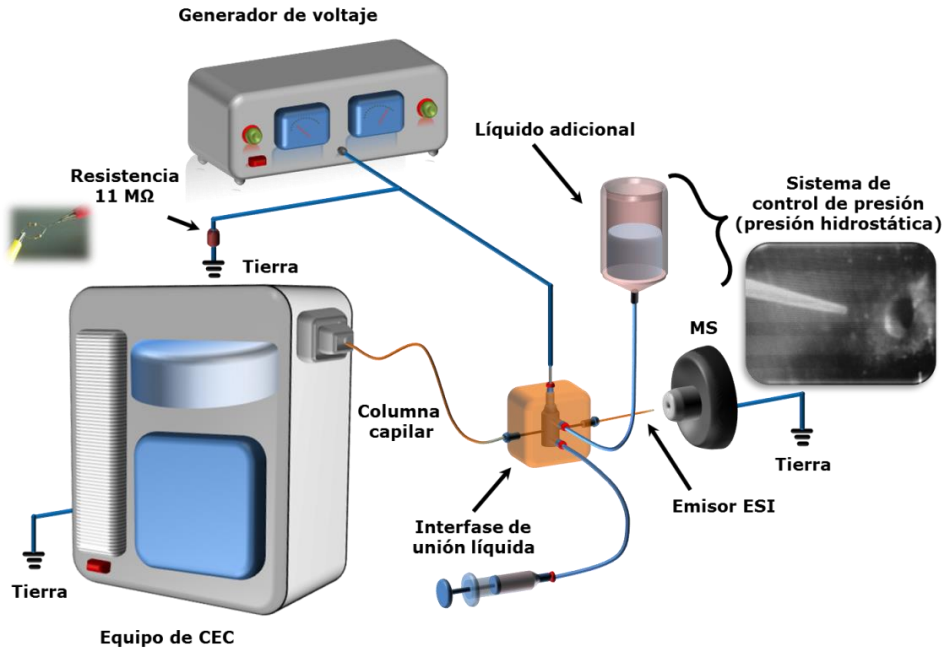

Figura I.22. Esquema de un sistema CEC-MS con interfase de unión líquida.

Este diseño permite un transporte adecuado de los analitos, y mantiene una elevada eficacia cromatográfica, al mismo tiempo que minimiza la dilución y, sobre todo, estabiliza la corriente de una forma eficaz, demostrando poca predisposición a la formación de burbujas.

En general, la determinación de estrógenos naturales, sintéticos y micoestrógenos mediante CEC ha sido muy escasa y se ha limitado sólo a dos trabajos de Liu y cols. (2005a; 2005b), en los que analizaron DES, HEX, y DS en pescado utilizando pCEC con detección amperométrica. Por tanto, y hasta el desarrollo de esta Tesis Doctoral, no existían en la bibliografía trabajos en los que se analizaran otros estrógenos mediante CEC, ni tampoco ningún trabajo en el que se analizara este tipo de compuestos mediante CEC-MS.

I.4. Métodos de extracción de compuestos con actividad estrogénica

En lo que se refiere a estrógenos naturales, sintéticos y micoestrógenos, la matriz alimentaria más analizada por la posible presencia de estas sustancias es la leche y sus derivados (Sonja y cols. 1998). Esta matriz contiene gran cantidad de proteínas y lípidos que son generalmente responsables de los procesos de adsorción sobre la fase estacionaria de las columnas cromatográficas, obstrucciones y supresión

iónica en el caso de utilizar detección mediante MS (Annesley, 2003). El pretratamiento de muestra más utilizado para este tipo de matrices implica, generalmente, llevar a cabo una desproteinización previa, bien sea por calentamiento, por adición de un ácido, una disolución tampón o un disolvente orgánico (Socas-Rodríguez y cols., 2013). Cuando se desea determinar la fracción conjugada de los estrógenos naturales, que pueden representar más del 85% del total de los estrógenos contenidos en la leche (Tso y Aga, 2010), la muestra se somete además a una hidrólisis enzimática mediante la adición de β-glucuronidasa/arilsulfatasa (Capriotti y cols, 2013).

En el caso del análisis de matrices grasas, como mantequilla, nata, yogur o queso, habitualmente se requiere la introducción de una etapa adicional para eliminar los lípidos, que con frecuencia se lleva a cabo realizando una extracción con un disolvente apolar como el *n*-hexano (Socas-Rodríguez y cols., 2014).

Los procedimientos clásicos de tratamiento de muestra son a menudo largos y laboriosos, requiriendo grandes cantidades de reactivos y disolventes. Por ello, en los últimos años, se ha introducido el concepto de *procedimientos analíticos sostenibles* (SAP), con la idea de desarrollar la preparación de muestra con un enfoque más sostenible, reduciendo el impacto ambiental, y en base a los siguientes principios (Płotka y cols., 2013):

1) Disminución y o eliminación de la cantidad de reactivos utilizados.
2) Reducción de las dimensiones del procedimiento de preparación de muestra.
3) Reciclaje de los disolventes y fases adsorbentes.
4) Empleo de disolventes verdes, como líquidos iónicos, fluidos supercríticos, fluidos presurizados (Richter y cols., 1996), etc.
5) Utilización de microondas o ultrasonidos para conseguir procesos de extracción más eficaces.
6) Facilidad de automatización.

En este contexto, la preparación de la muestra está focalizada hacia la miniaturización de los procesos de extracción (Koel y Kaljurand, 2006) y hacia el estudio de nuevas fases estacionarias para su uso en SPE. En este último caso, la investigación se ha centrado principalmente en el desarrollo de nuevos materiales adsorbentes que posean un elevado rango de interacción (mayor capacidad de carga), como los nano-materiales a base de carbono o nano-partículas magnéticas; o bien que generen una interacción altamente específica, como los adsorbentes inmunoselectivos o los polímeros de impresión molecular (MIPs). Con este tipo de adsorbentes se consigue una retención específica,

contribuyendo a la simplificación y reducción del tiempo de optimización de la etapa de limpieza (Płotka-Wasylkaa y cols., 2016).

I.4.1. Microextracción en fase líquida

El desarrollo de la microextracción en fase sólida (SPME) en 1989 por Belardi y Pawliszyn (1989) supuso un punto de inflexión importante en el desarrollo del pretratamiento de muestra, ya que abrió el campo de la investigación a la miniaturización.

Siguiendo esta tendencia, y a partir de la extracción líquido-líquido (LLE), caracterizada clásicamente por el empleo de grandes volúmenes de disolventes orgánicos (con su consecuente toxicidad) y procedimientos largos y tediosos, surgió su versión miniaturizada conocida como microextracción en fase líquida (LPME) (Psillakis y Kalogerakis 2003). La miniaturización de la LLE se basa en dos principios: i) la reducción notable de los volúmenes de la fase aceptora, normalmente un disolvente orgánico, y de la fase dadora, que es la muestra real en la que se encuentran disueltos los analitos; y ii) la inmiscibilidad de las dos fases en el proceso de extracción (Psillakis y Kalogerakis, 2003).

La LPME se ha llevado a cabo en diferentes modalidades de extracción, que se pueden clasificar en tres categorías principales: microextracción en gota (SDME), LPME en fibra hueca (HF-LPME) y microextracción líquido-líquido dispersiva (DLLME) (Asensio-Ramos y cols., 2011b).

I.4.1.1. Microextracción en gota

En 1996 Jeannot y Cantwell desarrollaron este procedimiento, utilizando como fase aceptora una gota de un disolvente inmiscible con la fase dadora acuosa como, por ejemplo, tolueno, *n*-hexano, ciclohexano, xilano o un líquido iónico (Jeannot y Cantwell, 1996). La gota, que cuelga de la aguja de una jeringa, se introduce en la muestra acuosa que contiene los analitos sometida a constante agitación. Transcurrido un cierto tiempo de extracción, la jeringa se retrae, pudiendo inyectar la gota directamente en el sistema cromatográfico, o bien evaporarla y reconstituirla en otro disolvente más adecuado.

Hasta la fecha, se han desarrollado diferentes modalidades de SDME para aumentar la eficacia de extracción, reducir las interferencias y permitir, sobre todo, una mejor limpieza (*clean-up*) de las matrices (Asensio-Ramos y cols., 2011b; Pena-Pereira y cols., 2010). En este sentido, la SDME se puede llevar a cabo en dos o tres fases. En los procedimientos en dos fases, la extracción tiene lugar entre la gota de disolvente y la muestra, pudiendo distinguirse la SDME con la gota inmersa en el interior de la muestra (DI-SDME), con la gota flotante no

soportada por la jeringa (DS-DME) (Yangcheng y cols., 2006), con la gota sumergida en un volumen muy pequeño (gota) de muestra (DDME) (Hui-Fen y cols., 2006) y en el seno de un flujo continuo (CF-SDME) (Liu y Lee, 2000). Por el contrario, en los procedimientos en tres fases, los analitos son extraídos en la gota a través de un disolvente que se encuentra en la fase dadora, pudiendo distinguirse la microextracción líquido-líquido-líquido (LLLME) y la SDME en espacio en cabeza (HS-DSME), ésta última empleada cuando los analitos son volátiles o semivolátiles (Theis y cols., 2001; Tankeviciute y cols. 2001). La Figura I.23 muestra las diferentes modalidades operacionales de la SDME.

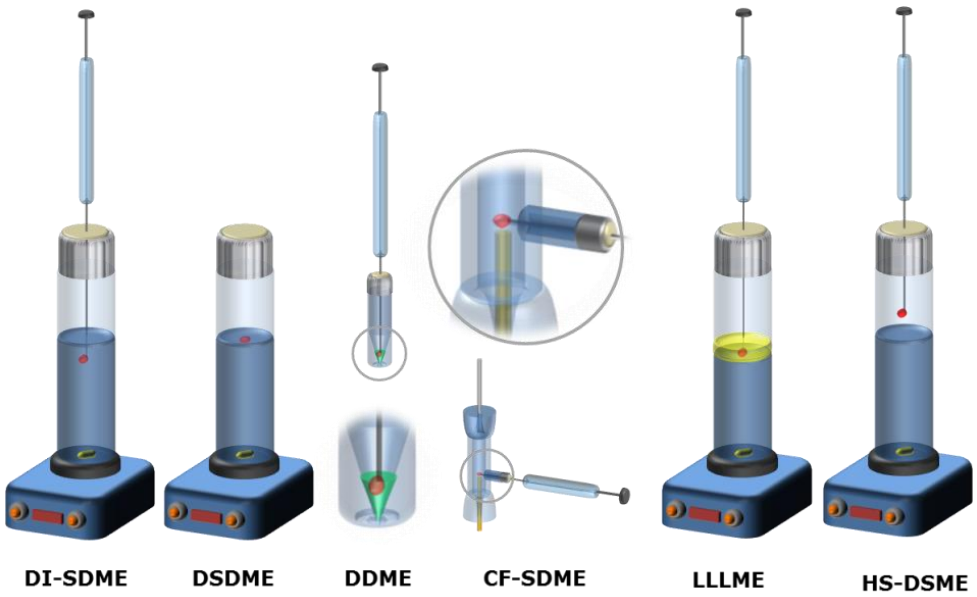

Figura I.23. Modalidades operacionales de la SDME.

La SDME se caracteriza por proporcionar un elevado factor de enriquecimiento debido a la elevada reducción del volumen de la fase aceptora, simplicidad de la instrumentación empleada, bajo coste y bajo impacto medioambiental. Sin embargo, el principal inconveniente de esta técnica es la variación de volumen e inestabilidad de la gota durante el proceso de extracción, que tiene una gran influencia en la repetibilidad de la extracción. Para solucionar parcialmente este problema, se puede utilizar un estándar interno (Romero y cols., 2007) y, cuando sea posible, emplear un disolvente con baja presión de vapor, como por ejemplo un líquido iónico (Vidal y cols., 2007).

I.4.1.2. Microextracción en fase líquida con fibra hueca

A finales de los años 90, Pedersen-Bjergaard y Rasmussen (1990) desarrollaron una nueva técnica microextractiva integrando las bases del funcionamiento de una membrana líquida soportada (SLM) con la LPME, utilizando una membrana hueca de polipropileno sujeta al extremo de la aguja de una jeringa. La definieron como LPME en fibra hueca (HF-LPME) (Pedersen-Bjergaard y Rasmussen, 1999; Rasmussen y cols., 2000) y de modo similar a la SDME, puede llevarse a cabo en dos o tres fases. La Figura I. 24 muestra las distintas modalidades operacionales de la HF-LPME.

En la HF-LPME en dos fases, el lumen y los poros de la fibra contienen la misma fase extractora, normalmente un disolvente orgánico inmiscible en la fase dadora como, por ejemplo, hexiléter, dihexiléter, dietiléter, cloroformo, octano, *n*-octanol o tolueno. El extremo de la fibra puede quedar abierto (Shen y Lee, 2002; Asensio-Ramos y cols, 2012) o bien puede sellarse mediante calor (King y cols., 2002). Cuando la fibra se introduce en la fase dadora, se establece un equilibrio de reparto entre ambas fases. En este caso, la eficacia de la extracción está garantizada para aquellos compuestos hidrofóbicos (Ho y cols., 2002). Este proceso de extracción se considera una evolución de la SDME, ya que la fase extractora se encuentra protegida por la fibra, lo que solventa la inestabilidad y, por tanto, la pérdida de la gota (Psillakis y Kalogerakis, 2003). El disolvente orgánico recuperado del lumen de la fibra mediante la retracción de la jeringa puede inyectarse directamente en los sistemas cromatográficos, o bien puede ser evaporado y reconstituido en el disolvente más compatible con la técnica de separación utilizada.

En lo que respecta a la HF-LPME en tres fases, la fibra se impregna con un disolvente orgánico inmiscible con la fase dadora y el lumen se rellena con una segunda fase acuosa. Esta modalidad también se conoce como microextracción líquido-líquido-líquido en fibra hueca (HF-LLLME). La fase orgánica contenida en los poros de la fibra actúa como una barrera, impidiendo la miscibilidad entre ambas fases acuosas, la dadora y la aceptora. Esta modalidad se caracteriza por una distribución combinada de los analitos basada en dos equilibrios de reparto: los existentes entre la fase orgánica y la fase dadora, y entre la fase aceptora y la fase orgánica. El éxito de este tipo de extracción se consigue variando adecuadamente la composición de las fases dadora y aceptora para favorecer el paso de una fase a otra de los analitos a través del disolvente orgánico, por ejemplo, mediante cambios de pH (Pedersen-Bjergaard y Rasmussen, 1999) o utilizando agentes de complejación denominados *carriers* (Zhao y cols., 2002). Una vez

finalizada la extracción, la fase aceptora acuosa puede inyectarse directamente en un equipo de HPLC o CE.

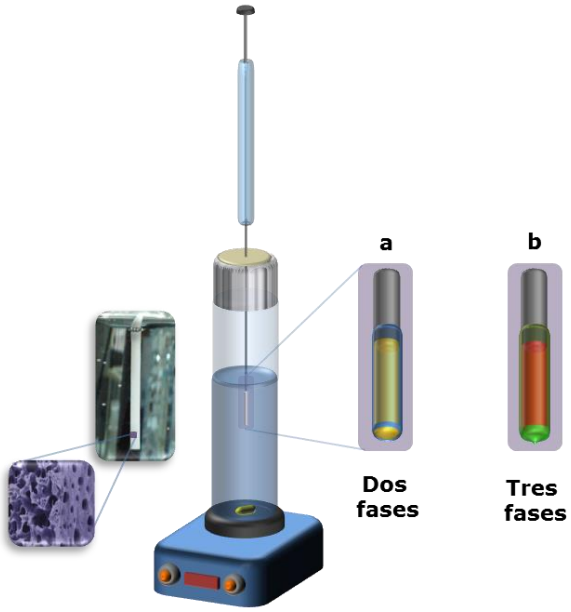

Figura I.24. Modalidades operacionales de la HF-LPME.

En general, la forma alargada de la fibra proporciona un aumento de la superficie de disolvente con respecto a la superficie de la gota en la SDME, aumentando así la capacidad de la extracción. Además, las pequeñas dimensiones de los poros (200-600 nm) permiten obtener extractos más limpios al actuar el poro como un tamiz molecular, lo que es una clara ventaja cuando se extraen matrices complejas.

Tal y como muestra la Tabla I.7, hasta el desarrollo de esta Tesis Doctoral, la HF-LPME se ha aplicado a la extracción de estrógenos naturales, sintéticos y micoestrógenos, fundamentalmente de matrices acuosas (sobre todo los estrógenos naturales), pero también se ha aplicado al análisis de muestras de leche, aunque en pocas ocasiones (Feng y cols., 2016; Liu y cols., 2010; Socas-Rodríguez y cols., 2013b; Xu y cols., 2013; Yang y cols., 2012), y sólo en una ocasión al análisis de derivados lácteos como el yogur y el queso (Socas-Rodríguez y cols., 2014a). Además, no existen trabajos en la bibliografía que describan la extracción de micotoxinas de la familia de la ZEN de leche y sus derivados.

I.4.1.3. Microextracción líquido-líquido dispersiva

En 2006, Rezaee y Assadi introdujeron esta nueva técnica de microextracción, basada también en los principios de la LLE (Rezaee y cols., 2006). La DLLME consiste en un sistema trifásico constituido por una fase dadora que contiene los analitos, un disolvente orgánico inmiscible con la fase acuosa (disolvente de extracción) y un disolvente orgánico miscible con la fase acuosa (disolvente dispersor). El procedimiento consta de dos etapas. En la primera de ellas, una mezcla compuesta por unos pocos microlitros del disolvente de extracción en algunos cientos de microlitros de disolvente dispersor se inyectan rápidamente en la fase dadora contenida en un tubo cónico, creando así una dispersión. En la segunda etapa, la mezcla se centrifuga y el disolvente de extracción, que contiene los analitos, se recupera con ayuda de una jeringa, pudiendo inyectarse directamente en el sistema cromatográfico, o bien ser evaporado y reconstituido en un disolvente diferente. La gran ventaja de esta técnica de extracción es su rapidez, debido a que la gran superficie de contacto entre el disolvente extractor y la fase dadora provoca que el equilibrio se alcance de inmediato.

La naturaleza del disolvente de extracción establece las diferentes modalidades operacionales de la DLLME. Así, si se emplean disolventes clorados, como clorobenzeno, diclorometano, tetracloruro de carbono, tetracloroetano o líquidos iónicos (Rezaee y cols., 2010), que poseen una densidad mayor que la del agua, la gota se recuperará en el fondo del tubo. Por el contrario, cuando se emplean disolventes con una densidad menor que aquella de la fase dadora como, por ejemplo, *n*-hexano o alcoholes de cadena larga como 1-octanol, 1-decanol, 1-undecanol, 1-dodecanol, 2-decanol, etc., la gota se recuperará en la superficie de la disolución, en lo que se conoce como DLLME con gota flotante (FO-DLLME) (Saraji y Boroujeni, 2014). En este último caso, la reproducibilidad del procedimiento puede ser baja; sin embargo, teniendo en cuenta el bajo punto de fusión que suelen tener estos disolventes de extracción, la gota se puede solidificar disminuyendo la temperatura y recuperarla en estado sólido. Esta modalidad se denomina DLLME con gota flotante sólida (SFO-DLLME) (Khalili-Zanjani y cols., 2007; Khalili-Zanjani y cols., 2008; Rezaee y cols., 2010) (Figura I.25).

La elección de los disolventes de extracción y de dispersión, su solubilidad en la fase acuosa, su volumen, el tiempo de extracción, el método de dispersión (con agitación mecánica, ultrasonidos (Jia y cols., 2010) o microondas (Gao y cols., 2010)) y el tiempo de centrifugación, son algunos de los parámetros que afectan a la repetibilidad de la metodología, al factor de enriquecimiento obtenido y a la recuperación de los analitos.

Tabla I.7. Aplicaciones de la HF-LPME al análisis de estrógenos naturales, sintéticos y micoestrógenos.

Analitos	Matriz	Tratamiento de muestra	Tipo de fibra	Modalidad	Disolvente de extracción	Disolvente de desorción	Técnica de análisis	Referencia
17β-E_2	Disolución patrón (10/90 v/v MeOH/H_2O)	-	Polietileno modificado por GMA y anti-ES anticuerpo	ES-IA fibra	-	-	ES-ELISA	Nishiyama y cols., 2002
E_1, 17β-E_2, EE_2, DES	Agua potable	-	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 1,2 cm, recubierta por DHPMM	PC-HF-LPME	CH_2Cl_2	-	GC-MS	Basheer y cols., 2005
DES, DS, HEX	Agua residual	-	- (500 µm x 300 µm x 0,2 µm) x 4,5 cm	HF-LPME	1-octanol	-	HPLC-UV	Liu y cols., 2008
DES, DS, HEX	Leche	Desproteini-zación: HCl y ACN, filtración y secado. Disolución en 5 mL MeOH	Polipropileno (150 µm x 330 µm x 0,47 µm) x 5 cm - copolímero con molde de DES	MIP-HFT	-	MeOH/ácido acético (99:1, v/v) y MeOH/ácido acético (9:1, v/v)	HPLC-UV	Liu y cols., 2010
E_1, 17β-E_2, EE_2	Hígado de pez cebra	Homogeniza-ción, HCl, ultrasonidos y agitación	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 1,2 cm	HF-LPME	Tolueno	-	GC-MS	Kanimozhi y cols., 2012

DES	Leche	MeOH y tampón Na_2HPO_4-ácido cítrico	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 0,6 cm y MWCNTs por reacción sol-gel	CNTs-HF-SPME	1-octanol	MeOH	HPLC-DAD	Yang y cols., 2012
E_1, 17β-E_2, EE_2	Cultivo celular de *Saccharomyces cerevisiae*	Digestión enzimática	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 0,8 cm	HF-LPME	Tolueno/ CH_2Cl_2 (50:50, v:v)	-	GC-MS	Kim y cols., 2012
E_1, 17β-E_2, E_3, EE_2, DES DS	Sedimentos y pescado	Sedimento: homogenización, extracción (MeOH), evaporación, reconstitución (agua Milli Q). Pescado: congelación y filtración	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 3,5 cm	HF-LLLME	Tolueno/ 1-octanol, (7:3, v/v) Fase aceptora: 0,15 M NaOH, 0,015 M β-CD	-	HPLC-UV)	Chen y cols., 2013
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS	Leche	Dilución (agua Milli-Q)	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 1,2 cm	HF-SEBLLME	1-octanol	HFBA/ACN, (1:5, v/v) (360 µL)	GC-MS	Xu y cols., 2013

(Continúa en la página siguiente)

Tabla I.7. (Continuación)

Analitos	Matriz	Tratamien-to de muestra	Tipo de fibra	Modalidad	Disolven-te de extrac-ción	Disolven te de deso-r-ción	Técnica de análisis	Referencia
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS, HEX, 2-OHE_2	Leche	Desproteiniz ación (ácido acético, ACN)	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 2,0 cm	HF-LPME	1-octanol	ACN	HPLC-DAD/FD	Socas-Rodríguez y cols., 2013b.
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS, HEX, 2-OHE_2	Leche, queso, yogurt	Desproteiniz ación (ácido acético, ACN), eliminación de la grasa (*n*-hexano)	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 2,0 cm	HF-LPME	1-octanol	ACN	HPLC-DAD/FD	Socas-Rodríguez y cols., 2014
E_1, EE_2, DES, HEX	Leche	Dilución (agua Milli-Q)	PVDF-HF (700 x 500 x 0,1 µm) x 1,0 cm	HFPIL-cápsula-SPME	-	ACN	HPLC-UV	Feng y cols., 2016.
17β-E_2, E_3, EE_2	Agua de río, de lago y grifo	-	Accurel Q3/2 (600 µm x 200 µm x 0,2 µm) x 2,7 cm	DLLME-HFCEP	DLLME: 1-decanol (extractor), EtOH (dispersor)	ACN	HPLC-DAD	Wang y cols., 2016

Tabla I.8. Algunas aplicaciones de la DLLME al análisis de estrógenos naturales, sintéticos y micoestrógenos.

Analitos	Matriz	Tratamiento de muestra	Modalidad	Disolvente de extracción	Disolvente dispersor	Técnica de análisis	Referencia
E_1, 17β-E_2, E_3, EE_2	Agua de río y de grifo	-	SFO-DLLME	1-undecanol (10 µL)	MeOH (200 µL)	UPLC-DAD	Chang y Huang, 2010
E_1, 17β-E_2, E_3, DES	Agua de río y de grifo	-	DLLME	CCl_4 (80 µL)	Acetona (1,25 mL)	HPLC-UV	Hadjmohammadi y Ghoreishi, 2011
E_1, 17β-E_2, E_3, EE_2, DES	Agua de río, de mar y residual	-	IL-DLLME	$[C_6MIM][PF_6]$ (20 µL)	Acetona (1 mL)	HPLC-DAD/FD	Wu y cols., 2012a
ZEN	Cerveza	Desgasificación (30 min), filtración (0,45 µm)	DLLME	$CHCl_3$ (75 µL)	ACN (0,25 mL)	HPLC-FD	Wu y cols., 2012b
17β-E_2, EE_2	Agua de río y agua de grifo	-	DLLME	Clorobenceno (200 µL)	Acetona (2 mL)	HPLC-FD	Lima y cols., 2013
ZEN	Cardo	QuEChERS	DLLME	$CHCl_3$ (620 µL)	ACN (0,95 mL)	UHPLC-MS/MS	Arroyo-Manzanares y cols., 2013
17α-E_2, 17β-E_2, E_3, EE_2, DES, DS, HEX, ZEN	Agua mineral y residual	-	IL-DLLME	$[PPIm][PF_6]$ (60 mg)	ACN (0,5 mL)	HPLC-DAD/FD	Socas-Rodríguez, cols., 2014b
17α-E_2, EE_2	Agua residual y superficial	-	DLLME	Clorobenceno (200 µL)	Acetona (2 mL)	ELISA	Lima y cols., 2014
17α-E_2	Agua de lago, agua potable	-	ILFOF-IL-DLLME	$[C_6MIM][BF_4]$ NH_4PF_6, NaCl (60 µL)	-	HPLC-FD	Zhang y cols., 2014

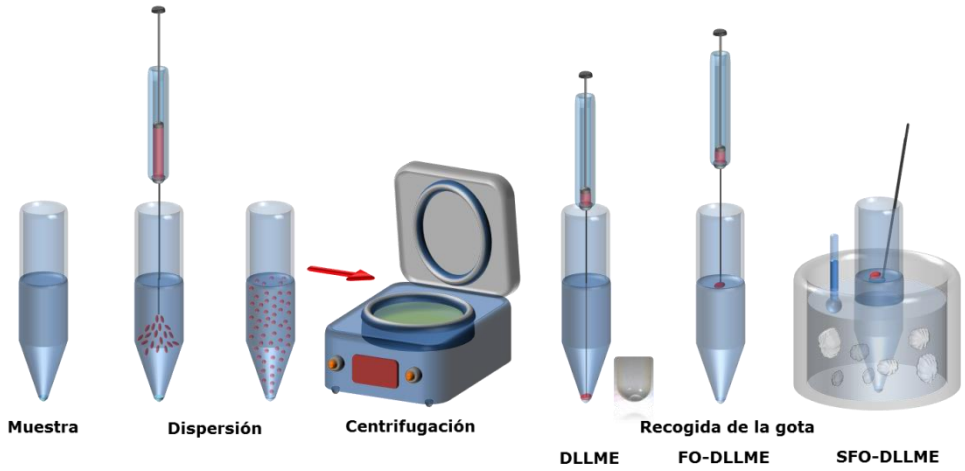

Figura I.25. Etapas y modalidades de la DLLME.

La DLLME es una técnica que se caracteriza por permitir alcanzar elevados factores de enriquecimiento, por su bajo coste, sencillez operativa y bajo impacto ambiental debido al empleo de pequeños volúmenes de disolventes orgánicos, lo que la convierte en una metodología microextractiva muy utilizada (quizás la más utilizada de las anteriores) y, sobre todo, sostenible.

Tal y como muestra la Tabla I.8, los compuestos con actividad estrogénica estudiados en esta Tesis Doctoral han sido extraídos de matrices acuosas y sólo en dos ocasiones de matrices alimentarias (Arroyo-Manzanares y cols., 2013; Wu y cols., 2012), en casi todos los casos, haciendo uso de la LC. Sin embargo, la técnica no ha sido aplicada a la extracción de leche y/o derivados lácteos.

I.4.2. Extracción en fase sólida con polímeros de impronta molecular

La SPE es probablemente la técnica más ampliamente utilizada para la extracción y preconcentración de los analitos de matrices o extractos acuosos, de ahí que también sea una de las técnicas que ha experimentado un mayor desarrollo. De hecho, con la intención de desarrollar metodologías de extracción más eficaces y selectivas, y de simplificar el procedimiento, se han implementado interesantes modificaciones, así como el uso de nuevos materiales adsorbentes.

En el primer caso, los procedimientos de SPE tradicionales se han sustituido en muchas ocasiones por su modalidad dispersiva (dSPE), ya que al no llevarse a cabo en columnas, cartuchos o discos, sino en el seno de la propia disolución, es más sencilla, rápida y menos tediosa que la SPE clásica (Socas-Rodríguez, 2015).

A pesar de los buenos resultados obtenidos, el mayor desarrollo de la técnica ha tenido lugar en lo que respecta al empleo de nuevos materiales adsorbentes. Éste es el caso de los nanotubos de carbono (CNTs) (Herrera-Herrera y cols., 2012), MIPs, materiales de acceso restringido, adsorbentes de inmunoafinidad y nanopartículas con o sin carácter magnético (Płotka-Wasylkaa y cols., 2016).

En el caso particular de los adsorbentes de inmunoafinidad, de los materiales de acceso restringido o los MIPs, se trata de adsorbentes altamente selectivos que se basan en un reconocimiento molecular, similar al que existe en los sistemas biológicos. De todos ellos, el uso de los MIPs ha experimentado un importante crecimiento en los últimos años hasta el punto de que en la actualidad se comercializa una amplia variedad de ellos para su aplicación en SPE, aunque no tanto como sería deseable. Sin embargo, también existen muchos ejemplos de síntesis en el propio laboratorio (Haupt, 2012).

En la MIP-SPE, empleada por primera vez en 1972 (Wulff y Sarhan, 1972), la fase adsorbente es una matriz polimérica que contiene cavidades con la forma de los grupos funcionales del analito de interés, también denominado "molécula diana", creando una interacción fuertemente específica. Su fabricación es relativamente sencilla y de bajo coste, sobre todo cuando se compara con la síntesis de los materiales de inmunoafinidad. Para ello, se lleva a cabo una reticulación polimérica inducida por reacciones radicalarias de un monómero enlazado a la molécula molde (normalmente el analito a extraer o similar) y un agente entrecruzante en presencia de un disolvente porógeno. Tras una intensa etapa de lavado, la molécula molde es eliminada, dejando cavidades complementarias en tamaño, forma y posición de sus grupos funcionales (Figura I.26).

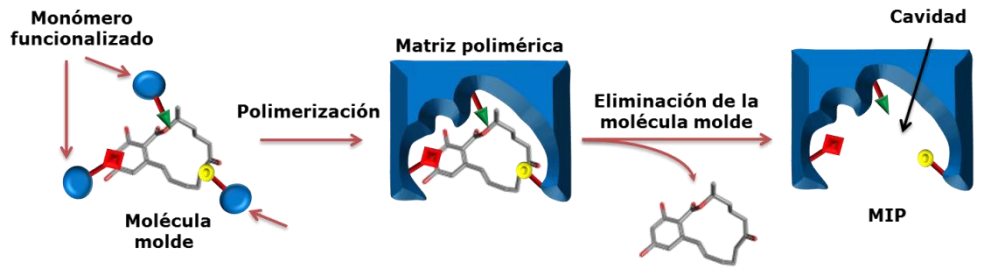

Figura I.26. Esquema del proceso de síntesis de un MIP.

La MIP-SPE se ha aplicado ampliamente a la extracción de una gran variedad de familias de compuestos de diferentes matrices (Pichon y

Chapuis-Hugon, 2008). En los pocos trabajos existentes en la bibliografía sobre la extracción de estrógenos naturales, sintéticos y micoestrógenos, se utilizan MIPs obtenidos utilizando el 17β-E_2 o la ZEN como moléculas molde (Urraca y cols., 2006; Lucci y cols., 2010).

Tal y como se puede apreciar en la Tabla I.9, la mayoría de los trabajos publicados en la bibliografía se han enfocado al análisis de muestras de agua y de leche, aunque siempre analizando un número bastante limitado de estos compuestos. Hasta la realización de esta Tesis Doctoral, no existe ningún trabajo en el que se extraigan simultáneamente estos tres grupos de compuestos mediante MIP-SPE.

Tabla I.9. Algunas aplicaciones de los MIP en SPE, SPME y MSPD al análisis de estrógenos naturales, sintéticos y micoestrógenos.

Analitos	Matriz	Procedimiento	Molécula molde o cartucho comercial utilizado	Técnica de análisis	Referencia
E_1, 17α-E_2, 17β-E_2, E_3, DES	Agua de lago	MIP-SPE	MIP (17β-E_2) MIP (DES)	HPLC-UV	Meng y cols., 2005
α-ZEL, ZEN	Cereales	MIP-SPE	MIP (ZEN)	HPLC-FD	Urraca y cols., 2006
E_1, E_2, DES	Agua de lago, río y grifo	MIP-SPE	MIP (E_1)	HPLC-UV	Wang y cols., 2008
17β-E_2, E_3, DES	Pescado y gamba	MIP-SPE	MIP (17β-E_2)	HPLC-FD	Jianga y cols., 2009
17β-E_2, E_3	Leche, yogur, carne de ternera, pollo y cerdo	MIP-SPE	MIP (17β-E_2)	HPLC-UV	Shi y cols., 2010
E_1, 17β-E_2, E_3, EE_2	Pescado y camarón	SPME-MIP	Fibra-MIP (17β-E_2)	HPLC-UV	Hu y cols., 2010
DES, DS, HEX	Leche	MIP-HFT	MIP (DES)	HPLC-UV	Liu y cols., 2010

(Continúa en la página siguiente)

Tabla I.9. (Continuación)

Analitos	Matriz	Procedimiento	Molécula molde o cartucho comercial utilizado	Técnica de análisis	Referencia
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS	Agua del grifo y de río	MIP-SPE	AFFINIMIP® SPE Estrogen(1)	UPLC-MS	Lucci y cols., 2011
17β-E_2	Leche de cabra	MIP-MSPD	MIP (17β-E_2)	HPLC-DAD	Gañán y cols., 2012
E_1, 17β-E_2, EE_2, DES	Orina, leche, tónico para la piel	DLLSME-MIPFs	MIPFs (17β-E_2)	HPLC-UV	Zhong y cols., 2012
17β-E_2	Agua	MIP-SPE	MIP (17β-E_2)	HPLC-UV	Jing y cols., 2012
E_1, 17β-E_2, E_3	Agua	MIP-SPE	MIP (17β-E_2)	HPLC-DAD	Sadowski y Gadzała-Kopciuch, 2013
E_1, 17β-E_2, E_3, DES	Leche en polvo	MMIP-SPME	MMIP (17β-E_2)	HPLC-UV	Lan y cols., 2014
DES, DS, HEX	Agua de estanque y leche	MMIP	MMIP (DES)	HPLC-DAD	Xie y cols., 2015

(1)POLYINTELL (Val de Reuil, France)

II. OBJETIVOS

II. OBJETIVOS

El objetivo principal de esta Tesis Doctoral es el desarrollo de metodologías analíticas sostenibles de bajo impacto medioambiental que permitan la extracción de estrógenos naturales, sintéticos, sus metabolitos más representativos y micoestrógenos de agua, leche y derivados lácteos. Para ello, se han utilizado diferentes procedimientos de extracción y microextracción combinados, en su mayoría, con técnicas analíticas miniaturizadas acopladas a la MS.

Para conseguir este objetivo principal se han planteado diferentes objetivos secundarios que incluyen:

1) Optimizar la separación analítica de diferentes grupos de compuestos con actividad estrogénica.

- Evaluar el uso del PFOA como tensioactivo volátil para acoplar eficazmente la MEKC con la MS.
- Investigar el uso de diferentes fases estacionarias para fabricar columnas capilares que proporcionen una adecuada separación analítica en nano-LC y CEC.
- Llevar a cabo el acoplamiento de la nano-LC y la CEC con la MS mediante el empleo de las interfases nano-spray y de unión líquida, respectivamente.
- Estudiar la reacción de derivatización de los compuestos estrogénicos con BSTFA para su análisis mediante GC-FID y GC-MS.
- Considerar la aplicación de métodos de preconcentración en línea en CE (métodos de *stacking*) y en nano-LC (*focusing*) con el objetivo de aumentar la sensibilidad del método.

2) Optimizar diferentes procedimientos de preparación de muestra.

- Estudiar la aplicación de técnicas de LPME, tales como la DLLME y la HF-LPME, para la extracción de compuestos con actividad estrogénica en muestras acuosas, leche y derivados lácteos, optimizando los diferentes parámetros que influyen en la microextracción así como el posible pretratamiento de la muestra (desproteinización, eliminación de lípidos, etc.).
- Evaluar la aplicación de un MIP comercial para la SPE de los compuestos seleccionados en muestras acuosas.
- Validar las metodologías desarrolladas en base a estudios de repetibilidad, calibración, precisión, exactitud y recuperación, así

como determinar la sensibilidad obtenida para cada uno de los compuestos estudiados.

- Demostrar el potencial de las metodologías desarrolladas para analizar muestras directamente relacionadas con la posible presencia de compuestos con actividad estrogénica, como aguas residuales, leche y derivados lácteos.

III. Resumen de los artículos científicos

III. RESUMEN DE LOS ARTÍCULOS CIENTÍFICOS

Tal y como se ha señalado en el capítulo introductorio, la presencia de agentes contaminantes en matrices alimentarias es un tema que ha suscitado una creciente preocupación, tanto en los productores como en los consumidores a nivel mundial.

En particular, la presencia de compuestos con actividad estrogénica como los estrógenos naturales, sintéticos o micoestrógenos en alimentos de origen animal y, en particular, en la leche y derivados lácteos es especialmente alarmante, dado el enorme consumo de estos productos a diario a nivel mundial. Todo esto, unido al hecho de que en muchos casos estos contaminantes no se eliminan completamente en las plantas depuradoras de aguas, hace que estos se liberen al medioambiente.

Para la determinación de este tipo de compuestos se hace necesario el empleo de metodologías analíticas muy sensibles, eficaces y selectivas. La etapa más crítica de este proceso es el tratamiento de muestra. En este sentido, las tendencias actuales se centran en el desarrollo de métodos de extracción miniaturizados, como la DLLME (*Artículos 1 y 2*) y la HF-LPME (*Artículo 4*), así como en el uso de materiales altamente selectivos como los MIPs en SPE (*Artículo 3*). Tal y como se mostró en las Tablas I.7 y I.8 de la Introducción, la DLLME y la HF-LPME se han aplicado solo en pocas ocasiones para la extracción de los compuestos objeto de estudio en esta Tesis Doctoral y, en ningún caso, para la determinación simultánea de los mismos en agua, leche y derivados.

Como se ha comentado previamente, el análisis de matrices tan complejas como la leche o el yogur requieren de una etapa previa de precipitación de proteínas y de eliminación de lípidos. En los artículos que constituyen esta Tesis Doctoral, la desproteinización se llevó a cabo mediante la utilización de un ácido en presencia de un disolvente orgánico (*Artículos 2 y 4*), mientras que la eliminación de grasas se realizó mediante LLE con *n*-hexano (*Artículo 2*). Por otra parte, en el caso del análisis de agua, se procedió a realizar un simple filtrado para eliminar posibles residuos sólidos presentes (*Artículos 1 y 3*).

En lo que respecta a los métodos de separación, y a pesar de que los compuestos con actividad estrogénica seleccionados han sido separados en un gran número de ocasiones mediante LC convencional, y en un menor número de casos mediante GC (*Tabla I.2*), la utilización de técnicas cromatográficas miniaturizadas ha sido escasa. Asimismo, para una detección unívoca de los compuestos analizados, un mejor aislamiento de los interferentes de la matriz y, sobre todo, por el

incremento de la sensibilidad alcanzado, en todos los casos la técnica de separación se ha acoplado con la MS.

Por una parte, y dado que los compuestos estrogénicos de interés no poseen carga en un amplio rango de pH, su separación se realizó mediante CE-MS utilizando la MEKC como modo de separación (*Artículos 1 y 2*). El acoplamiento con la MS se llevó a cabo empleando el PFOA como tensioactivo volátil, compatible con la ESI. Por otra parte, mediante el empleo de una columna de 25 cm de longitud y 75 µm de d.i. empaquetada con partículas de 3 µm de sílice funcionalizada con grupos fenilo, los compuestos objeto de estudio se separaron en un sistema nano-LC-MS con una interfase nano-spray, y en un sistema CEC-MS con una interfase de unión líquida (*Artículo 3*). Por último, su separación se llevó a cabo también mediante GC-MS/MS derivatizando los compuestos con BSTFA de forma previa a la inyección, dando lugar a TMS-derivados (*Artículo 4*).

Todas las metodologías propuestas fueron debidamente validadas, mediante estudios de repetibilidad, calibración, linealidad, exactitud y precisión y el cálculo de los LODs y LOQs tanto instrumentales como del método. Asimismo, éstas fueron aplicadas al análisis de muestras reales.

A continuación se presenta un breve resumen de la metodología aplicada y de los resultados obtenidos en los trabajos científicos publicados que forman parte de esta Tesis Doctoral por compendio de publicaciones, y que se encuentran en el Anexo I.

Artículo 1

Estrogenic compounds determination in water samples by dispersive liquid-liquid microextraction and micellar electrokinetic chromatography coupled to mass spectrometry

G. D'Orazio, M. Asensio-Ramos, J. Hernández-Borges, S. Fanali, M.A. Rodríguez-Delgado

Journal of Chromatography A 1344 (2014) 109-121

En este trabajo, un grupo de 12 compuestos con actividad estrogénica y que incluyen cuatro estrógenos naturales (E_1, 17α-E_2, 17β-E_2 y E_3), un exostrógeno (17α-EE_2), un estilbeno sintético (DS), una micotoxina (ZEN) y algunos de sus metabolitos principales (2-MeOE_2, α-ZAL, β-ZAL, α-ZEL y β-ZEL), fueron separados y determinados mediante el empleo de la MEKC acoplada a la MS utilizando una interfase ESI comercial. El tiempo de análisis obtenido fue de 11 min. Como BGE se utilizó una disolución acuosa a pH 9,0 al 10% en MeOH (modificador orgánico) conteniendo 45 mM de PFOA, un tensioactivo volátil compatible con la MS. Para aumentar la sensibilidad analítica, se aplicó un método de

preconcentración en línea, el NSM, inyectando hidrostáticamente durante 25 s la muestra disuelta en una disolución acuosa a pH 9,0 al 10% en MeOH conteniendo 11,5 mM de PFOA. Como método de extracción y preconcentración fuera de línea de los compuestos estudiados de muestras de aguas medioambientales (mineral, de escorrentía y residual) se aplicó la DLLME, empleando 110 µL de cloroformo y 500 µL de acetonitrilo (ACN) como disolventes de extracción y dispersión, respectivamente. El pH de las muestras se ajustó previamente a 3,0, conteniendo un 30% (w/v) NaCl. Se alcanzaron LODs entre 0,04 y 1,10 µg/L. La metodología DLLME-MEKC-MS/MS fue validada en términos de linealidad, precisión y recuperación, además de evaluar el efecto matriz para cada tipo de muestra. Se obtuvieron valores del coeficiente de determinación superiores a 0,992 y recuperaciones absolutas en el rango 43-91%.

Artículo 2

Evaluation of the combination of a dispersive liquid-liquid microextraction method with micellar electrokinetic chromatography coupled to mass-spectrometry for the determination of estrogenic compounds in milk and yogurt

G. D'Orazio, M. Asensio-Ramos, J. Hernández-Borges, M.A. Rodríguez-Delgado, S. Fanali

Electrophoresis 36 (2015) 615-625

En este otro trabajo, se evaluó la aplicación de la metodología DLLME-MEKC-MS anteriormente desarrollada para la extracción de los mismos 12 compuestos de tres tipos de leche diferentes (leche de vaca entera, leche de vaca desnatada y leche de cabra semidesnatada), y de yogur natural entero de vaca. Para ello, fue necesario realizar una etapa adicional de pretratamiento de muestra, y que consistió en una precipitación de las proteínas con ACN en medio ácido así como en la posterior adición de *n*-hexano para la eliminación de las grasas. La metodología se evaluó mediante los correspondientes estudios de calibración en la matriz, precisión y exactitud, obteniendo una buena linealidad con coeficientes de determinación superiores a 0,991 y LODs entre 3 y 32 µg/L.

Artículo 3

Capillary electrochromatography and nano-liquid chromatography coupled to nano-ESI interface for the separation and identification of estrogenic compounds

G. D'Orazio, J. Hernández-Borges, M. Asensio-Ramos, M.A. Rodríguez-Delgado, S. Fanali

Electrophoresis 27 (2016) 356-362

En este trabajo, se estudio el acoplamiento de la nano-LC y la CEC a la MS mediante las interfases nano-ESI y de unión líquida presurizada, respectivamente, para la separación y determinación simultánea de 11 compuestos con actividad estrogénica (E_1, 17α-E_2, 17β-E_2, E_3, 17α-EE_2, DS, ZEN, 2-MeOE_2, α-ZAL, β-ZAL y α-ZEL). Se estudiaron distintas fases estacionarias de relleno para las columnas capilares, tales como sílice con grupos fenilo, C18 y C18 bidentada, con el objetivo de obtener la mejor separación cromatográfica posible. Para ambas técnicas, la sílice con grupos fenilo resultó ser la mejor opción en lo que respecta a la eficacia de la separación, la selectividad, y la resolución. En las condiciones óptimas, se consiguió la separación completa de todos los analitos, incluyendo las formas isoméricas del E_2 y del ZAL en menos de 20 min en nano-LC-MS y en menos de 13 minutos en CEC-MS. Con el objetivo de aumentar la sensibilidad del método en nano-LC, se aplicó como método de preconcentración en línea la focalización en columna, obteniendo LODs instrumentales en el rango 1-55 ng/L. Los compuestos de interés fueron determinados en agua mineral mediante nano-LC-MS haciendo uso de la SPE con un MIP comercial apto para la determinación de zearalenonas. La metodología MIP-SPE-nano-LC-MS fue validada mediante un estudio de recuperaciones a dos niveles de concentración, obteniendo recuperaciones entre el 51 y el 78%.

Artículo 4

Determination of estrogenic compounds in milk and yogurt samples by hollow fiber liquid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry

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En este último artículo, se desarrolló un método de bajo impacto ambiental basado en la utilización de la HF-LPME para la extracción de 13 compuestos con actividad estrogénica (E_1, 17α-E_2, 17β-E_2, E_3, 17α-EE_2,

DS, HEX, ZEN, 2-MeOE$_2$, α-ZAL, β-ZAL, α-ZEL, β-ZEL) de leche de vaca entera, leche de cabra semidesnatada y yogur natural entero de vaca. Con el objetivo de determinar los analitos de interés mediante GC-MS/MS, se optimizó su derivatización mediante adición de BSTFA de forma previa a la inyección, dando lugar a TMS-derivados. Una vez más, las muestras se sometieron a la precipitación de proteínas mediante la adición de ACN en medio ácido y, una vez optimizadas las condiciones de extracción haciendo uso de la HF-LPME, la metodología completa fue validada, realizando estudios de calibración, precisión y exactitud. Los coeficientes de determinación fueron superiores a 0,991 para la calibración del método, mientras que los LODs variaron entre 0,04 y 3,73 µg/L.

IV. Conclusiones y perspectivas futuras

IV. CONCLUSIONES Y PERSPECTIVAS FUTURAS

IV.1. Conclusiones generales

En la presente Tesis Doctoral se han desarrollado diferentes metodologías analíticas sostenibles con un bajo impacto ambiental, permitiendo la extracción y determinación analítica de compuestos estrogénicos en aguas, leche y derivados lácteos. Para ello, se han aplicado métodos de microextracción y técnicas cromatográficas miniaturizadas acopladas a la MS.

En base a los objetivos que se describieron previamente, los resultados experimentales han permitido obtener las siguientes conclusiones:

- Se ha desarrollado un método MEKC-MS para la determinación de compuestos estrogénicos. En este sentido, un grupo de 12 estrógenos fue separado mediante MEKC utilizando el PFOA como tensioactivo volátil, lo que permitió una elevada compatibilidad de este modo de CE con la detección mediante MS.
- El empleo de la fase estacionaria Pinnacle^{TM} Phenyl con base de sílice permitió la separación de un amplio grupo de compuestos estrogénicos mediante nano-LC y CEC, incluyendo la separación de isómeros bajo las condiciones óptimas.
- El acoplamiento de la nano-LC y la CEC con la MS se llevó a cabo con éxito utilizando interfases ESI de nano-spray y unión-líquida, respectivamente.
- Se obtuvo un incremento importante de la sensibilidad utilizando el NSM como método de preconcentración en línea en MEKC y la focalización en columna en nano-LC.
- Se desarrolló un método selectivo de GS-MS (QqQ) utilizando BSTFA como agente derivatizante para la separación de un grupo de 13 compuestos estrogénicos.
- Se emplearon de forma eficaz procedimientos miniaturizados de extracción/preconcentración fuera de línea, como la DLLME y la HF-LPME, para extraer los compuestos de interés de matrices medioambientales y alimentarias.
- Se optimizó un método MIP-SPE para la extracción de compuestos estrogénicos de muestras de aguas utilizando cartuchos comerciales con el ZEN como molécula molde, permitiendo la extracción selectiva de los analitos en estudio.
- Las metodologías desarrolladas (DLLME-MEKC-MS/MS, MIP-SPE-nano-LC-MS y HF-LPME-GC-MS/MS) se validaron mediante estudios de repetibilidad, calibración, precisión, exactitud y recuperación en matrices acuosas, leche y derivados lácteos,

determinando la sensibilidad para cada uno de los compuestos en cada procedimiento analítico. Todo ello demuestra su posible aplicación al análisis de muestras reales.

IV.2. Perspectivas futuras

Los resultados obtenidos a partir de los trabajos desarrollados suponen un punto de partida para incrementar el número de metodologías analíticas que pueden ser utilizadas para la determinación de compuestos estrogénicos, de acuerdo con las tendencias de la Química Analítica verde. En este sentido, las perspectivas futuras con respecto al análisis de estos compuestos podrían enfocarse en los siguientes aspectos:

- Empaquetamiento de columnas capilares con partículas de d.i. inferiores a 2 µm para su empleo en nano-LC y CEC.
- Desarrollo de métodos rápidos nano-LC-MS, incluyendo la utilización de columnas capilares como emisores ESI.
- Combinación de diferentes fases estacionarias en la misma columna capilar para incrementar la capacidad de carga de la CEC con el objetivo de mejorar la sensibilidad analítica.
- Utilización de analizadores de masa más sensibles, como el QqQ o el de tiempo de vuelo (TOF), en el acoplamiento de la nano-LC y la CEC con la MS.
- Empleo de sorbentes nuevos y más selectivos en los procedimientos de extracción, como el carbón poroso grafitizado u otras nanopartículas.

V. English summary and conclusions

V. ENGLISH SUMMARY AND CONCLUSIONS

Hormones are natural substances produced by the organism to activate o deactivate certain functions. In this regard, the endocrine system coordinates and regulates vital activities through numerous hormones, including metabolism, reproduction or embryonic and fetal development. The endocrine glands, which are localized in different parts of the human body, react to external stimuli secreting hormones, which are transported by blood and in small amounts acts in some target cells that owns specific receptors to them.

Estrogens are, together with progestagens and androgens, sex steroid hormones involved in the menstrual cycle of humans and, in general, in the estrous cycle of mammalians. They stimulate the development of female reproductive apparatus and play an important role in mineral, fat, sugar and protein metabolization, being related with digestion, blood coagulation, cholesterol metabolization and some kidney functions.

Estrogenic hormones can be classified in two groups: natural/endoestrogens and synthetic/exoestrogens. Estrone (E_1), estradiol (E_2) and estriol (E_3) are natural organism-synthesized estrogens, also contained in milk, being E_2 the one that interacts with the estrogen receptors. On the other hand, exoestrogens are foreign compounds, either naturally or synthetically produced. Among them, ethynylestradiol (EE_2) is one of the most important, which is used for increasing the weight gain of animals. It owns, along with natural estrogens, an estrane-like structure, which has an aromatic ring possessing a hydroxyl group in position 3. The difference between them consists in the substituents on the tetracyclic ring. Synthetic stilbenes like diethylbestrol (DES), dienestrol (DS) and hexestrol (HEX) are also included in this group because of their growth promoter activity. They are also used as a treatment for estrogen-deficiency disorders in veterinary medicine, although they are non-steroidal compounds. This is also the case of the so-called xenobiotic estrogens like the zearalanone derivatives (*i.e.* zearalanol (ZAL) and zearalenol (ZEL)), also used for improving feed conversion efficiency as well as promoting growths rates in livestock.

Apart from the free forms of natural estrogens, their metabolites (methylated, hydroxilated, etc.) are also of great importance. Alternatively, estrogens can undergo a process of conjugation with sulfate and glucuronic acid. These conjugated forms are not biologically active, but when they reach the human gut, they can be transformed into their free active forms by bacterial sulfatases and by bacterial and/or

endogenous glucoronidases. Since both groups represent more than 85% of the total estrogen content in commercial milk, depending on the animal and on the gestation state, it is of great interest to determine both free and conjugated forms.

The main objective of this Ph.D. Thesis is the development of sustainable analytical methodologies with low environmental impact which allow the extraction of natural and synthetic estrogens, their metabolites and the most representative mycoestrogens in water, milk and dairy products. To this end, different extraction and microextraction procedures have been employed combining miniaturized analytical techniques with MS. While working on this main objective, different secondary issues have been raised. These include:

1) To optimize the analytical separation of different groups of compounds with estrogenic activity.

- To evaluate the use of PFOA as a volatile surfactant to effectively couple MEKC with MS.
- To investigate the use of different stationary phases to make capillary columns which provide adequate analytical separation in nano-LC and CEC.
- To carry out the coupling of nano-LC and CEC with MS by using nano-spray and liquid-junction interfaces, respectively.
- To study the derivatization reaction of estrogenic compounds with BSTFA for their analysis by GC-FID and GC-MS.
- To consider the application of on-line preconcentration methods in CE (stacking methods) and in nano-LC (focusing) in order to increase the sensitivity of the method.

2) To optimize different sample preparation procedures.

- To study the application of LPME techniques, such as DLLME and HF-LPME for the extraction of compounds with estrogenic activity from aqueous samples, milk and dairy products, optimizing the different parameters influencing the microextraction, as well as possible sample pre-treatments (deproteinization, lipid removal, etc.).
- To assess the implementation of a commercial MIP for the SPE of selected compounds in aqueous matrices.
- To validate the methodologies developed based on repeatability, calibration, precision, accuracy and recovery studies, as well as to

determine the sensitivity obtained for each of the studied compounds.

- To demonstrate the potential of the developed methodologies to analyse samples related to the possible presence of compounds with estrogenic activity, such as wastewater, milk and dairy products.

The presence of contaminants in food matrices is an issue that has aroused increasing concern, both for producers and consumers worldwide. In particular, the presence of compounds with estrogenic activity such as natural and synthetic estrogens or mycoestrogens in foods of animal origin, and in particular in milk and dairy products, is especially alarming, given the enormous daily consumption of these products worldwide. All this, along with the fact that in many cases these contaminants are not completely removed in wastewater treatment plants, makes their release into the environment.

For the determination of these compounds, the use of highly sensitive, effective and selective analytical methods is required. The most critical stage of this process is the sample preparation. In this respect, current trends are focused on the development of miniaturized extraction methods, such as DLLME (*Articles 1 and 2*) and HF-LPME (*Article 4*) and on the use of highly selective materials such as MIPs in SPE (*Article 3*). DLLME and HF-LPME have been applied only rarely for the extraction of the compounds studied in this Ph.D. Thesis and in no case for the simultaneous determination of them in water, milk and milk derivatives.

As previously discussed, the analysis of complex matrices such as milk or yogurt requires a previous step consisting in protein precipitation and delipidation. In the works that constitute this Thesis, deproteinization was carried out using an acid in the presence of an organic solvent (*Articles 2 and 4*), while fat removal was performed by LLE with *n*-hexane (*Article 2*). Moreover, in the case of water analysis, a simple filtration to remove any solid residues (*Articles 1 and 3*) was performed.

With regard to separation methods, and although compounds with estrogenic activity have been separated in a number of occasions by conventional LC, and in fewer cases by GC, the use of miniaturized chromatographic techniques has been scarce. Furthermore, for an unambiguous detection of the compounds, better isolation of interfering compounds from the matrix and, particularly, to increase the sensitivity, the separation technique has in all cases been coupled to MS.

On the one hand, and since the studied estrogenic compounds are not charged in a wide range of pH, their separation was performed by CE-

MS using MEKC as the separation mode (*Articles 1 and 2*). Coupling with MS was carried out using PFOA as an ESI compatible volatile surfactant.

On the other hand, the use of a 25 cm long and 75 μm i.d. column packed with 3 μm phenyl groups functionalized silica particles, allowed the separation of the target compounds in a nano-LC-MS system with a nano-spray interface, and in a CEC-MS system with a liquid-junction interface (*Article 3*). Finally, their separation was carried out also by GC-MS/MS derivatizing the compounds with BSTFA before the injection, resulting in TMS-derivatives (*Article 4*).

All the proposed methodologies were duly validated through repeatability, calibration, linearity, accuracy and precision studies, and calculating the LODs and LOQs, both of the instrument and of the whole methodology. They were also applied to the analysis of real samples.

V.1. General conclusions

In this Ph.D. Thesis, different sustainable analytical methodologies with low environmental impact have been developed, which allowed the extraction and analytical determination of estrogenic compounds in water, milk and dairy products. For this purpose, microextraction methods and miniaturized chromatographic techniques coupled to MS have been successfully applied.

Based on the aims that have been described at the beginning of this Ph.D. Thesis, the experimental results have permitted to obtain the following conclusions:

- A MEKC-MS method has been developed for the determination of estrogenic compounds. In this respect, a group of 12 estrogens were separated using MEKC with a volatile surfactant, PFOA, which allowed a high compatibility of this CE mode with MS detection.
- The use of silica-based Pinnacle™ II Phenyl stationary phase allowed the separation of a wide group of estrogenic compounds by nano-LC and CEC, being isomer separation possible under the best conditions.
- The coupling of nano-LC and CEC with MS was successfully achieved with a nano-spray and a liquid-junction ESI interface, respectively.
- An important increase in the sensitivity was obtained using NSM as on-line pre-concentration method in MEKC and on-column focusing in nano-LC.
- A selective GC-MS (QqQ) method using BSTFA as derivatization agent was developed for the separation of a group of 13 estrogenic compounds.

- Miniaturized off-line extraction/pre-concentration procedures, such as DLLME and HF-LPME, were efficiently employed to extract the target analytes from environmental and food matrices.
- A MIP-SPE method was optimized for extraction of estrogenic compounds from water samples using commercial cartridges based on a ZEN template, allowing a selective extraction of the target analytes.
- The methodologies developed, *i.e.* DLLME-MEKC-MS/MS, MIP-SPE-nano-LC-MS and HF-LPME-GC-MS/MS, were validated through repeatability, calibration, precision, accuracy and recovery studies in water, milk and dairy products matrices, determining the sensitivity achieved for each of the studied compounds in each analytical procedure, which demonstrates their effective applicability to analyze real samples.

V.2. Future perspectives

Results obtained in these works suppose a starting point to increase the number of analytical methodologies that can be used to determine estrogenic compounds, according to green Analytical Chemistry trends. In this respect, future prospects with respect to the analysis of these compounds might be focused on:

- Packing capillary columns with sub-2 μm particles to be employed in nano-LC and CEC.
- Developing of fast-nano-LC-MS methods, including the use of ESI-tip emitter capillary columns.
- Combining different stationary phases in the same capillary column to increase the load capacity of CEC in order to improve the analytical sensitivity.
- Using more sensitive mass analyzers, such as QqQ or TOF, in the coupling of nano-LC and CEC with MS.
- Employing new and more selective sorbents in the extraction procedures, such as porous graphitic carbon or other nanoparticles.

VI. Bibliografía

VI. BIBLIOGRAFÍA

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VII. Glosario

VII. GLOSARIO

2D	Dos dimensiones
2-$MeOE_2$	2-Metoxiestradiol
17α-E_2	17α-estradiol
17β-E_2	17β-estradiol

A

ACN	Acetonitrilo
α-ZAL	α-Zearalanol+
α-ZEL	α-Zearalenol
API	Ionización a presión atmosférica
AD	Detector amperométrico.
AOT	Sulfosuccinato sódico, octano, 1-butanol, CAPSO, tampón CAPS
Anti-ES	Anti-estrógeno

B

β-CD	β ciclodextrina
BGE	Electrolito de separación
BSFTA	N-O-bis-(trimetilsilil)-trifluoroacetamida
β-ZAL	β-Zearalanol
β-ZEL	β-Zearalenol

C

CE	Electroforesis capilar
CEC	Electrocromatografía capilar
CGE	Electroforesis capilar en gel
CIEF	Isoelectroenfoque capilar
CITP	Isotacoforesis capilar
CLC	Cromatografía líquida capilar
CNTs	Nanotubos de carbono
CTAB	Bromuro de cetiltrimetilamonio
CZE	Electroforesis capilar en zona libre
CF-FAB	Átomos rápidos en flujo continuo
CMC	Concentración micelar crítica
CAPSO	Ácido 3-(ciclohexilamino)-2-hidroxi-1-propanosulfónico acid
CAPS	Tampón, ácido ciclohexilamino-1-propanosulfónico
CF-SDME	Microextracción en gota en flujo continuo
CNTs-HF-SPME	Microextracción en fase sólida con fibra hueca reforzada con CNTs
$[C_8MIM][PF_6]$	Hexafluorofosfato de 1-octil-3-metilimidazolio
$[C_6MIM][PF_6]$	Hexafluorofosfato de 1-hexil-3-metilimidazolio
$[C_6MIM][BF_4]$	Tetrafluoroborato de 1-hexil-3-metilimidazolio.

D

d.i.	Diámetro interno
DDME	Microextracción en gota a gota
DI-SDME	Microextracción en gota de inmersión directa
DLLME	Microextracción líquido-líquido dispersiva
DS- DME	Microextracción en gota colgante

dSPE	Extracción en fase sólida dispersiva
DES	Dietilestilbestrol
DS	Dienestrol
DAD	Detector de diodos en serie
DHPMM	Polimetilmetacrilato hidroxilado
DLLME-HF CEP	DLLME-HF collection of extraction phase
DLLSME-MIPFs	Microextracción líquido-líquido-sólido dinámica con filamentos de MIP

E

ECD	Detector electroquímico.
EI	Impacto electrónico
EOF	Flujo electroosmótico
ESI	Ionización por electrospray
EU	Unión Europea
EPA	US Environmental Protection Agency
E1	Estrona
E_2	Estradiol
E_3	Estriol
EE_2	17β-etinilestradiol
EKC	Cromatografía electrocinética
ED	Detección electroquímica
ES-IA	Inmunoafinidad de estrógenos
ES-ELISA	Ensayo por inmunoadsorción (anticuerpo anti-estrógeno (ES))

F

FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FASS	Inyección electrocinética o amplificación del campo
FD	Detección por fluorescencia
FO-DLLME	Microextracción líquido-líquido dispersiva con gota flotante
FID	Detector de fotometría de llama

G

GC	Cromatografía de gases
GMA	Glicidil metacrilato

H

HF	Fibra hueca
HF-LPME	Microextracción en fase líquida con fibra hueca
HILIC	Cromatografía líquida de interacción hidrófila
HPLC	Cromatografía líquida de alta resolución
HS-SDME	Microextracción en gota en espacio en cabeza
HEX	Hexestrol
HF-SEBLLME	Microextracción líquido-líquido en fibra hueca con barra de agitación magnética
HF-LLLME	Microextracción líquido-líquido-líquido en fibra hueca
HFBA	Anhídrido heptafluorobutírico
HFPIL	Fibra hueca recubierta con un líquido iónico polimérico

I

IL	Líquido iónico
IT	Trampa de iones
ITP	Isotacoforesis
ILFOF-IL-DLLME	DLLME con flotación de espuma de líquido iónico

L

LC	Cromatografía líquida
LLE	Extracción líquido-líquido
LPME	Microextracción en fase líquida
LOD	Límite de detección
LOQ	Límite de cuantificación
LLLME	Microextracción en gota líquido-líquido-líquido
LLME	Microextracción líquido-líquido
LPME	Microextracción en fase líquida
LIF	Fluorescencia inducida por láser

K

K_a	Constante de disociación ácida

M

μ	Movilidad electroforética
MEKC	Cromatografía electrocinética micelar
MeOH	Metanol
MIP	Polímero de impresión molecular
MS	Espectrometría de masas
MSPD	Dispersión sólida en la matriz
Mw	Masa molecular
MWCNTs	Nanotubos de carbono de pared múltiple
MSTFA	N-metil-N-trimetilsililtrifluoroacetamida
MEEKC	Cromatografía electrocinética de microemulsión
MIP-HFT	Fibra hueca recubierta con un polímero de impronta molecular
MMIP-SPME	MIP magnético-SPME
MIP-MSPD	Dispersión sólida en la matriz con MIP
MIP-HFT	Fibra hueca recubierta con MIP

N

Nano-LC	Nanocromatografía líquida
NACE	Electroforesis capilar en medios no acuosos
NSM	*Stacking* en modo normal

P

PSA	Amina primaria/secundaria
PTFE	Politetrafluoroetileno
PSP	Pseudo-estacionarias.
PF	Partial filling
PFOA	Perfluorooctanoico

PFOS	Perfluorooctanosulfónico.
pCEC	CEC - modalidad presurizada
PC-HF-LPME	Microextracción en fase líquida con fibra hueca recubierta de polímero
PVDF	Fluoruro de polivinildeno

Q

Q	Cuadrupolo simple
QqQ	Triple cuadrupolo
QuEChERS	Método de preparación de muestra "*Quick, Easy, Cheap, Effective, Rugged* y *Safe*"

R

RSD	Desviación estándar relativa
RMM	Reverse-migrating micelles

S

S/N	Relación señal/ruido
SDME	Microextracción en gota
SDS	Dodecilsulfato sódico
SFE	Extracción con fluidos supercríticos
SFO-DLLME	Microextracción líquido-líquido dispersiva con gota flotante sólida
SLM	Membrana líquida soportada
SPME	Microextracción en fase sólida
SPE	Extracción en fase sólida
SWCNTs	Nanotubos de carbono de pared sencilla
SIM	Single ion monitoring

T

TOF	Tiempo de vuelo
TMIS	Trimetilyodosilano
TMCS	Trimetilclorosilano
TMS	Trimetilsilil

U

UAE	Extracción con disolvente asistida por ultrasonidos
UHPLC	Cromatografía líquida ultra rápida
UV	Ultra violeta

Z

ZEN	Zearalenona

Anexo I. Artículos científicos

Los artículos recopilados en esta Tesis Doctoral han sido reproducidos con el permiso de las editoriales correspondientes

Anexo I.1. - Artículo 1

Estrogenic compounds determination in water samples by dispersive liquid-liquid microextraction and micellar electrokinetic chromatography coupled to mass spectrometry

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Estrogenic compounds determination in water samples by dispersive liquid–liquid microextraction and micellar electrokinetic chromatography coupled to mass spectrometry☆

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ABSTRACT

In this work, a group of 12 estrogenic compounds, i.e., four natural sexual hormones (estrone, 17β-estradiol, 17α-estradiol and estriol), an exoestrogen (17α-ethynylestradiol), a synthetic stilbene (dienestrol), a mycotoxin (zearalenone) and some of their major metabolites (2-methoxyestradiol, α-zearalanol, β-zearalanol, α-zearalenol and β-zearalenol) have been separated and determined by micellar electrokinetic chromatography (MEKC) coupled to electrospray ion trap mass spectrometry. For this purpose, a background electrolyte containing an aqueous solution of 45 mM of perfluorooctanoic acid (PFOA) adjusted to pH 9.0 with an ammonia solution, as MS friendly surfactant, and methanol (10% (v/v)), as organic modifier, was used. To further increase the sensitivity, normal stacking mode was applied by injecting the sample dissolved in an aqueous solution of 11.5 mM of ammonium PFO (APFO) at pH 9.0 containing 10% (v/v) of methanol for 25 s. Dispersive liquid–liquid microextraction, using 110 μL of chloroform and 500 μL of acetonitrile as extraction and dispersion solvents, respectively, was employed to extract and preconcentrate the target analytes from different types of environmental water samples (mineral, run-off and wastewater) containing 30% (w/v) NaCl and adjusted to pH 3.0 with 1 M HCl. The limits of detection achieved were in the range 0.04–1.10 μg/L. The whole method was validated in terms of linearity, precision, recovery and matrix effect for each type of water, showing determination coefficients higher than 0.992 for matrix-matched calibration and absolute recoveries in the range 43–91%.

1. Introduction

Estrogens can be considered as global environmental pollutants, and their occurrence in aquatic systems has been demonstrated [1,2]. In particular it has been confirmed that they appear in the effluents from wastewater treatment plants since they are not entirely metabolized in the animal organism. In this way, natural occurring estrone (E_1), estradiol (E_2) and estriol (E_3), the synthetic derivative of E_2, 17α-ethynylestradiol (EE_2), widely used as contraceptive, dienestrol (DS) which is a growth promoter for cattle, or zearalenone (ZEN) derived mycotoxins present in cereal crops that might be consumed by mammals, are mainly excreted in urine. Furthermore, estrogenic compounds contained in animal wastes which are used in agricultural fields for fertilizing purposes may also reach run-off or groundwater.

Different methods have been developed so far for the determination of estrogens and their metabolites in diverse matrices [3,4]. Immunoassays were the first methods widely applied and although they are still used to obtain rapid and sensitive analysis of targeted estrogens, their disadvantages such as single analyte analysis, cross-reactivity or poor specificity have made the use of chromatographic techniques more reliable. In this sense, either liquid chromatography (LC), which is the preferred choice, or gas chromatography (GC) have been used [1–5]. In this last case, a derivatization step is required because of the low volatility of these

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compounds [6], a fact that clearly enlarges the analysis time and that may somehow complicate the sample pretreatment.

Regarding the use of capillary electromigration methods, which offer the typical advantages of any miniaturized technique, they have also been applied for the analysis of estrogens but in a lower number of occasions [7–19]. In these cases, and because they are neutral in a wide range of pH, they cannot be easily analyzed by capillary zone electrophoresis (CZE) [9] and therefore, micellar electrokinetic chromatography (MEKC) is preferred [8,11,13–19] though microemulsion electrokinetic chromatography (MEEKC) [10] and capillary electrochromatography (CEC) [7,20] have also been addressed for this purpose. In this sense, the use of non-volatile surfactants in MEKC has clearly precluded its combination with MS detection. Ion suppression effects as well as contamination of the ionization source are the main reasons and, as a result, estrogen analysis by MEKC has mostly used conventional detectors like UV/DAD [8,11,13–15,17] or electrochemical [18]. To the best of our knowledge, MEKC–MS analysis of estrogens has only been carried out in one occasion [21]. In that work, Sirén et al. analyzed a group of steroids in which only one estrogen, 17β-estradiol (17β-E_2), was included, using the partial filling technique (PF–MEKC–MS) to prevent the introduction of the micelles into the mass spectrometer. This is, in fact, the most employed alternative to avoid contamination of the mass spectrometer in MEKC–MS together with the use of reverse-migrating pseudostationary phases [22].

In order to achieve a direct coupling of MEKC with MS, some authors have suggested the use of alternative pseudostationary phases as, for example, the (semi)volatile surfactant ammonium perfluorooctanoate (APFO) [23–27]. It has been observed that its use provides similar results to standard systems based on sodium dodecyl sulphate and exceptional results regarding its high compatibility with MS [23,24]. This approach has been employed only for the separation of pharmaceutical drugs [24], pesticides [25,26] and, very recently, amino acids [27]. However, the use of APFO as a volatile surfactant in MEKC–MS for the analysis of estrogens has not yet been described.

Regarding the extraction of estrogens from aqueous samples, solid-phase extraction has been the preferred technique in the last decades [3] due to its simplicity of operation and high enrichment capacity in order to achieve concentrations in the low ng/L range. However, when a high preconcentration factor is required, high sample volumes are necessary (and therefore a relatively long sample retention time) as well as relatively high amounts of harmful organic solvents. To overcome this and other problems, miniaturized extraction techniques have been developed and widely used in recent years. In particular liquid-phase microextraction (LPME) in its different application modes – single drop microextraction, dispersive liquid–liquid microextraction (DLLME) and hollow fiber-LPME – has provided very good results in the extraction of organic compounds [28–30], because of their simplicity, effectiveness, rapidity and low consumption of organic solvents over classical techniques.

In general, DLLME has been the most exploited mode of LPME, but the number of applications in the estrogen field is still very low and only water samples have been analyzed [31–35] except for ZEN, which has been analyzed in beer [36] and milk thistle [37]. In this regard, a low number of analytes (no more than five) have been simultaneously extracted. Hadjmohammadi and Ghoreishi [31], for example, developed a method to extract E_1, E_2 and diethylstilbestrol (DES) from river, well and tap water using tetrachloromethane (CCl_4) and acetone as extraction and dispersion solvents. Similarly, Lima and co-workers [35] monitored E_2 and EE_2 in tap, surface and waste waters using chlorobenzene and acetone. Chang and Huang [34] and Liu et al. [32] studied the application of a DLLME method based on the solidification of a floating organic drop (SFO–DLLME) for the extraction of E_1 [34], E_2, E_3 and EE_2 [32,34] from different water samples using 1-undecanol and methanol (MeOH) as extractant and disperser solvent, respectively. Also, ionic liquid-based (IL)-DLLME using 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIm][PF_6]) as extraction solvent was used to extract E_1, E_2, E_3, EE_2 and DES from river, waste and sea water with the help of acetone as disperser solvent. In all the cited works, LC coupled to UV or fluorescence detection (FD) have been used; none of them has applied CE as separation technique. In fact, the combination of DLLME with CE for the analysis of organic compounds has only been achieved in a reduced number of occasions. Some examples can be found in [25,38,39].

As a result, the aim of this work is the development and validation of a DLLME–MEKC–MS method using APFO as a volatile BGE surfactant for the analysis of a group of twelve estrogenic compounds, i.e., four natural sexual hormones (E_1, 17β-E_2, 17α-E_2 and E_3), an exoestrogen (EE_2), a synthetic stilbene (dienestrol, DS), a mycotoxin (zearalenone, ZEN) and some of their major metabolites (2-methoxyestradiol, 2-$MeOE_2$, α-zearalanol, α-ZAL, β-zearalanol, β-ZAL, α-zearalenol, α-ZEL, and β-zearalenol, β-ZEL), in different types of water samples (mineral, run-off and waste waters). An on-line preconcentration technique (normal stacking mode) was also used to enhance sensitivity. To the best of our knowledge, this is the first time that MEKC–MS is used for the separation of the selected analytes and one of the very few applications of APFO as volatile surfactant in MEKC–MS. It is also the first time that DLLME is combined with CE for the analysis of estrogens, being also one of the few works that join the use of DLLME with CE.

2. Materials and methods

2.1. Chemicals and materials

All chemicals used in this study were of analytical reagent grade. MeOH was from VWR International Eurolab S.L (Barcelona, Spain), ethanol absolute (EtOH) was from Panreac Química (Barcelona, Spain); 2-propanol, dichloromethane (CH_2Cl_2), CCl_4, chloroform ($CHCl_3$), acetone, ACN of HPLC grade and hydrochloric acid (25% (w/w)) were obtained from Merck (Darmstadt, Germany); sodium hydroxide (NaOH) was from Scharlau Chemie S.A. (Barcelona, Spain), ammonia (25% (w/w)) from Riedel-de Haën (Seelze, Germany) and sodium chloride (purity > 99.5%) was purchased from Sigma–Aldrich Chemie (Madrid, Spain). Distilled water was deionized by a Milli-Q gradient system A10 from Millipore (Bedford, MA, USA). Perfluorooctanoic acid (PFOA, purity of 96%) was supplied by Sigma–Aldrich.

Analytical standards of DS (3,4-bis(4-hydroxyphenyl)-2,4-hexadiene), 17α-E_2 (1,3,5(10)-estratriene-3,17α-diol), 17β-E_2 (1,3,5-estratriene-3,17β-diol), E_3 (1,3,5(10)-estratriene-3,16α,17β-triol), E_1 (1,3,5(10)-estratrien-3-ol-17-one), EE_2 (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol), 2-$MeOE_2$ (1,3,5(10)-estratriene-2,3,17-triol 2-methyl ether), α-ZAL (2,4-dihydroxy-6-(6α,10-dihydroxyundecyl)benzoic acid μ-lactone), β-ZAL (2,4-dihydroxy-6-(6β,10-dihydroxyundecyl)benzoic acid μ-lactone), β-ZEL (2,4-dihydroxy-6-(6β,10-dihydroxy-trans-1-undecenyl)benzoic acid μ-lactone), α-ZEL (2,4-dihydroxy-6-(6α,10-dihydroxy-trans-1-undecenyl)benzoic acid μ-lactone), ZEN ((3S,11E)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione) were from Sigma–Aldrich Chemie and were used without further purification (purity ≥ 95%). Table 1 shows the structures and some of the properties of the studied compounds.

Individual stock solutions of the analytes were prepared in MeOH with the following concentrations: DS, 17β-E_2, 17α-E_2, E_3, E_1, EE_2 at 1000 mg/L; 2-$MeOE_2$, α-ZAL and β-ZAL at 100 mg/L;

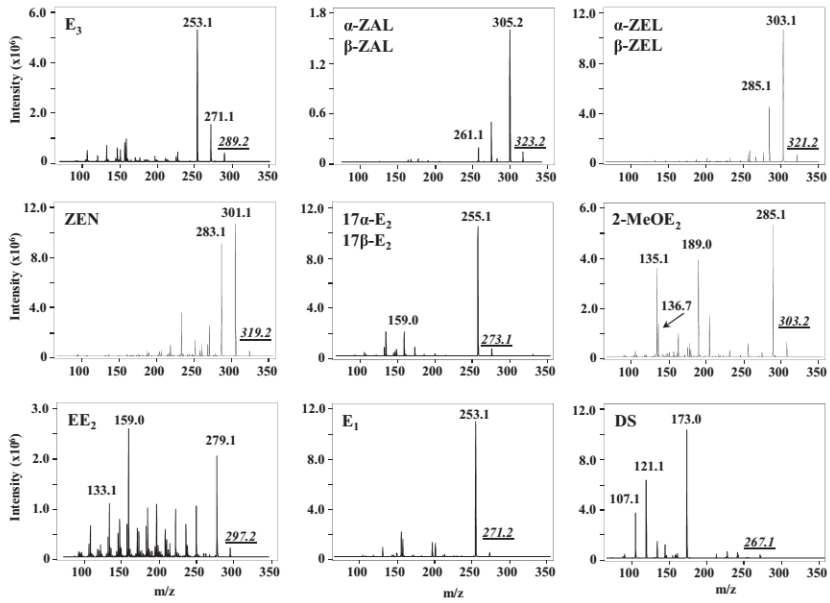

Fig. 1. MS/MS spectra of the standard estrogens. For experimental conditions, see Section 2.3.

α-ZEL and β-ZEL at 500 mg/L and ZEN at 180 mg/L. They were all stored in the freezer at −18 °C. The working standard mixtures were daily prepared by appropriate dilution in the sample medium (11.25 mM of APFO at pH 9 containing a 10% (v/v) of MeOH).

2.2. Apparatus, instrumentation and software

All CE–MS studies were performed with a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA) coupled with an amaZon SL ion trap (IT) mass spectrometer from Bruker Daltonik GmbH (Bremen, Germany). Karat Software (Beckman Instruments) was used for CE instrument control while all MS parameters and spectra data were controlled, collected and processed by using the Esquire NT software from Bruker Daltonik.

CE analyses were carried out using fused silica capillaries of 60 cm (50 μm i.d. × 363 μm o.d.) obtained from Composite Metal Services (Worcester, UK). The polyimide coating of the capillaries was removed from the capillary ends with a razor, checking the result with a Nikon Model SE Binocular optical microscope (Nikon Instruments Inc., Japan).

Measurements of pH were done with a Crison GLP 22 pH-meter (Barcelona, Spain) equipped with a combined electrode with a temperature sensor. The accurate measurement of pH was ensured by a two-point calibration with the appropriate certified buffer solutions at pH 4.00 and 7.00. Conductivity measurements were achieved using a Crison CM 35 portable conductimeter with a platinum cell and temperature correction capability. One-point calibration was done with a certified KCl solution of 1413 μS/cm.

Statgraphics Centurion XV Version 15.2.06 from Statistical Graphics (Rockville, MD, USA) was used for data processing.

2.3. CE–MS analysis

Before its first use, the capillary was activated by means of the following rinse sequence: 3 min of 1 M HCl, 3 min of Milli-Q water, 5 min of 0.1 M NaOH, 10 min of Milli-Q water, and finally 2 min of the BGE. In all cases, a pressure of 20 psi (137.9 kPa) was applied. The BGE was a solution of 45 mM APFO at pH 9.0 containing a 10% (v/v) of MeOH which was prepared by dissolving PFOA in Milli-Q water and adjusting the pH with 7 M ammonia solution.

In order to obtain a good repeatability between days, the capillary was flushed at the beginning of the working day with 7 M ammonia solution and Milli-Q water for 10 min each, and then with BGE for 2 min. Also, good repeatability was achieved by rinsing the capillary between injections with 7 M ammonia solution, Milli-Q water and BGE for 2 min each. At the end of the working day, the capillary was washed consecutively with 7 M ammonia solution, Milli-Q water and MeOH for 5 min each. All these washings steps were also done at a pressure of 20 psi. The capillary was left full of MeOH overnight.

Samples were injected hydrodynamically at 0.5 psi for 25 s. The electrophoretic separation was achieved using a voltage of +25 kV with an initial ramp time of 0.17 min. The temperature of the capillary inside the cartridge was 25 °C and the sample vial was kept constant at 15 °C before injection.

In order to couple the CE system to the mass spectrometer, the capillary was fixed into a coaxial sheath–liquid interface so that its outlet protruded 0.1 mm from the coaxial steel needle. The CE–MS system operated in the positive ionization mode. MS electropherograms were acquired in a range of 250–350 m/z and the MS tune was set in automatic mode, selecting 300 m/z as the target mass of the studied compounds (trap drive level of 100%).

Optimized ESI conditions were as follows: capillary voltage of −4600 V, end plate offset of −500 V, nebulizer gas (N_2) pressure

Table 1
Chemical structure and some properties of the studied compounds.

Analyte	CAS	Abbreviation	Structure	Mw (g/mol)	pK_a [a]	K_{ow} (log P) [b]	Solubility in water (g/L, 25 °C) [b]
Estriol	50-27-1	E_3		288.4	10.25	2.527	0.029 [c] 0.032 [d] 0.043 [e]
β-Zearalanol	42422-68-4	β-ZAL		322.4	8.08 [b]	4.648	0.55 [c] 12 [d] 387 [e]
β-Zearalenol	71030-11-0	β-ZEL		320.4	7.61 [b]	3.184	1.9 [c] 130 [d] 1000 [e]
α-Zearalenol	36455-72-8	α-ZEL		320.4	7.61 [b]	3.184	1.9 [c] 130 [d] 1000 [e]
17β-Estradiol	50-28-2	17β-E_2		272.4	10.27	4.146	3.0×10^{-3} [c] 3.0×10^{-3} [d] 4.6×10^{-3} [e]
17α-Estradiol	57-91-0	17α-E_2		272.4	10.27	4.146	3.0×10^{-3} [c] 3.0×10^{-3} [d] 4.6×10^{-3} [e]

Zearalenone	17924-92-4	ZEN		318.4	7.58[b]	2.765	0.96[c] 76[d] 1000[e]	
17α-Ethinylestradiol	57-63-6	EE_2		296.4	10.24	4.106	3.9×10^{-3} [c] 3.9×10^{-3} [d] 5.9×10^{-3} [e]	
α-Zearalanol	26538-44-3	α-ZAL		322.4	8.08[b]	4.648	0.55[c] 12[d] 387[e]	
2-Methoxyestradiol	362-07-2	2-MeOE_2		302.4	10.29	3.842	4.5×10^{-3} [c] 4.8×10^{-3} [d] 7.0×10^{-3} [e]	
Estrone	53-16-7	E_1		270.4	10.25	3.624	5.7×10^{-3} [c] 5.9×10^{-3} [d] 8.9×10^{-3} [e]	
Dienestrol	84-17-3	DS		266.3	9.21	4.920	5.6×10^{-3} [c] 9.6×10^{-3} [d] 0.088 [e]	

[a] Values taken from SciFinder database [40].
[b] This value corresponds to pK_{a1}.
[c] Solubility at pH 3.
[d] Solubility at pH 9.
[e] Solubility at pH 10.

Table 2
MS and MS/MS detection parameters of the studied estrogenic compounds.

Analyte	Precursor ion (m/z) $[M+H]^+$	Product ion (m/z)	Normalized collision energy
E_3	289.2	271.1	0.30
		253.1	
β-ZAL	323.2	305.2	0.25
		261.1	
β-ZEL	321.2	303.1	0.30
		285.1	
α-ZEL	321.2	303.1	0.30
		285.1	
17β-E_2	273.1	255.1	0.32
		159.0	
17α-E_2	273.1	255.1	0.32
		159.0	
ZEN	319.2	301.1	0.35
		283.1	
EE_2	297.2	279.1	0.35
		159.0	
		133.1	
α-ZAL	323.2	305.2	0.25
		261.1	
2-$MeOE_2$	303.2	285.1	0.32
		189.0	
E_1	271.2	253.1	0.32
DS	267.1	173.0	0.40
		121.1	
		107.1	

of 3.0 psi, dry gas (N_2) flow of 3.0 L/min and dry gas temperature of 200 °C. A sheath–liquid consisting in 100% 2-propanol at a flow rate of 1.7 μL/min was used to close the CE–MS electric circuit, to stabilize the CE-current and to assist the ionization. With regard to the IT parameters, the ion charge control (ICC) was set at 110,000, the maximum accumulation time at 200 ms with 6 averages per experiment and a rolling averaging of 7.

MS/MS experiments were performed by fragmentation of the protonated molecular ion $[M+H]^+$ which was selected as the precursor ion. In these last experiments, the set mass range was 70–350 m/z, the ICC was fixed at 10,000 and the maximum accumulation time at 200 ms with a single average per experiment. The m/z width was set at 1 and the collision energy was varied in the range 0.25–0.40. Table 2 shows the fragmentation data obtained for the studied analytes (m/z of the precursor and product ions, as well as the normalized collision energy). The obtained MS/MS spectra are shown in Fig. 1.

At the end of the working day, the ESI interface was cleaned with MeOH in order to remove the residues of the surfactant.

2.4. Water samples selection

In this work, three types of water samples (mineral, run-off and wastewater) were selected. Mineral water was acquired in a local supermarket, while the run-off and the two wastewater samples were collected in different areas of the island of Tenerife (Canary Islands, Spain). pH values for mineral, run-off, wastewater 1 and wastewater 2 were 7.84, 8.15, 8.37 and 8.26, respectively, while conductivity values were 0.18, 1.51, 1.44 and 1.79 mS/cm at 25 °C. Values of the chemical oxygen demand (COD) were 55 mg/L O_2 for wastewater 1 and 1100 mg/L O_2 for wastewater 2. All samples were filtered through a Chromafil® Xtra PET-20/25 filter before extraction.

2.5. DLLME procedure

An aliquot of 7.5 mL of a spiked or non-spiked water sample containing 30% (w/v) NaCl and adjusted to pH 3.0 with 1 M HCl was introduced in a 10 mL screw cap glass tube. Then a mixture containing 500 μL of ACN (as dispersion solvent) and 110 μL of $CHCl_3$ (as extraction solvent) was rapidly introduced into the aqueous sample with the help of a 1 mL micropipette, followed by 2 min of vortex-shaking. The dispersion was centrifuged at 4000 rpm ($2.5 \times g$) for 5 min, observing afterwards a settled phase at the bottom of the tube consisting in a droplet of $CHCl_3$ containing the target analytes. The upper aqueous phase was partially removed (about 4 mL) by means of a micropipette and the droplet (120 μL) was collected into a vial and evaporated to dryness with a gentle nitrogen stream. The residue was reconstituted in 75 μL of the sample medium (11.25 mM of APFO, pH 9 containing a 10% (v/v) of MeOH) and injected hydrodynamically in the CE–MS system. Analyses of blank samples were done in parallel to assure that no residues of the analytes were present in the samples.

3. Results and discussion

3.1. MEKC–MS method

3.1.1. Selection of the detection mode in MS

First of all, and before the optimization of the electrophoretic separation, preliminary studies of the MS determination of the selected group of estrogens were carried out. Although these compounds are generally non-charged in a wide pH range (see Table 1), they can be easily ionized under soft conditions in the ESI. In fact, they have been determined both in the negative [41] and positive mode [9,12,21,42,43].

An initial screening study was developed by filling the capillary with a mixture of the estrogens at a concentration of 10 mg/L in 50/50 (v/v) MeOH/45 mM APFO at pH 9. Then, an electrophoretic run was developed at +25 kV permitting the entrance of the analytes in the spectrometer. The ionization was supported by a sheath–liquid consisting of 90:10 (v/v) 2-propanol/water with 1% (v/v) formic acid or ammonia solution for positive or negative detection, respectively. In these first experiments, early ESI conditions, which were selected by varying each parameter using a step-by-step approach, were maintained as follows: capillary voltage of −4500 V, end plate offset of −500 V, nebulizer gas pressure of 6.0 psi, dry gas flow of 6.5 L/min and dry gas temperature of 200 °C. Regarding the IT, the ICC was set at 60,000, the maximum accumulation time at 200 ms with 6 averages per experiment and a rolling averaging of 7.

In the negative mode, the MS spectra of most of the analytes were not clear and they could not be correctly detected. Moreover, the presence of APFO, though a (semi)volatile surfactant, characterized the MS background spectra with three intense signals corresponding to the PFOA ion $[M-H]^-$ at 412.8 m/z, the loss of carbon dioxide at 368.9 m/z and the dimeric ion at 826.9 m/z, which generated an important ion suppression and a high spray current (≈2000 nA). The best results, indeed, were found in the positive mode, procuring characteristic MS spectra for each compound with high intensity at the time that the current spray did not exceed 25–30 nA.

3.1.2. Optimization of the MEKC–ESI–MS separation

For the optimization of the electrophoretic separation of the selected group of estrogenic analytes, the first experiments were carried out at +25 kV injecting at 0.5 psi for 5 s a mixture of them dissolved in different mediums at a concentration between 3 and 10 mg/L: MeOH (which was the solvent of the stock solutions), water, APFO solutions up to a concentration of 45 mM or mixtures of them, maintaining ESI and IT conditions as indicated in the previous section. In general, it could be observed that when using a BGE composed of different concentrations of APFO consistent results could be obtained when the sample was dissolved

in 80:20 (v/v) water/MeOH, that is, in the absence of surfactant. The use of MeOH alone, though also useful, provided some current breakdowns, while pure water caused low sensitivity due to the low solubility of the analytes.

For BGE composition optimization, initial experiments were carried out with APFO as buffer and surfactant solutions from the critical micellar concentration (CMC), which is 12 mM, to a concentration of 55 mM and pH between 7.5 and 9.5 adjusting it with ammonia solution. Though the use of a mass spectrometer does not require a complete separation of the analytes, efforts were made to separate them, in particular, E_2, ZAL and ZEL epimers (α- and β-), which share the same spectra in order to quantify them separately.

PFOA is a white powder with very low solubility in water at low pH (0.70 and 4.6 g/L at pH 2 and 3, respectively), which pK_a is 0.50 and its CMC is 12 mM (4.96 g/L) [40]. So when PFOA was added to water at the desired concentration (even at its CMC), still some powder was observed in suspension. However, the addition of ammonia solution led to the formation of the highly soluble salt, i.e., APFO, at the same time that the pH of the solution increased. Even increasing the BGE's pH up to 7.5, PFOA exhibited poor solubility. These experimental conditions only allowed studying concentrations of the additive between 40 and 55 mM. However, in general, a decrease in the pH value down to 7.5 produced a lower EOF with the corresponding increase of the analysis time (up to 13 min). Instead, at pH values higher than 9.0 the separation resulted to be poorer, while at pH 9.0, commonly selected condition as reported in the literature [24,26], a good compromise between analysis time (9 min) and resolution was achieved, especially for E_2, ZAL and ZEL epimers (α- and β-).

Following, and keeping the pH at 9.0, the concentration of APFO was varied. The CMC defines the lower end of the working range for a surfactant in MEKC. For the working PFOA/ammonia system, this concentration has been previously determined as 12 mM by surface tension measurements [24]. The effect was shown as a general increase in the migration times and resolution, as well as in some changes in the elution order. In general, concentrations lower than 45 mM did not allow an adequate resolution of the peaks due to a worsening of the peak shape and a loss in efficiency. Although 50 mM APFO allowed an improvement in the separation of the isomers of ZEL and ZAL, the presence of higher amounts of PFOA produced an increase of the background noise and a general reduction of the signal-to-noise ratio (S/N) for all analytes. The best results in terms of separation and sensitivity was achieved with 45 mM. Under these conditions, a partial separation of the selected analytes was obtained in 7.5 min, with good resolution for the isomers of E_2, ZAL and ZEL. However, co-migration of 17α-E_2, EE_2, ZEN and α-ZAL was still observed.

Finally, the effect of adding different organic solvents to the BGE was investigated. The addition of an organic modifier can produce changes in the viscosity of the medium and in the mobility of the analytes, as well as modifications in their partition coefficient. These factors can produce changes in selectivity, migration times, peak shape and also in the MS signal. The solvents tested were MeOH, ACN and 2-propanol at percentages between 5% and 10% (v/v). Both ACN and 2-propanol provided a worsening of the signal, as well as an overlapping of E_2 epimers. On the contrary, a 10% (v/v) of MeOH improved the peak shape with respect to the absence of organic modifier and maintained baseline separation of the ZAL, ZEL and E_2 epimers in less than 12 min. Furthermore, under these conditions, at an applied voltage of +25 kV, the electrophoretic current was kept lower than 25 μA, while the ESI current was about 30 nA, with a stable and intense MS signal and a total ion electropherogram similar to the base peak profile.

Under these conditions, the limits of detection (LODs) achieved, calculated as the concentration which provided a S/N ratio of three, were in the range 21.0–288 μg/L. Since estrogenic compounds are found in environmental waters in the ng/L range, such instrumental LODs are not satisfactory. In order to increase the sensitivity of the determination, two strategies were developed: the application of an on-line preconcentration procedure, namely normal stacking mode (NSM) as well as an off-line one, such as DLLME.

3.1.3. On-line preconcentration by normal stacking mode

As it is well known, when stacking is applied, the sample medium must have a lower conductivity than that of the BGE. As described by Quirino and Terabe [42,43], when using NSM with MEKC, the lower-conductivity sample solution with respect to the separation solution enhances the field in the sample zone, providing micelles with electrophoretic velocities bigger than those in the separation area. Then, in the concentration frontier, micelles decelerate and stack into sharp concentrated regions. For this purpose, different solutions of the analytes (40–100 μg/L) containing either water, BGE (0–45 mM), organic solvents (ACN, MeOH or 2-propanol at 0–50% (v/v)) as well as mixtures of them in different proportions were tested as the sample medium.

The optimum results in terms of resolution and sensitivity were obtained when the sample medium contained 11.5 mM APFO at pH 9.0, which is a concentration of APFO of approximately 4 times lower than that of the BGE. In addition, in order to improve the MS signal and analytes solubility, the effect of an organic solvent added to the sample solutions was studied. As an example of the improvement of the method performance, Fig. 2 shows the results achieved using MeOH, ACN and 2-propanol (panel A) and different concentrations of MeOH in the sample solutions (panel B).

The behavior of other analytes was very similar to that of 17-E_2 epimers reported in this figure. As can be seen, both ACN and 2-propanol caused a loss of 17-E_2 epimers resolution, while for other analyzed compounds resolution was only partly reduced (results not shown). On the contrary, a 10% (v/v) of MeOH improved the peak shape and sensitivity with respect to the absence of organic modifier and maintained baseline separation of the ZAL, ZEL and 17-E_2 epimers in less than 12 min. Summarizing the highest S/N ratios were obtained with the analytes dissolved in 11.5 mM APFO at pH 9.0 containing 10% MeOH (v/v).

Then, the volume of sample introduced into the capillary was studied through the injection time up to 40 s (theoretically equivalent to a sample volume of approx. 40 nL and a 3% of the capillary volume). The plot of the injection time vs the peak height (data not shown) revealed a linear relationship up to 25 s (approx. 25 nL and a 2% of the total capillary volume). Injection times higher than that value produced a decrease of the MS signal due to an increase of the band broadening with a reduction of the resolution. Therefore, for an injection of 25 s and in the extracted ion electropherogram acquisition mode, the instrumental LODs ranged from 2.04 to 59.0 μg/L, which resulted in an average improvement of 10 times with respect to normal injection under the assayed conditions (20.6–287.6 μg/L). These findings are in accordance to what Quirino and Terabe described when stacking neutral analytes in MEKC [44,45]. Besides, the application of this on-line preconcentration strategy also reduced the analysis time down to less than 10 min, which is usual.

3.1.4. Optimization of MS and MS/MS parameters

Once the optimum electrophoretic conditions were obtained, the optimization of the parameters that influence the ionization and MS/MS detection was performed in the positive mode. For this purpose, the standard mixture of the analytes was dissolved in the selected BGE (0.5–1.0 mg/L) and the ESI interface and IT parameters were changed by direct infusion voltage (during an electrophoretic run at +25 kV) in order to obtain the S/N for all the studied analytes.

First of all, and in order to obtain the highest ionization, different compositions of the sheath–liquid were studied. In this regard,

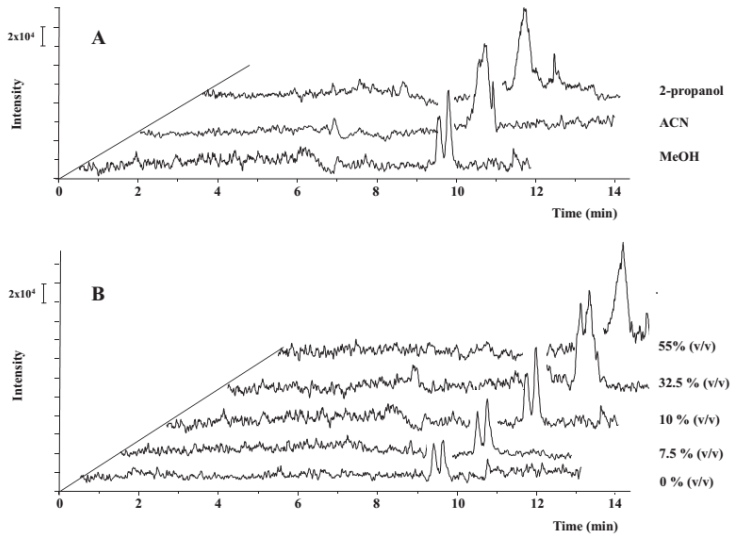

Fig. 2. Extracted ion electropherograms of 17-E_2 epimers (as representative compounds) showing the effect of the addition of a 10% (v/v) of different organic solvents in the sample medium (A) and the percentage of MeOH as the best of them (B) in the NSM study injecting 100 μg/L in 11.5 mM of PFOA at pH 9.0. Experimental conditions: separation capillary, 60 cm × 50 μm i.d. × 375 μm o.d.; BGE, 45 mM of PFOA at pH 9.0 containing 10% (v/v) of MeOH; injection, 0.5 psi for 20 s; applied voltage, +25 kV; temperature, 25 °C. For other experimental conditions and MS parameters, see Section 2.3.

diverse organic solvents (ACN, MeOH, ethanol and 2-propanol) mixed in different proportions with water (50–100% (v/v)) were tested at a constant flow rate of 2 μL/min using also formic or acetic acid as additives (0.1–1% (v/v)) to improve the ionization. The best results in terms of the S/N and stability of the electrophoretic and ESI spray currents were obtained using 100% of 2-propanol in the absence of any acidic additive.

Another important parameter of the CE–MS coupling is the sheath–liquid flow rate, so values between 1.0 and 2.5 μL/min were studied. Below 1.6 μL/min, the spray current was unstable and frequent interruption of the MS signal could be observed. On the contrary, at flow rates above 2 μL/min, the MS signal was dramatically reduced due to a higher dilution of the analytes and an increase of the background noise during the ionization. Furthermore, a deterioration of the electrophoretic separation, in terms of resolution and efficiency, was observed when the flow was high, probably due to a siphon effect, but also to the turbulent mixing of the sheath–liquid and the BGE which come at different velocities (μL/min vs nL/min), producing band broadening and partial mixing of the electrophoretic bands. In conclusion, the best result in terms of sensitivity and electrophoretic profile was obtained using a flow rate of 1.7 μL/min.

After the optimization of the sheath–liquid composition, the MS signal was maximized for each analyte tuning the following mass parameters in specific intervals: capillary voltage [(−4000) − (−5000) V], end plate offset [(−400) − (−600) V], nebulizer gas (N_2) pressure (2–5 psi) and drying gas (N_2) flow rate (1–5 L/min) and temperature (190–210 °C). Finally, a capillary voltage of −4600 V, an end plate offset of −500 V, a pressure of N_2 of 3.0 psi as nebulizer and a flow of 3.0 L/min of N_2 at 200 °C as the drying gas were selected as the best conditions. In order to obtain the maximum response, the value of the ICC was increased from 60,000 to 110,000, reducing the acquisition rate without affecting in any way the MS electrophoretic profile.

These optimization experiments were fundamental to obtain both a good electrophoretic separation in terms of resolution, efficiency and peak shape and excellent and similar total ion current and base peak electropherogram profiles with high S/N. Fig. 3 shows the separation of the selected compounds under the described conditions.

Following, MS/MS fragmentation experiments were developed for qualitative examination in order to confirm or deny the unequivocal presence of the analytes in the samples. A preliminary study was carried out by analyzing the fragmentation patterns of each analyte by direct infusion voltage as described above. Once the molecular peak $[M+H]^+$ was identified, the fragmentation energy was changed manually and optimized in order to obtain high intensity of the fragment ions leaving about a 10% of the precursor ion, comparing the results with those reported in the literature [12,42,43,46]. The presence or absence of all the analytes were unambiguously checked in the studied matrices by isolating and fragmenting the parent ion in the multiple reaction monitoring (MRM) mode. Table 2 shows the fragmentation data obtained for the studied analytes after running the MS/MS experiments while Fig. 1 shows the MS/MS spectra of the standard analytes. As can be seen, all the analytes lose a molecule of water, except DS, which structure is particularly different.

3.1.5. CE–MS repeatability and calibration

Under optimal stacking, separation and detection conditions, the MEKC–MS method was first validated by evaluating its repeatability in terms of the migration times and peak areas. For this purpose, five consecutive injections ($n = 5$) were developed in the same day and also in three consecutive days ($n = 15$) of the

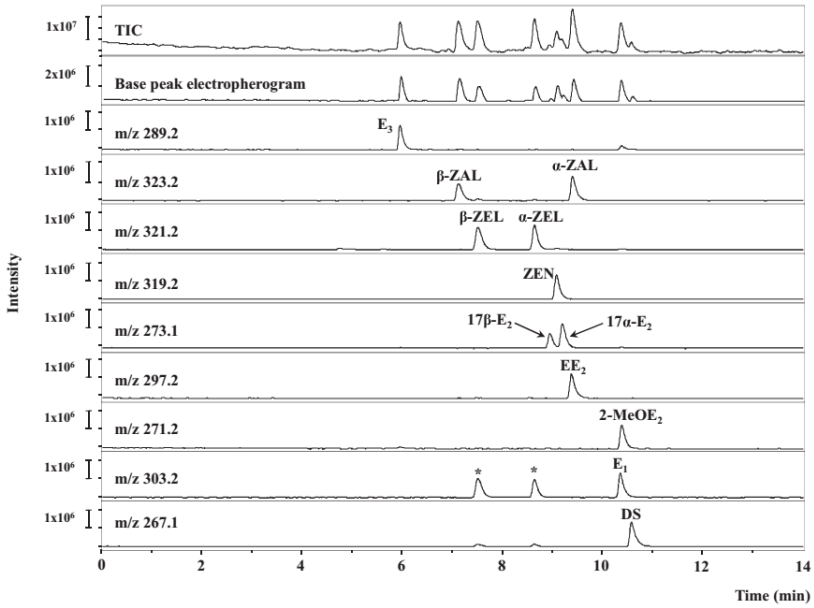

Fig. 3. Total ion current, base peak and extracted ion electropherograms of a standard mixture of the analytes injected in the MEKC–MS system. Experimental conditions: sample, 300–700 μg/L (depending on the analyte) in 11.5 mM of PFOA at pH 9.0 containing 10% (v/v) of MeOH; injection, 0.5 psi for 25 s. (*): $[321.2–H_2O]^+$. For other experimental conditions and MS parameters, see the legend of Fig. 2 and Section 2.3.

standard mixture at two concentration levels (60–200 μg/L and 120–400 μg/L). RSD values for migration times were in the range 0.5–1.8% and 0.8–2.1% for the same day and for different days, respectively. On the other hand, RSD values for the intra- and inter-day precision of the peak areas were in the range 2.4–8.3% and 10.0–15.2%, respectively.

Instrumental calibration curves of each estrogenic compound were obtained by injecting seven levels of concentration ($n = 7$) in triplicate. Results are reported in Table 3 and, as can be seen, good linearity was achieved with determination coefficients (R^2) higher than 0.991 in all cases. LODs and LOQs, calculated as the concentration that provided a S/N of 3 and 10, respectively, were in the range 2.04–59.0 μg/L for the first and 9.04–152 μg/L for the second.

3.2. Optimization of the DLLME procedure

Once the separation of the analytes was fully optimized and in order to extract them from environmental aqueous samples in a fast and environmentally friendly way, a DLLME method was studied for this purpose. Extraction efficiency of DLLME is influenced by several factors such as the extraction and disperser solvent type and volume, as well as the extraction time, pH and salt addition, which are varied and studied in order to favor the solubility of the target compounds in the extraction solvent. For this reason, the final conditions in DLLME depend a lot on the characteristics of the studied compounds, basically on their K_{OW} value. In general, high values of K_{OW} represent a compound which shows affinity for the organic phase.

According to our experience and based on the data reported in the literature [28], the best results in DLLME are obtained when using volumes between 5 and 10 mL of the donor phase. For this reason, 10 mL screw cap round-bottomed glass tubes were selected and considering all the solutions' volumes used for the extraction procedure, the maximum volume of water that could be used in this case (spiked with 0.6–2.0 μg/L of target analytes) was 7.5 mL. All the experiences were done in duplicate and analyses of blank samples were done in parallel to ensure that none of the analytes were present in the samples.

3.2.1. Selection of the extraction and dispersion solvents and other preliminary parameters

The extraction solvent must be capable of extracting the analytes in an effective way, as well as not being miscible with the aqueous donor phase. On the contrary, the dispersion solvent must be miscible with both the extraction solvent and the donor phase. The behavior of the analytes with the different tested solvents will depend on the equilibrium between the three phases (aqueous, extraction solvent and dispersion solvent), and on their solubility in each of them.

Different assays were initially carried out injecting a mixture of 80 μL of the extraction solvent (CCl_4, $CHCl_3$ and CH_2Cl_2) and 500 μL of the dispersion one (ACN, MeOH and ethanol) in 7.5 mL of Milli-Q water at pH 6. Results of these attempts showed that both CCl_4 and $CHCl_3$ formed a clear settled phase with all the solvents, while with CH_2Cl_2 the drop was not clearly distinguishable with any of the dispersion solvents. With CCl_4 the best results were obtained when it was combined with acetone but obtaining very low recoveries (4–47%). In contrast, when using $CHCl_3$, the highest extraction took place when it was combined with ACN (46–94%) vs the results obtained with acetone (37–72%), ethanol (11–53%)

Table 3
Calibration data of the selected group of estrogenic compounds from standards prepared in the final extract of each water sample after DLLME–MEKC–MS and assessment of the matrix effect.

Analyte	Studied linear range (μg/L)	Matrix	Regression equation ($n = 7$) $y = mx + n$		R^2	LOD[a] (μg/L)	LOQ[b] (μg/L)	Matrix effect
			$m \pm tS_m$ ($\times 10^3$)	$n \pm tS_n$ ($\times 10^3$)				
E_3	200–1000	Standard	11.46 ± 0.76	−162.16 ± 478.92	0.996	59.1	152	–
	200–1000	Mineral water	11.62 ± 0.76	99.10 ± 381.36	0.997	68.1	167	Yes
	200–1000	Run-off water	8.33 ± 0.73	129.92 ± 364.03	0.994	98.6	181	Yes
	220–1000	Wastewater	15.33 ± 1.14	−648.88 ± 533.03	0.997	110	220	Yes
β-ZAL	100–1000	Standard	17.91 ± 1.32	−805.39 ± 827.75	0.995	20.0	66.1	–
	100–1000	Mineral water	15.17 ± 0.71	−379.29 ± 356.19	0.998	27.4	79.1	Yes
	100–1000	Run-off water	10.56 ± 0.95	176.59 ± 476.76	0.994	32.4	72.4	Yes
	200–1000	Wastewater	16.31 ± 1.63	−1075.21 ± 819.09	0.993	95.9	195	Yes
β-ZEL	100–1000	Standard	28.13 ± 1.51	−261.05 ± 947.72	0.997	15.2	60.4	–
	100–1000	Mineral water	24.37 ± 1.44	−444.21 ± 719.63	0.997	17.1	88.1	Yes
	100–1000	Run-off water	17.52 ± 1.56	1044.07 ± 780.48	0.994	42.0	91.3	Yes
	150–1000	Wastewater	32.83 ± 1.64	−1054.62 ± 821.33	0.998	62.5	134	Yes
α-ZEL	100–1000	Standard	19.70 ± 1.53	−140.98 ± 961.13	0.995	11.6	57.2	–
	100–1000	Mineral water	18.75 ± 1.09	369.44 ± 547.40	0.997	18.2	89.1	No
	100–1000	Run-off water	15.06 ± 1.02	−106.60 ± 508.74	0.997	32.3	75.0	Yes
	200–1000	Wastewater	21.07 ± 1.83	−1185.48 ± 916.35	0.994	88.7	188	No
17β-E_2	100–1000	Standard	7.91 ± 0.41	−70.01 ± 255.69	0.998	46.4	90.1	–
	200–1000	Mineral water	9.29 ± 0.75	−205.10 ± 374.18	0.995	55.3	182	Yes
	200–1000	Run-off water	5.62 ± 0.19	130.51 ± 94.42	0.999	87.0	195	Yes
	220–1000	Wastewater	7.06 ± 0.67	−296.34 ± 336.43	0.993	92.1	220	Yes
17α-E_2	100–1000	Standard	14.24 ± 1.13	−292.62 ± 710.49	0.994	29.2	98.3	–
	110–1000	Mineral water	15.42 ± 1.27	−145.57 ± 636.64	0.995	35.2	103	Yes
	110–1000	Run-off water	8.61 ± 0.66	422.28 ± 333.10	0.999	51.6	110	Yes
	200–1000	Wastewater	15.97 ± 1.38	−686.56 ± 693.56	0.994	72.7	198	Yes
ZEN	50–1000	Standard	57.31 ± 4.24	−1265.32 ± 1435.41	0.995	11.3	36.5	–
	100–1000	Mineral water	56.22 ± 2.03	−250.05 ± 550.10	0.999	14.4	85.2	Yes
	110–1000	Run-off water	38.30 ± 3.48	99.06 ± 941.13	0.994	15.1	103	Yes
	110–1000	Wastewater	70.26 ± 5.84	−2726.75 ± 1579.19	0.995	18.3	108	Yes
EE_2	100–1000	Standard	14.84 ± 0.88	−303.23 ± 553.57	0.997	6.22	56.2	–
	200–1000	Mineral water	14.47 ± 0.86	48.92 ± 431.87	0.997	41.2	171	No
	200–1000	Run-off water	10.01 ± 0.92	−181.43 ± 459.40	0.994	89.5	180	Yes
	300–1000	Wastewater	15.89 ± 1.47	−893.05 ± 734.56	0.994	91.0	263	No
α-ZAL	100–1000	Standard	19.88 ± 1.43	−521.50 ± 898.16	0.995	7.00	70.4	–
	100–1000	Mineral water	18.00 ± 0.95	−371.41 ± 476.11	0.998	19.2	71.5	Yes
	100–1000	Run-off water	12.66 ± 1.18	−39.73 ± 592.96	0.993	38.1	88.2	Yes
	200–1000	Wastewater	17.38 ± 1.46	−623.15 ± 733.59	0.995	71.7	154	Yes
2-$MeOE_2$	10–400	Standard	132.43 ± 9.92	−553.02 ± 1865.72	0.995	2.04	9.04	–
	50–400	Mineral water	133.59 ± 9.34	−883.18 ± 1404.19	0.996	4.35	19.8	No
	50–400	Run-off water	89.53 ± 7.76	−104.35 ± 1167.16	0.996	9.50	25.4	Yes
	50–400	Wastewater	156.27 ± 16.41	−4570.69 ± 2467.44	0.992	12.1	30.2	Yes
E_1	100–1000	Standard	17.04 ± 1.51	−362.40 ± 949.93	0.993	29.2	98.2	–
	200–1000	Mineral water	18.92 ± 1.57	−252.16 ± 785.37	0.995	37.5	154	Yes
	200–1000	Run-off water	12.35 ± 0.93	−74.63 ± 464.82	0.996	67.3	160	Yes
	200–1000	Wastewater	23.33 ± 2.25	−1437.97 ± 1127.61	0.994	88.4	175	Yes
DS	50–400	Standard	30.73 ± 2.22	−515.66 ± 694.80	0.995	5.43	33.5	–
	100–400	Mineral water	22.09 ± 1.66	−390.58 ± 414.76	0.996	18.1	71.2	Yes
	100–400	Run-off water	13.68 ± 1.07	−238.85 ± 269.05	0.995	32.7	80.0	Yes
	200–400	Wastewater	21.02 ± 2.30	−466.16 ± 575.81	0.994	97.2	190	Yes

m, slope; S_m, standard deviation of the slope; n, intercept; S_n, standard deviation of the intercept; R^2, determination coefficient.
[a] Calculated as the concentration associated to a S/N ratio of 3.
[b] Calculated as the concentration associated to a S/N ratio of 10.

and MeOH (5–38%). In all cases and under the assayed conditions, E_3 could not be extracted. Such low extraction efficiency for this compound under these preliminary conditions can be withdrawn from its value of K_{OW} (Table 1) which is the lowest one. Taking into account these initial results, $CHCl_3$ was selected as the extraction solvent and ACN as the dispersion one.

Afterwards, and before the selection of the solvent volumes, agitation of the dispersion during the extraction by vortex or ultrasounds (1–3 min), extraction time (0–5 min) and centrifugation time (0–20 min) was also investigated. Vortex resulted to be much more effective than ultrasounds. In this respect, 2 min of vortex were enough to effectively assist the extraction, providing recoveries 3-fold higher than those obtained without it. Furthermore, it could be observed that the equilibrium was rapidly achieved and no significant differences were obtained in the recoveries of the analytes when the extraction time was extended. For this reason, after injecting the mixture of dispersive and extraction solvents in the sample, it was vortexed for 2 min and directly centrifuged.

Table 4
Mean recoveries percentages, RSD values, analyte concentration added to the sample and LODs of the method after the DLLME–MEKC–MS procedure.

Analytes	Mineral water			Run-off water			Wastewater		
	Level 1[a] Recovery[b] (RSD, %)	Level 2[a] Recovery[b] (RSD, %)	LOD_{method} (μg/L)	Level 1[a] Recovery[b] (RSD, %)	Level 2[a] Recovery[b] (RSD, %)	LOD_{method} (μg/L)	Level 1[a] Recovery[b] (RSD, %)	Level 2[a] Recovery[b] (RSD, %)	LOD_{method} (μg/L)
E_3	56 (3)	53 (3)	0.68	56 (13)	60 (10)	0.99	68 (10)	54 (14)	1.10
β-ZAL	82 (10)	80 (11)	0.27	69 (10)	79 (9)	0.32	57 (14)	64 (16)	0.96
β-ZEL	73 (16)	78 (10)	0.17	71 (10)	79 (7)	0.42	58 (17)	58 (17)	0.63
α-ZEL	80 (8)	83 (1)	0.18	69 (7)	82 (11)	0.32	61 (18)	56 (18)	0.89
17β-E_2	81 (14)	91 (10)	0.55	69 (7)	79 (11)	0.87	56 (15)	65 (13)	0.92
17α-E_2	73 (10)	73 (7)	0.35	74 (9)	80 (8)	0.52	55 (12)	69 (14)	0.73
ZEN	77 (13)	80 (6)	0.14	71 (11)	77 (13)	0.15	61 (17)	58 (15)	0.18
EE_2	79 (11)	78 (1)	0.41	74 (10)	73 (15)	0.90	57 (18)	59 (17)	0.91
α-ZAL	83 (15)	85 (11)	0.19	71 (7)	85 (9)	0.38	47 (11)	61 (16)	0.72
2-$MeOE_2$	72 (19)	76 (6)	0.04	66 (8)	73 (16)	0.10	64 (14)	73 (16)	0.12
E_1	69 (18)	71 (2)	0.38	69 (6)	75 (12)	0.67	74 (8)	75 (17)	0.88
DS	58 (15)	66 (17)	0.18	45 (15)	43 (12)	0.33	60 (17)	57 (17)	0.97

[a] Level 1: 60–200 μg/L; level 2: 200–667 μg/L.
[b] Mean of five extractions (n = 5), 7.5 mL of water.

Finally, the centrifugation time was studied (5–20 min), observing that results did not change when the sample was centrifuged for more than 5 min.

3.2.2. Extraction and dispersion solvent volume

The volumes of the solvents were optimized by means of a step by step approach in order to obtain good recovery values. The first experiments were carried out fixing the volume of ACN as the dispersion solvent at 500 μL and changing the volume of $CHCl_3$ between 60 and 130 μL. The extractions were carried out with 7.5 mL of Milli-Q water at pH 6.0. As can be seen in Fig. 1S of the Supplementary Material, an amount of 110 μL provided a maximum in the extraction recoveries for all analytes (71–96%), except for E_3 which was only recovered in a 6%.

Following, and maintaining constant the volume of the extraction solvent (110 μL), the volume of ACN was studied between 200 and 1200 μL (Fig. 2S of the Supplementary Material). In general, values higher than 500 μL caused a decrease in the recoveries, probably due to the partial solution of the analytes in the ACN that acted as the dispersion solvent. Also, with volumes lower than 500 μL, the dispersion was less effective, giving a slightly inferior extraction of the target analytes, in particular of E_3, β-ZAL, 2-$MeOE_2$, E_1 and DS which were around 60–81% for 200 μL of ACN. For this reason, the volume of ACN was finally fixed at 500 μL.

3.2.3. Effect of the pH and the ionic strength of the donor phase

Under the previously described conditions, the pH of the aqueous phase was varied between 2 and 10, since the analytes can be ionic or neutral depending on their pK_a and on the working pH value. If this value is higher than the pK_a, their solubility in water will be drastically affected, reducing the extraction efficiency (see Table 1 for pK_a values and solubility in water at different pH values). Fig. 3S shows the results of these studies observing that between pH 2 and 6 the recoveries did not vary significantly. However, at pH 3, the results were slightly better for all the analytes. On the contrary, at high pH values, the extraction decreased for some of the analytes, in particular for those with a p$K_a \sim 8$ (α-ZAL, β-ZAL, α-ZEL, β-ZEL and ZEN). This is justified by the increment of the solubility of the species in the aqueous phase when they are in their anionic form. At pH 3.0 mean recovery values were in the range 69% and 94% for all the analytes except, once more for E_3 which was around 6%.

A common practice in this type of microextraction techniques is the modification of the ionic strength of the donor phase in order to improve the extraction of some analytes taking advantage of a salting-out effect. For this reason, and especially with the purpose of improving the low extraction recovery of E_3, NaCl was added in concentrations from 0% to 30% (w/v). As can be seen in Fig. 4S, the increase of the ionic strength has an extraordinary important effect in the recovery value of E_3 which changed from 6%, when no salt was added, to 76% when the concentration was 30% (w/v). This behavior can be explained because E_3 becomes less soluble in water at high salt concentration, favoring its solubility in the organic solvent. For this reason, and because the recovery values for the rest of the analytes were sufficiently high and did not change significantly with the addition of NaCl, such concentration of NaCl in the aqueous phase was selected for further extractions.

3.3. Application of the DLLME–NSM–MEKC–MS method for the analysis of real water samples and validation

Under these optimized conditions, and in order to demonstrate the repeatability of the procedure, a recovery study was developed at one level of concentration (0.6–2.0 μg/L) for Milli-Q water (n = 5) obtaining recoveries in the range 76–96% with RSDs lower than 9%. In order to fully validate the method for the analysis of environmental water samples, the full procedure was applied to the extraction of the analytes from different types of water (mineral, run-off and wastewater) which were analyzed with the developed method in order to verify the presence of the target compounds. However, in none of them, residues of the studied estrogens were found.

3.3.1. Recovery study

The repeatability of the DLLME–NSM–MEKC–MS method was evaluated by the development of a recovery study at two concentration levels for the three types of water samples. All the extractions were carried out five times (n = 5) at each level, calculating the mean recovery values by comparison of the obtained peak areas with those of the standards. The results of this study, as well as the spiking levels, LODs and LOQs of the whole method are shown in Table 4.

In general, the recoveries slightly decreased as the complexity of the matrix increased, which is normal due to an evident matrix effect (which will be later considered). In this sense, recovery values were in the range 53–91% for mineral water, 43–85% for run-off water and 54–75% for wastewater 1, in all cases with RSDs values lower than 20%. The results for DS in run-off water are particularly low, probably due to a specific interaction between this analyte (which structure is somewhat different from the other selected compounds) and any component of the matrix.

In order to check if the methodology could be applied to more complex wastewater samples, which have a special interest in the

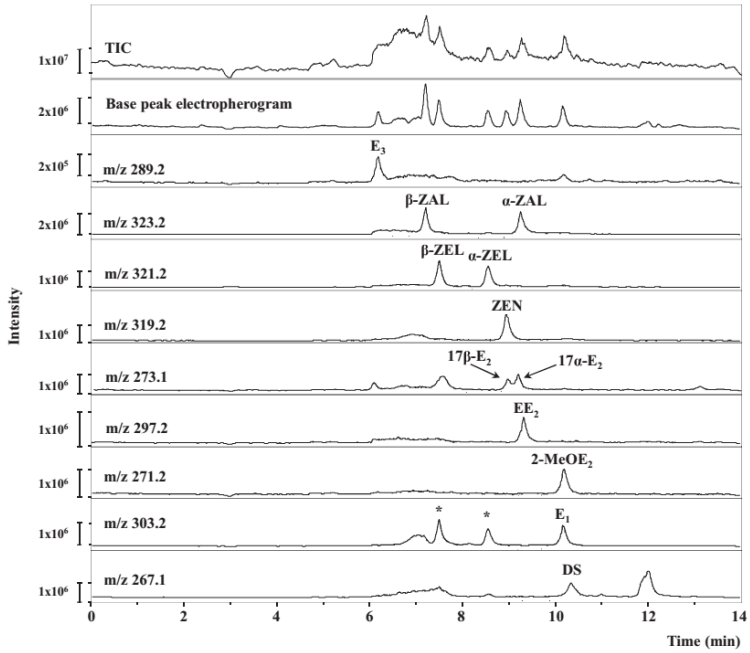

Fig. 4. Total ion current, base peak and extracted ion electropherograms of a spiked wastewater sample after the DLLME–MEKC–ESI–MS method. Experimental conditions: sample spiked at 0.60–2.00 μg/L (depending on the analyte). For other experimental conditions and MS parameters, see the legend of Fig. 2 and Section 2.3.

determination of estrogenic compounds, the recoveries were calculated also for a second wastewater which COD was 1100 mg/L O_2 (the wastewater selected for validation had a COD of 55 mg/L O_2). Recovery values for this new sample were also in the range 42–88% ($n = 3$), a result that confirms that the method may also be valid for the analysis of more complex wastewater samples.

LODs of the whole method were between 0.04 and 0.68 μg/L for mineral water, 0.10 and 0.99 μg/L for run-off water and 0.12 and 1.10 μg/L for wastewater. Concerning the articles that have previously analyzed estrogens in water samples by DLLME [31–37], the results in terms of LODs are in the same order of magnitude than the ones obtained in the present work, though it should be mentioned that in all of them, a lower number of analytes (no more than five) were extracted. For example, Chang et al. [34] obtained LODs in the range 0.8–3.1 μg/L for E_1, E_2, E_3 and EE_2 in river and tap water by SFO–DLLME while Wu et al. [33] achieved LODs between 0.08 and 0.50 μg/L for E_1, E_2, E_3, EE_2 and DES in river, sea and waste waters using IL–DLLME. However, these values are lower than those obtained by Lima et al. [35] (in the low ng/L range) who analyzed only two of the selected estrogenic compounds (E_2 and EE_2) in tap, surface and waste water samples. Such low LODs are explained by the use of HPLC–FD and by the obtainment of a high preconcentration factor as a result of the reconstitution of the dried final extract in 40 μL of acetonitrile, which could only be injected in the HPLC system just once. In the particular case of ZEN and its derivatives, only ZEN has been previously analyzed using DLLME [36,37]. The LOD obtained in this work (in the range 0.14–0.18 μg/L) is very similar to that obtained by Wu and co-workers [36] (0.12 μg/L) in beer and, though exact comparison cannot be made, lower than that obtained by Arroyo-Manzanares et al. [37] in milk thistle (6.05 μg/kg) as expected from a more complex sample.

It is also worth to mention that in the majority of these referenced works, separation of the analytes was carried out by HPLC [31,33–37] and one of them by GC [32]. For this reason, one of the biggest advantages and novelties of the proposed method is the coupling of DLLME with CE, characterized by short analysis time, extremely low consumption of reagents and samples and low operational costs.

3.3.2. Matrix-matched calibration

Alteration in the MS signal due to the presence of substances coming from the matrix that affect the ionization of the target analytes may be observed. In fact, the variations of the recoveries in the recovery study can give a clue in this respect, being probably caused by a non-disposable matrix effect. In order to assess such effect, it is highly recommendable to make a comparison between the calibration equations obtained from the standards dissolved in the pure injection medium and the ones obtained with the standards dissolved in the sample extracts (note that in both cases the solvent is the same: 11.5 mM APFO at pH 9.0 containing 10% MeOH (v/v)). If there are significant differences between the slope and/or intercept values obtained in both ways, then there is a matrix effect for the compound under study.

Table 3 also shows the calibration data of the standards dissolved in the injection medium (standard instrumental calibration) and in each type of water extract ($n = 7$). As can be seen, the response of the MS detector was linear in all cases in the tested range with determination coefficients (R^2) higher than 0.991. Statistical comparison between the calibration in the pure injection medium and in each type of water extract was carried out using a statistical program that calculates F and p values to compare the slopes and the intercepts (see Section 2.2). When the value of p for the comparison of the slopes or intercepts is ≤ 0.1, statistical differences exist, and thus, matrix effect is confirmed.

Only α-ZEL, EE_2 and 2-$MeOE_2$ in mineral water (the simplest matrix studied) and EE_2 in wastewater did not show matrix effect. The study for the rest of analytes in the three matrices demonstrated that there were significant differences between the intercepts and/or slopes. Therefore, and in order to achieve accurate results, quantification should be done using the calibration curves in the sample matrix.

Regarding the LODs of the matrix-matched calibrations, values in the ranges 4.35–68.1 μg/L, 9.50–98.6 μg/L and 12.1–110 μg/L were obtained for mineral, run-off and waste waters. Also in this case, the limits were calculated as the concentration that provided a S/N of three and were experimentally verified. In none of the water samples residues of the estrogenic compounds were found. Fig. 4 shows the MEKC–MS extracted ion electropherograms of all the analytes of a spiked wastewater sample after the DLLME–MEKC–MS method. As can be seen, the compounds could be perfectly identified in the sample. A similar electropherogram was also obtained for the other two samples.

4. Conclusions

A new MEKC–MS method has been optimized for the separation of a group of 12 estrogenic compounds using APFO as a volatile surfactant. A complete separation of the analytes was accomplished in 11 min and NSM was applied in order to enhance the sensitivity, obtaining also good efficiencies and repeatability.

DLLME was optimized for the extraction of the selected substances from mineral, run-off and waste water samples. Validation was carried out developing a recovery study at two levels of concentration for the three types of water obtaining acceptable results (43–91% for all the analytes in all the matrices) and LODs of the method in the range 0.04–1.10 μg/L. The assessment of the matrix effect was statistically studied through matrix-matched calibration in each type of water, revealing that the method was affected in general by the presence of the matrices.

The procedure proposed hereby constitutes a simple, fast and reliable method to determine the selected estrogenic compounds in this type of water samples by coupling DLLME with CE, which requires a very low consumption of reagents and samples as well as low operational costs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.005.

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Anexo I.2. - Artículo 2

Evaluation of the combination of a dispersive liquid-liquid microextraction method with micellar electrokinetic chromatography coupled to mass-spectrometry for the determination of estrogenic compounds in milk and yogurt.

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Research Paper

Evaluation of the combination of a dispersive liquid–liquid microextraction method with micellar electrokinetic chromatography coupled to mass spectrometry for the determination of estrogenic compounds in milk and yogurt

In this work, the suitability of a methodology based on dispersive liquid–liquid microextraction (DLLME) has been evaluated for the extraction of four endoestrogens (estriol, 17α-estradiol, 17β-estradiol, and estrone), an exoestrogen (17α-etynylestradiol), and a mycotoxin (zearalenone), together with some of their major metabolites (2-methoxyestradiol, α-zearalanol, β-zearalanol, α-zearalenol, and β-zearalenol) from different types of milk (whole and skimmed cow milk and semiskimmed goat milk) and whole natural yogurt. The methodology includes a previous protein precipitation with acidified ACN and a defatting step with *n*-hexane. Separation of the analytes, determination, and quantification were developed by MEKC coupled to ESI-MS using a BGE containing an aqueous solution of ammonium perfluorooctanoate as MS friendly surfactant. Calibration, precision, and accuracy studies of the described DLLME-MEKC-MS/MS method were evaluated obtaining a good linearity and LODs in the low micrograms per liter range.

Keywords:
Estrogens / Mass spectrometry / Micellar electrokinetic chromatography / Milk / Yogurt
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1 Introduction

All the technological evolution applied to improve the activities related with animal husbandry may involve the introduction into the environment, and especially into the food chain, of chemical substances that could threaten human health. Meat, milk, and dairy products are the main target products in this respect.

Regarding cow's milk, which is mostly consumed, annual production relative to the year 2012 was of more than 150 and 90 million tons in Europe and the United States, respectively (FAOSTAT, Food and Agriculture Organization of the United Nations, URL http://faostat.fao.org/, accessed September 5, 2014), with a worldwide consumption of about 85 kg/capita/year in 2009 (most recent data available) (FAOSTAT, Food and Agriculture Organization of the United Nations, URL http://faostat.fao.org/, accessed February 5, 2014). This means that this nourishment is not only a possible vehicle of chemical risk worldwide, but also that the levels of exposure for potential harmful substances are high and daily.

To satisfy global demand for milk, practices such as milk collection from pregnant cows and abusive or illegal use of synthetic estrogens, increase the exposure of humans to dairy products containing estrogenic hormones over recommended levels [1].

Additionally, humans are the only adult mammal who drinks milk after weaning; hence, this uncommon habit in the animal kingdom raises an important question about its necessity [2]. Although studies corroborate the importance of milk for the supply of nutrients in a healthy diet [3], its consumption has been related with a possible increased risk of various diseases in the Western world such as cardiovascular diseases, diabetes, or even cancer [2, 4, 5]. In particular, the high intake of endogenous or exogenous estrogenic hormones contained in dairy products may induce abnormalities in the human reproductive system [6, 7] and the possibility of the onset of more serious diseases such as testicular, breast, prostate, ovarian, and corpus uteri cancers, which seems quite confirmed [1, 4, 8–10].

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Abbreviations: 2-MeOE$_2$, 2-methoxyestradiol; **APFO**, ammonium perfluorooctanoate; **DES**, diethylstilbestrol; **DLLME**, dispersive liquid–liquid microextraction; **DS**, dienestrol; **E$_1$**, estrone; **E$_2$**, estradiol; **E$_3$**, estriol; **EE$_2$**, 17α-ethinylestradiol; **HEX**, hexestrol; **HF**, hollow fiber; **LPME**, liquid-phase microextraction; **MeOH**, methanol; **PFOA**, perfluorooctanoic acid; **TIC**, total ion current; **ZAL**, zearalanol; **ZEL**, zearalenol; **ZEN**, zearalenone

Regarding the European legislation, maximum residue limits are only established in milk (Commission Regulation (EU) No. 37/2010, Official Journal of the European Union L 15/1 of 20.1.2010) for certain hormones (e.g., dexamethasone, 0.3 μg/kg in bovine or caprine milk; prednisolone, 6 μg/kg in bovine milk). For this reason, there is a strong necessity of knowing if these compounds are present in these matrices or not, and at which concentrations.

Obviously, and due to the high concentrations of lipids, carbohydrates and proteins in dairy products (also vitamins and minerals in a lesser extent) sample pretreatment and analyte preconcentration are required. In this respect, liquid–liquid extraction (LLE) and SPE have been the main procedures employed for this purpose, in combination with different chromatographic methods [11].

One of the current trends in analytical methodologies is the development of easier and faster methods, involving a lower consumption of organic solvents such as microextraction procedures. For instance, and concerning the analysis of estrogens in milk, solid-phase microextraction has been used for their determination after a suitable deproteinization procedure. This is the case of the analysis of estrone (E_1), estradiol (E_2), ethinylestradiol (EE_2), diethylstilbestrol (DES), and hexestrol (HEX) by HPLC-UV [12]; DES, HEX, and dienestrol (DS) by HPLC-UV [13]; E_1, E_2, estriol (E_3), and DES by HPLC-UV [14]; or DES by HPLC-DAD [15]. Meanwhile, liquid-phase microextraction (LPME) has also been employed for this purpose, in particular hollow fiber (HF)-LPME. In this sense, Socas-Rodríguez et al. analyzed E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS, HEX, and 2-hydroxyestradiol in milk, yogurt, and cheese by HPLC-DAD/fluorescence detection after a previous deproteinization with ACN/acetic acid (cheese samples also required a second defatting step with *n*-hexane) [16, 17]. Also, Zhong et al. developed a dynamic liquid–liquid–solid microextraction HPLC-UV procedure using molecularly imprinted polymer filaments as solid phase for the determination of DES, dihydrodiethylstilbestrol, E_1, E_2, and EE_2 in complex matrices including milk, after deproteinization with ACN [18], while Xu et al. [19] analyzed 17α-E_2, 17β-E_2, E_3, and EE_2, among others, in milk by HF-based stirring extraction bar LLME-GC-MS after simple dilution.

Dispersive liquid–liquid microextraction (DLLME), which is another LPME mode, has been widely employed for food analysis [20]. In the case of milk or its derivatives, different methodologies have been proposed for the extraction of diverse analytes as, for example, antibiotics [21, 22] polybrominated diphenyl ethers [23, 24], cholesterol [25] nonsteroidal anti-inflammatory drugs [26], etc. However, up to now, this technique has not been employed for the determination of estrogenic hormones in these matrices.

In a previous work recently developed by our group [27], a method based on the use of DLLME was optimized and validated for the extraction and preconcentration of a group of four natural estrogens (E_3, 17β-E_2, 17α-E_2, and E_1), an exoestrogen (EE_2), a synthetic stilbene (DS), a mycotoxin (ZEN), and some of their major metabolites (2-methoxyestradiol, 2-MeOE_2; α-zearalanol, α-ZAL; β-zearalanol, β-ZAL; α-zearalenol, α-ZEL; and β-zearalenol, β-ZEL) from different types of water samples. The determination of the target analytes was carried out MEKC-MS using ammonium perfluorooctanoate (APFO) as a MS friendly surfactant that it has been recently used for determination of 17 N-methylcarbamates [28] amino acids [29] and 5-nitroimidazole [30] by MEKC-MS. The whole methodology showed a worthy performance with LODs in the low micrograms per liter range. The proposed procedure was simple, selective, effective, and with low consumption of organic solvents.

The main objective of the present paper is to evaluate the suitability of our previous method for the extraction of estrogenic compounds residues in different types of milk samples and also yogurt as a different dairy product matrix. In order to adapt the methodology, a deproteinization step followed by a suitable defatting was necessary to obtain good extraction yields for all the analytes. Also, a minor change in the sheath liquid was required with respect to previous MS conditions, in order to remove some matrix components from the ESI interface, which hindered obtaining a stable analysis. To the best of our knowledge, this is the first time that estrogenic compounds have been extracted from milk or dairy products by DLLME and the first work reporting the analysis of these estrogenic compounds in milk or dairy products by MEKC-MS.

2 Materials and methods

2.1 Chemicals and materials

All chemicals were of analytical reagent grade and used as received without filtration or further purification. Methanol (MeOH) and *n*-hexane were from VWR International Eurolab S.L. (Barcelona, Spain) and formic acid (98% v/v) was from Panreac Química (Barcelona, Spain). ACN of HPLC grade, 2-propanol, chloroform ($CHCl_3$), hydrochloric acid (25% v/v), and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was from Scharlau Chemie S.A. (Barcelona, Spain), ammonia solution (25%, v/v) from Riedel-de Haën (Seelze, Germany), and sodium chloride (NaCl) (purity > 99.5%) was purchased from Sigma-Aldrich Chemie (Madrid, Spain). Bondesil primary/secondary amine (40 μm) bulk sorbent was from Agilent Technologies (Waldbronn, Germany).

Ultrapure water was DI by a Milli-Q gradient system A10 from Millipore (Bedford, MA, USA). The BGE was an aqueous solution of APFO at pH 9.0 prepared using perfluorooctanoic acid (PFOA) (96% w/w) from Sigma-Aldrich and 7 M ammonium hydroxide solution for pH adjustment.

Analytical standards, namely 17α-E_2 (1,3,5(10)-estratriene-3,17α-diol), 17β-E_2 (1,3,5-estratriene-3,17β-diol), E_3 (1,3,5(10)-estratriene-3,16α,17β-triol), E_1 (1,3,5(10)-estra trien-3-ol-17-one), EE_2 ((17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol), 2-MeOE_2 (1,3,5(10)-estratriene-2,3,17-triol 2-methyl ether), α-ZAL (2,4-dihydroxy-6-(6α,10-dihydroxy undecyl)benzoic acid μ-lactone), β-ZAL (2,4-dihydroxy-6-(6β,

10-dihydroxyundecyl)benzoic acid μ-lactone), α-ZEL (2,4-dihydroxy-6-(6α,10-dihydroxy-*trans*-1-undecenyl)benzoic acid μ-lactone), β-ZEL (2,4-dihydroxy-6-(6β,10-dihydroxy-*trans*-1-undecenyl)benzoic acid μ-lactone), ZEN ((3S,11E)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione) were purchased from Sigma-Aldrich Chemie (Madrid, Spain) and were used without further purification (purity ≥ 95%).

Stock solutions of each analyte were prepared in MeOH with the following concentrations: 17β-E_2, 17α-E_2, E_3, E_1, and EE_2 at 1000 mg/L; 2-MeOE_2, α-ZAL, and β-ZAL at 100 mg/L; α-ZEL and β-ZEL at 500 mg/L; and ZEN at 180 mg/L. They were all stored in the freezer at −18°C. The working standard mixtures were daily prepared by appropriate dilution in the sample medium (11.25 mM of APFO at pH 9 in 10% v/v MeOH). All samples, standard solutions, and extracted samples were stored at 4°C and kept in the dark prior to analysis.

2.2 Apparatus and software

MEKC-MS experiments were performed with a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA) coupled with an AmaZon SL IT mass spectrometer from Bruker Daltonik (Bremen, Germany) through a CE-MS coaxial sheath-liquid electrospray interface. Karat Software (Beckman Instruments) was used for controlling the CE instrument while all MS parameters, spectra data, and chromatograms were controlled, collected, and processed by Esquire NT software from Bruker Daltonik.

Fused-silica capillaries of 60 cm (50 μm id × 363 μm id), obtained from Composite Metal Services (Worcester, UK) were used to carry out CE analyses. A few millimeters of the thin layer of polyimide coating was carefully removed from the capillary ends with a razor and with the help of a Nikon Model SE binocular optical microscope (Nikon Instruments, Japan).

A Crison GLP 22 pH-meter (Barcelona, Spain) equipped with a combined electrode and a temperature sensor was used for pH measurements.

2.3 MEKC-MS conditions

Before its first use, the separation capillary was activated with the following rinse sequence: 3 min of 1 M HCl, 3 min of Milli-Q water, 5 min of 0.1 M NaOH, 10 min of Milli-Q water, and finally 2 min of the BGE. In all cases, a pressure of 20 psi (137.9 kPa) was applied. The BGE was a solution of 45 mM APFO at pH 9.0 containing a 10% v/v of MeOH prepared dissolving PFOA in Milli-Q water and adjusting the pH with 7 M ammonium hydroxide solution.

Good repeatability between days was obtained flushing the capillary with 7 M ammonium hydroxide solution and Milli-Q water for 10 min each at the beginning of the working day, and then with BGE for 2 min. Between injections the capillary was washed with 7 M ammonium hydroxide solution, Milli-Q water, and BGE for 2 min each. At the end of the working day, it was rinsed consecutively with 7 M ammonium hydroxide solution, Milli-Q water, and MeOH for 5 min each. All these washings steps were also done at a pressure of 20 psi. The capillary was left full of MeOH overnight.

Samples were injected hydrodynamically at 0.5 psi for 25 s. The chromatographic separation was achieved using a voltage of +25 kV with an initial ramp time of 0.17 min. The capillary temperature that was controlled by an air conditioning system was set at 25°C and the sample vial was kept at 15°C before injection.

Coupling of the CE system to the mass spectrometer was achieved fixing the capillary into a coaxial sheath-liquid interface so that its outlet protruded 0.1 mm from the coaxial steel needle. The MEKC-MS system worked in the positive ionization mode. Chromatograms were acquired in a range of 250–350 m/z and the MS tune was set in automatic mode, selecting 300 m/z as the target mass of the studied compounds (trap drive level of 100%).

Optimized ESI conditions were as follows: capillary voltage of −4600 V, end plate offset of −500 V, nebulizer gas (N_2) pressure of 3.0 psi, dry gas (N_2) flow of 3.0 L/min, and dry gas temperature of 200°C. The sheath liquid was a solution of 96:4 v/v 2-propanol/water at a flow rate of 1.7 μL/min. Regarding IT parameters, the ion charge control was set at 110 000, the maximum accumulation time at 200 ms with six averages per experiment, and a rolling averaging of 7.

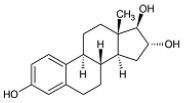
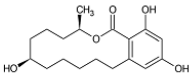
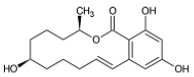
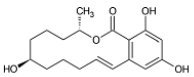
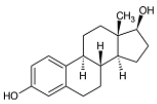
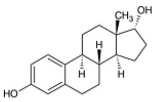
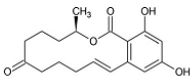
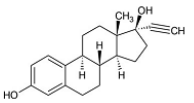
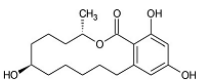
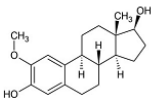
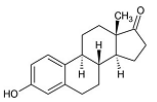
MS/MS experiments were performed by fragmentation of the protonated molecular ion $[M + H]^+$, which was selected as the precursor ion. The mass range was 70–350 m/z, the ion charge control was fixed at 10 000, and the maximum accumulation time at 200 ms with a single average per experiment. The m/z width was established at 1 and the collision energy was varied in the range 0.25–0.40, depending on the analyte. Table 1 shows the structures and the fragmentation data obtained for the studied analytes (m/z of the precursor and product ions, as well as the normalized collision energy).

At the end of the working day, the ESI interface was cleaned with MeOH and water in order to remove the residues of the surfactant and matrix residue of real sample.

2.4 Milk and yogurt sample selection, deproteinization, and defatting

In this work, three types of ultrahigh-temperature processed milk (whole cow, skimmed cow, and semiskimmed goat milk) and whole cow natural yogurt (liquid yogurt) were selected for validation of the methodology. The content of proteins, carbohydrates, and fats were 3.0 g/100 mL, 4.5 g/100 mL, and 3.6 g/100 mL for the first; 3.1 g/100 mL, 4.7 g/100 mL, and 0.3 g/100 mL for the second; 3.4 g/100 mL, 4.5 g/100 mL, and 1.6 g/100 mL for the third; and 3.5 g/100 g, 4.5 g/100 g, and 3.6 g/100 g for the fourth, respectively. All the samples analyzed in this study were purchased in local supermarkets of Tenerife, Canary Islands, Spain.

Table 1. Characteristics and MS and MS/MS detection parameters of the studied estrogenic compounds

Analyte	Abbreviation	Structure	*M* (g/mol)	Production (*m/z*)	NCE[a)]
Estriol	E_3		288.4	271.1 253.1	0.30
β-Zearalanol	β-ZAL		322.4	305.2 261.1	0.25
β-Zearalenol	β-ZEL		320.4	303.1 285.1	0.30
α-Zearalenol	α-ZEL		320.4	303.1 285.1	0.30
17β-Estradiol	17β-E_2		272.4	255.1 159.0	0.32
17α-Estradiol	17α-E_2		272.4	255.1 159.0	0.32
Zearalenone	ZEN		318.4	301.1 283.1	0.35
17α-Ethinylestradiol	EE_2		296.4	279.1 159.0 133.1	0.35
α-Zearalanol	α-ZAL		322.4	305.2 261.1	0.25
2-Methoxyestradiol	2-Me0E_2		302.4	285.1 189.0	0.32
Estrone	E_1		270.4	253.1	0.32

a) Normalized collision energy

The samples were left an hour at room temperature and homogenized. Two milliliters of spiked or nonspiked sample were introduced in a 15 mL polypropylene centrifuge tube from VWR (Barcelona, Spain). Subsequently, 4 mL of ACN and 100 μL of acetic acid were added to the sample and it was vortex shaken in a *Lab Dancer* vortex from VWR (2800 rpm) for 2 min. The mixture was kept for 15 min in the darkness in order to produce complete protein precipitation. Afterward it

was centrifuged at 4400 rpm (3000 × *g*) for 10 min in a 5702 centrifuge from Eppendorf (Hamburg, Germany). Following, the supernatant was transferred into a new 10 mL polypropylene centrifuge tube and treated with 2 mL of *n*-hexane to induce a defatting of the extract. The suspension was vigorously mixed for 2 min with vortex shaking and centrifuged again at 4400 rpm (3000 × *g*) for 10 min. The *n*-hexane layer containing the fat fraction was carefully removed and the remaining solution was later evaporated at 40°C and 180 mbar using a rotavapor R-200 equipped with a V-800 vacuum controller and a 500 vacuum pump, all of them from Büchi Labortechnik (Flawil, Switzerland). The obtained aqueous residue (approx. 1.5 mL) was ready for the microextraction procedure (see Section 2.5).

2.5 DLLME procedure

The previous milk/yogurt extract was diluted to a final volume of 7.5 mL with Milli-Q water and NaCl was added to a final concentration of 30% w/v. Afterwards, the solution was filtered through a 0.45 μm Minisart SRP 15 PTFE filter from Sartorius (Goettingen, Germany) into a 10 mL screw cap glass tube. A mixture containing 500 μL of ACN (as dispersion solvent) and 110 μL of $CHCl_3$ (as extraction solvent) was rapidly introduced into the aqueous extract with the help of a 1 mL micropipette, followed by 2 min of vortex shaking. The dispersion was centrifuged at 4000 rpm (2500 × *g*) for 5 min, observing later a droplet of $CHCl_3$ in the bottom of the tube. The upper aqueous phase was partially removed (about 4 mL) using a micropipette and the droplet was transferred to a vial and evaporated to dryness with a gentle nitrogen stream. The residue was reconstituted in 75 μL of the sample medium (11.25 mM of APFO, pH 9 containing a 10% v/v of MeOH) and injected hydrodynamically in the MEKC-MS system. Analyses of blank samples were done in parallel to assure that no residues of the analytes were present in the samples.

3 Results and discussion

As previously indicated, in a work recently done by our group [27], we proposed and optimized the separation and extraction of a group of 12 estrogenic compounds, that is, four natural sexual hormones (as E_3, 17β-E_2, 17α-E_2, and E_1), an exoestrogen as 17α-EE_2, a synthetic stilbene as DS, a mycotoxyin as ZEN, and some of their major metabolites (as 2-MeOE_2, α-ZAL, β-ZAL, α-ZEL, and β-ZEL). Separation was carried out by MEKC coupled to ESI-IT-MS, using APFO as an MS compatible surfactant, and normal stacking mode as an online preconcentration technique to enhance the sensitivity. A DLLME procedure was optimized for the extraction of the selected substances from mineral, runoff, and waste water samples, which was found to be very simple, quick, and effective. With the aim of extending this methodology to the analysis of more complex matrices (milk and yogurt), the method must be slightly modified, introducing a deproteinization and a defatting steps previous to their DLLME-MEKC-MS analysis.

In our first work [27], we discussed in depth the optimization of MEKC and MS conditions. In this case, when the final extracts obtained after sample treatment were injected, it was experimentally observed that milk/yogurt matrices had no effect in the chromatographic separation. However, the MS signal was affected by an increase of the background noise (and its subsequent loss of sensitivity) and frequent current breakdowns during the runs at a migration time just when the first compound was about to reach the detector (i.e., migration time of the EOF or higher). One possible explanation is that the matrix, which is not completely removed during sample preparation, produced an instability and interruption of the electrospray during the ionization of the analytes in the ESI interface. In order to solve this problem, the composition of the sheath liquid was varied with respect to our previous work (which was 2-propanol) [27], studying the behavior of the total ion current (TIC) and the stability of the MS signal. It was observed that the addition of a small percentage of water managed to increase the S/N ratio and maintain the MS signal stable during the chromatographic run. For this reason, from now on, a sheath liquid consisting of 96:4 v/v water/2-propanol was used.

3.1 Optimization of the deproteinization/defatting procedure

As an starting point, and taking into account the experience that our group has in the sample treatment of milk and milk derivatives for the extraction of antibiotics [31–33] and estrogenic compounds [16, 17], first experiments were developed by treating simultaneously spiked whole cow milk samples and whole cow natural yogurt samples (since these are the more complex matrices under study) keeping in mind the deproteinization procedures carried out by Socas-Rodríguez et al. [16, 17], due to the similarity among the compounds studied. In these last works, a mixture of ACN/acetic acid to induce protein precipitation was used. Later, the analysis of the estrogenic compounds in the aqueous extract was accomplished by HF-LPME and separation by HPLC-fluorescence detection/DAD, resulting in an overall effective analytical method.

Taking into account that the effect of the matrix is directly related to the extraction and separation technique that will be used later (in this case DLLME-MEKC-MS), initial screening experiments were developed using MeOH or ACN in different proportions with respect to the sample (1:2 v/v, 1:3 v/v, 1:4 v/v milk or yogurt/solvent) varying the type and volume of acid (acetic or formic acid, 50–150 μL) and using 2 mL of sample. The mixture was vortexed for 2 min and maintained 15 min in the darkness to favor precipitation [16, 17, 32, 34]. In order to achieve suitable phase separation, a brief study of the centrifugation time showed that 10 min at 4400 rpm resulted enough. Then, the supernatant was evaporated in a rotavapor and the remaining residue was reconstituted with

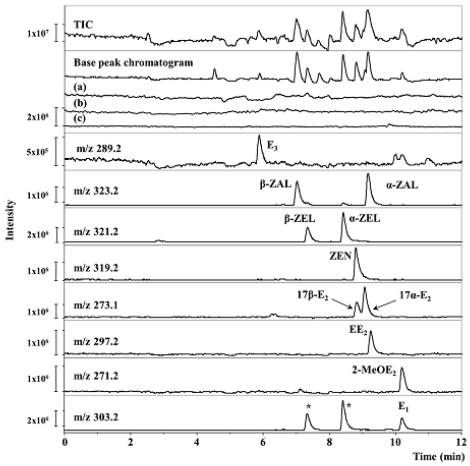


Figure 1. Total ion current (TIC), base peak, and extracted ion chromatograms of a spiked whole cow milk sample, and base peak chromatograms of nonspiked samples of whole cow (a), skimmed cow (b), and semiskimmed goat (c) milk after the DLLME-MEKC-ESI-MS method. Experimental conditions: separation capillary, 60 cm × 50 μm id × 375 μm od; BGE, 45 mM of PFOA at pH 9.0 containing 10% v/v of MeOH; sample, 36–609 μg/L (depending on the analyte) in 11.5 mM of PFOA at pH 9.0 containing 10% v/v of MeOH; injection, 0.5 psi for 25 s; applied voltage, +25 kV; temperature, 25°C. (*): $[321.2 - H_2O]^+$. For other experimental conditions and MS parameters, see Section 2.

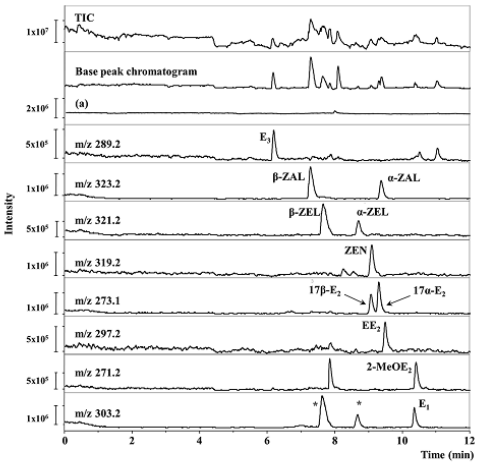


Figure 2. TIC, base peak, and extracted ion chromatograms of a spiked whole yogurt sample, and base peak chromatogram of a nonspiked whole yogurt sample after the DLLME-MEKC-ESI-MS method. Experimental conditions: sample, 36–609 μg/L (depending on the analyte). For other experimental conditions and MS parameters, see Fig. 1.

Milli-Q water up to 7.5 mL. Finally, the DLLME procedure described in our previous work [27] was carried out in this aqueous extract.

In general, MeOH was not able to produce suitable precipitation as also observed in one of our previous works [16]. On the contrary, of all the studied combinations, 4 mL of ACN (ratio 1:2 v/v sample/ACN) together with 100 μL of acetic acid was observed to produce good precipitation of the proteins and phase separation for both milk and yogurt. Regarding the acid, even though formic acid provided comparable results to acetic acid, the latter directly assured a pH value of 3 for all the matrices studied, which is, in fact, the pH value required for optimum DLLME of the selected analytes [27]. Under these conditions, and after carrying out the DLLME procedure described in Section 2.5, extraction recoveries ranged between 10 and 50% for all the analytes, except for α-ZEL, ZEN, and EE_2, which were around 5%.

Apart from proteins, milk and yogurt have a high content of carbohydrates and fats (as shown in their commercial nutritional values), which can produce MS signal suppression and a reduction in the performance of the extraction method. For this reason and in an effort to increase the recovery values and taking into account the high success of primary/secondary amine in the clean-up of matrices with high content of carbohydrates [35] some experiments were carried out adding amounts between 200 and 500 mg of this sorbent to the supernatant obtained after precipitation of the proteins. After 2 min of vortex and centrifugation for 10 min at 4400 rpm (3000 × *g*), the supernatant was evaporated, reconstituted, and submitted to the DLLME procedure. While a slight improvement of the baseline signal was perceived, an increase in the extraction recoveries was not observed at all. Due to the small benefit and in order to avoid long treatment time, this step was not considered. On the other hand, and regarding the good results obtained in the literature after defatting steps with *n*-hexane when considering fatty matrices [17, 36, 37], such procedure was considered after the deproteinization step. For this purpose, *n*-hexane was added to the ACN supernatant layer obtained after deproteinization and after 2 min of vortex and centrifugation for 10 min at 4400 rpm (3000 × *g*), the lipid phase (upper phase) was discarded, and the extract was evaporated and subjected to the DLLME procedure. The volume of defatting solvent was studied between 1 and 5 mL, experimentally verifying that 2 mL were necessary and enough to produce appropriate defatting of the supernatant, resulting in an improvement of the extraction recoveries up to 10%, depending on the analyte.

Finally, the amount of sample was studied between 2 and 4 mL, adapting all the reagents proportionally. This study showed that volumes higher than 2 mL did not produced the sedimentation of the $CHCl_3$ phase in the bottom of the tube, but a layer of the solvent remained suspended over the aqueous phase. In this way, the solvent could not be recovered with repeatability. In fact, one of the aspects that must be carefully taken into account in DLLME is the possible inversion of the

Table 2. Method calibration data of the selected compounds in the different milk derivatives after the DLLME-MEKC-MS method

Analyte	Linear range (μg/L)	Sample	Regression equation ($n = 6$) $y = mx + n$		R^2	LOD[b] (μg/L)
			$m \pm tS_m$ (10^4)	$n \pm tS_n$ (10^5)		
E_3	30–180	Whole cow milk	8.99 ± 0.81	7.36 ± 7.82	0.995	14
	20–180	Skimmed cow milk	8.72 ± 0.94	4.40 ± 9.62	0.994	3
	30–180	Semiskimmed goat milk	13.05 ± 1.73	9.25 ± 17.96	0.993	7
	30–180	Natural yogurt	4.69 ± 0.59	15.06 ± 6.13	0.992	12
β-ZAL	30–750	Whole cow milk	5.90 ± 0.60	39.34 ± 24.10	0.993	6
	20–750	Skimmed cow milk	7.85 ± 0.90	0.96 ± 38.40	0.993	5
	50–750	Semiskimmed goat milk	7.67 ± 1.16	6.02 ± 51.19	0.991	15
	30–750	Natural yogurt	0.94 ± 0.12	86.26 ± 5.03	0.992	10
β-ZEL	40–750	Whole cow milk	6.58 ± 0.69	52.74 ± 27.79	0.993	16
	30–750	Skimmed cow milk	9.45 ± 1.16	52.29 ± 49.30	0.992	9
	70–750	Semiskimmed goat milk	7.32 ± 1.08	37.17 ± 46.78	0.992	20
	30–750	Natural yogurt	0.93 ± 0.11	91.69 ± 4.76	0.993	9
α-ZEL	250–2500	Whole cow milk	1.06 ± 0.11	32.40 ± 22.29	0.993	71
	150–2500	Skimmed cow milk	1.83 ± 0.23	14.91 ± 48.12	0.992	40
	350–2500	Semiskimmed goat milk	1.00 ± 0.10	8.11 ± 22.51	0.996	104
	275–2500	Natural yogurt	0.29 ± 0.03	6.84 ± 7.41	0.993	77
17β-E_2	120-1220	Whole cow milk	1.29 ± 0.15	12.42 ± 14.47	0.992	19
	100–1220	Skimmed cow milk	1.80 ± 0.13	29.17 ± 13.02	0.998	21
	210–1220	Semiskimmed goat milk	1.16 ± 0.17	18.12 ± 17.66	0.992	61
	120–1220	Natural yogurt	0.16 ± 0.02	41.78 ± 1.79	0.994	26
17α-E_2	100-1220	Whole cow milk	2.76 ± 0.33	3.66 ± 32.23	0.991	19
	50–1220	Skimmed cow milk	3.41 ± 0.39	54.89 ± 40.28	0.993	13
	120–1220	Semiskimmed goat milk	2.18 ± 0.18	21.66 ± 18.98	0.997	36
	100–1220	Natural yogurt	0.49 ± 0.05	36.51 ± 5.33	0.995	19
ZEN	45–900	Whole cow milk	4.06 ± 0.48	17.21 ± 36.62	0.993	12
	45–900	Skimmed cow milk	6.41 ± 0.66	35.00 ± 50.42	0.995	9
	90–900	Semiskimmed goat milk	3.28 ± 0.45	27.76 ± 34.60	0.993	22
	135–900	Natural yogurt	0.76 ± 0.08	16.44 ± 5.93	0.995	29
EE_2	450–2013	Whole cow milk	0.52 ± 0.04	3.99 ± 6.99	0.996	72
	250–2013	Skimmed cow milk	0.96 ± 0.09	1.32 ± 16.21	0.995	64
	750–2013	Semiskimmed goat milk	0.34 ± 0.05	6.43 ± 8.12	0.993	220
	500–2013	Natural yogurt	0.18 ± 0.02	4.76 ± 4.11	0.992	140
α-ZAL	50–1000	Whole cow milk	2.66 ± 0.31	10.92 ± 24.93	0.991	13
	50–1000	Skimmed cow milk	3.14 ± 0.37	38.75 ± 31.25	0.993	9
	100–1000	Semiskimmed goat milk	2.37 ± 0.34	14.78 ± 29.75	0.992	26
	100–1000	Natural yogurt	0.75 ± 0.09	12.92 ± 7.85	0.993	15
2-Me0E_2	10–100	Whole cow milk	23.75 ± 2.52	23.17 ± 20.31	0.993	3
	10–100	Skimmed cow milk	35.14 ± 3.66	14.27 ± 31.21	0.994	1
	15–100	Semiskimmed goat milk	21.05 ± 2.79	4.85 ± 24.05	0.993	4
	15–100	Natural yogurt	5.64 ± 0.71	17.17 ± 6.18	0.992	5
E_1	60–407	Whole cow milk	4.40 ± 0.43	10.47 ± 13.97	0.994	32
	30–407	Skimmed cow milk	5.02 ± 0.46	36.89 ± 16.07	0.996	8
	65–407	Semiskimmed goat milk	4.13 ± 0.63	26.32 ± 22.03	0.991	19
	50–407	Natural yogurt	0.98 ± 0.12	19.85 ± 4.11	0.993	14

a) SD of the slope (S_m) and intercept (S_n).
b) Calculated as a S/N of 3.

phases due to density variations of them. Density of $CHCl_3$ is 1.48 g/mL at 25°C, and if that of water containing 30% w/v NaCl and the matrix exceeds it, the droplet remains in suspension or in the upper part of the aqueous phase. For this reason, 2 mL of sample was maintained in all cases to carry out the final methodology. Finally, the methodology was also applied to skimmed cow milk, semiskimmed goat milk, and whole natural yogurt, obtaining similar results.

3.2 Validation of the method and analysis of real milk and yogurt samples

Validation of the method was carried out after it had been adapted for the extraction of the selected group of analytes from skimmed and whole cow milk, semiskimmed goat milk, and whole natural yogurt. Figure 1 shows the MEKC-MS TIC chromatogram, base peak chromatogram, and extracted ion

Table 3. Results of the precision and accuracy study of the DLLME-MEKC-MS method for the selected compounds in milk and yogurt

Peak	Analyte	Type of milk or yogurt	Spiked level (μg/L)	Found[a)] (μg/L)	Accuracy (%) (RSD)	$t^{b)}$
1	E_3	Whole cow milk	36	37 ± 7	103 (7)	0.82
			72	80 ± 6	112 (6)	2.70
		Skimmed cow milk	36	35 ± 9	97 (16)	0.41
			72	76 ± 7	106 (18)	0.68
		Semiskimmed goat milk	36	32 ± 11	89 (11)	2.16
			72	72 ± 10	100 (7)	0.05
		Whole natural yogurt	36	40 ± 10	112 (11)	1.85
			72	78 ± 8	108 (13)	1.29
2	β-ZAL	Whole cow milk	150	157 ± 32	105 (10)	0.96
			300	279 ± 28	93 (17)	1.02
		Skimmed cow milk	150	165 ± 37	110 (6)	2.70
			300	326 ± 31	109 (19)	0.73
		Semiskimmed goat milk	150	127 ± 55	85 (10)	2.68
			300	286 ± 47	95 (8)	1.49
		Whole natural yogurt	150	127 ± 42	85 (11)	2.60
			300	317 ± 35	106 (7)	1.75
3	β-ZEL	Whole cow milk	150	146 ± 33	98 (12)	0.47
			300	272 ± 29	81 (19)	1.10
		Skimmed cow milk	150	152 ± 40	101 (14)	0.20
			300	308 ± 33	102 (20)	0.17
		Semiskimmed goat milk	150	123 ± 53	82 (19)	2.25
			300	285 ± 45	95 (19)	1.02
		Whole natural yogurt	150	156 ± 39	104 (13)	0.56
			300	330 ± 33	110 (5)	2.68
4	α-ZEL	Whole cow milk	750	700 ± 85	93 (15)	0.99
			1500	1494 ± 76	100 (7)	0.12
		Skimmed cow milk	750	762 ± 56	102 (13)	0.26
			1500	1603 ± 46	107 (20)	0.23
		Semiskimmed goat milk	750	855 ± 114	114 (16)	1.50
			1500	1675 ± 104	97 (13)	1.09
		Whole natural yogurt	750	845 ± 106	113 (18)	1.09
			1500	1622 ± 74	108 (8)	1.81
5	17β-E_2	Whole cow milk	366	372 ± 87	102 (10)	0.35
			732	664 ± 77	91 (13)	1.54
		Skimmed cow milk	366	353 ± 56	96 (13)	0.61
			732	733 ± 46	100 (19)	0.02
		Semiskimmed goat milk	366	377 ± 120	103 (15)	0.38
			732	708 ± 106	97 (13)	0.54
		Whole natural yogurt	366	322 ± 88	88 (17)	1.61
			732	621 ± 76	85 (12)	2.50
6	17α-E_2	Whole cow milk	366	406 ± 89	111 (12)	1.89
			732	682 ± 80	93 (11)	1.50
		Skimmed cow milk	366	359 ± 91	98 (15)	0.28
			732	700 ± 76	96 (19)	0.54
		Semiskimmed goat milk	366	349 ± 70	95 (9)	1.10
			732	722 ± 60	99 (6)	0.57
		Whole natural yogurt	366	356 ± 84	97 (11)	0.53
			732	789 ± 71	108 (10)	1.27
7	ZEN	Whole cow milk	270	232 ± 70	86 (16)	2.04
			540	481 ± 61	89 (19)	0.97
		Skimmed cow milk	270	270 ± 601	100 (14)	0.01
			540	508 ± 52	94 (16)	0.81
		Semiskimmed goat milk	270	248 ± 85	92 (17)	1.02
			540	589 ± 72	109 (18)	0.62
		Whole natural yogurt	270	277 ± 60	102 (12)	0.41
			540	583 ± 51	108 (10)	1.45
8	EE_2	Whole cow milk	609	511 ± 112	84 (18)	2.09
			1218	1218 ± 95	100 (15)	0.01

Table 3. Continued

Peak	Analyte	Type of milk or yogurt	Spiked level (μg/L)	Found[a)] (μg/L)	Accuracy (%) (RSD)	$t^{b)}$
		Skimmed cow milk	609	626 ± 130	103 (14)	0.43
			1218	1187 ± 112	95 (17)	0.33
		Semiskimmed goat milk	609	704 ± 182	116 (14)	1.94
			1218	1314 ± 162	108 (9)	1.70
		Whole natural yogurt	609	529 ± 176	87 (14)	2.14
			1218	1272 ± 145	104 (12)	0.69
9	α-ZAL	Whole cow milk	300	276 ± 76	92 (12)	1.46
			600	504 ± 68	84 (19)	1.98
		Skimmed cow milk	300	328 ± 75	109 (11)	1.65
			600	567 ± 67	95 (17)	0.66
		Semiskimmed goat milk	300	306 ± 99	102 (11)	0.37
			600	563 ± 88	94 (15)	0.87
		Whole natural yogurt	300	296 ± 81	99 (11)	0.22
			600	634 ± 95	106 (11)	1.01
10	2-MeOE$_2$	Whole cow milk	30	26 ± 7	85 (15)	2.27
			60	53 ± 6	89 (10)	2.39
		Skimmed cow milk	30	34 ± 7	114 (17)	1.59
			60	60 ± 6	100 (14)	0.01
		Semiskimmed goat milk	30	25 ± 9	83 (11)	2.59
			60	65 ± 8	109 (18)	0.90
		Whole natural yogurt	30	26 ± 9	88 (14)	1.93
			60	68 ± 10	114 (9)	2.70
11	E$_1$	Whole cow milk	122	127 ± 25	104 (19)	0.29
			244	228 ± 22	93 (19)	0.74
		Skimmed cow milk	122	127 ± 24	104 (10)	0.85
			244	245 ± 21	100 (19)	0.03
		Semiskimmed goat milk	122	133 ± 42	109 (19)	0.86
			244	206 ± 40	84 (19)	1.63
		Whole natural yogurt	1212	126 ± 32	103 (18)	0.33
			244	226 ± 44	93 (6)	2.43

a) Average value ± confidence interval (five determinations, 0.05 of confidence).
b) t_{tab} (Student's *t*-test) = 2.78, $\alpha = 0.05$.

chromatograms of all the analytes of a spiked whole cow milk sample after the DLLME-MEKC-MS method, as well as the base peak chromatograms of blank samples of whole cow milk (a), skimmed cow milk (b), and semiskimmed goat milk (c). Figure 2 shows the same results for the whole natural yogurt. As can be seen, all the analyzed compounds were clearly identified in the samples and quantified without any problem. It is worth noting the presence of two peaks (tagged with an asterisk) in the extracted ion chromatogram at m/z 303.3 was also observed in the injection of the standard mixture of the analytes. Since their migration times match with those of α- and β-ZEL (m/z 321.2), it can be deduced that they are a result of the loss of a water molecule during ESI of the structure of these analytes ($[321.2 - H_2O]^+$).

Calibration of the method was carried out by spiking each matrix with six increasing concentrations of the target analytes ($n = 6$). The results are shown in Table 2, observing good linearity for all the matrices with determination coefficients (R^2) higher than 0.991 in all cases. The LODs and LOQs were calculated as the concentration of analyte that gave a S/N of 3 and 10, respectively, and were experimentally checked. LODs ranged between 3 and 32 μg/L for whole cow milk, between 1 and 21 μg/L for skimmed cow milk, between 4 and 61 μg/L for semiskimmed goat milk, and between 5 and 29 μg/L for whole natural yogurt, except for 17β-E$_2$ (40–104 μg/L) and EE$_2$ (64–220 μg/L), which were slightly higher. These results in the micrograms per liter range are in general comparable to those obtained with the use of microextraction techniques for the analysis of estrogens from milk [12–17].

Following, a study to evaluate the precision and the accuracy of the method was carried out spiking each matrix with the 11 analytes at two concentration levels and performing the extraction five times ($n = 5$). The concentration calculated from the previous calibration curves was compared by a Student's *t*-test with the added one. Table 3 shows the results of this study, observing that all experimental t values were lower than the tabulated one ($t_4 = 2.78$ for $n = 5$, $p = 0.05$), and that good accuracy percentages, between 81 and 116% and RSDs lower than 20% in all cases were obtained. Accordingly, the developed method can be successfully employed for the analysis of this group of estrogenic compounds in the selected matrices, with high repeatability and obtaining good relative recovery values with acceptable RSDs.

3.3 Application of the method for the analysis of commercial samples

In order to fully demonstrate the potential of the proposed methodology for the analysis of the selected group of analytes in milk and yogurt, a total of 15 milk samples and three yogurt samples were bought in local supermarkets and submitted to the described treatment. The nutritional information of the analyzed samples was quite similar to the ones previously analyzed. In none of the samples, residues of the estrogenic compounds analyzed were found over the LODs of the method. MS/MS experiments were developed in order to confirm the absence of the analytes.

4 Concluding remarks

In the present work, a DLLME-MEKC-MS method previously developed for the analysis of a group of compounds with estrogenic activity in water samples has been adapted for its application to more complex matrices. This method allows achieving very good selectivity and sensitivity employing a nonvolatile surfactant as an additive of the BGE (APFO) without any interference in the MS signal. In this way, after a suitable deproteinization and defatting of whole cow, skimmed cow, and semiskimmed goat milk and whole cow natural yogurt, the methodology resulted suitable for the analysis of the target compounds.

The methodology was validated by means of method calibration, precision, and accuracy studies, obtaining good results. LODs were in the micrograms per liter level, values that are in the same order of magnitude with respect to similar works. A screening analysis of 18 milk and yogurt samples currently commercialized in Spain was carried out, not detecting any of the estrogenic substances in any of the analyzed samples.

This research represents the first application of DLLME for the analysis of estrogenic compounds in milk and yogurt. It is also worth mentioning that it is also the first time that DLLME is coupled with MEKC-MS for this purpose. The whole methodology involves a low consumption of organic solvents and samples, has a relatively low cost, and is very simple.

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Anexo I.3. - Artículo 3

Capillary electrochromatography and nano-liquid chromatography coupled to nano-ESI interface for the separation and identification of estrogenic compounds

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Research Article

Capillary electrochromatography and nano-liquid chromatography coupled to nano-electrospray ionization interface for the separation and identification of estrogenic compounds

Nano-LC and CEC were coupled to MS through a nanospray or a pressurized liquid-junction interface for the simultaneous separation and determination of 11 estrogenic compounds. Different stationary phases, that is, phenyl, C18, and C18 bidentate silica hydrate, were studied. For both techniques, the phenyl stationary phase was the best option, considering separation efficiency, selectivity, and resolution. Under the optimized conditions, the baseline separation of the target compounds (including estradiol and zearalanol epimers) was achieved in less than 20 min in nano-LC-MS and less than 13 min in CEC-MS. Molecular imprinted polymer SPE was used for extracting the target compounds from mineral water samples with the analysis of nano-LC-MS. The whole molecular imprinted polymer SPE nano-LC-MS method was validated through a recovery study at two levels of concentration. Sensitivity was improved by on-column focusing technique obtaining LODs in the range 1.4–55.4 ng/L.

Keywords:
Electrochromatography / Estrogens / Molecular imprinted polymer / MS / Nano-LC
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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Recent trends in the field of Analytical Chemistry focus on the development of approaches that consider environmental aspects [1]. One way to achieve the so-called "Green Analytical Chemistry" is definitely the miniaturization of the separation system. In this respect, electromigration techniques, that is, CE and CEC, and miniaturized chromatography, that is, capillary LC (CLC) and nano-LC, have become important in the last decades. On the one side, column downscaling (internal diameter $\leq 100\ \mu m$) entails an important advantage over conventional tools as reduced sample and mobile phase volumes, use of small amounts of stationary phase, and lower costs analysis; and on the other side, it offers higher separation efficiency and resolution, and shorter analysis duration. Finally, working at low flow rates (nL/min), these miniaturized techniques can be easily coupled to MS using mainly nano- ESI interfaces. Although commercial solutions are available, such as sheath liquid (SL) interfaces for CZE [2] and nanospray for CLC/nano-LC [3, 4], the hyphenation of CEC with MS is still mainly focused in the research field.

The use of traditional SL flow interface for CEC-MS may cause a current instability and limited sensitivity, on the one hand, and a worse chromatographic performance in terms of the selectivity resolution and a long analysis time due to the use of a long capillary column [5] on the other hand. Good results have been obtained employing nanospray MS interfaces as sheath-less [6] and the liquid junction [7]. In the latter solution, a hybrid design emerges as a low sheath-flow [8] between the sheath-less and the sheath-flow interface due to the presence of the SL, which offers versatile applicability [9–11] without any assisted pressure. Despite the good results achieved, it has not been widely applied in other important fields such as environmental analysis.

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Abbreviations: BP, base peak; **CLC**, capillary LC; **DS**, dienestrol; **EIC**, extracted ion chromatogram; **L-J**, liquid-junction; **MIP**, molecular imprinted polymer; **SL**, sheath liquid; **TIC**, total ion current; **ZAL**, Zearalanol

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In this respect, estrogens analysis has been described [12–14] using conventional chromatographic techniques (GC and HPLC). On the contrary, only few studies have described the use of miniaturized techniques [2, 15–20]. In this sense, Kuehnbaum and Britz-McKibbin [15] developed a CE-TOF/MS method to analyze free and conjugated estradiol (E_2) and estriol (E_3) in human urine. Sirén et al. [16] used the partial filling technique in micellar electrokinetic chromatography (PF-MEKC) MS to determine metabolic profiles by analyzing endogenous low-hydrophilic steroids, including 17β-E_2. In addition, MEKC-MS was applied in our laboratories [2, 17] to determine 12 estrogenic compounds in water, milk, and yogurt using a BGE-containing perfluorooctanoic acid as an MS volatile surfactant. Pressurized CEC with UV detection or end-column amperometric detection was used for the analysis of diethylstilbestrol, hexestrol, and dienestrol (DS) in fish muscle [18, 19]. Kozlík et al. [20] used CLC-MS/MS to separate and quantify trace concentrations of five estrogens (E_1, E_3, 17α-E_2, 17β-E_2, and EE_2) in aqueous samples, while Farlow et al. [21] also used 300 μm id column for CLC-MS/MS to determine total and free estrogens (i.e., E_1, E_3, 17α-E_2) and estrogens metabolites in commercial milk products.

Although SPE has been the most historically employed extraction and preconcentration technique in many fields for the analysis of estrogens in environmental waters [12, 22, 23], nowadays there is a growing use of more selective sorbents, such as molecularly imprinted polymers (MIPs). In this sense, MIP-SPE has been applied for the removal of estrogenic pollutants from wastewaters [24] and also in the trace analysis of estrogens [25, 26]. In this respect natural and synthetic estrogens have been extracted from river and tap water using a laboratory-made [27, 28] and commercial MIP, such as AFFINIMIP® SPE Estrogens [29], a mixture of AFFINIMIP® SPE sorbents (i.e., estrogens, bisphenol A, and phenolics) [30], and very recently AFFINIMIP® SPE ZEN Zearalenone [31].

This work proposes the separation of 11 estrogenic compounds by nano-LC and nonpressurized CEC. The hyphenation with MS by nanospray or pressurized liquid-junction (L-J) interface is a main novelty. This is the first time that such a large group of estrogenic compounds are studied by these miniaturized techniques. The selected compounds were analyzed utilizing capillaries packed with different silica-based stationary phases. In order to improve the sensitivity, online preconcentration techniques were applied. Mineral water samples were analyzed after a simple sample preparation based on MIP-SPE using AFFINIMIP® SPE ZEN commercial cartridges. To the best of our knowledge, this is the first time that CEC-MS with an L-J interface is used for the separation of estrogenic compounds. Although MIP-SPE has been already combined in one occasion with CE for the analysis of estrogens [32], this is the first time that MIP-SPE has been combined with nano-LC-MS for the same purpose.

2 Materials and methods

2.1 Chemicals and materials

All chemicals used in this study were of analytical reagent grade. Formic acid (HFo), glacial acetic acid (HAc, >99%, m/m), hydrochloric acid (HCl, 37%, m/m), methanol (MeOH), and propan-2-ol (Pr-2OH) were purchased from Carlo Erba (Rodano, Milan, Italy); ACN was from VWR (International PBI S.r.l., Milan, Italy) while ammonia solution (30%, m/m) was from Riedel-de Heän (Seelze, Germany). Pure and DI water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

Analytical standards, namely DS (peak #10), 17α-estradiol (17α-E_2, peak #6), 17β-estradiol (17β-E_2, peak #4), estriol (E_3, peak #1), estrone (E_1, peak #9), 17α-ethinylestradiol (EE_2, peak #8), 2-methoxyestradiol (2-MeOE_2, peak #7), α-Zearalanol (α-ZAL, peak #3), β-Zearalanol (β-ZAL, peak #2), α-Zearalenol (α-ZEL, peak #5), Zearalenone (ZEN, peak #11) were from Sigma-Aldrich Chemie and were used without further purification (purity ≥ 95%).

Stock solutions of the analytes were prepared dissolving the appropriate amount of each in MeOH at a final concentration of 1000 mg/L, except α-ZAL at 500 mg/L. Ammonium formate and acetate buffer solutions were prepared by titrating appropriate volumes of formic or acetic acid with 1 M NH_4OH to the desired pH. The standard stock solutions were stored at −20°C for no more than 2 months, while working standards, buffer solutions, and extracted samples were stored at 4°C and kept in the dark prior to analysis.

AFFINIMIP® SPE ZEN commercial cartridges were provided by POLYINTELL (Valde Reuil, France). Each cartridge contained 100 mg of MIP in a 3 mL plastic tube.

2.2 Instrumentation

A basic 20 pH (Crison, Barcelona, Spain) was used for pH measurement. An ultrasonic bath, model FS 100b Decon (Hove, UK) was used to sonicate solutions and capillary columns during the packing procedure.

Polyimide-coated fused silica capillaries from Polymicro Technologies™ (CM Scientific, Silsden, UK) with 375 μm od and 25, 50, and 75 μm id were used to prepare capillary columns and tip emitters. The columns were packed in our laboratory following a packing procedure published previously [11, 33], which was based on the slurry packing method. The following stationary phases were studied: Pinnacle™ II C18 (3 μm, pore size 11 nm, carbon load 13%) and Pinnacle™ II phenyl (3 μm, pore size 11 nm, carbon load 6%), both purchased in bulk from Restek (Bellefonte, PA, USA), and RP-C18, Cogent Bidentate C18™ (TYPE-C™, 4 μm, pore size 10 nm, carbon load 16.5%) kindly donated by Microsolv Technology (Eatontown, NJ, USA). Columns were packed for 25.0 cm, with an effective and total length of 26.5 and 35.0 cm.

2.3 Nano-LC

Nano-LC experiments were carried out using a laboratory-assembled instrument previously described [10]: an LC Agilent 1100 series LC micropump (G1376A) with a passive split system to reduce the flow rate from microliter per minute to nanoliter per minute, a modified injector for nanoliter injection (Enantiosep, Műnster, Germany) [34, 35] and a Spectra 100 UV apparatus, set at 214 nm (Thermo Separation Products, San Jose, CA, USA) with an on-column detector cell (data acquisition and rise time were fixed at 20 Hz and 1.0 s, respectively). After these preliminary experiments, the capillary column was then cut at the outlet frit and connected to the nano-ESI interface for MS coupling. The HPLC pump was controlled by the Chemstation software (Rev.A.09.01, Agilent Technologies), while the UV detector data were acquired and analyzed with the ChromQuest version 3.0 software (Thermo Finnigan).

2.4 CEC

A ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) was employed for CEC experiments. During the CEC-DAD experiments, the sample was electrokinetically injected applying +15 kV × 15, 40, or 120 s at the anodic end or hydrodynamically injected for 10 bar × 30 s, and then separated at +25 kV (column temperature, 25°C) pressurizing both ends. The detection wavelength was set at 214 nm.

For CEC-MS experiments, the capillary column was cut at the UV detection window and, following the protocol described in the previous paper, it was introduced into the L-J interface [36]. At the end of the working day, the inlet reservoir was immersed in an aqueous–organic mixture (H_2O:MeOH, 1/99, v/v, 5 mM ammonium acetate pH 4.5). Chemstation software (Rev. A.09.01, Agilent Technologies) was used for managing the instrument and to handle the obtained data.

2.5 MS and electrospray interface

The nano-LC system was coupled to the MS through a commercial suitably modified nanospray interface (Thermo Finnigan) as described in our previous paper [10]. A laboratory-made L-J ESI interface was used for coupling CEC with MS as fully described in a previous work [36].

The MS S/N was optimized changing the MS parameters. The MS tune, in negative detection, was set in an automatic mode by direct infusion of a 1 μg/mL 17β-E_2 standard solution in mobile phase, considering the base peak (BP) at 271.4 m/z. The following MS parameters were set up: capillary voltage, −11.45 V; capillary temperature, 200°C; tube lens voltage, 0 V; automatic gain control, 3×10^7; number of micro scans: 3; and injection time, 70 ms. Mass spectra were acquired in the full scan mode in the range 200–350 m/z.

For method validation, retention times, peak heights, and peak areas were taken from the extracted ion chromatograms (EICs) for each studied compound. The mass spectra and ion- and electrochromatograms (TIC, BP, and extracted ions) were acquired and reprocessed using Xcalibur™ 1.3 software (Thermo-Finnigan).

2.6 Nano-LC-MS and CEC-MS optimum conditions

The best separation was achieved using the columns packed with Pinnacle™ II phenyl stationary phase following the experimental conditions listed below. For CEC: mobile phase, 5 mM NH_4Ac at pH 4.5 in 60/40 ACN/H_2O v/v; separation voltage, +15 kV; sample medium, 20/80 ACN/H_2O v/v; electrokinetic injection, +15 kV × 40 s; spray liquid, MeOH/H_2O, 60:40 (v/v) with 0.1% (v/v) NH_4OH delivered at a pressure of ~4.0 kPa; for nano-LC: mobile phase, 50/10/40, ACN/MeOH/H_2O v/v/v at 100 nL/min; injection volume: 700 nL; injection medium, MeOH/H_2O, 10/90 v/v. For nano-LC, and in order to obtain a better repeatability between injections, the column was flushed consecutively with MeOH and ACN for 5 min each, and then reconditioned with mobile phase. At the end of the working day, the column was washed with ACN for 15 min.

2.7 Extraction and clean-up using MIP-SPE

Mineral water sample was acquired from a local supermarket. Its pH value and conductivity at 20°C as well as the fixed residue at 180°C were 7.8, 118 μS/cm, and 80.2 mg/L respectively.

AFFINIMIP® SPE-ZEN cartridges were conditioned with 5 mL of ACN followed by 5 mL of Milli-Q water. For the MIP-SPE experiments, 100 mL of spiked or nonspiked Milli-Q or mineral water were adjusted to pH 7.0 with 0.1 M HCl or NaOH. The samples were then percolated through the MIP-SPE cartridge at a flow rate of 1 mL/min. After washing with 4 mL of ACN/H_2O, 80:20 v/v, and 2 mL of water, the sorbent was dried by applying full vacuum. Estrogenic compounds were eluted from the cartridges with 5 mL of MeOH, which was evaporated at 40°C using a rotavapor R-200 from Bűchi Labortechnik (Flawil, Switzerland) equipped with a KNF LABOPORT diaphragm vacuum pump (KNF NEUBERGER, Trenton, NJ, USA). The residues were reconstituted in 200 μL H_2O/MeOH 90:10 v/v and injected into the nano-LC-MS system.

3 Results and discussion

Before coupling the miniaturized techniques to MS, experiments were carried out using a UV detector. In this respect, parameters such as the best stationary phase, experimental conditions, and sensitivity were evaluated first in CEC and subsequently applied to nano-LC. CEC conditions were selected taking into account that the MS coupling through the L-J interface might be affected by bubble formation.

3.1 Study of the stationary phase

The selected analytes are noncharged in a wide pH range (pK_a>7.5 [17]) and are characterized by their hydrophobic molecular structure. For these reasons, the analytical separation of estrogenic compounds can occur mainly by an MEKC [2] or a RP chromatographic mechanism [13]. Following the last of them, three different stationary phases with free silanol groups were investigated changing different parameters (injection mode, applied voltage, and mobile phase composition and solvent medium), reaching the best chromatographic separation in terms of selectivity, isomeric resolution, analysis time, and peak shape of all estrogenic compounds.

The column packed with RP-C18 Cogent Bidentate provided a poor resolution of the analytes (eight of 11 identified peaks) and was not able to separate 17α- and 17β-E_2. For this reason, this stationary phase was discarded for subsequent studies. On the contrary, Pinnacle™ phases proved to be very selective, especially for the resolution of ZAL and E_2 epimers, obtaining the complete separation of nine over 11 peaks. Due to the high carbon load value of Pinnacle™ II C18, the strong hydrophobic and secondary interactions toward 2-$MeOE_2$ resulted in a clear peak tailing (peak #7, asymmetry 0.63) that was hardly quantifiable in MS experiments. For this reason, this column was set aside for this study. Finally, the phenyl substituents existent on the Pinnacle™ Phenyl silica promoted π–π interactions with the aromatic moieties and the planar structure of the studied compounds, a fact that resulted in a better chromatographic profile in terms of separation efficiency (116 000–140 000 theoretical plates per meter), peak shape (asymmetry: 0.96–1.05), selectivity, and analysis time (about 12 min); see Supporting Information Fig. 1.

Preliminary experiments in nano-LC-UV were assayed under the best conditions for CEC separation for each capillary column. RP-C18 Cogent Bidentate and Pinnacle™ II C18 stationary phases were discarded for the same reasons previously expressed in the CEC experiments. Taking all these results into account, the column packed with Pinnacle™ II Phenyl stationary phase was selected for further experiments.

3.2 Increasing the sensitivity: on-column preconcentration

Sample stacking and/or focusing strategies [37, 38] with different compositions of sample medium were studied by loading a quite large injection volume of the standard mixture by an electrokinetic injection for CEC (+15 kV × 30 s) and a high pressure injection for nano-LC (400 nL). Optimum results regarding separation efficiency, resolution, shape, and height for each analyte were achieved when the sample was dissolved in ACN/H_2O (20:80, v/v) and MeOH/H_2O (10:90, v/v) for CEC and nano-LC, respectively. Under these conditions, different sample volumes were injected in the systems, by electrokinetic injection in the range 5–60 s for CEC and by high pressure injection with volumes in the range 100–800 nL for nano-LC. Analyzing the ratio of peak height and peak width at half height ($H/w_{1/2}$) versus the injected sample volumes, the highest compression peak effect was interpolated at the last volume/time of a linear trend ($R^2 = 0.98$–0.99) identified with 40 s by electrokinetic injection for CEC and 700 nL in nano-LC (see Supporting Information Fig. 2).

3.3 Optimization of nano-LC-ESI-MS and CEC-ESI-MS separations

Once the electro/chromatographic conditions were optimized, both miniaturized techniques were coupled to MS. In nano-LC, as the nanospray interface is not designed to use a make-up liquid for ionization, the composition of the mobile phase had to be slightly modified to assist the ionization. In the positive detection mode, although no change in the chromatographic separation was observed under acidic conditions (0.1–1% v/v of formic acid or TFA or ammonium formate pH 2.5), a good MS signal was obtained only for ZAL, ZEL, ZEN, 2-$MeOE_2$, and E_1, while the other analytes were below the LOD signal. In the negative mode, no signal was observed using basic conditions (0.1% of NH_4OH v/v or ammonium acetate pH 8.0). In contrast, in the negative mode, when the mobile phase was only an organic mixture (ACN/H_2O 60:40, v/v), TIC and EICs for all the studied compounds were collected with a clear and a unique signal. Finally, an improvement of the peak shapes and resolution of E_2 epimers was obtained when a 10% of MeOH v/v was added (see Supporting Information Fig. 3).

When the column was coupled to MS detector, it was not possible to pressurize both ends of the column as in CEC-UV experiments. A useful solution to prevent bubbles formation was lowering the applied voltage and thus the current. Afterwards, by decreasing the separation voltage from +25 to +15 kV (current decreased from 2.1 to 1.4 μA), the separation was achieved in less than 13 min (MS negative detection), with a separation efficiency in the range 73 000–125 000 plates/m and epimers resolution of ZAL and E_2 of 4.8 and 1.5, respectively.

In order to obtain a stable spray and the highest S/N ratio for each analyte, the spray liquid composition and the hydrostatic pressure were studied [36, 39]. In general, a dramatic ion suppression (with the exception of ZAL, ZEL, and DS) occurred when a 0.1% (v/v) formic acid or 5 mM ammonium formate pH 2.5 was aqueous-organic (MeOH/H_2O, 50:50; data not shown). A slightly better MS response was obtained under basic conditions (ammonia solution), which suppressed the positive charge of the majority of the analytes. Subsequently, among ACN, MeOH, and Pr-2OH, in a ratio 50:50 v/v with water, the second resulted to guarantee in general the highest S/N value and better spray stability (data not shown). Finally, a high MeOH content led to a higher spray signal, but lower S/N ratios, while in contrast a high water content increased the surface tension, which together with the lowest volatility of the mixture resulted in a unstable

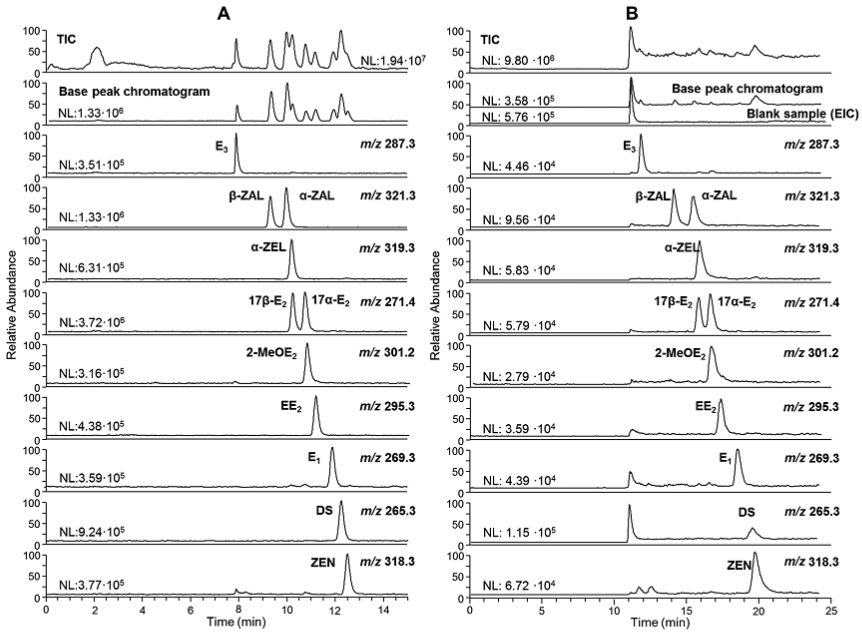

Figure 1. TIC, BP, and EICs of a standard mixture of the analytes injected in (A) CEC-MS and (B) MIP-SPE-nano-LC-MS method. Capillary column, 75 μm id; packed length, 25.0 cm; MS interface length, 25.5 cm; stationary phase, Pinnacle™ II Phenyl; (A) sample, 100–1000 μg/L in ACN/H_2O (20:80, v/v); mobile phase, 5 mM NH_4Ac pH 4.5 in ACN/H_2O (60:40, v/v); applied voltage, +15 kV; electrokinetic injection at +15 kV for 40 s; temperature, 25°C; spray liquid, MeOH/H_2O (60:40, v/v) with 0.1% (v/v) ammonia solution; hydrostatic pressure ~4.0 kPa (50.0 cm). (B) sample spiked at 40–400 ng/L in MeOH/H_2O (10:90, v/v); mobile phase, ACN/MeOH/H_2O (50:10:40, v/v/v); flow rate: 100 nL/min; injection volume: 700 nL; temperature, 25°C. For peak identification see Section 2.1. For MS experimental conditions see Section 2.5.

spray with a decrease of the ionization (shown in Supporting Information Fig. 4). Therefore, a 60% (v/v) of MeOH with 0.1% (v/v) ammonia allowed obtaining the highest S/N value for almost all analytes.

The height of the reservoir with respect to the column-tip liquid junction was changed in the range 25–55 cm, giving a pressure of ~2.1–4.5 kPa. As it has been previously observed [36] no influence on the chromatographic separation was shown. The increase of the pressure produced an enhancement of the flow into the emitter-tip, giving a higher S/N, up to ~4.0 kPa (shown in Supporting Information Fig. 5). A higher flow of the spray liquid made the evaporation of the solvent surrounding the analyte molecules difficult, hindering the electrospray phenomenon and observing a reduced ionization, spray instability, sample dilution, and increase of the noise level. In addition, the spray liquid flow between the column and the tip emitter in the liquid junction produced a turbulence that influenced the chromatographic separation in terms of band broadening (data not shown). From these data it can be concluded that the highest sensitivity for all the studied analytes was achieved at ~4.0 kPa (corresponding to a height of 50 cm), providing a flow of the spray liquid of about 200 nL/min into the tip emitter tip. Figure 1A shows the TIC, BP, and extract ion electrochromatograms of the separation of the 11 analytes under the optimized conditions.

3.4 Instrumental validation of the method

For the repeatability study, a standard solution containing the 11 selected compounds was consecutively injected five times in the same day ($n = 5$) for 3 days ($d = 3$, $n = 15$). In nano-LC-MS, 700 nL at a concentration of 10–100 ng/mL, were injected obtaining an intraday precision (RSD, %) in the range 1.5–2.3% and 5.1–12.1% for migration times and peak areas, respectively, while interday precision was in the range 5.2–7.3% and 7.4–15.3%, for the same chromatographic parameters, respectively. In the case of CEC-MS, for an electrokinetic injection of 40 s at +15 kV at a concentration of 1–10 μg/mL, retention times precision was in the range 0.7–0.9% and 5.8–6.3%, while peak areas precision was in the

Table 1. Mean recovery percentages, RSD values, analyte concentration added to the sample, LODs, and LOQs of the method after the SPE-MIP-nano-LC-MS procedure

Analytes	Milli-Q water	Mineral water			
	Level 2[a] recovery (%)[b],[c] (RSD, %)	Level 1[b] recovery (%)[b],[c] (RSD, %)	Level 2[b] recovery (%)[b],[c] (RSD, %)	LOD_{method} (ng/L)	LOQ_{method} (ng/L)
E_3	57 (13)	58 (15)	55 (16)	5.46	18.8
β-ZAL	69 (15)	65 (12)	61 (13)	1.73	4.42
α-ZAL	69 (16)	67 (13)	65 (12)	1.61	5.20
17β-E_2	64 (13)	62 (12)	55 (11)	13.8	47.2
α-ZEL	78 (15)	71 (13)	75 (12)	1.42	4.25
17α-E_2	67 (12)	58 (13)	63 (14)	15.8	41.4
2-Me0E_2	65 (17)	62 (15)	58 (15)	55.4	143
EE_2	64 (13)	61 (12)	59 (13)	19.2	73.5
E_1	58 (14)	52 (14)	55 (16)	18.5	66.7
DS	51 (16)	45 (16)	41 (17)	14.6	62.5
ZEN	73 (15)	71 (14)	68 (14)	5.63	18.2

a)Level 1: 10–100 μg/L; level 2: 20–200 μg/L.
b)Mean of five extractions ($n = 5$), 100 mL of water.
c)Recovery data: A_{EIC} (SD in sample)/A_{EIC} (SD in solvent).

range 10.8-14.4% and 13.5-19.8% for the same day and for different days, respectively.

Calibration curves were obtained plotting the values of the peak areas, measured in the EICs, versus the concentration of each analyte. Results of the linear dynamic range and sensitivity were reported in Supporting Information Table 1. Despite the use of on-column focusing/stacking, less satisfactory LOD and LOQ results were obtained in CEC-MS. In this respect, on the one hand, the injection can be considered small in comparison with nano-LC, while on the other, the presence of the buffer in the mobile phase, necessary for electrical conductivity, probably suppresses the MS signal, as also observed when the mobile phase was studied in nano-LC. Besides, the spray liquid was not able to provide an ionization comparable to that obtained by the nanospray interface.

3.5 Application to water samples

Once the separation was fully optimized, the estrogenic compounds were extracted from aqueous samples and analyzed by nano-LC-MS. The optimization of the extraction procedure was performed using 10 mL of spiked Milli-Q water at a concentration in the range 20–200 μg/L. Following the procedure indicated in the AFFINIMIP® SPE ZEN instruction sheet and from the literature for the extraction of a similar group of compounds [31], the presence of acetic acid in the elution solvent produced an ion suppression effect and then an overall disturbed MS background signal and low S/N ratio was obtained. The recovery values were lower than 10%. Hence, the extractive procedure was applied in the absence of acid, obtaining an enhanced MS signal for all the analytes, reaching recovery values in the range of 35–65%. This fact demonstrated that acetic acid led to an ion suppression effect, likely due to the increase of the surface tension that change the efficiency of droplet formation or evaporation and competition with negative charged ions, which in turn affects the amount of charged ion in the gas phase and then ions not compatible with the developed analytical methodology.

A repeatability study of the MIP-SPE-nano-LC-MS methodology was carried out with 100 mL of mineral water at two concentration levels, and recovery data of LODs and LOQs of the method were reported in Table 1. Figure 1B shows the TIC, BP, and EICs of a spiked mineral water sample after being subjected to the MIP-SPE-nano-LC-MS method. No residues of the estrogenic compounds were found in the analyzed samples over the LOD of the method.

Comparing the results obtained by the present method with other previously published studies that analyzed estrogenic compounds in water samples using SPE-MIP coupled to classic chromatographic techniques, LODs are of the same order of magnitude. For instance, Lucci et al. [29] used AFFINIMIP® SPE Estrogens cartridges, analyzing the target compounds (E_1, 17β-E_2, 17α-E_2, E_3, EE_2, DS, and diethylstilbestrol) by UPLC-APCI-QqQ-MS. For 100 mL of water sample, LODs and LOQs were in the range 4.5–9.8 ng/L and 14.9–32.6 ng/L, respectively. Also, Matějíček et al. [30] obtained LODs and LOQs in the range 0.7–1.9 and 2.5–6.4 ng/L, respectively, using as sorbent a mixture of AFFINIMIP® SPE Estrogens, bisphenol A, and phenolics and HPLC-ESI-IT-MS for the analysis of E_1, 17β-E_2, E_3, EE_2.

4 Concluding remarks

Nano-LC and CEC techniques coupled to both UV and MS were optimized for the separation of 11 estrogenic compounds, achieving baseline E_2 and ZAL epimers separation in less than 20 and 13 min, respectively. Different types of stationary phases were studied for column packing, obtaining the best results for Pinnacle™ II Phenyl silica support

concerning separation efficiency, selectivity, resolution, and EOF (in the case of CEC).

In order to increase the method sensitivity, on-column focusing/stacking mode was studied, as well as MS hyphenation parameters. The optimized method was validated through retention/migration time and area repeatability, calibration, and sensitivity.

A simple MIP-SPE, using a commercial MIP cartridge, was optimized for the extraction of the 11 selected substances from mineral water, carrying out the analysis by nano-LC-MS. The methodology was validated through a recovery study in Milli-Q and mineral water, obtaining acceptable results for mineral water and LODs and LOQs of the whole method between 1.4–19.2 and 4.2–143.7 ng/L, respectively. The analysis of mineral water samples did not reveal the presence of any residues of the studied estrogenic compounds over the LOD of the developed method.

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The authors declare no conflict of interest.

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Anexo I.4. - Artículo 4

Determination of estrogenic compounds in milk and yogurt samples by hollow fiber liquid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry

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RESEARCH PAPER

Determination of estrogenic compounds in milk and yogurt samples by hollow-fibre liquid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry

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Abstract An environmentally friendly method based on hollow-fibre liquid-phase microextraction (HF-LPME) was developed for the extraction of selected estrogenic compounds (i.e. four natural sexual hormones: estrone, 17β-estradiol, 17α-estradiol and estriol; two exoestrogens: 17α-ethynylestradiol and 2-methoxyestradiol; two synthetic stilbenes: dienestrol and hexestrol; and five resorcylic acid lactones: zearalenone, α-zearalanol, β-zearalanol, α-zearalenol and β-zearalenol), from whole cow and semi-skimmed goat milk and whole natural yogurt. After the optimization of the sample preparation procedure, spiked extracts were derivatized to their trimethylsilyl products using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide reagent and then analyzed by gas chromatography–tandem mass spectrometry (GC-MS/MS). Once optimum extraction conditions were established (protein precipitation with acetonitrile, extraction and the back-extraction in acetonitrile following the HF-LPME procedure), the method was validated and the calibration range, precision and accuracy were studied. The RSD values for the intra- and inter-day precision of the peak areas were in the range 0.65–9.69 and 1.00–11.47 %, respectively. The determination coefficients were higher than 0.991 for method calibration curves while LOD and LOQ values were between 0.06–2.55 and 0.16–6.11 μg/L for whole cow milk, 0.04–1.70 and 0.11–4.86 μg/L for semi-skimmed goat milk and 0.07–3.73 and 0.23–9.81 μg/L for natural yogurt, respectively. Finally, the accuracy and precision of the method were evaluated, obtaining a value in the range 84 81–119 % and RSD values lower than 20 % in all cases.

Keywords Estrogens · Hollow-fibre liquid-phase microextraction · Gas chromatography-triple quadrupole mass spectrometry · Milk · Yogurt

Abbreviations

2-MeOE$_2$	2-Methoxyestradiol
DES	Diethylstilbestrol
DS	Dienestrol
E$_1$	Estrone
E$_2$	Estradiol
E$_3$	Estriol
EE$_2$	17α-Ethinylestradiol
HEX	Hexestrol
ZAL	Zearalanol
ZEL	Zearalenol
ZEN	Zearalenone

Introduction

Lately, substances exhibiting natural or synthetic estrogenic activity have become of concern, since they can be introduced

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in the environment but also in the human food chain. These compounds may mimic the activity of endogenous hormones or even interfere with them [1, 2].

Regarding their introduction in the human diet, those estrogenic compounds that can be introduced through meat, milk or dairy products ingestion [3–5] are of high interest. In particular, milk collection from pregnant cows and the abusive or illegal use of synthetic estrogens as growth promoters are common practices that involve the presence of estrogenic hormones in dairy products over the recommended levels [6, 7]. It is a matter of concern that high intakes of dairy products containing estrogenic hormones can induce problems in humans, such as cancer [8–10].

The European Commission has forbidden the use of substances with hormonal activity for fattening livestock in Directive 2003/74/EC (Official Journal of the European Communities, L 262/17 of 14.10.2003) and establishes maximum residue limits (MRLs) for certain hormones in meat (Commission Regulation (EU) N° 37/2010, Official Journal of the European Union L 15/1 of 20.1.2010). Although the EU legislation on food safety in this issue is still lacking, scientific research continues to focus its efforts on monitoring and development of more efficient, sensitive and environmentally friendly analytical methods in order to increase the security in their consumption.

Although HPLC and UHPLC are the analytical techniques most applied to the determination of estrogenic compounds and their metabolites in milk and dairy products [11–13], miniaturized LC separation systems have also been used [14]. This is the case of the work of Farlow et al. who used a 300-μm ID column in a capillary LC (cLC)-MS/MS system to determinate total and free estrogens (i.e. E_1, E_3, 17α-E_2) and estrogens metabolites in milk and buttermilk [14].

Furthermore, a relatively low number of LC analytical methods have also been developed to detect other compounds with estrogenic activity such as resorcylic acid lactones (RALs) like the mycotoxin ZEN produced by *Fusarium* species and its metabolites, α- and β-ZAL and ZEL, in milk and dairy products in particular, by HPLC-MS/MS [15–18] and UHPLC-MS/MS [19–21].

GC is another alternative for the determination of these compounds; however, it has only been used for this purpose in very few occasions [22–28]. Among these works, it is worth mentioning the application of Choi et al. [22] who developed a method for the determination of natural estrogens and mycotoxins in breast milk by means of GC-MS or the work of Azzouz et al. [23] in which a SPE procedure was used in combination with GC-MS for the determination of EE_2, E_1, 17β-E_2 in milk samples [23]. In addition, recently, a stir-bar sorptive extraction procedure, in which a stainless steel wire was confined inside a hollow fibre (HF) impregnated with solvent, was applied to the extraction of hormones, including E_1, E_2, E_3, EE_2, from milk samples by GC-MS [28].

Due to the low concentrations at which these compounds appear in food matrices, sample preparation procedures is one of the most important parts of their determination. In this sense, traditional extraction, pre-concentration and clean-up procedures like liquid–liquid extraction (LLE) or solid-phase extraction (SPE) have been widely used [12] despite the fact that, depending on the application, they might use relatively high amounts of solvents, in particular, LLE.

In the course of recent years, together with the use of traditional extractive methodologies, trends in sample pretreatment have been focused on the use of simple, rapid and miniaturized techniques with low solvent consumption. In this respect, liquid-phase microextraction (LPME), and specially HF-LPME, combined with HPLC or GC has been successfully applied for the extraction of a wide variety of analytes from environmental [29–31], biological fluids [32, 33] and also food samples [34]. In particular, HF-LPME has been applied in different cases for the determination of some natural and synthetic estrogenic compounds like 17α-E_2, E_1, 17β-E_2, E_3, EE_2, HEX or DS in milk and dairy products [28, 35–39].

In this work, a HF-LPME-GC-MS/MS method has been developed and validated using *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) as silylating agent for the analysis of the TMS derivatives of 13 estrogenic compounds, i.e. natural hormones (17α-E_2, E_1, 17β-E_2, E_3) and metabolites (2-MeOE_2), exoestrogens (EE_2), synthetic stilbenes (HEX, DS), a mycotoxin (ZEN) and some of its major metabolites (α-ZAL, β-ZAL, α-ZEL and β-ZEL), in whole cow milk, semi-skimmed goat milk and natural yogurt. 17β-Estradiol-d_5 was used as internal standard. To the best of our knowledge, this is the first time that HF-LPME is applied to the determination of α-ZAL, β-ZAL, α-ZEL, β-ZEL and ZEN, also to their simultaneous determination with the natural and synthetic estrogenic compounds previously described. It is also the application in which the higher number of natural and synthetic estrogenic hormones and mycotoxins has been analyzed and the first time that GC-MS/MS using a triple quadrupole mass analyzer is combined with HF-LPME to analyze the target analytes in milk/yogurt.

Materials and methods

Chemicals and materials

All chemicals were used as received without filtration or further purification. Methanol (MeOH) and *n*-hexane (HPLC grade) were purchased from VWR International Eurolab S.L. (Barcelona, Spain) and formic acid (98 %, *w/w*) from Panreac Química (Barcelona, Spain). ACN of HPLC grade, hydrochloric acid (25 %, *w/w*), acetone and anhydrous glacial acetic acid (100 %, *w/w*) were from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was from Scharlau

Chemie S.A. (Barcelona, Spain), while 1-octanol, cyclohexane, sodium chloride (NaCl) (purity > 99.5 %) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) for GC derivatization were purchased from Sigma-Aldrich Chemie (Madrid, Spain). The Accurel Q3/2 polypropylene HF membrane (600 μm ID, 200 μm wall thickness and 0.2 μm pore size) was acquired from Membrana GmbH (Obernburg, Germany). Ultrapure water was deionized by a Milli-Q gradient system A10 from Millipore (Bedford, MA, USA).

Analytical standards, namely 3,4-bis(4-hydroxyphenyl)-2,4-hexadiene (DS, dienestrol), 4,4 0-(1,2-diethylethylene)diphenol (HEX, hexestrol), (1,3,5(10)-estratriene-3,17α-diol (17α-E_2, 17α-estradiol), 1,3,5-estratriene-3,17β-diol (17β-E_2, 17β estradiol), 1,3,5(10)-estratriene-3,16α,17β-triol (E_3, estriol), 1,3,5(10)-estratrien-3-ol-17-one (E_1, estrone), (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol (EE_2, 17α-ethinylestradiol), 1,3,5(10)-estratriene-2,3,17-triol 2-methyl ether (2-MeOE_2, 2-methoxyestradiol), 2,4-dihydroxy-6-(6α,10-dihydroxyundecyl)benzoic acid μ-lactone (α-ZAL, α-Zearalanol), 2,4-dihydroxy-6-(6β,10-dihydroxyundecyl]benzoic acid μ-lactone (β-ZAL, β-Zearalanol), 2,4-dihydroxy-6-(6α,10-dihydroxy-trans-1-undecenyl)benzoic acid μ-lactone (α-ZEL, α-Zearalenol), 2,4-dihydroxy-6-(6β,10-dihydroxy-trans-1-undecenyl)benzoic acid μ-lactone (β-ZEL, β-Zearalenol), (3S,11E)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1*H*-2-benzoxacyclotetradecine-1,7(8H)-dione (ZEN, Zearalenone) were from Sigma-Aldrich Chemie and were used without further purification (purity ≥ 95 %) (see Electronic Supplementary Material (ESM) Table S1). Furthermore, for GC-MS/MS method validation, 17β-estradiol-d_5 (17β-E_2-d_5) (Sigma-Aldrich Chemie) was employed as internal standard (IS).

Stock solutions of each compound were prepared in MeOH and stored at −18 °C: DS, HEX, 17β-E_2, 17α-E_2, E_3, E_1 and EE_2 at 1000 mg/L; 2-MeOE_2, α-ZAL and β-ZAL at 100 mg/L; α-ZEL and β-ZEL at 500 mg/L and ZEN at 100 mg/L. The standard mixtures were prepared each working day by dilution in MeOH. Standard solutions, the derivatization reagent, samples and sample extracts were stored in the dark at 4 °C prior to analysis.

Apparatus and software

GC-FID analysis was performed with a Varian 3800 GC system (Walnut Creek, CA, USA) equipped with a FID detector and a Combi PAL autosampler. Separation was achieved using a poly (5 % diphenyl/95 % dimethylsiloxane)-bonded fused silica capillary column (Equity™-5; 30 m × 0.25 mm, 0.25-μm film thickness, Supelco™, Bellefonte, PA, USA). Nitrogen was used as carrier gas at a flow rate of 0.7 mL/min. Two microlitres of a standard or sample solution was injected (10 μL syringe Microliter® Pal System, Hamilton company, Reno, NV, USA) by means of a CP-1177 injector valve at 280 °C. Split/splitless injection was applied: Initially, the split state was in off position, at 3 min the split state was set at 1:75 and held for all analysis time. The 13 TMS-*O*-estrogenic compounds were resolved nearly to the baseline in 19 min following the next GC oven program: The column temperature was initially set at 55 °C followed by a 25 °C/min ramp to 255 °C and soon after a 3 °C/min ramp to 275 °C was applied and held for 5 min; finally, the temperature was increased to 300 °C at 30 °C/min and held for 4 min more. The temperature of the FID was maintained at 300 °C. The GC, autosampler and FID detector were controlled by the Varian Star chromatography Workstation (version 6.41, Varian). This equipment was used for the optimization of the extraction and derivatization procedures.

For compound identification and method quantification, an Agilent 7890B GC coupled to a triple quadrupole (QqQ) mass spectrometer 7000B MS with an electron impact interface was used (Agilent 7000C Triple Quadrupole GC/MS System, Agilent Technologies, Waldbronn, Germany). Two (5 % phenyl)-methylpolysiloxane bonded fused silica capillary columns (HP-5 MS; 15 m × 0.25 mm, 0.25-μm film thickness, Agilent Technologies) were connected by means of a backflush valve. He flows in the first and second column were set at 0.7 and 0.9 mL/min, respectively. Separation was carried out by the same gradient of temperature used in the GC-FID system due to the equivalence of columns and flows. The QqQ was operated in multiple reaction monitoring (MRM) mode with two transitions for each analyte as listed in Table 1. The mass spectrometer was used under the following conditions: transfer line temperature 250 °C, source temperature 300 °C, electron ionization energy at −70 eV; the temperature of the first and second quadrupole was 180 °C and the collision cell gases were nitrogen (1.5 mL/min) and helium (2.25 mL/min). Depending on the specific transitions, the collision energy and dwell times were adjusted in the range 5–37.5 eV and 25–40 s, respectively; the time window was set at 3.3 cycles s^{-1} (Hz) (see Table 1).

The mass spectra and the ion chromatograms were collected and processed using the MassHunter software. During method validation, all chromatographic parameters, i.e., retention time, peak height, peak area and noise level, were acquired for each studied compound and processed using the MassHunter quantitative analysis software v. B.04.04 (Agilent Technologies).

Samples

Different types of ultra-heat treated milk (whole cow and semi-skimmed goat milk) and whole cow natural yogurt were selected. The content of proteins, carbohydrates and fats were

Table 1 Diagnostic ions for all studied O-TMS estrogenic compounds and their internal standard

Peak #	Abbreviation	Retention time (min)	Mw (g/mol)	Mw TMS-derivatized (g/mol)	Precursor ion (*m/z*)	MS/MS quantification			MS/MS qualification		
						Product ions (*m/z*)	Collision energy (eV)	Dwell (s)	Product ions (*m/z*)	Collision energy (eV)	Dwell (s)
1	HEX	11.1	270.4	414	207	191	10	25	179	10	25
2	DS	11.2	266.3	410	410	381	15	25	395	15	25
3	17α-E_2	14.0	272.4	416	416	326	7.5	40	285	15	40
4	E_1	14.0	270.4	342	342	244	15	40	257	15	40
5	17β-E_2	14.5	272.4	416	416	326	7.5	40	285	15	40
IS	17β-E_2-D_5	14.5	277.4	421	421	331	15	40	287	15	40
6	EE_2	15.0	296.4	368	368	205	35	40	285	15	40
7	2-$MeOE_2$	16.3	302.4	446	446	416	15	30	315	20	30
8	α-ZAL	16.5	322.4	538	433	309	17	30	389	10	30
9	β-ZAL	16.7	322.4	538	433	309	17	30	389	10	30
10	ZEN	17.0	318.4	462	462	444	5	30	151	10	30
11	α-ZEL	17.5	320.4	536	536	317	37.5	30	446	7.5	30
12	E_3	17.6	288.4	504	504	386	10	30	414	5	30
13	β-ZEL	17.8	320.4	536	536	317	37.5	30	446	7.5	30

in range 4.2–4.5 g/100 mL, 3.0–3.4 g/100 mL, 1.0–3.6 g/100 mL, respectively. All the analyzed samples were purchased in local supermarkets of Tenerife, Canary Islands (Spain).

Sample preparation

A deproteinization procedure, previously used by our group for the analysis of milk/yogurt samples [40], was adapted to our analytical system (GC-FID and GC-MS).

Briefly, a standard mixture including the IS (10 μg/L) was introduced in a 10-mL glass centrifuge tube and the solvent was evaporated to dryness with a gentle nitrogen stream. Then, 2 ml/g of non-spiked milk/yogurt sample at room temperature was introduced into a glass centrifuge tube. Just after, the sample was vortex shaken (Lab Dancer vortex from VWR) at 2800 rpm for 1 min and left at rest for 5 min. Afterwards, 4 mL of ACN was added to the spiked or non-spiked milk and it was vortex shaken at 2800 rpm for 2 min to ensure complete protein precipitation. The organic mixture was kept for 15 min in the darkness. Regarding yogurt samples, 2 g (about 2 mL) was accurately weighted and treated with 4 mL of ACN and 100 μL of acetic acid and subjected to the same procedure performed for milk samples. Both types of samples were then centrifuged on a 5702 centrifuge from Eppendorf (Hamburg, Germany) at 4400 rpm (3000×*g*) for 10 min. Then, the supernatant was evaporated at 40 °C and 180 mbar using a rotary evaporator R-200 (Buchi Labortechnik, Flawil, Switzerland) equipped with a V-800 vacuum controller and a 500 vacuum pump. The obtained aqueous residue (approx. 1.5 mL) was ready for the HF-LPME extraction procedure.

HF-LPME procedure for milk/yogurt samples

Before the application of the HF-LPME procedure, the sample extract was diluted to a final volume of 10 mL with Milli-Q water and NaCl was added to achieve a final concentration of 10 % (*w/v*). After the complete dissolution by ultrasounds (3510 Branson, Danbury, CT, USA), the solution was transferred into a 10 mL polypropylene/polyethylene syringe (Injekt®, B. Braun Melsungen AG, Mesungen, Germany) and filtered through a 0.45-μm Chromafil® Xtra PET-45/25 polyester filter (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) into a 22-mL crew cap amber glass vial (Supelco™). The pH was adjusted to 6.0 with 8 M NaOH.

The HF-LPME procedure used was slightly modified from a previous work reported by our group [36]. In order to remove impurities a 2.0 cm of HF was pre-rinsed sequentially with acetonitrile and acetone, sonicated for 5 min and dried under an air stream. The fibre was then inserted into the tip of a needle of a 25-μL syringe (Hamilton) that was previously filled with 20 μL 1-octanol. Afterwards, the solvent was slowly introduced into the lumen of the HF until the syringe was completely empty and the same fibre was carefully impregnated. The syringe-fibre system was quickly placed into amber glass vial containing the aqueous extract in such a way that the fibre was situated at the centre of the sample. Extraction time was performed for 60 min at room temperature with a constant magnetic agitation at 1250 rpm (Agimatic-E, JP

SELECTA S.A., Barcelona, Spain). Subsequently, the HF (connected with the syringe) was introduced/submerged in a conical glass insert (Supelco™) containing 320 μL of ACN and introduced in an ultrasonic bath (Selecta S.A., Barcelona, Spain) for 10 min (back-extraction took place). Finally, the solvent was evaporated to dryness with a gentle nitrogen stream. A small volume of 1-octanol (approx. 20 μL) was left in the glass insert. The extract was ready to be subjected to the derivatization procedure.

Derivatization procedure

Following the derivatization procedure most frequently described in the literature [41], exact volumes of each working standard solution were added to the glass conical insert and then evaporated to dryness under a gentle stream of nitrogen. The analytes were derivatized by adding 20 μL of 1-octanol, 40 μL of cyclohexane and 40 μL of silylating agent for the standards, while for the samples extracted by HF-LPME (which also contained about 20 μL of 1-octanol) only cyclohexane and the derivatizing reagent were added. The final volume (100 μL) was mixed, and the insert was introduced in amber screw top glass vial with PTFE/silicone cap (Supelco™) and heated at 70 °C for 80 min. After cooling, the TMS derivatives of the selected estrogenic compounds were ready for GC-FID or GC-MS/MS analysis.

MS quantification procedure

As dictated by Commission Decision 2002/657/EC (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32002D0657), the quantification method was based on the selection of specific ions for each studied compound: one precursor and two product ions as quantification and qualifier ion (see Table 1). Furthermore, the principle established in the SANCO Guide for analysis of pesticide residues in food [42], according to which the MS response ratio between two product fragments must satisfy a range of ±30 % of maximum tolerance as strict criteria diagnostic of their presence, was adopted. Quantification study was based on peak area of the most intense product ion of the analyte relative to that one of the internal standard (IS).

Results and discussion

The analytical methodology used for the determination of estrogenic compounds was optimized in order to obtain the best conditions (including the derivatization reaction and the HF-LPME procedure) in Milli-Q water and then applied to spiked milk/yogurt samples.

Evaluation of derivatization conditions

Although estrogens can be directly analyzed by GC [43, 44], their conversion into more volatile and thermo-stable products is usually preferred [12, 41]. For this purpose, it is very common to use *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as silylating agent [23].

It is worth mentioning that although the silylation procedure improves the properties of MS measurements with the formation of more favourable diagnostic fragmentation patterns, which allows achieving a more specific identification and better sensitivity [45], the reaction with BSTFA could yield more than one -O-TMS derivative. Indeed, the presence of multiple hydroxyl groups on the molecular structure, as it happens in this case, implies that multiple TMS compounds are synthesized and that a more complicated GC profile, also for a simple standard mixture, may occur. For this reason, the derivatizing solution (BSTFA) was acquired containing 1 % (*v*/*v*) of trimethylchlorosilane (TMCS) as a catalyst to increase the derivative efficiency for multiple hydroxyl analytes. This fact results in an easier interpretation of MS spectra of TMS-derivatives.

It is important to remark that the HF-LPME procedure used in this work [34] requires the impregnation of the fibre in 1-octanol, as it will be later shown. After extraction, the fibre is introduced/submerged in ACN for 10 min (back-extraction takes place) and evaporated to dryness with a gentle stream of nitrogen. Since 1-octanol has a low volatility, its complete evaporation is time consuming. Therefore, the derivatization procedure was tested including the presence of 20 μL of 1-octanol, since the presence of other solvents may also influence the derivatization reaction and the chromatographic profile [34, 46].

For the optimization of the derivatization reaction conditions, 20 μL of 1-octanol and 40 μL of cyclohexane were mixed. A large excess of BSTFA respect to the analytes/mixture (40 μL BSTFA with 1 % TMCS solution) was also added, while the temperature was maintained at 70 °C, as previously reported [41]. The reaction time was changed in the range 20–90 min (data not shown). Between 20 and 60 min, average peak areas ($n = 3$) increased significantly, while 80–90 min were long enough times to obtain a stable response factor, resulting in the complete derivatization reaction. Thus, 80 min was selected as the optimum derivatization time. Under the final derivatization conditions, batch-to-batch repeatability was evaluated. Peak areas RSD values of four derivatization batches were in the range 5.3–9.1 % (see ESM Table S2), which clearly demonstrates the repeatability of the derivatization procedure.

HF-LPME optimization

In one of our previous work [35], a HF-LPME procedure was applied to the extraction of HEX, DS, E_2, E_1, EE_2 and E_3 from milk samples. After a previous deproteinization step, the

procedure was applied to the extraction of a 10-mL aqueous sample. Such procedure consisted on the use of a 2 cm of a PP HF impregnated with 1-octanol, 10 mL of water at pH = 6 containing 10 % *w/v* of NaCl and 60 min of extraction at 1250 rpm at room temperature.

Despite the good performance of the method, its suitability for the analysis of 2-$MeOE_2$, α-ZAL, β-ZAL, ZEN, α-ZEL and β-ZEL, the rest of the analytes used in this work, was still unknown. Therefore, and in order to verify the suitability of such procedure, 10 mL of Milli Q water, as donor phase, was fortified at 20 μg/L and the pH and ionic strength of the aqueous phase, as well as the extraction time and the time of the back-extraction of the HF-LPME procedure were studied.

The 2-cm HF impregnated with 1-octanol was immersed in Milli-Q water at pH 2, 6 and 8 for 60 min with a constant stirring at 1250 rpm at room temperature. Figure 1a shows the results of this study. As can be observed, considering the standard deviation, the recoveries at pH 2 and 6 did not vary meaningfully while only a slight decrease of the recovery values was observed at pH 8 for α-ZAL, β-ZAL, ZEN, α-ZEL and β-ZEL. This trend can be explained considering the similar values of the experimental conditions. pH and RALs pKa increase the solubility of the analytes that are present in their anionic form in the aqueous phase with respect to the hydrophobic phase. Based on the obtained results, pH 6 was maintained for further experiments, as previously described in [35].

Maintaining the previous selected conditions (2 cm of HF impregnated with 1-octanol, 10 mL of spiked water at pH = 6 and 60 min of extraction at 1250 rpm and room temperature), the donor phase ionic strength was studied by changing the percentage of NaCl in a range of between 5 and 15 % (*w/v*). As can be seen in Fig. 1b, a slight improvement was observed for natural and synthetic estrogens by the addition of 10 % (*w/v*) of salt while the recovery values did not change significantly for the mycotoxins.

Afterwards, the extraction time varied between 30 and 80 min. An important increase of the percentage of extraction was observed in the range of 21–38 to 29–54 % at 30 and 60 min of extraction time, respectively. Increasing the extraction time up to 80 min, recovery values slightly increased were showed but affected by a considerable standard deviation, therefore not statistically relevant. Taking into account these results, 60 min was considered as a compromise between recovery and precision values and used for further experiments as also found in our previous work [35] (data not shown).

Finally, and in order to complete the optimization of the extraction procedure, the HF was submitted to the back-extraction step. Taking into account the previous results

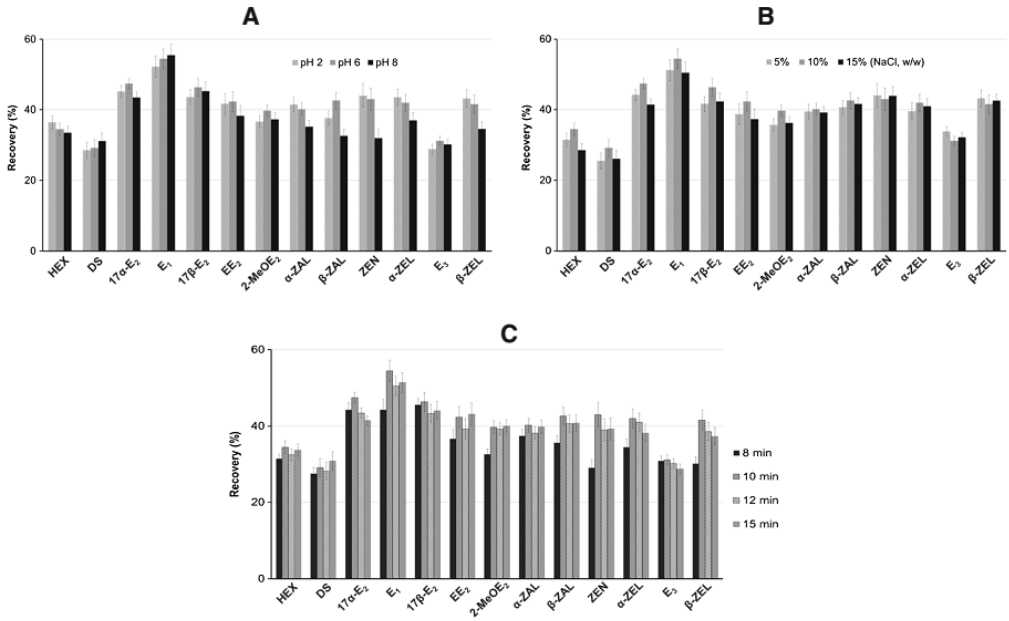

Fig. 1 Effect of (**A**) pH, (**B**) ionic strength expressed as percentage (*w/v*) NaCl of donor phase and (**C**) the back-extraction time in the HF-LPME procedure on the absolute recovery of fortified Milli-Q water sample with selected estrogens by GC-FID system. Experimental conditions: 10 μg/L of the analytes; 2.0 cm HF, 1-octanol as extraction solvent, 60 min extraction time. For other experimental conditions, see HF-LPME optimization

obtained by our group for the extraction of natural and synthetic estrogens [35, 36], ACN was selected as back-extraction solvent. In the GC micro vial, later used as reactor for the TMS-derivatization, 320 μL of ACN (the maximum volume of the vial insert) covered the HF along its entire length. This volume, despite being different from that used in the previous manuscript [36], was suitable for a satisfactory back-extraction of all the analytes. Regarding back-extraction time (assisted by sonication), it was investigated in the range of 8–15 min. The study showed that for a time up to 10 min provided the best efficiency while beyond had changes in which recovery value was not related to any trend for improvement considering the standard deviation (Fig. 1c). Therefore, 10 min was set for all real samples. Moreover, a better background signal was obtained when the fibre was previously washed with an organic solvent as fully described in the HF-LPME procedure for milk/yogurt samples section.

Mass spectra of the derivatives

Table 1 shows MS/MS acquisition parameters used to generate MS fragments as diagnostic ions of the selected TMS-O-estrogenic compounds including the internal standard. In general, the full scan MS data showed that the base peaks were the molecular ions corresponding to the molecular weight of the selected estrogens derivatized with the highest number of TMS groups. Cases apart are the derivatives of the estrogen ZAL and HEX. A slight fragmentation in the source could be the cause of the most intense peak. Among these are exceptions as the TMS-O-ZAL and TMS-O–HEX. Likely due to a EI fragmentation source, the intense base peak of TMS-O-ZAL corresponding to *m/z* 433 could be the result of consecutive of loss of methyl $[M-15]^+$ and trimethylsilanol $[M-90]^+$ [41], while *m/z* 207 of TMS-O–HEX as result of a possible molecular symmetry breaking.

All MS spectra of the derivatives yielded an ion at *m/z* 73, $[(CH_3)_3Si]^+$, which was characteristic of the TMS group.

In MS/MS experiments, the base peak of each TMS-O-estrogen was fragmented and in each fragmentation pattern MS ions with the highest relative abundance, charge–mass ratio, time stability and, in particular, those that satisfy the diagnostic criteria previously described European Commission, SANCO/12571/2011, were selected.

Similar results were obtained when fortified milk and yogurt samples were later analyzed.

Application of the HF-LPME-GC-MS/MS method for the analysis of milk and yogurt samples and validation

Once the HF-LPME procedure was studied, the complete methodology was extended to more complex matrices such as milk and yogurt samples.

In this respect, and because of the need to obtain an aqueous sample compatible with the extraction procedure, a previous deproteinization step was introduced to remove the protein fraction that strongly influences the recovery of the target analytes.

Optimization of the deproteinization

As a starting point, and on the base of our previous experience [35, 36, 40], a mixture of ACN and acetic or formic acid was tested to induce protein precipitation. Screening experiments were developed with 2 mL of sample by adding ACN in different proportions in relation to the sample (1:2 *v/v* or 1:4 *v/v* milk or yogurt/solvent) changing the type and volume of acid (acetic or formic acid, 100–400 μL). Finally, 4 mL of ACN (1:2 *v/v* sample/ACN) without acid addition offered a good protein precipitation and phase separation for milk samples.

GC-MS/MS validation of the method and analysis of real milk and yogurt samples

Under optimal separation and detection conditions, the HF-LPME-GC-MS/MS method was validated evaluating some parameters such as repeatability of the peak areas, linear calibration range and LODs and LOQs of the method. For the evaluation of the repeatability, spiked whole cow milk extracts at two concentration levels (level 1, 10 μg/L and level 2, 20 μg/L) were injected six times each ($n = 6$) in three consecutive days ($n = 18$). As reported in Table S3 in the ESM, RSD values for the intra- and inter-day precision of the peak areas were in the range 0.65–9.69 and 1.00–11.47 %, respectively.

As it is well known, the calibration of the standards in pure solvent could give biased results by suppressing or by increasing the chromatographic signal [47]. In this regard, concerning the performance of analytical methods, the European guidelines recommend the use of matrix-matched calibration (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32002D0657). This study was tackled evaluating the matrix effect percentage, estimated as the difference between the slope value of the matrix-match calibration curve and the pure solvent one [48]. Under optimal experimental conditions, calibration standard mixtures of the 13 analytes were prepared in pure solvent and in the matrix extract (whole cow milk extract). Each calibration curve comprised five concentration levels, within the range of 25–1000 μg/L, and each level was injected in triplicate.

In general, an increase in the slope of the matrix-matched calibration curve was observed with respect to the calibration in pure solvent. The relative change of the two slopes was evaluated in a range of 10–60 % with relative standard deviations lower than 15 %. Considering this, a medium/strong matrix effect was obtained, and for this reason, all experiments

Table 2 Calibration data of the selected group of estrogenic compounds in spiked real samples before the sample pretreatment HF-LPME and analyzed by GC-MS/MS. For experimental conditions, see 'Material and methods' section

Analyte	Linear dynamic range (μg/L)	Sample	Regression equation ($n = 7$)				
			y=mx+n				
			$m \pm t \cdot S_m$ (10^2)	$n \pm t \cdot S_n$ (10^2)	R^2	LOD^a method (μg/L)	LOQ^b method (μg/L)
HEX	1.25–50.00	Whole cow milk	29.74 ± 3.35	3.13 ± 4.14	0.9918	0.51	1.24
	1.25–50.00	Semi-skimmed goat milk	24.82 ± 2.81	7.69 ± 9.23	0.9937	0.32	1.17
	1.25–50.00	Natural yogurt	39.58 ± 6.16	−7.05 ± 17.08	0.9921	0.43	1.21
	1.25–50.00	Whole cow milk	3.63 ± 0.34	−2.67 ± 9.62	0.9943	0.33	1.10
DS	1.25–50.00	Semi-skimmed goat milk	2.91 ± 0.29	1.92 ± 8.15	0.9966	0.24	0.79
	1.25–50.00	Natural yogurt	6.18 ± 0.67	−6.07 ± 18.68	0.9939	0.28	0.72
	1.25–50.00	Whole cow milk	14.15 ± 1.32	−19.71 ± 37.08	0.9944	0.17	0.54
17α-E_2	1.25–50.00	Semi-skimmed goat milk	13.66 ± 1.27	−19.31 ± 35.62	0.9944	0.10	0.35
	1.25–50.00	Natural yogurt	12.25 ± 0.80	−8.72 ± 22.35	0.9978	0.18	0.66
	1.25–50.00	Whole cow milk	29.09 ± 2.21	−38.84 ± 62.12	0.9963	0.06	0.16
E_1	1.25–50.00	Semi-skimmed goat milk	28.64 ± 3.33	−21.24 ± 23.74	0.9913	0.04	0.11
	1.25–50.00	Natural yogurt	24.19 ± 1.21	−16.4 ± 33.75	0.9987	0.07	0.23
	1.25–50.00	Whole cow milk	13.02 ± 0.98	−12.75 ± 27.63	0.9963	0.16	0.52
17β-E_2	1.25–50.00	Semi-skimmed goat milk	12.65 ± 0.95	−12.74 ± 26.70	0.9964	0.17	0.60
	1.25–50.00	Natural yogurt	11.57 ± 0.74	−5.89 ± 20.61	0.9979	0.20	0.56
	1.25–50.0	Whole cow milk	2.41 ± 0.19	−5.06 ± 5.34	0.9960	0.36	1.01
EE_2	1.25–50.0	Semi-skimmed goat milk	2.89 ± 0.26	−6.56 ± 7.28	0.9948	0.26	0.78
	2.50–50.00	Natural yogurt	1.80 ± 0.18	−1.29 ± 3.51	0.9945	0.64	1.75
	1.25–50.00	Whole cow milk	9.71 ± 0.95	−20.49 ± 26.76	0.9938	0.11	0.36
2-$MeOE_2$	1.25–50.00	Semi-skimmed goat milk	10.13 ± 1.10	−17.47 ± 30.83	0.9925	0.07	0.21
	1.25–50.00	Natural yogurt	7.22 ± 0.87	−22.05 ± 24.29	0.9924	0.14	0.38
	1.25–50.00	Whole cow milk	3.62 ± 0.37	−9.26 ± 10.34	0.9933	0.37	1.24
α-ZAL	1.25–50.00	Semi-skimmed goat milk	3.67 ± 0.41	−7.36 ± 11.49	0.9920	0.29	1.00
	2.50–50.00	Natural yogurt	2.97 ± 0.34	−2.69 ± 9.44	0.9932	0.69	1.68
	2.50–50.00	Whole cow milk	3.80 ± 0.36	−10.51 ± 12.01	0.9943	0.39	1.33
b-ZAL	1.25–50.00	Semi-skimmed goat milk	3.48 ± 0.34	−7.58 ± 9.58	0.9938	0.31	1.12
	2.50–50.00	Natural yogurt	2.65 ± 0.29	−3.05 ± 8.23	0.9936	0.80	2.02
	10.00–50.00	Whole cow milk	0.12 ± 0.01	−0.27 ± 0.32	0.9946	2.55	6.11
ZEN	5.00–50.00	Semi-skimmed goat milk	0.14 ± 0.01	−0.40 ± 0.48	0.9939	1.58	4.86
	10.00–50.00	Natural yogurt	0.10 ± 0.01	−0.10 ± 0.36	0.9920	3.73	9.81
	10.00–50.00	Whole cow milk	0.24 ± 0.02	−0.48 ± 0.63	0.9945	2.16	5.43
α-ZEL	5.00–50.00	Semi-skimmed goat milk	0.26 ± 0.03	−0.69 ± 0.81	0.9920	1.70	4.64
	10.00–50.00	Natural yogurt	0.22 ± 0.02	−0.25 ± 0.48	0.9967	2.62	8.43
	1.25–50.00	Whole cow milk	2.37 ± 0.19	−3.65 ± 5.36	0.9958	0.32	0.73
E_3	1.25–50.00	Semi-skimmed goat milk	2.22 ± 0.25	−3.40 ± 6.89	0.9922	0.18	0.59
	1.25–50.00	Natural yogurt	1.78 ± 0.11	−1.91 ± 2.99	0.9981	0.38	1.07
	1.25–50.00	Whole cow milk	1.32 ± 0.11	−2.00 ± 3.12	0.9954	0.34	0.96
β-ZEL	1.25–50.00	Semi-skimmed goat milk	1.41 ± 0.14	−3.15 ± 3.93	0.9937	0.24	0.91
	2.50–50.00	Natural yogurt	1.03 ± 0.12	−1.25 ± 3.33	0.9930	0.80	2.18

m slope, *Sm* standard deviation of the slope, *n* intercept, *Sn* standard deviation of the intercept, R^2 determination coefficient.; t = (Student's t test) = 2.78, $\alpha = 0.05$

[a] Calculated as the concentration associated to a S/N ratio of 3

[b] Calculated as the concentration associated to a S/N ratio of 10

Table 3 Results of the precision and accuracy study of the HF-LPME GC-MS/MS method for the selected compounds in milk and yogurt

Peak	Analyte	Sample	Spiked level (μg/L)	Found[a] (μg/L)	Accuracy (%) (RSD)	t[b]
1	HEX	Whole cow milk	10	9.44 ± 2.51	94 (18)	1.60
			20	18.16 ± 4.04	91 (15)	0.70
		Semi-skimmed goat milk	10	10.76 ± 3.31	108 (18)	2.69
			20	21.38 ± 4.72	107 (15)	0.63
		Whole natural yogurt	10	9.39 ± 2.97	94 (14)	0.31
			20	19.23 ± 4.82	96 (15)	0.75
2	DS	Whole cow milk	10	10.67 ± 2.46	107(17)	0.82
			20	23.40 ± 3.54	117 (19)	0.98
		Semi-skimmed goat milk	10	11.36 ± 2.52	114 (16)	1.70
			20	21.73 ± 3.81	109 (14)	1.08
		Whole natural yogurt	10	8.87 ± 2.62	89 (17)	0.67
			20	17.53 ± 3.62	88 (13)	2.02
3	17α-E_2	Whole cow milk	10	10.99 ± 2.42	110 (13)	2.66
			20	21.31 ± 5.04	107 (14)	0.27
		Semi-skimmed goat milk	10	11.10 ± 3.19	111 (11)	2.11
			20	21.49 ± 4.21	107 (16)	1.43
		Whole natural yogurt	10	11.84 ± 1.71	118(12)	0.23
			20	22.85 ± 4.74	114 (18)	2.09
4	E_1	Whole cow milk	10	10.91 ± 1.97	109 (15)	1.12
			20	19.23 ± 4.12	96 (11)	0.25
		Semi-skimmed goat milk	10	11.62 ± 3.34	116 (18)	1.59
			20	22.56 ± 4.92	113 (13)	1.25
		Whole natural yogurt	10	11.23 ± 1.46	112 (11)	2.31
			20	23.11 ± 5.26	116 (15)	2.35
5	17β-E_2	Whole cow milk	10	11.50 ± 1.95	117 (13)	2.58
			20	20.78 ± 3.85	104 (12)	0.15
		Semi-skimmed goat milk	10	11.95 ± 2.14	119 (10)	2.31
			20	23.39 ± 3.84	117 (18)	1.87
		Whole natural yogurt	10	11.70 ± 1.67	117 (16)	2.42
			20	22.31 ± 6.30	112 (12)	1.16
6	EE_2	Whole cow milk	10	11.48 ± 2.03	115 (14)	2.07
			20	21.54 ± 3.57	108 (16)	0.17
		Semi-skimmed goat milk	10	9.91 ± 1.75	99 (15)	0.06
			20	19.10 ± 3.26	96 (12)	0.29
		Whole natural yogurt	10	9.26 ± 2.03	93 (16)	0.59
			20	18.23 ± 4.62	91 (12)	0.40
7	2-MeOE_2	Whole cow milk	10	8.16 ± 2.64	82 (17)	2.68
			20	18.49 ± 4.74	92 (18)	0.27
		Semi-skimmed goat milk	10	11.19 ± 2.13	112 (15)	1.40
			20	22.40 ± 4.02	112 (17)	1.40
		Whole natural yogurt	10	9.82 ± 2.83	98 (12)	1.42
			20	17.34 ± 4.41	87 (13)	2.14
8	β-ZAL	Whole cow milk	10	9.46 ± 2.52	95 (19)	0.58
			20	22.84 ± 5.42	114 (15)	0.14
		Semi-skimmed goat milk	10	8.61 ± 2.95	86 (17)	1.18
			20	16.32 ± 3.65	84(13)	1.57
		Whole natural yogurt	10	11.02 ± 2.95	110 (11)	0.88
			20	22.52 ± 3.52	113 (16)	0.76

Table 3 (continued)

Peak	Analyte	Sample	Spiked level (μg/L)	Found[a] (μg/L)	Accuracy (%) (RSD)	t[b]
9	α-ZAL	Whole cow milk	10	9.66 ± 2.47	97 (14)	0.46
			20	21.11 ± 5.25	106 (17)	0.05
		Semi-skimmed goat milk	10	10.16 ± 2.58	102 (16)	0.19
			20	19.17 ± 4.63	96(17)	0.51
		Whole natural yogurt	10	10.23 ± 2.16	102 (12)	0.22
			20	23.27 ± 4.12	116 (18)	0.89
10	ZEN	Whole cow milk	10	9.55 ± 2.43	95 (19)	0.50
			20	17.34 ± 5.05	87 (16)	0.67
		Semi-skimmed goat milk	10	10.49 ± 1.73	105 (13)	0.40
			20	20.01 ± 2.98	100 (17)	1.42
		Whole natural yogurt	10	8.06 ± 2.85	81 (16)	0.70
			20	17.21 ± 5.63	86 (14)	0.60
11	α-ZEL	Whole cow milk	10	11.39 ± 2.39	114 (13)	0.90
			20	21.80 ± 4.87	109 (14)	0.34
		Semi-skimmed goat milk	10	9.35 ± 1.34	93 (16)	2.51
			20	17.53 ± 4.52	88 (18)	0.96
		Whole natural yogurt	10	9.16 ± 2.14	92 (12)	0.82
			20	19.13 ± 5.52	96 (14)	1.98
12	E_3	Whole cow milk	10	11.45 ± 2.07	114 (12)	2.60
			20	19.27 ± 4.67	96 (17)	0.03
		Semi-skimmed goat milk	10	8.92 ± 1.66	89 (13)	0.73
			20	16.76 ± 3.74	84 (16)	1.09
		Whole natural yogurt	10	11.53 ± 1.68	115 (16)	1.38
			20	17.35 ± 5.19	87 (14)	1.25
13	β-ZEL	Whole cow milk	10	11.60 ± 2.17	116 (18)	1.29
			20	22.41 ± 4.37	112 (16)	0.12
		Semi-skimmed goat milk	10	10.55 ± 2.22	106 (19)	0.36
			20	19.89 ± 4.97	99 (14)	0.04
		Whole natural yogurt	10	11.25 ± 3.22	113 (14)	1.71
			20	18.94 ± 4.82	95 (11)	0.38

[a] Average value ± confidence interval (five determinations, 95 % confidence value)

[b] t_{tab} (Student's t test) = 2.78; $\alpha = 0.05$

comprised in the validation study were performed using the matrix-matched calibration.

Once the matrix effect was evaluated, and since the HF-LMPE extraction is a non-equilibrium method, the calibration curve of the whole method using the IS was obtained in order to quantify and then evaluate the precision and accuracy of the whole method. Therefore, calibration method curves of each estrogenic compound were established by extracting spiked samples (including the IS) at seven levels of concentration ($n = 7$) following the HF-LPME method and derivatizing the final extract. Each level was in triplicate and injected three times in the GC system. Results, which are reported in Table 2, showed a linear response of the MS signal in the tested range, with determination coefficients (R^2) higher than 0.991 in all cases. Moreover, the sensitivity of the whole method was also reported in Table 2. In this respect, LODs and LOQs values, evaluated as the concentration that provided a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10) respectively, were between 0.06–2.55 and 0.16–6.11 μg/L for whole cow milk, 0.04–1.70 and 0.11–4.86 μg/L for semi-skimmed goat milk and 0.07–3.73 and 0.23–9.81 μg/L for natural yogurt, respectively. These results are comparable to those obtained with the use of microextraction techniques by other authors for the analysis of estrogens in this type of matrices [28, 36–39].

Finally, the precision and accuracy of the method were evaluated. In this respect, each matrix was spiked with the 13 analytes at two concentration levels and the extraction was performed in five times ($n = 5$, see Table 3). The concentration calculated from the previous calibration curves (Table 2) was compared by a Student's t test, comparing the

experimental t value with the tabulated one for $n = 5$ ($t_4 = 2.78$ for $n = 5$, $p = 0.05$). As it is shown in Table 3, all experimental t values were lower than t_4, with good with accuracy percentages in the range 81–119 % and RSDs lower than 20 % in all cases.

Accordingly, precisely for being highly repeatable and giving good relative recovery values with acceptable RSDs, the method hereby optimized can be effectively employed for the analysis of these selected estrogenic compounds in the matrices selected. Figure 2 shows the extracted ion chromatogram of all analytes spiked in whole cow milk after the HF-LPME-GC-MS/MS. As can be seen, each compound (such as TMS-O-estrogenic compounds) could be perfectly identified through two selected MS-transitions (qualification and quantification) in the sample. Analogous chromatograms were obtained for semi-skimmed goat milk and whole cow natural yogurt.

In the literature, estrogenic compounds have been extracted from milk and yogurt samples in some occasions, for instance using HPLC-UV/DAD or fluorescence detection combined with HF-LPME [35, 36], vortex-assisted HF-LPME (HF-VA-LPME) [37], carbon nanotube-reinforced HF solid-phase microextraction (CNTs-HF-SPME) [38] or molecularly imprinted polymer-coated polypropylene HF tube (MIP-HFT) [39], obtaining slightly worse sensitivities than in the presented work. Recently, interesting results have been described by Xu et al. [28], who developed a GC-MS methodology based on the extraction of hormones, including only 4 natural hormones and one exoestrogen (E_1, 17α-E_2, 17β-E_2, E_3, and EE_2) in milk obtaining LODs and LOQs in the range 0.02–0.06 and 0.07–0.19 ng/mL, respectively, with recovery values in the range 93.6–104.6 %. However, such methodology implied a HF-based stirring extraction bar liquid–liquid microextraction with similarities with stir bar sorptive extraction, and thus, results are not comparable.

Moreover, regarding the analysis of RALs, such as the mycotoxin ZEN and its metabolites, ZAL and ZEL, in milk and dairy products, a number of methods have been developed employing conventional SPE and LC-MS [15–21] or GC-MS [22]. In the latter work, TMS derivatives were separated in less than 25 min achieving LOQs values of just a few ng/mL in breast milk.

Finally, and although a micro-extraction technique as DLLME has been already combined in one occasion with MEKC-MS for the analysis of estrogens and RALs [36, 40], the presented article represents the first time that HF-LPME combined with GC-MS/MS is used for this purpose.

Conclusions

In the present work, a GC-MS/MS method was applied to the separation of 13 estrogenic compounds previously transformed into volatile TMS derivative by means of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). Baseline separation of all studied compounds, including their isomeric

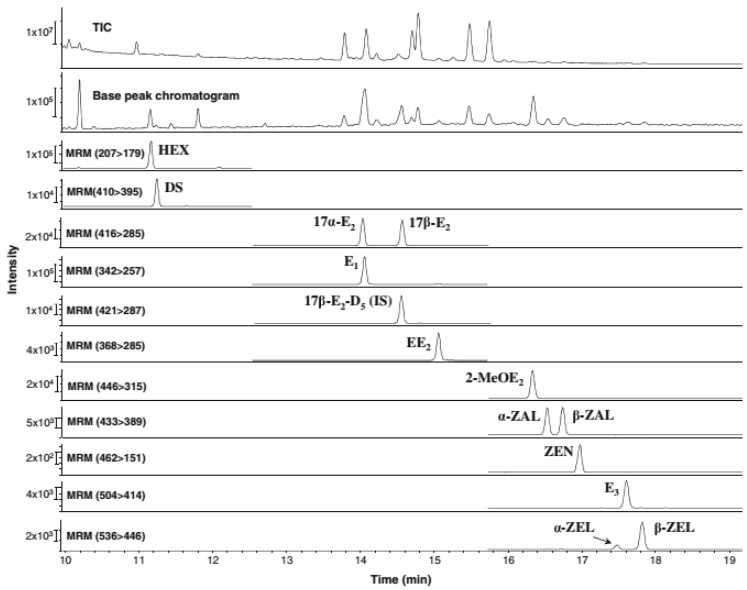

Fig. 2 TIC, base peak and MS quantitation transition of spiked whole cow milk after the HF-LPME-GC-MS/MS method. Experimental conditions: TMS-sample of 20 μg/L. For further information, see material and methods section

forms, was accomplished in less than 20 min obtaining good day-to-day precision.

After an appropriate deproteinization of cow and goat milk and natural yogurt, a HF-LPME procedure was applied to fortified samples and the methodology was validated in MRM mode as suggested by Commission Decision 2002/657/EC, using a QqQ mass analyzer.

The method was validated obtaining good results with LOQ values below 10 μg/L and an accuracy and precision in the range 81–119 % with a RDSs lower than 20 %. The proposed procedure is simple, selective, repeatable and effective, as demonstrated by the validation study carried out.

Finally, and to the best of our knowledge, this is the first time that a HF-LPME procedure is applied to the determination and quantification of α-ZAL, β-ZAL, α-ZEL, β-ZEL and ZEN. In addition, this is the first time that GC-MS/MS is combined with HF-LPME for the analysis of 13 estrogenic compounds in milk/yogurt samples.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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Nuevas Aportaciones al Análisis de Estrógenos y Derivados mediante el uso de Técnicas Analíticas Miniaturizadas

En esta Tesis Doctoral se han desarrollado metodologías analíticas de bajo impacto medioambiental que permiten llevar a cabo la extracción y determinación de estrógenos naturales, sintéticos, sus metabolitos más representativos, así como micoestrógenos en matrices de diferente naturaleza, como el agua, la leche y derivados lácteos. Para ello, se han utilizado técnicas de extracción y/o microextracción tales como la microextracción líquido-líquido dispersiva, la microextracción en fase líquida con fibra hueca, o la extracción en fase sólida con polímeros de impronta molecular. Estos procedimientos se han combinado con técnicas analíticas miniaturizadas, como la cromatografía electrocinética micelar, la nano-cromatografía líquida y la electrocromatografía capilar, así como con la cromatografía de gases, acopladas a la espectrometría de masas.

New Contributions to the Analysis of Estrogens and their Derivatives using Miniaturized Analytical Techniques

In this PhD Thesis, different analytical methodologies with low environmental impact have been developed, allowing the extraction and determination of natural and synthetic estrogens, their most representative metabolites and mycoestrogens in matrices of different nature, such as water, milk and dairy products. For this purpose, extraction and microextraction techniques such as dispersive liquid-liquid microextraction, hollow-fiber liquid-phase microextraction or solid phase extraction with molecularly imprinted polymers have been used. These procedures have been combined with miniaturized analytical techniques, such as micellar electrokinetic chromatography, nano-liquid chromatography and capillary electrochromatography, as well as gas chromatography coupled to mass spectrometry.

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