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MICROEXTRACCIÓN EN FASE LÍQUIDA USANDO MEMBRANAS LÍQUIDAS SOPORTADAS SOBRE FIBRAS HUECAS (HF-LPME, *Hollow Fiber Based Liquid Phase Microextraction*) COMO PROCEDIMIENTO DE PRECONCENTRACIÓN Y LIMPIEZA PARA LA DETERMINACIÓN DE FÁRMACOS EN MATRICES BIOLÓGICAS Y AMBIENTALES

TESIS DOCTORAL

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CERTIFICA: Que el presente trabajo de investigación titulado "**Microextracción en fase líquida usando membranas líquidas soportadas sobre fibras huecas (HF-LPME*, Hollow Fiber Based Liquid Phase Microextraction*) como procedimiento de preconcentración y limpieza para la determinación de fármacos en matrices biológicas y ambientales**", que constituye la Memoria presentada por María Dolores Ramos Payán para aspirar al grado de Doctor en Química con Mención Europea, se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad de Sevilla, bajo la dirección de los Doctores Manuel Callejón Mochón, Miguel Ángel Bello López y Rut Fernández Torres, profesores de este Departamento, reuniendo a mi juicio las condiciones exigidas para este tipo de trabajos.

Y para que conste, expido y firmo el presente certificado en Sevilla, Octubre de 2011.

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ÍNDICE

ÍNDICE GENERAL

OBJETO DE LA TESIS

El objetivo de esta Tesis Doctoral es el estudio y puesta a punto de diferentes metodologías de microextracción que implican el uso de membranas líquidas soportadas (*supported liquid membrane*, SLM) sobre fibras huecas de polipropileno, y su aplicación a la determinación de diversos principios con actividad farmacológica en matrices biológicas y ambientales.

Se analizarán principios activos pertenecientes a las familias de los antiinflamatorios no esteroideos, sulfonamidas y fluoroquinolonas; optimizándose para ello, procedimientos de microextracción en fase líquida (*hollow fiber liquid phase microextraction,* HF-LPME) en configuración de tres fases, como procedimientos basados en el uso de electromembranas (*electromembrane extraction*, EME). Para su determinación, se utilizarán técnicas analíticas de inyección en flujo (FIA) con detección luminiscente, cromatografía líquida con diversos tipos de detectores: diodos en fila, fluorescencia y de espectrometría de masas con triple cuadrupolo, así como electroforesis capilar con detección por diodos en fila.

Las metodologías desarrolladas se aplicarán a la determinación de los analitos en muestras de orina, humana y animal, y en aguas medioambientales (aguas residuales urbanas y aguas superficiales), esperándose obtener unos buenos resultados en base al excelente *clean-up* y preconcentraciones que suele proporcionar el empleo de SLM.

Se realizará, asimismo, una revisión exhaustiva de las aplicaciones analíticas que emplean membranas líquidas soportadas sobre fibras huecas publicadas hasta la fecha.

The main goal of this PhD Thesis is the development of different microextraction methodologies involving the use of supported liquid membranes (SLM) on polypropylene hollow fiber and their use for determining some pharmacologically active ingredients in biological and environmental matrices.

Several compounds belonging to the family of non steroideal anti-inflammatory drugs (NSAIDs), such as sulfonamides and fluoroquinolones will be analyzed. Accordingly, optimized methodologies involving extraction procedures based on liquid phase microextraction (hollow fiber liquid phase microextraction in three-phase configuration) as well as electromembrane extraction procedures (EME) will be optimized. Several analytical techniques will be used for determination purposes, such as flow injection analysis (FIA) with luminescence detection, liquid chromatography with different types of detectors (diode array, fluorescence and triple cuadrupole mass spectrometry) and capillary electrophoresis with diode array detection.

The optimized methodologies will be applied to the determination of active ingredients in different human and animal urine samples and in environmental water samples. Good results based on the excellent clean-up and preconcentrations are expected.

A state of the art comprehensive review on analytical applications of supported liquid membranes on hollow fibers will be given also.

CAPÍTULO 1

INTRODUCCIÓN

El proceso de "caza de brujas" iniciado en la población de Salem (Nueva Inglaterra) en 1692 tras episodios de alucinaciones e histeria generalizados entre su población, conduce a que, en mayo de ese mismo año, se produzca la ejecución de Bridget Bishop, primera de una serie de más de una veintena de muertes y de múltiples encarcelamientos y torturas.

Por encima de fuertes intrigas políticosociales y económicas y de un fanatismo religioso exacerbado, una posible intoxicación alimentaria podría haber sido el desencadenante último del tristemente conocido "proceso de Salem".

Algunos autores atribuyen esas manifestaciones a episodios de ergotismo, una enfermedad causada por la ingesta de alimentos contaminados por micotoxinas, concretamente en este caso por harina de centeno contaminada por cornezuelo (*Claviceps purpurea*), que contiene polipéptidos derivados del ácido lisérgico.

De haber sido posible, un análisis de los alimentos y de las personas infectadas, hubiera podido dejar sin argumentos a los magistrados locales que ordenaron el arresto, tortura y ejecución de muchas personas. Hoy sabemos de los efectos del ácido lisérgico y de sus derivados, siendo el más conocido su dietilamina o LSD, sintetizada por Albert Hoffman en 1938, y que por encima de sus usos médicos, ha pasado a la historia como el estandarte de una generación al servir las alucinaciones que produce como inspiración artística y dando lugar a un tipo de arte con nombre propio: el Arte Psicodélico.

Sabemos que la etapa más crítica en el proceso analítico es, con frecuencia, disponer del analito en la forma más adecuada para su análisis; es aquí donde selectividad y sensibilidad cobran protagonismo y donde un adecuado pretratamiento de la muestra puede hacer viable una determinación concreta. Muestras biológicas y ambientales presentan frecuentemente una complejidad tal que hace necesario el uso de eficaces métodos de *clean-up* y preconcentración.

La sofisticación y la capacidad de identificación y/o cuantificación de la instrumentación actual contrastan con la lentitud y laboriosidad de los procedimientos de tratamiento de muestra convencionales, por lo que en los últimos años se han producido grandes avances en procedimientos alternativos.

Las técnicas de preparación de la muestra se encuentran en continua evolución debido al elevado consumo de tiempo, muestra y reactivos, en algunos casos caros y/o tóxicos, y es la causa más frecuente de pérdida y contaminaciones. Por ello, la tendencia actual es eliminar todos o parte de estos problemas o, al menos, reducirlos, por lo que se ido incrementado el desarrollo de nuevas técnicas de preparación de la muestra o la modificación de las ya existentes con la finalidad de hacerlas más rápidas, potentes y versátiles.

Dos de las tendencias que más se han desarrollado durante los últimos años son la automatización [1,2] y la miniaturización [3,4] en la preparación de la muestra. La miniaturización se ha convertido en una tendencia dominante en la Química Analítica, ya que presenta numerosas ventajas frente a la preparación de la muestra tradicional, y al minimizar la cantidad de muestra, se disminuye el consumo de reactivos y de disolventes caros y tóxicos, lo que está más acorde con las tendencias actuales hacia una "Química Verde".

Se ha producido el desarrollo a microescala de técnicas de separación como la microextracción en fase sólida (SPME) [5], la microextracción en fase líquida (LPME) [6], la electroforesis capilar (EC) [7] así como de las técnicas cromatográficas [8]. En los últimos años también se ha comenzado a miniaturizar los sistemas de detección, por ejemplo, espectrómetros de masas [9] y plasmas [10,11], entre otros.

1. TÉCNICAS DE EXTRACCIÓN

Sin tener en cuenta la lixiviación, la extracción líquido-líquido (*liquid-liquid extraction*, LLE) es la técnica convencional más antigua de extracción con disolventes [12]. Para muestras acuosas, la LLE, es probablemente, la técnica de preparación de muestras más aceptada, utilizada y versátil, y ha sido empleada en numerosos métodos de análisis [13,14]. Sin embargo, la LLE convencional consume grandes cantidades de disolventes orgánicos de alto costo y potencialmente peligrosos. Se han desarrollado técnicas de extracción miniaturizadas evitando, de este modo, algunos de esos inconvenientes. La miniaturización se ha llevado a cabo, fundamentalmente, de dos modos: reducción de las dimensiones de la técnica tradicional (extracción en vial) y, a través del desarrollo de nuevas modalidades de extracción (microextración en fase líquida (LPME)). Estas técnicas miniaturizadas han adquirido una enorme popularidad debido a que los volúmenes de disolvente orgánico empleados son muy pequeños y la manipulación de la muestra es mínima.

A finales de los 70, se introdujo un procedimiento de preparación de muestras conocido como extracción en fase sólida (*solid phase extraction*, SPE), que en esencia, se podría considerar como una modalidad cromatográfica en modo discontinuo. La técnica de extracción en fase sólida ha sido ampliamente utilizada para la preparación de la muestra [12,16] hasta nuestros días.

La SPE posee un gran número de ventajas comparada con otras técnicas de extracción desarrolladas anteriormente, como son una instrumentación económica, sencilla, con un consumo de disolvente relativamente bajo, de fácil automatización y sin formación de emulsiones. Han sido empleadas diferentes tipos de fases estacionarias, siendo las utilizadas con mayor frecuencia aquellas que contienen fases apolares: octilsilano (C8) y octadecilsilano (C18). También han sido empleadas fases estacionarias del tipo sílice enlazada, así como resinas modificadas, y polímeros, entre otras.

Uno de los principales inconvenientes de la SPE reside en que un componente de la matriz impida o dificulte la interacción entre el analito y el sorbente, lo que con frecuencia proporciona una baja recuperación de los analitos de interés. También puede

ocurrir que algunos componentes oleosos y/o sólidos de la muestra obstruyan los canales del cartucho o del disco impidiendo la retención de los analitos. Sin embargo, la SPE se ha aplicado exhaustivamente en análisis medioambiental, biomédico, toxicológico, bromatológico, eliminación de interferencias y preconcentración de analitos.

Arthur y Pawlisyzn [17] introdujeron en 1990 un nuevo procedimiento de pretratamiento de la muestra conocido como microextracción en fase sólida (*solid phase microextraction*, SPME). Esta técnica, surge como alternativa para reducir algunas de las limitaciones de la extracción en fase sólida, presentando un avance significativo en el ámbito de la preparación de la muestra. Una fibra de sílice fundida y revestida con material adsorbente hace que se concentren y extraigan analitos de amplio rango de polaridad. La geometría de la SPME permite la colocación de la fibra recubierta directamente en la muestra (matrices gaseosas o acuosas), en el espacio de cabeza situado sobre la muestra para extraer los analitos volátiles o se recubre con una membrana de protección cuando se coloca directamente en muestras acuosas con matrices sucias. El gran número de recubrimientos de las fibras disponibles hoy día ha contribuido a ampliar el tipo de analitos que pueden ser separados con esta técnica. Algunas de las fibras disponibles incluyen la apolar polidimetilsixolano (PDMS), las semipolares polidimetilsiloxanodivinilbenceno (PDMS-DVB), carboxen-polidimetilsiloxano (carboxen-PDMS) y divinilbenceno-carboxen-polidimetilsiloxano (DVB-carboxen-PDMS) y las polares poliacrilato (PA), carbowax-divinilbenceno (CW-DVB) y carbowaxresina templada (CW-TPR) [12,16]. Recientemente han sido desarrolladas nuevas fases absorbentes para recubrir las fibras de SPME, por ejemplo, los polímeros de impresión molecular [18,19,20], nanotubos de carbono [21,22] o líquidos iónicos [23,24].

Entre las ventajas de la SPME, destaca su sencillez de uso, fácil automatización, bajo consumo de materiales y mayor versatilidad en el tipo de muestras. Además, ofrece una rápida transferencia de masa durante la extracción y la desorción, previene las obstrucciones que se pueden producir en la SPE, reduce el consumo de disolventes, los tiempos de preconcentración largos y facilita el manejo e introducción directa en los instrumentos analíticos de medida. Las principales desventajas que presenta esta técnica son la fragilidad de la fibra, pues puede ser dañada fácilmente disminuyendo su tiempo

de vida, la cantidad reducida de fase sorbente, su elevado coste y los posibles efectos memoria. Las aplicaciones descritas son semejantes a la SPE, además de permitir su uso en muestras sólidas, líquidas y gaseosas.

Simultáneamente se han ido desarrollando procedimientos alternativos de extracción, entre los que destaca el uso de membranas líquidas soportadas (*supported liquid membrane*, SLM). En general, todas las técnicas que comparten estas características de membranas liquidas se suelen agrupar bajo la denominación de LPME (*liquid phase microextraction*, LPME), una alternativa a la SPE. Estas técnicas miniaturizadas han adquirido una enorme popularidad debido a que los volúmenes de disolvente orgánico empleados son muy pequeños y la manipulación de la muestra es mínima.

El funcionamiento general de una membrana líquida soportada, consiste inmovilizar en el interior de los poros de un soporte microporoso la fase orgánica o extractante, y por el exterior de esta membrana se hacen circular la fase acuosa. El principio de funcionamiento de esta técnica de extracción puede observarse en la Figura 1. La muestra se encuentra en contacto con un lado de la membrana y la membrana actúa como una barrera selectiva. Los analitos pasan desde la fase donadora a través de la membrana hasta la fase aceptora.

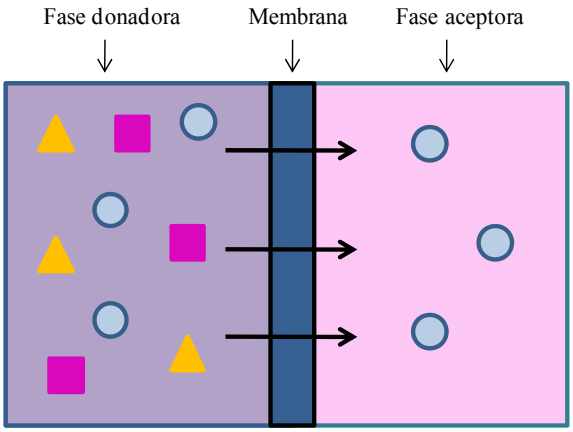


Figura 1. Principio de funcionamiento de separación mediante membrana líquida.

La técnica de microextracción en gota (*single drop microextraction*, SDME) se puede considerar como la primera modalidad descrita de LPME. En esencia consiste en el empleo de una gota de disolvente como fase extractante [25-32]. Jeannot y Cantwell [26] introdujeron una modalidad de microextracción en gota en la que una microgota se encuentra suspendida en la punta de una aguja [27]. Más adelante, He y Lee [28] introducen por primera vez el término estático y dinámico aplicado a la LPME en 1997, derivando recientemente hacia la automatización de la modalidades dinámicas, incluso con la introducción de la microextracción en flujo continuo. No obstante, la SDME ha sufrido recientemente varias modificaciones con objeto de eliminar los problemas de estabilidad de la gota suspendida cuando se agita vigorosamente la muestra, que se recogen bajo el término de microextraccion en gota directamente suspendida (DSDME) [33]. Se han introducido nuevos dispositivos que ayudan a la formación de la gota [34-36], algunos sistemas han sido automatizados [37-39] y se ha empezado a utilizar nuevas fases extractantes como microgotas acuosas [40] y líquidos iónicos [36,41].

Al objeto de solucionar algunos de los inconvenientes de las técnicas anteriores, Audunsson [42] introdujo en 1986 un concepto alternativo de LPME mejorado por Thordarson [43] y desarrollado posteriormente por Pedersen-Bjergaard y Rasmussen en 1999 [44]. Esta nueva técnica, denominada microextracción en fibra hueca (*hollow fiber liquid phase microextraction*, HF-LMPE) está basada en el uso de fibras huecas como como soporte de fases orgánicas inmiscibles con una fase acuosa que contiene el analito.

En HF-LPME los poros de una fibra hueca se impregnan con un disolvente, normalmente orgánico, y existen dos modalidades esenciales: en dos y tres fases, cuya diferencia reside en la existencia de una o dos interfases entre líquidos diferentes. En el sistema de dos fases el extracto final que se obtiene es una fase orgánica compatible con técnicas analíticas tales como cromatografía de gases o cromatografía líquida. En el sistema de tres fases, el analito es extraído desde una fase acuosa (fase donadora) a través del disolvente orgánico inmovilizado en los poros de la fibra hueca (fase orgánica) hasta otra fase acuosa (fase aceptora) que se encuentra en el interior de la fibra. La fase orgánica en este último caso actúa como una barrera entre las disoluciones acuosas dadora y aceptora, impidiendo la mezcla de ambas fases. Esta modalidad suele asociarse con las técnicas de cromatografía líquida o electroforesis capilar. Mediante

este sistema, las muestras pueden ser agitadas fuertemente sin pérdida de la fase orgánica durante la microextracción. Por lo tanto, se considera la HF-LPME como una técnica fiable y robusta que puede proporcionar elevados enriquecimientos, por tanto, bajos límites de detección, requiere un equipamiento sencillo y de bajo costo, y permite obtener muestras muy "limpias".

Con el fin de favorecer y optimizar la extracción de una amplia gama de compuestos hidrofílicos y con diferentes propiedades y funcionalidades, se ha desarrollado una nueva modalidad de HF-LPME añadiendo ciertas sustancias, bien a la disolución acuosa que contiene el analito, o al disolvente inmovilizado en los poros de la fibra, facilitando así el paso del analito a través de la membrana líquida. Estas sustancias adicionadas reciben el nombre de transportadores o "*carriers*" [45,46]. En general, se trabaja con dos modalidades diferentes, y en ambos casos el resultado es la formación de una molécula neutra que facilita el paso del analito inicialmente ionizado, desde la fase donadora hacia la fase aceptora.

Más recientemente, en 2006, Pedersen-Bjergaard y Rasmussen, proponen por primera vez, el uso de campos eléctricos para favorecer la extracción del analito a través de la membrana líquida soportada (SLM) [47]. Este sistema se denomina extracción mediante electromembranas (*electromembrane microextraction*, EME) en el que los analitos son extraídos desde una solución acuosa (donadora) a una solución acuosa aceptora (lumen de la fibra hueca) a través de un disolvente orgánico inmovilizado en la pared de una fibra hueca de polipropileno porosa, con la ayuda de un campo eléctrico producida al aplicar una diferencia de potencial entre electrodos inmersos en las fases donadora y aceptora.

El conocimiento profundo de esta técnica aún es muy limitado, pero ya se ha investigado sobre los factores que influyen en la extracción, así como se ha propuesto un modelo teórico para describir el proceso de extracción. Como se puede apreciar, la LMPE ha experimentado un rápido desarrollo debido a la mejora que proporciona en el enriquecimiento de los analitos, la fácil manipulación, la rapidez, su bajo coste, su carácter ecológico y su amplio campo de aplicación. Además, el hecho de proporcionar un extracto listo para su análisis instrumental ha sido sin duda, clave para su rápido desarrollo.

2. MICROEXTRACCIÓN EN FIBRA HUECA

2.1 Modalidades de trabajo

Dependiendo de la disposición de la fibra hueca, es posible trabajar adoptando diferentes configuraciones.

a) La primera configuración utiliza una configuración en U (cuyos entremos de la fibra puede sujetarse o no, con dos agujas de jeringa convencionales (Figura 2). En el caso de no emplear agujas, los extremos de la fibra son sellados una vez introducida la fase aceptora.

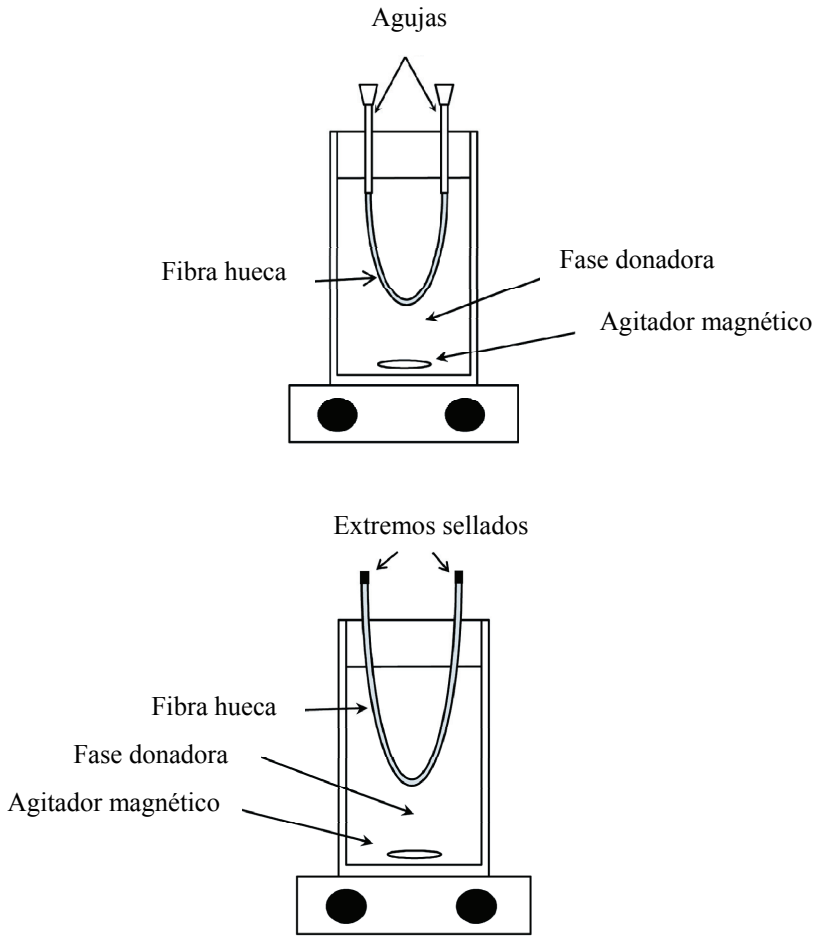


Figura 2. HF-LPME en una configuración en U empleando dos agujas o sellada en los extremos.

b) En la segunda configuración únicamente un extremo de la fibra es utilizado para la inyección y recolección de la fase aceptora mientras que el otro extremo queda expuesto a la disolución donadora (Figura 3). Este extremo es sellado previamente a la inmersión de la fibra en el disolvente orgánico y se procede a la introducción de la fase aceptora como última etapa. En este caso, la jeringa soporta la fibra durante la extracción.

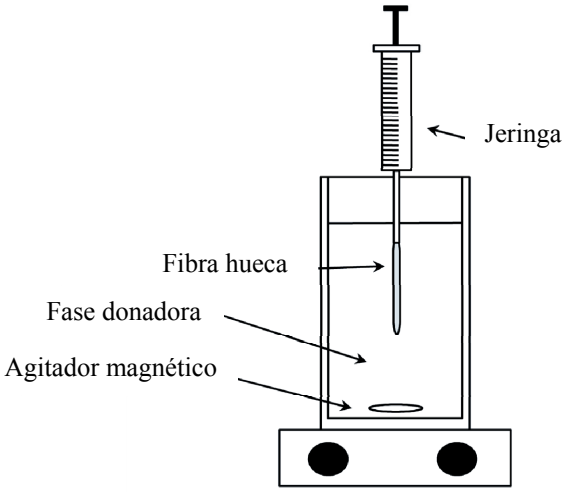


Figura 3. HF-LPME empleando una sola jeringa.

c) Otra modalidad de HF-LPME es la que se conoce como *solvent bar microextraction* (SBME) [48]. A diferencia de las otras modalidades, ambos extremos de la fibra son finalmente sellados (uno de ellos previo a la inmersión de la fibra en el disolvente seleccionado y el otro una vez que todos los poros queden impregnados). Finalmente, la barra se introduce en la muestra (Figura 4), y una vez concluida la extracción, uno de los extremos es abierto para recoger la fase aceptora.

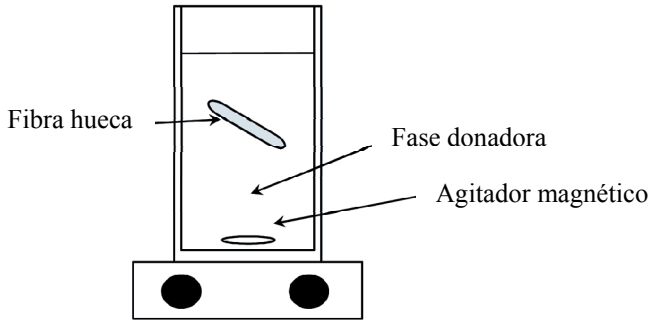


Figura 4. HF-LPME en modalidad *solvent bar microextraction*.

2.2 Configuración en dos fases

En la configuración de dos fases los analitos se extraen desde una disolución acuosa (donadora) hacia una fase orgánica (fase aceptora) a través de un disolvente inmiscible que se encuentra inmovilizado en los poros de una fibra hueca (Figura 5).

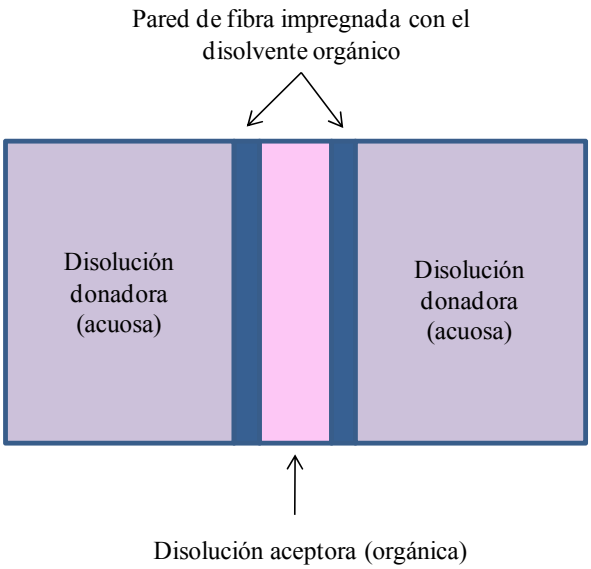


Figura 5. Esquema de modalidad en dos fases.

La eficacia de la extracción depende fundamentalmente del pH y del tipo de disolvente orgánico con el que se impregna la fibra. Para que se lleve a cabo la extracción, el analito ha de encontrarse en forma neutra en la fase acuosa (donadora). La fase orgánica aceptora debe ser compatible con la técnica analítica a emplear, y caso de no serlo se debe realizar una evaporación previa y una reconstitución en el medio adecuado.

La elección del disolvente orgánico más adecuado es uno de los parámetros más importantes cuando se trabaja en una configuración de dos fases, ya que el disolvente orgánico actúa de disolvente extractante y como fase aceptora. Dependiendo del tipo de compuestos que se pretenda extraer, se prueban varios disolventes inmiscibles en agua de diferente polaridad. La elección final del disolvente debe basarse en la comparación de la selectividad, eficiencia de la extracción, capacidad de retención en los poros de la fibra y nivel de toxicidad.

2.2.1 Características cinéticas y modelos matemáticos

El modelo matemático que explica y profundiza en las características cinéticas del proceso se fundamenta sobre la base de que los analitos son extraídos desde la fase acuosa (fase donadora) hasta el interior de la fase orgánica (fase aceptora) mediante un mecanismo de difusión pasiva [49,50].

El proceso de microextracción se basa en las diferencias de concentración de los analitos entre las dos fases líquidas inmiscibles (acuosa y orgánica). La transferencia de masa del analito desde la fase acuosa (fase donadora) a la fase orgánica (fase aceptora) continúa hasta que se alcanza el equilibrio termodinámico o se interrumpe la extracción.

$$A_d \rightleftharpoons A_a$$

El modelo establece un balance de masas dinámico para el analito entre las diferentes fases. De acuerdo a la expresión,

$$C_d \cdot V_d + C_a \cdot V_a = C_d^0 \cdot V_d \qquad (1)$$

donde,

C_d y C_a son las concentraciones del analito en la fase donadora (acuosa) y en la fase aceptora (orgánica) en un instante dado, respectivamente,

V_d y V_a son el volumen de la fase donadora (acuosa) y de la fase aceptora (orgánica), respectivamente,

C_d^0 es la concentración inicial del analito en la fase donadora.

En condiciones de equilibrio, el coeficiente de partición del analito se define como:

$$K = \frac{C_{eq,a}}{C_{eq,d}} \qquad (2)$$

donde,

$C_{eq,a}$, es la concentración del analito en la fase aceptora en el equilibrio,

$C_{eq,d}$ es la concentración del analito en la fase donadora en el equilibrio.

La concentración del analito en la fase aceptora (orgánica) en las condiciones de equilibrio, viene dada por:

$$C_{eq,a} = KC_{eq,d} = \frac{KC_d^0}{1+K(V_a/V_d)} \quad (3)$$

El balance de masas dinámico del analito en la extracción por fibra puede expresarse en función del tiempo de extracción:

$$\frac{d(C_a V_a)}{dt} = \beta_a A_i [KC_d - C_a] \quad (4)$$

donde,

A_i es el área interfacial,

K es el coeficiente de partición en el equilibrio,

β_a es el coeficiente de transferencia de masa total del analito con respecto a la fase orgánica.

Si se considera la transferencia de masa a través de la interfase líquido-líquido, el coeficiente de transferencia de masa total viene dado por la siguiente expresión:

$$\frac{1}{\beta_a} = \frac{1}{k_a} + \frac{K}{k_d} \quad (5)$$

donde,

k_a y k_d son los coeficientes de transferencia de masa para el analito en la fase orgánica (fase aceptora) y fase acuosa (fase donadora),

β_a es el coeficiente de transferencia de masa total con respecto a la fase extractante.

Si se asume que el volumen de la fase aceptora (fase orgánica), V_a, permanece constante, A_i también será constante. Combinando las ecuaciones (1) y (4) se puede expresar C_a en función del tiempo, de forma que:

$$C_a(t) = C_{eq,a}(1 - e^{-kt}) \quad (6)$$

donde,

$C_{eq,a}$ es la concentración del analito en la fase aceptora en el equilibrio y,

k es la constante de velocidad observada (s^{-1}).

Ambas variables vienen definidas según las expresiones:

$$C_{eq,a} = \frac{KC_d^0 V_a}{V_d + KV_a} \quad (7)$$

$$k = \frac{\beta_a A_i}{V_a}\left(1 + \frac{KV_a}{V_d}\right) \quad (8)$$

Jeannot y Cantwell verificaron experimentalmente la validez de la ecuación (6) que representa la fracción de analito extraída en un instante dado de la reacción [26,27]. Las ecuaciones (5) y (8) muestran los parámetros experimentales que influyen en el tiempo requerido para que la extracción se complete. Por otro lado, el modelo descrito sugiere que maximizando β_a y A_i combinado con la minimización de V_d y V_a se obtienen mayores velocidades de extracción y, por lo tanto, menor es el tiempo de extracción. La máxima área superficial se puede conseguir aumentando la longitud de la fibra, mientras que el máximo de β_a se puede alcanzar aumentando el valor de k_d , ya que k_a es, a menudo, más grande que k_d y, por lo tanto, la resistencia a la transferencia de masa en la fase acuosa controla la velocidad total de transferencia.

De acuerdo a la teoría de la doble película desarrollada por Withman, el coeficiente de transferencia de masa bien en la muestra (fase donadora) o en la fase extractante (fase aceptora), está relacionada con el coeficiente de difusión del analito "D" y con el espesor de la película inmovilizada en los poros de la fibra correspondiente, "δ", también conocida como la película de difusión de Nernst. La relación entre ellas presenta la forma:

$$\beta = \frac{D}{\delta} \quad (9)$$

Esta ecuación sugiere que se puede conseguir un aumento en β reduciendo δ, y esto último se puede alcanzar aumentando la velocidad de agitación en el recipiente que contiene la muestra.

2.2.2 Aplicaciones

Existen en la bibliografía numerosas aplicaciones que hacen uso de la microextracción en fase líquida mediante fibra hueca en configuración de dos fases.

El disolvente orgánico más adecuado cuando se trabaja en una configuración de dos fases suele ser de naturaleza apolar, como tolueno o decano (Tabla 1-4). Además, se emplean pequeños volúmenes de fase extractante (generalmente entre 1-50µl), que junto con la buena volatilidad de estos disolventes, da lugar a extractos compatibles para ser inyectados directamente en un cromatógrafo de gases, siendo ésta la técnica de análisis más habitual cuando se trabaja en esta configuración de dos fases.

La mayoría de los análisis se llevan a cabo mediante cromatografía de gases sin derivatización, aunque cuando esta etapa es necesaria puede llevarse a cabo previamente a la extracción [51,52], posteriormente a la extracción [53,54] o simultáneamente durante la extracción, adicionando el agente derivatizador a la fase donadora [55-59].

El tipo de fibra que se emplea es generalmente una fibra de polipropileno tipo Q3/2 (0.2µm de tamaño de poro, 200µm de espesor y 600µm de diámetro interno). Sin embargo, algunos autores, han empleado otras fibras de polipropileno del tipo (0.2µm de tamaño de poro, 200µm de espesor y 1200µm de diámetro interno) [60,61], o fibras de tipo 150/330 (0.2µm de tamaño de poro, 150µm de espesor y 300µm de diámetro interno) [62-64]. De manera excepcional y poco usual se ha empleado el difluoro polivinilideno como alternativa al polipropileno [65,66].

La microextracción mediante fibra hueca en dos fases funciona muy bien en la extracción de sustancias apolares, por lo que la técnica se ha aplicado para la determinación de diferentes compuestos orgánicos, como hidrocarburos aromáticos, incluidos hidrocarburos aromáticos policíclicos (PAHs), fenoles, etc. (Tabla 1). Además, en los últimos años, ha sido ampliamente aplicada a la determinación de pesticidas. Como se puede observar en la Tabla 2, esta técnica se ha aplicado principalmente a los carbamatos, organoclorados (OCP), organofosforados (OPP) y organosulfurados (OSP), plaguicidas, bifenilos policlorados (PCB) y herbicidas

triazinas, entre otros. Algunos autores han propuesto métodos que pueden aplicarse a la extracción y determinación de plaguicidas de diferentes familias [67-73].

En la tabla 3, se observa que ésta técnica se ha aplicado también a la determinación de productos con actividad farmacológica y otras sustancias de interés clínico (como la nicotina o drogas de abuso) en fluidos biológicos, productos farmacéuticos y muestras ambientales. Se han empleado también, para la determinación de sustancias empleadas en guerra química y sus productos de degradación como agente nervioso VX, gas mostaza o ácidos fosfónicos [74-77].

En análisis de alimentos, esta técnica se ha aplicado a la determinación de los colorantes Sudán I-IV [78], de componentes activos de envasado de alimentos [63,64], de conservantes y antioxidantes [79], de caproato de etilo en bebidas alcohólicas [80] y micotoxinas [81]. Por último, la técnica se ha aplicado a la determinación de compuestos organometálicos [82,52].

Recientemente se ha descrito, el uso de líquidos iónicos como fase aceptora a fin de hacer la técnica directamente compatible con HPLC [36], no obstante, no parece que hasta el momento se hayan obtenido resultados muy satisfactorios debido, esencialmente, a la relativamente alta solubilidad de los líquidos iónicos en agua.

Tabla 1. Aplicaciones de HF-LPME en dos fases para la determinación de compuestos orgánicos.

Analito/Matriz	SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (µl)	E_f	LOD (µg L^{-1})	Técnica analítica
Hidrocarburos aromáticos								
Plasma y orina humana [172]	Tolueno	2	700	n.d.	n.d.	n.d.	n.d.	GC-MS
Agua de grifo y nieve [173]	di-butil ftalato/Octanol	n.d.	n.d.	n.d.	n.d.	112 – 260	0.06 – 0.22	
Agua de río y residual [174]	Octanol[d]	25	800	5	3	41.47 – 128.01	0.005 – 0.03[c]	GC-FID
Benzofenonas								
Orina humana [58]	Tolueno[d]	20	104[i]	(Water/HCl/NaCl)	2	130-251	0.01 - 0.05	GC–MS
Orina humana [175]	Tolueno[d]	15	500	1.1 (pH 6.8)	n.d.	n.d.	5 -10[a]	GC-MS
Clorofenoles								
Orina humana [57]	Tolueno[d]	15	500	1	3	n.d.	0.1 – 0.2	GC-MS
Fango [176]	Octanol[d]	10	700	10	3	70 - 110	0.028 – 0.36[b]	GC-ECD
Orina humana [177]	Octanol						5 - 20	
Agua [178]	Octanol[d]	300	1200	500 (1 mM tampón fosfato)	10	n.d.	n.d.	HPLC-UV
Agua de grifo y superficiales [56]	Dodecano[d]	20	n.d.	11 (NaOH 0.1 M)	5	n.d.	0.02	GC-MS
PAHs								
Agua de lluvia [156]	Tolueno[d]	35	700	5 (pH 9, 30% NaCl)	5	47 - 167	0.002 – 0.059	GC-MS
Agua residual y de río [179]	Tolueno[d]	15	1000	5 (2.5% NaCl)	3	n.d.	0.005 – 0.011	GC-MS
Suelo [180]	Octanol[d]	8	800	22 (Acetona/Agua (7:15))	8	80.1 – 170.7	0.13 – 0.22	GC-FID
Agua [61]	Octanol[e]	45	750	10	20	123 - 176	n.d.	GC-MS
Agujas de pino [181]	Tolueno[d]	20	1250	5 (agua/acetona (22:7), 5% NaCl)	3	n.d.	0.01 – 0.95[b]	GC-MS
Agua [182]	Octanol[d]	10	1000	3	3	42 - 75	n.d.	GC-MS

Fenoles									
Aguas costeras [53]		Tolueno[d]	30	697	5 (pH 2, 30% NaCl)	5	84 - 162	0.005 – 0.015	GC-MS
Orina humana [59]		Tolueno[d]	15	500	1	n.d.	n.d.	0.02	GC-MS
Agua [178]		Octanol[d]	300	1200	500 (1 mM tampón fosfato)	10	n.d.	n.d.	HPLC-UV
Ésteres de ftalatos									
Agua de grifo y mineral [183]		Tolueno[d]	20	1000	5	3	n.d.	0.005 – 0.1	GC-MS
Agua de rio [184]		Tolueno	n.d.	600	n.d.	n.d.	19.8 - 221	0.07 – 0.81	GC
Trihalometanos									
Agua de lago [185]		Octanol[d,f]	60	n.d.	15	160 , 50	21.6 – 34.8	0.03 – 0.05	GC ECD
Agua de grifo y mineral [186]		Octanol[d]	30	n.d.	10	40	28 - 62	0.01 – 0.2	GC-ECD
Otros									
Antraquinonas	[66]	Octanol[g]	60	800	(2 mM HCl, 50% metanol)	n.d.	21 - 47	0.20 – 0.35	HPLC-UV
Cloruro de bencilo	Agua contaminada [187]	Metilbenceno	15			3		0.5	GC-MS
Parabenos	Orina [188]	Clorobenceno	40	n.d.	(40% NaCl)	n.d.	21 - 154	0.01 – 0.20[c]	n.d.
Petroleo	Aceites [189]	Hexano[g]	30	700	n.d.	4	n.d.	n.d.	GC–MS, GC–IRMS
PBDEs	Suero humano [190]	Decano[d]	20	1000	(Agua/Metanol (70:30))	4	30 - 83	15.2 – 40.5[a]	GC-ICP-MS
Aminas primarias	Agua de río [55]	Tolueno[d]	30	600	20 (pH 10.5)	3	172 - 244	0.29 – 0.44	GC-MS
Haloéteres	Agua de lago [191]	Tolueno[d]	30	450		4	103 - 335	0.11 – 4.28	GC-FID, GC-ECD
Explosivos nitroaromáticos	Agua de río y superficial [192]	Tolueno[d]	20	1000	5	3	n.d.	0.29 – 0.87	GC-MS
Triésteres organofosforados	Plasma sanguíneo humano [62]	Hexano/MTBE[h]	7	n.d.	0.1 (50% ácido fórmico)	n.d.	n.d.	0.2 – 36	GC-NPD, GC-MS

[a](ng L^{-1}), [b](ng g^{-1}), [c](mg L^{-1}), [d](PP Q3/2) (i.d. 600 μm, espesor 200 μm, tamaño de poro 0.2 μm), [e](KM (i.d. 1200 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [f](PP S6/2 (i.d. 1800 μm, espesor 150 μm)), [g](PVDF (i.d. 500 μm, espesor 150 μm, tamaño de poro 0.2 μm)), [h](PP 150/330 (i.d. 330 μm, espesor 150 μm), MTBE (metil-t-butiléter)

Tabla 2. Aplicaciones de HF-LPME en dos fases para la determinación de pesticidas.

Analito/Matriz	SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (µl)	E_f	LOD (µg L^{-1})	Técnica Analítica
Pesticidas organofosforados								
Agua [153]	1-Octanol/decano[d] 40:60	30	80	n.d.	50	294 - 873	0.0010 – 5.5	HPLC-UV
Vino tinto [60]	1-Octanol[e]	90	750	4	20	n.d.	n.d.	GC-MS
Agua de pozo [154]	Tolueno	20	720	4.5	n.d.	>45	1 – 5	HPLC-UV
OCPs								
Agua de grifo, pozo y mar [155]	Tolueno[d]	30	900	10 (pH 2)	5	63 - 139	0.013 – 0.059	GC-MS
Agua de lluvia [156]	Tolueno[d]	35	700	5 (pH 9, 30% NaCl)	5	47 - 167	0.002 – 0.059	GC-MS
Agua de río, tomate y fresa [157]	1-Octanol[d]	60	n.d.	15 (NaCl, pH 2 y 4)	20	n.d.	2.7 – 20[a]	GC-ECD
Vino [158]	*n*-Tetradecano[d]	30	600	20	2	990	0.3-249.2[a]	GC-MS/MS
Hojas y té instantaneo [159]	1-Octanol[d]	40	800	5	3	34 - 297	0.031 – 0.164	GC-ECD
Agua potable [160]	Tolueno[d]	15	480	20 (pH 6.5)	3		n.d.	GC-MS
OPPs								
Agua residual [161]	Tolueno:hexano[d] (1:1)	35	700	10	5	73 - 204	0.3 – 11.4[a]	GC-MS
Agua de lago y subterranea [162]	Undecano[d]	4320		1125	9.4 - 11	n.d.	15 – 80[a]	GC-MS
Agua de lago [163]	Ciclohexano[d]	50	700	20 (15% NaCl)	3.5	n.d.	0.006 – 0.2	GC-MS
Muestras vegetales [164]	Dodecano[d]	30	1360	acetona	3	n.d.	0.099 – 0.128	GC-ECD

OSPs								
Agua, suelo, bebidas [165]	*o*-xileno[d]	35	1200	5	5	27 - 530	1.16 – 48.48	GC-FPD
PCBs								
Plasma humano [166]	Tolueno[d]	30	700	5 (pH 10.5, 20% NaCl)	3	67 - 241	0.05 – 0.94	GC-MS
Agua [167]	Nonano[d]	60	500	200 (pH 7.4)	10	n.d.	n.d.	GC-ECD
Agua de río [168]	Tolueno[f]	15	500	10	3	718 - 840	13 – 41	GC-MS
Herbicidas de triazina								
Agua superficial [169]	Octanod[d]	180	115	250	12	n.d.	0.1 – 1	HPLC-UV
Fango [170]	Tolueno[d]	20	1000	3 (10% NaCl)	3	42 - 208	0.007 – 0.063	GC-MS
Combinación de péptidos								
Sedimentos marinos [70]	Tolueno[d]	20	700	10	5	n.d.	0.1 – 0.7[b]	GC-MS
Vino, cerveza [73]	1-Octanol[d]	45	90	15			0.01 – 5.61[c]	UHPLC MS/MS
Agua de estanque, fango [68]	Tolueno[d]		700 - 1000	4	3	30 - 490	0.01 – 5.1	GC-MS
Agua potable y de río [71]	Tolueno[d]	20	800	5 (pH 5.5, 5% NaCl)	3	n.d.	1 – 74[a]	GC-FTD
Agua [72]	Tolueno[d]	15	500	10	3	140 - 390	0.1 – 1.3	GC-MS
Agua de granja [69]	Tolueno[d]	20	870	3 (pH 4)	3	135 - 213	0.004 – 0.025	GC-ECD
Aguas medioambientales [171]	Tolueno[d]	20	850	4	4	314-329	3 – 7[a]	GC-ECD

[a](ng L^{-1}), [b](ng g^{-1}), [c](mg L^{-1}), [d](PP Q3/2) (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm), [e](KM (i.d. 1200 µm, espesor 200 µm, tamaño de poro 0.2 µm)), [f](PP (i.d. 550 µm, espesor 50 µm, tamaño de poro 0.2 µm))

Tabla 3. Aplicaciones de HF-LPME en dos fases en análisis clínico y farmacéutico.

Analito/Matriz		**SLM**	**t_{ext} (min)**	**v_{ag} (rpm)**	**V_{Fd} (mL)**	**V_{Fa} (µl)**	**E_f**	**LOD (µg L^{-1})**	**Técnica analítica**
Flunitrazepam [65]	a) Orina humana	a) p-xileno[e]	a)30	a)450	a)4.0 (pH 9.5)	20	a)167	a) 0.001	GC-MS/MS
	b) Plasma humano	b) p-xileno/Octanol[e]	b)45	b)375	b)4.0 (pH 8)		b)13	b) 0.025	
Cocaina y metabolitos [193]	Orina humana	Cloroformo[d]	3	1600	8	14	10.5 – 29.1	11 - 48	GC-PDHID
Cocaina y metabolitos [194]	Saliva humana	Cloroformo[d]	10	2000	2 (pH 10.5)	10	16.2 – 58.6	6 – 28	GC-PDHID
Ibuprofeno, naproxeno y ketoprofeno [195]	Agua residual	Octanol[d]	20		20	4	133-272	1 – 2[a]	GC-FID
Drogas protéicas [196]	Sangre de pollo	Octanol[g]	60	600	15 (pH 7.4)	25	n.d.		HPLC-UV
Acetato de clorhexidina [197]	Supositorios	Octanol	20	n.d.	7.5	n.d.	24	n.d.	HPLC-UV
Dehydroandrographolide [198]	Compmrimidos chuanxinlian	Octanol	n.d.	n.d.	Metanol	n.d.	n.d.		HPLC-UV
Progesterona [175]	Suero humano	Tolueno[d]	20	500	1.5	n.d.	n.d.	0.5	GC-MS
Vinclozolina [199]	Sedimentos	Tolueno	20	800	5	3	150.4	0.5[b]	GC-ECD
Esteroides anabólicos no conjugados [200]	Orina humana	Octanol[d]	30	1250	4 (pH 7)	20	72.6 – 82.7	2	GC-MS
Clenbuterol, metopropol y propanolol [201]	Orina humana	Metilbenzol:MSTFA[d]	20	925	(pH 12, NaCl 14%)	4	n.d.	0.08 – 0.10	GC–MS
Clenbuterol [202]	Cerdo	Tolueno	20	1000	(pH 11)	n.d.	78	2.4[b]	HPLC
Macrolidos [203]	Leche	Tolueno	20	1000	(pH 8.5)	n.d.	34 - 52	2 – 5	HPLC
Mirtazapina [204]	Plasma humano	Tolueno[d]	30	n.d.	4 (1.75 M NaOH)	22	n.d.	6.25	HPLC-UV
Diazepam and prazepam [83]	Plasma humano	Octanol[d]	30	1000	1.5 (pH 5.5)	15	69 - 103	2	GC-NPD
Óxido de trifenilfosfina [205]	Ingredientes farmacéuticos	Octanol[d]	60	80	15 (0.1 M HCl)	40	101	n.d.	HPLC-UV
Nicotina [206]	Productos alimenticios	Tolueno[d]	10	n.d.	0.03	1.5	19	2[b]	GC-MS
Diuréticos [207]	Orina humana	Octanol[d]	40	1010	7.5 (pH 2, 15% NaCl)	25	0.8 – 144.8	0.3 – 6.7	GC-MS/MS
Diazepam, *N*-desmetildiazepam [208]	a) Orina humana b) Plasma humano	a)Acetato[d] de butilo/Octanol b) DHE/Octanol[d]	a)50 b)15	600	a)3.8 (pH 7) b)3.2 (6.25% metanol)	25	a)104 b)64 - 88	0.020- 0.115	GC-NPD
Tramadol [209]	Agua de río, orina y plasma humano	Tolueno	15	300	4 (250 mM NaOH)	n.d.		0.01 – 0.05[c]	GC-FID
Phenotiazinas [210]	Orina humana	Tolueno[d]	10	1000	(pH 9)	4	98-141	n.d.	GC-FID
Ibuprofeno, naproxeno y ketoprofeno, ácido clofíbrico [54]	Agua de río y agua residual	Octanol[d]	20	994	(Agua/HCl/NaCl)	n.d.	n.d.	n.d.	GC–MS

[a](ng L^{-1}), [b](ng g^{-1}), [c](mg L^{-1}), [f](nmol L^{-1}), [d](PP Q3/2) (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm), [e](PVDF (i.d. 1200 µm, espesor 300 µm, tamaño de poro 0.2 µm)), [g](i.d. 1000 µm, tamaño de poro 0.08 µm), DHE (Dihexiléter), MSTFA (N-Metil-N-(trimetilsilil)trifluoroacetamida)

Tabla 4. Aplicaciones de HF-LPME en dos fases para la determinación de pesticidas.

Analito/Matriz		SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (µl)	E_f	LOD (µg L^{-1})	Técnica analítica
Componentes de residuos de embalaje									
Residuos de embalaje [63]		Tolueno[e]	20	300	20	50	83 - 338	0.33 – 1.66[b]	GC-MS
Residuos de embalake [64]		Tolueno[e]	20	n.d.	(a) agua, (b) 3% AcOH, (c) 10% metanol	50	4 - 189	0.01 – 0.21[b]	GC - MS
Agentes químicos de guerra									
Agua [74]		Tricloroetileno[d]	15	1000	5 (30% NaCl)	1	n.d.	0.1 – 10	GC-MS
Agua, fango [75]		Cloroformo[d]	30	1250	20 (30 % NaCl)	5	n.d.	0.03 – 0.20	GC-MS
Agua [76]		Cloroformo[d]	45	1000	3.3 (pH 0, 33% NaCl)	n.d.	2 - 216	0.01 – 0.54[c]	GC-MS
Agua [77]		Cloroformo[d]	30	1000	3 (pH 12, 30% Na_2SO_4)	5	n.d.	0.04 – 0.36	GC-MS
Agua [51]		Tricloroetileno[d]	30	n.d.	1.5	8	n.d.	0.10 – 0.75[c]	GC-MS
Otros									
Caproato de etilo [80]	Licor	Hexano[d]	20	1000		4	n.d.	0.25[c]	GC-MS
Metilmercurio[82]	Pelo humano, lodos	Tolueno[d]	10	1300	(pH 6)	4	55	0.4	GFAAS
Antioxidantes [79]	Alimentos	Tolueno[d]	15	1200	(15% Na_2SO_4)	5	n.d.	0.002 – 8.0[b]	GC-MS
Selenoaminoácidos[52]	Ajo, col, champiñones	Tolueno/Cloroformo 3:1[d]	5	1000	2.5 (10% NaCl)	5	n.d.	11 – 23[a]	GC – ICP - MS
Colorantes [78]	Salsa de fresa, chile, huevos y pimientos	Octanol[d]	40	1000	15 (pH 2)	12	37 - 186	0.09 – 0.95	HPLC-UV/MS
Ocratoxina A, toxina T-2 [81]	Vino, cerveza	Octanol[d]	360	90	15 (pH 2, 10% NaCl)	n.d.	4.1 – 8.3	0.02 – 0.09	UHPLC – MS/MS

[a](ng L^{-1}), [b](ng g^{-1}), [c](mg L^{-1}), [d](PP Q3/2) (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm), [e](PP 150/330 (i.d. 330 µm, espesor 150 µm)

2.3 Configuración en tres fases

En la modalidad de tres fases, como ya se ha comentado, los analitos se extraen desde una fase acuosa (fase donadora) hacia otra fase acuosa (fase aceptora), atravesando los poros de una fibra hueca que soporta una membrana líquida (fase orgánica), actuando de barrera entre la solución donadora y la fase de aceptora (Figura 6).

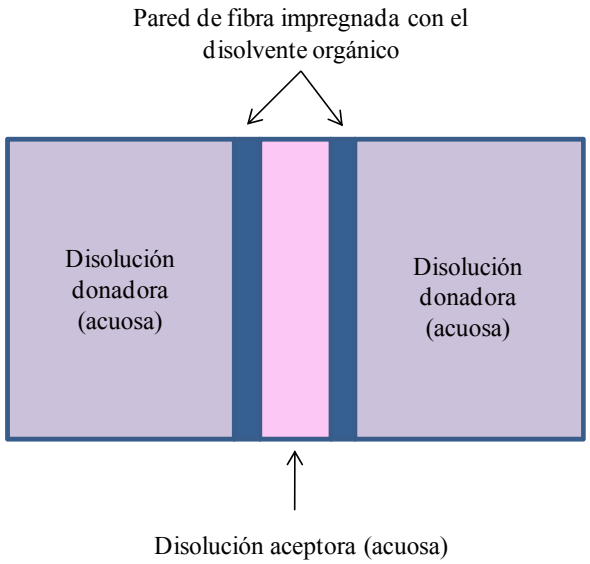


Figura 6. Esquema de modalidad en tres fases.

A diferencia de lo que ocurre en una configuración en dos fases, el procedimiento de extracción depende tanto del pH de la fase donadora como de la fase aceptora, así como del disolvente orgánico que se encuentra inmovilizado en el interior de los poros de la fibra. El disolvente orgánico elegido como extractante debe ser de polaridad adecuada según el tipo de analito que se pretende extraer.

Esta modalidad de extracción se emplea cuando se pretende extraer analitos básicos o ácidos con grupos ionizables; de esta forma el analito se encuentra en la forma neutra en la fase donadora, e ionizado en la fase aceptora. En el caso de extracción de compuestos básicos, se emplea como fase aceptora una solución acuosa ácida, mientras que si se pretende extraer compuestos ácidos, es necesario emplear como fase aceptora una solución alcalina. En ambos casos, una vez se extraen los analitos a la fase aceptora, está muy desfavorecido el hecho de que vuelvan a la fase donadora.

La fase aceptora ha de ser compatible con la posterior técnica analítica de análisis o, en su defecto, debe hacerse compatible.

Al igual que en dos fases, es posible trabajar con líquidos iónicos como disolvente inmovilizado en los poros; sin embargo, se han obtenido pobres resultados debido a la relativamente alta solubilidad de los líquidos iónicos en soluciones acuosas.

2.3.1 Características cinéticas y modelos matemáticos

En la modalidad de tres fases, los analitos son extraídos desde la fase acuosa (fase donadora) hasta el interior de la fase acuosa (fase aceptora) mediante un mecanismo de difusión pasiva. A continuación se describe un modelo matemático de la cinética del proceso propuesto por Rasmussen y Pedersen [49,50].

El proceso de microextracción se basa en las diferencias de concentración de los analitos entre las tres fases líquidas inmiscibles (acuosa-orgánica-acuosa). La transferencia de masa del analito desde la fase acuosa donadora a la fase acuosa aceptora continúa hasta que se alcanza el equilibrio termodinámico o se interrumpe la extracción.

El modelo establece un balance de masas dinámico para el analito (A) entre las diferentes fases.

$$A_d \underset{K_2}{\overset{k_1}{\rightleftharpoons}} A_m \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_a$$

donde,

A_d, A_m y A_a hacen referencia al analito en la fase donadora, en la membrana y en la fase aceptora, respectivamente

k_1, k_2, k_3, y k_4 son las constantes de velocidad de extracción de primer orden

De acuerdo a esto,

$$C_d \cdot V_d + C_m \cdot V_m + C_a \cdot V_a = C_d^0 \cdot V_d \quad (10)$$

donde,

C_d , C_m y C_a son las concentraciones del analito en la fase donadora (acuosa), en la membrana (fase orgánica) y en la fase aceptora (acuosa) en un instante dado, respectivamente

V_d, V_m y V_a son el volumen de la fase donadora (acuosa), del disolvente orgánico en la membrana (fase orgánica) y de la fase aceptora (acuosa), respectivamente

C_d^0 es la concentración inicial del analito en la muestra

Atendiendo al equilibrio entre las tres fases, el coeficiente de partición del analito entre la fase aceptora y donadora se define como:

$$K = \frac{C_{eq,a}}{C_{eq,d}} = K_{m/d}\, K_{a/m} \quad (11)$$

donde,

$C_{eq,a}$, es la concentración del analito en la fase aceptora en el equilibrio

$C_{eq,d}$ es la concentración del analito en la fase donadora en el equilibrio

$K_{m/d}$ es el coeficiente de partición del analito entre la membrana y la fase donadora

$K_{a/m}$ es el coeficiente de partición del analito entre la fase aceptora y la membrana

La concentración del analito en la fase aceptora (acuosa) en las condiciones de equilibrio, viene dada por:

$$C_{eq,a} = \frac{K_{m/d} C_d^0}{K_{a/m} + K_{m/d} K_{a/m} (V_m/V_d) + K_{m/d} (V_a/V_d)} \quad (12)$$

donde,

V_d, V_m y V_a son el volumen de la fase donadora (acuosa), del disolvente orgánico en la membrana (fase orgánica) y de la fase aceptora (acuosa), respectivamente

C_d^0 es la concentración inicial del analito en la muestra

$K_{m/d}$ es el coeficiente de partición del analito entre la membrana y la fase donadora

$K_{a/m}$ es el coeficiente de partición del analito entre la fase aceptora y la membrana

Sin embargo, en la práctica, al igual que ocurre cuando se trabaja en una configuración de dos fases, las condiciones de equilibrio rara vez se alcanzan cuando se emplea esta técnica de extracción, ya que implica tiempos de extracción excesivamente largos. Por lo tanto, la concentración del analito en la fase aceptora, puede expresarse en función del tiempo de microextracción, según [13]:

$$C_a(\mathrm{t}) = \mathrm{C_d^0}\ \frac{\mathrm{V_d}}{\mathrm{V_a}}\left[\frac{\mathrm{k_1 k_3}}{\lambda_2\lambda_3} + \frac{\mathrm{k_1 k_3}}{\lambda_2(\lambda_2-\lambda_3)}\ \mathrm{e}^{-\lambda_2 \mathrm{t}} + \frac{\mathrm{k_1 k_3}}{\lambda_3(\lambda_2-\lambda_3)}\ \mathrm{e}^{-\lambda_3 \mathrm{t}}\right] \quad (13)$$

donde,

$\mathrm{C_d^0}$ es la concentración inicial del analito en la muestra y,

$\mathrm{k_1}$, $\mathrm{k_2}$, $\mathrm{k_3}$, y $\mathrm{k_4}$ son las constantes de velocidad observadas ($\mathrm{s^{-1}}$).

λ_2 y λ_3 vienen definidas por las siguientes expresiones:

$$\lambda_2 = \frac{1}{2}\left[(\mathrm{k_1+k_2+k_3+k_4}) + \left[(\mathrm{k_1+k_2+k_3+k_4})^2 - 4(\mathrm{k_1k_3+k_2k_3+k_1k_4})\right]^{1/2}\right] \quad (14)$$

$$\lambda_3 = \frac{1}{2}\left[(\mathrm{k_1+k_2+k_3+k_4}) - \left[(\mathrm{k_1+k_2+k_3+k_4})^2 - 4(\mathrm{k_1k_3+k_2k_3+k_1k_4})\right]^{1/2}\right] \quad (15)$$

La constante de primer orden, está relacionada con las constantes individuales de velocidad de la siguiente forma:

$$k \approx \frac{\mathrm{k_1 k_3}}{\mathrm{k_2+k_3}} = \frac{A_{md}A_{am}\mathrm{K_{m/d}}\beta_\mathrm{m}\beta_\mathrm{a}}{V^d\left(A_{md}\mathrm{K_{a/m}}\beta_\mathrm{m}+A_{am}\beta_\mathrm{a}\right)} \quad (16)$$

donde,

β_m y β_a son los coeficientes totales de transferencia de masa, que describen la velocidad de transferencia del analito desde la fase donadora hacia la membrana líquida orgánica, y desde la fase orgánica hasta la fase aceptora,

A_{md} y A_{am} son las áreas interfaciales entre la fase orgánica y fase donadora, y entre la fase acuosa extractante y la fase orgánica, respectivamente.

2.3.2 Aplicaciones

Como se puede observar en las tablas desde la 5 a la 8, la técnica analítica más utilizada tras la extracción en sistemas de tres fases es la cromatografía líquida de alta resolución con detección ultravioleta o espectrometría de masas, y la electroforesis capilar. No obstante, se han empleado otras técnicas como la cromatografía de gases [83], espectrometría de absorción atómica [82], análisis por inyección en flujo con detector de quimioluminiscencia [84] o de espectrometría de masas [85], y análisis en flujo electrocinético con detección espectrofotométrica [86].

Los disolventes orgánicos empleados para formar la membrana líquida en una configuración de tres fases tienen, en general, mayor polaridad que los empleados en una configuración de dos fases, siendo el octanol y dihexiléter los más habituales. En algunos casos, se ha empleado un líquido iónico como soporte en la membrana, hexafluorofosfato 1-octil-3-metilimidazolio [omim][PF6] [87] o 1 hexafluorofosfato-butil-3-metilimidazolio ([bmim]PF6) [88], aunque los factores de enriquecimiento no son especialmente elevados.

En la mayoría de los casos la fase donadora y aceptora presentan valores de pH muy diferentes, empleando generalmente ácido clorhídrico para acidificar e hidróxido sódico como base fuerte para alcalinizar. En algunos casos, la extracción se lleva a cabo con un valor de pH neutro en la fase donadora, que se consigue empleando un tampón adecuado [83,86,89-92], o bien ajustando el pH de la muestra [87,93].

Como ocurre en la configuración de dos fases, la fibra de tipo Q3/2 es la más empleada. No obstante, se han empleado otras como por ejemplo, de tipo KM [89,94-98], otras de 150 μm de espesor de la pared en lugar de 200μm [99] empleadas en la determinación de anilinas, e incluso otras de tipo 50/280 (40% de porosidad, de 50 μm de espesor de pared y 280 μ m de diámetro interior) [100-102].

Además se han utilizado otras fibras de polipropileno, de características de tamaño de poro, espesor de la pared y diámetro interior diferentes [103-109]. Por

último, se han utilizado poliacrilonitrilo [111] y difluoruro de polivinilideno [93, 111, 112] como alternativas al polipropileno.

Como puede verse en las tablas desde la 5 hasta la 8, esta técnica de extracción, se ha aplicado a la determinación de múltiples tipos de analitos como fármacos: antidepresivos, medicamentos anti-inflamatorios no esteroideos (AINEs) y las anfetaminas (Tabla 5), entre otros (Tabla 5). Al igual que HF-LPME en dos fases, la técnica se ha aplicado a la determinación de compuestos orgánicos (por lo general de naturaleza fenólica (Tabla 6,7) y pesticidas (Tabla 8). También se ha aplicado a la determinación de compuestos organomercuriales, análisis de alimentos o la determinación de alcaloides (Tabla 8).

Tabla 5. Aplicaciones de HF-LPME en tres fases para la determinación de compuestos orgánicos nitrogenados.

Analito/Matriz	SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (µl)	E_f	LOD (µg L^{-1})	Técnica analítica
Anilinas								
Agua de grifo [211]	Octanol[e]	20	1000	4 (0.1 M NaOH)	5 (05 M HCl)	60 - 140	0.008 – 0.02[c]	CE
Agua [99]	Alcochol bencílico / Etil acetato[f]	20	900	3 (pH 13)	3 (pH 2)	106.43 -286.33	1.0 – 2.5	HPLC-UV
Agua de río, subterranea y de grifo [212]	Tolueno[e]	10	1200	6 (1 M NaOH, 10 % NaCl)	6 (0.5 M HCl, 600 mM)	258.9 – 674.2	0.01 – 0.1	HPLC-UV
Aminas aromáticas								
Agua [213]	Alcochol bencílico / Etil acetato[g]	30 + 20	900	(pH 13)	5 (pH 2)	6091.83 – 17093.77	10 – 250	HPLC-UV
Agua superficial y de grifo [182]	DHE[e]	30	1000	4 (0.1 M NaOH, 20% NaCl)	4 0.5 M HCl	240 - 510	0.05 – 0.10	HPLC-UV
Dinitrofenoles								
Agua de mar y de lluvia [214]	DHE[e]	90	1000	6, (pH 2)	50 (28% TOPO)	70 - 115	19.4 - 52.7[a]	HPLC-UV
Plasma humano [101]	DHE[h]	n.d.	n.d.	2 (5% isopropanol, pH 2, 186 mM NaCl)	(pH 10)	n.d.	0.05 – 0.1[c]	HPLC-MS
Plasma humano [102]	DHE[h]	n.d.	n.d.	2 (5% isopropanol, pH 2, 88 mM NaCl)	(pH 10)	n.d.	0.05 – 0.1[c]	HPLC-MS
[215]	Octanol[e]	20	700	4 (pH 1)	4 (pH 13)	190 - 347	0.45 – 0.98	HPLC-UV
Agua de río [216]	Undecano / Tolueno[e]	45	880	6 (pH 10)	25-27 (pH 2)	200 - 250	98 – 506[a]	HPLC-UV

Nitrofenoles									
Plasma humano [100]		DHE[e]	45	400	1 (pH 10.0)	9 (pH 3)		0.02 – 0.03	CE
[215]		Octanol[e]	20	700	4 (pH 1)	4 (pH 13)	190 - 347	0.45 – 0.98	HPLC-UV
Agua [217]		Octanol[e]	25	1050	14 (pH 3.0)	40 (pH 12.0)	336 - 398	0.01 – 0.04[c]	CE
Agua de mar [118]		Octanol[e]	50	1000	2.5 (1 M HCl)	2 (0.1 M NaOH)	236 - 380	0.5 – 1.0	HPLC-UV
Otros									
Hidrocarburos alifáticos y aromáticos [87]	Agua de lluvia	[bmim]PF6[e]	40	105 rad/s	10 (pH 4.2 – 4.6)	5 (Tolueno)	53 - 210	1 – 7[a]	GC-MS
Amino alcohols [104]	Orina humana	Octanol[i]	50	1000	4 (1 M NaOH)	5 (0.1 M HCl)	72 - 110	0.08 – 0.5[c]	CE
Antraquinonas[111]	Ruibarbo	1-hexanol[k]	50	1500	(Metanol)	(1 mM NaoH)	10 - 200	2.9 – 20.0	HPLC-UV
Ácidos carboxílicos aromáticos [219]	Alimentos	2-octanona[e]	15	1200	(10 mM HCl)	30 (10 mM NaOH)	n.d.	n.d.	CE
Clorofenoles [88]	Agua residual, de grifo, río y suelo	[omim][PF6][e]	60	n.d.	15 (0.1 M HCl, 20% NaCl)	10 (1 M NaOH)	n.d.	0.5 – 1.0	HPLC-UV
Aminas heterocíclicas [92]	Orina humana	Octanol[e]	90	850	3 (pH 5.5)	40 (0.1 M H_2SO_4)	n.d.	0.002 – 0.050[b]	HPLC-MS/MS
Compuestos hidroxi-aromáticos [220]	Agua de río	Nonanol / DHE 1:3[e]	50	800	10 (1 M HCl)	5 (1 M NaOH)	339 - 630	2 – 51.2[d]	HPLC-UV
Ácidos hidroxibenzoicos [112]	kaki	Heptanol[k]	35	1200	(HCl 5 mM)	(NH_3 80 mM)	107.6	0. 09 – 30	n.d.
Fenoles[114]	Agua	Octanol[e]	20	1000	50 (2 mM HCl)	150 (8 mM NaOH)	904 - 2682	2.5 -8.0	CE

[a](ng L^{-1}), [b](ng g^{-1}), [c](mg L^{-1}), [d](µmol L^{-1}), [e]((PP Q3/2) (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm)), [f](PP (i.d. 1200 µm, espesor 150 µm, tamaño de poro 0.2 µm)), [g](PP (espesor 150 µm, tamaño de poro 0.2 µm)), [h](PP (i.d. 280 µm, espesor 50 µm,)), [i](PP (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.64 µm)), [k](PVDF: polifluoruro de vinilideno), DHE (Dihexiléter), ([bmim]PF6) (1 hexafluorofosfato-butil-3-metilimidazolio), ([omim]PF6) (hexafluorofosfato 1-octil-3-metilimidazolio)

Tabla 6. Aplicaciones de HF-LPME en tres fases en análisis clínico y farmacéutico: anfetaminas, antidepresivos y AINEs.

Analito/Matriz	SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (μl)	E_f	LOD (μg L^{-1})	Técnica analítica
Anfetaminas								
Orina humana, plasma humano [103]	Dihexiléter[c]	15 (urine and plasma), 30 (whole blood)	1500	4 (625 mM NaOH)	25 (0.1 M HCl)	60 -105	n.d.	CE
Orina y sangre [85]	Dihexiléter	15	n.d.	1 (0.5 M NaOH)/ 4 (0.0625 M NaOH)	25 (0.01 M HCl)	n.d.	0.4 – 100	FIA-MS/MS
Orina y plasma humano [221]	Octanol[c]	45	400	2.5 (0.1 M NaoH)	25 (0.1 M HCl)	75	5	CE
Orina y plasma humano [83]	Octanol	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 - 3	n.d.
Fármacos antidepresivos								
Plasma humano [222]	Dihexiléter[c]	45	1500	0.75 (0.5 M NaoH)	25 (0.01 M HCl)	n.d.	12.5	CE
Plasma humano [223]	Dodecil acetato[d]	45	1500	1.5 (130 mM NaOH)	15 (pH 2.75)	19 - 31	1.4 – 3.4	CE
Leche[94]	Aceite de silicona[d]	60	1500	1.5 (260 mM NaOH)	15 (HCl 10 mM)	n.d.	50	CE
Orina y plasma humano [224]	Dodecano[c]	45	700	11 (pH 12.0)	24 (pH 2.1)	298 – 315	0.5 – 2.3	HPLC-UV
Plasma humano [225]	Dihexiléter[c]	40	1400	5 (pH 14.0)	20 (HCl 20 mM)		5	HPLC FL
Orina humana, plasma humano [103]	Dihexiléter[c]	15 (urine and plasma), 30 (whole blood)	1500	4 (625 mM NaOH)	25 (0.1 M HCl)	60 -105	n.d.	CE
Plasma humano [226]	Dihexiléter[c]	60	1200	4 (125 mM NaOH)	25 (pH 2.75)	25 - 30	5 – 5.5	CE

Orina humana, plasma humano [95]	Dodecil acetato[d]	30	1500	1.5 (130 mM NaOH)	15 (200 mM ácido fórmico)	n.d.	1 - 106	HPLC-MS
Agua residual [104]	Dihexiléter[e]	120	800	100 / 1100 (pH 11.8)	20 (10 mM HCl)	19250 - 26950	6 – 31[a]	HPLC-MS
Plasma humano [227]	Dihexiléter[c]	45	400	1 (pH 10.0)	9 (pH 3)	n.d.	0.02 – 0.03[b]	CE
Plasma humano [90]	Dihexiléter[c]	45	n.d.	4 (pH 8, 15% NaCl)	20 (0.01 M AcOH)	n.d.	1.25	HPLC-MS/MS
Orina humana [228]	Dihexiléter	45	n.d.	3 (pH 11, 15% NaCl)	(0.01 M AcOH)	n.d.	62.5	CE
Orina y plasma humano [44]	Octanol[c]	45	400	(0.1 M HCl)	25 (0.1 M NaOH)	75	5	CE
Orina y plasma humano [83]	Octanol[c]	30 - 45	1000		25 (0.01 M HCl)	30 - 125	0.7 - 3	HPLC, GC, CE
Aguas residuales [107]	Dihexiléter[e]	120	800	1000 (pH 11.8)	20 (pH 2)	n.d.	17 – 618[a]	HPLC-MS
AINEs								
Agua residual [108]	Dihexiléter[f]	45	n.d.	(pH 1.5 – 2.0)	(pH 9.5)	270 - 880	0.01 - 0.05	HPLC-UV/FL
Orina humana [84]	Dihexiléter	n.d.	n.d.	n.d.	n.d.	n.d.	1.9 – 52.9	n.d.
Orina humana [261]	Dihexiléter[c]	15	300	50 (pH 2)	50 (pH 10)	n.d.	0.03	FIA CL
Agua residual [262]	Dihexiléter[c]	15	300	50 (pH 2.0)	50 (pH 12.5)	n.d.	0.02 -0.3	HPLC-MS/MS
Agua [263]	Dihexiléter[c]	45	400	2.5 (0.1 M HCl)	25 (10 mM NaOH)	75 - 100	1	CE

[a](pg L^{-}), [b] (μmol L^{-1}), [c](PP Q3/2) (i.d. 600 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [d](KM (i.d. 1200 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [e](PP (i.d. 330 μm, espesor 140 μm, tamaño de poro 0.4 μm)), [f](PP (i.d. 30 μm, espesor 240 μm, tamaño de poro 0.1 μm))

Tabla 7. Aplicaciones de HF-LPME en tres fases en análisis clínico y farmacéutico: otras drogas.

Analito/Matriz	SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (μl)	E_f	LOD (μg L^{-1})	Técnica Analítica
Medicamentos contra la diabetes								
Orina y plasma humano [229]	Dihexiléter[c]	30	600	10 (pH 9.5)	15 (0.1 M HCl)	n.d.	0.18 / 2.83	HPLC-UV / CE
Agua de grifo, orina y plasma humano [230]	Dihexiléter[c]	30	500	10 (pH 8.0, NaCl 10%)	24 (pH 2.2)	180	1.0	HPLC-UV
Medicamentos contra la malaria								
Plasma de rata [90]	Octanol[c]	30	1500	4 (pH 11)	20 (0.1 M TFA)	n.d.	4.0 – 4.7	HPLC-MS
Orina humana [231]	Octanol[c]	40	1200	4 (125 mM NaOH, 10% NaCl)	50 (100 mM HCl)	n.d.	10 – 21	CE
Drogas básicas								
Plasma humano [97]	a)Dodecil acetato[c] b)Octanol	60	1500	a)1.5 (130 mM NaOH) b)0.1 (pH 7.0)	a)15 (10 mM HCl) b)20 (50 mM HCl)	n.d.	n.d.	CE
Orina y plasma humano [96]	Aceite de palma[d]	45	1200	1 (0.2 M NaOH)	20 (10 mM AcOH)	n.d.	n.d.	CE
Nicotina								
Orina humana [232]		n.d.	n.d.	(Alcalino)	5 (Acido)	158, 6	9.7 - 194.5	HPLC-UV
Orina humana [233]		n.d.	n.d.	n.d.	n.d.	n.d.	3	HPLC
Plasma humano [234]		n.d.	n.d.	(Alcalino)	(10 mM KH_2PO_4)	n.d.	0.05[b]	HPLC

Otros

Drogas ácidas [235]	Agua residual	Octanol[c]	45	500	22 (pH 2)	20 (10 mM carbonato amónico)	38 - 234	0.5 – 42[a]	LC-MS/MS
Glucurónidos [236]	Orina y plasma humano	Octanol[c]	60	n.d.	4 (pH 2)	50 (250 mM NH_3, 20% MeOH)	n.d.	2 - 250	HPLC-MS
Anticoagulantes [237]	Orina y plasma humano	Dihexiléter[c]	45	1250	8.5 (2 M HCl)	24 (pH 9.1)	95	0.2	HPLC-UV
Antifungal drugs[238]	Agua de grifo, orina y plasma humano	Dihexiléter[c]	45	800	10 (pH 11.0, 5% NaCl)	25 (pH 2.5)	127.9 - 71	0.9 – 4.0	HPLC-UV
Benzodiazepinas [89]	Sangre humana	Nonanol, Decanol, Dodecanol	n.d.	17	10 - 60	1.5 (pH 7.5)	n.d.	n.d.	HPLC-UV
Clorpromazina [239]	Agua de río, orina y suero humano	Dodecane[c]	60	1000	11 (0.01 M NaOH)	20 (pH 2.0)	250	0.5	HPLC-UV
Clenbuterol [240]	Orina humana	Octanol[c]	30	1000	7.5 (1 M NaoH)	10 (5 M ácido formico)	79	7[a]	HPLC-MS
Diureticos [241]	Orina humana	Octanol[c]	50	250	6 (0.20 M HCl) / 6 (2.0 M NaoH)	12 (0.12 M NaOH) / 0.04M H_3PO_4)	59-175	0.5 – 2.0	HPLC-UV
Melamina [242]	Productos lácteos	Tri-butil-fosfato	30	1000	(pH 8.0)	(pH 5)	n.d.	2.5	HPLC
Oximatrina, matrina[110]	Sophora	Alcochol isopropílico	30	1500	2 (pH 9)	(pH 4)	n.d.	1[b]	HPLC
Pentazocina [243]	Orina y plasma humano	Octanol[c]	25	900	3 (pH 9.0)	3 (0.5M AcOH)	104	2	HPLC-MS
Drogas protéicas[244]	Plasma humano	Dihexiléter[c]	10	1500	1 (125 mM NaOH)	25 (10 mM HCl)	n.d.	n.d.	CE
Estrógenos sintéticos [106]	Agua residual	Octanol[e]	40	1200	10 (pH 1.5, 20% NaCl)	10 (0.5 M NaOH)	302.8 – 336.0	0.25 - 0.50	HPLC-UV
Tetrandina, fangchinolina[246]	Plasma humano	Octanol[c]	60	1100	4.5 (pH 8.5)	20 (pH 3.3)	23 - 25	2.0 - 3.0	HPLC-UV

[a](ng L^{-1}), [b](mg L^{-1}), [c]((PP Q3/2) (i.d. 600 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [d]((PP (i.d. 1200 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [e](PP (i.d. 500 μm, espesor 300 μm, tamaño de poro 0.2 μm))

Tabla 8. Aplicaciones de HF-LPME en tres fases para la determinación de pesticidas y compuestos variados.

Analito/Matriz		SLM	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (µl)	E_f	LOD (µg L^{-1})	Técnica Analítica
Pesticidas									
Pesticidas organofosforados [109]	Vegetales y fruta	Dodecanol[c]	15	800	23 (pH 7.5)	0.2 (30 mM Metilamonio hidroclururo, pH 11.6)	1100 - 1410	0.004 – 0.01	CE
Pesticidas organofosforados [86]	Vegetales	Dodecanol[d]	n.d.	n.d.	5 (pH 7.5)	10 (0.3M NaOH)	n.d.	2[a]	EFA-UV
Ácido clorofenoxiacético [247]	Agua de río	Octanol / DHE[b]	40	800	50 (0.5 HCl)	15 (1M NaOH)	438 - 553	0.75 – 1.9	HPLC-UV
Doxepina, amitriptilina, clomipramina, mianserin[98]	Agua	Dihexiléter[f]	45	1050	1 (pH 10.8)	15 (10 mM AcOH)	n.d.	n.d.	HPLC-UV
Fungicidas[248]	Zumo de naranja	2-octanona[b]	30	1000	3.5 (120 mM NaOH)	20 (HCl 10 mM)	n.d.	0.05 - 0.10	HPLC-MS
Herbicidas [249]	Leche bovina	Octanol[b]	60	1250	8 (0.5 M HCl)	7 (0.1 M NaOH)	261 - 952	0.5	HPLC-UV
Ácido clorofenoxiacético [250]	Agua de río	Octanol[b]	60	750	10 (0.5 M HCl, 15% NaCl)	5 (0.5 M NaOH)	129 - 400	0.2 – 2.8	HPLC-UV
Herbicidas de triazina [93]	Agua residual y subterránea	Octanol[e]	30	n.d.	50 (pH 7.0, 20% NaCl)	8 (5 M HCl)	44 - 51	0.5 – 1.0	HPLC-UV
Imidacloprid[251]	Arroz, hojas, agua y suelo	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	HPLC-UV

Otros

Alcaloides [252]	Corydalis yanhusuo	n.d.	n.d.	n.d.	10 (alcalina)	Ácido	100 - 184	10.0 – 13.7	HPLC-UV
Aminas biogénicas [253]	Salsa de camarón y de tomate	Dihexiléter[b]	30	800	10 (pH 9.5)	25 (0.1 M HCl)	47 - 546	0.01 – 0.03	HPLC-UV
Metilmercurio [82]	Pelo humano, lodos	Tolueno[b]	10	1300	pH 6	4 (4% (m/v) tiourea)	204	0.1	GFAAS
Methilmercurio, ethilmercurio, fenilmercurio [254]	Agua de lago y productos del mar	Tolueno[b]	25	1500	3.8 (1 M HCl)	7.6 (0.1M Na_2SO_3)	120 – 350	0.03 - 3.8	HPLC-UV
Methilmercurio, ethilmercurio, fenilmercurio [106]	Pescado y cabello humano	Bromobenceno[b]	n.d.	1000	12 (pH 3.0)	15 (0.02 % L-cisteina)	261 – 458	0.03 – 0.14	CE
Productos de degradación de agentes nerviosos [255]	Agua	Octanol[b]	60	500	6 (pH 1, NaCl 10%)	8 (pH 14)	11 -135	0.1 – 500 [1]	HPLC-MS/MS
Fitohormonas [256]	Zumo de coco	Etil fenil éter	n.d.	n.d.	n.d.	n.d.	n.d.	0.0009 – 8.8 [1]	n.d.
Estricnina, brucina [257]	Orina humana	Octanol[b]	40	1500	4 (0.5 M NaOH)	0.1 M H_3PO_4	n.d.	1 - 2	CE
Strychnos alcaloides [258]	Orina humana	Octanol[b]	40	1500	4 (0.5 M NaOH)	0.1 M H_3PO_4	n.d.	1 - 2	CE
Carvacrol, timol, eugenol[219]	Alimentos	Cloropentano[b]	25	1200	(10 mM HCl)	30 (100 mM NaOH)	n.d.	n.d.	CE

[a](ng g^{-1}), [b]((PP Q3/2 (i.d. 600 μm, espesor 200 μm, tamaño de poro 0.2 μm)), [c](PP (i.d. 400 μm, espesor 70 μm, tamaño de poro 0.2 μm)), [d](PP (tamaño de poro 0.45 μm)), [e](PP (i.d. 400 μm, espesor 125 μm, tamaño de poro 2.5 μm)), [f](PP (i.d. 1200 μm, espesor 200 μm, tamaño de poro 0.2 μm))

2.4 Microextracción en fase líquida con empleo de *carrier*

De forma paralela, con el fin de mejorar la extracción de drogas hidrofílicas, se ha desarrollado una variante de microextracción en fibra hueca en una configuración de tres fases añadiendo ciertas sustancias, bien a la disolución acuosa que contiene el analito [113-115], o bien al disolvente inmovilizado en los poros de la fibra [116,117], facilitando así el paso del analito a través de la membrana líquida. Estas sustancias que se adicionan reciben el nombre de transportadores o "*carriers*". En ambos casos, el resultado es la formación de una molécula neutra que facilita el paso del analito inicialmente ionizado desde la fase donadora hacia la fase aceptora (Figura 7).

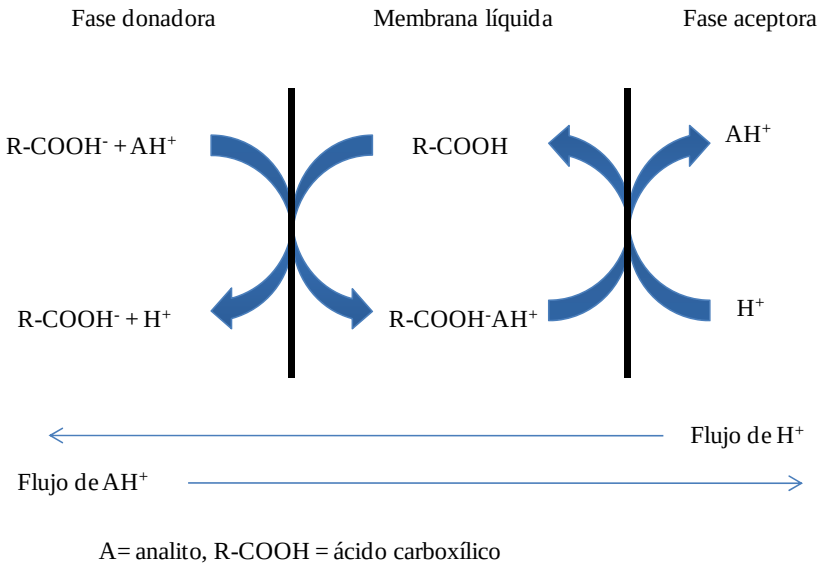


Figura 7. Esquema de HF-LPME en tres fases empleando *carrier*.

En una de sus variantes, el *carrier* se encuentra disuelto en el disolvente orgánico inmovilizado en los poros de la fibra que separa la fase donadora y aceptora. El analito y el *carrier* (disuelto en el disolvente orgánico) forman un complejo neutro en la membrana líquida, manteniendo de esta manera la neutralidad en la membrana apolar, y a continuación, pasa a través la membrana hacia la fase aceptora donde el analito es intercambiado por el correspondiente contraión.

Una segunda variante, consiste en añadir directamente el *carrier* (formador de par iónico) en la fase donadora donde el analito se encuentra previamente ionizado, y en

consecuencia, forma un par iónico hidrofóbico que atraviesa la membrana orgánica, para finalmente ser de nuevo intercambiado por el contraión presente en la fase aceptora.

En ambas variantes, los contraiones presentes en exceso en la fase aceptora forman un par iónico con el *carrier* en la superficie de la membrana que separa ambas fases, por lo que el nuevo complejo neutro formado vuelve a la fase donadora, dando lugar a un ciclo que se repite de forma continua. En definitiva, se pretende facilitar el transporte del analito inicialmente ionizado en la fase donadora, hacia la fase aceptora; y por otro lado asegurar la transferencia de contraiones en la dirección opuesta.

La optimización del pH en ambas fases, así como la elección adecuada del disolvente orgánico inmovilizado en los poros de la fibra y del *carrier* son fundamentales para que la extracción se lleve a cabo con éxito. El valor del pH en la fase donadora será aquel en el que en analito se encuentre inicialmente ionizado, y forme un complejo con el *carrier* que actúa de contraión. Los analitos catiónicos se extraen empleando *carriers* aniónicos, usándose *carriers* catiónicos para analitos aniónicos.

2.4.1 Características cinéticas y modelos matemáticos

Como ocurre en la modalidad de tres fases, los analitos se extraen desde una disolución acuosa donadora hasta el interior de una fase acuosa aceptora atravesando la membrana líquida orgánica soportada en la fibra mediante un mecanismo de difusión pasiva, en el que las reacciones implicadas son idénticas a las de una configuración en tres fases.

El empleo de *carriers* podría considerarse una variante de la configuración en dos o tres fases, ya que se han empleado tanto disolventes orgánicos, como, disoluciones acuosas como fase aceptora. Por tanto, los modelos matemáticos podrían ser similares a los previamente descritos con anterioridad para configuraciones en dos fases y tres fases.

2.4.2 Aplicaciones

La HF-LPME favorecida por *carrier* puede llevarse a cabo, como se ha descrito, de dos formas, empleando un *carrier* disuelto en la membrana líquida o bien disuelto en la fase donadora, y ambas alternativas se pueden realizar en configuraciones de dos y tres fases. Las tablas 9 y 10 recogen las aplicaciones descritas usando las cuatro posibles combinaciones.

Formador de par iónico en la fase donadora

La extracción mediante *carriers* en una configuración de dos fases se ha aplicado principalmente a la extracción de cationes y aniones que, debido a su carga y su naturaleza inorgánica, necesitan formar un par iónico para que sea posible extraerlos en una fase orgánica. Como puede observase en la Tabla 9 el *carrier* que se emplea en esos casos es generalmente una sal de tetrabutilamonio (TBA) o la sal de amonio del ditiocarbamato de pirrolidina (PDTC).

Las técnicas que se emplean para la determinación de los iones inorgánicos no llevan asociado un paso de separación previa: espectroscopia de absorción atómica electrotérmica para la determinación de arsénico [118], níquel y plomo [119] y selenio y teluro [120]; ICP-MS para la determinación de arsénico [121], selenio [122], cobre, cinc, paladio, cadmio, mercurio, plomo y bismuto [123]; ICP-OES para la determinación de vanadio [124]; y análisis por inyección en flujo con detector MS/MS para la determinación de perclorato [125]. También se ha desarrollado un método para la determinación de selenio mediante HPLC-UV [126].

Se ha conseguido aplicar a la determinación de sustancias orgánicas, como pesticidas ácidos [127], al metabolito de la marihuana (ácido 9-tetrahidrocannabinol-9-carboxílico 11-nor-Δ) [113], al tensioactivo cloruro de dicocodimetilamonio [128] y a ácidos haloacéticos [129].

Además, se han determinado drogas polares cuya alta naturaleza hidrofílica les impedía entrar en la fase orgánica formando un par iónico en la fase donadora [114]. De

esta manera, se han conseguido determinar, en fluidos biológicos, drogas polares como la morfina, practolol y anfetamina, entre otras [97, 114, 115,130].

Carrier disuelto en la membrana líquida

Como se observa en la tabla 9, se ha aplicado en el campo de la biomedicina para la determinación de sulfonamidas, como sulfadiazina, sulfamerazina, sulfametazina, sulfadimetoxina y sulfametoxazol [131]; tetraciclinas, como tetraciclina, oxitetraciclina y doxiciclina [132]; receptores antagonistas β2-adrenérgicos, como el salbutamol y terbutalina [133]; hormonas estrogénicas, como 17 β-estradiol, estrona y 17 α-etinilestradiol [134]. También se ha aplicado a la determinación de pesticidas [135-137], sustancias aromáticas como los fenoles [136], aminas aromáticas [138] y, en algunos casos, aunque minoritarios, en la determinación de especies inorgánicas como el mercurio [116], cobre [117] y cadmio [139].

Cuando el *carrier* se emplea disuelto en la fase orgánica, el óxido de trioctilfosfina (TOPO), y el cloruro de N-metil-N,N-dioctiloctan-1-amonio (Aliquat 336, Cognis Corp) son los más empleados, independientemente de trabajar en configuración de dos o tres fases (Tabla 10).

Tabla 9. Aplicaciones de HF-LPME mediante *carrier* formador de par iónico.

Analito/Matriz		**SLM**	***Carrier***	**t_{ext} (min)**	**v_{ag} (rpm)**	**V_{Fd} (mL)**	**V_{Fa} (μl)**	**E_f**	**LOD (μg L^{-1})**	**Técnica analítica**
DOS FASES										
Inorgánicos										
As (III), As (V)[118]	Pelo humano, agua de grifo, de estanque y de río	Tolueno[b]	PDTC de amonio	10	1500	2.7 (pH 3.5)	4 (Tolueno)	78	0.12	ET-AA
As (III), As (V)[121]	Agua de lago y de grifo	Nitrobenceno[b]	PDTC de amonio	15	700	10 (pH 3.0)	10 (Nitrobenceno)	220	0.32[a]	ETV-ICP-MS
ClO_4^- [125]	Aguas superficiales	Octanol[b]	Amonio di-n-hexil acetato	40	300	40 (pH 7.0)	100 (Octanol)		0.5	FIA-MS-MS
Cu, Zn, Pd, Cd, Hg, Pb and Bi[123]	Agua de lago y de río	CCl_4[b]	DDTC	15	1000	2.5 (pH 8.0)	4 (CCl_4)	20 - 305	1.6 -29[a]	ICP-MS
Ni^{2+}, Pb^{2+}[119]	Tejido de ostras, aguas de río, de grifo y subterráneas	[C8mim][PF6][b]	PDTC de amonio	12 - 15	1100	3 (pH 3.0)	8 [C8MIM][PF6]	60 - 75	0.02 – 0.03	ET-AAS
Se (IV)[126]	Orina y plasma humano, agua de grifo y de pozo	Octanol[b]	*o*-fenilendiamina	30	500	a)11 (30% NaCl) (b) 130 (30% NaCl)	20 (Octanol)	(a) 50, (b) 130	(a) 0.1, (b) 0.02	HPLC-UV
Se (IV), Se (VI)[122]	Agua de río, de lago y de piscina	CCl_4[b]	PDTC de amonio	20	800	2.5	4 (CCl_4)	410	0.50 – 0.56 [a]	ETV-ICP-MS
Te (IV), Te (VI), Se (IV), Se (IV)[120]	Aguas mediambientales y muestras de suelo	Tolueno[b]	PDTC de amonio		1200	10 (pH 4)	10 (Tolueno)	480-520	4 – 5[a]	ETAAS
V (IV), V (V)[124]	Aguas ambientales, de lago y de mar, muestras del suelo y partículas del tubo de escape de vehículos	CCl_4[b]	PDTC de amonio	12		3.5 (pH 5)	(CCl_4)	74	71 – 86[a]	ETV-ICP-OES

Compuestos orgánicos										
Ácido 11-nor-Δ^9-tetrahidro cannabinol-9-carboxilico	Orina humana	N,O-bis(trimethilsilil) trifluoroacetamida/ octanol[b]	Tetrabutilamonio hidrógeno sulfato	8	1540	8 (pH 8.0)	20 (N,O-bis(trimethylsilyl) trifluoroacetamide/ octane 5:1)	34.4 – 60.4	1	GC
Herbicidas ácidos [127]	Aguas de estanque y drenaje	Octanol[b]	Cloruro de tetrabutilamonio	40	459	10 (pH 7)	4 (Octanol)	n.d.	0.51 – 13.7 [a]	GC-MS
Cloruro de dicocodimethilammonium [128]	Agua de grifo	Octanol[b]	Octanoato	1020	600	250 (pH 10, 50 mM carbonato)	6.6 (Octanol)	120 - 400	0.9	HPLC-MS
Ácidos halocéticos [129]	Agua de grifo y de piscina	Decano[b]	Tetrabutilamonio hidrógeno sulfato	60	700	10 (40% Na_2SO_4)	5 (Decano)	n.d.	0.3 – 15	GC-MS
TRES FASES ***Drogas polares***										
	Orina y plasma humano [114]	Octanol[b]	Octanoato sódico	45	1500	4 (pH 7)	25 (50 mM HCl)	14 - 67	n.d.	CE
	Plasma humano [115]	Octanol[b]	Octanoato sódico	60	1500	0.1 (pH 7)	20 (50 mM HCl)	n.d.	25 – 50	HPLC-MS
	Plasma humano [130]	Octanol, Aceite de menta	Azul de bromotimol	60	n.d.	n.d.	20 (50 mM HCl)	n.d.	n.d.	n.d.
	Plasma humano [97]	Octanol[b]	Ácido octanoico	60	1500	0.1 (pH 7)	20 (50 mM HCl)	n.d.	n.d.	CE

[a](ng L^{-1}), [b](PP Q3/2 (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm), PDTC (Ditiocarbamato de porrolidina), DDTC (Dietil ditiocarbamato), [C8mim][PF6] (hexafluorofosfato de 1-octil-3-metilimidazolio)

Tabla 10. Aplicaciones de HF-LPME con *carrier* disuelto en la membrana líquida.

Analito/Matriz		***Carrier*/SLM**	**t_{ext} (min)**	**v_{ag} (rpm)**	**V_{Fd} (mL)**	**V_{Fa} (µL)**	**E_f**	**LOD (µg L^{-1})**	**Técnica Analítica**
DOS FASES									
Estrógenos [134]	Agua residual y de grifo	10% TOPO/DHE[c]	120	1100	100 (10% NaCl)	10 (DHE)	1500 - 3400	1.6 – 10[a]	GC-MS
Hg (II)[116]	Agua del mar	*N*-benzoil-*N'*,*N'*-diheptadeciltiourea Decalina/Cumena[e]	1320	800	($NaNO_3$ 0.25M, pH 2)	Decalina/Cumeno 80:20	9.2 – 15.3	n.d.	ICP-AES
Herbicidas fenoxi ácidos y fenoles [136]	Agua de mar, de lago y subterránea	10% TOPO /DHE[f]	240	n.d.	50 (pH 1.3, 5% NaCl)	(DHE)	156 - 442	0.4 – 1.2	HPLC-UV
Pesticidas[137]	Agua natural y de grifo	TOPO (10%) + TBP (10%)/DHE[d]	320	100	250 (pH 8.0)	(DHE)	n.d.	0.026 – 0.081[b]	HPLC-MS/MS
TRES FASES									
Pesticidas aminofosfóricos [135]	Aguas subterráneas	Aliquat-336/DHE[c]	60	n.d.	40 (pH 9.0)	20 (1 M KCl)	136 - 853	0.22 – 3.40	HPLC-FL
Aminas aromáticas [138]	Agua resicual, de río, de lago y de pozo	8% TOPO/DHE[d]	80	200	100 (0.1 M NaOH, 1M Na_2SO_4)	10 (8 M HCl)	407 – 2000	0.5 – 1.5	HPLC-UV
Receptores de los agonistas β2-adrenergic [133]	Comprimidos, agua y orina humana	20% Aliquat-336/DHE[c]	60	500	11 (pH 11.7)	24 (1 M NaBr)	52.9 - 213.1	2.5 – 5.0[a]	HPLC-UV, HPLC-MS
Cd (II)[139]	Agua de mar	Ácido oléico y ditiazona/octanol[c]	30	n.d.	100 (pH 5.2)	10 (0.05 M HNO_3)	387	0.8[a]	ET-AA
Cu (II)[117]	Agua	1,10-dibenzil-1,10-diaza-18-corona-6 en ácido oléico / DHE[d]	75	125	100	(pH 8.7, 0.15 mM 4-(piridil-2-azo)resorcinol	n.d.	5	Espectrofotometría
Sulfonamidas[131]	Agua residual y de arroz	14% TOPO/[C8mim][PF6][c]	480	300	4 (pH 4.5. 2M Na_2SO_4)	25 (pH 13)	58 - 135	0.1 – 0.4	HPLC-UV
Tetraciclinas[132]	Leche bovina, plasma humano y agua	Aliquat-336/1-octanol[c]	35	900	11 (pH 0.05M Na_2HPO_4)	24 (pH 1.6, 1 M NaCl)	109 - 196	0.5 – 1.0	HPLC-UV

[a](ng L^{-1}), [b](mg L^{-1}), [c](PP Q3/2) (i.d. 600 µm, espesor 200 µm, tamaño de poro 0.2 µm)), [d](PP (i.d. 280 µm, espesor 50 µm)), [e](PP-2E) , [f](X (i.d. 240 µm, espesor 30 µm)), TOPO (óxido de trioctilfosfina), DHE (Dihexiléter), Aliquat 336 (N-metil-N,N-dioctiloctan-1-amonio), TBP (Tributilfosfato), [C8mim][PF6] (hexafluorofosfato de 1-octil-3-metilimidazolio)

2.5 Microextracción en fase líquida usando electromembranas

La microextracción en fase líquida mediante electromembranas (*electromembrane microextraction*, EME) surge esencialmente, con el objetivo de disminuir el tiempo de extracción, y el sistema que se emplea es en esencia, el mismo descrito para la extracción de tres fases, a excepción de la adición de dos electrodos y una fuente de alimentación que genera corriente continua (Figura 8).

En esta configuración, uno de los electrodos, de platino, se coloca en la fase donadora acuosa, mientras que el otro electrodo de platino se coloca en la fase aceptora acuosa, dentro del interior de la fibra, garantizando así el transporte del analito hacia la fase aceptora a través de la membrana líquida soportada. El analito ha de encontrarse ionizado en ambas fases acuosas.

Los analitos básicos se extraen colocando el electrodo positivo en la fase donadora (Figura 8) y el negativo en la fase aceptora [140-142], invirtiendo la configuración para extraer analitos ácidos [143].

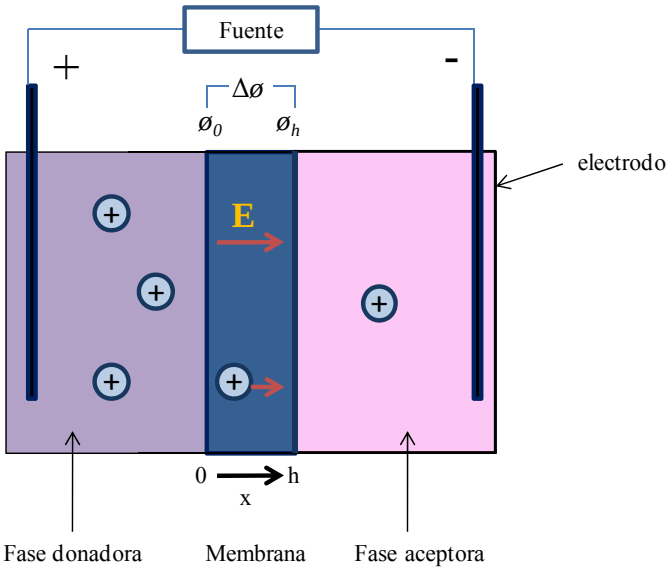


Figura 8. Esquema de HF-LPME mediante electromigración.

Teniendo en cuenta los parámetros que influyen durante la extracción, el flujo de analitos a través de la membrana líquida hacia la fase aceptora se controla con una elección adecuada del pH, el empleo de un disolvente orgánico adecuado al tipo de

analito que se pretende extraer y que a su vez permita dejar el paso de la corriente, y además por la agitación constante y vigorosa en todo el sistema. El voltaje aplicado es uno de los parámetros más importantes ya que de éste dependerá en gran medida la transferencia de masa del analito y selectividad en el sistema, cuyo objetivo es favorecer y reforzar el flujo de analitos a través de la membrana líquida. De esta manera, ofrece una mayor flexibilidad y velocidad, reduciendo así el tiempo de extracción.

Esta técnica, al igual que configuraciones anteriormente descritas, proporciona extractos limpios cuando se trabaja con matrices complejas, además de elevadas recuperaciones partiendo de pequeños volúmenes de muestra, por lo que se espera que sea una opción interesante para la preparación de muestras sobre todo en sistemas de análisis miniaturizados.

2.5.1 Características cinéticas y modelos matemáticos

Pedersen y Rasmussen [144] han propuesto un modelo matemático para la extracción con electromembranas. El modelo matemático se desarrolla atendiendo a la ecuación de Nerst-Planck y al flujo de analitos a través de la membrana líquida como consecuencia del campo eléctrico aplicado. El flujo en el estado estacionario, J_j, de un compuesto iónico (j) que atraviesa la membrana líquida soportada se puede describir mediante la ecuación de flujo de Nerst-Planck:

$$J_j = -D_j \frac{dc_j}{dx} + \frac{D_j z_j e E c_j}{kT} \qquad (17)$$

donde,

D_j es el coeficiente de difusión para el ión,

z_j es la carga del ión,

c_j es la concentración del ión en la membrana líquida,

x es la distancia de la interfase entre la fase aceptora y la membrana líquida,

k es la constante de Boltzmann,

T es la temperatura absoluta.

El primer término de la ecuación (17) describe la difusión del analito iónico en la membrana líquida, mientras que el segundo término describe la electromigración. En

la muestra pueden estar presentes otros iones diferentes al analito y el flujo para cada uno de esos iones puede describirse a través de esta misma ecuación (17).

Además de la ecuación (17), la ecuación de Poisson explica las características del campo eléctrico en la membrana líquida, de tal forma que:

$$\frac{d^2\phi}{dx^2} = \frac{p(x)}{\varepsilon} \tag{18}$$

donde,

ϕ es el potencial eléctrico a través de la membrana líquida

ε es la permitividad del disolvente orgánico en la membrana líquida

$p(x) = e\Sigma_j z_j c_j$ es la densidad de carga en toda la membrana líquida

En general, las ecuaciónes (17) y (18) describen el transporte iónico dentro de la membrana líquida. Para resolver estas ecuaciones se puede aplicar la aproximación de Planck [145] o la de Goldman [146]. La aproximación de Planck asume que todos los puntos en la membrana líquida son eléctricamente neutros a escala microscópica, y es válido en el caso de membranas de espesor considerable, mientras que para membranas de poco espesor, la aproximación no es válida ya que las cargas superficiales en cada pared de la membrana están relativamente cerca. Por otro lado, la aproximación de Goldman considera el campo eléctrico constante en cualquier punto de la membrana líquida y funciona para membranas relativamente delgadas o bien, cuando la concentración iónica total a cada lado de la membrana difiere considerablemente. En EME, se emplean fibras de pequeño espesor (200µm), por tanto, aplicando la condición de neutralidad según la aproximación de Planck, se obtiene que:

$$\sum_{i=1}^{M} z_i c_i + \sum_{k=1}^{N} z_k c_k = 0 \tag{19}$$

donde,

z_i es la carga del ión catiónico

c_i es la concentración del ión catiónico dentro de la membrana líquida

z_k es la carga del ión aniónico

c_k es la concentración del ión aniónico dentro de la membrana líquida

Esta ecuación puede reemplazarse por la ecuación (18) y sustituirse en la ecuación (17) para cada una de las especies del sistema. El primer término de la ecuación (19) representa las sustancias catiónicas "M" en el sistema, mientras que el segundo término hace referencia a los aniónicas. Si se considera que todas las especies presentes en el sistema están ionizadas con una sola carga y que la membrana no está cargada, el flujo de especies catiónicas J_i a través de la membrana en el estado estacionario, se puede calcular como [147]:

$$J_i = -\frac{D_i}{h}\left(1 + \frac{v}{\ln \chi}\right)\left(\frac{\chi - 1}{\chi - \exp(-v)}\right)(c_i - c_{i0}\exp(-v)) \tag{20}$$

donde,

D_i es el coeficiente de difusión para el ión

h es el espesor de la membrana

v es la fuerza del campo (definido en la ecuación (21))

χ es la relación entre la conconcentración iónica total en la fase donadora y la fase aceptora (balance de iones), y se define según la ecuación (22)

$$v = \frac{z_i e \Delta\phi}{kT} \tag{21}$$

$$\chi = \frac{\sum_i c_{ih} + \sum_k c^*_{kh}}{\sum_i c_{i0} + \sum_k c^*_{k0}} \tag{22}$$

donde,

c^*_{kh} es la concentración de la especie aniónica en la fase donadora

c^*_{k0} es la concentración de la especie aniónica en la fase aceptora

El último término de la ecuación (20) tiene en cuenta tanto la concentración de las sustancias catiónicas en la interfase entre la fase donadora y la membrana líquida, como la concentración en la interfase entre la fase aceptora y la membrana líquida. Si se asume que en el estado estacionario el coeficiente de partición para las sustancias catiónicas es la unidad; entonces se emplean c_{ih} y c_{i0} para expresar la concentración de especies libres en la fase donadora y aceptora, respectivamente, como se define más adelante (29)(30).

La intensidad de corriente eléctrica que circula a través de la membrana líquida en un momento dado, puede calcularse según la ecuación:

$$I = \sum_i J_i z_i F + \sum_k J_k z_k F \qquad (23)$$

donde,

F es la constante de Faraday

J_i es el flujo de las especies catiónicas

J_k es el flujo de las especies aniónicas

z_i es la carga del ión catiónico

z_k es la carga del ión aniónico

Sustituyendo en la ecuación (20), tanto para sustancias catiónicas como aniónicas [147], tenemos:

$$I = \frac{|z|F}{h}\frac{\chi - 1}{ln\chi}\left\{ln\left(\frac{\chi}{\xi}\right)\left(\frac{V_h - \xi V_0}{\chi - \xi}\right) - ln(\xi\chi)\left(\frac{\xi U_h - U_0}{\xi\chi - 1}\right)\right\} \qquad (24)$$

La ecuación (24) predice que el campo generado a través de la membrana líquida depende de forma no lineal del voltaje aplicado, y esto se debe a que durante la extracción, el contenido de especies ionizadas en la membrana líquida está cambiando continuamente debido a las diferencias de concentración entre las fases aceptora y donadora. Asímismo la intensidad de corriente dependerá del espesor de la membrana, de la fuerza electromotriz y de la relación entre las concentraciones iónicas totales de las fases donadora y aceptora, entre otros [147].

Cuando el potencial y la concentración que se emplea durante la extracción permanecen constantes en el interior de la membrana líquida, es posible calcular el flujo de iones aplicando la ecuación (20). Sin embargo, durante la extracción, tanto el potencial como la concentración cambian significativamente en las dos interfases de la membrana, y, por tanto, la aproximación que se hace en la ecuación (20) no es válida, por lo que deben considerarse los coeficientes de partición para cada una de las especies que participan.

Este modelo se puede desarrollar tanto para analitos ácidos como básicos. En el caso de que el analito sea básico (A^+) y que el ácido clorhídrico sea el que se emplee como fase donadora, las sustancias catiónicas en el sistema son (A^+) y (H^+), mientras que las únicas especies aniónicas serán los cloruros (Cl^-). Considerando el potencial electroquímico el mismo en ambos lados de la membrana, se pueden definir los correspondientes coeficientes de partición (K), atendiendo a las siguientes ecuaciones [148]:

$$K_{H^+} = \frac{[H^+]_m}{[H^+]_{da}} = \left(\frac{\alpha}{\beta\gamma}\right)^{1/4} \tag{25}$$

$$K_{A^+} = \frac{[A^+]_m}{[A^+]_{da}} = \left(\frac{\alpha\beta^3}{\gamma}\right)^{1/4} \tag{26}$$

$$K_{Cl^-} = \frac{[Cl^-]_m}{[Cl^-]_{da}} = \left(\frac{\alpha\gamma}{\beta}\right)^{1/4} \tag{27}$$

La diferencia de potencial generada en el sistema entre la membrana ("m") y las fases donadora y aceptora ("da"), viene dada por la expresión:

$$\Delta\phi_{m,da} = \phi_m - \phi_{da} = \frac{1}{3F}\left\{\Delta_H + \Delta_A - \Delta_{Cl} + \frac{RT}{4} ln\left(\frac{\gamma^3}{\alpha\beta^3}\right)\right\} \tag{28}$$

El flujo de analitos (A^+) a través de la membrana se puede calcular ahora a partir de la ecuación (20), teniendo en cuenta las concentraciones en la fase aceptora y donadora, así como los coeficientes de partición que se han definido anteriormente en las ecuaciones (25-27). Además, la diferencia de potencial real en el sistema se calcula a partir de la ecuación (28). υ y χ se calculan según las ecuaciones (29) y (30), respectivamente [147], teniendo en cuenta la concentración de analito protonado [A^+], la concentración de protones [H^+] y de iones cloruro [Cl^-] en la interfase entre la fase aceptora y la membrana líquida ("0") y dichas concentraciones en la interfase entre la fase donadora y la membrana líquida ("h"):

$$v = \frac{e\Delta\phi}{kT} + \frac{1}{4} ln\left(\frac{\gamma_{x=h}}{\gamma_{x=0}}\right) \tag{29}$$

$$\chi = \frac{[A^+]_{ad,h} K_{A,h} + [H^+]_{ad,h} K_{H,h} + [Cl^-]_{ad,h} K_{Cl,h}}{[A^+]_{ad,0} K_{A,0} + [H^+]_{ad,0} K_{H,0} + [Cl^-]_{ad,0} K_{Cl,0}} \tag{30}$$

2.5.2 Aplicaciones

Las aplicaciones usando EME son, hasta el momento, muy escasas (Tabla 11). La mayoría de trabajos sobre esta técnica están aplicados a la determinación de compuestos básicos, donde destacan el haloperidol, nortriptilina, la metadona y la loperamida. Gjelstad [149] y Middelthon-Bruer [141] ofrecen la gama más amplia de aplicaciones, que consisten en el estudio de hasta treinta y cinco compuestos básicos diferentes. No obstante, también se han determinado compuestos ácidos, como el ibuprofeno, diclofenaco y naproxeno, entre otros [143], y péptidos del tipo angiotensinas, entre otros [150-151].

El voltaje aplicado varía entre 10 y 300V y depende en gran medida del disolvente orgánico en el que se impregna la fibra, entre otros factores. Los disolventes orgánicos empleados en la membrana suelen ser distintos de las que normalmente se emplean para los otros tipos de configuraciones. Como se puede observar en la tabla 4, el octanol o heptanol, disolventes con cierto carácter polar, se emplean en muy pocos casos; mientras que los derivados de nitrobenceno o nitrofeniléteres son los más empleados. En cualquier caso, para llevar a cabo la extracción mediante electromembranas es imprescindible elegir un disolvente orgánico que conduzca la corriente eléctrica, por lo que disolventes muy apolares se consideran inadecuados.

En todos los casos, el tipo de fibra hueca que se emplea es de polipropileno del tipo Q3/2, y los tiempos de extracción se encuentran entre los 5-10 minutos. Estos tiempos son notablemente más cortos que los requeridos para la mayoría de las técnicas de microextracción en fibra hueca, que por lo general son del orden de 15-60 minutos. Eibak y colaboradores [152] han conseguido reducir el tiempo de extracción a un minuto para la determinación de amitriptilina, citalopram, fluoxetina y fluvoxamina en fluidos biológicos, alcanzando límites de detección entre 0,4 y 2,43 µg/L.

Tabla 11. Aplicaciones de HF-LPME mediante electromembranas.

Analito/matriz	SLM	Potencial (V)	t_{ext} (min)	v_{ag} (rpm)	V_{Fd} (mL)	V_{Fa} (μl)	E_f	LOD (μg L^{-1})	Técnica analítica
Drogas ácidas									
Agua [143]	Heptanol[a]	50	5	1200	0.3 (pH 12.0)	30 (pH 12.0)	0.8 – 10.0		CE
Pb^{2+}									
Suero y orina humana, liquido amniótico y lápiz labial [266]	Tolueno[a]	300	15	700	10	50 (pH 8.1)	557		CE
Péptidos									
Agua [150]	15% di-(2-etilhexil) fosfato en 1-octanol[a]	50	5	1050	1 (pH 3.0)	25 (100 mM HCl)	0.4 – 10.6		HPLC-UV
Plasma humano [151]	8% di-(2-etilhexil) fosfato en 1-octanol[a]	15	10	n.d.	0.5 (pH 3)	25 (50 mM HCl)	n.d.		CE, HPLC-MS
Agua [264]	10% di-(2-ethilhexil) fosfato en 1-octanol/di-isobutilcetona[a]	50	5	1050	0.5 (1 mM HCl)	25 (50 mM HCl)	n.d.		CE
Agua [265]	a) Eugenol[a] b) 10% di-(2-etilhexil) fosfato en 1-octanol/di-isobutilcetona[a] c) 5% 15-corona-5 éter en 1-octanol [a]	25	15	900	0.5 (1 mM formic)	25 (100 mM fórmico)	n.d.		HPLC- MS/MS
Drogas básicas									
Plasma humano [152]	1-etil-2-nitrobenceno[a]	9	1	n.d.	0.07	30 (10 mM HCOOH)	n.d.		LC - MS
Orina y plasma humano [267]	2-nitrofenil octil éter[a]	150	15	700	7 8100 mM HCl)	25 (100 mM HCl)	144 - 190		HPLC - UV
Agua [149]	2- nitrofenil pentil éter / 25% di-(2-etilhexil) fosfato en 2-nitrofenil pentil éter[a]	300	5	1200	0.3 (10 mM HCl)	30 (10 mM HCl)	n.d.		CE
Agua [259]	2-nitrofenil pentil éter[a]	300	10	900	0.15	25 (10 mM HCl)	n.d.		CE
Agua [144]	2-nitrofenil octil éter[a]	300	0 - 15	1000	0.5 (10 mM HCl)	25 (10 mM HCl)	n.d.		HPLC - UV
Plasma y sangre humana [142]	1-etil-2-nitrobenceno[a]	10				n.d.			
Plasma y orina humana, leche [146]	1-isopropil-4-nitrobenceno[a]	10	5	1000	1 (10 mM pH 4)	25 (10 mM HCl)	20 - 37		CE
Agua [143]	2-nitrofenil pentil éter / 10% di-(2-etilhexil) fosfato en 2-nitrofenil pentil éter[a]	50	5	1200	1 (0.1 - 10 mM HCl)	25 (10 mM HCl)	n.d.		CE
Orina y plasma humano [260]	2-nitrofenil octil éter[a]	300	5	1200	0.3 (10 mM HCl)	30 (10 mM HCl)	7.0 – 7.9		CE

[a](PP Q3/2) (i.d. 600 μm, espesor 200 μm, tamaño de poro 0.2 μm)

3. CARACTERÍSTICAS DE LOS COMPUESTOS ESTUDIADOS

Los hábitos de consumo actuales están generando una serie de residuos y microcontaminantes que hace tan solo unos años no existían, o sus niveles no eran, en absoluto, “preocupantes”; estos "contaminantes emergentes", constituyen un nuevo problema medioambiental. Entre estos contaminantes se encuentran los surfactantes, plásticos, diversos aditivos industriales, cosméticos y principios activos farmacológicos, entre otros.

Los principios activos farmacológicos son un amplio grupo de compuestos químicos con diversa actividad biológica cuya aplicación se extiende a la industria alimentaria, empleándose para combatir enfermedades y como promotores de crecimiento en animales de granja y piscifactorías, y, por su propia naturaleza, su presencia en el medio ambiente es problemática debido a su intensa actividad biológica inherente. Como consecuencia de ello, los principios activos farmacológicos están siendo continuamente introducidos en el medioambiente, dando lugar a la presencia de un enorme espectro de componentes activos cuya finalidad biológica es en parte desconocida. En la actualidad, una disminución en su uso, o la sustitución por otros componentes más biodegradables, se plantea como una solución complicada.

Tras su ingesta, estas sustancias se transforman a través de los procesos metabólicos y, una vez liberadas, pueden permanecer como tal o bien transformarse nuevos productos, que en muchos casos poseen una actividad biológica incluso mayor que los productos de partida. Ello hace que aparezcan en el medio ambiente fármacos en su forma activa y parcialmente metabolizados, pudiendo ser estos últimos incluso más tóxicos que las sustancias de partida [268].

Existen diferentes vías a través de las cuales estos principios activos entran en contacto con el medio ambiente (Figura 9). Para identificarlas es necesario dividir los fármacos entre los empleados en usos terapéuticos (Medicina y Veterinaria) y en otros usos, ya que, aunque en muchos casos los principios activos empleados sean los mismos, el destino de estos, como consecuencia de los efectos que causan sobre el ecosistema, son distintos.

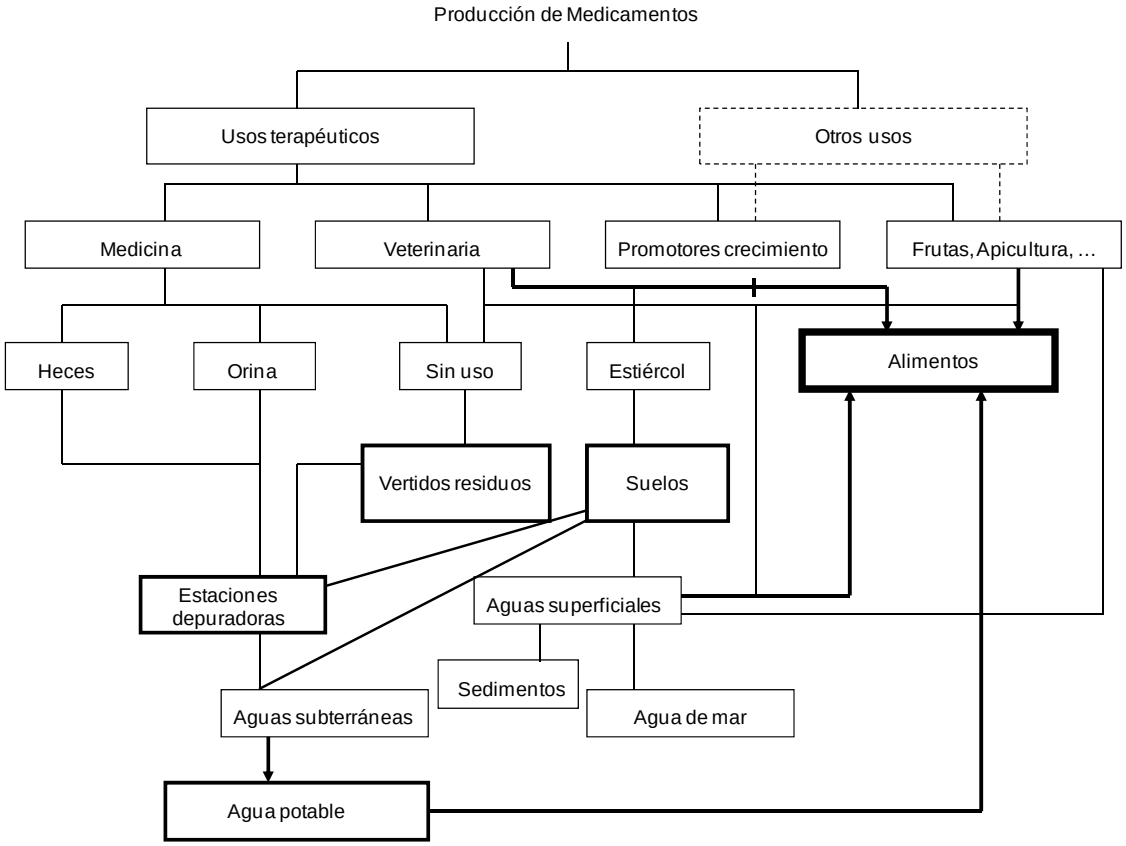


Figura 9. Mecanismos de introducción de principios activos farmacológicos en el medio ambiente.

Tras su administración, muchos medicamentos son metabolizados en gran medida antes de su excreción, mientras que otros quedan sólo parcialmente o no metabolizados y otros, como los medios de contraste, se excretan completamente intactos. Así, los productos farmacéuticos utilizados en medicina y sus metabolitos llegan a la red de aguas residuales y a las plantas de tratamiento. Si las drogas y sus metabolitos no se eliminan durante el tratamiento de las aguas residuales, pueden entrar en el medio ambiente acuático y, eventualmente, llegar a las aguas potables.

Los medicamentos caducados representan, asimismo, un gran problema debido a que con frecuencia son eliminados en los hogares a través de los desagües. Se ha informado que aproximadamente un tercio del total de volumen de los productos farmacéuticos vendidos en Alemania [269] y aproximadamente el 25% de los que se venden en Austria [270] se eliminan con los residuos domésticos o por el desagüe, y entran al medio ambiente intactos.

Entre los fármacos empleados en Veterinaria, los antimicrobianos y antiinflamatorios son los compuestos más usados; se utilizan con fines terapéuticos o como promotores del crecimiento, sobre todo cuando se trata de la cría y engorde de animales de granja y piscifactoría a gran escala [271]. Estos fármacos utilizados en la cría de animales y tratamiento de ganadería intensiva y sus metabolitos son excretados con el estiércol. Los agricultores utilizan estiércol y los lodos de depuradora para fertilizar los campos, por tanto, los residuos de las drogas se introducen en el suelo. Los productos farmacéuticos veterinarios pueden llegar a las aguas superficiales como consecuencia de la caída de fuertes lluvias.

La posibilidad de que los principios activos se introduzcan en el medio ambiente es conocida por la comunidad científica desde hace décadas, pero no es hasta 1990 cuando se hace posible su determinación y control en tales muestras, ya que, a las bajas concentraciones a las que estas sustancias se encuentran presentes en el medio ambiente, se le suma la complejidad de tales muestras, lo que hace necesario el uso de técnicas con elevada capacidad de separación y detección. Es necesario, sobre todo, disponer de métodos rápidos y robustos que permitan su empleo en análisis de rutina permitiendo determinar gran variedad de principios activos farmacológicos de distinta naturaleza.

Como se ha comentado anteriormente, los medicamentos que presentan un mayor interés en la acuicultura son los fármacos antibacterianos (antibióticos) y los antiparasitarios. Los antibióticos de mayor difusión en acuicultura son las tetraciclinas, penicilinas, sulfamidas, timetoprim, quinolonas, fluoroquinolonas y otros antibacterianos como el florfenicol y tiamfenicol; y en la alimentación del ganado vacuno penicilinas, cefalosporinas, tetraciclinas, aminoglucósidos, macrólidos y fluoroquinolonas, además de ciertos antiinflamatorios. La principal consecuencia de la existencia de restos de estos antibióticos en el medio ambiente es la aparición de cepas resistentes que podrían infectar a seres humanos y animales; el posible efecto puede verse incrementado con la ingestión de alimentos que contengan restos de los mismos.

Se ha observado que los fármacos antiinflamatorios no esteroideos dañan las enzimas que protegen a las células contra el estrés oxidativo, lo que ocasiona que se

oxiden las membranas celulares; asimismo sufren daño parte de las bases que constituyen el material genético.

En cualquier caso, siempre se trata de compuestos regulados, y los residuos de los mismos no deben sobrepasar los Límites Máximos Recomendados (LMR) regulados por la Unión Europea por el Reglamento (UE) nº 37/2010 de la Comisión de 22 de diciembre de 2009 [272], sobre los límites máximos de residuos de medicamentos veterinarios en alimentos de origen animal y que ha derogado el Reglamento (CE) 2377/90 del Consejo, aunque actualizando sus anexos. El establecimiento de los LMR resulta complicado debido a la constante aparición de nuevos medicamentos, lo que se refleja en las múltiples modificaciones que han sufrido el Reglamento (CE) 2377/90 y el Reglamento (UE) 37/2010, cuyos anexos se han ido actualizando conforme han ido apareciendo nuevos fármacos y se han conocido mejor los ya existentes en el mercado, siendo la última modificación registrada en el Boletín Oficial de la Comunidades Europeas el Reglamento (UE) n ° 914/2010 de la Comisión, de 12 de octubre de 2010 [273]. Por motivos de claridad y de adaptación a la nueva normativa, se establecen dos cuadros diferentes en el Anexo, agrupando el primero a aquellas de los Anexos I, II y III del derogado 2377/90 (Sustancias farmacológicas para las que hay un LMR establecido, sustancias para las cuales no es necesario establecer un LMR y sustancias farmacológicas con LMR provisionales) y otro que se corresponde con el antiguo Anexo IV (sustancias farmacológicas para las que no puede establecerse un LMR y por ende queda prohibida su administración a animales productores de alimentos).

En esta Tesis se estudiarán algunos de los antibióticos y antiinflamatorios de mayor consumo humano y veterinario, y por tanto, con mayor presencia en el medio ambiente, como son diversos antibióticos pertenecientes a las familias de las **fluoroquinolonas**: marbofloxacina, norfloxacina, ciprofloxacina, danofloxacina, enrofloxacina, gatifloxacina, grepafloxacina y flumequina, y la la familia de las **sulfonamidas**: sulfamerazina, sulfametazina, sulfadiazina, sulfametoxazole y sus correspondientes N^4- acetil metabolitos. Se estudiarán, asimismo, las sustancias más representativas, por uso y consumo, de la familia de los **antiinflamatorios no esteroideos**: ketoprofeno, naproxeno, ibuprofeno, ácido salicílico, ketorolaco y diclofenaco.

3.1 Antiinflamatorios no esteroideos

Los antiinflamatorios no esteroideos (AINEs) son un grupo variado y químicamente heterogéneo de fármacos con efectos antiinflamatorios, analgésicos y antipiréticos.

El AINE prototipo es el ácido acetilsalicílico y le acompañan una gran variedad de ácidos orgánicos, incluyendo derivados del ácido propílico (como el ibuprofeno y naproxeno), derivados del ácido acético (como la indometacina) y ácidos enólicos (como el piroxicam), todos competidores con el ácido araquidónico por el sitio activo de la ciclooxigenasa. El paracetamol se incluye entre los AINEs, a pesar de su escasa acción antiinflamatoria.

Todos ejercen sus efectos por acción de la inhibición de la enzima ciclooxigenasa. Los antiinflamatorios naturales, segregados por el propio organismo, son los derivados de los corticoides, sustancias de origen esteroideo de potente acción antiinflamatoria pero que cursan con importantes efectos secundarios. En oposición a los corticoides, el término "no esteroideo" se aplica a los AINE para recalcar su estructura química no esteroidea y la menor cantidad de efectos secundarios. Como analgésicos se caracterizan por no pertenecer a la clase de los narcóticos y actuar bloqueando la síntesis de prostaglandinas.

3.1.1 Mecanismo de acción

La acción principal de todos los AINEs es la inhibición de la ciclooxigenasa, una enzima que convierte el ácido araquidónico en endoperóxidos cíclicos, los cuales se transforman en prostaglandinas y en tromboxanos mediadores de la inflamación y factores biológicos locales, no circulantes llamados autacoides, incluyendo los eicosanoides. La inhibición de la síntesis de prostaglandinas y tromboxanos por los AINEs sería la responsable de su actividad terapéutica y de los efectos tóxicos de este grupo de fármacos. Esta inhibición puede ocurrir por distintos mecanismos:

- Inhibición irreversible, como en el caso de la aspirina
- Inhibición competitiva, como en el caso del ibuprofeno
- Inhibición reversible no competitiva, como el paracetamol

Los antiinflamatorios no esteroideos disponibles en el mercado inhiben la actividad tanto de la ciclooxigenasa-1 (COX-1) como a la ciclooxigenasa-2 (COX-2) y, por lo tanto, la síntesis de prostaglandinas y tromboxanos. Al inhibir a la ciclooxigenasa y la subsecuente síntesis de prostaglandinas, se reduce la liberación de sustancias y mediadores inflamatorios, previniéndose la activación de los nociceptores terminales, de modo que los AINEs alivian el dolor asociado con la inflamación. Los AINEs actuales no pueden inhibir la vía de la lipooxigenasa, por lo que continúan formándose leucotrienos y otros mediadores activos, lo que explica la limitación de éstos fármacos para controlar los procesos en los que intervengan numerosos mediadores.

La ciclooxigenasa tiene dos isoformas, la ciclooxigenasa-1 (COX-1) (presente en la mayoría de los tejidos que sintetizan prostaglandinas como el riñón, la mucosa del estómago, duodeno y plaquetas) y la ciclooxigenasa-2 (COX-2) (presente en los tejidos donde se monta una respuesta inflamatoria como el cerebro, pulmón, páncreas, placenta y ovarios). La inhibición sobre la actividad enzimática de las isoformas de las ciclooxigenasas depende del fármaco en cuestión. Existen estudios que parecen demostrar que la actividad analgésica de los AINE se realiza también a través de la COX-1. No obstante, también se han propuesto nuevos mecanismos independientes de la COX por los que los AINEs actúan como analgésicos. Se propone que el gen *c-Fos*, que se expresa durante el proceso nociceptivo es reprimido por el ketoprofeno, la indometacina y nuevos AINEs como el lornoxicam, con lo que disminuye el dolor y la inflamación. Por último, el hecho de que los AINEs no produzcan sueño, ni alteren el humor, ni provoquen alteraciones en la conciencia hace postular que su sitio de acción sea el hipotálamo.

Existen otros mecanismos de acción sugeridos para los AINEs:

1. Interferencia con la activación de neutrófilos
2. Estimulación de la vía óxido nítrico-GMPc
3. Bloqueo de las citocinas
4. Disminución en la expresión de canales iónicos sensibles a ácido (ASICs)

3.1.2 Farmacocinética

Se ha demostrado una relación entre la concentración del medicamento en el plasma sanguíneo y la respuesta antiinflamatoria en pacientes que toman antiinflamatorios no esteroideos, como aquellos con artritis reumatoide o con dolor postoperatorio. Se han observado, asimismo, diferencias farmacocinéticas entre un paciente y otro a la misma dosis. Existe también una relación directa entre la dosis administrada y el riesgo de perforación o sangrado en el tracto gastrointestinal superior.

Diclofenaco, ibuprofeno, naproxeno e indometacina son ácidos carboxílicos, mientras que otros, como fenilbutazona, son ácidos enólicos. La nabumetona es una cetona administrada como un profármaco que luego es metabolizado a su forma activa ácida.

La vía de elección para la administración de algunos AINEs en el tratamiento del dolor agudo es la intravenosa dado que permite un rápido comienzo de acción siendo conveniente una dosis de carga, que dependerá del fármaco, para llegar a la ventana terapéutica rápidamente con efectos secundarios minimos. Por vía oral, poseen una rápida y buena absorción, incluyendo pacientes veterinarios. Presentan una elevada unión a proteínas plasmáticas y una buena distribución por difusión pasiva pH dependiente, así como gran liposolubilidad.

Los AINEs se metabolizan por vía hepática, tienen buena biodisponibilidad (las excepciones notables son el diclofenaco: 54% y la aspirina: 70%), la unión proteica es reversible y extensa por lo que los volúmenes de distribución son muy pequeños, su excreción es fundamentalmente renal y, en su mayoría, en forma de metabolitos. La velocidad de absorción de los AINEs tiende a verse reducida con los alimentos, aunque en la mayoría de los casos se recomienda su administración con alimentos o antiácidos para minimizar el efecto negativo sobre la mucosa gástrica, sobre todo en tratamientos prolongados. A pesar de que tarda más en absorberse el medicamento con comida, el total absorbido no disminuye. Debido a que la unión proteica es saturable, a medida que la dosis aumenta, la concentración plasmática de naproxeno, salicilato, fenilbutazona y posiblemente de ibuprofeno aumentan de manera no proporcional a como lo hacen a dosis bajas.

3.1.3 Efectos adversos

El amplio uso de los antiinflamatorios no esteroideos significa un incremento en la prevalencia de los efectos adversos de estos medicamentos, que por lo general son muy seguros. Los efectos secundarios más frecuentes se relacionan con el sistema gastrointestinal y los riñones. Estos efectos son dependientes de la dosis administrada y, en muchos casos, lo suficientemente severos en ciertos grupos en la población, como para poner en riesgo sus vidas. Se estima que entre un 10 y 20% de los pacientes que toman AINEs presentan indigestión y que los efectos adversos causados por la administración de AINEs conllevan a más de 100 mil hospitalizaciones y unas 16 mil muertes cada año en los Estados Unidos.

3.1.4 Tipos y clasificación de los AINEs

La vida media de los AINEs es muy variable, razón por la que se los divide en tres grupos (Tabla 4).

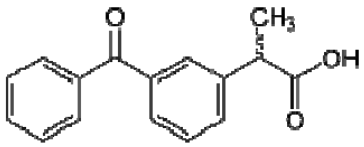
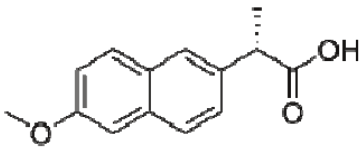
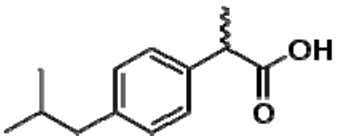
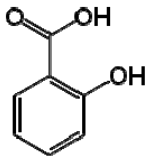
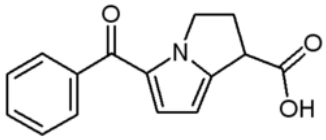
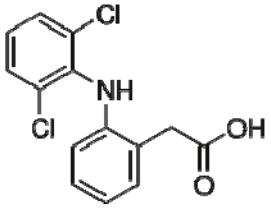
Tabla 12. Clasificación de los AINEs.

AINEs de vida media corta (<6 h)		
Aspirina	Diclofenaco	Etodolaco
Fenoprofeno	Ibuprofeno	Indometacina
Ketoprofeno		
AINEs de vida media intermedia (entre 6 y 10 h)		
Diflunisal	Fenbufen	Carprofeno
AINEs de vida media larga (>10 h)		
Nabumetona	Naproxeno	Fenilbutazona
Piroxicam	Sulindac	

3.1.5 Analitos estudiados

En esta Tesis se han puesto a punto diferentes procedimientos para la determinación de los siguientes antiinflamatorios no esteroideos (Tabla 5): ketoprofeno, naproxeno, ibuprofeno, ácido salicílico, ketorolaco y diclofenaco.

Tabla 13. Estructuras y propiedades de los AINEs.

Estructura	Características
	Ketoprofeno *(RS)2-(3-benzoylphenyl)-propionic acid* $C_{16}H_{14}O_3$ 254.281 g/mol [22071-15-4]
	Naproxeno *(+)-(S)-2-(6-methoxynaphthalen-2-yl) propanoic acid* $C_{14}H_{14}O_3$ 230,26 g/mol [22204-53-1]
	Ibuprofeno *(RS)-2-(4-(2-methylpropyl)phenyl)propanoic acid* $C_{13}H_{18}O_2$ 206,26 g / mol [15687-27-1]
	Ácido Salicílico *2-Hydroxybenzoic acid* $C_7H_6O_3$ 138,12 g/mol [69-72-7]
	Ketorolaco *(±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1 carboxylicacid, 2-amino-2-(hydroxymethyl)-1,3-propanediol* $C_{15}H_{13}NO_3$ 255.27 g/mol [74103-06-3]
	Diclofenaco *2-(2-(2,6-diclorophenylamino) phenyl)acetic acid* $C_{14}H_{11}NCl_2O_2$ 296.148 g/mol [15307-86-5]

3.1.6 Procedimientos analíticos previamente descritos

En la bibliografía se han encontrado que las técnicas analíticas más empleadas en la determinación de este tipo de compuestos, son, la cromatografía líquida [86, 274-276], cromatografía de gases [277,278] y electroforesis capilar (CE) [279-282].

3.2 Sulfonamidas

En 1932, trabajando con colorantes para teñir al *Estafilococo Aureus,* el científico alemán Gerhard Domagk descubrió que un colorante rojo (llamado posteriormente Prontosil Rubrum) protegía a los ratones y conejos contra dosis letales de estafilococos y estreptococos hemolíticos. Este Prontosil era un derivado de la sulfanilamida (p-aminobenzenosulfonamida) que había sido sintetizada por el químico vienés Gelmo en 1908.

Domagk no estaba seguro de que los resultados pudieran ser aplicables a los seres humanos, sin embargo, su propia hija se enfermó gravemente de una infección estafilocóccica y Domagk en un momento de desesperación decidió administrarle una dosis de Prontosil que le permitió recuperarse completamente.

En el año 1935 se realizaron experimentos clínicos controlados y se descubrió que el Prontosil era metabolizado a sulfanilamida (Figura 10), un compuesto con una excelente actividad antibacteriana en humanos y con esta base se desarrollaron posteriormente nuevos fármacos que se englobaron dentro del grupo de las “sulfas”.

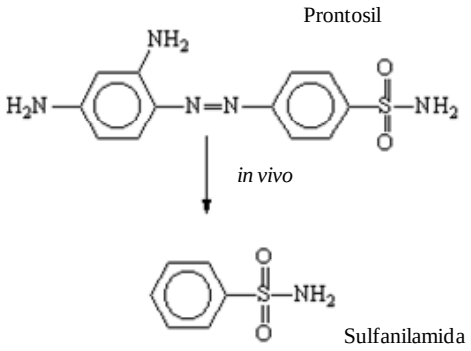


Figura 10. Estructura de la sulfanilamida.

Las sulfonamidas son quimioterápicos sintéticos derivados de la para-aminobencenosulfonamida (sulfanilamida) caracterizados por un núcleo bencénico con un grupo sulfonamido (SO_2NH_2) y otro amino (NH_2) que le confiere a la mólecula su actividad antibacteriana (Figura 11). Para mantener la actividad antibacteriana es

esencial que el grupo amino en posición 4 quede libre. Las sustituciones en el radical sulfónido (-SO_2-) no alteran la actividad bacteriostática sino que modifican las propiedades farmacocinéticas.

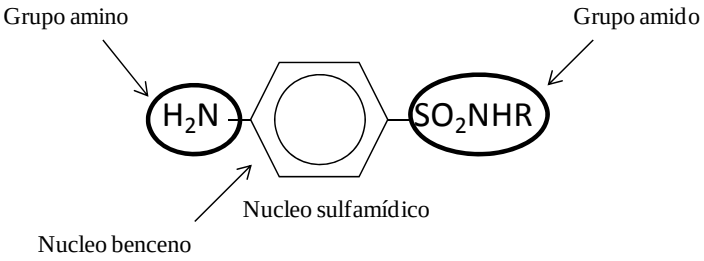


Figura 11. Estructura y configuración de las sulfonamidas.

3.2.1 Farmacocinética

En general, presentan buena absorción por vía oral (entre 70 y 100%) y las concentraciones máximas en plasma se obtienen entre 2 y 6 horas tras su administración. Se unen en diferente grado a las proteínas plasmáticas, especialmente a la albúmina, y se distribuyen por el agua corporal y todos los tejidos del cuerpo penetrando los espacios pleural, peritoneal, sinovial y ocular en concentraciones cercanas a las séricas. La sulfadiazina y el sulfisoxazol penetran el líquido cefalorraquídeo. Las sulfonamidas atraviesan la placenta y pasan a la circulación fetal. Sufren metabolismo principalmente hepático, produciendo metabolitos no activos pero que sí poseen toxicidad y son eliminadas principalmente por el riñón ya sea sin ser metabolizadas o como metabolitos inactivos, mientras que pequeñas cantidades son eliminadas por las heces y bilis.

La mayoría de las sulfamidas se absorben rápidamente en el tubo digestivo (estómago e intestino, sobre todo delgado) en forma no ionizada. Por otra vía (rectal, piel o mucosas), la absorción es reducida, aunque se detectan niveles en sangre con la administración tópica cutánea. Tras la administración oral se alcanzan concentraciones máximas en sangre al cabo de 2- 4 horas, con valores de 50-150 mg/l.

En general, las sulfamidas se distribuyen bien por todos los tejidos y líquidos, incluyendo LCR, sinovial, pleural y peritoneal, alcanzando el 30-80 % de los niveles plasmáticos. Atraviesan la barrera placentaria con niveles detectables en sangre fetal y líquido amniótico. Se detectan pequeñas cantidades en bilis, secreción prostática, saliva, sudor, lágrimas y leche.

La unión a proteínas es muy variable, desde el 22% para el sulfatiazol hasta el 98% para la sulfadoxina, siendo generalmente menor en las sulfamidas de semivida corta. Sufren metabolización hepática mediante N-acetilación, glucuronidación e hidroxilación; tanto el fármaco activo como sus metabolitos se eliminan por orina.

Las sulfamidas son activas frente a un amplio espectro de bacterias, tanto grampositivas como gramnegativas, así como frente a *Chlamydia, Plasmodium, Toxoplasma, Mycobacterium leprae, Histoplasma capsulatum y Paracoccidioides brasiliensis.* Las Sulfonamidas son activas contra: el *Estreptococo Piógenes, Neumococo, Hemophilus Influenzae, Hemophilus Ducrei, Clamidia Trachomatis, Nocardia, Actinomyces, Calymmatobacterium Granulomatis, Toxoplasma* y *Plasmodium*.

Son de elección en el tratamiento de nocardiosis a dosis altas (6-8 g/día durante 4-6 meses o más). Aunque no son los fármacos de elección, se pueden utilizar también en infecciones producidas por *Chlamydia H. influenzae*, dermatitis herpetiforme y en asociación con otros fármacos en infecciones por protozoos *Plasmodium y Pneumocystis carinii*.

La sulfasalazina se usa en el tratamiento de la colitis ulcerosa. El mafénido y la sulfadiazina argéntica se usan tópicamente en quemaduras. La vía de elección es la oral; por vía intravenosa producen con frecuencia flebitis y por vía intramuscular irritación local importante. También pueden aplicarse localmente en piel y mucosas, 3-4 veces al día en la zona afecta. Están contraindicadas en casos de hipersensibilidad a las sulfamidas, fracaso renal, insuficiencia hepática, últimos trimestres del embarazo, neonatos, prematuros, déficit de G-6-PD y hemoglobinopatías.

3.2.2 Sulfonamidas estudiadas

En esta Tesis se han puesto a punto procedimientos analíticos para la determinación de sulfamerazina, sulfadiazina, sulfametazina, sulfametoxazol y sus correspondientes N^4-acetil metabolitos (Tabla 14).

Tabla 14. Estructuras y propiedades de las sulfonamidas estudiadas y correspondientes metabolitos.

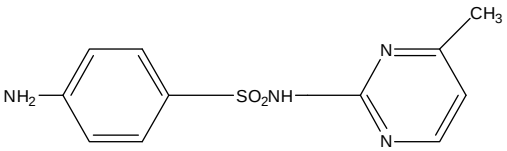
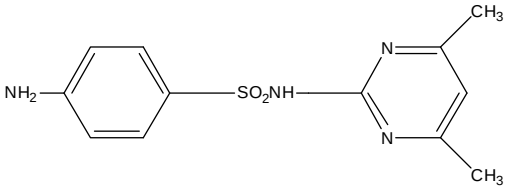
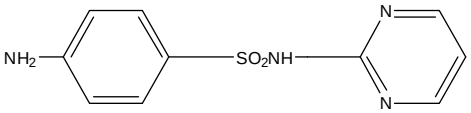
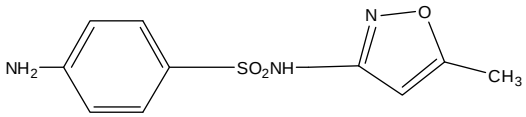
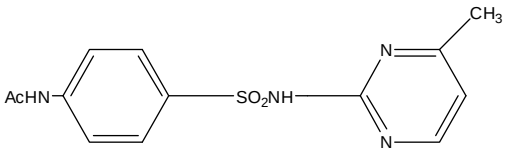
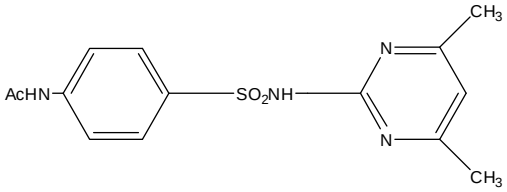
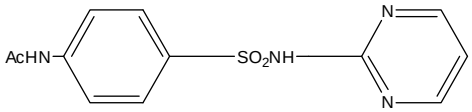
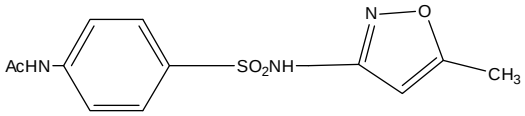
Estructura	Características
	Sulfamerazina *Benzenesulfonamide, 4-amino-N-(4-methyl-2-pyrimidinyl)* $C_{11}H_{12}N_4O_2S$ 264.30 g/mol [127-79-7]
	Sulfametazina *Benzenesulfonamide, 4-amino-N-(4,6-dimethyl-2-yrimidinyl)* $C_{12}\ H_{14}\ N_4\ O_2\ S$ 278.33 g/mol [57-68-1]
	Sulfadiazina *Benzenesulfonamide, 4-amino-N-2-pyrimidinyl* $C_{10}\ H_{10}\ N_4\ O_2\ S$ 250.28 g/mol [68-35-9]
	Sulfametoxazole *Benzenesulfonamide, 4-amino-N-(5-methyl-3-isoxazolyl)* $C_{10}\ H_{11}\ N_3\ O_3\ S$ 253.28 g/mol [723-46-6]

Tabla 14. Continuación.

Estructura	Características
	N^4-acetilsulfamerazina *Acetamide, N-[4-[[(4-methyl-2 pyrimidinyl)amino]sulfonyl] phenyl]-* $C_{13}H_{14}N_4O_3S$ 306.34 g/mol [127-73-1]
	N^4-acetilsulfametazina *Acetamide, N-[4-[[(4,6-dimethyl-2 pyrimidinyl)amino] sulfonyl]phenyl]-* $C_{14}H_{16}N_4O_3S$ 320.37 g/mol [100-90-3]
	N^4-acetilsulfadiazina *Acetamide, N-[4-[(2-pyrimidinylamino)sulfonyl]phenyl]-* $C_{12}H_{12}N_4O_3S$ 292.31 g/mol [127-74-2]
	N^4-acetilsulfametoxazol *Acetamide, N-[4-[[(5-methyl-3-isoxazolyl)amino] sulfonyl]phenyl]-* $C_{12}H_{13}N_3O_4S$ 295.31 g/mol [21312-10-7]

3.2.3 Procedimientos analíticos previamente descritos

Se han descrito numeroso métodos para la determinación de sulfonamidas: fotométricos [283], el método de Bratton-Marshall [284-285], métodos de análisis volumétrico [286], cromatografía en capa fina [287], FIA con detección amperométrica [288], ELISA [289], cromatografía de líquidos con detección UV y fluorescencia [290-294], cromatografía de gases y cromatografía líquida acoplada a espectrometría de masas [295-297] y electroforesis capilar [298-300].

3.3 Fluoroquinolonas

Las fluoroquinolonas son un grupo de compuestos relacionados con el ácido nalidíxico, fármaco sintetizado a partir de la cloroquina en 1962; desde entonces se han desarrollado y comercializado un gran número de sustancias pertenecientes a esta familia. A finales de los 70, se empezaron a usar las denominadas quinolonas de segunda generación, que contenían un átomo de flúor, siendo compuestos más activos y con mayor espectro de acción.

En 1978 se comercializó la primera fluoroquinolona, la norfloxacina, de mayor potencia y espectro antibacteriano que las quinolonas originales. Con posterioridad surgieron ciprofloxacina (1987), ofloxacina (1991), enoxacina, lomefloxacina y temafloxacina (1992), levofloxacina y sparfloxacina (1997), trovafloxacina y grepafloxacina (1998), gatifloxacina y moxifloxacina (1999). Algunas tuvieron que ser retiradas del mercado debido a sus efectos tóxicos (sparfloxacina, trovafloxacina, grepafloxacina). Hoy en día se está definiendo una tercera generación de quinolonas bi y trifluoradas.

La Figura 12 presenta la estructura general de una fluoroquinolona. Las quinolonas tienen como núcleo común la 4-oxo-1,4-dihidroquinoleína. El sustituyente R (carbono 7) es, generalmente, un anillo de piperazina, o derivado, en el caso de una fluoroquinolona.

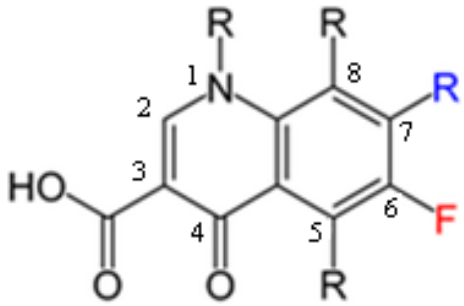


Figura 12. Estructura y configuración de las fluoroquinolonas.

El nitrógeno en posición 1 y el grupo carboxilado en posición 3 son indispensables para la actividad antibacteriana. Las fluoroquinolonas poseen un átomo de flúor en posición 6 y un anillo de piperacina (norfloxacino, ciprofloxacino, enoxacino) o de metilpiperacina (ofloxacino, pefloxacino, amifloxacino) en posición 7. Se diferencian entre sí por el radical en la posición N-1 del núcleo principal y por el radical unido al grupo piperacina. Las posiciones 5 y 8 pueden no estar sustituidas, o soportar grupos pequeños, metilo o aminas en posición 5, un halógeno (lomefloxacino, fleroxacino, esparfloxacino) o un grupo metoxi en posición 8; este lugar puede estar ocupado por un átomo de nitrógeno, de forma que el núcleo quinolona se transforma en naftiridona (ácido nalidíxico, enoxacino, trovafloxacino).

Algunos de esos radicales se relacionan directamente con diferentes características de cada molécula. El grupo carboxilo en posición 3 y la cetona en la posición 4 son los lugares de unión al ADN y a la ADN-girasa. El flúor en posición 6 aumenta la capacidad de penetración al interior de la bacteria y mejora la afinidad por la girasa. Los sustituyentes en posición 7 amplían el espectro, sobre todo frente a bacterias gramnegativas y especialmente frente a *P. aeruginosa*; si este sustituyente es el grupo 3-azabiciclo se incrementa de forma considerable la actividad frente a cocos grampositivos (trovafloxacino). El grupo difluorofenil en posición N-1 confiere buena actividad sobre bacterias anaerobias. También se ha relacionado algunos de estos radicales con diferencias farmacocinéticas; así, las quinolonas con un grupo metilpiperacina en posición 7 o las que poseen un núcleo naftiridona presentan vidas medias de eliminación más prolongadas. El anillo piperacina en posición 7 es el punto principal de degradación del fármaco a través de las rutas metabólicas. La neurotoxicidad se ha relacionado con el anillo piperacina sin ningún sustituyente en posición 7 del núcleo principal (ciprofloxacino, enoxacino, norfloxacino) y la fotosensibilidad, fundamentalmente con el halógeno situado en posición 8.

3.3.1 Mecanismo de acción

Las fluoroquinolonas son antibióticos diana de la topoisomerasa II (ADN girasa) y la topoisomerasa IV. Para muchas bacterias grampositivas como el *estafilococo aureus*, la topoisomerasa IV es la diana para las fluoroquinolonas. En contraste, para muchas bacterias gramnegativas como *escherichia coli*, la ADN girasa

es el blanco primario de la fluoroquinolona. La ADN girasa, en condiciones normales, es la que permite la relajación de la cadena helicoidal de ADN para su transcripción y duplicación; la topoisomerasa IV es la encargada de la separación del cromosoma replicado durante la división celular. Las fluoroquinolonas interfieren con la acción de estas dos enzimas produciendo acción bactericida sobre los organismos susceptibles.

Actúan en el interior de la bacteria penetrando a través del canal acuoso de las porinas. Son los únicos agentes antibacterianos que ejercen su actividad bactericida uniéndose a topoisomerasas bacterianas (enzimas que controlan el superenrollamiento y desenrollamiento del ADN bacteriano) e inhibiéndolas, aunque éste no sería el único mecanismo de acción. La compleja interacción de las quinolonas con las topoisomerasas es la base de su diferente espectro antibacteriano.

Las quinolonas de primera generación son activas frente a microorganismos gramnegativos, con excepción de *Pseudomonas* spp. y otros bacilos gramnegativos no fermentadores. Las quinolonas de segunda generación son fármacos predominantemente activos frente a bacterias gramnegativas; también tienen buena actividad contra algunos gérmenes grampositivos y micobacterias. La ciprofloxacina es la más activa contra *Pseudomonas aeruginosa*, sin embargo, su actividad frente a *Acinetobacter* y *S. maltophilia* es moderada. Estas fluoroquinolonas son activas contra *S. aureus*, pero tiene escasa actividad frente a *S. pneumoniae* y otras especies de *Streptococcus*. Su actividad es escasa contra *Enterococcus* spp y tienen baja actividad contra anaerobios. Las de tercera y cuarta generación mantienen la buena actividad de las de segunda generación frente a gramnegativos y micobacterias, pero presentan mejor actividad frente a grampositivos, anaerobios y patógenos "atípicos". Las quinolonas más recientes (levofloxacina y moxifloxacina) tienen buena actividad frente a cocos grampositivos, incluyendo cepas de *S. pneumoniae* resistentes a penicilina y *S. Aureus* meticilinosensible.

Aunque las primeras quinolonas tenían actividad sólo contra bacterias aerobias gramnegativas y eran eficaces para tratar infecciones gastrointestinales y urinarias, las nuevas quinolonas actúan contra un mayor número de infecciones gracias a su amplio espectro de actividad, su buena biodisponibilidad y penetración tisular. Como consecuencia de su uso extensivo, en los últimos años se ha observado un incremento

progresivo de cepas resistentes. El amplio espectro de actividad de las fluoroquinolonas permite su uso en una amplia variedad de infecciones de aparato urinario, piel, partes blandas, hueso y aparato respiratorio.

3.3.2 Farmacocinética

Las fluoroquinolonas se absorben bien en el tracto gastrointestinal superior después de su administración oral, mostrando marcada reducción de la biodisponibilidad cuando se las coadministra por vía oral con antiácidos que contienen aluminio, magnesio o calcio, y con sales de hierro o zinc, debido a la formación de complejos catión-quinolona que se absorben escasamente. Las fluoroquinolonas tienen una biodisponibilidad que supera el 50% en todos los compuestos y se aproxima a 100% en algunos. Así, la norfloxacina y ciprofloxacina solo se absorben el 50 y 70%, respectivamente, mientras que el resto de fluoroquinolonas presentan una absorción entre el 97 y el 100%. Esta alta biodisponibilidad permite el tratamiento por vía oral o el rápido cambio de administración de vía parenteral a oral cuando las condiciones del paciente lo permiten.

Por lo general se alcanzan las mayores concentraciones séricas entre 1 a 3 horas después de haber sido administrada. Los alimentos no reducen de manera sustancial la absorción de las quinolonas, pero pueden prolongar el tiempo en que se alcanza la concentración sérica máxima, por lo que es necesario separar su administración al menos en 2 horas con la ingestión de alimentos.

La eliminación se produce principalmente por vía renal, como fármaco inalterado, en el caso de ofloxacina y lomefloxacina. La eliminación biliointestinal es predominante en el caso de pefloxacina. Algunos de los metabolitos pueden sufrir circulación entero-hepática. Ciprofloxacina, enoxacina, fleroxacina y norfloxacina presentan una eliminación mixta, renal y biliar. Como consecuencia, en casi todos los casos se alcanzan altos niveles urinarios.

3.3.3 Resistencia

Se ha observado un rápido desarrollo de cepas resistentes a las fluoroquinolonas de segunda generación, especialmente en c*ampylobacter jejuni* y el gonococo, pero también en cocos grampositivos como el MRSA, *Pseudomonas*, y *Serratia*. Los mecanismos de resistencia incluyen la acumulación intracelular disminuida del principio activo por la producción de bombas de flujo o cambios en la estrictitas de las porinas (en el caso de los Gramnegativos). Los mecanismos de bomba de flujo extracelular parecen ser responsables de la resistencia de las cepas de *micobacteria tuberculosis, estafilococo aureus*, y *estreptococo pneumoniae*. Cambios en la sensibilidad de la enzima "diana" por las mutaciones en las regiones de unión en las fluoroquinolonas confieren resistencia a ciertos organismos específicos en contra de estos fármacos.

3.3.4 Uso clínico

Las fluoroquinolonas son efectivas en el tratamiento de infecciones urogenitales y del tracto genital causadas por organismos gramnegativos que incluyen el *gonococo*, e*scherichia coli*, k*lebsiella pneumoniae*, c*ampylobacter jejuni*, e*nterobacter, pseudomonas aeruginosa*, *salmonella*, y s*higuella*. Han sido también usadas ampliamente en el tratamiento de infecciones de tracto respiratorio, piel, e infecciones de tejido suave, pero su efectividad es actualmente muy variable por los ya comentados fenómenos de resistencia que se producen.

Ciprofloxacina y ofloxacina son alternativas a las cefalosporinas de tercera generación en el tratamiento de la gonorrea siendo administradas en dosis oral única. Levofloxacina tiene buena actividad frente a organismos asociados con la neumonía hospitalaria, que incluye agentes como el *m. pneumoniae*. La esparfloxacina tiene actividad incrementada contra los grampositivos, incluyendo el *pneumococo* resistente a penicilina, pero tiene efectos mínimos en contra de las infecciones con pseudomonas. moxifloxacina y trovafloxacina, fluoroquinolonas de cuarta generación, tienen mayor espectro de acción, incluyendo a bacterias grampositivas y gramnegativas y bacterias anaeróbicas.

3.3.5 Toxicidad

Las fluoroquinolonas, en sentido general, son fármacos bien tolerados. El efecto adverso más común es el distress gastrointestinal, donde 3-17% de los pacientes llegan a mostrar nausea moderada, vómitos, y/o disconfort abdominal. Pueden producir también rashes, dolores de cabeza, mareos, insomnio, función hepática anormal, fototoxicidad, y tendinitis y ruptura de tendón. No se suelen usar en los niños o durante el embarazo porque causan problemas con los cartílagos en desarrollo.

Las fluoroquinolonas pueden incrementar los niveles plasmáticos de teofilina y otras metilxantinas, aumentando su toxicidad. La esparfloxacina prolonga los intervalos de las ondas QT con posible riesgo de arritmia cardíaca, y está asociada a alta incidencia de fotosensibilidad. El trovafloxacina tiene potencial hepatotóxico. La grepafloxacina, una fluoroquinolona de tercera generación, fue retirada de su uso clínico en Estados Unidos por su seria cardiotoxicidad.

3.3.6 Efectos adversos

Inicialmente fueron considerados antibióticos muy seguros, pero varios efectos adversos se hicieron evidentes con su uso, entre los que se puede destacar la aparición de numerosos casos de rotura espontánea de tendones, especialmente cuando se usaban conjuntamente con corticoides. El uso de fluoroquinolonas en aves de corral y ganado hizo posible que cepas de bacterias resistentes a los antibióticos y agentes patógenos en los animales pasasen a los humanos.

3.3.7 Tipos y clasificación

Las fluoroquinolonas han sido clasificadas por generación basadas en su actividad antimicrobiana dependiendo de su espectro. Las quinolonas se dividen en dos grandes grupos: las viejas quinolonas representadas por el ácido nalidíxico y cinoxacino y las nuevas quinolonas o fluorquinolonas. Una clasificación más actual, divide a las fluoroquinolonas en cuatro generaciones (Tabla 15):

Tabla 15. Clasificación de las Fluoroquinolonas.

Fluoroquinolonas de primera generación		
Flumequina		
Fluoroquinolonas de segunda generación		
Norfloxacina	Ciprofloxacina	Pefloxacina
Enoxacina	Fleroxacina	Rufloxacina
Lomefloxacina	Ofloxacina	
Fluoroquinolonas de tercera generación		
Tosufloxacina	Levofloxacina	Esparfloxacina
Gatifloxacina	Pazufloxacina	Grepafloxacina
Fluoroquinolonas de cuarta generación		
Clinafloxacina	Moxifloxacina	Trovafloxacina
Sitafloxacina	Gemifloxacina	

3.3.8 Analitos estudiados

En esta Tesis se han puesto a punto procedimientos analíticos para la determinación de las siguientes fluoroquinolonas (Tabla 16): marbofloxacina, norfloxacina, ciprofloxacina, danofloxacina, enrofloxacina, gatifloxacina, grepafloxacina y flumequina. La flumequina es una fluoroquinolona de primera generación, con amplio espectro, y suele utilizarse en la medicina veterinaria para el tratamiento de las infecciones entéricas. A veces es utilizada en medicina humana para el tratamiento de infecciones del tracto urinario. La ciprofloxacina es una fluoroquinolona de segunda generación, y su uso está limitado a medicina veterinaria. La marbofloxacina se utiliza en veterinaria bajo el nombre de Marbocyl y Zeniquin y puede ser suministrada tanto por vía oral como por vía tópica, es especialmente utilizada para las infecciones de la piel, el sistema respiratorio y las glándulas mamarias. La danofloxacina se utiliza en el tratamiento de enfermedades respiratorias en pollos, ganado y cerdos; no está destinada para su uso en vacas lecheras que producen leche para consumo humano. La enrofloxacina tiene actividad antibacteriana contra un amplio espectro de bacterias gramnegativas y grampositivas. El norfloxacino presenta un amplio espectro, indicado casi exclusivamente en el tratamiento de las infecciones

urinarias como las cistitis y pielonefritis, aunque a veces se indica en el tratamiento de ciertas infecciones estomacales, gonorrea no complicada y prostatitis. El gatifloxacino pertenece al grupo de las quinolonas de tercera generación y está indicado en el tratamiento de infecciones causadas por bacterias, incluyendo ciertos tipos de gonorrea y ciertas infecciones en los pulmones, senos paranasales, piel y tracto urinario. La grepafloxacina es una fluoroquinolona que fue retirada del mercado de Estados Unidos en 1999.

Tabla 16. Estructura de las fluoroquinolonas estudiadas.

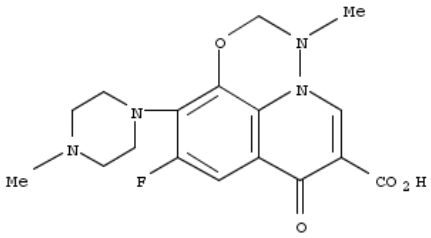
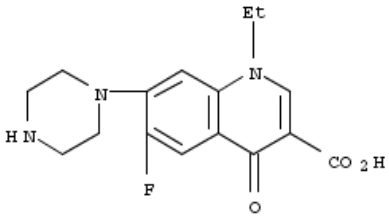
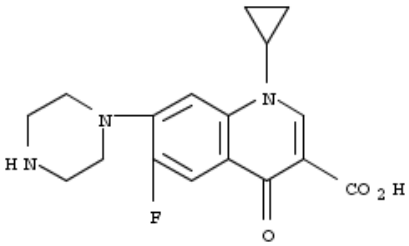
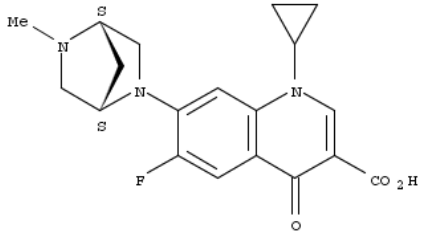
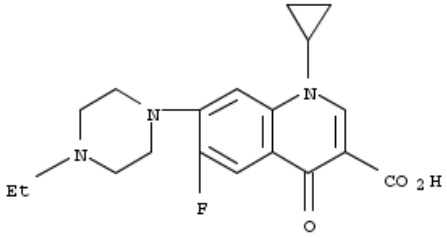
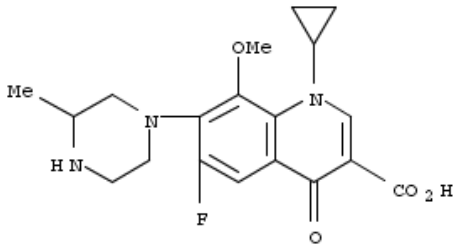
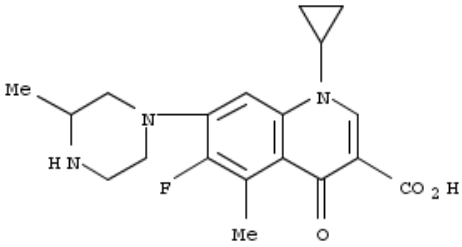
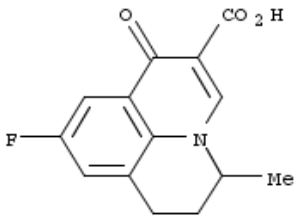
Estructura	Características
	Marbofloxacina *9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1 piperazinyl)-7-oxo-7H-pyrido[3,2,1-ij][4,1,2]benzoxadiazine-6-carboxylic acid* $C_{17}H_{19}FN_4O_4$ 362.36 g/mol [115550-35-1]
	Norfloxacina *1-Ethyl-6-fluoro-1,4-dihydro-7-(1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid* $C_{16}H_{18}FN_3O_3$ 319.34 g/mol [70458-96-7]
	Ciprofloxacina *1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid* $C_{17}H_{18}FN_3O_3$ 331.346 g / mol [85721-33-1]
	Danofloxacina *1-cyclopropyl-6-fluoro-1,4-dihydro-7-[(1S,4S)-5-methyl-2,5-diazabicyclo[2.2.1]hept-2-yl]-4-oxo-3-quinolinecarboxylic acid* $C_{19}H_{20}FN_3O_3$ 357.37 g/mol [112398-08-0]

Tabla 16. Continuación.

Estructura	Características
	Enrofloxacina *1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid* $C_{19}H_{22}FN_3O_3$ 359.4 g/mol [93106-60-6]
	Gatifloxacina *1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid* $C_{19}H_{22}FN_3O_4$ 375.40 g/mol [112811-59-3]
	Grepafloxacina *1-cyclopropyl-6-fluoro-1,4-dihydro-5-methyl-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid* $C_{19}H_{22}FN_3O_3$ 359.4 g/mol [119914-60-2]
	Flumequina *9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-Benzo[ij]quinolizine-2-carboxylic acid* $C_{14}H_{12}FNO_3$ 261.25 g/mol [42835-25-6]

3.3.9 Procedimientos analíticos previamente descritos

Se ha encontrado en la bibliografía aplicaciones analíticas para la determinación de drogas en muestras biológicas, llevadas a cabo principalmente por cromatografía líquida de alta resolución con detección UV [301,309], fluorescencia [302-309], detección electroquímica [310], o acoplada a espectrómetros de masas [306, 311-315]. Además de diversos procedimientos de limpieza previa a su análisis, como por ejemplo, extracción en fase sólida [309,312, 315-319], extracción en fase sólida on-line [311], extracción acelerada con disolventes [320-322] o mediante polímeros con impresión molecular [313,314].

CAPÍTULO 2

MATERIALES Y REACTIVOS

A continuación se relacionan los reactivos y disolventes empleados así como los instrumentos y equipos usados para la determinación de los principios activos estudiados y los metabolitos sintetizados.

1. REACTIVOS

- Acetonitrilo HPLC gradient grade (Prolabo)

- Metanol HPLC gradient grade (Prolabo)

- Etanol (Merck)

- Ácido fórmico 98-100% GR P.A. (Merck)

- Ácido clorhídrico 37% P.A. (Merck)

- Ácido fosfórico P.A. (Panreac)

- Ácido nítrico GR P.A. (Merck)

- Ácido sulfúrico GR P.A. (Merck)

- Agua destilada calidad Alpha Q. (Millipore, Milford, MA, USA)

- Amoniaco 30% P.A. (Panreac)

- Diclorometano ROMIL-Sps™ Super Purity Solvent

- Piridina (Sigma-Aldrich)

- Ninhidrina (Sigma-Aldrich)

- Anhídrido acético (Sigma-Aldrich)

- Glicerina (Sigma-Aldrich)

- Bicarbonato sódico (Sigma-Aldrich)

- Sulfato de magnesio (Sigma-Aldrich)

- Sulfito sódico (Sigma)

- Permanganato potásico P.A. (Merck)

- Sulfato sódico P.A. (Aldrich)

- Helio 99,98% (Air Liquide)

- Nitrógeno N-50 (Alpha gaz)

- Hidróxido sódico P.A. (Merck)

- 2-Propanol (Merck)

- 1-Octanol (Fluka-Sigma-Aldrich)

- Dihexiléter (Fluka-Sigma-Aldrich)

- Nitrofenil octil eter (Fluka-Sigma-Aldrich)

- 1-heptanol (Fluka-Sigma-Aldrich)

- Alcohol bencílico (Fluka-Sigma-Aldrich)

- Ciclohexanol (Fluka-Sigma-Aldrich)

- Ácido nonafluoropentanoico (DEHPA) 97% (Fluka)

- Aliquat 336 (Sigma-Aldrich)

2. MATERIALES

- Agitador magnético FB15107 (Fisher Scientific, Pittsburgh, PA, USA).

- Agitador magnético ANS-00/1 Science Basic Solutions (Rubí, Barcelona, España).

- Agitador magnético Variomag Electronicruher, RO 10 power; (IKA-Werke, Staufen, Alemania).

- Balanza analítica de 0.1 mg de precisión (Sartorius, Goettingen, Alemania).

- Bomba de vacío (Millipore, Billerica, MA, USA).

- Baño de ultrasonido con termostato, Ultrasons (Selecta, Barcelona, España).

- Electrodos de platino 0.25 mm.

- Columnas cromatográficas:

  - Eclipse® XDB-C18 3.5µm (150mm×3.0mm i.d.) (Agilent., Palo Alto, CA (USA)).

  - Pursuit® XRs Ultra 2.8µm C18 (100×2.0mm i.d.) (Varian Inc., Palo Alto, CA, USA).

  - Monolithic silica type HPLC column Chromolith® Performance RP-18e (100–4.6mm i.d.) (VWR, Darmstadt, Germany).

  - Phenomenex® Prodigy ODS(3) 100 Å de 250 x 4.6 mm I.D. (5 µm de tamaño)

  - LiChroCART® 250-4 LiCrospher® 100 RP-18 de 250 x 4 mm I.D. (5 µm de tamaño) (Merck®).

  - Phenomenex® con relleno Gemini C-18 (110 Å) de 150 x 4.6 mm I.D. (5 µm de tamaño de partícula).

  - LiChroCART® 75-4 Purosphere® STAR RP-18e 3 µm (75 x 4.0 mm i.d.) (VWR, Darmstadt, Germany).

  - Kromasil C8 5 µm (150 x 3.0 mm i.d.) (Merck®).

  - RP-C18 5 µm (250 x 4.6 mm i.d.) (ACE).

  - Genesis AQ 120A 4µm (50 x 2.1 mm i.d.) (Grace Vydac-Jones Chromatography, Brockville, Ontario, Canada).

- Congelador (con temperatura de congelación mínima de -80ºC).

- Sistema de evaporación por corriente de nitrógeno.

- Conjunto de microfiltración entre 1-10 µm (Whatman, Mainstone, UK) y 0.45 µm (Pall Corporation, Ann Arbor, MI, USA).

- Equipo de filtración (Supelco).

- Equipo de purificación de agua Milli-Q (Millipore, Bedford, MA; USA).

- Jeringas estériles de polipropileno de 1 ml (Acofarma).

- Microjeringa (Hamilton Bonaduz).

- Embudos de decantación.

- Micropipetas graduadas de 1-10 µl, 1-100 µl y 100-1000 µl.

- Papel de filtro Albet (20 – 25 µm de tamaño de poro).

- Pipetas graduadas de 1, 5 y 10 mL.

- Pipetas Pasteur.

- Viales de 10 ml (VWR International, West Chester, Pensilvania, USA).

- Fibra hueca de polipropileno Accurel Q 3/2 (Membrana, Wuppertal, Alemania).

- Fibra hueca de polipropileno Accurel S6/2 (Membrana, Wuppertal, Alemania).

Todo el material volumétrico empleado se mantiene durante 24 horas sumergido en mezcla oxidante y luego se lava repetidas veces con agua desionizada de calidad P.A.

3. INSTRUMENTACIÓN

Además de las instalaciones generales del laboratorio (campanas extractoras, instalaciones de gases, sistema de vacío, etc.) se utilizan los siguientes equipos y aparatos:

- Cromatógrafo líquido de alta resolución LaChrom®VWR-Hitachi (Barcelona, Spain) equipado con una bomba cuaternaria L-2130, un compartimiento termostatizado de columna, un detector UV-DAD y un detector de Fluorescencia programable con

disposición en línea. El inyector fue de tipo Rheodyne (Model 7725i) con un loop de muestra de 20 μL. Se usaron diferentes columnas cromatográficas para la optimización y separación de las diferentes familias estudiadas equipadas con una precolumna previa.

- Espectrofotómetro de luminiscencia Modelo Varian Cary Eclipse. El equipo consta de dos monocromadores Czerny-Turner (excitación-emisión), una lámpara de Xenón, selectores de filtro, atenuadores y dos tubos fotomultiplicadores como detectores. El equipo se conecta mediante una tarjeta GPIB IEE-488 a un PC.

- Espectrofotómetro de absorción UNICAM UV 500. ThermoSpectrometric.

- Sistema cromatográfico acoplado a un espectrómetro de masas de triple cuadrupolo API 2000 (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipado con una fuente Turbo HS-602, y una fuente de ionización Electrospray (ESI). Modos de ionización: positivo y negativo.

- Sistema FIA acoplado con una bomba peristáltica Miniplus 3 (Gilson, Inc., Middleton, WI, USA) y un detector de quimioluminiscencia (Model CL2 (Camspec, Cambridge, UK)).

- Fuente de electrophoresis Power Source 300V (VWR International, West Chester, Pensilvania, USA).

- Sistema cromatográfico acoplado a un espectrómetro de masas de trampa iónica LCQ, Spectra Physics P2000 y una fuente de ionización Electrospray (Thermo Finnigan, San Jose, CA; USA).

- Cromatógrafo líquido de alta resolución Agilent- Technologies Serie 1100 equipado con bomba, sistema desgasificador, compartimiento termostatizado de columna, detector UV-DAD y detector de Fluorescencia programable con disposición en línea.

- Sistema de electroforesis capilar Beckman P/ACE MDQ equipado con un detector de diodo array (Beckman-Coulter, Fullerton, CA, USA).

- pH-metro PHM 210 (Meterlab).

4. DISOLUCIONES PATRÓN

Todos los principios activos (entre el 97 y 100% de pureza) fueron suministrados por Sigma-Aldrich (Steinheim, Alemania).

Los principales metabolitos de las sulfonamidas: N^4-acetilsulfamerazina, N^4-acetilsulfametazina, N^4-acetilsulfadiazina y N^4-acetilsulfametoxazol se sintetizaron en nuestro laboratotio.

5. SÍNTESIS DE METABOLITOS

Se sintetizaron los N^4-acetil derivados de las sulfonamidas estudiadas:

El procedimiento seguido para la síntesis de dichos metabolitos se basa en el descrito por Pfeifer y Briefly [323], aunque con algunas modificaciones. El procedimiento consiste en pesar en un matraz de fondo redondo aproximadamente 100 mg del principio activo correspondiente al metabolito que se pretende obtener y se le añade 1.5 ml de piridina y 1.5 ml de anhídrido acético. Se agita y calienta a una temperatura de 40 ºC en un baño de glicerina, durante 6 horas y media, 7 horas, 3 horas y 6 horas para los casos de sulfadiazina, sulfamerazina, sulfametazina y sulfametoxazole, respectivamente.

Tras la reacción se le añade agua-hielo y se realiza una extracción líquido-líquido con 20 ml de diclorometano agitando vigorosamente. Se deja decantar y separar ambas fases. El proceso se repite dos veces más con nuevas cantidades de diclorometano y finalmente se reúnen los extractos orgánicos.

Los extractos orgánicos se lavan con dos porciones de 20 ml de H_2SO_4 2N y una porción de 20 ml de $NaHCO_3$ saturado. El extracto orgánico se seca con $MgSO_4$. La disolución se filtra y se concentra a vacío.

El proceso de reacción, extracción y limpieza fue controlado mediante cromatografía en capa fina sobre sílice, usando como eluyente una mezcla diclorometano:metanol (10:1) y posterior detección de los compuestos por exposición

de las placas a una fuente de luz UV (λ=254 nm) y mediante revelado por inmersión en disolución de ninhidrina al 0.1% en etanol y calentamiento a 100ºC.

Las fracciones de extracto obtenidas se purifican mediante cromatografía en columna. Se usó una columna con relleno de silica gel y posterior elución con una mezcla diclorometano:metanol (50:1) recogiendo las fracciones de interés para su posterior concentración y evaporación del disolvente, primero en rotavapor y después bajo corriente de nitrógeno. El proceso se controló mediante cromatografía en capa fina utilizando placas de sílica, al igual que en el proceso de reacción y extracción.

La confirmación de la composición y pureza de los compuestos sintetizados se realizó mediante espectrometría de masas por bombardeo con átomos rápidos (FAB) en el Servicio de Espectrometría de Masas del Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS).

En la Figura 13 se muestran las reacciones globales de síntesis de los N^4-acetil metabolitos.

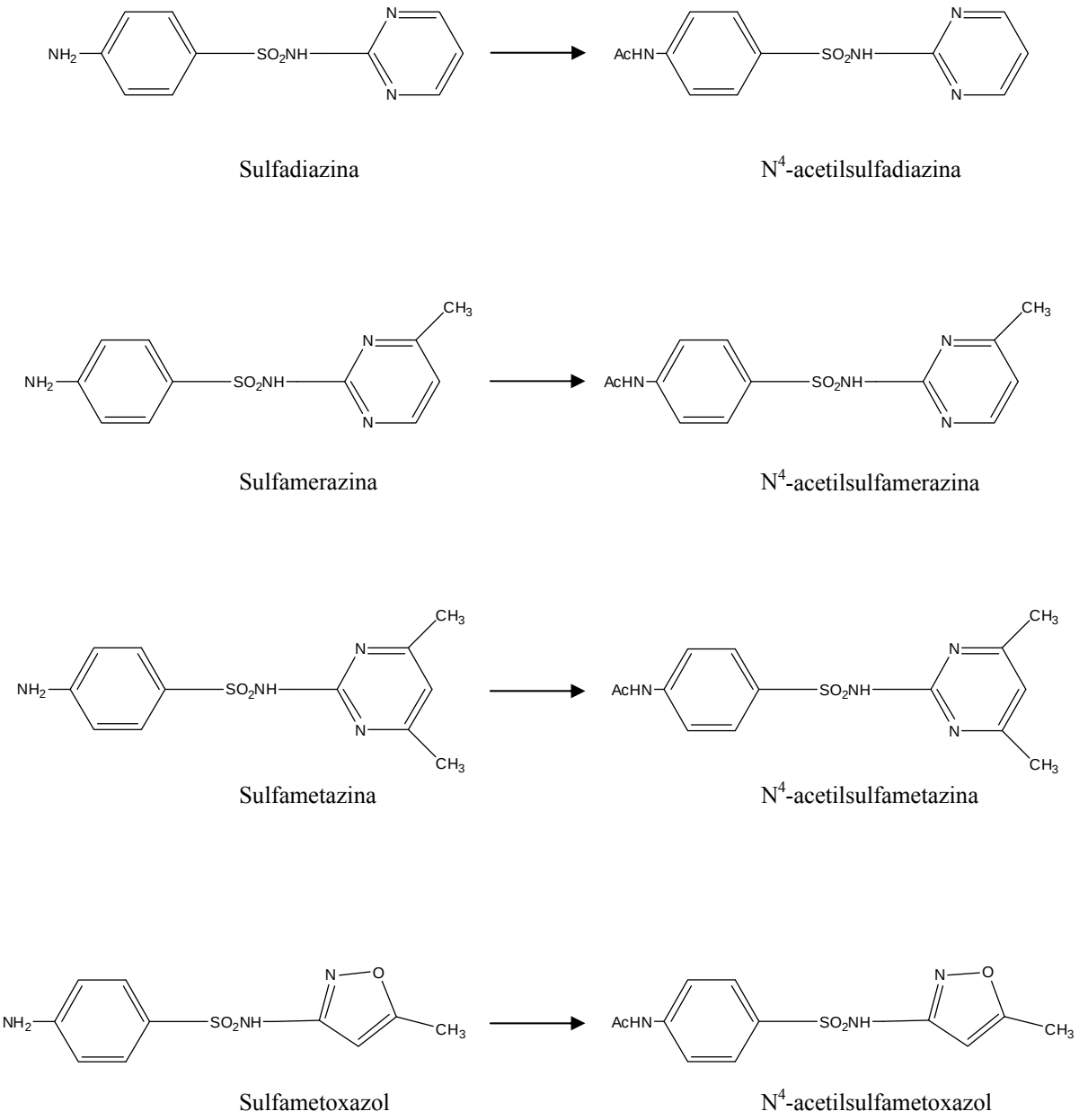


Figura 13. Reacción global de la síntesis de los N^4-acetil metabolitos de las sulfonamidas.

CAPÍTULO 3

RESULTADOS

En esta memoria se han puesto a punto nuevos procedimientos de extracción empleando la microextracción en fibra hueca en configuración de tres fases y mediante electromembranas. Se han estudiado compuestos pertenecientes a diferentes familias: Antiinflamatorios no esteroideos, sulfonamidas y fluoroquinolonas.

Todos los métodos propuestos están enfocados a la miniaturización de la preparación de la muestra y al desarrollo de métodos ecológicos, económicos, rápidos, sencillos, sensibles y precisos. Las diferencias principales entre ellos son los tipos de fases extractantes empleados, analitos, muestras, modalidad de extracción, métodos de optimización y sistemas de separación y detección. La metodología general de trabajo seguida en los trabajos conlleva la optimización de todas las variables que influyen en la extracción, la validación del método, y por último, la aplicación del método a muestras biológicas y medioambientales.

Los resultados obtenidos han sido publicados en diferentes revistas científicas de alto índice de impacto, o bien se encuentran en proceso de revisión editorial. A continuación se reproducen las publicaciones correspondientes.

ANTIINFLAMATORIOS NO ESTEROIDEOS

Hollow fiber-based liquid phase microextraction (HF-LPME) of ibuprofen followed by FIA-chemiluminescence determination using the acidic permanganate-sulfite system

En este trabajo se propone y optimiza la extracción y posterior determinación de Ibuprofeno mediante microextracción en fibra hueca en una configuración de tres fases y el análisis por FIA y con detección por quimioluminiscencia.

Se emplearon corrientes de permanganato potásico 1,5 10^{-4}M en medio ácido 5 10^{-2}M y sulfito sódico 1,5 10^{-3}M, a un flujo de 1.5mL/min para el analizador FIA.

La membrana líquida de dihexiléter fue soportada sobre una fibra de polipropileno Accurel Q3/2. Se empleó como fase donadora una solución acuosa de pH 2 (ajustada con HCl), y una solución acuosa de NaOH de pH 10 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de quince minutos con una agitación de 300 rpm.

El procedimiento propuesto permite un límites de detección de 0.03 µg/L y reproducibilidad del 1.6%.

El procedimiento se aplicó satisfactoriamente a la determinación de ibuprofeno en orina humana y a diferentes preparaciones farmacéuticas, obteniendo recuperaciones de prácticamente el 100%.

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Hollow fiber-based liquid-phase microextraction (HF-LPME) of ibuprofen followed by FIA-chemiluminescence determination using the acidic permanganate–sulfite system

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ABSTRACT

Hollow fiber-based liquid-phase microextraction (HF-LPME) is a relatively new technique employed in analytical chemistry for sample pretreatment which offers more selectivity and sensitivity than any traditional extraction technique. This paper describes a three-phase HF-LPME method for ibuprofen using a polypropylene membrane supporting dihexyl ether followed by a chemiluminescence (CL) determination using the CL enhancement on the acidic permanganate–sulfite system in a FIA configuration which is the first time that both techniques have been combined for analytical purposes. The CL intensity (peak area) was proportional to the log of ibuprofen concentration in the donor phase over the range 0.1–20 μg mL^{-1}. The detection limit was 0.03 μg mL^{-1} of ibuprofen in the donor phase. The method was satisfactory reproducible and has been applied to the ibuprofen determination in pharmaceuticals and in real human urine samples.

1. Introduction

In the last years, there has been an increasing interest in developing new sample pretreatment approaches to determine all type of analytes in several matrices; this is of special importance in the analysis of biological and environmental samples. Liquid–liquid extraction (LLE) is a classical and common technique used for the preconcentration and cleanup prior to chromatographic or electrophoretic analysis that requires large organic solvent consumption. It is also tedious and analyte-loss is frequent due to multi-stage operations that cannot be neglected.

Some interest has been focused on the miniaturising of analytical LLE. The main idea behind this has been to facilitate automation, to speed up extractions, and to reduce the consumption of organic solvents. Liquid-phase microextraction (LPME), based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) [1–2], is a simple, inexpensive, fast, effective and virtually solvent-free sample pretreatment technique. However, SDME is not very robust, and the droplets may be lost from the needle tip of the microsyringe during extraction. This is especially the case when samples are stirred vigorously to speed up the extraction process. More importantly, it requires careful and elaborate manual operations; however, the sensitivity and the precision of the SDME methods developed are relatively poor. This is why prolonged extraction time and faster stirring rates are not recommended.

Audunsson [3] introduced an alternative concept for LPME that was developed by Thordarson et al. [4] and for Pedersen-Bjergaard and Rasmussen [5] based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber. In hollow fiber liquid-phase microextraction (HF-LPME), the organic phase is protected by the fiber, and it appears that the hollow fiber decelerates the process of organic solvent dissolution into the bulk solution. Another factor contributing to the improved sensitivity of LPME is that the surface area for the rod-like configuration of the two-phase HF-LPME system is larger than the spherical one adopted by the drop-based SDME method. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor solution from entering the accepting phase, and at the same time, most macromolecules do not enter the hollow fiber because they are not soluble

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in the organic phase present in the pores, thus yielding very clean extracts. HF-LPME has been widely applied to a variety of environmental and biological samples. Several reviews that focus on basic extraction principles, technical setup, recovery, enrichment, extraction speed, selectivity, applications and future trends in hollow fiber-based LPME have been reported. [6–9]

There are two modes used: two-phase HF-LPME and three-phase HF-LPME. In two-phase HF-LPME, the analytes are extracted by passive diffusion from the sample into the hydrophobic organic solvent supported by the fiber, and in three-phase HF-LPME the analytes were extracted through an organic solvent immobilized in the pores of fiber and further into a new aqueous phase in the lumen of fiber.

Compared with LLE and SPE, HF-LPME gives a comparable and satisfactory sensitivity and much better enrichment of analytes, and the consumption of solvent is significantly reduced by up to several hundred or several thousand times. The LPME technique is simple, fast, and inexpensive. Due to the small volume of the extracting solvent, the extracted samples do not require further concentration prior to analysis and thus total analysis time considerably decreases in comparison to traditional LLE procedure. Additional advantages of LPME also make the technique attractive. Since, LPME tolerates a wide pH range; it can be used in applications that would not be suitable for solid-phase extraction (SPE) or solid-phase microextraction (SPME). Sample carryover can be avoided because the hollow fibers are inexpensive enough to be single-used and disposed. In cases, where a large number of samples are prepared by SPE, an equivalent LPME preparation procedure could also be more economical. It has been proven that HF-LPME is very useful for extraction of drugs and metabolites from biological matrices and pollutants from environmental samples with simultaneous cleanup of the extracts [10–12].

On the other hand, in opposition with chromatographic or electrophoretic methods, analytical methods combining chemiluminescence (CL) with flow-injection techniques have the advantages of simplicity, rapidity, use of non-expensive instrumentation. CL methods have found extensive applications in many interesting areas, but their main disadvantages are usually related, in general, with their poor selectivity. So HF-LPME can be an excellent sample pretreatment alternative for FIA-CL methods and their application to analytes into complex matrices as environmental and biological samples.

In this work, the CL determination has been carried on the basis of the known CL system acidic permanganate–sulfite. Hidson and Barnettt [13] published in 2001 a wide review about the analytical applications of this CL system. Stauff and Jaeschke [14] studied the oxidation of sulfur dioxide with acidic potassium permanganate and proposed a mechanism to explain the CL reaction which involves the sulfite oxidation to produce an excited molecule of sulfur dioxide, which emits radiation in the range of 300–550 nm [15]. The energy of the excited molecule can be transferred to a fluorescent molecule added into the system [16] in order to obtain a higher quantum efficiency which can allow its sensitive determination. Another way to enhance the CL emission is the use of non-fluorescent compounds but which are capable of amplifying the emission (sensitizers) through several mechanisms [17–19].

We have verified that the presence of ibuprofen ((*R*,*S*)-2-(4-isobutylphenyl)-propionic acid) (Fig. 1), a non-steroidal anti-inflammatory drug widely used, enhances the CL of the system acidic potassium permanganate/sodium sulfite. On this basis, a flow-injection method was developed for the determination of ibuprofen which has been directly applied to its rapid determination in pharmaceuticals. This method cannot be directly applied to the ibuprofen determination in human urine due to the high CL of the sample blanks, but the use of a previous HF-LPME overcomes this selectivity problem and allowed an adequate procedure for the ibuprofen determination in urine.

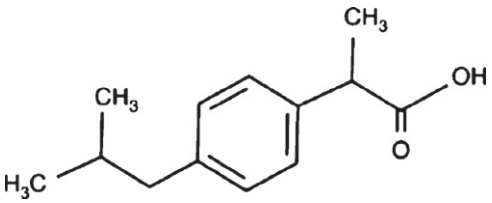

Fig. 1. Chemical structure for ibuprofen.

Ibuprofen has a pK_a value of 4.30 so a three-phase HF-LPME has been developed using a acid donor phase and a basic solution as acceptor phase which was directly injected into the FIA system.

All the HF-LPME and FIA parameters have been optimized in order to propose a rapid and simply determination of ibuprofen which is the first analytical procedure proposed that combines both techniques. The method has been applied to the determination of ibuprofen in human urine.

2. Experimental

2.1. Chemicals and materials

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water (Milli-Q, Milipore, Bedford, MA). Ibuprofen, dihexyl ether and 1-octanol were purchased from Fluka–Sigma–Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany). Working solution of 1.5×10^{-4} M $KMnO_4$ in 5×10^{-2} M H_3PO_4 was prepared daily by diluting the 3×10^{-3} M $KMnO_4$ stock solution. The solution of 1.5×10^{-3} M Na_2SO_3 was prepared daily. Working solutions of ibuprofen were prepared by adequate dilutions from a 200 μg mL^{-1} ibuprofen stock solution. Q3/2 Accurel KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). Human urine samples were collected from healthy volunteers.

2.2. Equipment

The FIA system used in this work is shown in Fig. 2. To deliver flow streams, a peristaltic pump Minipuls 3 from Gilson (Gilson, Inc., Middleton, WI, USA) was used. Polytetrafluoroethylene (PTFE) tubing (0.8 mm i.d.) was used to connect all components in the flow system. 100 or 20 μl loops were placed in the injection valve. The CL signal was measured by a ChemLab Chemiluminescence Detector model CL2 (Camspec, Cambridge, UK) where the carrier streams were mixed through a Y-shaped element previously to the 60 μl/5 mm path length glass flow cell. CL data were acquired with a personal computer using Clarity Lite software (DataApex Ltd., Prague, The Czech Republic).

Sample stirring were performed on an ANS-00/1 magnetic stirrer from Science Basic Solutions (Rubí, Barcelona, Spain).

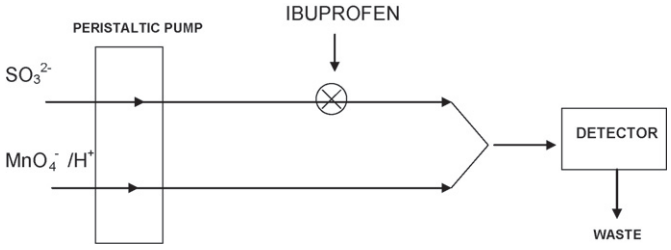

Fig. 2. Schematic diagram of the FIA system used for the ibuprofen determination.

A HPLC equipment (Merck-Hitachi) consisted of a L-7100 pump, a Rheodyne (Cotati, CA, USA) model 7725i injection valve with a 20-μl sample loop, a Lichrospher® 100 RP-18 column (125 mm × 4 mm i.d., 5 μm) (Merck, Darmstadt, Germany), and a model L-7455 diode array detector controlled by a Merck-Hitachi D-7000 interface equipped with a HPLC System Manager® software was used to realize chromatographic determinations.

2.3. FIA analysis procedure

By keeping the six-way valve in washing position, acidic permanganate and sulfite solutions were continuously pumped into the manifold at a flow rate of 1.5 mL min^{-1}. Ibuprofen solutions were injected into the sulfite stream from the valve loop. The content of ibuprofen was determined from the calibration plot of CL emission intensity versus log of ibuprofen concentration.

2.4. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in ultrasonic bath and dried. The fiber was soaked in the membrane phase (dihexyl ether) during 5 s to impregnate pores of the support, and rinsed with water on the outside by placing into an ultrasonic bath for 30 s in order to remove the excess of organic solvent. The lumen of the prepared fiber piece was filled with 50 μL of acceptor phase (pH 10 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of hot soldering tool and adhesive tape. During extraction, the membrane portion that contains the acceptor phase (Fig. 3) was immersed in the 50 mL sample solution (pH 2) contained in a 50 mL glass beaker. The sample was stirred for 15 min by means of a magnetic stirrer at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extract using a HPLC syringe and injected into the FIA system through the 20 μL loop.

2.5. Preparation of real samples

Pharmaceuticals were dispersed into pH 8 aqueous solutions. The non-soluble fraction was removed by filtration or centrifugation, and the samples were diluted with water. Suitable aliquots were taken and diluted with water for their measurements.

Spiked and real urine samples were simply diluted 1:10 with HCl pH 2 aqueous solution and submitted to HF-LPME procedure.

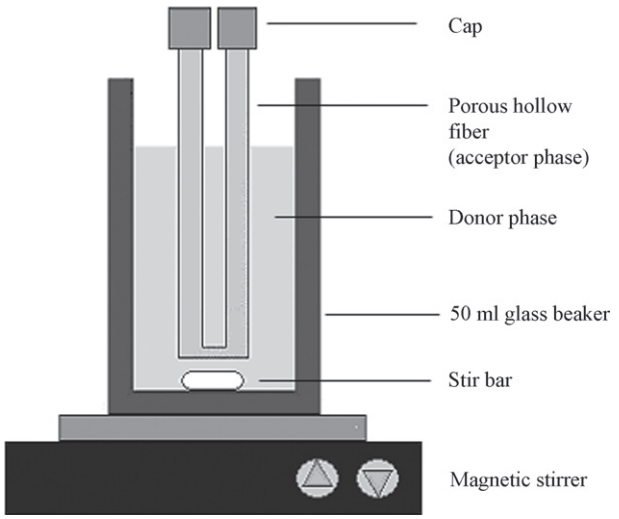

Fig. 3. Schematic diagram of the HF-LPME procedure.

3. Results and discussion

3.1. Optimization of experimental variables for FIA-CL

The CL that results from the acidic permanganate and sulfite reaction has a low intensity. When ibuprofen was added into the system, the CL increases which indicates that it is a sensitive enhancer of the CL of this reaction. The sensitizing effect of ibuprofen is also related to the acid, permanganate and sulfite concentrations. Thus, several tests were performed to choose the best experimental conditions in order to obtain maximum CL signals.

Variable concentrations of HCl, HNO_3, H_2SO_4 and H_3PO_4 were tested, and the results obtained indicated that the strongest CL occur if H_3PO_4 is used; so, it was chosen to obtain acid pH; H_2SO_4 produced slightly lower CL intensity than H_3PO_4. CL intensity increases with the H_3PO_4 concentration reaching a maximum value between 2.5×10^{-2} and 6.5×10^{-2} mol L^{-1} when 5.0×10^{-5} M permanganate and 5.0×10^{-4} M sulfite at a flow rate of 1.0 mL min^{-1} were used. A H_3PO_4 concentration of 5×10^{-2} M was chosen as the optimum acidic medium for the sulfite/permanganate/ibuprofen reaction.

The effect of permanganate concentration was checked in the range 1×10^{-5} to 3.0×10^{-3} mol L^{-1} keeping a H_3PO_4 concentration of 5×10^{-2} M, sulfite concentration of 5.0×10^{-4} M and flow rate of 1.0 mL × min^{-1} were also kept constant. The CL increases with the permanganate concentration reaching a maximum value from 6×10^{-5} M to 2×10^{-4} M; higher permanganate concentrations resulted in a decrease of the emission intensity, which could be due to a permanganate absorption [19,20]. A permanganate concentration of 1.5×10^{-4} M was chosen as optimum.

When the effect of sulfite concentration was checked at previously chosen H_3PO_4 and permanganate concentrations at a flow rate of 1.0 mL min^{-1}, maximum CL was obtained when sulfite concentration was between 8×10^{-4} and 1.75×10^{-3} mol L^{-1}, the decrease at higher sulfite concentrations can be due to the resulting sulfite/permanganate relation. A 1.5×10^{-3} M Na_2S0_3 solution was chosen as optimum.

The effect of the flow rate was tested keeping the H_3PO_4, permanganate and sulfite concentrations at the chosen values; the CL signal increases up to 1.3 mL min^{-1} and remains stable from this value up to 4.5 mL min^{-1}. A flow rate of 1.5 mL min^{-1} was selected as the optimum in order to minimize reagents consumption.

3.2. Linearity, sensitivity and precision

The chemiluminescence intensity (peak area) using the optimized conditions previously described was proportional to the log of concentration of ibuprofen over the range 2–100 μg mL^{-1}. The detection limit calculated as three times the standard deviation of the background signal (3σ) was 0.5 μg mL^{-1} of ibuprofen. The relative standard deviation for 15 repetitive determinations of 30 μg mL^{-1} ibuprofen (intraday repeatability) was 0.8%. The interday repeatability ($n = 3$; 5 days) was 1.6%.

3.3. Kinetic characteristics of CL reaction

The CL reaction between permanganate, sulfite and ibuprofen occurs immediately after the mixing and is very fast. It is a flash-type emission and is apparently controlled by the mixing speed. The possible mechanism of this CL reaction has been previously established [21].

3.4. Determination of ibuprofen in pharmaceuticals

The proposed FIA-CL method was applied to the analysis of several pharmaceuticals: Gelofeno® (tablets) (Lab. Gelos, S.L.),

Table 1
Ibuprofen determination in several pharmaceuticals by the FIA-CL proposed procedure and compared with content labeled and obtained by another HPLC method.

Pharmaceutical	Label content (mg)	Proposed method[a]	HPLC method[a]
Gelofeno®	200	195 ± 3	198 ± 3
	400	399 ± 1	402 ± 2
	600	597 ± 4	595 ± 4
Neobrufen®	600	592 ± 6	601 ± 2
Ibuprofeno Cinfa®	600	600 ± 2	597 ± 3

[a] Average ± standard deviation (n = 5).

Neobrufen® (effervescet powder) (Aboot Laboratories) and Ibuprofeno Cinfa® (tablets) (Lab. Cinfa). Results obtained were compared with labeled contents and those from an HPLC method [19]. Table 1 shows the results obtained. As can be seen, good agreement between labeled, HPLC and FIA-CL contents was obtained.

3.5. Optimization of experimental conditions for HF-LPME extraction

First, several tests with donor phases pH 1–2 and acceptor phases pH 10–11 were carried out in order to choose the more adequate liquid supported on the polypropilene membrane; dihexyl ether and 1-octanol were checked, and the best results were obtained with dihexyl ether, so this was the liquid support selected.

Based on the pK_a value for ibuprofen (4.30), donor HCl aqueous solutions within 1–4 pH range were tested using 50 mL of aqueous ibuprofen of 5.0 mg L^{-1} extracted at 300 rpm during 30 min and 50 μL of aqueous pH 10 NaOH solution was used as acceptor phase; ibuprofen extraction decreases a pH value higher than 2.5. pH 2 aqueous solutions were selected as donor phase and then NaOH aqueous solutions with pH values between 8 and 12 were tested as acceptor phase; maximum ibuprofen extraction was carried out with acceptor phases with pH higher than 9.5; accordingly, pH 10 solutions were selected as acceptor phase.

Using the selected donor and acceptor phases, the influence of the stirring time was checked; as can be seen in Fig. 4 maximum extraction was obtained for values higher than 10 min, so a stirring time of 15 min at 300 rpm was selected as optimum value.

3.6. Linearity, sensitivity and precision for HF-LPME extraction

Using the selected HF-LPME conditions, several aqueous pH 2 solutions with different ibuprofen concentrations were submitted to the liquid microextraction procedure and analyzed according to the described experimental procedure. The CL intensity (peak area) was proportional to the log of ibuprofen concentration in the donor phase over the range 0.1–20 μg mL^{-1}. The detection limit calculated as three times the standard deviation of the background signal (3σ) was 0.03 μg mL^{-1} of ibuprofen. The relative standard deviations for

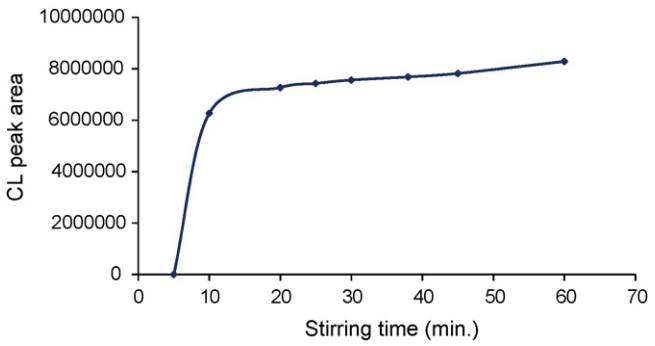

Fig. 4. Influence of the stirring time (300 rpm) on the HF-LPME.

Table 2
Ibuprofen recoveries using HF-LPME/FIA-CL from spiked human urine samples.

Spiked concentration (μg mL^{-1})	Measured concentration (μg mL^{-1})[a]
5.0	4.97 ± 0.06
10.0	9.84 ± 0.19
15.0	15.05 ± 0.12

[a] Average ± standard deviation (n = 5).

Table 3
Ibuprofen contents after the oral administration of a 600 mg dose for the analyzed real urine samples using HF-LPME/FIA-CL proposed method and compared with another HPLC method.

Time (min)	Proposed method (μg mL^{-1})[a]	HPLC method (μg mL^{-1})[a]
30	1.23 ± 0.12	1.30 ± 0.07
60	2.65 ± 0.16	2.59 ± 0.10
90	6.31 ± 0.09	6.40 ± 0.08
120	18.93 ± 0.10	19.05 ± 0.07
150	22.35 ± 0.06	22.28 ± 0.11
180	12.38 ± 0.14	12.40 ± 0.09
210	3.07 ± 0.14	3.12 ± 0.09

[a] Average ± standard deviation (n = 5).

15 repetitive determinations of 1 and 10 μg mL^{-1} ibuprofen (intraday repeatability) were 1.2% and 0.8%, respectively. The interday repeatabilities (n = 3; 5 days) were 1.8% and 1.1% for those concentration values.

3.7. Human urine analysis using HF-LPME extraction

Direct analysis of ibuprofen in urine samples using the FIA-CL proposed method is not possible due to matrix interference that produces very high CL signal for blank samples; so, the proposed HF-LPME procedure was applied for this purpose.

Recovery assays were performed using spiked human urine samples from volunteers to obtain ibuprofen concentrations of 5, 10 and 15 μg mL^{-1} which were submitted to the HF-LPME extraction and FIA-CL determination procedure described in the experimental section. The results obtained are shown in Table 2, with good agreement between spiked and measured quantities.

Human real urine samples from a volunteer were collected at intervals of 30 min after the administration of a 600 mg oral dose of ibuprofen. Table 3 shows the concentration values obtained by the HF-LPME/FIA-CL proposed procedure and by an HPLC method [22]. As can be seen, a good agreement between both analytical methods is evident.

4. Conclusions

This study presents a rapid hollow fiber-based liquid-phase microextraction (HF-LPME) method for ibuprofen combined with the chemiluminescence determination using its sensitizing effect on the acidic permanganate/sulfite reaction into a FIA configuration. The applicability of the method for urine samples has been demonstrated. The simplicity of the extraction process and analysis procedure, including the use of aqueous solutions only, makes the developed method as an attractive alternative to other methods. The FIA-CL method has been also successfully applied to the determination of ibuprofen in pharmaceuticals.

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HPLC determination of ibuprofen, diclofenac and salicylic acid using hollow fiber-based liquid phase microextraction (HF-LPME)

En este trabajo se propone y optimiza la extracción y posterior determinación de tres compuestos pertenecientes a la familia de los antiinflamatorios no esteroideos: ibuprofeno, diclofenaco y ácido salicílico. La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior se realiza usando detectores de diodo array y fluorescencia conectados en serie.

La membrana líquida de dihexiléter fue soportada sobre una fibra de polipropileno Accurel Q3/2. Se empleó como fase donadora una solución acuosa de pH 2 (ajustada con HCl) y una solución acuosa de NaOH de pH 12.5 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de quince minutos con una agitación de 300 rpm.

La separación cromatográfica se realizó empleando una columna monolítica Chromolith® Performance RP-18e (100-4.6mm i.d.) y un gradiente de ácido fórmico 0,1%/Metanol a un flujo de 2.5 ml/min. En estas condiciones el cromatograma completo se desarrolla en tan solo 10 minutos, mostrando una buena resolución y una separación adecuada de los picos cromatográficos. Este tipo de columnas presenta una elevada compacidad y permite trabajar con flujos relativamente altos sin alcanzar presiones muy elevadas, resolviéndose los cromatogramas en tiempos relativamente cortos.

El factor de enriquecimiento obtenido varía entre 70 y 1000, los límites de cuantificación obtenidos para los analitos estudiados, varían entre 41-135 µg/L mediante DAD y entre 6-23 ng/L en el caso de detección por fluorescencia. Las reproducibilidades obtenidas son bastante buenas y oscilan entre el 1.1% y el 2.3%. La evaluación de la robustez del método se ha realizado mediante un diseño experimental de tres factores a dos niveles, comprobando a su vez el efecto y la importancia de las variables experimentales optimizadas de forma independiente y conjunta sobre el resultado final y se ha podido comprobar que la variable experimental correspondiente a la fase aceptora es, en este caso, una variable crítica.

El procedimiento ha sido aplicado satisfactoriamente a la determinación directa de los analitos en muestras de orina, obteniendo recuperaciones superiores al 82% en todos los casos. Además, el procedimiento propuesto se puede aplicar a la

determinación de sus correspondientes metabolitos, en los que cabe esperar factores de enriquecimiento similares a los analitos estudiados, por lo que el método propuesto podría ser adecuado para la determinación de los analitos y sus correspondientes metabolitos presentes en bajas concentraciones en otras muestras biológicas o matrices medioambientales.

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HPLC determination of ibuprofen, diclofenac and salicylic acid using hollow fiber-based liquid phase microextraction (HF-LPME)

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ABSTRACT

This paper describes an extraction method using a polypropylene membrane supporting dihexyl ether (three-phase hollow fiber-based liquid phase microextraction (HF-LPME)) for the analysis of several pharmaceuticals (salicylic acid (SAC), ibuprofen (IBU) and diclofenac (DIC)) followed by a HPLC determination using a monolithic silica type HPLC column, that allows lower retention times than the usual packed columns with adequate resolution. Detection was realized by means of a coupled in series diode array (DAD) and fluorescence (FLD) detectors. HF-LPME is a relatively new technique employed in analytical chemistry for sample pretreatment which offers more selectivity and sensitivity than any traditional extraction technique. Detection limits by DAD are 12, 53 and 40 ng mL^{-1} for salicylic acid, diclofenac and ibuprofen, respectively and by FLD 7 and 2 ng mL^{-1} for salicylic acid, and ibuprofen. The method has been successfully applied to their direct determination in human urine and the results obtained demonstrated that could be also applied to the determination of the corresponding metabolites.

1. Introduction

In the last years, there has been an increasing interest in developing new sample pretreatment approaches to determine all type of analytes in several matrices; this is of special importance in the analysis of biological and environmental samples. Liquid–liquid extraction (LLE) is a classical and common technique used for the preconcentration and clean-up prior to chromatographic or electrophoretic analysis that requires large organic solvent consumption. It is also tedious and analyte-loss is frequent due to multi-stage operations that cannot be neglected.

Liquid phase microextraction (LPME), based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) [1,2], is a simple, inexpensive, fast, effective and virtually solvent-free sample pretreatment technique. However, SDME is not very robust, and the droplets may be lost from the needle tip of the microsyringe during extraction.

Audunsson [3] introduced an alternative concept for LPME that was developed by Thordarson et al. [4] and for Pedersen-Bjergaard and Rasmussen [5] based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber. In hollow fiber liquid phase microextraction (HF-LPME), the organic phase is protected by the fiber, and it appears that the hollow fiber decelerates the process of organic solvent dissolution into the bulk solution. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor solution from entering the accepting phase and, at the same time, most macromolecules do not enter the hollow fiber because they are not soluble in the organic phase present in the pores, thus yielding very clean extracts. Several reviews that focus on basic extraction principles, technical setup, recovery, enrichment, extraction speed, selectivity, applications and future trends in hollow fiber-based LPME have been reported [6–9].

There are two modes used: two-phase HF-LPME and three-phase HF-LPME. In two-phase HF-LPME, the analytes are extracted by passive diffusion from the sample into the hydrophobic organic solvent supported by the fiber, and in three-phase HF-LPME the analytes were extracted through an organic solvent immobilized in the pores of fiber and further into a new aqueous phase in the lumen of fiber.

Compared with LLE and SPE, HF-LPME gives a comparable and satisfactory sensitivity and much better enrichment of analytes, and the consumption of solvent is significantly reduced by up to

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Table 1
Efficiency and selectivity chromatographic parameters for the proposed HPLC procedure (for abbreviation see text).

	t_R (min)	RSD-t_R (%)	$W_{1/2}$ (min)	T	N	K'	α	R_s
SAC	2.00	0.015	0.1056	1.29	2,153	199	6.44	34.57
DIC	9.07	0.006	0.1357	1.06	24,749	903	1.04	2.06
IBU	9.47	0.019	0.1333	1.04	27,961	946	1.04	2.06
Critical values		<1%		<1.5	>2,000	>2		>1.5

Table 2
HPLC calibration parameters and LOD for the analytes.

	Regression coef. (r^2)	Linearity (%)	Linear range ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)
DAD				
SAC	0.9993	99.02	518.3–20,000	155.5
DIC	0.9972	98.02	1047–20,000	314.1
IBU	0.9941	98.87	2244.5–40,000	673.4
FLD				
SAC	0.9993	98.81	240.3–15,000	83.1
IBU	0.9991	99.02	131.8–9000	41.6

several hundred or several thousand times. The LPME technique is simple, fast, and inexpensive. Due to the small volume of the extracting solvent, the extracted samples do not require further concentration prior to analysis and thus total analysis time considerably decreases in comparison to traditional LLE procedure. Additional advantages of LPME also make the technique attractive. Since, LPME tolerates a wide pH range; it can be used in applications that would not be suitable for solid-phase extraction (SPE) or solid-phase microextraction (SPME). Sample carryover can be avoided because the hollow fibers are inexpensive enough to be single-used and disposed. In cases, where a large number of samples are prepared by SPE, an equivalent LPME preparation procedure could also be more economical. It has been proven that HF-LPME is very useful for extraction of drugs and metabolites from biological matrices and pollutants from environmental samples with simultaneous clean-up of the extracts [10–13].

In this work, an HPLC method combined with prior HF-LPME was developed for the determination of three widely used drugs: salicylic acid (2-hydroxy-benzoic acid), the hydrolysis product of the well known acetylsalicylic acid (2-(acetyloxy)-benzoic acid) and two non-steroidal anti-inflammatory drug widely used, diclofenac (2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid) and ibuprofen ((*R*,*S*)-2-(4-isobutylphenyl)-propionic acid). All the HF-LPME and HPLC parameters have been optimized in order to propose a rapid, simply and sensitive determination of those drugs. The method has been satisfactorily applied to their determination in

Table 3
Experimental and coded values (between parentheses) for the full factorial design.

Run #	Donor pH: experimental (coded)	Acceptor pH: experimental (coded)	Extraction time (min): experimental (coded)
1	1.5 (−1)	12 (−1)	10 (−1)
2	1.5 (−1)	12 (−1)	20 (+1)
3	1.5 (−1)	13 (+1)	10 (−1)
4	1.5 (−1)	13 (+1)	20 (+1)
5	2.5 (+1)	12 (−1)	10 (−1)
6	2.5 (+1)	12 (−1)	20 (+1)
7	2.5 (+1)	13 (+1)	10 (−1)
8	2.5 (+1)	13 (+1)	20 (+1)

Table 4
Results from the full factorial design.

		t calculated		
		Donor pH	Acceptor pH	Stirring time
SAC	$Y = 2.1295 - 0.1511b_1 + 1.7413b_2 + 0.0026b_3$	1.4629	4.9361	0.3331
DIC	$Y = 1.0629 + 0.3033b_1 - 1.7939b_2 + 0.0663b_3$	2.6079	0.7496	0.0089
IBU	$Y = 1.3855 + 0.4327b_1 - 1.7573b_2 + 0.029b_3$	1.1617	4.6717	0.3553

Critical Value for t ($P = 0.05$, $n = 4$) 2.78.

Table 5
HF-LPME/HPLC calibration parameters and LOD for the analytes.

	Regression coef. (r^2)	Linearity (%)	Linear range (ng mL^{-1})	LOD (ng mL^{-1})
DAD				
SAC	0.9998	99.81	41.0–10,000	12.3
DIC	0.9989	99.57	176.6–10,000	52.9
IBU	0.9994	99.68	135.3–10,000	40.6
FLD				
SAC	0.9991	98.93	23.8–500	7.1
IBU	0.9988	98.43	6.3–50	1.9

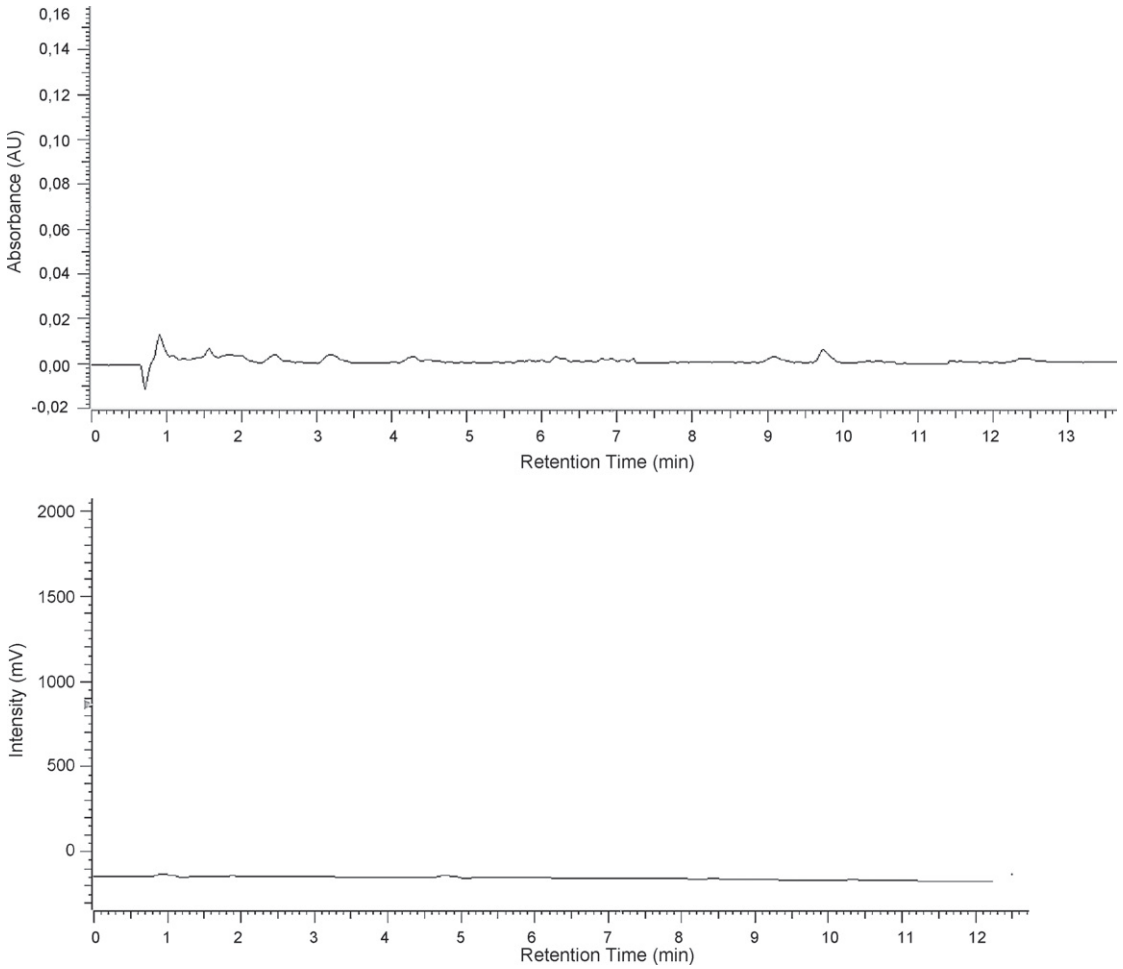

Fig. 1. HF-LPME/HPLC-DAD and FLD chromatograms of blank urine samples.

human urine. The results obtained in the analysis of real human urine samples show that it could be applied to the determination of the corresponding metabolites.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water form a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). SAC, DIC, IBU dihexyl ether and 1-octanol were purchased from Fluka–Sigma–Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany).

Working solutions of SAC, DIC and IBU were daily prepared by adequate dilutions from 200 μg mL^{-1} stock solutions. Q3/2 Accurel KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). Human urine samples were collected from healthy volunteers.

2.2. Chromatographic conditions

Chromatography was performed using a LaChrom® instrument Merck-Hitachi (Barcelona, Spain) with a quaternary L-7100 pump a L-7455 diode array detection system and a L-7485 programmable fluorescence detector. The injector was a Rheodyne manual injection valve Model 7725i, fitted with a 20-μL sample loop. Separations were carried out using a monolithic silica type HPLC column Chromolith® Performance RP-18e (100–4.6 mm i.d.) (VWR, Darmstadt, Germany) preceded by a guard column Chromolith® RP-18e (5–4.6 mm i.d.) (VWR, Darmstadt, Germany). The system was controlled through an interface module D-7000 and a personal computer. Chromatograms were processed by a HPLC-System-Manager HSM D-7000 (Merck-Hitachi).

The mobile phase consisted of 0.1% formic acid (component A) and methanol (component B). A linear elution gradient was programmed from 40% to 70% B in 10 min, at a flow rate of 2.5 mL min^{-1}. Five minutes were waited between injections. The wavelengths used for DAD were 235, 280 and 225 nm for SAC, DIC and IBU, respectively; for FLD detection excitation wavelengths of 230 and 220 nm were used for SAC and IBU, respectively, and their corresponding emission wavelengths were 445 and 290 nm. To confirm the identification of the substances, the UV and fluorescence spectra of the chromatographic peaks in urine samples and standard solutions were compared.

2.3. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in ultrasonic bath and dried. The fiber was soaked in the membrane phase (dihexyl ether) for 5 s to impregnate pores of the support, and rinsed with water on the outside by placing into an ultrasonic bath for 30 s in order to remove the excess of organic solvent. The lumen of the prepared fiber piece was filled with 50 μL of accep-

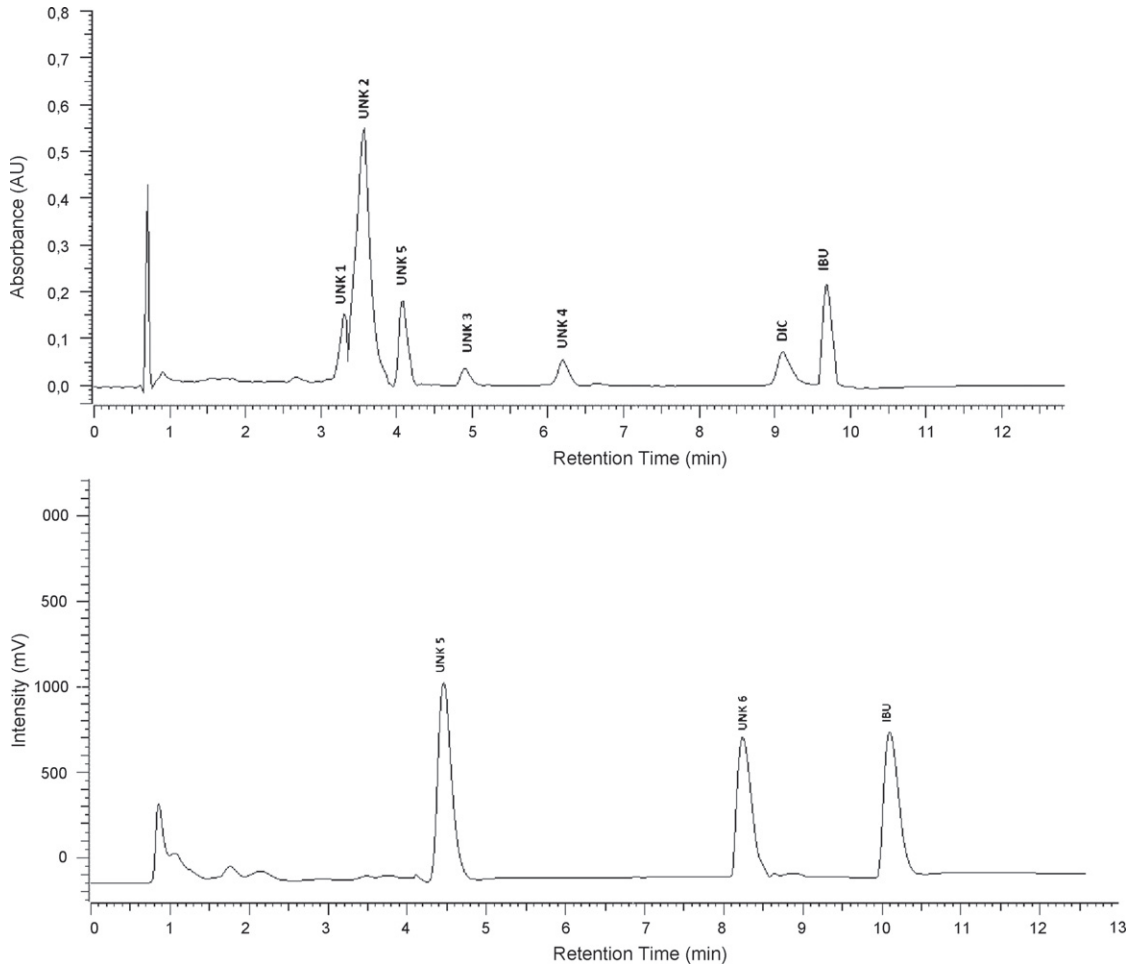

Fig. 2. Representative HF-LPME/HPLC-DAD and FLD chromatograms obtained after the administration of oral doses of ibuprofen and diclofenac (for unknown peaks see text).

tor phase (pH 12.5 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of hot soldering tool and adhesive tape. During extraction the membrane portion that contains the acceptor phase was immersed in the 50 mL sample solution (pH 2) contained into a 50 mL glass beaker. The sample was stirred for 15 min by means of a magnetic stirrer (ANS-00/1 Science Basic Solutions (Rubí, Barcelona, Spain) at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extract using a HPLC syringe and injected into the HPLC-system.

2.4. Preparation of real samples

Spiked and real urine samples were directly extracted by the HF-LPME procedure after HCl addition just obtain pH 2.

3. Results and discussion

3.1. Chromatographic conditions

Monolithic silica type HPLC column was selected as working column due to its characteristics that make it possible the use of high flow-rates without pressure increase that reduces the time of analysis; also good peak symmetry was achieved.

The mobile phase consisted of 0.1% formic acid and methanol. Different gradient elution conditions were tested in order to save time of analysis without sacrificing peak shape. The gradient elution program described in Section 2.2, was the best option in terms of time of analysis, shape of the peaks and reproducibility.

Table 1 shows several efficiency and selectivity chromatographic parameters for the proposed HPLC procedure: t_R (retention time), RSD-t_R (relative standard deviation for retention time), *N*

Table 6
SAC, DIC and IBU recoveries (%) using HF-LPME/HPLC from spiked human urine samples.

Concentration ($\mu g\,mL^{-1}$)	DAD			FLD	
	SAC[a]	DIC[a]	IBU[a]	SAC[a]	IBU[a]
0.025	–	–	–	–	82.3 ± 1.2
0.2	94.0 ± 1.1	99.0 ± 0.7	85.3 ± 1.0	95.3 ± 0.9	–
1.0	95.8 ± 0.9	98.2 ± 0.5	84.8 ± 0.6	–	–
2.0	95.5 ± 0.8	97.1 ± 0.8	83.2 ± 0.5	–	–

(–) Out of linear range.
[a] Average of three determinations ± standard deviation.

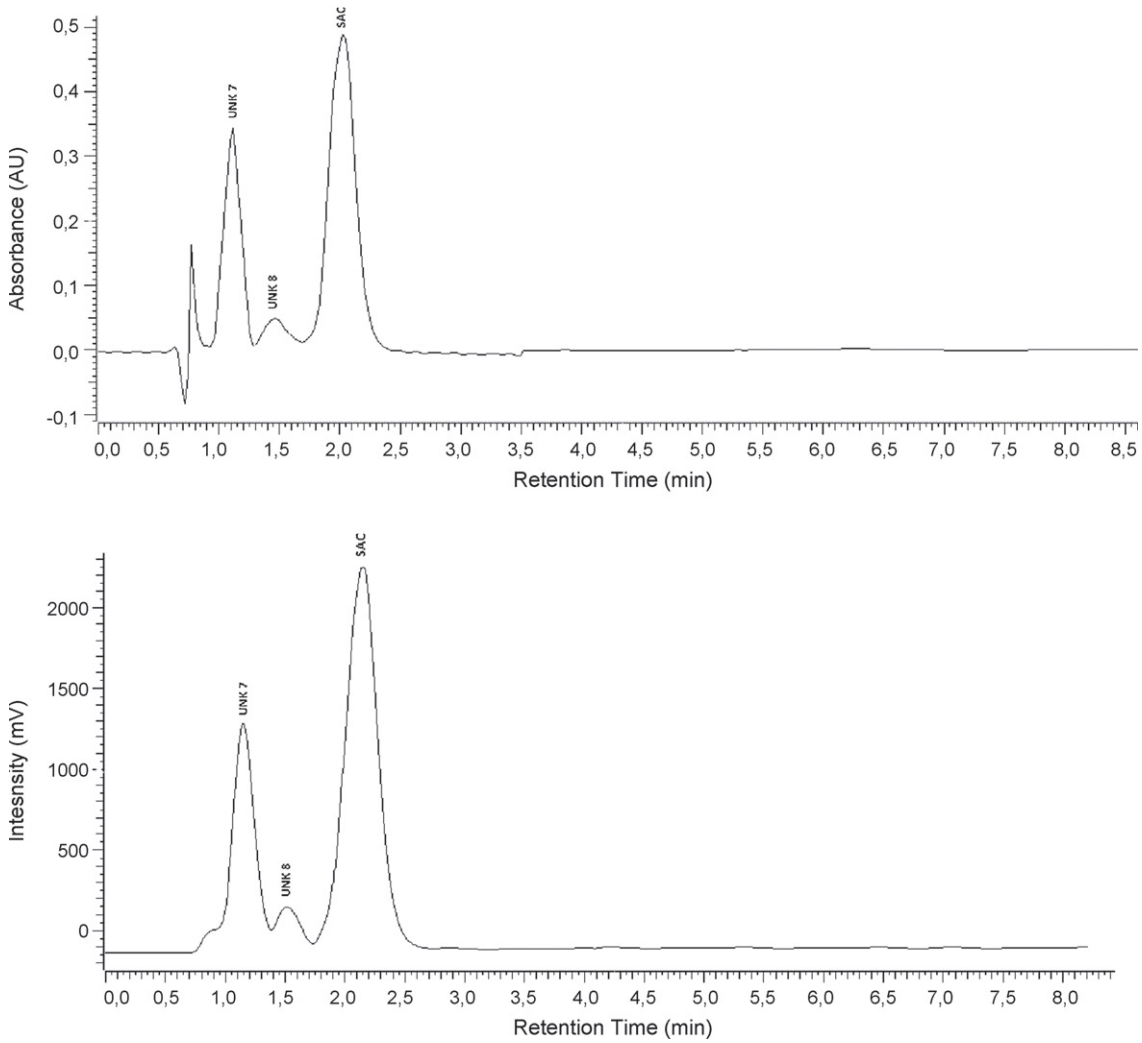

Fig. 3. Representative HF-LPME/HPLC-DAD and FLD chromatograms obtained after the administration of a oral dose of acetylsalicylic acid (for unknown peaks see text).

(number of theoretical plates), T (asymmetry factor), $W_{1/2}$ (peak half-width), K' (capacity factor), α (selectivity factor), R_s (peak resolution). The DAD and FLD peak areas were proportional to the SAC, DIC and IBU concentrations.

Table 2 shows the corresponding DAD and FLD calibration parameters and the detection limits calculated as three times the standard deviation of the background signal (3σ). For DAD, the relative standard deviation for 15 repetitive determinations of 5 $\mu g\,mL^{-1}$ of SAC, DIC and IBU (intraday repeatability) were 1.4, 1.1 and 0.7%, respectively. The interday repeatability ($n = 3$; 5 days) were 1.3, 1.3 and 0.9%. For FLD, the relative standard deviation for 15 repetitive determinations of 0.1 $\mu g\,mL^{-1}$ of SAC and 0.025 $\mu g\,mL^{-1}$ of IBU (intraday repeatability) were 1.3 and 1.4%, respectively. The interday repeatability ($n = 3$; 5 days) were 1.8 and 1.9%, respectively.

3.2. Optimization and evaluation of experimental conditions for HF-LPME

First, several tests with donor phases pH 1–2 and acceptor phases pH 10–11 were carried out in order to choose the more adequate liquid supported on the polypropylene membrane; dihexyl ether and 1-octanol were checked and the best results were obtained with dihexyl ether, so this was the liquid supported selected.

Due to the pK_a values for SAC, DIC and IBU, donor HCl aqueous solutions within 1–4 pH range were tested using 50 mL of aqueous solutions containing 1 $\mu g\,mL^{-1}$ SAC, 5 $\mu g\,mL^{-1}$ DIC and 10.0 $\mu g\,mL^{-1}$ IBU that were extracted at 300 rpm during 30 min and 50 μL of aqueous pH 10 NaOH solution was used as acceptor phase; SAC, DIC and IBU extraction decreases at pH values upper than 2, so pH 2 aqueous solutions were selected as donor phase and then NaOH aqueous solutions with pH values between 8 and 13 were tested as acceptor phase; due to the different SAC, DIC and IBU extraction factors pH 12.5 solutions were selected as acceptor phase that allows adequate extraction for all the analytes (see suplementary electronic material).

Using the selected donor and acceptor phases, the influence of the stirring time was checked; maximum extraction was obtained for values higher than 15 min, so a stirring time of 15 min at 300 rpm was selected as optimum value in order to reduce the processing time.

The use of a full factorial design was realized to establish the effects of the main variables and, in addition, the cross effects or two/three factors interaction (AB/AC/BC and ABC). The extraction procedure is controlled by the donor/acceptor pH and the extraction time (factors). A full factorial design [14,15] for three factors and two levels involving eight experiments (2^3) has been used to determine the effect and importance of the mentioned variables on the final result. The effects were differential quantities that are related to the response change as a result of changes in one or more factors/variables.

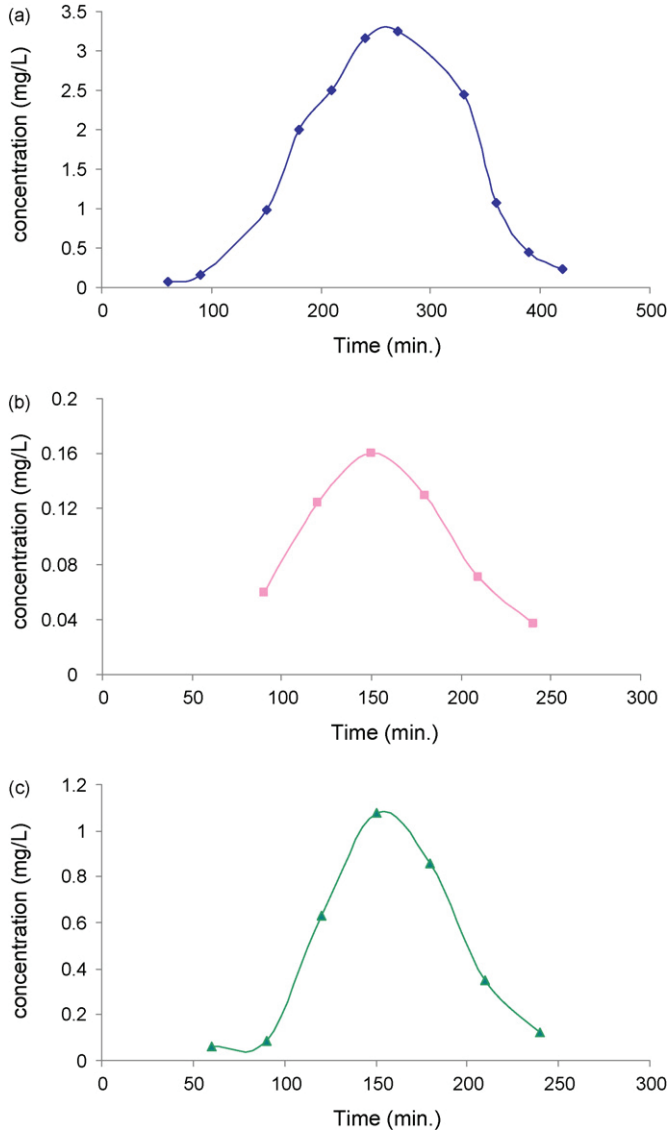

Fig. 4. Concentration in urine vs. time after the administration of oral doses of 500 mg of acetylsalicylic acid (a), 100 mg of diclofenac (b) and 600 mg of ibuprofen (c).

The corresponding concentrations calculated from DAD peak area for the analytes at a middle concentration level is the response value. In a first stage, a nominal procedure has been defined based on the results of preliminary results: donor pH 2, acceptor pH 12.5, and extraction time 15 min. Based on the nominal levels a high (coded value +1) and low (coded value −1) levels are defined for each factor, so the full factorial design comprises 8 experiments (2^3) as shown in Table 3 with the corresponding peaks area.

Table 4 shows the equations obtained from the experimental data and the calculated *t*-values for each of the factors and analytes where b_1, b_2 and b_3 are the donor pH, acceptor pH and stirring time, respectively.

As can be seen the main factor is the acceptor pH for all the analytes. For DIC and IBU, an increase in the acceptor pH leads to better results in the extraction, while in the case of SAC leads to a decrease in the signal; so this pH value is a critical parameter. The donor pH value is less critical than the acceptor pH, as could be expected considering the extraction procedure and the involved chemical processes. Again, the SAC behaviour is the opposite respect the DIC and IBU. Time seems to be the less critical factor and always with a positive effect on the extraction procedure.

From the results it is possible to consider the acceptor pH as a critical factor that must be carefully controlled, while with respect donor pH and time the extraction procedure can be considered a robust extraction procedure.

3.3. Linearity, sensitivity and precision for HF-LPME extraction

Using the selected HF-LPME conditions, several aqueous pH 2 solutions with different SAC, DIC and IBU concentrations were submitted to the liquid microextraction procedure and analyzed according to the described HPLC procedure. The DAD and FLD peak areas were proportional to the SAC, DIC and IBU concentrations in the donor phase. Table 5 shows the corresponding DAD and FLD calibration parameters and the detection limits calculated as three times the standard deviation of the background signal (3σ). The enrichment factors obtained with HF-LPME are 70, 1060 and 200 for SAC, DIC and IBU, respectively. For DAD, the relative standard deviation for 15 repetitive determinations of 5 μg mL^{-1} of SAC, DIC and IBU (intraday repeatability) were 1.2, 1.3 and 0.8%, respectively. The interday repeatability (n = 3; 5 days) were 1.8, 1.7 and 1.1%. For FLD, the relative standard deviation for 15 repetitive determinations of 0.1 μg mL^{-1} of SAC and 0.025 μg mL^{-1} of IBU (intraday repeatability) were 1.5 and 1.8%, respectively. The interday repeatability (n = 3; 5 days) were 1.8 and 2.3%, respectively.

3.4. Human urine analysis using HF-LPME extraction

Urine samples are complex matrices that usually require previous clean-up procedures; SPE procedures are the most frequently clean-up method used. SPE cartridges are, depending on the stationary phase used, a non-low-cost procedure and that requires several conditioning and elution steps. Low precision values are frequently obtained in analytical methods that use SPE as clean-up and/or preconcentration procedure. This work proposes HF-LPME extraction as a simple, accurate and low-cost procedure for the clean-up and preconcentration of SAC, DIC and IBU in human urine. Fig. 1 shows the DAD and FLD chromatograms obtained after the HF-LPME extraction and HPLC determination of a blank human urine sample; as can be seen, the chromatograms show excellent baselines with peak absence that enhances that HF-LPME can be an adequate clean-up procedure for urine samples.

Recovery assays were performed using spiked human urine samples from volunteers to obtain several concentration levels of SAC, DIC and IBU that were submitted to the HF-LPME extraction and HPLC determination procedure described in the experimental section. The results obtained are shown in Table 6, with good agreement between spiked and measured quantities for SAC and DIC; the recoveries for IBU are around 85%.

Two sets of human real urine samples from two healthy volunteers were collected at intervals of 30 min. The first set of samples was collected after the administration of oral doses of 600 mg IBU and 100 mg diclofenac. The second set of samples corresponds to the collection after the administration of a 500 mg oral dose of acetylsalicylic acid; it is well known that this compound is quickly hydrolyzed in the stomach to salicylic acid.

Figs. 2 and 3 show the corresponding chromatograms where, as can be seen, additionally to the SAC, DIC and IBU peaks other unknown peaks appear. According to their absorption and fluorescence spectra and bibliographic data these peaks can be assigned to their main metabolites. The specific identification was not possible because the corresponding metabolite standards were not available. So, peaks UNK1 to UNK4 can be assignated to several or all the main five diclofenac metabolites (4′-hydroxy-, 5-hydroxy-, 3′-hydroxy-, 4′,5-dihydroxy- and 3′-hydroxy-4′-methoxy diclofenac);

peaks UNK5 and UNK6 can be assignated to the two main ibuprofen metabolites (2-hydroxyibuprofen and 2-carboxyibuprofen). For the urine samples collected after the administration of acetylsalicylic acid, the unknown peaks UNK7 and UNK8 can be assignated to several or all of the three main metabolites: salicyl acyl glucuronide, salicyluric acid and gentisic acid.

Fig. 4 shows the SAC, DIC and IBU urine concentration vs. time after the administration of the above described oral doses; as can be seen maximum urine concentrations are obtained at 140 min for diclofenac and ibuprofen and around 200 min for salicylic acid.

4. Conclusions

This study presents a rapid hollow fiber-based liquid phase microextraction (HF-LPME) method combined with a HPLC determination using a monolithic silica type column that allows a simple, low-cost, fast, accurate and selective methodology for the determination of salicylic acid, ibuprofen and diclofenac in urine samples. According to the data obtained, it is possible that the proposed procedure can be also applied to the determination of their corresponding main metabolites and can be expected similar enrichment factors to the corresponding analytes, which improves the HF-LPME/HPLC method proposed. Likewise, the high enrichment obtained with the HF-LPME procedure can be adequate for the determination of the analytes and metabolites at very low levels and could be used for their analysis in other biological or environmental matrices.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2009.09.018.

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Application of hollow fiber-based liquid-phase microextraction (HF-LPME) for the determination of acidic pharmaceuticals in wastewaters

En este trabajo se propone y optimiza la extracción y posterior determinación de tres compuestos pertenecientes a la familia de las aintiinflamatorios no esteroideos: ibuprofeno, diclofenaco y ácido salicílico, mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior empleando espectrometría de masas con detector de triple cuadrupolo.

La membrana líquida de dihexiléter fue soportada sobre una fibra de polipropileno Q3/2 Accurel KM. Se empleó como fase donadora una solución acuosa de pH 2 (ajustada con HCl) y una solución acuosa de NaOH de pH 12.5 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de quince minutos con una agitación de 300 rpm.

La separación cromatográfica se consiguió en diez minutos, empleando una columna de alto empaquetamiento Pursuit® XRs Ultra 2.8µm C18 (100×2.0mm i.d.) y un gradiente de ácido fórmico 0,1%/Metanol a un flujo de 0.2 ml/min. Las columnas de alto empaquetamiento permiten obtener una elevada resolución empleando flujos relativamente bajos haciendola compatible con la técnica de detección que se emplea, así como una buena simetría en los picos y tiempos de desarrollo pequeños.

Los límites de cuantificación obtenidos varían entre 0.1-0.50 µg/L y se obtienen reproducibilidades entre el 1.5% y el 2.1%. La evaluación de la robustez se ha realizado mediante un diseño experimental de tres factores a dos niveles, para ver cuál es la variable experimental más crítica en el procedimiento de extracción, así como posibles interacciones que hay entre ellas.

El procedimiento ha sido aplicado muy satisfactoriamente a la determinación directa de los analitos en aguas residuales urbanas y en agua de río, con recuperaciones en torno al 100%, excepto para ibuprofeno y diclofenaco, en aguas residuales, con recuperaciones del 50 y 70%, respectivamente.

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Short communication

Application of hollow fiber-based liquid-phase microextraction (HF-LPME) for the determination of acidic pharmaceuticals in wastewaters

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ABSTRACT

The presence of pharmaceuticals in the environment is a very important problem that requires analytical solutions. The wide variety of matrices and, usually, the low pharmaceuticals levels in the environmental samples requires high sensitive and selective analytical procedures. Wastewaters are one of the more important sources of environmental pollutants but they are very complex matrices that need clean-up procedures prior the analysis. Hollow fiber-based liquid-phase microextraction (HF-LPME) is a relatively new technique used in analytical chemistry for sample pre-treatment that offers high selectivity and sensitivity compared to most traditional extraction techniques. The low organic solvent consumption derived from the use of HF-LPME is according to the current trends to a "Green Chemistry", and Analytical Chemistry should follow these environmental good practices. This paper describes an extraction method using a polypropylene membrane supporting dihexyl ether (three-phase hollow fiber-based liquid-phase microextraction (HF-LPME)) for the direct analysis of three pharmaceuticals (salicylic acid (SAC), ibuprofen (IBU) and diclofenac (DIC)) in raw and treated wastewaters followed by a HPLC/MS-MS determination using a highly packed Pursuit® XRs Ultra 2.8 μm C18 column that allows high resolution using low flow-rates and, simultaneously, short retention times. Detection limits were 20, 100 and 300 ng L^{-1} for salicylic acid, diclofenac and ibuprofen, respectively.

1. Introduction

Pharmaceutical products are a broad and diverse group of chemicals developed and used to produce specific biological effects in humans and animals health care or livestock farming. The growing worldwide consumption of pharmaceuticals and their proved occurrence in the environment has become an important issue in recent years, and in the last decade, the focus of environmental research has been extended from more classical environmental pollutants as PCBs, PAHs or pesticides to pharmaceuticals and personal care products; pharmaceuticals are, in some way, dangerous because they have been designed to be biologically active. The amount of human pharmaceuticals reaching the environment depends on the consumption amount, and excretion rate via faeces and urine. Effluents of wastewater treatment plants (WWTPs) are considered the principal source of drugs in the aquatic environment. A smaller contribution to the presence of pharmaceuticals in the environment is due to the disposal of outdated medicines down household drains [1] and to the pharmaceutical industry waste [2,3].

Raw and treated wastewater are complex matrices that difficult their analysis. Several extraction procedures have been applied to wastewaters and recent results have been reviewed for molecular imprinted polymers [4,5] or stir bar sorptive extraction [6]. However, solid-phase extraction (SPE), using several sorbent types has been the preferred sample preparation technique to extract pharmaceuticals from environmental waters [7–9]; although the sorbents usually show poor selectivity and this will constitute a problem when a selective extraction from complex matrices must be performed. Additionally, SPE involves intensive sample handling and needs several time consuming steps. Liquid–liquid extraction (LLE) is a classical and common technique used for preconcentration and cleanup prior to chromatographic or electrophoretic analysis that requires large organic solvent consumption. It is also tedious and analyte-loss is frequent due to multi-stage operations that cannot be neglected. Liquid-phase microextraction (LPME), based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) [10,11], is a simple, inexpensive, fast, effective and virtually solvent-free sample pre-treatment technique. However, SDME is not very robust, and the droplets may

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be lost from the needle tip of the microsyringe during extraction.

Audunsson [12] introduced an alternative concept for LPME that was developed by Thordarson et al. [13] and Pedersen-Bjergaard and Rasmussen [14] based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber. In hollow fiber liquid-phase microextraction (HF-LPME), the organic phase is protected by the fiber, and it appears that the hollow fiber decelerates the process of organic solvent dissolution into the bulk solution. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor solution from entering the accepting phase and, at the same time, most macromolecules do not enter the hollow fiber because they are not soluble in the organic phase present in the pores, thus yielding very clean extracts. Several reviews that focus on basic extraction principles, technical setup, recovery, enrichment, extraction speed, selectivity, applications and future trends in hollow fiber-based LPME have been reported [15–18].

There are two modes used: two-phase HF-LPME and three-phase HF-LPME. In two-phase HF-LPME, the analytes are extracted by passive diffusion from the sample into the hydrophobic organic solvent supported by the fiber, and in three-phase HF-LPME the analytes are extracted through an organic solvent immobilized in the pores of the fiber and further into a new aqueous phase in the lumen of the fiber.

Compared with LLE and SPE, HF-LPME gives, at least, a comparable and satisfactory sensitivity and in many cases better enrichment for the analytes; the consumption of solvent is significantly reduced by up to several hundred or several thousand times. The LPME technique is simple, fast, and inexpensive. Due to the small volume of the extracting solvent, the extracted samples do not require further concentration prior to analysis and thus total analysis time considerably decreases in comparison to traditional LLE procedures. Additional advantages of LPME also make the technique attractive. Since, LPME tolerates a wide pH range; it can be used in applications that would not be suitable for solid-phase extraction (SPE) or solid-phase microextraction (SPME). Sample carryover can be avoided because the hollow fibers are enough cheap to be single-used and disposed. It has been demonstrated that HF-LPME is very useful for the extraction of acidic drugs and, in some cases their metabolites, from biological matrices and from environmental samples with a simultaneous cleanup and preconcentration of the extracts [19–30]. This extraction technique has been used for the analysis of ibuprofen using HPLC [23–25], gas chromatography [26,27], capillary electrophoresis (CE) [28,29] and FIA with chemiluminescence detection [30]. Diclofenac [23,25] and salicylic acid [25] have been also analyzed using HPLC previous HF-LPME treatment.

The aim of this work was to develop of an alternative extraction method for pharmaceuticals applicable to wastewaters which avoid some analytical problems that usually overcomes with SPE technique when mass spectrometry is used as detection system coupled to HPLC: important matrix and ionic suppression effects. The simplicity of the HF-LPME could be an interesting way to obtain enough sensitivity due to the low levels of these drugs in wastewaters. Likewise, the consumption of organic solvents can be reduced to several microliters using HF-LPME in contrast to SPE procedures.

In this work, a HPLC/MS–MS method combined with prior HF-LPME was developed for the determination of three widely used drugs: salicylic acid (2-hydroxy-benzoic acid) (SAC), the hydrolysis product of the well known acetylsalicylic acid (2-(acetyloxy)-benzoic acid) and two non-steroidal anti-inflammatory drugs widely used, diclofenac (2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid) (DIC) and ibuprofen ((R,S)-2-(4-isobutylphenyl)-propionic acid) (IBU); the method was applied to their determination in wastewater. The HF-LPME provides very clean extracts that can be directly injected into the chromatographic system allowing excellent baselines. Additionally, HF-LPME also provides sample preconcentration that enhances the applicability of the proposed method.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water form a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). SAC, DIC, IBU, dihexyl ether and 1-octanol were purchased from Fluka-Sigma–Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany).

Aqueous working solutions of SAC, DIC and IBU were daily prepared by adequate dilutions from aqueous 200 $\mu g\,mL^{-1}$ stock solutions. Q3/2 Accurel KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Chromatographic conditions

The chromatographic separation was performed at 20 °C using a LaChrom® Elite VWR-Hitachi (Barcelona, Spain) with a quaternary L-2130 pump. The injector was a Rheodyne manual injection valve Model 7725i, fitted with a 20-μL sample loop. Separations were carried out using a Pursuit® XRs Ultra 2.8 μm C18 (100 × 2.0 mm i.d.) (Varian Inc., Palo Alto, CA, USA) preceded by a guard column Kromasil® 100 Å, C18, 5 μm, (15 × 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain).

The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and methanol (component B) at a flow rate of 0.2 $mL\,min^{-1}$. An initial 20% component A was used in isocratic mode for 3 min and then a linear elution gradient was programmed from 20% to 0% A for another 7 min. Three minutes were waited between injections which allowed re-equilibration of the column to the initial conditions.

2.3. Mass spectrometry detection

For the MS/MS detection an API 2000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipped with a Turbo HS-602 source housing was used. Nitrogen was used as collision gas at 4 psi. The ion source and curtain gases were set at 30 psi in both cases. The electrospray voltage was −4500 V. Acquisition was performed in selected reaction monitoring (SRM) mode and the protonated molecular ion of each compound was chosen as precursor ion. Sciex Analyst 4.0 software was used for data acquisition and handling. The optimisation of MS parameters (declustering potential, entrance potential, for precursor ions and collision energy, and collision cell exit potential for product ions) was performed by flow injection analysis for each compound. Table 1 shows the values of the parameters optimised and the SRM transitions selected. Quantitative analysis was performed using external calibration.

2.4. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked with dihexyl

Table 1
Optimised parameters for the MS/MS analysis of the selected compounds.

Compound	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (v)	Entrance potential (v)	Collision energy (eV)	Collision cell exit potential (v)
SAC	137	92.9	−45	−7	−25	−10
DIC	294.2	250	−50	−9	−16	−15
IBU	205.2	161.1	−45	−9	−12	−15

Table 2
Efficiency and selectivity chromatographic parameters for the proposed HPLC procedure.

	t_R (min)	RSD-t_R (%)	$W_{1/2}$ (min)	T	N	$K\tilde{i}$	α	R_s
SAC	2.25	0.021	0.1273	1.39	1731	130	3.01	10.37
DIC	4.76	0.003	0.1583	1.29	5009	258	1.91	2.64
IBU	5.41	0.007	0.1321	1.18	9292	282	1.17	2.64
Critical values		<1%		<1.5		>2		>1.5

t_R, retention time; RSD-t_R, relative standard deviation for retention time; N, number of theoretical plates; T, asymmetry factor; $W_{1/2}$, peak half-width; K', capacity factor; α, selectivity factor; R_s, peak resolution.

ether during 5 s to impregnate the pores and rinsed with water on the outside by placing it into an ultrasonic bath for 30 s in order to remove the excess of organic solvent. The lumen of the prepared fiber piece was filled with 50 μL of acceptor phase (pH 12.5 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and adhesive tape. During extraction the membrane portion that contains the acceptor phase was immersed in the 50 mL sample solution (pH 2) contained into a 50 mL glass beaker. The sample was stirred for 15 min by means of a magnetic stirrer (ANS-00/1 Science Basic Solutions (Rubí, Barcelona, SPAIN) at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extracted using a HPLC syringe and injected into the HPLC system.

2.5. Preparation of wastewater samples

Samples were obtained from "Guadalquivir"-ALJARAFESA Wastewater Treatment Plant (WWTP) which is located in Palomares del Río, Seville, Spain. The plant essentially receives urban wastewaters. The capacity of this WWTP is 100,000 inhabitants and the discharged flow is 12,433,313 m^3/year (2008 data).

Grab samples of the influent (raw water, WWR), after the primary sedimentation tank (WW1), after the aeration tank (WW2) and the effluent (treated water after anaerobic digestion, WWT) were collected in 13th May 2009. All samples were filtered through a GDU1 glass fibre filter bed (10–1 μm) (Whatman, Mainstone, UK) and through Pall Nylaflo™ nylon membrane filter 0.45 μm (Pall Corporation, Ann Arbor, MI, USA) and adjusted to pH 2 with HCl. Filtered samples were stored in the dark at 4 °C prior to HF-LPME extraction.

3. Results and discussion

3.1. Chromatographic conditions

Looking for a fast and high resolution separation a Pursuit® XRs Ultra (2.8 μm) was selected as working column. This column is a highly packed HPLC column that allows high resolution separations using low flow-rates compatible with MS detection coupled to conventional HPLC equipment. The selected column provides good resolution and good peak symmetry.

The mobile phase consisted of 0.1% formic acid and methanol. Different gradient elution conditions were tested searching for the shortest time of analysis without sacrificing peak shape. The gradient elution program described in Section 2.2, was the best option in terms of time of analysis, shape of the peaks and reproducibility.

The efficiency and selectivity chromatographic parameters of the proposed procedure are shown in Table 2. As it can be seen, all resolutions are above the critical value >1.5, and peaks show good symmetry.

3.2. Optimization and evaluation of experimental conditions for HF-LPME extraction

Optimal experimental conditions for the HF-LPME extraction are fully described in our previous paper [25] where aqueous HCl solutions within 1–4 pH range were tested as donor, NaOH aqueous solutions with pH values between 8 and 13 were assayed as acceptor phase and stirring times between 5 and 30 min at 300 rpm were also tested. Optimum values of pH 2 (donor phase), pH 12.5 (acceptor phase) and 15 min of stirring time (300 rpm) were fixed. As in our previous paper [25], a full factorial design [31,32] for three factors and two levels involving eight experiments (2^3) has been used to determine the effect and importance of the mentioned variables on the final result.

Table 3 shows the equations obtained from the experimental data and the calculated t-values for each of the factors and analytes where b_1, b_2 and b_3 are the donor pH, acceptor pH and stirring time, respectively.

As it can be seen, the main factor is the acceptor pH for all the analytes. For DIC and IBU, an increase in the acceptor pH leads to better results in the extraction, while in the case of SAC leads to a decrease in the signal; so this pH value is a critical parameter. The donor pH value is less critical than the acceptor pH, as could be expected considering the extraction procedure and the involved chemical processes. Again, SAC behaviour is the opposite respect DIC and IBU. Time seems to be the less critical factor and always with a positive effect on the extraction procedure.

From the results obtained it is possible to consider the acceptor pH as a critical factor that must be carefully controlled, while with respect to donor pH and time, the extraction procedure can be considered a robust extraction procedure.

3.3. Linearity, sensitivity and precision for HF-LPME extraction

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve was constructed using a least-square linear regression analysis of standards mixtures of the analytes at different concentrations. Using the selected HF-LPME conditions, aqueous pH 2 solutions with different SAC, DIC and IBU concentrations were submitted to the liquid microextraction procedure and analyzed according to the described HPLC procedure. Peak areas of SAC, DIC and IBU were proportional

Table 3
Results from the full factorial design.

		t calculated		
		Donor pH	Acceptor pH	Stirring time
SAC	$Y = 2.1358 - 0.1612b_1 + 1.8433b_2 + 0.0018b_3$	1.4733	4.8216	0.2935
DIC	$Y = 1.0735 + 0.2851b_1 - 1.6885b_2 + 0.0523b_3$	2.6285	0.7685	0.0081
IBU	$Y = 1.3766 + 0.4845b_1 - 1.7768b_2 + 0.0036b_3$	1.1523	4.6582	0.3623

Critical value for t ($P = 0.05$, $n = 4$) 2.78.

Table 4
HPLC calibration parameters and instrumental detection limits (ILOD) for the analytes.

	Regression coef. (r^2)	Linearity (%)	Linear range ($\mu g\,L^{-1}$)	ILOD ($\mu g\,L^{-1}$)
SAC	0.9993	99.32	0.5–300	0.3
DIC	0.9993	98.34	1–300	0.5
IBU	0.9999	99.37	5–300	1.0

Table 5
HF-LPME/HPLC calibration parameters and method detection limit (MLOD) for the analytes.

	Regression coef. (r^2)	Linearity (%)	Linear range ($\mu g\,L^{-1}$)	MLOD ($\mu g\,L^{-1}$)
SAC	0.9998	99.81	0.1–50	0.02
DIC	0.9989	99.57	0.25–50	0.10
IBU	0.9994	99.68	0.50–50	0.30

to concentrations in the donor phase. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ (figures depicted in Tables 3 and 5) and the calibration curves obtained showed no changes over the course of one month.

Detection and quantitation limits were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratios are 3 and 10, respectively. Instrumental limits (ILOD and ILOQ) are listed in Table 4 and method limits (MLOD and MLOQ) are listed in Table 5. As it can be seen in tables method limits are lower than instrumental limits due to the preconcentration suffered by the analytes during the extraction procedure.

To evaluate the repeatability and the intermediate precision, spiked samples (validation standards) at three concentrations levels 0.25, 10 and 30 $\mu g\,mL^{-1}$ of SAC and 0.5, 10 and 30 $\mu g\,mL^{-1}$ of DIC and IBU in triplicate were subjected to the entire analytical procedure and measured in one single day and one day per week during two months, respectively. Intermediate precision was performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the $m \times p \times n$ design (m = analytical levels, p = days and n = replications). From the corresponding ANOVA, the intermediate precision was computed [33]. The repeatability, expressed as relative standard deviation, was in the range 1.1–1.6%. Intermediate precision also expressed as relative standard deviation, was in the range 1.5–2.1%.

3.4. Wastewater analysis using HF-LPME

3.4.1. Recovery assays on spiked wastewater

Recovery assays were performed on the four different wastewater samples at three concentration levels 0.25, 10 and 30 $\mu g\,L^{-1}$ for SAC and 1, 10 and 30 $\mu g\,L^{-1}$ for DIC and IBU; results obtained are shown in Table 6. As it can be seen, extraction effectiveness for the analyzed substances are unrelated to the type of wastewater, remaining practically constants (about 100% SAC, 71% DIC and 52% IBU). The decrease in the recoveries of DIC and IBU can be due to the high surfactant concentrations in the wastewaters, even after their depuration, which modifies the behaviour of the supported liquid membrane on the polypropylene fiber. This behaviour seems to be more pronounced when the polarity of the extracted substances decreases. The behaviour modification has been checked in our laboratory with several experiments testing the effect of the addition of several surfactants to standards (even at low concentrations). Despite the fact that DIC and IBU recovery decreases, the excellent clean-up obtained implies a great advantage over other sample treatment procedures which justifies the HF-LPME extraction.

Fig. 1 shows representative chromatograms obtained from spiked (5 $\mu g\,L^{-1}$ of SAC, DIC and IBU) wastewater samples (raw, WWR and treated, WWT); as it can be seen, both chromatograms show excellent baselines and well-defined peaks corresponding only to the spiked substances, similar chromatograms were obtained for WW1 and WW2 spiked samples. This fact demonstrates that HF-LPME is an adequate clean-up procedure for wastewater samples. Chromatograms from wastewaters without SAC, DIC and IBU show horizontal baselines without peaks.

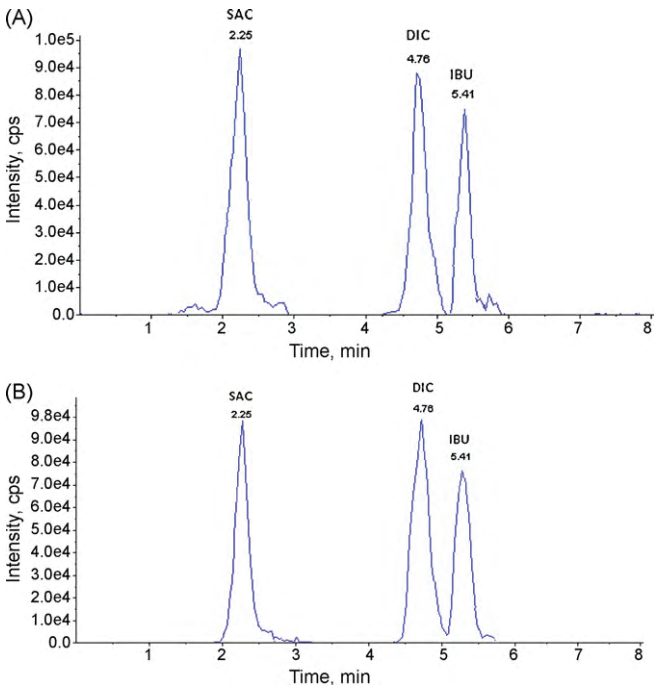

Fig. 1. Representative HPLC/MS–MS chromatograms for extracted raw (A) and treated (B) spiked wastewaters. (5 $\mu g\,L^{-1}$ of SAC, DIC and IBU).

Table 6
HF-LPME/HPLC/MS–MS recoveries (average of three determinations ± standard deviation) from spiked wastewaters.

Wastewater	Spiked level ($\mu g\,L^{-1}$)	SAC	DIC	IBU
WWR	1 (0.25*)	100.3 ± 0.3	71.0 ± 0.7	53.0 ± 1.8
	10	99.3 ± 0.4	70.8 ± 0.9	50.0 ± 1.5
	30	100.1 ± 0.5	72.2 ± 0.6	52.8 ± 1.2
WW1	1 (0.25*)	100.3 ± 0.7	70.8 ± 1.2	50.9 ± 1.1
	10	99.0 ± 0.6	72.9 ± 1.0	51.3 ± 0.9
	30	100.2 ± 0.8	71.9 ± 0.8	50.4 ± 0.9
WW2	1 (0.25*)	99.3 ± 0.2	71.3 ± 0.8	52.8 ± 1.6
	10	99.8 ± 0.1	72.1 ± 0.3	50.9 ± 1.2
	30	100.3 ± 0.5	71.8 ± 0.6	52.1 ± 0.8
WWT	1 (0.25*)	99.8 ± 0.1	71.5 ± 0.9	52.6 ± 1.0
	10	100.0 ± 0.4	72.7 ± 1.0	51.8 ± 0.5
	30	99.6 ± 0.5	71.9 ± 0.8	52.0 ± 1.2

* Spiked level for SAC.

Table 7
Concentration ($\mu g\,L^{-1}$) of the pharmaceuticals in the analyzed wastewater samples.

Compound	WWR	WW1	WW2	WWT
SAC	*	*	–	–
DIC	*	–	–	–
IBU	0.65	*	*	–

–, Bellow method detection limits.
*, MLOD < Concentration < MLOQ.

3.4.2. Analysis of real samples

The results from the application of the proposed HF-LPME procedure to the wastewater samples are shown in Table 7; as it can be seen, only raw wastewater (WWR) sample showed IBU levels that allows its determination; in this sample, SAC and DIC were only detected. In the other wastewater samples only SAC and/or IBU were detected.

These results obtained are according to several bibliographic data [34–37], and the high ibuprofen levels reflect its large consumption.

4. Conclusions

Wastewater samples are complex matrices that require previous clean-up procedures like SPE that are the most frequently used nowadays. SPE requires several conditioning and elution steps which sometimes traduce in low precision values. Besides, wastewaters extracts obtained by SPE usually produce important matrix effects and ionic suppression when MS detection is used.

This study presents a rapid hollow fiber-based liquid-phase microextraction (HF-LPME) method combined with an HPLC–MS/MS determination using a highly packed chromatographic column that allows a simple, low-cost, fast, accurate, sensitive and selective methodology for the determination of salicylic acid, ibuprofen and diclofenac in wastewater samples. The proposed extraction procedure has a very low (several μLs) organic solvent consumption. The excellent clean-up obtained implies a great advantage over other sample treatment procedures.

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Capillary electrophoresis determination of nonsteroidal anti-inflammatory drugs in wastewater using hollow fiber liquid-phase microextraction

En este trabajo se emplea como técnica analítica de separación un sistema de electroforesis capilar empleando un detector de diodo array, para la determinación de siete antiinflamatorios no esteroideos: ibuprofeno, naproxeno, ketoprofeno, diclofenaco, ketorolaco, aceclofenaco y ácido salicílico, previamente extraídos mediante HF-LPME.

El electrolito de separación, es un tampón acetato 30mM a pH4 al 25% de acetonitrilo. El voltaje aplicado fue de 25KV y la temperatura del capilar se mantiene constante a 20ºC. Esta técnica permite inyectar del orden de nanolitros, por lo que se pueden llevar a cabo extracciones con fibras de menor longitud.

La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases, empleando dihexiléter como membrana líquida sobre una fibra de polipropileno Accurel Q3/2 de 13cm. La fase donadora y aceptora consisten en una solución acuosa a pH 2 y pH 12 respectivamente. La muestra se agita a 300 rpm y se obtienen enriquecimientos de 86 a 320, tras veinte minutos de agitación.

Los límites de detección obtenidos para los analitos estudiados varían entre 0.25-0.86 µg/L, con reproducibilidades entre 2.1 y 3.2 %.

El procedimiento ha sido aplicado satisfactoriamente a la determinación de los analitos en aguas residuales urbanas y agua de río, con recuperaciones entre el 58-100%.

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Research Article

Capillary electrophoresis determination of nonsteroidal anti-inflammatory drugs in wastewater using hollow fiber liquid-phase microextraction

The presence of pharmaceuticals in the environment due to growing worldwide consumption has become an important problem that requires analytical solutions. This paper describes a CE determination for several nonsteroidal anti-inflammatory drugs (ibuprofen, naproxen, ketoprofen, diclofenac, ketorolac, aceclofenac and salicylic acid) in environmental waters using hollow fiber membrane liquid-phase microextraction. The extraction was carried out using a polypropylene membrane supporting dihexyl ether and the electrophoretic separation was performed in acetate buffer (30 mM, pH 4) using ACN as the organic modifier. Detection limits between 0.25 and 0.86 ng/mL were obtained, respectively. The method could be applied to the direct determination of the seven anti-inflammatories in wastewaters, and five of them have been determined or detected in different urban wastewaters.

Keywords:
CE / Hollow fiber liquid-phase microextraction / Nonsteroidal anti-inflammatory drugs / Wastewater
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1 Introduction

Pharmaceuticals are a class of emerging environmental pollutants that are widely used both in human and veterinary medicine. After their use, large amounts of them are discharged into the water environment, and as a result, they have been detected in wastewater, surface water and groundwater [1–6]. Furthermore, pharmaceuticals are known to have potential risk to the aquatic ecosystems such as endocrine disrupting and severe side effects because they originally cause specific biological effects [7–11]. For these reasons, pharmaceutical pollution has become an emerging environmental problem worldwide. The main route of pharmaceuticals to the environment is through discharged effluents from wastewater treatment plants as a result of excretion from humans and animals, as well as from domestic disposal of medicinal products [3]. Among these pharmaceuticals, nonsteroidal anti-inflammatory drugs (NSAIDs) and lipid regulators are some of the most commonly detected, in concentrations ranging from the low ng/L up to the μg/L level [12–15]. NSAIDs are a group of pharmaceutical compounds frequently employed in many fields; they have analgesic, antipyretic and platelet-inhibitory actions. Ibuprofen (IBU), naproxen (NAX), ketoprofen (KTP), diclofenac (DIC), ketorolac (KTR), aceclofenac (ACE) and salicylic acid (SAC) are the NSAIDs more frequently consumed. Many methods have been developed over the decades for the determination of NSAIDs. Among them, the methods based on LC [16–19], GC [20–22] and CE [23–26] are the most efficient. A few manuscripts for the determination of acidic drugs by hollow fiber-based liquid-phase microextraction (HF-LPME) and CE determination have been reported in the recent years [27–29].

Raw and treated wastewaters are complex matrices that are difficult to analyze. Several extraction procedures have been applied to wastewater and recent results have been reviewed for molecular imprinted polymers [30, 31] or stir bar sorptive extraction [32]. However, solid-phase extraction (SPE) has been the preferred extraction technique for pharmaceuticals in environmental waters [33–35], but this technique exhibits from our point of view some disadvantages such as being time consuming, relatively expensive and hazardous to health due to the large amount of solvents used. In contrast, liquid-phase microextraction (LPME) is a relatively recent technique that combines extraction, clean-up and preconcentration in one step. Normally, this technique is carried out by using a membrane as interface

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Abbreviations: ACE, aceclofenac; **DIC,** diclofenac; **EF,** enrichment factor; **HF-LPME,** hollow fiber-based liquid-phase microextraction; **IBU,** ibuprofen; **KTP,** ketoprofen; **KTR,** ketorolac; **NAX,** naproxen; **NSAID,** nonsteroidal anti-inflammatory drugs; **SAC,** salicylic acid; **WW1,** wastewater from the primary sedimentation tank; **WWR,** raw wastewater; **WWT,** treated water after anaerobic digestion

between the sample (donor) and the organic solvent (acceptor), which avoids mixing of the two phases and other problems encountered in classical liquid–liquid extraction [36, 37]. The main advantages of LPME are very low organic solvent consumption and low cost.

Audunsson [38] introduced the concept for LPME that was developed by Thordarson et al. [39] and for Pedersen-Bjergaard and Rasmussen [40] based in a three-phase system. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microliters) immobilized within the pores of a porous HF, and into an acceptor solution adjusted to the adequate pH inside the lumen of the HF [24, 41]. Several reviews that focus on the basic extraction principles, technical setup, recovery, enrichment, extraction speed, selectivity, applications and future trends in HF-based LPME have been reported [36, 37, 42, 43].

Compared with LLE and SPE, HF-LPME gives a satisfactory sensitivity and similar enrichment of analytes, and the consumption of solvent is significantly reduced up to several hundred or several thousand times. Owing to the small volume of the extracting solvent, the extracted samples do not require further concentration prior to analysis and the use of CE presents a great advantage versus HPLC, which needs more sample volume to inject [44]. LPME sample treatment is an adequate procedure for CE analysis due to the low injection volume required.

With respect to the previously published HF-LPME procedures for the NSAIDs determination, the one developed by Quintana et al. [12] uses 1-octanol as the supported liquid membrane with extraction times of 45 min, using HPLC-MS analysis for the determination of KTR, NAX, fenoprofen, IBU and DIC with enrichment factors (EFs) of 196, 186, 154, 118 and 70, respectively. This procedure was applied for their determination in wastewaters. Pederesen-Bjergaard and Rasmussen [27] describe an HF-LPME/CE procedure for the determination of IBU, NAX and KTP in pure water and urine using dihexyl ether with extraction times of 45 min. LOD for NAX was 1 ng/mL and needs the use of internal standard in order to improve the repeatability. EFs in the 75–100 range were reported. Rasmussen et al. [28] describe an HF-LPME/CE procedure for the determination of NAX, using 1-octanol at 45-min extraction time that allows LOD of 1 ng/mL and the procedure was applied for the NAX determination in human urine samples with EF of 125. Nozal et al. [29] describe an in-line LPME for the analysis of IBU, fenbufen and KTP with LODs of 1.7, 1.5 and 1.2 ng/mL, respectively, which was applied to human urine samples.

The aim of this work was to develop a simple and low-cost method for pharmaceuticals applicable to wastewater, which does not involve cumbersome clean-up procedures. A CE method combined with HF-LPME was developed for the determination of seven NSAIDs: IBU, NAX, KTP, DIC, KTR, ACE and SAC. All the HF-LPME and CE parameters have been optimized. The method has been satisfactorily applied for the determination of these compounds in different kinds of wastewaters.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were of analytical-reagent grade. All solutions and dilutions were prepared in ultrapure water from a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). IBU, NAX, KTP, DIC, KTR, ACE, SAC and dihexyl ether were purchased from Fluka-Sigma-Aldrich (Madrid, Spain) and the rest of the reagents were supplied by VWR (Barcelona, Spain).

Working solutions of NSAIDs were prepared daily by adequate dilutions from a 100 μg/mL stock solution. All standard solutions were stored at 4°C and were stable for at least 1 month. Q3/2 Accurel® KM polypropylene HF (600 μm id, 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2 Apparatus

All measurements were made with a Beckman P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA, USA) equipped with a diode array detector (DAD). Uncoated fused-silica capillaries (Beckman-Coulter) of 75 μm id and an effective length of 50 cm (total length of 57 cm) were used. The magnetic stirrer was an ANS-00/1 Science Basic Solutions (Rubí, Barcelona, Spain).

2.3. Operating conditions

Daily, before use, the capillary was successively rinsed with 0.1 M NaOH, water and the running buffer for 5 min each. Between runs, it was also conditioned with 0.1 M NaOH (2 min), water (3 min) and the running buffer (5 min). The separation was performed using acetate buffer (30 mM, pH = 4, 25% ACN), a separation voltage of 25 kKV and the temperature of the capillary was set to 20°C. Injections were performed in pressure mode, setting the injection time at 7 s and 0.7 psi. The wavelengths used for DAD were 220 nm for IBU, DIC, ACE, SAC; 225 nm for NAX; 253 for KTP and 318 nm for KTR.

2.4 Supported liquid membrane preparation and extraction procedure

HFs were cut into 13-cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked in the membrane phase (dihexyl ether) for 5 s to impregnate pores of the support and rinsed with water on the outside by placing into an ultrasonic bath for 25 s in order to remove the excess of organic solvent. The lumen of the prepared fiber was filled with 30 μL of the acceptor phase (pH 12, aqueous solution adjusted with NaOH) using an HPLC syringe. Both open ends of the fiber were closed by means of

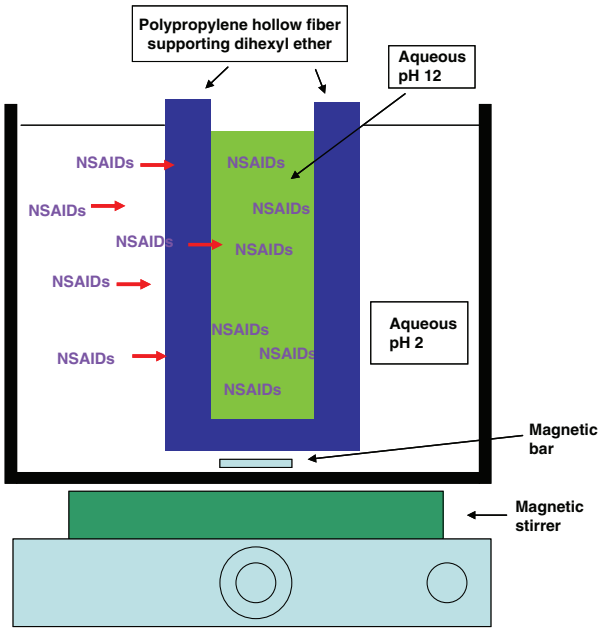

Figure 1. Scheme of the experimental HF-LPME setup.

a hot soldering tool and an adhesive tape. During extraction, the HF was immersed into 50 mL sample solution (pH 2, aqueous solution adjusted with HCl) contained into a 50-mL glass beaker. The sample was stirred for 20 min by means of a magnetic stirred at 300 rpm. Figure 1 shows a scheme of the experimental setup. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extracted using an HPLC syringe, transferred into a microvial and injected into the CE-system.

2.5 Preparation of water samples

Wastewater samples were obtained from the Wastewater Treatment Plant Punta Umbría-AQUALIA which is located in Punta Umbría (Huelva, Spain). The plant essentially receives urban wastewaters. The discharged flow is 21 250 m^3/day. Samples for the influent (raw water, WWR), after the primary sedimentation tank (WW1) and the effluent (treated water after anaerobic digestion, WWT) were analyzed.

All samples were filtered through a GDU1 glass fibre filter bed (10–1 µm) (Whatman, Mainstone, UK) and through Pall Nylaflo^{TM} nylon membrane filter of 0.45 µm (Pall, Ann Arbor, MI, USA). Filtered samples were stored in dark at 4°C prior to HF-LPME extraction.

3 Results and discussion

3.1 Optimization of the separation conditions

The pH of the running buffer exhibits a strong effect on the ionization of the studied compounds and the magnitude of EOF, through which it influences both the resolution and the migration time. The effect of the buffer pH was examined over the 2–10 range using phosphate buffer, borate buffer and acetate buffer, obtaining the best results with acetate buffer at pH 4. The influence of the acetate concentration was tested over the 10–60 mM range at pH 4. Raising the acetate concentration, an increased is observed on the resolution but an increment of current and migration time was obtained, so the optimum concentration of acetate buffer was set at 30 mM, obtaining a good resolution for each compound under these conditions. The addition of organic modifiers to the running buffer was considered on account of their effects on various properties including viscosity, dielectric constant, zeta potential, migration time and peak resolution [45]. ACN was tested as the organic modifier over the 5–40% range, obtaining the best resolution with 25% of ACN. The influence of the applied voltage was studied in the 10–30 kV range and the best results in terms of resolution and time of analysis were obtained when 25 kV was used. Controlling the capillary temperature in CE is important in order to avoid undesirable changes in EOF, efficiency, viscosity, electrophoretic mobility and migration time. The effect of the capillary temperature was studied between 15 and 40°C; a temperature of 20°C provides the best compromise between resolution and time of analysis. Finally, the influence of injection time was also studied because it affects to width and height of the peak. Samples were hydrodynamically injected at 0.5 psi for 3–15 s. Peak areas increased with the injection time; however, times higher than 7 s led to distorted peaks and decreased resolution as a result; so 7 s was selected as the optimum value.

3.2 Optimization of experimental conditions for HF-LPME

Several preliminary tests with donor phases pH 1–2 and acceptor phases pH 10–11 were carried out in order to choose the more adequate liquid supported on the polypropylene membrane; dihexyl ether and 1-octanol were checked with better EFs (between 5- and 12-fold better) for dihexyl ether, so this was the liquid supported selected for further optimization.

Owing to the pK_a values for the NSAIDs studied, donor HCl aqueous solution within 1–5 pH range were tested using 50 mL of aqueous solutions containing 20 ng/mL of each analyte and they were extracted at 300 rpm during 30 min using 30 µL of aqueous pH 10 solution as the acceptor phase. NSAID extraction decrease at pH values higher than 2, as can be observed in Fig. 2, so pH 2 aqueous solutions were selected as the donor phase and then NaOH aqueous solutions with pH values between 9.5 and 12 were tested as the acceptor phase. The extraction efficiency increases with the pH of the acceptor phase (Fig. 3), being higher for NAX and ACE, so pH 12 was selected as the optimum value. Acceptor solutions with pH values higher

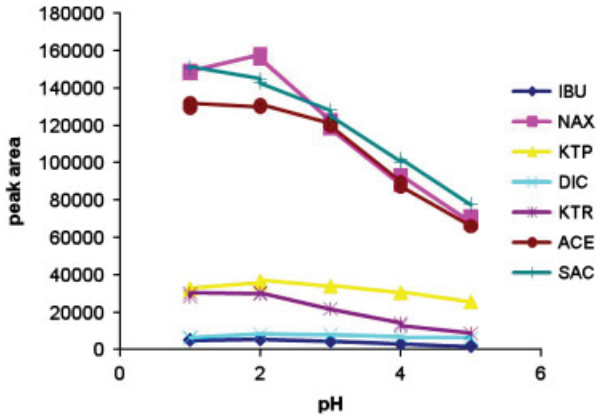

Figure 2. Optimization of the pH of the donor phase.

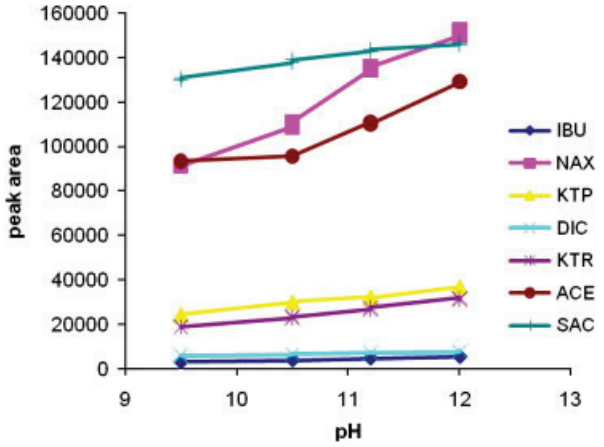

Figure 3. Optimization of the pH of the acceptor phase.

than 12 cannot be used because the degradation of some of the analytes has been observed.

Using the selected donor and acceptor phases, the influence of the stirring time was checked. It has been observed that the extraction efficiency increases with the stirring speed, so the maximum value that does not produce turbulences, which dramatically decrease the extraction, has been selected. Maximum extraction was obtained at 20 min of stirring time except for SAC (Fig. 4), for which maximum extraction occurs at a time greater than 30 min, when a decrease in the extraction efficiency comes up for the other studied drugs. A stirred time of 20 min provides the best compromise between SAC and the other NSAIDs in order to reduce the processing time. On the other hand, the addition of salts (NaCl or Na_2SO_4) does not produce changes on the extraction. The temperature has not been controlled in order to simplify the experimental system; however, only a slight effect of temperature on the extraction efficiency has been observed in some preliminary tests.

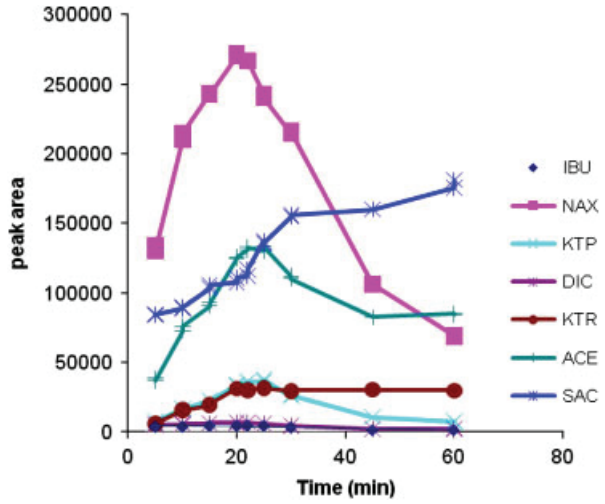

Figure 4. Optimization of the stirring time.

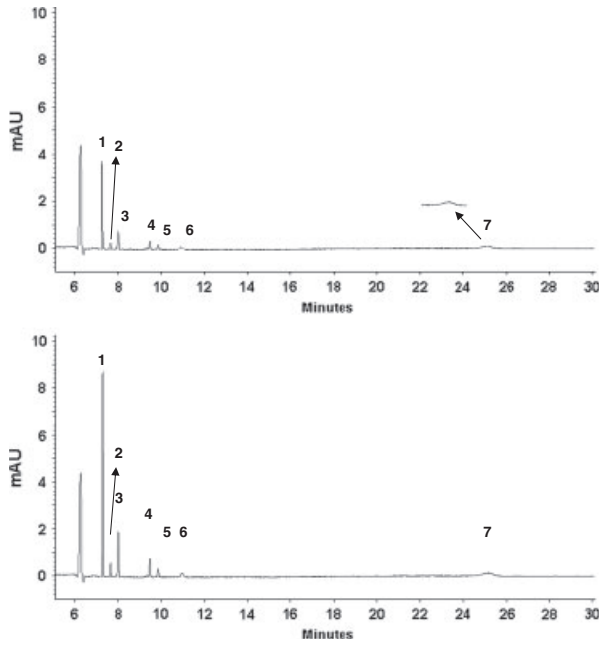

Figure 5. Electropherogram corresponding to the HF-LPME/CE analysis of an aqueous solution of standards (3 and 10 ng/mL) (1, NAX; 2,KTP; 3, IBU; 4, DIC; 5, KTR; 6, ACE; 7, SAC).

3.3 Linearity, sensitivity and precision for the proposed method

Using the optimal HF-LPME conditions, several aqueous pH 2 solutions with different NSAIDs concentrations were submitted to the extraction procedure described in the previous section and analyzed according to the described CE procedure. Figure 5 shows the electropherogram corresponding to an aqueous standard solution submitted to the HF-LPME procedure. As can be seen, well-defined and

Table 1. Regression coefficients, linearity, linear range, detection limits and enrichment factor of the proposed method

NSAIDs	Reg. coef. (r^2)	Linearity[a)] (%)	Linear range (ng/mL)	LOD (ng/mL)	EF
NAX	0.9987	99.57	0.85–200	0.25	240
KTP	0.9992	99.80	1.39–200	0.42	180
IBU	0.9992	99.86	1.05–200	0.31	320
DIC	0.9989	99.61	1.45–200	0.43	300
KTR	0.9994	99.90	1.72–200	0.50	90
ACE	0.9988	99.54	2.87–200	0.86	86
SAC	0.9996	99.92	1.31–200	0.39	130

a) Calculated as $(1-S_b/b)*100$, where b is the slope and S_b its corresponding standard deviation.

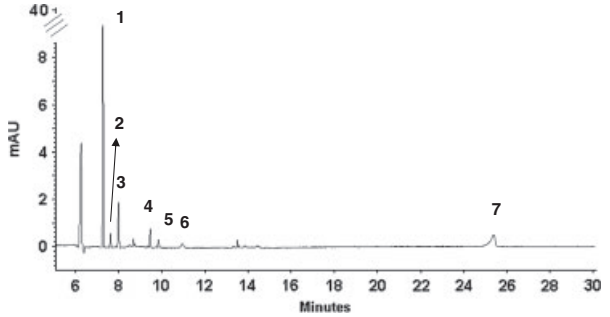

Figure 6. Electropherogram corresponding to the HF-LPME/CE analysis of spiked (10 ng/mL) raw wastewater sample (WWR). (1, NAX; 2, KTP; 3, IBU; 4, DIC; 5, KTR; 6, ACE; 7, SAC).

resolved peaks were obtained. The DAD peak areas were proportional to the NSAIDs concentrations in the donor phase. Table 1 shows the calibration parameters, the LODs for each analyte calculated as three times the standard deviation of the background signal (3σ) and the EFs obtained with HF-LPME, defined as the relationship between the acceptor and the donor phase concentrations, were between 86 for ACE and 320 for IBU. The precision of the proposed method was assessed in terms of repeatability and intermediate precision. Repeatability determined from six consecutive injections of 20 ng/L of the seven NSAIDs studied was between 1.5 and 2.3%. Intermediate precision studied over a period of 6 days was between 2.1 and 3.2%. The proposed method is therefore highly repeatable and reproducible for the determination of the seven NSAIDs.

3.4 Water analysis using HF-LPME extraction and CE determination

3.4.1 Recovery assays on spiked water

Recovery assays were performed on the different kinds of wastewater samples at three concentration levels of the seven NSAIDs that were submitted to the HF-LPME extraction and CE determination procedure described in Section 2. Figure 6 shows the electropherogram corresponding to the spiked raw wastewater sample at a level close to the lower quantitation limits and submitted to the HF-LPME procedure. As can be seen, the electropherogram obtained for the more complex sample shows an excellent baseline and well-defined and resolved peaks as the corresponding aqueous standard one. All samples were analyzed in triplicate with the proposed method and the recoveries obtained, calculated from the corresponding external HF-LPME calibration data, were in the range of 58.8–93.2% (Table 2). Wastewaters contain extremely high surfactant concentrations and those probably modify the behavior of the supported liquid membrane [41],] which leads to recovery decreases. Despite this fact, the preconcentration and clean-up obtained implies a great advantage over other sample treatments, which justifies the use of the proposed methodology.

3.4.2 Analysis of water samples

Wastewater samples described in Section 2.5 were submitted to the proposed method. Some of the NSAIDs were detected in some wastewater analyzed (IBU, DIC and ACE in WWR and WW1 and IBU in WWT) and only contents of NAX and SAC were measured (Table 3).

In Fig. 7, an electropherogram corresponding to the HF-LPME/CE determination of WWR sample is shown. The excellent baseline shows that HF-LPME is an adequate extraction and clean-up procedure for the analysis of wastewater sampler.

4 Concluding remarks

Wastewaters are, in general, complex matrices that usually require previous clean-up procedures; SPE procedures are the most frequently used clean-up methods. This study presents a rapid and simple HF-LPME method combined with CE determination using uncoated fused-silica capillaries that allow an accurate and selective methodology for the determination of IBU, NAX, KTP, DIC, KTR, ACE and SAC in wastewater samples. Low organic solvent consumption and low amount of samples are needed for the proposed HF-LPME procedure with CE-DAD determination; also, an excellent clean-up and high enrichments were obtained for these compounds, which imply a great advantage over other sample treatment procedures.

Table 2. Recovery (%) of the analyzed NSAIDs in spiked wastewaters at three different levels

Water	Concentration (ng/mL) ($n = 3$)	NAX	KTP	IBU	DIC	KTR	ACE	SAC
WWR	10	59.9 ± 2.6	69.8 ± 2.1	58.8 ± 2.9	88.9 ± 2.7	76.6 ± 2.5	68.8 ± 2.8	81.3 ± 2.7
	20	60.1 ± 3.1	73.4 ± 2.8	60.0 ± 2.3	89.3 ± 3.0	78.1 ± 2.9	67.3 ± 2.4	81.4 ± 3.3
	50	62.3 ± 2.7	71.3 ± 3.1	63.3 ± 2.4	91.5 ± 2.9	75.6 ± 3.0	67.9 ± 2.3	82.2 ± 2.5
WW1	10	63.4 ± 2.9	69.9 ± 3.2	61.5 ± 2.9	90.3 ± 3.4	75.7 ± 3.5	67.5 ± 2.8	86.3 ± 2.1
	20	61.1 ± 3.3	74.8 ± 3.1	62.7 ± 3.1	90.0 ± 3.0	77.4 ± 3.2	68.0 ± 2.7	85.9 ± 2.3
	50	63.2 ± 2.6	75.1 ± 2.9	61.8 ± 3.0	93.1 ± 2.9	78.1 ± 3.0	67.9 ± 2.6	83.3 ± 2.1
WWT	10	69.8 ± 2.9	77.7 ± 3.0	71.3 ± 2.8	92.5 ± 2.2	79.3 ± 2.8	70.3 ± 2.2	88.6 ± 2.0
	20	67.8 ± 2.7	78.4 ± 3.1	69.8 ± 2.7	92.8 ± 2.6	71.2 ± 3.0	71.1 ± 2.8	89.4 ± 1.9
	50	70.1 ± 3.2	80.2 ± 3.3	70.8 ± 2.9	93.2 ± 2.1	72.6 ± 2.9	73.8 ± 2.7	90.1 ± 2.2

Table 3. Results obtained from HF-LPME-CE analysis of wastewater samples

Water	Concentration of NSAIDs (ng/mL) ($n = 3$)						
	NAX	KTP	IBU	DIC	KTR	ACE	SAC
WWR	2.12 ± 0.11	–	*	*	–	*	2.87 ± 0.21
WW1	2.09 ± 0.13	–	*	*	–	*	2.59 ± 0.21
WWT	1.43 ± 0.09	–	*	–	–	–	1.98 ± 0.17

– Indicates lower than method detection limits; * indicates detected.

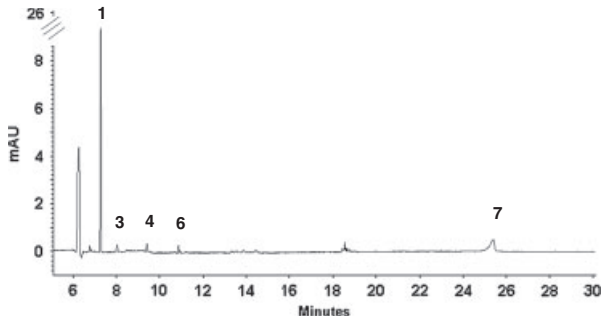

Figure 7. Electropherogram obtained after the HF-LPME of the raw wastewater sample (WWR). (1, NAX; 3, IBU; 4, DIC; 6, ACE; 7, SAC).

With respect to previously reported HF-LPME procedures for the determination of NSAIDs, those that use CE analysis [27–29] have been applied for the determination of a lower number of analytes and with, in general, similar or lower EFs and detection limits than those obtained in the present work. Additionally, they have been applied to urine or pure water versus the direct application to wastewater described in this work. The HF-LPME developed by Quintana et al. [12] was developed for the HPLC/MS determination of NSAIDs in wastewater with higher EFs and extraction time than those obtained in the present work and their better LODs should be only attributed to the detection system used.

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The authors have declared no conflict of interest.

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Electromembrane extraction (EME) and HPLC determination of non-steroidal anti inflammatory drugs (NSAIDs) in wastewater samples

Se propone y optimiza la extracción mediante electromembranas y posterior determinación cromatográfica usando detectores de diodo array y fluorescencia conectados en serie, para la determinación de seis compuestos de la familia de antiinflamatorios no esteroideos: ácido salicílico, ketorolaco, ketoprofeno, naproxeno, diclofenaco e ibuprofeno.

La separación cromatográfica se realizó empleando una columna LiChroCART® 75-4 Purosphere® STAR RP-18e 3 μm (75 x 4.0 mm i.d.), y un gradiente de ácido fórmico 0,1%/acetonitrilo a un flujo de 0,8 ml/min. La columna empleada permite obtener cromatogramas bien resueltos en tiempos relativamente cortos; en este caso el cromatograma se resuelve en 12 minutos.

Los analitos se extraen mediante empleando una fibra hueca de polipropileno Accurel S6/2 que soporta una membrana líquida de 1-octanol. Tanto la fase donadora como aceptora consisten en una disolución acuosa de pH 12 (ajustada con NaOH) y en ambas se encuentra sumergidos electrodos de platino. En estas condiciones, aplicando un voltaje de 10V y agitando a 600 rpm, se obtuvieron los mejores resultados tras 10 minutos de extracción, con factores de enriquecimiento entre 28-49. Las variables experimentales que afectan significativamente a la extracción son optimizadas mediante un diseño experimental central compuesto para cuatro factores a tres niveles, manteniendo fija la concentración en la fase aceptora, ya que se considera una variable crítica en la extracción.

Los límites de cuantificación para los analitos estudiados son bajos, y varían entre 0.29 y 11.1 μg/L, y entre 0.003 y 3.1 μg/L para DAD y FLD, respectivamente, con reproducibilidades entre el 2.7 y el 5.3%.

El procedimiento ha sido aplicado muy satisfactoriamente a la determinación de los analitos en aguas residuales urbanas y otras aguas ambientales, con, recuperaciones superiores al 60% para todos los analitos, excepto para el ibuprofeno y el diclofenaco con recuperaciones del 75 y 100%, respectivamente; obteniendo, asimismo excelentes líneas bases.

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Electromembrane extraction (EME) and HPLC determination of non-steroidal anti-inflammatory drugs (NSAIDs) in wastewater samples

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ABSTRACT

In this paper, an electromembrane extraction (EME) combined with a HPLC procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of six widely used non-steroidal anti-inflammatory drugs (NSAIDs): salicylic acid (SAC), ketorolac (KTR), ketoprofen (KTP), naproxen (NAX), diclofenac (DIC) and ibuprofen (IBU). The drugs were extracted from basic aqueous sample solutions, through a supported liquid membrane (SLM) consisting of 1-octanol impregnated in the walls of a S6/2 Accurel® polypropylene hollow fiber, and into a basic aqueous acceptor solution resent inside the lumen of the hollow fiber with a potential difference of 10 V applied over the SLM. Extractions that were carried out in 10 min using a potential of 10 V from pH 12 NaOH aqueous solutions shown concentration enrichments factors of 28–49 in a pH 12 NaOH aqueous acceptor solution. The proposed method was successfully applied to urban wastewaters. Excellent selectivity was demonstrated as no interfering peaks were detected. The procedure allows very low detection and quantitation limits of 0.0009–9.0 and 0.003–11.1 $\mu g\,L^{-1}$, respectively.

1. Introduction

The analysis of complex samples and the analytes detection or quantitation at very low levels are nowadays two of the main analytical problems. The analytical complexity increases in those cases where both problems are present. The use of clean-up procedures is an old analytical tool that in the last years has undergone very important developments. Traditionally, liquid–liquid extraction (LLE) has been an important sample-preparation technique prior to chemical analysis. Recently, solid phase extraction (SPE), using several sorbent types, has been the preferred sample-preparation technique to extract pharmaceuticals from several environmental and biological matrices [1–3], but in the last years, there has been a high interest in developing new extraction and clean-up procedures.

Liquid phase microextraction (LPME), also known as supported liquid membranes (SLM) extraction, is an attractive alternative to the widely used solid phase extraction (SPE). Audunsson [4] introduced an alternative concept for LPME that was developed by Thordarson et al. [5], and Pedersen-Bjergaard and Rasmussen [6], based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene (HF-LPME) that allows in most cases, not only a efficient clean-up procedure, but it can also produce high degree of pre-concentration. Additionally, the low organic solvent consumption makes HF-LPME an interesting and environmental friendly analytical procedure. Some reviews on hollow fiber-based LPME have been reported [7–10].

The ionic nature of several of the interesting analytes in liquid extraction procedures led to some authors to propose the use of electrical fields to enhance and to manipulate LLE. Early attempts of analytical LLE driven by an external electrical field (from 1 to 15 kV dc) were reported by van der Vlis et al. between 1994 and 1996 [11–13]. After this period, no other papers emerged on the electro extraction concept. In 2005, Arrigan and co-workers proposed another approach to LLE driven by electrical potential with the development of "electrochemically modulated LLE of ions" [14–16]; where the analytes were extracted from a flowing aqueous phase of a flow-injection system with a stationary organo-gel phase (pseudo-liquid) using electrical potentials in the −1 to +1 V range.

In 2006, Pedersen-Bjergaard and Rasmussen demonstrated, for the first time, that an electrical potential produces analytical extraction through a supported liquid membrane (SLM) [17,18]. This system was termed electromembrane extraction (EME) and the analytes were extracted from an aqueous sample through an organic solvent (2-nitrophenyl octylether, NPOE) immobilized as SLM in the wall of a polypropylene porous hollow fiber to an aqueous acceptor solution placed inside the lumen of the hollow

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fiber. Essentially, it is similar to a HF-LPME where the migration through the SLM is forced by the electrical field generated from two electrodes placed outside the fiber and inside the lumen (electromigration, EMI). In order to ensure efficient electrokinetic mobility in the EME system, pH must be adjusted to provide total ionization of the analytes in the two aqueous solutions. In these first works, basic analytes were analyzed using acid pHs and electrical potential of 300 V.

In 2007, the same authors publish an article [19] where EME was discussed in more detail from a theorical point of view. It was demonstrated that the flux of analytes across the membrane can be described using a mathematical model based on the Nernst–Planck equation. The model demonstrated that the magnitude of the electrical potential difference, the ion balance of the system, and the absolute temperature influenced the flux of analyte through the SLM. These conclusions were verified by experimental data with five basic drugs and NPOE as SLM. Other articles related to the extraction of basic drugs using EME have also been published by these authors [20–25] using lower voltages than those used in the first published papers.

The electromembrane extraction procedure using hollow fibers as support for the SLM has been also applied to the separation of peptides [26,27]. Other EME configuration, called by the authors drop-to-drop [28], shown poor recoveries and repeatability.

There is only a single previous article about the electrokinetic migration of acidic drugs through SLM in a hollow fiber configuration [29] where the influence of some parameters (pH of donor and acceptor solutions, voltage, extraction time and agitation) on the electromigration process was studied. Some of the drugs analyzed in the present work were studied with recoveries between 25% and 82% and enrichment factors of 2.5–8.2. Repeatability and linearity was briefly tested for some of the acidic drugs analyzed with not very good results; authors attribute this fact to the experiments were performed with home-built equipment.

In this work, a HPLC/DAD-FLD method combined with prior EME was developed for the determination of six widely used non-steroidal anti-inflammatory drugs (NSAIDs): salicylic acid (SAC), ketorolac (KTR), ketoprofen (KTP), naproxen (NAX), diclofenac (DIC) and ibuprofen (IBU). The method was applied to their determination in urban wastewaters. The EME provides very clean extracts that can be directly injected into the chromatographic system allowing excellent baselines. The proposed EME is an easy and rapid sample pretreatment procedure that additionally, provides high sample preconcentration which enhances the applicability of the proposed method. EME reduces the organic solvents consumption to several microlitres in contrast to other clean-up/preconcentration alternatives which is according to the current trends to a “Green Chemistry”.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water form a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). SAC, KTR, KTP, NAX, DIC and IBU, dihexyl ether, 2-nitrophenyl octyl ether (NPOE), 1-heptanol, benzyl alcohol, cyclohexanol, and 1-octanol were purchased from Fluka–Sigma–Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany).

Aqueous working solutions of NSAIDs were daily prepared by adequate dilutions from methanolic (KTP, NAX) and aqueous (SAC, KTR, DIC, IBU) 100 mg L^{-1} stock solutions.

2.2. Chromatographic conditions

The chromatographic separation was performed at 10 °C using a LaChrom® VWR-Hitachi (Barcelona, Spain) liquid chromatograph with a quaternary L-2130 pump. The injector was a autosampler L-2200. Separations were carried out at 10 °C (VWR Refrigerated Circ Model 1160S, West Chester, Pennsylvania, USA) using a LiChroCART® 75-4 Purosphere® STAR RP-18e 3 μm (75 mm × 4.0 mm i.d.) (VWR, Darmstadt, Germany) preceded by a guard column Kromasil® 100 Å, C18, 5 μm (15 mm × 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain).

The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and acetonitrile (component B) at a flow rate of 0.8 mL min^{-1}. An initial 60% component A was used in isocratic mode for 5 min and then a linear elution gradient was programmed from 60% to 0% A for other 5 min, finally an isocratic mode for 2 min was programmed. Five minutes were waited between injections which allowed re-equilibration of the column to the initial conditions. Table 1 shows the monitoring wavelengths for DAD and fluorescence detection (FLD) detections and the retention times for the analyzed compounds.

2.3. Supported liquid membrane preparation and extraction procedure

The electrical equipment consisted in a dc power supply model Power Source 300 V (VWR International, West Chester, Pennsylvania, USA) with programmable voltage in the range 2–300 V, providing currents in the range 4–500 mA. Simple platinum wires with a diameter of 0.25 mm were used as electrodes in the sample and acceptor solutions with an average inter-electrode distance of 2 mm which resulted in an electrical field of 50 V cm^{-1} (for a typical 10 V dc). As sample compartment, 10 mL glass vials were used with a height of 48 mm and with an internal diameter of 20 mm (VWR International, West Chester, Pennsylvania, USA). Hollow fiber used for immobilization of the supported liquid membrane and for housing the acceptor solution was a S6/2 Accurel® polypropylene hollow fiber (1800 μm i.d., 450 μm wall thickness and 0.2 μm pore size) that was purchased from Membrana (Wuppertal, Germany). The sample solution was stirred with a FB15107 Magnetic Stirrer (Fisher Scientific, Pittsburgh, PA, USA).

Hollow fibers were cut into 24 mm pieces, washed with acetone in an ultrasonic bath and dried; the fiber was closed in the lower end by thermal and mechanical pressure, whereas the upper end was connected to a piece of 8 mm length from a pipette tip of polypropylene (Rodelab, Seville, Spain) as a guiding tube. The fiber was soaked with 1-octanol during 5 s to impregnate the pores, and the excess of organic solvent was removed with a medical wipe. The lumen of the prepared fiber piece was filled with 50 μL of acceptor phase (pH 12 aqueous solution) using a HPLC syringe and the positive electrode was placed in the acceptor solution. Hollow fiber with acceptor solution was placed into the 10 mL sample solution (pH 12) and voltage (10 V) was applied for 10 min during the sample stirring at 600 rpm.

After electromigration, the fiber was taken out and the acceptor phase was extracted using a HPLC syringe and placed into a HPLC microvial to be injected (20 μL) into the HPLC system through the autosampler unit.

2.4. Preparation of environmental water samples

Wastewater samples were obtained from E.D.A.R. Punta Umbría-AQUALIA Wastewater Treatment Plant which is located in Punta Umbría, Huelva, Spain. The WWTP essentially receives urban wastewaters. The capacity of this WWTP is 140,000 inhabitants and

Table 1
Monitoring wavelengths and retention times.

	DAD			FLD			
	λ_{max} (nm)	t_R (min)	SD (min)	λ_{exc} (nm)	λ_{em} (nm)	t_R (min)	SD (min)
SAC	235	2.72	0.021	230	445	3.00	0.024
KTL	315	3.73	0.009	–	–	–	0.011
KTP	255	6.45	0.014	–	–	–	0.013
NPX	230	7.01	0.008	239	251	7.24	0.015
DIC	280	10.27	0.018	–	–	–	0.017
IBU	224	10.41	0.017	224	290	10.65	0.022

the discharged flow is 21,250 m^3/day. Samples from the influent (raw water, WWR), after the primary sedimentation tank (WW1) and the effluent (treated water after anaerobic digestion, WWT) were analyzed.

All samples were filtered through a GDU1 glass fibre filter bed (10–1 μm) (Whatman, Mainstone, UK) and through Pall Nylaflo™ nylon membrane filter 0.45 μm (Pall Corporation, Ann Arbor, Michigan, USA) and adjusted to pH 4 with HCl. Filtered samples were stored in the dark at 4 °C prior to electromigration procedure. Water samples, were directly analyzed after NaOH addition just to obtain pH 12 prior to be submitted to the electromigration procedure.

3. Results and discussion

3.1. Chromatographic conditions

Looking for a fast and high resolution separation a LiChroCART® 75-4 Purosphere® STAR RP-18e 3 μm was selected as working column. This column is a small size particle HPLC column that allows high resolution separations and lower retention times than other 5 μm ones. The selected column also provides good resolution and good peak symmetry.

Table 2
Four-level factorial design (X_1: donor pH, X_2: voltage, X_3: extraction time and X_4: stirring speed).

X_1	X_2	X_3	X_4
+1	+1	+1	+1
+1	+1	+1	−1
+1	+1	−1	+1
+1	+1	−1	−1
+1	−1	+1	+1
+1	−1	+1	−1
+1	−1	−1	+1
+1	−1	−1	−1
−1	+1	+1	+1
−1	+1	+1	−1
−1	+1	−1	+1
−1	+1	−1	−1
−1	−1	+1	+1
−1	−1	+1	−1
−1	−1	−1	+1
−1	−1	−1	−1
α	0	0	0
$-\alpha$	0	0	0
0	α	0	0
0	$-\alpha$	0	0
0	0	α	0
0	0	$-\alpha$	0
0	0	0	α
0	0	0	$-\alpha$
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0

The mobile phase consisted of 0.1% formic acid and acetonitrile. Different gradient elution conditions were tested searching for the shortest time of analysis without sacrificing peak shape. The gradient elution program described in the previous section was the best option in terms of time of analysis, shape of the peaks and reproducibility. The peak resolution for the analyzed substances oscillates between 1.60 and 12.33 (all above the critical value, 1.5). Additionally, asymmetry factors, retention factors, and selectivity factors for the obtained peak are according to their critical values.

3.2. Optimization and evaluation of experimental conditions for electromigration

First, some preliminary tests were carried out in order to check the electromembrane extraction behaviour according to the organic solvent used as supported liquid membrane; the solvents checked were dihexyl ether, 2-nitrophenyl octyl ether (NPOE), 1-heptanol, benzyl alcohol, cyclohexanol, 1-octanol and 1:1, 1:2, 1:3 and 1:5 dihexyl ether: 1-octanol mixtures. Dihexyl ether can not be used as supported liquid membrane due to produces an interruption in the electrical conduction. Much better results were obtained when 1-octanol was used as SLM; the rest of solvents and mixtures only lead to recoveries of 2–20% respect to the 1-octanol one. Additionally, an impregnation time of 5 s was established as optimum.

The inter-electrode distance was also checked between 1 and 5 mm and an optimum distance of 2 mm was obtained. Lower a higher inter-electrode distances lead to a general decrease in the analytes extraction.

Some tests concerning to the effect of the ionic strength of the donor phase in the electromigration process were realized. Sodium sulfate concentrations between 0.1 and 1.5 M were checked without relevant results. It is remarkable that sodium sulfate concentrations over 1 M produce the burning of the fiber.

Some preliminary tests related to the pH effect on the electromigration efficiency were carried out showing that electromigration increases for all the analytes with the increase of the pH of acceptor phase. However, pH values higher than 12 produce analytes degradation, so pH 12 was fixed in the acceptor phase for the later optimization of the rest of experimental variables that, as it is described below, was realized using an experimental design.

On the other hand, we have checked that, in the experimental conditions, if no electrical power is applied the extraction of the analytes is null, which shows that there is not liquid phase

Table 3
Coded factors used for the experimental design.

Levels					
Factors	−2	−1	0	1	2
X_1 (pH)	9	9.75	10.5	11.25	12
X_2 (V)	2	6	10	14	18
X_3 (time) min	5	7.5	10	12.5	15
X_4 (speed) rpm	150	300	450	600	750

Table 4
Detection limits, linear ranges, % linearity and enrichment factors for the HPLC method (DAD and FLD detection) combined with prior EME.

	DAD			FLD			Enrichment
	LOD[a]	Linear range [a]	% Linearity	LOD[a]	Linear range[a]	% Linearity	
SAC	0.16	0.55–100	99.45	0.12	0.41–100	99.87	40
KTR	0.18	0.61–100	99.75	–	–	99.71	35
KTP	0.12	0.40–100	99.67	–	–	99.63	43
NAX	0.08	0.29–100	99.89	0.0009	0.003–30	99.79	32
DIC	0.23	0.77–100	99.91	–	–	99.84	49
IBU	3.36	11.1–100	99.73	0.94	3.1–50	99.65	28

[a] $\mu g\,L^{-1}$.

microextraction (LPME) contribution to the proposed electromembrane extraction procedure.

In order to optimize the experimental extraction parameters an experimental design was applied. The fundamental objectives of the experimental planning are to identify controllable factors that significantly influence the outcome of the experiment, minimizing the effects of uncontrollable factors and optimizing the objective function to get the best response. It is interesting to obtain as much information as possible with the least number of experiences.The influence of the experimental variables to find the best conditions to quantify the six NSADs studied (donor phase pH (X_1), voltage (X_2), electromigration time (X_3) and stirring speed (X_4)) has been considered.

As it has been previously described that the pH of the acceptor phase is a critical parameter during the extraction so it was not considered a variable to optimize in the experimental design and it was fixed at pH 12.The optimization has been carried out using a central composite design (CCD) for four factors at two levels. These designs account for the main factors and binary interactions that influence the signal, with a low number of assays. The design matrix corresponds to four factors and thirty experiments, as illustrated in Table 2. The thirty runs are split into three groups: sixteen runs on the basis of levels +1 or −1, eight runs on the basis of levels $+\alpha$ or $-\alpha$ and six runs at the center of design. Table 3 shows the coded levels of selected factors ($-\alpha$, −1, 0, 1, α) where $\alpha = 2$.The computer program used on the experimental design was ECHIP ver. 6.4.1 (Velocity Pointe, Wilmington, DE, USA). After a scrutiny of the optimal conditions, it was considered a slight modification of some of them in order to favor the extraction for those compounds with higher detection limits (analytical optimal conditions). So, the optimal conditions used were those described in Section 2.3, pH 12 for the donor and acceptor, a voltage of 10 V, an electromigration time of 10 min and a stirring speed of 600 rpm. Respecting to the optimum time and voltage, it has been observed that the optimum value voltage is 10 whatever the value taken by any other of the variables. On the other hand, most of the compounds get better enrichment factor 10 min after extraction. After 10 min extraction, the enrichments are constant or lower for some of the compounds.

3.3. Linearity, sensitivity, precision and robustness for the electromigration

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve, was constructed using a least-square linear regression analysis of standards mixtures of the analytes at different concentrations. Using the selected electromigration conditions, aqueous pH 12 solutions with different analytes concentrations were submitted to the electromigration procedure and analyzed according to the described HPLC procedure. Peak areas are proportional to concentrations in the

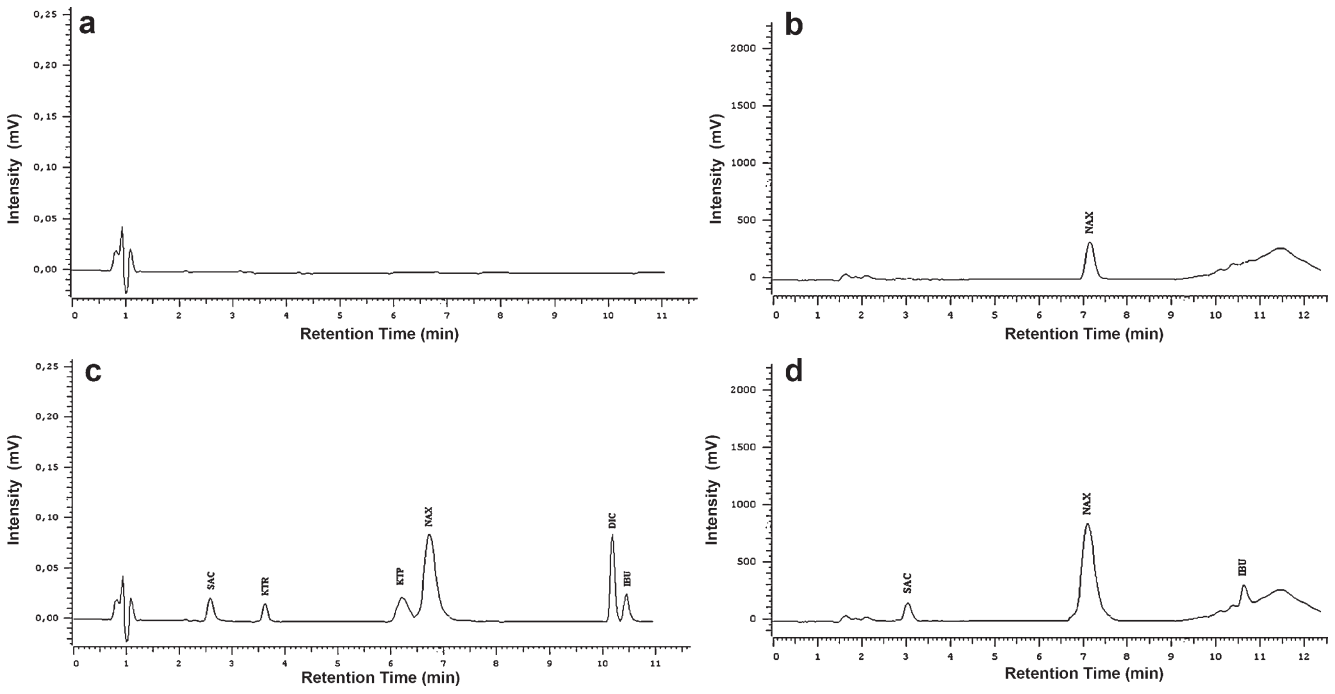

Fig. 1. DAD (a) and FLD (b) chromatograms from blank and spiked (DAD: all 5 ng mL^{-1} except IBU 20 ng mL^{-1} and FLD: SAC 2 ng mL^{-1}, NPX 0.05 ng mL^{-1}, IBU 5 ng mL^{-1}) wastewater (WWR) sample.

donor phase. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ and the calibration curves obtained showed no changes over the course of 1 month. Detection and quantitation limits were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio is 3 and 10, respectively. Enrichment factor was calculated as the increase in the concentration between the aqueous external donor phase and the internal (acceptor) phase. Table 4 shows the corresponding data and, as can be seen, the high enrichment values obtained for all the analytes allow their determination at low concentration levels.

To evaluate the repeatability and the intermediate precision, aqueous samples at three concentrations levels 0.8 (0.1 for IBU), 5 and 20 $\mu g\,L^{-1}$ (in triplicate) were subjected to the entire analytical procedure and measured in one single day and 1 day per week during 2 months, respectively. Intermediate precision was performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the *mxpxn* design (*m* = analytical levels, *p* = days and *n* = replications). From the corresponding ANOVA, the intermediate precision was computed [30]. The repeatability, expressed as relative standard deviation, was in the range 2.4–5.2%. Intermediate precision also expressed as relative standard deviation, was in the range 2.7–5.3%.

The robustness study is based on the procedure suggested by Youden [31]. A design matrix with two factors in eight experiments was used where the +1 and −1 levels correspond to slight modifications in the more critical variables: pH values of donor and acceptor phases and stirring time.

The effect of a given factor, say x_i can be estimated as the difference of result averages at levels +1 and −1:

$$D(x_i) = \frac{1}{4}\left[\sum R_{(x_i=+1)} - \sum R_{(x_i=-1)}\right]$$

where R is the corresponding experimental result obtained.

A significance *t*-test [32] was used to determine whether variations have a significant effect on the result,

$$t(x_i) = \frac{\sqrt{2}|D(x_i)|}{S_{IP}}$$

where S_{IP} is the standard deviation of the intermediate precision, evaluated in the precision study. The $t(x_i)$ values were compared with the corresponding critical t values ($n = 4$) at 5% significance level and three degrees of freedom. The results obtained indicated that the procedure can be considered robust against the considered factors for all the analyzed compounds.

3.4. Wastewater samples analysis

Wastewater samples described in Section 2.4 were submitted to the EME proposed procedure and analyzed. Some of the NSAIDs were detected in the samples analyzed (SAC in WWR and WWT, NAX in WWT, DIC in WWR and WW1, and IBU in WWR) and only contents of NAX could be measured in the samples WWR (0.043 $\mu g\,L^{-1}$) and WW1 (0.023 $\mu g\,L^{-1}$).

In order to check the suitability of the proposed procedure, spiked samples at some concentration levels: 0.8, 2, 5 and 20 $\mu g\,L^{-1}$ (plus 0.1 $\mu g\,L^{-1}$ for NAX and 50 $\mu g\,L^{-1}$ for IBU) were analyzed. Results obtained are shown in Table 5. Wastewater samples are, in general, complex samples from the analytical point of view, that frequently require complex clean-up processes. The direct application of the proposed HF-LPME procedure to the different kind of wastewater samples analyzed show, in general, good results in terms of recovery, approximately of 60% and high for SAC, KTR, KTP and NAX in all samples; 70% and high for IBU and nearly 100% for DIC. No significant differences have been observed in relation to the depuration process suffered by wastewaters. Urban wastewaters have extremely high surfactants concentrations that could modify the supported liquid membrane behaviour [33] which could lead to recovery decreases. Despite this fact, the good preconcentration and clean-up obtained implies a great advantage over other sample treatment procedures which could justify the use of the proposed EME procedure.

Table 5
Recoveries (%) using EME/HPLC from wastewater spiked samples. (Average of three determinations ± standard deviation).

	Spiked level[a]	Wastewater sample ([b])		
		WWR	WW1	WWT
SAC	0.8	66 ± 5.3	67 ± 6.4	69 ± 6.2
	2	67 ± 7.1	66 ± 6.8	69 ± 6.3
	5	65 ± 6.1	65 ± 7.6	66 ± 6.0
	20	68 ± 4.6	69 ± 5.9	70 ± 4.8
KTR	0.8	55 ± 6.8	56 ± 7.2	57 ± 5.3
	2	55 ± 3.1	56 ± 4.5	57 ± 4.4
	5	55 ± 3.6	57 ± 3.9	56 ± 3.6
	20	57 ± 2.6	57 ± 3.1	59 ± 2.5
KTP	0.8	62 ± 4.8	63 ± 5.2	65 ± 3.7
	2	62 ± 4.0	62 ± 4.3	63 ± 3.5
	5	63 ± 4.3	61 ± 4.4	64 ± 4.1
	20	62 ± 4.4	64 ± 4.7	62 ± 5.0
NAX	0.1	58 ± 4.2	58 ± 4.3	60 ± 4.1
	0.8	59 ± 4.3	60 ± 4.4	60 ± 4.5
	2	59 ± 4.1	61 ± 4.1	61 ± 3.6
	5	59 ± 3.7	58 ± 3.3	60 ± 2.8
	20	61 ± 3.5	60 ± 3.1	62 ± 3.3
DIC	0.8	100 ± 2.4	99 ± 2.5	100 ± 2.5
	2	99 ± 2.6	99 ± 2.6	99 ± 2.8
	5	100 ± 2.7	99 ± 2.7	100 ± 2.7
	20	100 ± 2.7	100 ± 2.5	100 ± 2.6
IBU	5	74 ± 3.4	73 ± 3.6	74 ± 3.9
	20	74 ± 4.5	75 ± 4.0	73 ± 4.1
	50	75 ± 3.8	75 ± 3.5	76 ± 3.8

[a] ($\mu g\,L^{-1}$).
[b] Average recovery (%) ± standard deviation ($n = 3$).

Fig. 1 shows representative DAD and FLD chromatograms obtained from blank and spiked wastewater (WWR) sample. This samples has been selected as the more complex wastewater. As it can be seen, both FLD chromatograms show excellent baselines, showing the blank FLD chromatogram the NAX peak above mentioned. Spiked chromatograms show well defined peaks corresponding only to the added substances.

4. Conclusions

This study presents a hollow fiber-based electromembrane microextraction method combined with an HPLC (DAD-FLD) determination using a small size particle chromatographic column that allows a rapid, simple, low-cost, accurate, high sensitive and selective methodology for the determination of six widely used non-steroidal anti-inflammatory drugs. The proposed extraction procedure has a very low (few μL) organic solvent consumption. The excellent clean-up obtained implies a great advantage over other sample treatment procedures.

The proposed procedure has been demonstrated adequate for the determination of the analytes in urban wastewater samples that usually require tedious clean-up and preconcentration steps.

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Determination of nonsteroideal antiinflammatories in human urine using hollow fiber liquid phase microextraction (HF-LPME) and capillary electrophoresis

En este trabajo se emplea como técnica analítica de separación un sistema de electroforesis capilar con detector de diodo array para la determinación de siete antiinflamatorios no esteroideos: ibuprofeno, naproxeno, ketoprofeno, diclofenaco, ketorolaco, aceclofenaco y ácido salicílico.

El electrolito de separación es un tampón acuoso de acetato 30mM de pH4 al 25% de acetonitrilo. El voltaje aplicado fue de 25KV y la temperatura del capilar se mantiene constante a 20ºC. Esta técnica permite inyectar del orden de nanolitros, por lo que se pueden llevar a cabo extracciones con fibras de corta longitud.

La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases, empleando dihexiléter como membrana líquida sobre una fibra de polipropileno Accurel Q3/2 de 13cm de longitud. La fase donadora y aceptora consisten en una solución acuosa a pH 2 y pH 12 respectivamente. La muestra se agita a 300 rpm y se obtienen enriquecimientos de 86 a 320 tras veinte minutos de agitación.

Los límites de detección obtenidos para los analitos estudiados varían entre 0.25-0.86 µg/L, con reproducibilidades entre 2.1 y 3.2 %.

El procedimiento ha sido aplicado satisfactoriamente a la determinación de los analitos en orina humana, obteniéndose recuperaciones superiores al 81%, permitiendo incluso la determinación de algunos de sus metabolitos.

Determination of nonsteroideal antiinflammatories in human urine using hollow fiber liquid phase microextraction (HF-LPME) and capillary electrophoresis

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Abstract

In this paper a new method based on hollow fiber membrane liquid phase microextraction (HF-LPME) followed by capillary electrophoresis (CE) determination for several non steroidal anti-inflammatory drugs (Ibuprofen (IBU), Naproxen (NAX), Ketoprofen (KTP), Diclofenac (DIC), Ketorolac (KTR), Aceclofenac (ACE) and Salicylic Acid (SAC)) has been developed. Hollow fiber liquid phase microextraction (HF-LPME) is a technique widely employed in recent years in analytical chemistry because it provides a high selectivity and sensitivity. Low organic solvent and low volume of sample are required, so the use of this technique combined with capillary electrophoresis becomes a good alternative to HPLC methods that generally requires more sample volume and higher solvent consumption. The electrophoretic separation was performed in acetate buffer (30 mM, pH 4) using acetonitrile as organic modifier (25 %). The separation voltage was 25 kV and the temperature of the capillary was set to 20 ºC. Detection limits are between 0.25 ng mL^{-1} and 0.86 ng mL^{-1} for naproxen and aceclofenac, respectively. The method has been successfully applied to the determination of the seven anti-inflammatories in human urine.

Keywords: Hollow fiber liquid-phase microextraction; nonsteroideal antiinflamatories; capillary electrophoresis; human urine.

1. Introduction

In the last years, there has been an increasing interest in developing new sample pretreatment approaches to determine all type of analytes in several matrices with high sensitivity and selectivity. It often happens that patients are treated simultaneously with combinations of pharmaceuticals, for example, patients whose postoperative course was complicated or during a hospital stay. As a result of this, hospitals are more often demanding fast and simple methods that permit the determination of a wide range of drugs in just one analysis for both screening or quantitative purposes to dosing adjustment or for patients with unknown treatments to avoid combinations of drugs which are not recommended to be used simultaneously. Therefore it is necessary to develop analytical procedures, which could determine various drugs simultaneously in the shortest time. In this study, the selection of drugs was made according to the most often prescribed NSAIDs for common affections. Many methods have been developed over decades for the determination of NSAIDs. Among them, the methods based on liquid chromatography (LC) (Kang and Kim 2008; Marco-Urrea et al. 2010; Ramos Payán et al. 2009; Aresta et al. 2006), gas chromatography (GC) (Selke et al. 2010; Alkatheeri et al. 1999; Weigel et al. 2004) and capillary electrophoresis (CE) (Gómez et al. 2003; Goto et al. 1998; Orlandini et al. 2004; Macià et al. 2007) are the most efficient. Nonsteroideal anti-inflammatory drugs (NSAIDs) are a group of pharmaceutical compounds frequently employed in many fields; they have analgesic, antipyretic and platelet-inhibitory actions.Ibuprofen, Naproxen, Ketoprofen, Diclofenac, Ketorolac, Aceclofenac and Salicylic Acid are the NSAIDs more frequently administered.

Several extraction procedures have been applied prior to the determination of these compounds. Conventional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) techniques exhibit disadvantages such as time consuming, expensive and health hazardous due to large amount of solvents used. Liquid phase microextraction (LPME) based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) (Liu and Dasgupta 1996; Jeannot and Cantwell 1996) emerged as an attractive alternative for sample preparation, because this technique was fast and simple and eliminate the disadvantages of conventional extraction methods, such as time consuming operation

and using specialized apparatus, LPME combines extraction, concentration and sample introduction in one step, however, SDME is not very robust, and the droplets may be lost from the needle tip of the microsyringe during the extraction. Audunsson (1986) introduced an alternative concept for LPME that was developed by Thordarson et al. (1996) and by Pedersen-Bjergaard and Rasmussen (1999) based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber (Ramos Payán et al. 2009; Ramos Payán et al. 2010). Several reviews that focus on basic extraction principles, technical setup, recovery, enrichment, extraction speed, selectivity, applications and future trends in hollow fiber-based LPME have been reported (Psillakis and Kalogerakis 2003; Rasmussen and Pedersen-Bjergaard 2004; Pedersen-Bjergaard and Rasmussen 2005; Pedersen-Bjergaard and Rasmussen 2008).

Compared with LLE and SPE, HF-LPME gives a satisfactory sensitivity and similar or better enrichment of analytes, and the consumption of solvent is significantly reduced by up to several hundred or several thousand times. Due to the small volume of the extracting solvent, the extracted samples do not required further concentration prior analysis and the use of capillary electrophoresis presents a great advantage versus to HPLC which needs more sample volume to inject (Varcárcel et al. 2001). It has been proven that HF-LPME is very useful for extraction of drugs and metabolites from biological matrices with simultaneous clean-up of the extracts (Zorita et al. 2007; Zorita el al. 2007; Zorita et al. 2008; Ramos Payán et al. 2009).

The aim of this work was to develop a simple and low-cost method, which does not involve cumbersome clean-up procedures. A CE method combined with HF-LPME was developed for the determination of seven NSAIDs: Ibuprofen (IBU), Naproxen (NAX), Ketoprofen (KTP), Diclofenac (DIC), Ketorolac (KTR), Aceclofenac (ACE) and Salicylic Acid (SAC), that to our best knowledge there has not been so far reported before. All the HF-LPME and CE parameters have been optimized. The method has been satisfactorily applied to the determination of these compounds in human urine. Simple and effective sample preparation for analyses as well as relatively short time of these analyses prove its usefulness and applicability in clinical laboratories.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical –reagent grade. All solutions and dilutions were prepared in ultrapure water from a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). IBU, NAX, KTP, DIC, KTR, ACE, SAC and dihexyl ether were purchased from Fluka-Sigma-Aldrich (Madrid, Spain) and the rest of the reagents were supplied by VWR (Barcelona, Spain).

Working solutions of NSAIDs were daily prepared by adequate dilutions from a 100 μg mL^{-1} stock solution. All standard solutions were stored at 4 ˚C and were stable for at least one month. For validation of the method, human urine was obtained from pooled samples collected from healthy volunteers and stored at −18 ◦C before use. Q3/2 Accurel ® KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Instrumentation

All measurements were made with a Beckman P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA, USA) equipped with a diode array detector (DAD). Uncoated fused-silica capillaries (Beckman-Coulter, Fullerton, CA, USA) of 75 μm i.d. and an effective length of 50 cm (total length of 57 cm) were used. The magnetic stirrer was an ANS-00/1 Science Basic Solutions (Rubí, Barcelona, Spain).

2.3. Operating conditions.

Daily before use, the capillary was successively rinsed with 0.1M NaOH, water and the running buffer for 5 min each. Between runs, it was also conditioned with 0.1M NaOH (2 min), water (3min) and the running buffer (5min). The separation was performed using acetate buffer (30 mM, pH= 4), a separation voltage of 25 KV and the temperature of the capillary was set at 20 ºC. Injections were performed in hydrodynamic mode, setting the injection time at 7 s and 0.7 psi. The wavelengths used for DAD were 220 nm for IBU, DIC, ACE, SAC; 225 nm for NAX; 253 for KTP and 318 nm for KTR.

2.4. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 13 cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked in the membrane phase (dihexyl ether) for 5s to impregnate pores of the support, and rinsed with water on the outside by placing into an ultrasonic bath for 25 s in order to remove the excess of organic solvent. The lumen of the prepared fiber was filled with 30 μL of acceptor phase (pH 12 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and adhesive tape. During extraction the hollow fiber was immersed in 50 mL sample solution (pH 2) contained into a 50 mL glass beaker. The sample was stirred for 20 minutes by means of a magnetic stirred at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was withdrawn using a HPLC syringe and injected into the CE-system.

2.5. Preparation of urine samples

Spiked samples and samples from healthy volunteers were prepared by dilution 1:25 or suitable dilution of the urine and extracted by the HF-LPME procedure described in previous section after adjusting the pH to 2 by HCl addition.

3. Results and discussion

3.1. Optimization of the separation conditions

Buffer pH is important in capillary electrophoresis because it affects both the charge of the analytes and the strength of electro-osmotic flow (EOF). The influence of buffer pH from 2 to 10 was tested by using phosphate buffer, borate buffer and acetate buffer, obtaining the best results with acetate buffer at pH 4. The influence of buffer concentration on the separation of the studied drugs was also examined in the range 10-60 mM. When the concentration of acetate increased, a longer migration time and an increment of current was obtained so the optimum concentration of acetate buffer was set at 30 mM obtaining a good resolution for each compound under this conditions. The addition of organic modifiers to the running buffer was considered on account of their effects on various properties including viscosity, dielectric constant, zeta potential,

migration time, peak symmetry and resolution. Test with methanol and acetonitrile at concentration over 5-35% range. Best results were obtained when 25 % of acetonitrile was used.

Separation voltage was studied in the range 10 to 30 kV and the best results in terms of resolution and time of analysis were obtained when 25 kV was used. Capillary temperature was tested between 15 and 40 ºC obtaining the best results at 20 ºC. Finally, the influence of the injection time was evaluated between 3 and 15 s obtaining the best results at 7 s at 0.7 psi.

3.2. Optimization and evaluation of experimental conditions for HF-LPME

Several preliminary tests with donor phases pH 1-2 and acceptor phases pH 10-11 were carried out in order to choose the more adequate liquid supported on the polypropylene membrane; dihexyl ether and 1-octanol were checked and the best results were obtained with dihexyl ether, so it was used for further optimisation.

Due to the pK_a values for the NSAIDs studied, donor HCl aqueous solution within 1-5 pH range were tested using 50 mL of aqueous solutions containing 20 ng mL^{-1} of each analyte and they were extracted at 300 rpm during 30 min using 30 µL of aqueous pH 10 solution as acceptor phase. NSAIDs extraction decrease at pH values upper than 2 as it can be observed in Figure 1, so pH 2 aqueous solutions were selected as donor phase. NaOH aqueous solutions with pH values between 9.5 and 12 were tested as acceptor phase. Best results were obtained when pH of acceptor phase was 12 (Figure 2). Acceptor solutions with pH values upper 12 cannot be used due to the degradation of some of the analytes has been observed.

Using the selected donor and acceptor phases, the influence of the stirring time was checked; maximum extraction was obtained at 20 minutes of stirring time except for SAC (Figure 3) which maximum extraction takes place at times longer 30 minutes, when a decrease on the extraction efficiency comes up for the other studied drugs , so a stirring time of 20 min at 300 rpm was selected as optimum value in order to reduce the processing time.

3.3. Linearity, sensitivity and precision for HF-LPME extraction

Using the optimal HF-LPME conditions, several aqueous pH 2 solutions with different NSAIDs concentrations were subjected to the extraction described in the previous section and analysed according to the described CE procedure. The DAD peak areas were proportional to the NSAIDs concentrations in the donor phase. Table 1 shows the calibration parameters, the detection limits (LOD) for each analyte calculated as three times the standard deviation of the background signal (3σ) and the enrichment factors (Er) obtained with HF-LPME that were between 86 for ACE and 320 for IBU. The relative standard deviations for 6 repetitive determinations of 20 ng mL^{-1} of the seven NSAIDs studied were between 1.5 and 2.3 % (intraday repeatability). The interdays repeatability (n=3, 6 days) were between 2.1 and 3.2 %.

3.4. Human urine analysis using HF-LPME extraction

Urine samples are complex matrices that usually require previous clean-up procedures; SPE procedures are the most frequently clean-up methods used. This work proposes an alternatively methodology based on HF-LPME extraction as a simple, accurate and low-cost procedure for the clean-up and preconcentration of IBU, NAX, KTP, DIC, KTR, ACE and SAC in human urine, just in one step.

Recovery assays were performed using spiked human urine samples from volunteers to obtain three concentrations levels of the seven NSAIDs that were subjected to the HF-LPME extraction and CE determination procedure described in the experimental section. The recoveries obtained were in the range 81-105 % (Table 2). In Figure 4 a DAD electropherogram obtained after the HF-LPME and CE determination of a blank of human urine sample is shown. The electropherogram shows an excellent baseline with peak absence, which enhances that HF-LPME can be an adequate extraction and clean-up procedure for the analysis of urine samples.

Urine samples from volunteers under treatment, which inform consent was obtained, were collected after the administration of oral doses of each NSAID. Two sets of samples were collected; the first set of samples was collected at 120 minutes after the administration of the NSAID and the second one after 240 minutes. Table 3 shows the doses administrated and the values obtained by the treatment procedure described in

previous section. Figure 5 (a-f) show the electropherograms obtained from the volunteers urine after administration of IBU (a), NAX (b), KTP (c), DIC (d), ACE (e) and acetylsalicylic acid (f); it is well known that this latest compound is quickly hydrolysed in the stomach to salicylic acid (SAC). Additionally to the NSAIDs peaks, other unknown peaks appear in each electropherogram, as can be seen in Figure 5. According to their absorption spectra and bibliographic data these peaks might be assigned to any of their main metabolites; for an adequate metabolite identifications it should be necessary the corresponding standards or a mass spectrometry detection system. So, peak UNK1 could be assigned to one of the two main metabolites of ibuprofen (2-hydroxyibuprofen and 2-carboxyibuprofen) (Moraes de Oliveira et al. 2005, Chai et al. 1988), peak UNK2 might be assigned to *O*-desmethylnaproxen, the main metabolite of the drug Naproxen [Aresta et al. 2006; Selke et al. 2010; Konstantianos et al. 199), while UNK3 could be assigned to 3- or 4-hydroxybezolketoprofen [Marco-Urrea et al. 2010; Alkatheeri et al. 1999). For the urine samples collected after administration of diclofenac, the unknown peak (UNK4) could be assigned to any or all of the main five diclofenac metabolites (4-hydroxy-, 5-hydroxy-, 3-hydroxy-, 4,5-dihydroxy-, and 3-hydroxy-4-metoxy diclofenac) (Zecca et al. 1991; Schmitz et al. 1993). 4-hydroxyaceclofenac, diclofenac and 4-hydroxydiclofenac are the main metabolites of aceclofenac, UNK5 could be assigned to 4-hydroxyaceclofenac or 4-hydroxydiclofenac [1]. UNK6 might be one of the three main metabolites of acetylsalicylic acid: salicyl acyl glucuronide, salicyluric acid and gentisic acid (Rumble and Roberts 1981; O`Kruk et al. 1984).

4. Conclusions

This study presents a rapid and simple hollow fiber-based liquid phase microextraction (HF-LPME) method combined with CE determination using uncoated fused-silica capillaries that allow an accurate and selective methodology for the determination of Ibuprofen, Naproxen, Ketoprofen, Diclofenac, Ketolorac, Aceclofenac and Salicylic Acid in human urine samples. Low organic solvent consumption and low amount of samples are needed for the proposed HF-LPME procedure with CE-DAD determination, also, high enrichments were obtained for these compounds. This procedure can be adequate for the determination of the analytes at very low levels and

may help to underlying processes of drug excretion when therapeutic goals are not being achieved or even to determine if a patient is under any kind of treatment when communication is not possible; which can prevent incompatibilities between drugs.

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Table 1. Regression coefficients, linearity, linear range, detection limits and enrichment factor of the proposed method.

NSAIDs	Reg. coef. (r^2)	Linearity (%)	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	EF
IBU	0.9992	99.86	1.05-200	0.31	320
NAX	0.9987	99.57	0.85-200	0.25	240
KTP	0.9992	99.80	1.39-200	0.42	180
DIC	0.9989	99.61	1.45-200	0.43	300
KTR	0.9994	99.90	1.72-200	0.50	90
ACE	0.9988	99.54	2.87-200	0.86	86
SAC	0.9996	99.92	1.31-200	0.39	130

Table 2. Recovery of NSAIDs in spiked urine at three different levels.

Concentration (ng mL^{-1}) (n=3)	IBU	NAX	KTP	DIC	KTR	ACE	SAC
10	87.8 ± 1.9	97.8 ± 1.6	99.8 ± 1.1	88.0 ± 1.7	104.6 ± 1.5	104.5 ± 1.8	97.3 ± 2.7
20	91.0 ± 2.3	92.3 ± 1.9	93.4 ± 1.8	83.3 ± 3.0	101.1 ± 1.9	97.8 ± 2.4	90.4 ± 3.3
50	81.3 ± 2.4	83.3 ± 1.7	81.3 ± 3.1	81.5 ± 1.7	96.6 ± 2.0	102.7 ± 2.3	92.2 ± 2.5

Table 3. Concentration of NSAIDs in urine samples.

Time of collection after administration	Concentration of NSAIDs (ng mL^{-1})(n=3)					
	IBU (Oral dose of 600 mg)	NAX (Oral dose of 250 mg)	KTP (Oral dose of 50 mg)	DIC (Oral dose of 100 mg)	ACE (Oral dose of 100 mg)	SAC (Oral dose of 500 mg)
120 min	839.9±7.3	185.2±4.1	1557±18	257.3±5.1	2765±15	8345±17
240 min	157.3±3.2	1122±12	9849±16	456.2±8.3	8097±19	11876±19

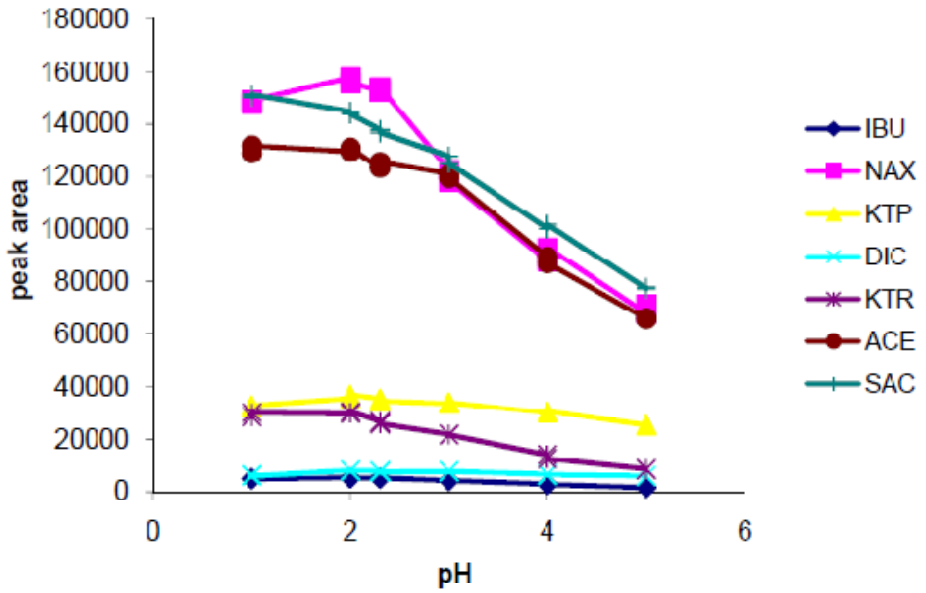

Figure 1. Optimization of pH of donor phase.

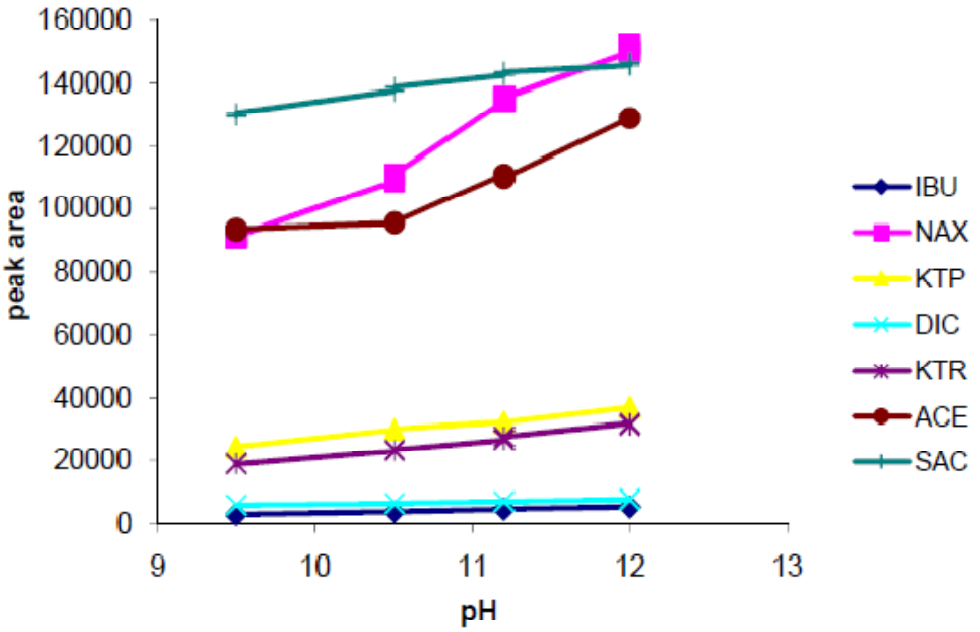

Figure 2. Optimization of pH of acceptor phase.

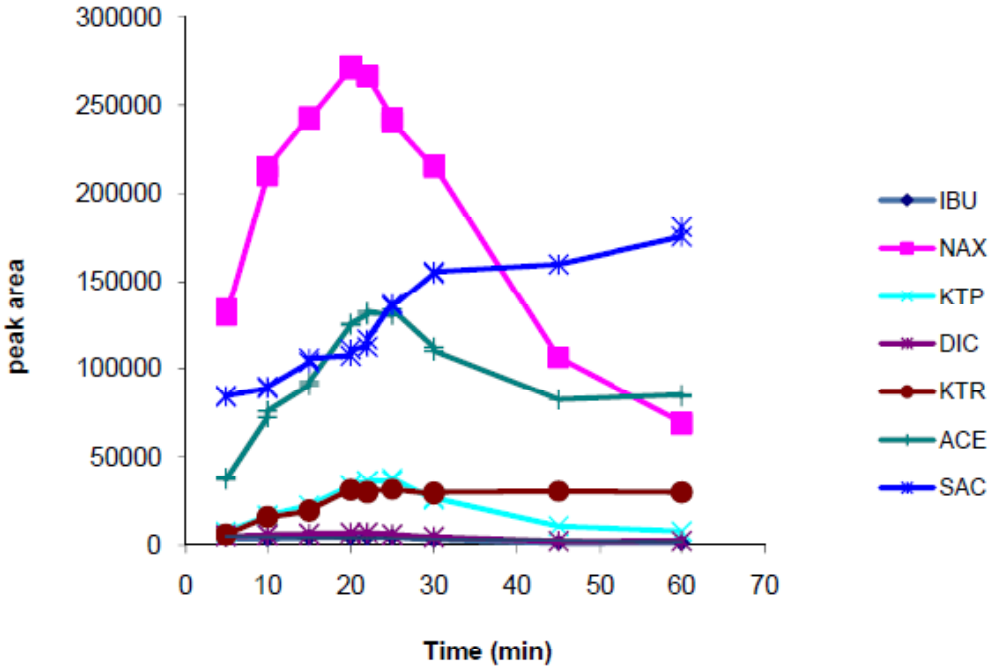

Figure 3. Optimization of stirring time.

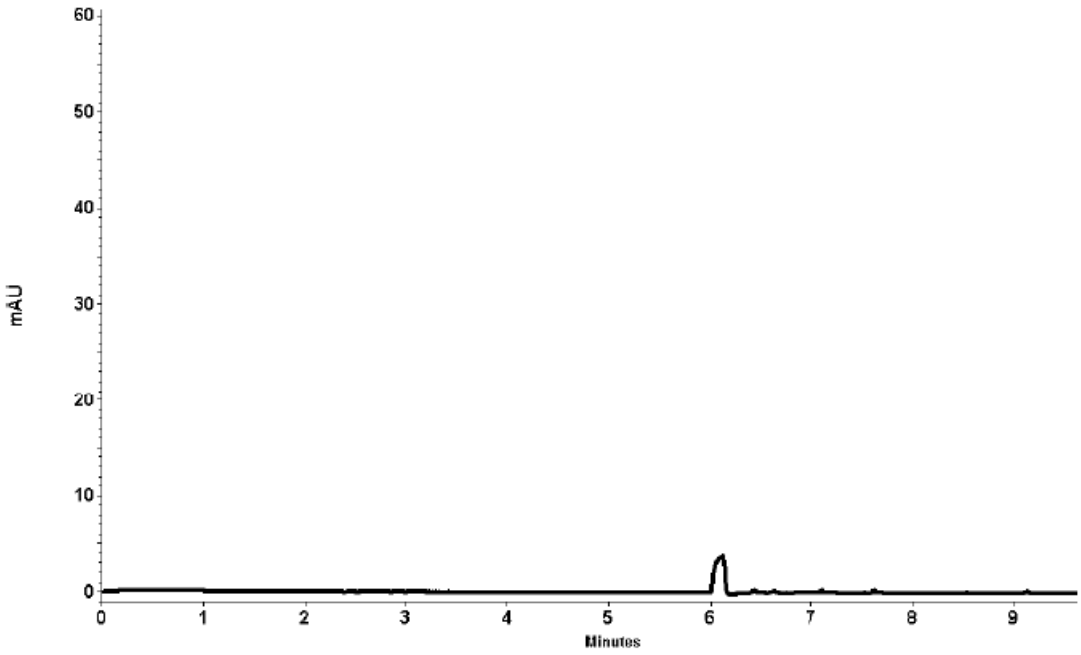

Figure 4. Electrophoregram of a blank of urine sample.

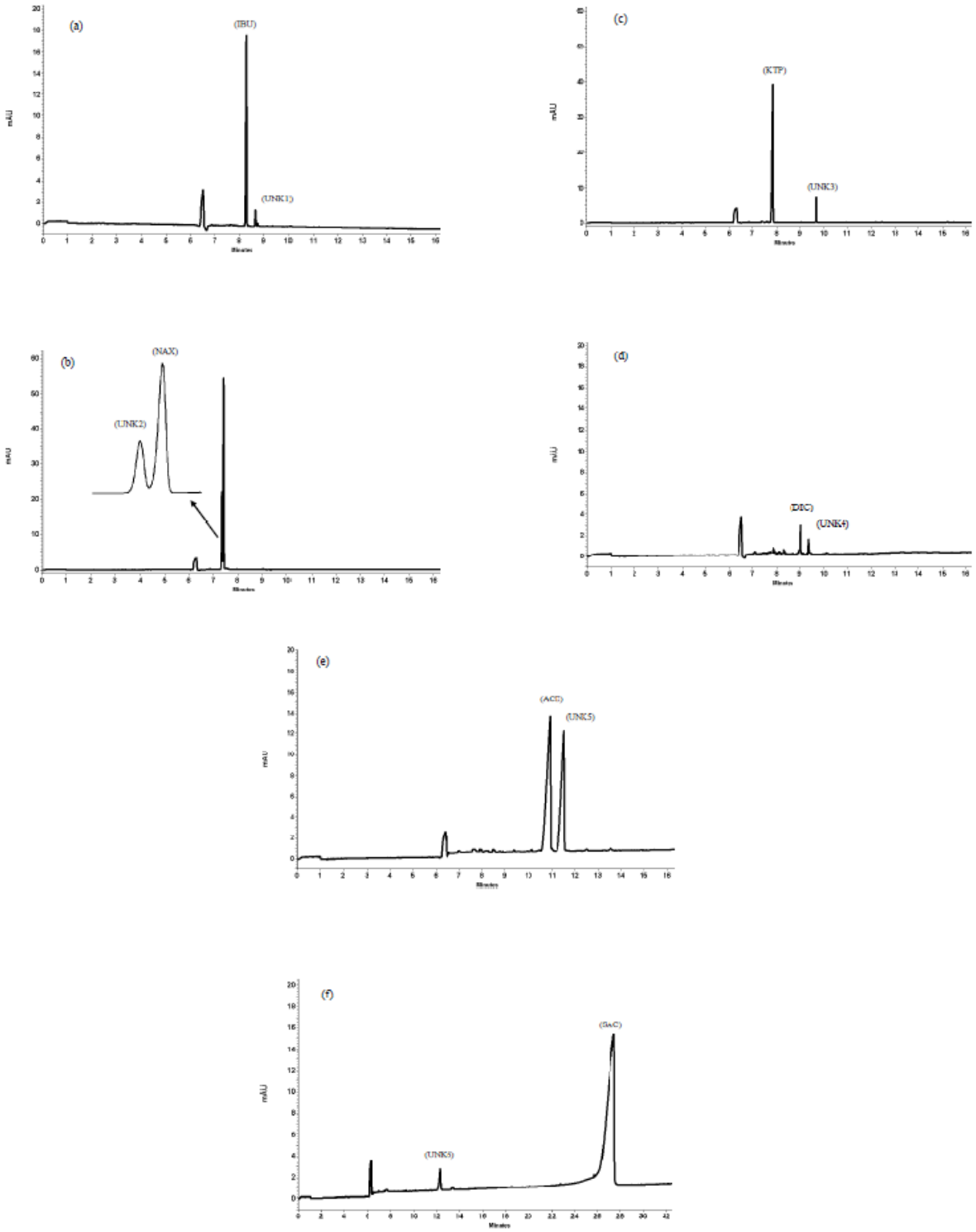

Figure 5: Electropherogram of urine sample after administration of NSAIDs: a) Ibuprofen, b) Naproxen, c) Ketoprofen, d) Diclofenac, e) Aceclofenac and f) Acetylsalicylic acid.

SULFONAMIDAS

Hollow fiber-based liquid phase microextraction (HF-LPME) for a highly sensitive HPLC determination of sulfonamides and their main metabolites

En este trabajo se propone y optimiza, la extracción y posterior determinación de ocho compuestos de la familia de las sulfonamidas. La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior se realiza usando detectores de diodo array y fluorescencia conectados en serie.

Se han determinado cuatro sulfonamidas ampliamente usadas y sus correspondientes N^4- acetil metabolitos: sulfadiazina, sulfamerazina, sulfametazina y sulfametoxazole, y sus correspondientes metabolitos: N^4-acetil-sulfadiazina, N^4- acetil-sulfamerazina, N^4- acetil-sulfametazina y N^4- acetil-sulfametoxazole. Los principios activos fueron suministrados por Sigma-Aldrich, mientras que los correspondientes metabolitos fueron sintetizados en el laboratorio, como se expone en capitulo 2.

La membrana líquida de 1-octanol fue soportada sobre fibras de polipropileno Accurel Q3/2. Se empleó como fase donadora una solución acuosa de Na_2SO_4 de pH4 (ajustada con HCl) y una solución acuosa de NaOH de pH 12 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de seis horas con una agitación de 300 rpm. La separación cromatográfica se realizó empleando una columna Eclipse® XDB-C18 3.5µm (150mm×3.0mm i.d.) y un gradiente de ácido fórmico 0,1%/acetonitrilo a un flujo de 0,4 ml/min. En estas condiciones el cromatograma completo se desarrolla en 30 minutos.

El procedimiento propuesto permite unos bajos límites de cuantificación para los analitos estudiados 3-50 ng/L y entre 0.9-100 ng/L para DAD y FLD, respectivamente, y una reproducibilidad de entre 1 y 1.8 %.

El procedimiento se ha aplicado satisfactoriamente a la determinación de los analitos en aguas residuales urbanas, agua de río, agua de lago y agua potable, con recuperaciones cercanas al 100% en el caso de agua de rio, agua de lago y agua potable, y con recuperaciones entre el 33-90% en aguas residuales, excepto para la sulfametazina, y los metabolitos de la sulfamerazina y sulfametoxazol, cuyas recuperaciones están en torno al 100%. Cabe destacar las excelentes reproducibilidades y líneas bases que se obtienen.

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Hollow fiber-based liquid phase microextraction (HF-LPME) for a highly sensitive HPLC determination of sulfonamides and their main metabolites

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ABSTRACT

In this paper, three phase-hollow fiber-based liquid phase microextraction (HF-LPME) combined with a HPLC procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of four widely used sulfonamides: sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and their main metabolites, the corresponding N^4-acetyl derivatives: N^4-acetyl-sulfadiazine, N^4-acetyl-sulfamerazine, N^4-acetyl-sulfamethazine, N^4-acetyl-sulfamethoxazole. A Q3/2 Accurel KM polypropylene hollow fiber supporting 1-octanol was used between a 2 M Na_2SO_4 aqueous solution (pH 4) as a donor phase and aqueous solution (pH 12) as an acceptor phase. The procedure allows very low detection and quantitation limits of 0.3–33 ng L^{-1} and 0.9–100 ng L^{-1}, respectively. The proposed method was applied to the determination of the analytes in environmental water samples (surface, tap and wastewater).

1. Introduction

The analysis of complex samples and the analyte detection or quantitation at very low levels are nowadays two of the main analytical problems. The analytical complexity increases in those cases where both problems are present. The use of clean-up procedures is an old analytical tool that, in the last years has undergone very important developments. Liquid phase microextraction (LPME), also known as supported liquid membranes extraction (SLM), is an attractive alternative to the widely used solid phase extraction (SPE). The use of hollow fibers for LPME (HF-LPME) not only allows in most cases an efficient clean-up procedure, but also produces high degree of pre-concentration. Additionally, the low cost of the polypropylene fibers used and the low organic solvent consumption make HF-LPME an interesting and environmental friendly analytical procedure.

The growing worldwide consumption of pharmaceuticals and their proved occurrence in the environment has become an important issue in recent years, and in the last decade, the focus in environmental research has been extended from more classical environmental pollutants as PCBs, PAHs or pesticides to pharmaceuticals and personal care products. The amount of human pharmaceuticals reaching the environment depends on the consumption amount, and excretion rate via faeces and urine. Effluents of wastewater treatment plants (WWTPs) are considered the principal source of drugs in the aquatic environment. A smaller contribution to the presence of pharmaceuticals in the environment is due to the disposal of outdated medicines down household drains [1] and to the pharmaceutical industry waste [2,3]. The low levels expected and the matrix complexities make it necessary to use adequate preconcentration and clean-up procedures.

Solid phase extraction (SPE), using several sorbent types, has been the preferred sample preparation technique to extract pharmaceuticals from environmental waters [4–6] but in the last years there has been a high interest in developing new clean-up procedures.

Liquid–liquid extraction (LLE) is a classical and common technique used for preconcentration and clean-up prior to chromatographic or electrophoretic analysis that leads to large organic solvent consumption. It is also tedious and the frequently analyte is lost due to the multi-stage operations that cannot be neglected. Liquid-phase microextraction (LPME) based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) [7,8], is a simple, inexpensive, fast, effective and virtually solvent-free sample pretreatment technique. However, SDME is not very robust, and the droplets may be lost from the needle tip of the microsyringe during extraction.

Audunsson [9] introduced an alternative concept for LPME that was developed by Thordarson et al. [10], and Pedersen-Bjergaard and Rasmussen [11], based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this

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concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilised within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber; when acceptor phase is an aqueous phase the procedure is known as three-phase HF-LPME. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor solution from entering the accepting phase and, at the same time, most macromolecules do not enter the hollow fiber because they are not soluble in the organic phase present in the pores in the extraction conditions, thus yielding very clean extracts [12]. Several reviews on hollow fiber-based LPME have been reported [13–16].

Sulfonamides play an important role as effective chemotherapeutics of bacterial and protozoal diseases and also exhibit growth-promoting properties in veterinary medicine [17,18]. For several authors, sulfonamides have been implicated in the growing prevalence of antibiotic resistance in humans [19–21] so their possible presence in foods (products of animal origin, essentially) and in the environment is a public health concern.

Several methods have been reported for the analysis of sulfonamides, using many kinds of analytical tools that include photometric methods [22], the Bratton–Marshall method [23,24], titrimetric assay methods [25], thin layer chromatography [26], FIA with amperometric detection [27], ELISA [28], high-performance liquid chromatography [29–33], gas chromatography and gas chromatography–mass spectrometry [34–36] and capillary electrophoresis (CE) [37–39]. Generally, for the application of these methods to real complex samples, a high number of extraction, concentration and purification steps are necessary, usually solid phase extraction (SPE) or more laborious approaches.

Two methods that use liquid phase microextraction for the determination of sulfonamides have been previously published. Msagati and Muzi-Nindi [40] use a porous PTFE membrane (FG type Millipore filter) impregnated with 5% (w/v) tri-n-octylphosphine oxide (TOPO) dissolved in hexyl amine and placed between two circular polyvinylidene difluoride (PVDF) blocks in a continuous configuration with two separated channels controlled by two peristaltic pumps. The extracts were analysed by HPLC–MS allowing the simultaneous determination of 16 sulfonamides in spiked water, urine, milk and animal tissues with recoveries between 34 and 93% (34–70% for sulfamerazine, sulfamethazine and sulfamethoxazole) and detection limits between 1.8 and 24.3 $\mu g\,L^{-1}$. Yong et al. [41] use an ionic liquid (1-octyl-3-methylimidazolium hexafluorophosphate [C_8MIMI]) as liquid membrane and 14% (w/v) TOPO as additive in a three phases HF-LPME configuration using a Q3/2 Accurel KM polypropylene hollow fiber. The procedure allows the HPLC-UV determination of five sulfonamides (sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and sulfadimethoxine) in aqueous samples (farm wastewaters) with recoveries >82%, detection limits between 0.1 and 0.4 $\mu g\,L^{-1}$ and quantitation limits of 1.0 $\mu g\,L^{-1}$.

The aim of this work was to develop a highly sensitive determination of sulfonamides and their metabolites that can be easily applicable to environmental waters (including wastewaters). The organic solvent consumption of several microlitres, in contrast to other clean-up/preconcentration alternatives like SPE is according to the current trends to a "Green Chemistry".

In this work, a HPLC DAD-FLD method combined with preceding HF-LPME was developed for the highly sensitive determination of four sulfonamides: sulfadiazine (SDI), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMX) and their main metabolites N^4-acetyl-sulfadiazine (NSDI), N^4-acetyl-sulfamerazine (NSMR), N^4-acetyl-sulfamethazine (NSMZ) and N^4-acetyl-sulfamethoxazole (NSMX). (Table 1 shows their structures, pK_a and IUPAC names.) The method has been successfully applied to their determination in several water types: wastewaters from the different treatment steps of a WWTP, and water samples from river, lake and tap water.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water form a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). SDI, SMR, SMZ, SMX, dihexyl ether and 1-octanol were purchased from Fluka-Sigma-Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany). N^4-acetyl sulfonamides metabolites (NSDI, NSMR, NSMZ and NSMX) were synthesised according to Pfeifer et al. [42].

Methanolic working solutions of SDI, SMR, SMZ, SMX, NSDI, NSMR, NSMZ and NSMX were daily prepared by adequate dilutions from methanolic 100 $mg\,L^{-1}$ stock solutions. Q3/2 Accurel KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Chromatographic conditions

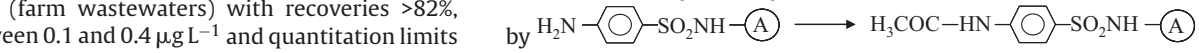
The chromatographic separation was performed at 10 °C using a LaChrom® VWR-Hitachi (Barcelona, Spain) liquid chromatograph with a quaternary L-2130 pump. The injector was a Rheodyne manual injection valve Model 7725i, fitted with a 20-μL sample loop. Separations were carried out using a Eclipse® XDB-C18 3.5 μm (150 mm × 3.0 mm i.d.) (Agilent., Palo Alto, CA (USA)) column preceded by a guard column Kromasil® 100 Å, C18, 5 μm, (15 mm × 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain).

The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and acetonitrile (component B) at a flow rate of 0.4 $mL\,min^{-1}$. An initial 90% component A was used in isocratic mode for 10 min and then a linear elution gradient was programmed from 90% to 85% A for another 5 min, finally an elution gradient was programmed from 85% to 60% A for another 15 min. Three minutes were waited between injections which allowed re-equilibration of the column to the initial conditions.

Table 2 shows the monitoring wavelengths for DAD and FLD detections and the retention times for the analysed compounds.

2.3. Synthesis of the metabolites

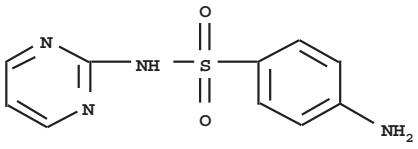
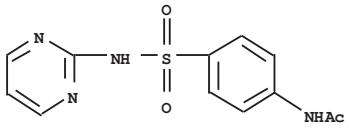
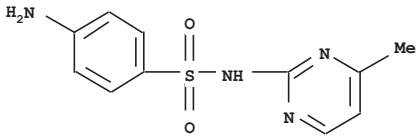
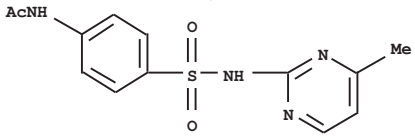
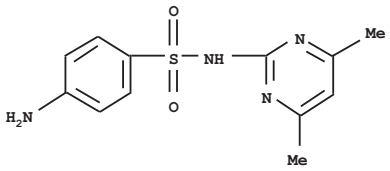
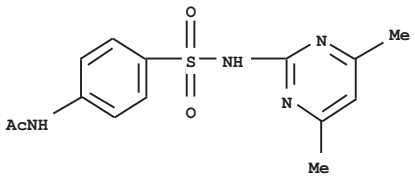
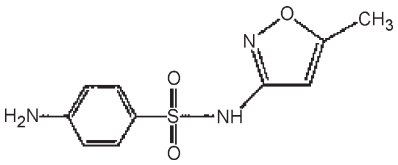
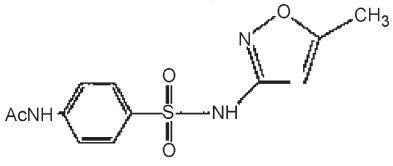
The main sulfonamides metabolites are their corresponding N^4-acetyl derivatives [43,44] and they were synthesised in our laboratory. The synthesis reaction can be schematised by



where (A) is the corresponding heterocyclic rest for each sulfonamide.

The synthesis of the sulfonamides metabolites was carried out by slight modifications of the procedure proposed by Pfeifer et al. [42]. Briefly the procedure consisted on: 100 mg of sulfonamide was mixed with 1.5 mL of pyridine and 1.5 mL of acetic anhydride, and the mix was stirred and heated under reflux at 40 °C in a glycerin bath for a variable time depending on the sulfonamide: 6.30 h for SDI, 7 h for SMR, 3 h for SMZ and 6 h for SMX. After reaction the mix was cooled into an ice-bath and extracted with three portions of 20 mL of dichloromethane. Organic extracts were washed with two portions of 200 mL 2 N H_2SO_4 and 20 mL of saturated $NaHCO_3$. The resulting organic extract was dried with $MgSO_4$, filtered and concentrated under vacuum.

Table 1
Structure and IUPAC name of the examined antibiotics and their corresponding N^4-acetyl metabolites.

Drug	Structure	IUPAC name
SDI		Benzenesulfonamide, 4-amino-N-2-pyrimidinyl pK_{a1} = 1.6; pK_{a2} = 6.5
NSDI		Acetamide, N-[4-[(2-pyrimidinylamino)sulfonyl]phenyl]
SMR		Benzenesulfonamide, 4-amino-N-(4-methyl-2-pyrimidinyl) pK_{a1} = 1.58; pK_{a2} = 6.90
NSMR		Acetamide, N-[4-[[(4-methyl-2-pyrimidinyl)amino]sulfonyl]phenyl]
SMZ		Benzenesulfonamide, 4-amino-N-(4,6-dimethyl-2-pyrimidinyl) pK_{a1} = 2.07; pK_{a2} = 7.49
NSMZ		Acetamide, N-[4-[[(4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl]phenyl]
SMX		Benzenesulfonamide, 4-amino-N-(5-methyl-3-isoxazolyl)- pK_{a1} = 1.85; pK_{a2} = 5.60
NSMX		Acetamide, N-[4-[[(5-methyl-3-isoxazolyl)amino]sulfonyl]phenyl]-

The concentrated extract was purified by chromatography using a silica column and dichloromethane:methanol (50:1) as mobile phase and the purity of the collected fractions were controlled by thin layer chromatography (silica as a stationary phase and dichloromethane:methanol (10:1) as a mobile phase). Detection was carried out with ethanolic 1% ninhydrine or UV irradiation (254 nm). Purified sulfonamide metabolite fraction was identified by mass spectrometry.

2.4. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked with 1-octanol during 10 s to impregnate the pores, and rinsed with water on the outside by placing it into the ultrasonic bath for 30 s in order to remove the excess of organic solvent. The lumen of the prepared

Table 2
Monitoring wavelengths and retention times.

	DAD			FLD			
	λ_{max} (nm)	t_R (min)	S.D. (min)	λ_{exc} (nm)	λ_{em} (nm)	t_R (min)	S.D. (min)
SDI	270	8.69	0.008	280	450	9.21	0.003
SMR	270	12.45	0.006	275	445	13.18	0.012
NSDI	262	15.52	0.01	270	356	15.98	0.009
SMZ	270	16.99	0.005	275	445	17.51	0.006
NSMR	262	18.96	0.011	275	351	19.38	0.008
NSMZ	262	21.84	0.007	275	351	22.21	0.011
SMX	270	28.91	0.013	275	382	29.30	0.002
NSMX	262	29.94	0.014	280	351	30.41	0.004

fiber piece was filled with 50 μL of acceptor phase (pH 12 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and a plastic film (Parafilm®, Pechiney Plastic Packaging Company, Chicago, IL, USA). During extraction the membrane portion that contains the acceptor phase was immersed in the 50 mL sample solution (pH 4, Na_2SO_4 2 M) contained into a 50 mL glass beaker. The sample was stirred for 6 h by means of a magnetic stirrer (ANS-00/1 Science Basic Solutions; Rubí, Barcelona, SPAIN) at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extracted using a HPLC syringe and injected into the HPLC system.

2.5. Preparation of environmental water samples

Wastewater samples were obtained from "Guadalquivir"-ALJARAFESA Wastewater Treatment Plant which is located in Palomares del Río, Seville, SPAIN. The WWTP essentially receives urban wastewaters. The capacity of this WWTP is 100.000 inhabitants and the discharged flow is 12,433,313 m^3/year (2008 data). Grab samples of the influent (raw water, WWR), after the primary sedimentation tank (WW1), after the aeration tank (WW2) and the effluent (treated water after anaerobic digestion, WWT) were collected in 11 January 2010.

Two samples from Guadalquivir River were analysed. One (RIVER1) from Coria del Río, Seville, 2 km downstream the WWTP previously mentioned and other sample (RIVER2) was taken at the mouth of Guadalquivir River (Sanlúcar de Barrameda, Cádiz) where water has a high seawater proportion. Lake water samples (LAKE) were obtained from "Lagos del Serrano" (Guillena, Seville). Tap water sample (TAP) was obtained directly from the laboratory tap.

All samples, except tap water, were filtered through a GDU1 glass fiber filter bed (10–1 μm) (Whatman, Mainstone, UK) and through Pall Nylaflo™ nylon membrane filter 0.45 μm (Pall Corporation, Ann Arbor, MI, USA) and adjusted to pH 4 with HCl. Filtered samples were stored in the dark at 4 °C prior to HF-LPME extraction.

Water samples, were directly analysed after Na_2SO_4 addition for a 2 M final concentration; HCl was added just to obtain pH 4 prior to be submitted to the HF-LPME procedure.

3. Results and discussion

3.1. Chromatographic conditions

Looking for a fast and high resolution separation an Eclipse® XDV-C18 3.5 μm was selected as working column. This column is a high packing HPLC column that allows high resolution separations using low flow-rates which implies low solvent consumption. The selected column provides good resolution and good peak symmetry.

The mobile phase consisted of 0.1% formic acid and acetonitrile. Different gradient elution conditions were tested searching for the shortest time of analysis without sacrificing peak shape. The gradient elution program described in Section 2.2, was the best option in terms of time of analysis, shape of the peaks and reproducibility.

N^4-acetyl metabolites are not stable at high pH values like the used for the acceptor phase and they are transformed to the corresponding N^4-hydroxy derivatives; we have checked that a complete transformation carry out in 4 h at pH 12, so the measured chromatographic peaks for the N^4-metabolites correspond to their N^4-hydroxy derivatives, and the chromatographic conditions were accordingly optimised for these compounds. It is remarkable that N^4-hydroxy derivatives only can be generated from the corresponding N^4-acetyl metabolites. Fig. 1 shows representative chromatograms from aqueous standards submitted to the HF-LPME procedure.

The efficiency and selectivity chromatographic parameters of the proposed procedure are shown in Table 3, N (number of theoretical plates), T (asymmetry factor), $W_{1/2}$ (peak half-width), k (retention factor), α (selectivity factor) and R_s (peak resolution). As it can be seen, all parameter values are adequate according to their critical values.

3.2. Optimization and evaluation of experimental conditions for HF-LPME extraction

First, several tests with donor phases pHs 3–5 and acceptor phases pHs 10–12 were carried out in order to choose the more adequate liquid supported on the polypropylene membrane; dihexyl ether and 1-octanol were checked and the best results

Table 3
Efficiency and selectivity chromatographic parameters for the proposed HPLC procedure (for abbreviation see text).

	$W_{1/2}$ (min)	T	N	k	α	R_s
SDI	0.2577	1.21	6299	3.35	1.56	7.56
SMR	0.3290	1.33	7933	5.23	1.93	5.03
NSDI	0.3919	1.44	8688	6.76	1.11	2.14
SMZ	0.4178	1.41	9161	7.50	1.13	2.84
NSMR	0.4017	1.40	12,342	8.48	1.17	4.45
NSMZ	0.3613	1.36	20,243	9.92	1.36	10.34
SMX	0.4453	1.44	23,351	13.46	1.04	1.66
NSMX	0.2873	1.28	60,164	13.97	1.04	1.66
Critical values		<1.5	>2000	>2	>1	>1.5

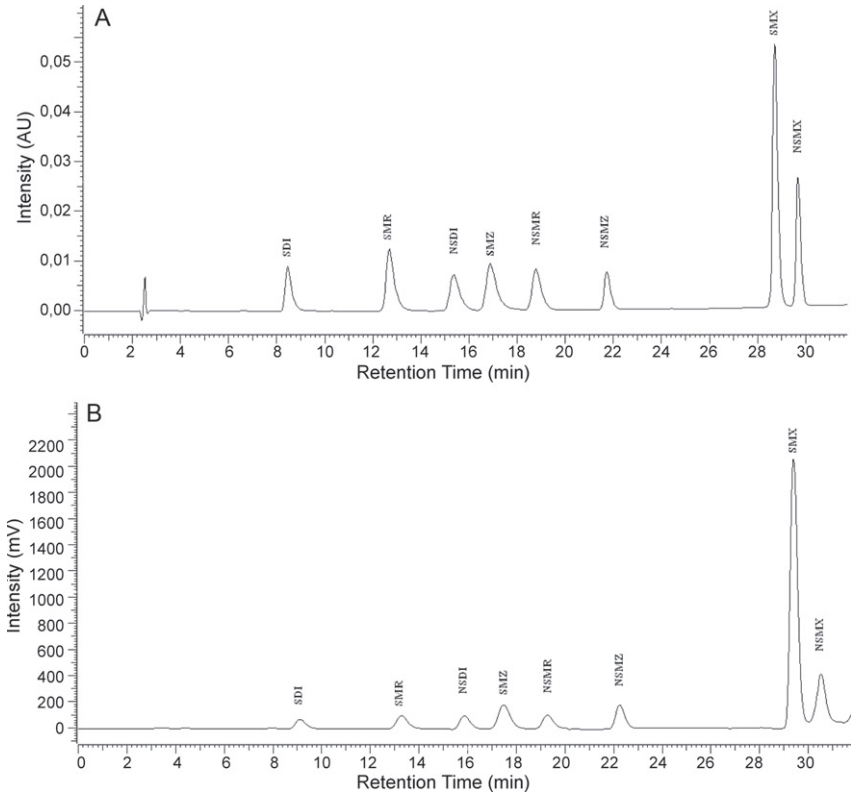


Fig. 1. DAD (a) and FLD (b) chromatograms from standard aqueous solutions (10 and 5 ng L^{-1}, respectively).

were obtained with 1-octanol, so this was the liquid supported selected.

Sulfonamides have two dissociation constants. A sulfonamide contains one basic amine group (–NH_2) and one acidic group (–NH–SO_2–) which correspond to pK_{a1} and pK_{a2}, respectively. The amine group is able to gain a proton, while the amide group is able to release a proton under specific pH conditions. When pH is adjusted to the average of pK_{a1} and pK_{a2} the neutral molecule form is the dominant specie. When pH is above pK_{a2} sulfonamides become neutral and then changed to anionic forms. N^4-acetyl metabolites have pK_a values slightly lower than the corresponding sulfonamide ones. For these reasons donor HCl aqueous solutions within 3.3–5 pH range were tested using 50 mL of aqueous solutions containing 10 μg L^{-1} of each analyte. The extraction was carried out 300 rpm during 30 min and 50 μL of aqueous pH 11 NaOH solution was used as acceptor phase. In general, maximum recoveries were observed when donor pH varies between 3.5 and 4.3, so a pH 4 aqueous solution was selected as donor phase. When pH values between 10 and 12.5 (adjusted with aqueous NaOH solutions) were tested as acceptor phase, maximum extraction efficiencies were observed within 11.5 and 12.2. Thus, pH 12 was selected as optimum for acceptor phase.

Once the donor and acceptor pHs were optimised, a possible influence of salting out effect was tested. Aqueous pH 4 solutions containing NaCl (2–6 M) or Na_2SO_4 (0.5 M to saturation) were checked as donor phases using a pH 12 acceptor phase and stirring at 300 rpm during 30 min. In general, salting out allows an increase in the extraction efficiency with the salt concentration that is more pronounced with Na_2SO_4. Aqueous saturated (approximately 2 M) Na_2SO_4 pH 4 solution was selected as optimum donor phase.

Using the selected optimum donor and acceptor phases, the influence of the stirring time was checked between 3 and 8 h; maximum extraction was obtained for time values around 6 h for all the analytes, so a stirring time of 6 h at 300 rpm was selected as an optimum value.

All the figures corresponding to the optimization and evaluation of experimental conditions for the proposed HF-LPME extraction have been supplied as Supplementary Electronic Material.

3.3. Linearity, sensitivity, precision and robustness for the HF-LPME extraction

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve, was constructed using a least-square linear regression analysis of standards mixtures of the analytes at different concentrations. Using the selected HF-LPME conditions, aqueous pH 4 solutions with different analyte concentrations were submitted to the liquid microextraction procedure and analysed according to the described HPLC procedure. Peak areas were proportional to concentrations in the donor phase. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ and the calibration curves obtained showed no changes over the course of one month. Detection and quantitation limits were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio is 3 and 10, respectively. Enrichment factor was calculated as the increase in the concentration between the aqueous external donor phase and the internal (acceptor) phase. Table 4 shows the corresponding data and, as can be seen, the high enrichment values obtained for all the analytes allow the detection and determination of low concentration levels.

To evaluate the repeatability and the intermediate precision, aqueous samples at three concentrations levels 10^2 (25 for SMX and NSMX), 10^3 and 10^5 ng L^{-1} (in triplicate) were subjected to the entire analytical procedure and measured in one single day and one day per week during two months, respectively. Intermediate precision was performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the $m \times p \times n$ design (m = analytical levels, p = days and n = replications). From the corresponding ANOVA, the intermediate precision was

Table 4
Detection limits, linear ranges, % linearity and enrichment factors for the HPLC method (DAD and FLD detection) combined with prior HF-LPME.

	DAD			FLD			Enrichment
	LOD ($ng\,L^{-1}$)	Linear range ($ng\,L^{-1}$)	% linearity	LOD ($ng\,L^{-1}$)	Linear range ($ng\,L^{-1}$)	% linearity	
SDI	15	50–10^6	99.81	15	50–10^5	99.91	200
SMR	5	15–10^6	99.67	15	50–10^5	99.63	1000
NSDI	15	50–10^6	99.59	8	25–10^5	99.96	400
SMZ	15	50–10^6	99.86	15	50–10^5	99.83	250
NSMR	15	50–10^6	99.69	8	25–10^5	99.97	500
NSMZ	15	50–10^6	99.92	33	100–10^5	99.88	175
SMX	1	3–10^6	99.77	0.3	0.9–10^5	99.95	1000
NSMX	3.5	10–10^6	99.84	8	25–10^5	99.90	600

computed [45]. The repeatability, expressed as relative standard deviation, was in the range 0.8–1.2%. Intermediate precision also expressed as relative standard deviation, was in the range 1.0–1.8%.

The robustness study is based on the procedure suggested by Youden [46]. A design matrix with two factors in eight experiments was used when the +1 and −1 levels correspond to high and low pH values (4.5 and 3.5 for donor phase and 12.5 and 11.5 for the acceptor phase). Stirring time is not considered as a variable for robustness study due to its high optimum value (6 h) and the fact that variations in the order of minutes do not have significant effects in the extraction efficiency.

A significance *t*-test [47] was used to determine whether variations have a significant effect on the result, and the calculated *t* values were compared with the corresponding critical *t* values ($n = 4$) at 5% significance level and three degrees of freedom. The results obtained indicated that the procedure can be considered robust against the considered factors for all the analysed compounds.

3.4. Environmental water sample analysis

The different water samples were selected taking into account the maximum variability with respect to provenance and matrix composition

First, the different water samples were submitted to the HF-LPME proposed procedure and analysed. None of the sulfonamides and their corresponding N^4-acetyl metabolites were detected in the water samples. In order to check the suitability of the proposed procedure, spiked samples at three concentration levels: 100 (15 for SMX and NSMX), 500 and 5000 $ng\,L^{-1}$, were analysed. Results obtained are shown in Table 5. In the "more simple" water samples (RIVER1, RIVER2, LAKE and TAP), recoveries for all the compounds are within 93 and 101%. Wastewater samples are, in general, complex samples from the analytical point of view, that frequently require complex clean-up processes. The direct application of the proposed HF-LPME procedure to the different wastewater types analyses shows, in general, excellent results with recoveries within 99 and 101% for SMZ, NSMR and NSMX, however the rest of analysed compounds show variable recoveries within 33 and 90%. It is remarkable that, in general, recoveries slowly increase with the depuration process, showing the better values for the WWT sample with recoveries higher than 72% except for SMR (56–56%) and NSMZ (40–44%). Urban wastewaters have extremely high surfactants concentrations that could modify the supported liquid membrane behaviour [48] and that could lead to recovery decreases but remain low standard deviations. Despite the recovery decreases, the excellent preconcentration and clean-up obtained implies a great advantage over other sample treatment procedures which can justifies the use of the proposed HF-LPME procedure.

Figs. 2 and 3 show representative DAD and FLD chromatograms obtained from blank and spiked (500 $ng\,L^{-1}$) wastewater (WWR) and river (RIVER1) samples. These samples have been selected as the more complex wastewater (raw wastewater) and surface water. As it can be seen, RIVER1 blank chromatograms show excellent baselines that are a little poor for the WWR ones. Spiked RIVER1 chromatograms only show well defined peaks corresponding to

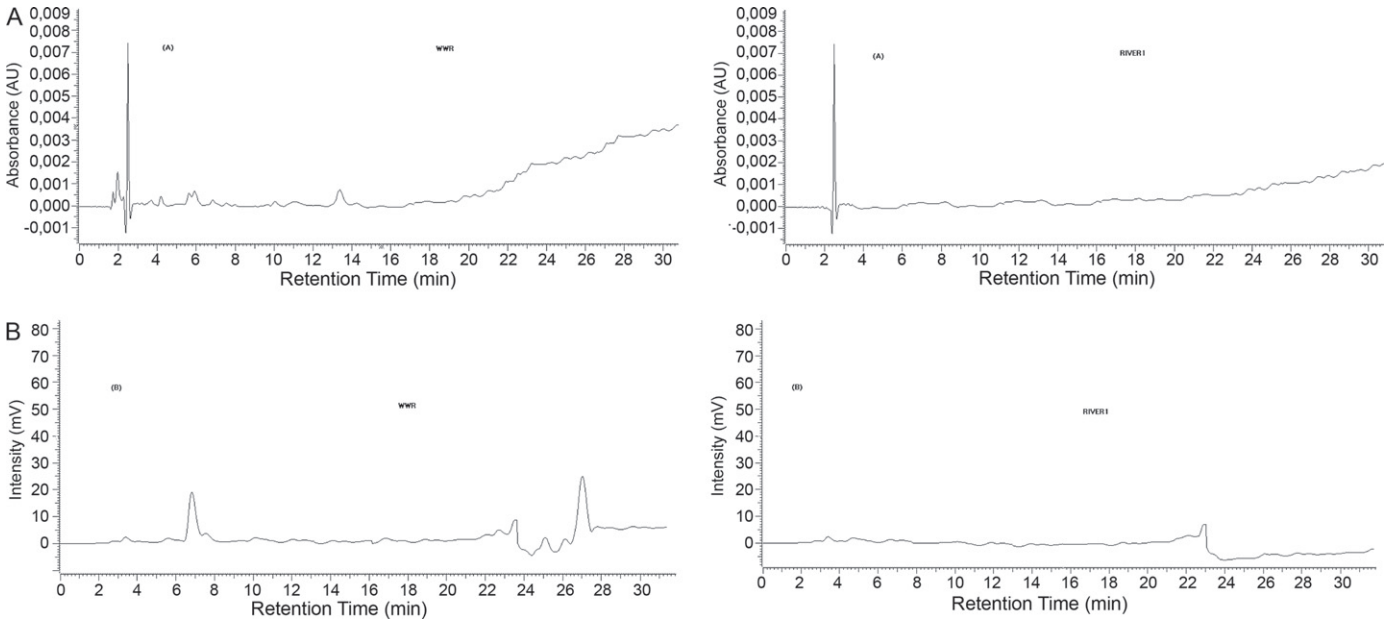


Fig. 2. DAD (a) and FLD (b) chromatograms of blank wastewater (WWR) and river (RIVER1) samples.

Table 5
Recoveries (%) using HF-LPME/HPLC from water spiked samples (average of three determinations ± standard deviation).

	Spiked level ($ng\,L^{-1}$)	Water sample[a]							
		WWR	WW1	WW2	WWT	RIVER1	RIVER2	Lake	Tap
SDI	100	63.6 ± 2.2	68.7 ± 1.8	70.4 ± 1.8	78.2 ± 1.8	99.7 ± 0.6	98.6 ± 1.6	99.7 ± 2.2	99.9 ± 1.6
	500	66.7 ± 1.8	69.1 ± 1.8	71.0 ± 1.8	81.3 ± 1.2	99.8 ± 0.2	98.3 ± 0.4	100.2 ± 1.6	100.0 ± 1.6
	5000	71.3 ± 2.4	68.9 ± 1.6	70.9 ± 2.2	82.2 ± 1.2	100.0 ± 0.2	99.1 ± 0.4	99.9 ± 1.0	100.0 ± 0.8
SMR	100	32.6 ± 3.8	34.2 ± 2.2	35.2 ± 1.8	56.2 ± 3.0	99.9 ± 2.2	99.4 ± 1.2	99.6 ± 1.8	99.6 ± 1.6
	500	33.7 ± 3.0	35.2 ± 0.8	34.6 ± 1.6	58.8 ± 1.8	98.4 ± 1.8	99.6 ± 0.8	99.9 ± 0.8	99.3 ± 1.8
	5000	36.2 ± 1.6	35.1 ± 0.8	34.7 ± 1.6	58.3 ± 1.6	100.0 ± 1.0	99.9 ± 0.6	100.1 ± 0.8	100.0 ± 1.2
NSDI	100	51.9 ± 1.8	52.2 ± 1.6	54.5 ± 1.0	72.5 ± 1.6	99.6 ± 1.8	98.3 ± 0.6	100.30 ± 0.4	99.49 ± 0.8
	500	52.4 ± 1.6	52.4 ± 1.4	54.2 ± 0.8	72.1 ± 1.0	99.7 ± 1.6	98.7 ± 0.6	99.56 ± 0.2	100.21 ± 0.8
	5000	53.2 ± 0.4	52.7 ± 0.8	54.8 ± 0.2	74.2 ± 1.0	100.0 ± 1.6	99.1 ± 0.6	99.77 ± 0.2	100.16 ± 0.2
SMZ	100	98.9 ± 2.2	98.9 ± 1.6	98.6 ± 0.15	99.1 ± 3.2	99.4 ± 2.8	98.3 ± 1.4	99.7 ± 1.4	99.9 ± 2.2
	500	100.0 ± 1.2	100.0 ± 1.6	99.9 ± 1.2	100.0 ± 1.8	99.8 ± 1.6	98.1 ± 1.0	99.9 ± 0.6	100.1 ± 2.0
	5000	99.7 ± 1.6	99.6 ± 2.4	100.0 ± 1.2	100.1 ± 3.0	99.9 ± 1.2	98.3 ± 0.8	99.7 ± 0.2	100.0 ± 1.6
NSMR	100	97.5 ± 1.0	98.4 ± 1.2	99.9 ± 1.6	100.2 ± 0.8	99.6 ± 1.8	99.75 ± 1.8	100.1 ± 1.6	99.8 ± 1.2
	500	100.0 ± 1.0	99.6 ± 0.8	99.6 ± 0.8	99.9 ± 0.8	100.0 ± 1.0	101.01 ± 1.6	99.9 ± 1.6	99.0 ± 1.2
	5000	100.1 ± 0.6	100.6 ± 0.8	99.3 ± 0.6	99.47 ± 0.4	99.7 ± 1.0	99.89 ± 1.6	100.2 ± 0.6	99.8 ± 1.0
NSMZ	100	Detected	Detected	Detected	Detected	95.6 ± 1.0	93.21 ± 1.6	98.4 ± 2.2	99.2 ± 1.8
	500	33.1 ± 1.6	34.0 ± 1.8	38.2 ± 1.6	41.0 ± 2.2	95.4 ± 1.0	96.15 ± 1.6	99.5 ± 0.8	99.8 ± 1.8
	5000	35.2 ± 1.6	35.7 ± 1.2	38.8 ± 1.2	44.2 ± 1.8	99.8 ± 0.6	99.0 ± 0.8	100.2 ± 0.8	100.1 ± 1.2
SMX	15	62.5 ± 1.8	62.5 ± 1.8	83.2 ± 1.6	88.7 ± 1.6	100.0 ± 1.4	100.2 ± 2.2	100.0 ± 1.8	100.1 ± 2.4
	500	61.8 ± 1.8	61.8 ± 1.8	88.2 ± 1.6	90.0 ± 1.8	100.1 ± 1.0	100.1 ± 1.8	99.8 ± 1.6	100.0 ± 1.6
	5000	63.7 ± 0.2	63.7 ± 0.2	89.7 ± 1.2	91.0 ± 0.8	100.0 ± 1.4	99.9 ± 1.8	100.1 ± 1.6	99.9 ± 0.05
NSMX	15	100.0 ± 2.2	100.0 ± 1.6	99.84 ± 3.0	99.9 ± 2.2	100.2 ± 0.6	99.9 ± 0.1	99.8 ± 1.6	99.9 ± 1.8
	500	100.0 ± 1.8	99.9 ± 1.0	99.96 ± 0.8	100.2 ± 1.0	100.1 ± 0.6	99.7 ± 1.8	99.9 ± 1.0	100.2 ± 1.8
	5000	99.9 ± 1.2	100.2 ± 1.0	100.08 ± 0.8	100.0 ± 0.6	100.0 ± 0.2	100.4 ± 0.6	100.2 ± 1.0	100.0 ± 0.8

[a] Average recovery (%) ± standard deviation ($n = 3$).

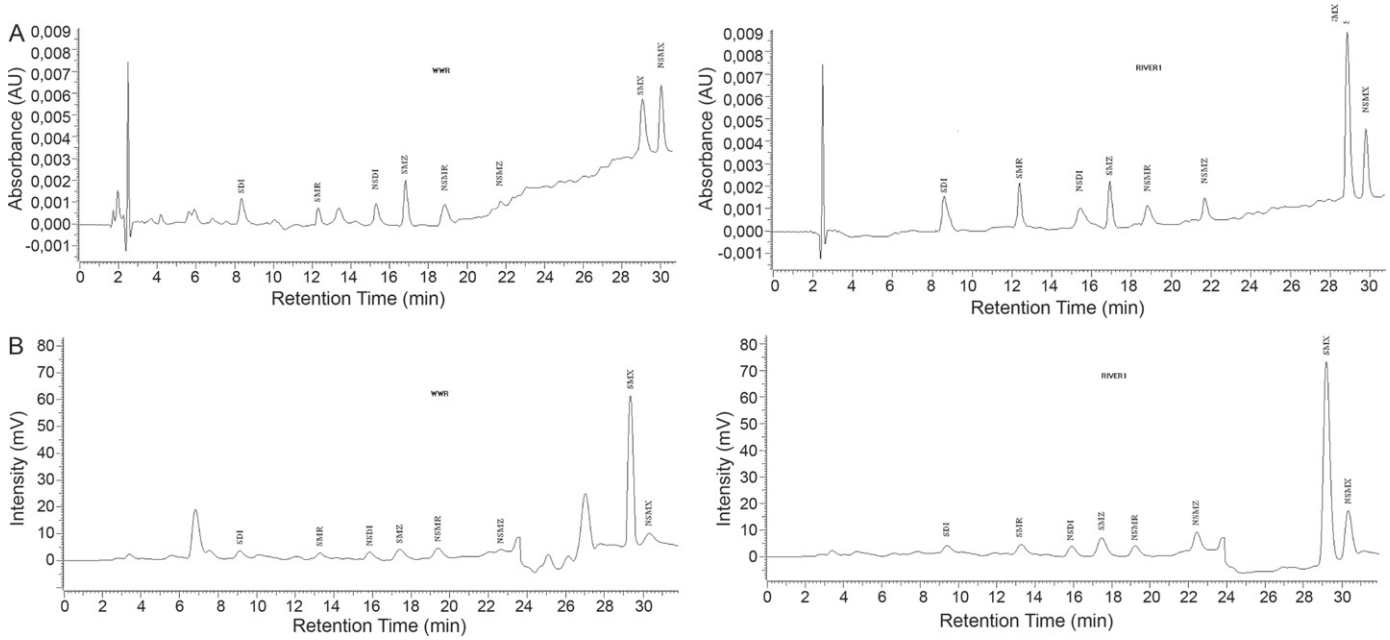


Fig. 3. DAD (a) and FLD (b) chromatograms from spiked ($500\,ng\,L^{-1}$) wastewater (WWR) and river (RIVER1) samples.

the added substances, and for WWR, the low peaks observed in the blank chromatograms do not interfere with the corresponding spiked ones.

4. Conclusions

This study presents a hollow fiber-based liquid phase microextraction (HF-LPME) method combined with an HPLC (DAD-FLD) determination using a high packing chromatographic column that allows a simple, low-cost, accurate, highly sensitive and selective methodology for the determination of four widely used sulfonamides and their corresponding N^4-acetyl metabolites. The proposed extraction procedure has a very low (few microlitres) organic solvent consumption. The excellent preconcentration and clean-up obtained implies a great advantage over other sample treatment procedures including a previously described HF-LPME method that uses a mixture ionic liquid/TOPO as supported liquid membrane, with high solvent cost; our procedure shows an

improvement in the detection and quantitation limits and it has been demonstrated their applicability to the analysis of the main sulfonamide metabolites.

The proposed procedure has been demonstrated adequate for the determination of the analytes in environmental samples including urban wastewaters that usually require tedious clean-up and preconcentration steps.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.12.006.

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Sensitive HPLC determination of sulfonamides and their main metabolites in urine samples using hollow fiber-based liquid phase microextraction (HF-LPME)

En este trabajo se propone y optimiza, la extracción y posterior determinación de ocho compuestos de la familia de las sulfonamidas en muestras de orina. La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior se realiza usando detectores de diodo array y fluorescencia conectados en serie.

La membrana líquida de 1-octanol fue soportada sobre fibras de polipropileno Accurel Q3/2. Se empleó como fase donadora una solución acuosa de Na_2SO_4 de pH4 (ajustada con HCl) y una solución acuosa de NaOH de pH 12 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de seis horas con una agitación de 300 rpm.

El procedimiento propuesto permite unos bajos límites de cuantificación para los analitos estudiados 3-50 ng/L y entre 0.9-100 ng/L para DAD y FLD, respectivamente, y una reproducibilidad de entre 1 y 1.8 %.

El procedimiento ha sido satisfactoriamente aplicado a la determinación de los analitos y sus correspondientes metabolitos en orina humana, obteniéndose recuperaciones del 100%, y excelentes líneas bases.

Sensitive HPLC determination of sulfonamides and their main metabolites in urine samples using hollow fiber-based liquid phase microextraction (HF-LPME)

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ABSTRACT: In this paper, an Accurel® Q3/2 PP polypropylene hollow fiber supporting 1-octanol was used between a 2M Na_2SO_4 aqueous solution (pH 4) as donor phase and aqueous solution (pH 12) as acceptor phase for a three phase-hollow fiber-based liquid phase microextraction (HF-LPME) configuration. This system leads to the direct extraction from human urine samples of four widely used sulfonamides: sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and their main metabolites, the corresponding N^4-acetyl derivatives: N^4-acetyl-sulfadiazine, N^4-acetyl-sulfamerazine, N^4-acetyl-sulfamethazine, N^4-acetyl-sulfamethoxazole. The determination was carried out with a HPLC procedure using diode array (DAD) and fluorescence detection (FLD). The procedure allows very low detection and quantitation limits of 0.3-33 ng L^{-1} and 0.9-100 ng L^{-1}, respectively.

Keywords: Hollow fiber liquid phase microextraction; HF-LPME; sulfonamides; metabolites; urine; HPLC

INTRODUCTION

Sulfonamides play an important role as effective chemoterapeutics of bacterial and protozoal diseases and also exhibit growth-promoting properties in veterinary medicine (Dixon and Katz, 1991; Jones and Davidson, 2000). For several authors, sulfonamides have been implicated in the growing prevalence of antibiotic resistance in humans (Ferber, 2000; Wegener, 1999; Fey et al., 2000).

Several methods have been reported for the analysis of sulfonamides in urine samples using many kinds of analytical tools that include photometric (Funk, 1967) and fluorimetric (Cardoso et al., 2004; Diez et al. 2007) methods, the Bratton–Marshall method (Koupparis and Anagnostopoulou, 1988; Salinas et al., 1993), thin layer chromatography (Kilinc et al., 2009), FIA with different detection systems (Median et al., 2002; Flores et al., 2007; Lattanzio et al., 2008; Zhang et al., 2008), ELISA (Hong, 1999), [1]micellar liquid chromatography (Simó et al., 1995; Yang and Khaledi, 1995), high performance liquid chromatography with fluorescence detector (Morita et al., 2004; Zotou and Vasiliadou, 2009), high performance liquid chromatography–mass spectrometry (Heinig and Henion, 1999; Msagati and Nindi, 2004), capillary electrophoresis (CE) (Johansson et al., 1991; Veraart et al., 1998 & 1999), radioimmunoassay methods (Lin et al., 2006), electrochemical methods (Ali, 1994; Sabry, 2007) or indirect atomic absorption spectrometry (Montero et al., 1988). Generally, for the application of these methods a high number of extraction, concentration and purification steps are necessary that usually use solid phase extraction (SPE) or more laborious approaches.

Liquid phase microextraction (LPME), also known as supported liquid membranes extraction (SLM), is an attractive alternative to the widely used solid phase extraction (SPE). Audunsson (1986) introduced an alternative concept for LPME that was developed by Thordarson et al. (1996), and Pedersen-Bjergaard and Rasmussen (1999), based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene (HF-LPME) that allows in most cases, not only a efficient clean-up procedure, but it can also produces high degree of pre-concentration. Additionally, the low organic solvent consumption makes HF-LPME an interesting and environmental friendly analytical procedure. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilised within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber; when acceptor phase is an aqueous phase the procedure is known as three-phase HF-LPME. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor

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solution from entering the accepting phase and, at the same time, most macromolecules do not enter the hollow fiber because they are not soluble in the organic phase present in the pores in the extraction conditions, thus yielding very clean extracts (Ramos et al. 2009). Several reviews on hollow fiber-based LPME have been reported (Psillakis and Kalogerakis, 2003; Rasmussen and Pedersen-Bjergaard, 2004, 2005 & 2008).

In this work, a HPLC DAD-FLD method combined with preceding HF-LPME previously developed by the authors (Ramos et al., 2011) was applied for the highly sensitive determination of four sulfonamides in spiked and from human patients urine samples: sulfadiazine (SDI), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMX) and their main metabolites N^4-acetyl-sulfadiazine (NSDI), N^4-acetyl-sulfamerazine (NSMR), N^4-acetyl-sulfamethazine (NSMZ) and N^4-acetyl-sulfamethoxazole (NSMX).

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water form a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). SDI, SMR, SMZ, SMX, dihexyl ether and 1-octanol were purchased from Fluka-Sigma-Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany). N^4-acetyl sulfonamides metabolites (NSDI, NSMR, NSMZ and NSMX) were synthesised by the authors (Ramos et al., 2011) modifying the proposed by Pfeifer et al. (2002).

Methanolic working solutions of SDI, SMR, SMZ, SMX, NSDI, NSMR, NSMZ and NSMX were daily prepared by adequate dilutions from methanolic 100 $mg \cdot L^{-1}$ stock solutions. Q3/2 Accurel KM polypropylene hollow fiber (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

Chromatographic conditions

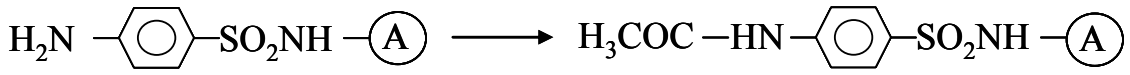
The chromatographic separation was performed at 10º C using a LaChrom® VWR-Hitachi (Barcelona, Spain) liquid chromatograph with a quaternary L-2130 pump. The injector was a Rheodyne manual injection valve Model 7725i, fitted with a 20-µL sample loop. Separations were carried out using a Eclipse® XDB-C18 3.5 µm (150 x 3.0 mm i.d.) (Agilent., Palo Alto, CA (USA)) column preceded by a guard column Kromasil® 100Å, C18, 5µm, (15 x 4.6mm i.d.) (Scharlab S.L., Barcelona, Spain).

The mobile phase consisted of 0.1 % formic acid (pH 2.6) (component A) and acetonitrile (component B) at a flow rate of 0.4 $mL \cdot min^{-1}$. An initial 90% component A was used in isocratic mode for 10 minutes and then a linear elution gradient was programmed from 90% to 85 % A for another 5 minutes; finally an elution gradient was programmed from 85% to 60% A for another 15 minutes. Three minutes were waited between injections which allowed re-equilibration of the column to the initial conditions.

Table 1 shows the monitoring wavelengths for DAD and FLD detections and the retention times for the analysed compounds.

Synthesis of the metabolites

The main sulfonamides metabolites are their corresponding N^4-acetyl derivatives (Grondel et al., 1986;Vree et al., 1990) and they were synthesised in our laboratory (Ramos el al., 2011). The synthesis reaction can be schematised by



where (A) is the corresponding heterocyclic rest for each sulfonamide.

Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked with 1-octanol during 10 seconds to impregnate the pores, and rinsed with water on the outside by placing it into the ultrasonic bath for 30 seconds in order to remove the excess of organic solvent. The lumen of the prepared fiber piece was filled with 50 μL of acceptor phase (pH 12 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and a plastic film (Parafilm®, Pechiney Plastic Packaging Company, Chicago, IL, USA). During extraction the membrane portion that contains the acceptor phase was immersed in the 50 mL sample solution (pH 4, Na_2SO_4 2M) contained into a 50 mL glass beaker. The sample was stirred for 6 hours by means of a magnetic stirrer (ANS-00/1 Science Basic Solutions (Rubí, Barcelona, SPAIN) at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extracted using a HPLC syringe and injected into the HPLC system.

Preparation of urine samples

Urine samples from healthy volunteers were directly extracted by the HF-LPME procedure after their 1:500 dilution in aqueous 2 M Na_2SO_4 solution and HCl addition to obtain pH 4. If necessary, urine samples were stored in the dark at 4ºC no more than few hours prior to HF-LPME extraction.

RESULTS AND DISCUSSION

Chromatographic conditions

Looking for a fast and high resolution separation an Eclipse® XDV-C18 3.5 μm was selected as working column. The mobile phase consisted of 0.1 % formic acid and acetonitrile. Different gradient elution conditions were tested searching for the shortest time of analysis without sacrificing peak shape. The gradient elution program described

in the previous section was the best option in terms of time of analysis, shape of the peaks and reproducibility.

N^4-acetyl metabolites are not stable at high pH values like the used for the acceptor phase and they are transformed to the corresponding N^4-hydroxy derivatives; we have checked that a complete transformation carry out in 4 hours at pH 12, so the measured chromatographic peaks for the N^4- metabolites correspond to their N^4-hydroxy derivatives, and the chromatographic conditions were accordingly optimised for these compounds. It is remarkable that N^4-hydroxy derivatives only can be generated from the corresponding N^4-acetyl metabolites.

Linearity, sensitivity, precision and robustness for the HF-LPME extraction

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve, was constructed using a least-square linear regression analysis of standards mixtures of the analytes at different concentrations. Using the optimised selected HF-LPME conditions (Ramos et al., 2011), aqueous pH 4 solutions with different analytes concentrations were submitted to the liquid microextraction procedure and analysed according to the described HPLC procedure. Peak areas were proportional to concentrations in the donor phase. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ and the calibration curves obtained showed no changes over the course of one month. Detection and quantitation limits were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio is 3 and 10, respectively. Enrichment factor was calculated as the increase in the concentration between the aqueous external donor phase and the internal (acceptor) phase. Table 2 shows the corresponding data and, as can be seen, the high enrichment values obtained for all the analytes allow the detection and determination of low concentration levels.

To evaluate the repeatability and the intermediate precision, aqueous samples at three concentrations levels 10^2 (25 for SMX and NSMX), 10^3 and 10^5 ng L^{-1} (in triplicate) were subjected to the entire analytical procedure and measured. The repeatability (one single day), expressed as relative standard deviation, was in the range

0.8 % - 1.2%. Intermediate precision (one day per week during two months) also expressed as relative standard deviation, was in the range 1.0 %- 1.8%. (Ramos et al., 2011)

The results obtained (Ramos et al., 2011) from a robustness study indicated that the procedure can be considered robust against the experimental factors for all the analysed compounds.

Urine analysis

Recovery assays on spiked urine

A urine pool from three healthy volunteers was spiked with the studied sulfonamides and their corresponding N^4-acetyl metabolites at three concentration levels (0.5, 10 and 50 μg mL^{-1}) and submitted to the HF-LPME procedure described in the experimental section. Representative DAD and FLD chromatograms can be observed in Figure 1. Figure 2 shows the corresponding blank chromatograms. As it can be seen, the peaks have good resolution and excellent baselines were obtained. Table 3 shows the corresponding recoveries obtained and, as it can be seen, the values obtained were within the range 99-101 %, which demonstrates that the proposed HF-LPME procedure is adequate for urine samples analysis.

Analysis of samples from patients

Urine samples from two patients under treatment, who informed consent was obtained, after the ingestion of a dose of Septrin® tablets from UCB Pharma (sulfamethoxazole 400 mg; Trimetropin 80 mg; potato starch, sodium glycolate 9.5 mg) and Sulfadiazina tablets from Reig Jofre Laboratory (sulfadiazine 500 mg; manitol) were collected at intervals of time and analysed according to the chromatographic method after applying the HF-LPME procedure. Figures 3 and 4 show the typical chromatograms obtained; as it can be seen the sulfonamides and their corresponding metabolite peaks show good resolution and excellent baselines. Trimetropin (2,4-Pyrimidinediamine, 5-[(3,4,5-trimethoxyphenyl)methyl]) usually is co administered with sulfamethoxazole and it does not interfere due to it is not extracted in the HF-LPME conditions applied.

Figure 5 shows the compounds concentration versus time in the urine samples. It is remarkable that the high preconcentration and clean-up of HF-LPME procedure allows the determination of very low levels of the analysed compounds

CONCLUSIONS

This study presents a hollow fiber-based liquid phase microextraction (HF-LPME) method combined with an HPLC (DAD-FLD) determination using a small size particle chromatographic column that allows a simple, low-cost, accurate, highly sensitive and selective methodology for the determination of four widely used sulfonamides and their corresponding N^4-acetyl metabolites. The proposed extraction procedure has a very low (few μL) organic solvent consumption in contrast to other clean-up / preconcentration alternatives like SPE which is according to the current trends to a "Green Chemistry". The procedure has been demonstrated adequate for the analytes determination at the usual levels in human urine samples.

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Table 1. Monitoring wavelengths and retention times

	DAD			FLD			
	λ max (nm)	**t_R (min)**	**S.D. (min)**	**λexc(nm)**	**λem (nm)**	**t_R (min)**	**S.D. (min)**
SDI	270	8.69	0.008	280	450	9.21	0.003
SMR	270	12.45	0.006	275	445	13.18	0.012
NSDI	262	15.52	0.01	270	356	15.98	0.009
SMZ	270	16.99	0.005	275	445	17.51	0.006
NSMR	262	18.96	0.011	275	351	19.38	0.008
NSMZ	262	21.84	0.007	275	351	22.21	0.011
SMX	270	28.91	0.013	275	382	29.30	0.002
NSMX	262	29.94	0.014	280	351	30.41	0.004

Table 2. Detection limits, linear ranges, % linearity and enrichment factors for the HPLC method (DAD and FLD detection) combined with prior HF-LPME.

	DAD			**FLD**			**Enrichment**
	LOD*	**Linear range ***	**% linearity**	**LOD***	**Linear range ***	**% linearity**	
SDI	15	$50\text{-}10^6$	99.81	15	$50\text{-}10^5$	99.91	200
SMR	5	$15\text{-}10^6$	99.67	15	$50\text{-}10^5$	99.63	1000
NSDI	15	$50\text{-}10^6$	99.59	8	$25\text{-}10^5$	99.96	400
SMZ	15	$50\text{-}10^6$	99.86	15	$50\text{-}10^5$	99.83	250
NSMR	15	$50\text{-}10^6$	99.69	8	$25\text{-}10^5$	99.97	500
NSMZ	15	$50\text{-}10^6$	99.92	33	$100\text{-}10^5$	99.88	175
SMX	1	$3\text{-}10^6$	99.77	0.3	$0.9\text{-}10^5$	99.95	1000
NSMX	3.5	$10\text{-}10^6$	99.84	8	$25\text{-}10^5$	99.90	600

* (ng L^{-1})

Table 3. Recoveries (%) using HF-LPME/HPLC from spiked human urine samples. (Average of three determinations ± standard deviation).

	Urine spiked level (mg L^{-1})		
	0.5	**10**	**50**
SDI	99.5 ± 1.2	100.3 ± 1.3	101.1 ± 1.8
SMR	100.4 ± 1.6	100.2 ± 1.4	99.8 ± 1.3
NSDI	99.7 ± 1.5	100.5 ± 1.5	99.7 ± 1.5
SMZ	99.7 ± 1.6	100.3 ± 1.2	99.6 ± 1.5
NSMR	100.2 ± 1.9	99.8 ± 2.1	99.7 ± 1.8
NSMZ	99.6 ± 1.1	100.1 ± 1.7	99.9 ± 1.3
SMX	100.3 ± 1.8	100.2 ± 1.8	99.8 ± 1.3
NSMX	99.9 ± 1.8	100.0 ± 1.7	100.2 ± 1.6

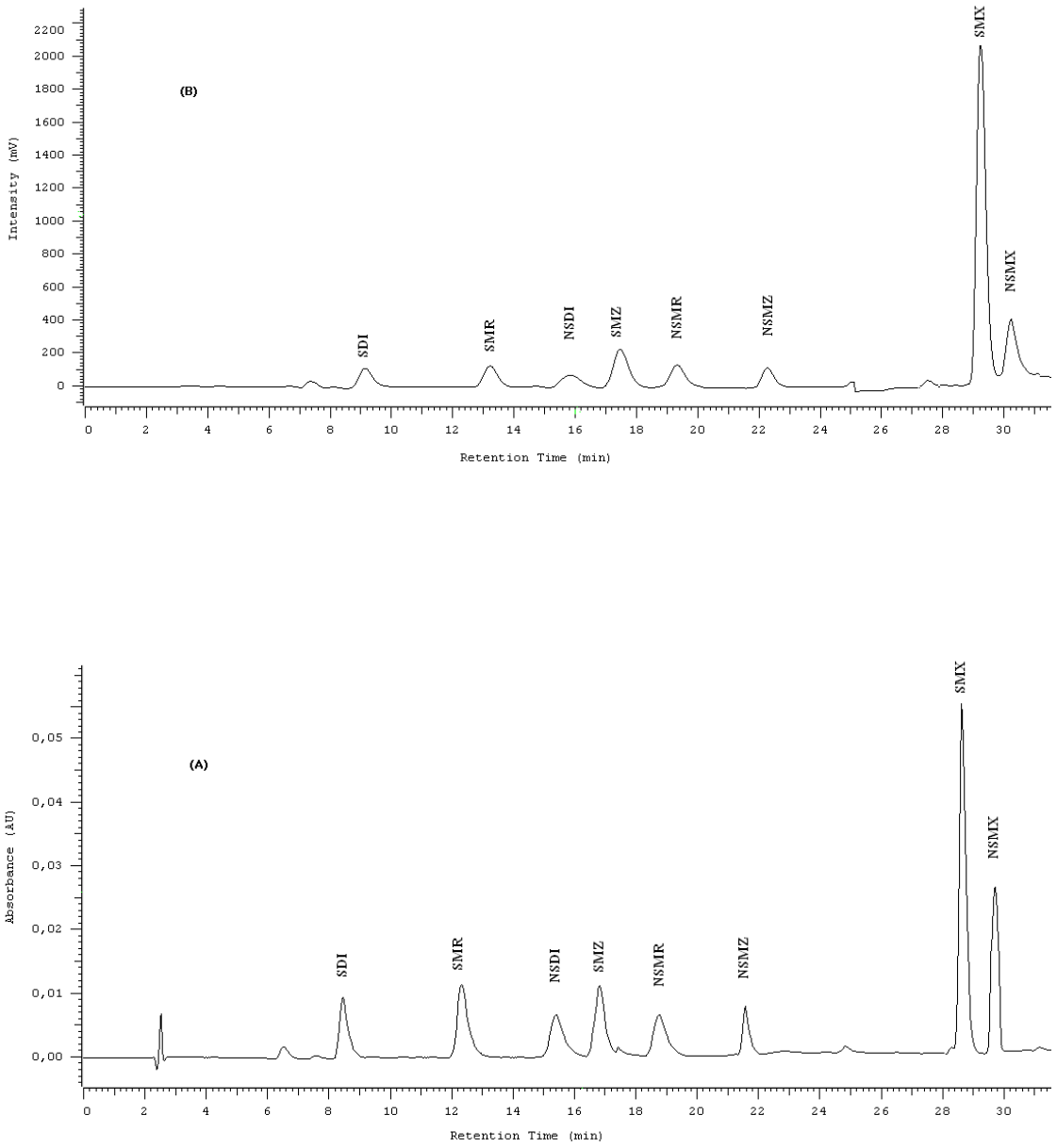


Figure 1. DAD (a) and FLD (b) chromatograms from spiked (10 and 5 ng L^{-1}, respectively) urine samples.

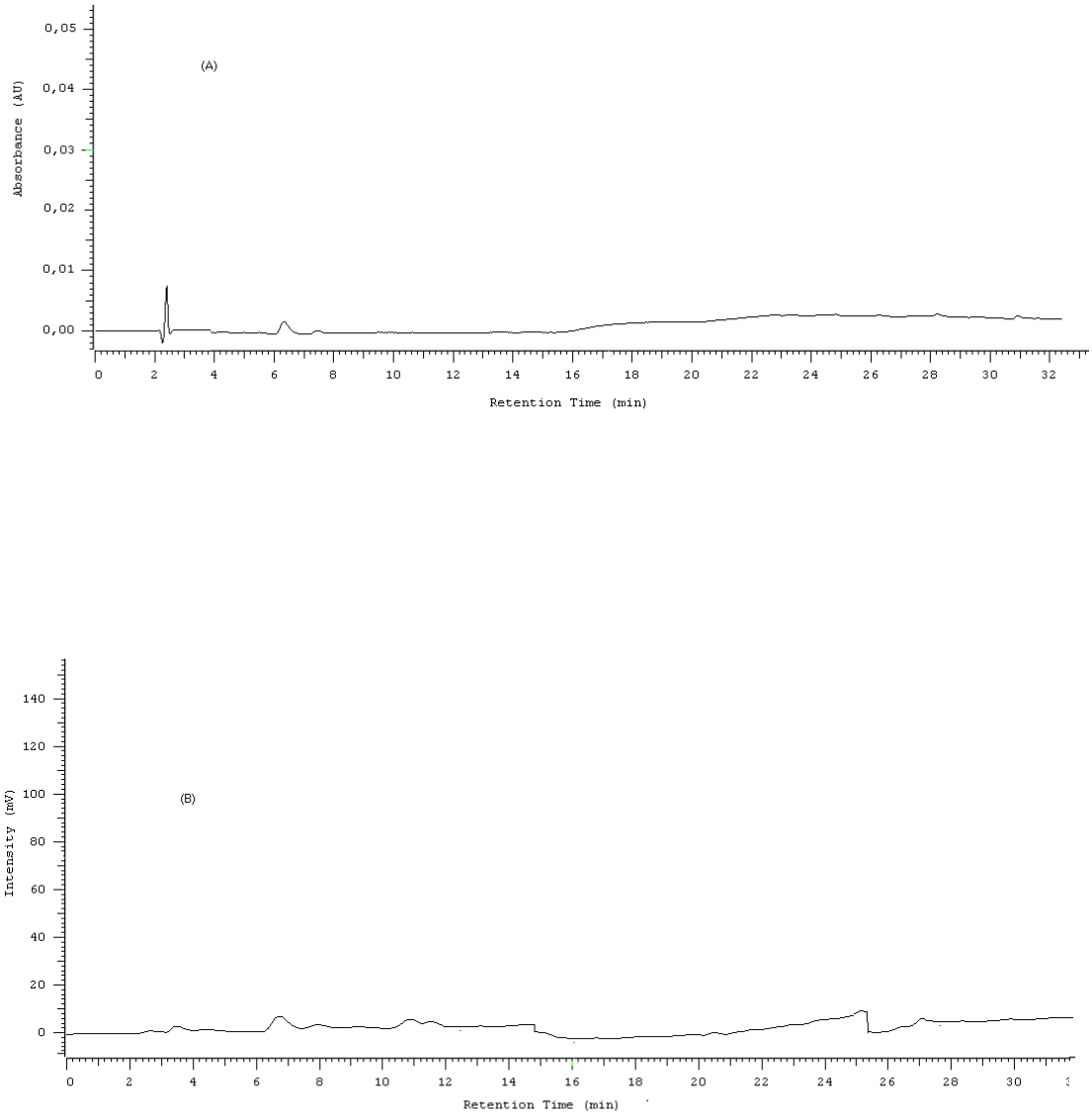


Figure 2. DAD (a) and FLD (b) chromatograms of blank urine sample.

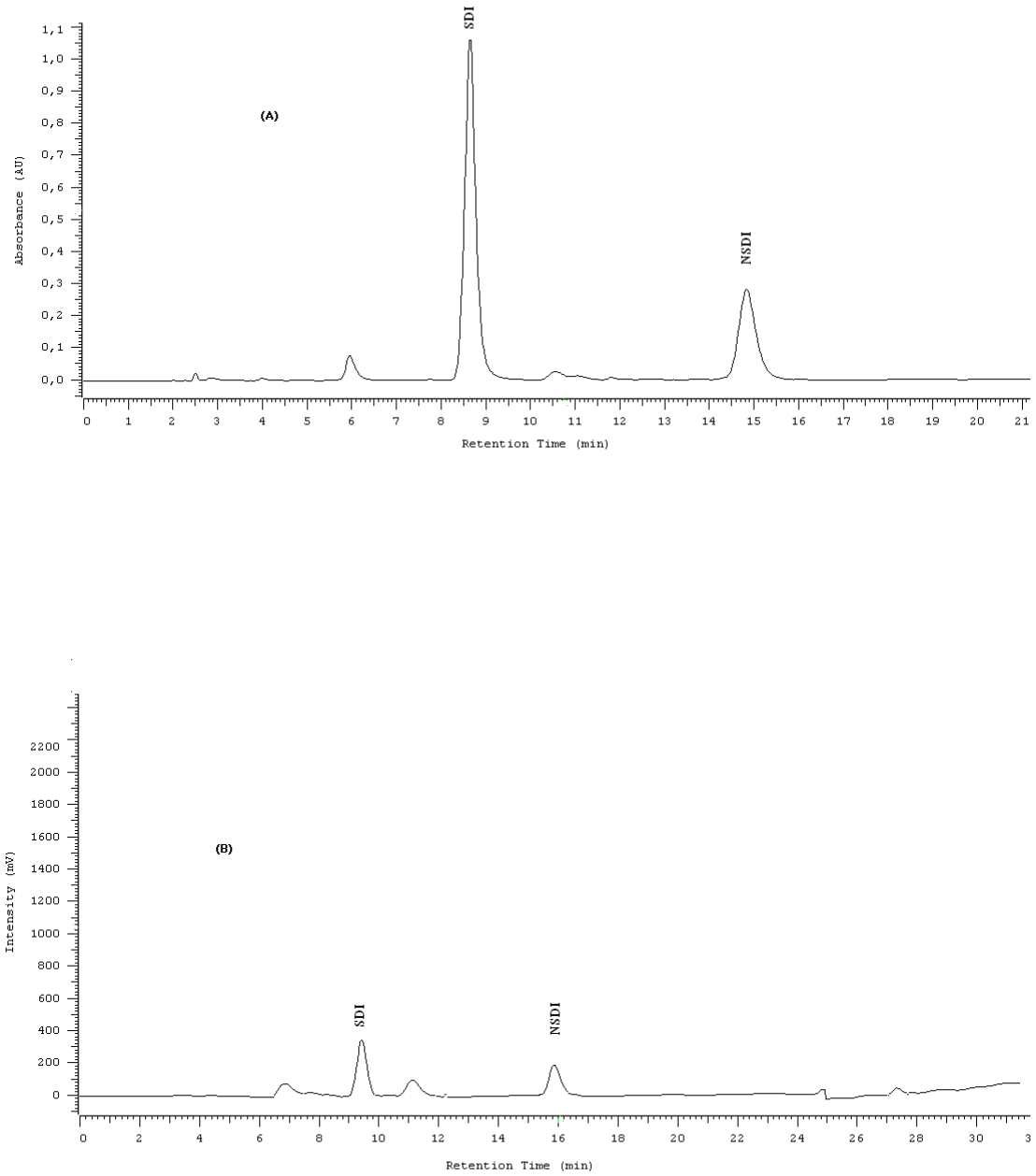


Figure 3. Representative DAD (a) and FLD (b) chromatograms from a urine sample after the ingestion of sulfadiazine (for details see text).

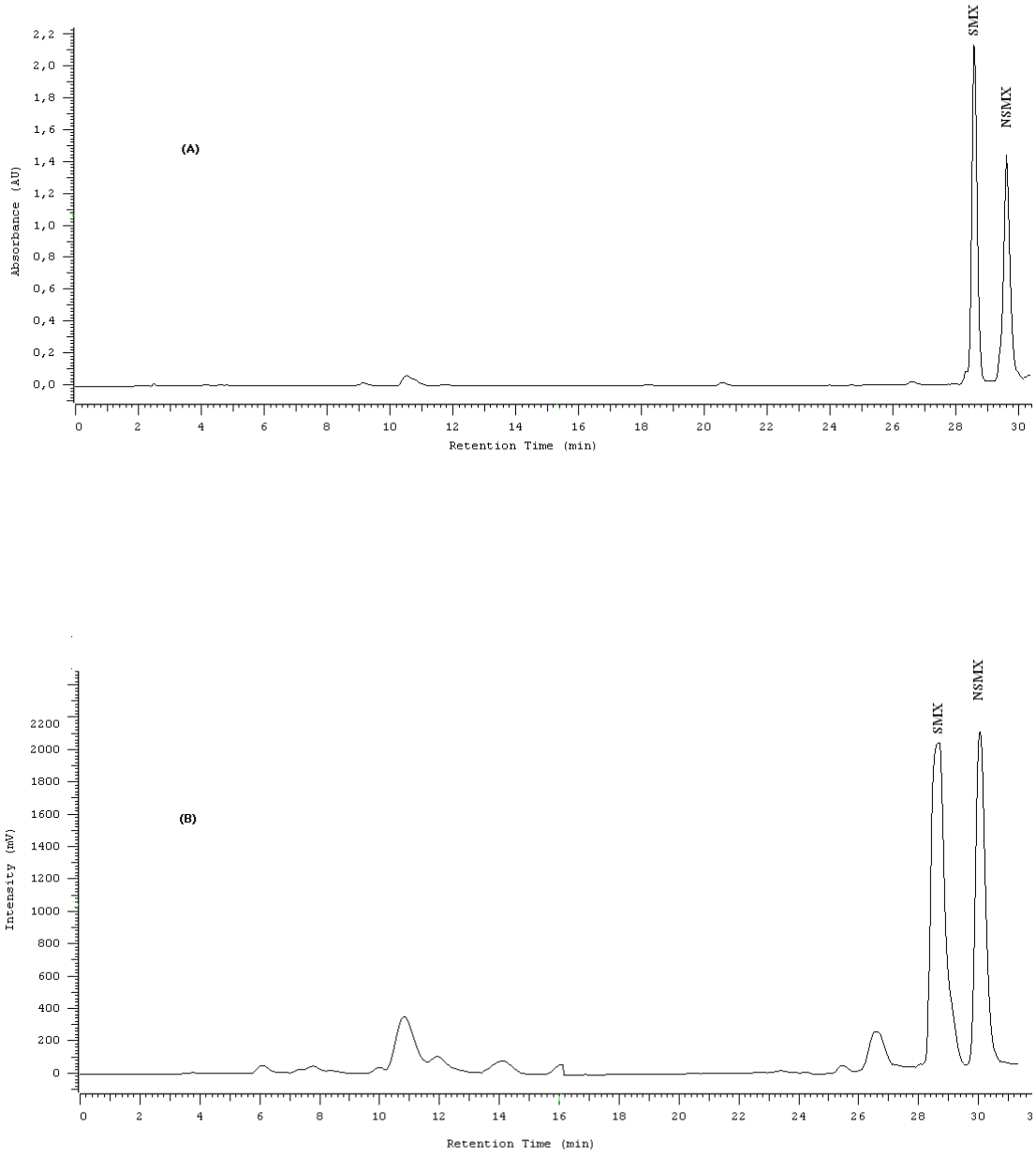
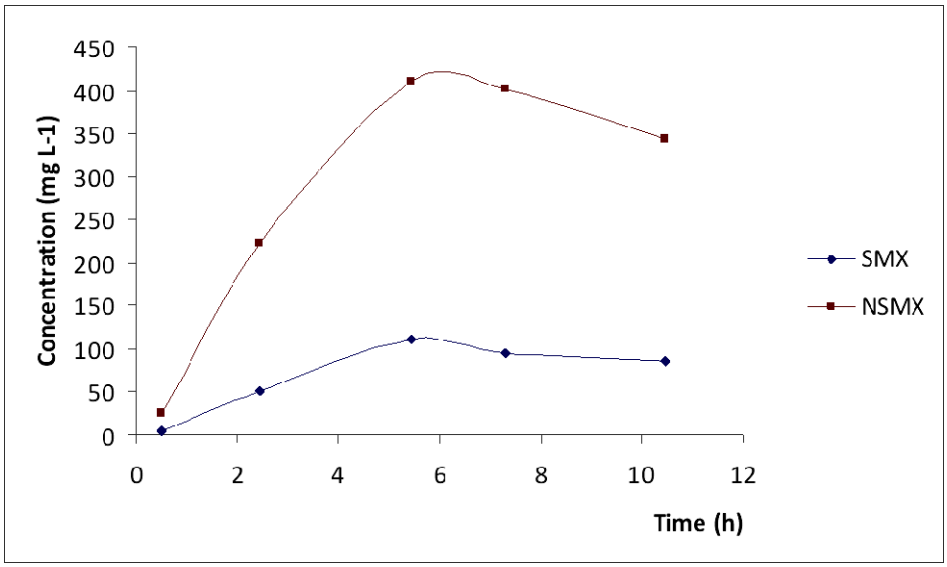


Figure 4. Representative DAD (a) and FLD (b) chromatograms from a urine sample after the ingestion of sulfamethoxazole (for details see text).



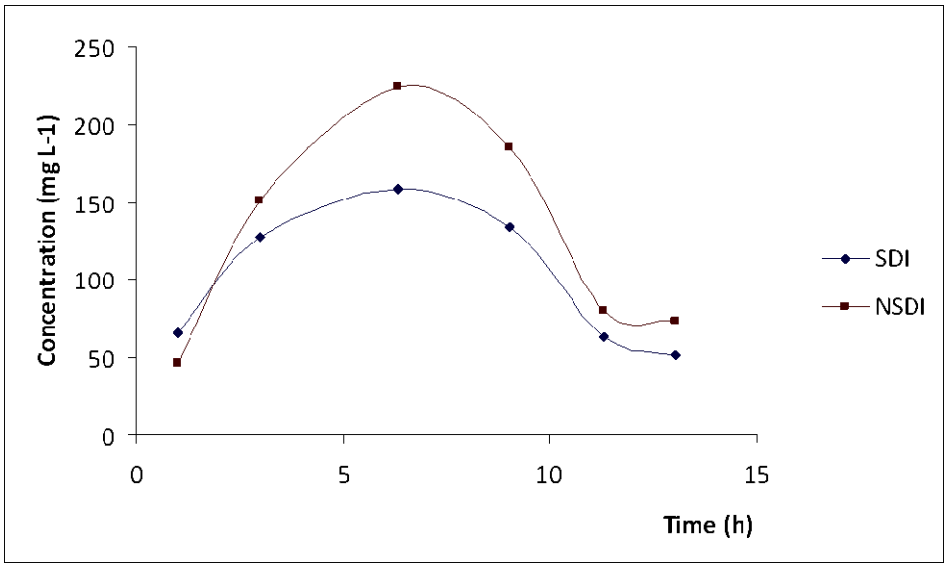


Figure 5. Compounds concentration versus time in the real urine samples after the ingestion of sulfadiazine (500 mg) and sulfamethoxazole (400 mg).

FLUOROQUINOLONAS

Hollow fiber-based liquid phase microextraction (HF-LPME) as a new approach for the HPLC determination of fluoroquinolones in biological and environmental matrices

En este trabajo se propone y optimiza, la extracción y posterior determinación de ocho compuestos de la familia de las fluoroquinolonas. La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior se realiza usando detectores de diodo array y fluorescencia conectados en serie.

Se han determinado ocho fluoroquinolonas ampliamente usadas, como son: marbofloxacina, norfloxacina, ciprofloxacina, danofloxacina, enrofloxacina, gatifloxacina, grepafloxacina, flumequina, y el metabolito de la enrofloxacina: ciprofloxacina.

La separación cromatográfica se realizó empleando una columna LiChroCART® 75-4 Purosphere® STAR RP-18e 3 μm (75 x 4.0 mm i.d.) y un gradiente de ácido fórmico 0,1%/acetonitrilo a un flujo de 0,8 ml/min. En estas condiciones el cromatograma completo se desarrolla en 20 minutos.

La membrana líquida de 1-octanol fue soportada sobre una fibra de polipropileno Accurel Q3/2. Se empleó como fase donadora una solución acuosa de 2M Na_2SO_4 de pH 7 y una solución acuosa de NaOH de pH 12 como fase aceptora. Se obtuvo un tiempo óptimo de extracción de cinco horas y media con una agitación de 300 rpm. Las variables experimentales que afectan significativamente a la extracción fueron optimizadas mediante un diseño experimental central compuesto para tres factores a tres niveles.

El procedimiento propuesto permite límites de cuantificación para los analitos estudiados entre 1-50 ng/L, y una reproducibilidad de entre el 1 y el 1.8 %.

El procedimiento ha sido aplicado satisfactoriamente a la determinación de los analitos en aguas medioambientales, con recuperaciones, superiores al 90%, obteniéndose excelentes líneas bases. Además se ha aplicado a muestras de orina bovina, obteniéndose extractos limpios, con recuperaciones en torno al 100% excepto para la flumequina, con recuperaciones del 75%; y buenas líneas bases. Se han determinado enrofloxacina y su metabolito, ciprofloxacina, en muestras de orina procedentes de animales a los que se les suministró el fármaco (ALSIR® 5%).

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Hollow fiber-based liquid phase microextraction (HF-LPME) as a new approach for the HPLC determination of fluoroquinolones in biological and environmental matrices

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ABSTRACT

In this paper, a three phase hollow fiber-based liquid phase microextraction (HF-LPME) combined with a HPLC procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of eight widely used fluoroquinolones: marbofloxacin (MRB), norfloxacin (NRF), ciprofloxacin (CPR), danofloxacin (DNF), enrofloxacin (ENR), gatifloxacin (GTF), grepafloxacin (GRP) and flumequine (FLM). A Q3/2 Accurel PP polypropylene hollow fiber supporting 1-octanol was used between a 2 M Na_2SO_4 aqueous solution (pH 7) as donor phase and aqueous solution (pH 12) as acceptor phase. The microextraction parameters were optimised from an experimental central composite design. The procedure allows very low detection and quantitation limits of 0.3–16 ng L^{-1} and 1–50 ng L^{-1}, respectively. The proposed method was applied to the determination of the analytes in bovine urine and in environmental water samples (surface, tap and wastewater).

1. Introduction

Fluoroquinolones (FQs) are antibacterial agents widely used due to their broad spectrum activity against both gram-positive and gram-negative bacteria through inhibition of their DNA gyrase. They have good oral absorption and are applied as both human and veterinary medicine; at sub-therapeutic levels FQs, like other antibiotics, promote animal's growth. The FQs administered to humans or animals are almost excreted as unchanged compounds in urine, and are mainly effluent from the wastewater treatment plants (WWTPs) [1]. FQs are rather resistant to microbial degradation [2–4], and these compounds might be persisting within environmental waters because of their strong sorption properties. It is well-known that bacteria exposed to antibiotics may acquire resistances [5] and surface waters can be an adequate propagation vector for resistant diseases. For these and other reasons, it is necessary to develop simple and sensitive methods for enabling the determination of these antibiotics at naturally occurring levels and on matrices of variable complexity.

The use of clean-up procedures is an old analytical tool that, in the last years, has suffered very important developments in order to resolve the analytical problems derived from the analysis of complex samples or the quantitation/detection at very low levels. Solid phase extraction (SPE) is the most used clean-up analytical procedure, however, in the last years there has been a highly interest in developing new clean-up procedures.

Liquid–liquid extraction (LLE) is a classical and common technique used for preconcentration and clean-up prior to chromatographic or electrophoretic analysis that requires large organic solvent consumption. It is also tedious and analyte-loss is frequent due to multi-stage operations that cannot be neglected. Liquid-phase microextraction (LPME), based on a droplet of water-immiscible organic solvent hanging at the end of a microsyringe needle (single drop microextraction, SDME) [6,7], is a simple, inexpensive, fast, effective and virtually solvent-free sample pretreatment technique. However, SDME is not very robust, and the droplets may be lost from the needle tip of the microsyringe during extraction.

Audunsson [8] introduced an alternative concept for LPME that was developed by Thordarson et al. [9] and for Pedersen-Bjergaard and Rasmussen [10] based on the use of single, low-cost, disposable, porous, hollow fibers made of polypropylene. In this concept, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber; when acceptor phase is an aqueous phase the procedure is known as three-phase HF-LPME. The disposable nature of the hollow fiber totally eliminates

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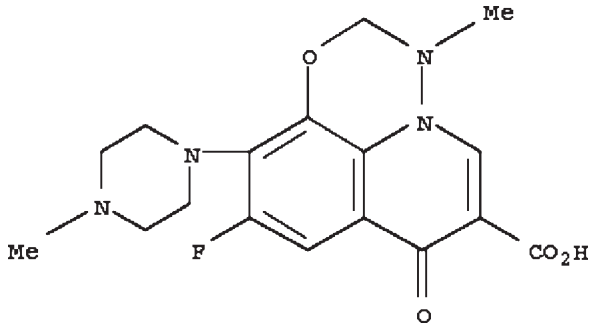
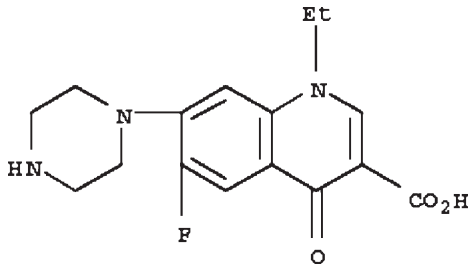
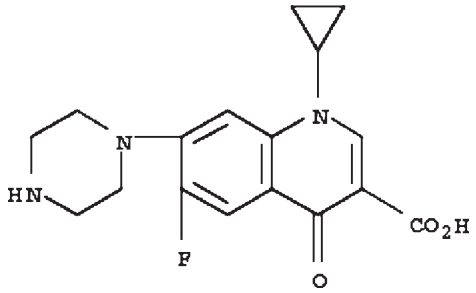
Table 1
Structure and IUPAC name of the examined fluoroquinolones.



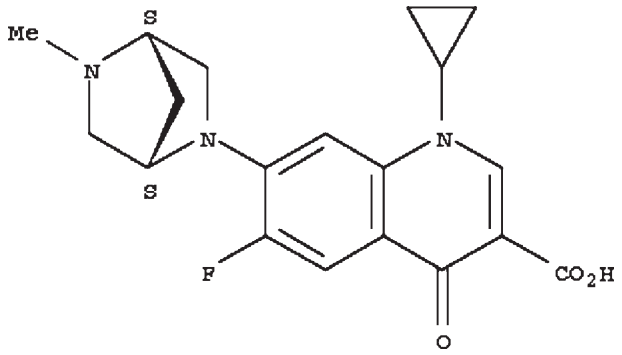
Marbofloxacin
9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[3,2,1-ij][4,1,2] benzoxadiazine-6-carboxylic acid



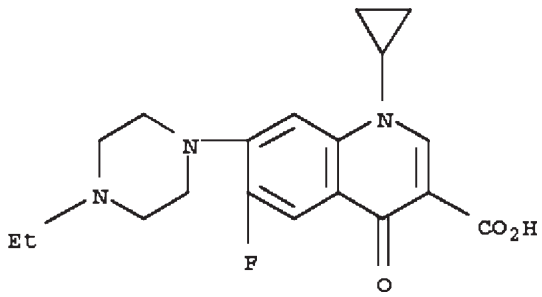
Norfloxacin
1-Ethyl-6-fluoro-1,4-dihydro-7-(1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid



Ciprofloxacin
1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid

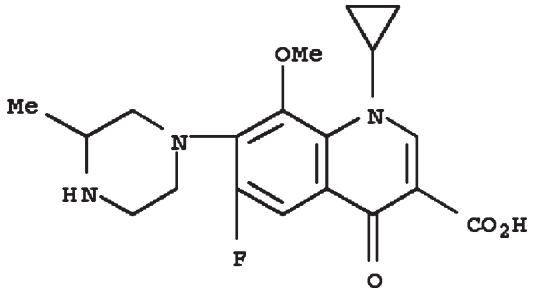


Danofloxacin
1-Cyclopropyl-6-fluoro-1,4-dihydro-7-[(1S,4S)-5-methyl-2,5-diazabicyclo[2.2.1]hept-2-yl]-4-oxo-3-quinolinecarboxylic acid

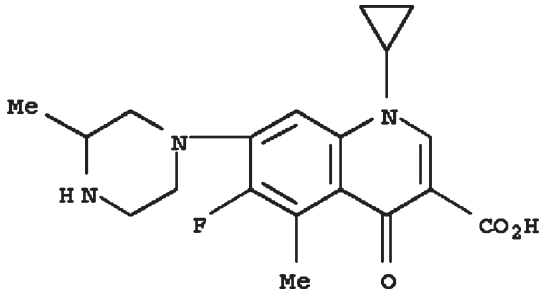


Enrofloxacin
1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid

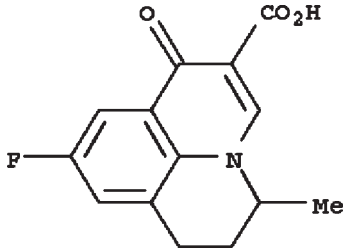
Table 1 (*Continued*)



Gatifloxacin
1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic
acid



Grepafloxacin
1-Cyclopropyl-6-fluoro-1,4-dihydro-5-methyl-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic
acid



Flumequine
9-Fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic
acid

the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents large molecules and particles present in the donor solution from entering the accepting phase and, at the same time, most macromolecules do not enter the hollow fiber because they are not soluble in the organic phase present in the pores, thus yielding very clean extracts [11]. Several reviews on hollow fiber-based LPME have been reported [12–15].

Analysis of FQs for the drug monitoring in body fluids samples and their determination in aqueous samples have been carried out mainly by high-performance liquid chromatography (HPLC) with UV [16–18] fluorescence [19–22], electrochemical detection [23], or HPLC coupled to mass spectrometers for detection [19,24–28]. The methods of analysis are coupled with diverse clean-up procedures like off-line SPE [22,25,28–32], on-line SPE [18,24], pressurized liquid extraction [33–35] or molecularly imprinted polymer extraction [26,27].

The aim of this work was the development of a sensitive and environmental friendly HF-LPME combined with HPLC diode array-fluorescence detection (DAD–FLD) for the determination of fluoroquinolones, that can be easily applicable to several matrices like urine or environmental waters (including wastewaters) at the naturally occurring levels. HF-LPME reduces the organic solvents consumption to several microlitres in contrast to another clean-up/preconcentration alternatives like SPE which is according to the current trends to a "Green Chemistry".

Recently a method that uses liquid phase microextraction for the determination of fluoroquinolones has been published by Poliwoda et al. [36]. Authors analysed four fluoroquinolone antibiotics: ciprofloxacin, enrofloxacin, norfloxacin and danofloxacin, in river water samples. The proposed method involves their HPLC determination with UV (270 nm) detection previous extraction using a Q3/2 Accurel PP polypropylene hollow fiber membrane supporting 20% (w/w) di-(2-ethylhexyl) phosphoric acid in di-*n*-hexyl ether as liquid membrane. Aqueous pH 6 and 0.1 M HCl were used as donor and acceptor phases, respectively. Recoveries in the 93–120% range were obtained from diluted (1:1) river water samples with detection limits between 10 and 25 ng L^{-1}.

In this work, a HPLC DAD–FLD method combined with prior HF-LPME was developed for the sensitive determination of eight widely used fluoroquinolones: marbofloxacin (MRB), norfloxacin (NRF), ciprofloxacin (CPR), danofloxacin (DNF), enrofloxacin (ENR), gatifloxacin (GTF), grepafloxacin (GRP) and flumequine (FLM) (Table 1 shows their structures and IUPAC names). The method has been successfully applied to their determination in spiked and urine samples from treated dairy cows (Jersey breed) and on several environmental water samples: wastewaters from the different treatments steps of a WWTP, and water samples from river, lake and water supply network. A previous similar article has been published with the emphasis to develop a method to determine sulfonamides in environmental waters [37].

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water from a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). MRB, NRF, CPR, DNF, ENR, GTF, GRP, FLM, dihexyl ether and 1-octanol were purchased from Fluka–Sigma–Aldrich (Madrid, Spain) and the rest of products were obtained from Merck (Darmstadt, Germany).

Methanolic working solutions of MRB, NRF, CPR, DNF, ENR, GTF, GRP and FLM were daily prepared by adequate dilutions

Table 2
Monitoring wavelengths and retention times.

	DAD			FLD			
	λ max (nm)	t_R (min)	S.D. (min)	λexc (nm)	λem (nm)	t_R (min)	S.D. (min)
MRB	300	3.57	0.006	300	515	4.02	0.009
NRF	274	4.56	0.012	278	445	5.15	0.011
CPR	280	5.15	0.009	280	456	5.79	0.014
DNF	280	6.69	0.014	280	456	7.52	0.016
ENR	280	7.55	0.011	280	456	8.15	0.012
GTF	287	10.61	0.012	292	484	11.03	0.014
GRP	280	13.25	0.013	330	441	13.80	0.012
FLM	315	16.21	0.008	315	368	16.79	0.007

Table 3
Efficiency and selectivity chromatographic parameters for the proposed HPLC procedure (for abbreviation see text).

	$W_{1/2}$ (min)	T	N	k	α	R_s
MRB	0.1365	1.47	3789	2.16	1.41	4.04
NRF	0.1527	1.37	4940	3.04	1.17	2.11
CPR	0.1767	1.46	4705	3.56	1.38	4.72
DNF	0.2083	1.44	5714	4.92	1.15	2.34
ENR	0.2250	1.36	6237	5.68	1.48	6.86
GTF	0.3015	1.47	6860	8.39	1.28	8.25
GRP	0.0760	1.36	168,389	10.73	1.24	21.95
FLM	0.0831	1.00	210,801	13.35	1.24	21.95
Critical values		<1.5	>2000	>2	>1	>1.5

from methanolic 100 (g mL^{-1} stock solutions. Q3/2 Accurel PP polypropylene hollow fiber (600 μm i.d., 200 (m wall thickness and 0.2 (m pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Chromatographic conditions

The chromatographic separation was performed at 10 °C using a LaChrom® VWR-Hitachi (Barcelona, Spain) with a quaternary L-2130 pump. The injector was a Rheodyne manual injection valve Model 7725i, fitted with a 20-μL sample loop. Separations were carried out using an LichroCART® 75-4 Purosphere® STAR RP-18e 3 μm (75 mm × 4.0 mm i.d.) (VWR, Darmstadt, Germany) preceded by a guard column Kromasil® 100 Å, C18, 5 μm (15 mm × 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain).

The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and acetonitrile (component B) at a flow rate of 0.8 mL min^{-1}. An initial composition 86–14% (A–B) was used in isocratic mode for 8 min and then a linear elution gradient was programmed from 86% to 20% A for another 12 min. Three minutes were waited between injections which allowed re-equilibration of the column to the initial conditions.

Table 2 shows the monitoring wavelengths for DAD and FLD detection, the retention times and the corresponding standard deviations for the analyzed compounds.

2.3. Supported liquid membrane preparation and extraction procedure

Hollow fibers were cut into 27 cm pieces, washed with acetone in an ultrasonic bath and dried. The fiber was soaked with 1-octanol during 10 s to impregnate the pores, and rinsed with water on the outside by placing it into the ultrasonic bath for 30 s in order to remove the excess of organic solvent. The lumen of the prepared fiber piece was filled with 50 μL of acceptor phase (pH 12 aqueous solution) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and a plastic film (Parafilm®, Pechiney Plastic Packaging Company, Chicago, IL, USA). During extraction the membrane portion that contains the acceptor phase was immersed in the 50 mL sample solution (pH 7, Na_2SO_4 2 M) contained into a 50 mL glass beaker. The sample was stirred for 5.5 h by means of a magnetic stirrer ANS-00/1 Science Basic Solutions (Rubí, Barcelona, SPAIN) at 300 rpm. After extraction, the fiber was taken out, one of the ends was cut and the acceptor phase was extracted using a HPLC syringe and injected into the HPLC system.

2.4. Preparation of urine cow samples

Spiked and real urine samples from Jersey cows were directly extracted by the HF-LPME procedure after their 1:50 dilutions (1:1000 to avoid saturation in the FLD detector) in aqueous 2 M Na_2SO_4 solution and NaOH addition just to obtain pH 7. If necessary, urine samples were stored in the dark at 4 °C no more than several hours prior to HF-LPME extraction.

2.5. Preparation of environmental water samples

Wastewater samples were obtained from "Guadalquivir"-ALJARAFESA Wastewater Treatment Plant which is located in Palomares del Río, Seville, Spain. The WWTP essentially receives urban wastewaters. The capacity of this WWTP is 100,000 inhabitants and the discharged flow is 12,433,313 m^3/year (2008 data). Grab samples of the influent (raw water, WWR), after the primary sedimentation tank (WW1), after the aeration tank (WW2) and the effluent (treated water after anaerobic digestion, WWT) were collected in 11th January 2010.

Two samples from Guadalquivir River were analysed. One (RIVER1) from Coria del Río, Seville, 2 km downstream the WWTP previously mentioned and other sample (RIVER2) was taken at the mouth of Guadalquivir River (Sanlúcar de Barrameda, Cádiz) where water has a high seawater proportion. Lake water sample (LAKE) comes from "Lagos del Serrano" (Guillena, Seville). Tap water sample (TAP) was obtained directly from the laboratory tap.

All samples, except tap water, were filtered through a GDU1 glass fibre filter bed (10–1 μm) (Whatman, Mainstone, UK) and through Pall Nylaflo™ nylon membrane filter 0.45 μm (Pall Corporation, Ann Arbor, MI, USA) and adjusted to pH 2 with HCl. Filtered samples were stored in the dark at 4 °C prior to HF-LPME extraction, no more than one week.

Water samples, were directly analysed after Na_2SO_4 addition for a 2 M final concentration; NaOH was added just to obtain pH 7 prior to be submitted to the HF-LPME procedure.

3. Results and discussion

3.1. Chromatographic conditions

Looking for a fast and high resolution separation a LiChroCART® 75-4 Purosphere® STAR RP-18e (3 μm) was selected as working column. This column is a highly packed HPLC column that allows to higher resolution separations using usual flow-rates. The selected column provides good resolution and good peak symmetry.

The mobile phase consisted of 0.1% formic acid and acetonitrile. Different gradient elution conditions were tested in order to save time of analysis without sacrificing peak shape. The gradient elution program described in Section 2.2 was the best option in terms of time of analysis, shape of the peaks and reproducibility.

Fig. 1 shows representative chromatograms from aqueous standards submitted to the HF-LPME procedure.

The efficiency and selectivity chromatographic parameters of the proposed procedure are shown in Table 3, N (number of theoretical plates), T (asymmetry factor), $W_{1/2}$ (peak half-width), k (retention factor), α (selectivity factor), R_s (peak resolution). As it can be seen, all parameters are according to their critical values.

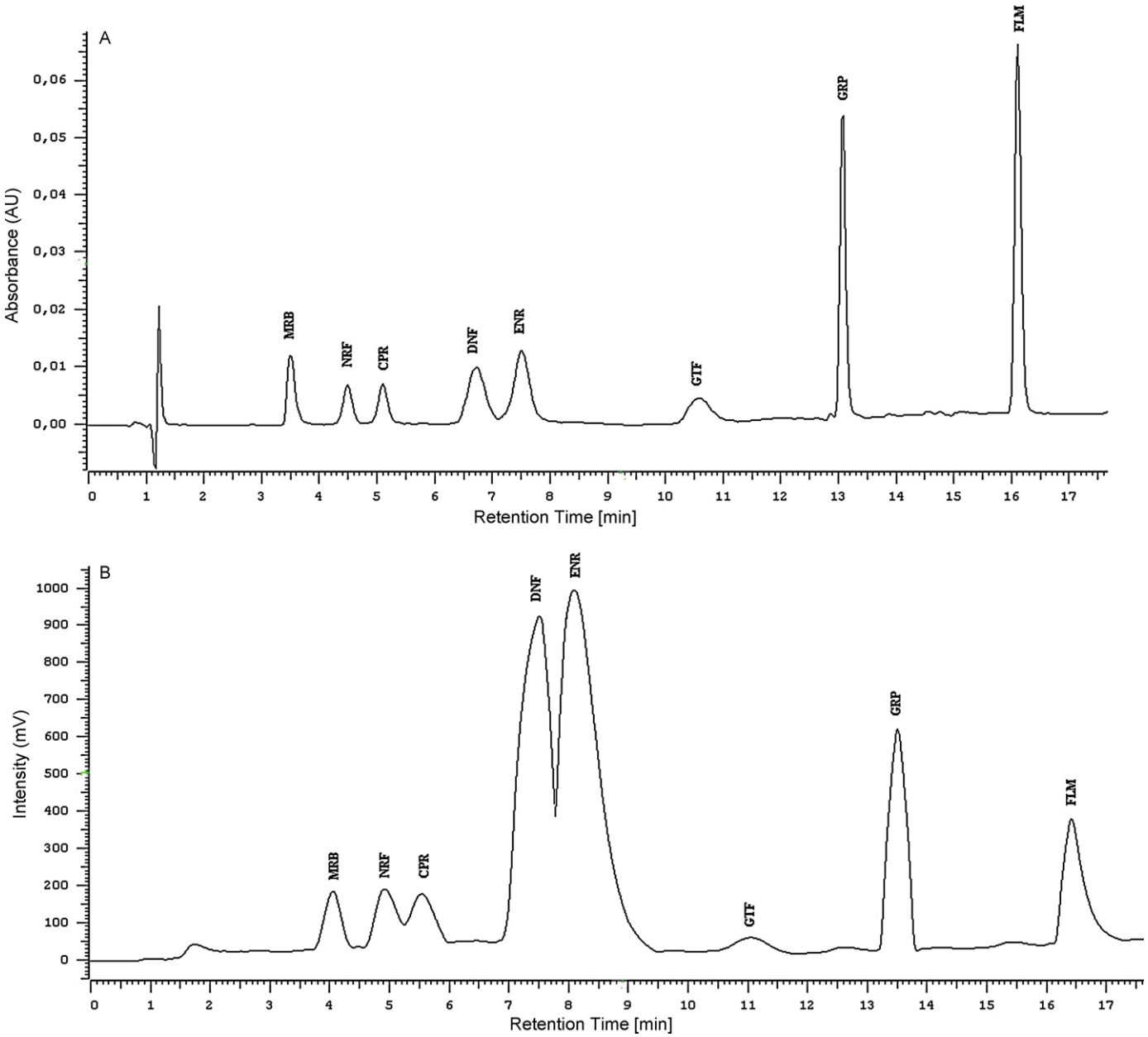


Fig. 1. DAD (a) and FLD (b) chromatograms from standard aqueous solutions (10 and 5 ng L^{-1}, respectively).

3.2. Optimization and evaluation of experimental conditions for HF-LPME extraction

First, several tests with donor phases pH 5–7 and acceptor phases pH 10–12 were carried out in order to choose the more adequate liquid supported on the polypropylene membrane; dihexyl ether and 1-octanol were checked and the best results were obtained with 1-octanol, so this was the liquid supported selected.

On the other hand, donor phases solutions containing NaCl (2–6 M) or Na_2SO_4 (0.5 M to saturation) were checked and, in general, salting out allows an increase in the extraction efficiency with the salt concentration that is more pronounced with Na_2SO_4 so aqueous saturated (approx. 2 M) Na_2SO_4 solutions were selected as optimum donor phases.

In order to optimise the experimental extraction parameters an experimental design was applied. The objective of the experimental designs is to get as much information as possible with the least number of experiences. To apply the experimental design, the fundamental objectives of the experimental planning are to identify controllable factors that significantly influence the outcome of the experiment, minimizing the effects of uncontrollable factors and secondly to optimize the objective function to get the best response.The influence of the experimental variables (called factors in experimental design), namely pH donor phase (X_1), pH acceptor phase (X_2) and time stirring (X_3) has been considered to find the best conditions for the eight compounds studied in this work. The optimization has been carried out by using a central composite design (CCD) for three factors at two levels. These designs account for the main factors and binary interactions that influence the signal, with a low number of assays.

The design matrix corresponds to three factors and twenty experiments. The design consists of three distinct sets of experimental runs: eight runs on the basis of levels +1 or −1, four runs

Table 4
Real and coded factors.

Factors	Levels				
	−1.68	−1	0	1	1.68
X_1 (pH)	5	5.8	7	8.2	9
X_2 (pH)	11	11.4	12	12.6	13
X_3 (time)	2 h	3 h	4.5 h	6 h	7 h

on the basis of levels +1.68 or −1.68 and six runs at the center of design (see supplementary electronic material).

Table 4 shows the coded levels of selected factors (−168, −1, 0, 1, 1.68), as well as their uncoded values.The computer program used on the experimental design was ECHIP ver. 6.4.1 (Velocity Pointe, Wilmington, DE, USA). The result is contour maps and response surfaces for each of the compounds, and the optimal combination for all of them in both cases. The figures of contour maps and surface response for each of the compounds, the contour map for the optimum combination for all the compounds and the three-factor response surface for all compounds are included as supplementary electronic material.

After a scrutiny of the optimal conditions, it was considered a slight modification of the optimal level of factor X_3 (stirring time) in order to favor the extraction for those less sensitive compounds (analytical optimal conditions). So, the optimal conditions used were those described in Section 2.3.

3.3. Linearity, sensitivity, precision and robustness for the HF-LPME extraction

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve, was constructed using a least-square linear regression analysis of standards mixtures of the analytes at different concentrations. Using the selected HF-LPME conditions, aqueous pH 7 solutions with different analytes concentrations were submitted to the liquid microextraction procedure and analysed according to the described HPLC procedure. Peak areas were proportional to concentrations in the donor phase. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ and the calibration curves obtained showed no changes over the course of one month. Detection and quantitation limits were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio is 3 and 10, respectively. Enrichment factor was calculated as the increase in the concentration between the aqueous external donor phase and the internal (acceptor) phase. Table 5 shows the corresponding data and, as can be seen, the high enrichment values obtained for all the analytes allow the detection and determination of very lows levels. In fact, detection and quantitation limits were of the same, even better, magnitude order that other methods that use mass spectrometry detection [19,21,22,24,26,35].

Table 5
Detection limits, linear ranges, % linearity and enrichment factors for the HPLC method (DAD and FLD detection) combined with prior HF-LPME.

	DAD			FLD			Enrichment
	LOD[a]	Linear range[a]	R^2[b]	LOD[a]	Linear range[a]	R^2[b]	
MRB	16	50–10^6	0.9997	16	50–10^5	0.9996	95
NRF	20	60–10^6	0.9998	5	15–10^5	0.9997	60
CPR	10	30–10^6	0.9996	3	8–10^5	0.9996	50
DNF	7	20–10^6	0.9997	0.7	2–10^3	0.9996	200
ENR	7	20–10^6	0.9997	1.3	4–10^3	0.9997	200
GTF	20	60–10^6	0.9996	13	40–10^5	0.9996	100
GRP	13	40–10^6	0.9997	1.7	5–10^4	0.9997	600
FLM	7	20–10^6	0.9997	0.3	1–10^3	0.9997	900

[a] ng L^{-1}.
[b] Correlation coefficient.

Table 6
Recoveries (%) using the proposed HF-LPME/HPLC method from spiked bovine urine samples (average of three determinations ± standard deviation).

	Urine spiked level (mg L^{-1})		
	0.025	0.5	5
MRB	95.7 ± 1.0	96.4 ± 0.9	100.2 ± 1.0
NRF	99.6 ± 0.9	100.2 ± 1.0	99.9 ± 1.0
CPR	99.8 ± 1.1	100.5 ± 1.4	99.7 ± 1.5
DNF	99.7 ± 0.5	99.9 ± 1.9	99.9 ± 0.9
ENR	99.0 ± 0.6	99.9 ± 1.0	99.9 ± 1.4
GTF	99.4 ± 2.1	99.7 ± 0.6	99.9 ± 0.9
GRP	98.1 ± 1.1	99.3 ± 0.9	99.3 ± 0.6
FLM	76.0 ± 1.9	76.2 ± 1.5	79.0 ± 2.4

To evaluate the repeatability and the intermediate precision, aqueous samples at three concentrations levels 10^2, 10^3 and 10^5 ng L^{-1} (in triplicate) were subjected to the entire analytical procedure and measured in one single day and one day per week during two months, respectively. Intermediate precision was performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the $m \times p \times n$ design (m = analytical levels, p = days and n = replications). From the corresponding ANOVA, the intermediate precision was computed [38]. The repeatability, expressed as relative standard deviation, was in the range 0.6–1.2%. Intermediate precision also expressed as relative standard deviation, was in the range 1.0–1.8%.

The robustness study is based on a landmark procedure suggested by Youden [39]. A design matrix (two factors in eight experiments) was used (see supplementary electronic material). The levels +1 or −1 correspond to high and low pH values, 7.5 and 6.5 for donor phase and 12.5 and 11.5 for the acceptor phase. Stirring time is not considered as a variable for robustness study due to its high optimum value (5.5 h) and the fact that variations in the order of minutes do not have significant effects in the extraction efficiency.

The effect of a given factor, say x_i can be estimated as the difference of result averages at levels +1 and −1:

$$D(x_i) = \frac{1}{4}\left[\sum R_{(x_i=+1)} - \sum R_{(x_i=-1)}\right]$$

when R is the corresponding experimental result obtained.

A significance t-test is used [40] to determine whether variations have a significant effect on the result,

$$t(x_i) = \frac{\sqrt{2}|D(x_i)|}{S_{IP}}$$

where S_{IP} is the standard deviation of the intermediate precision, evaluated in the precision study. The $t(x_i)$ values were compared with the corresponding critical t values (n = 4) at 5% significance level and three degrees of freedom. Results obtained (see supplementary electronic material) that t values calculated for each factor are lower than the tabulated one (3.18), so the procedure can be considered robust against the considered factors for all the analysed compounds.

3.4. Cow urine analysis

3.4.1. Recovery assays on spiked urine

A urine pool from two cows was spiked with the studied fluoroquinolones at three concentration levels (0.025, 0.5 and 5 (g mL^{-1}) and submitted to the HF-LPME procedure described in Section 2. Representative DAD and FLD chromatograms can be observed in Fig. 2 including the corresponding blank chromatograms. As it can be seen, the peaks have good resolution and good baselines were

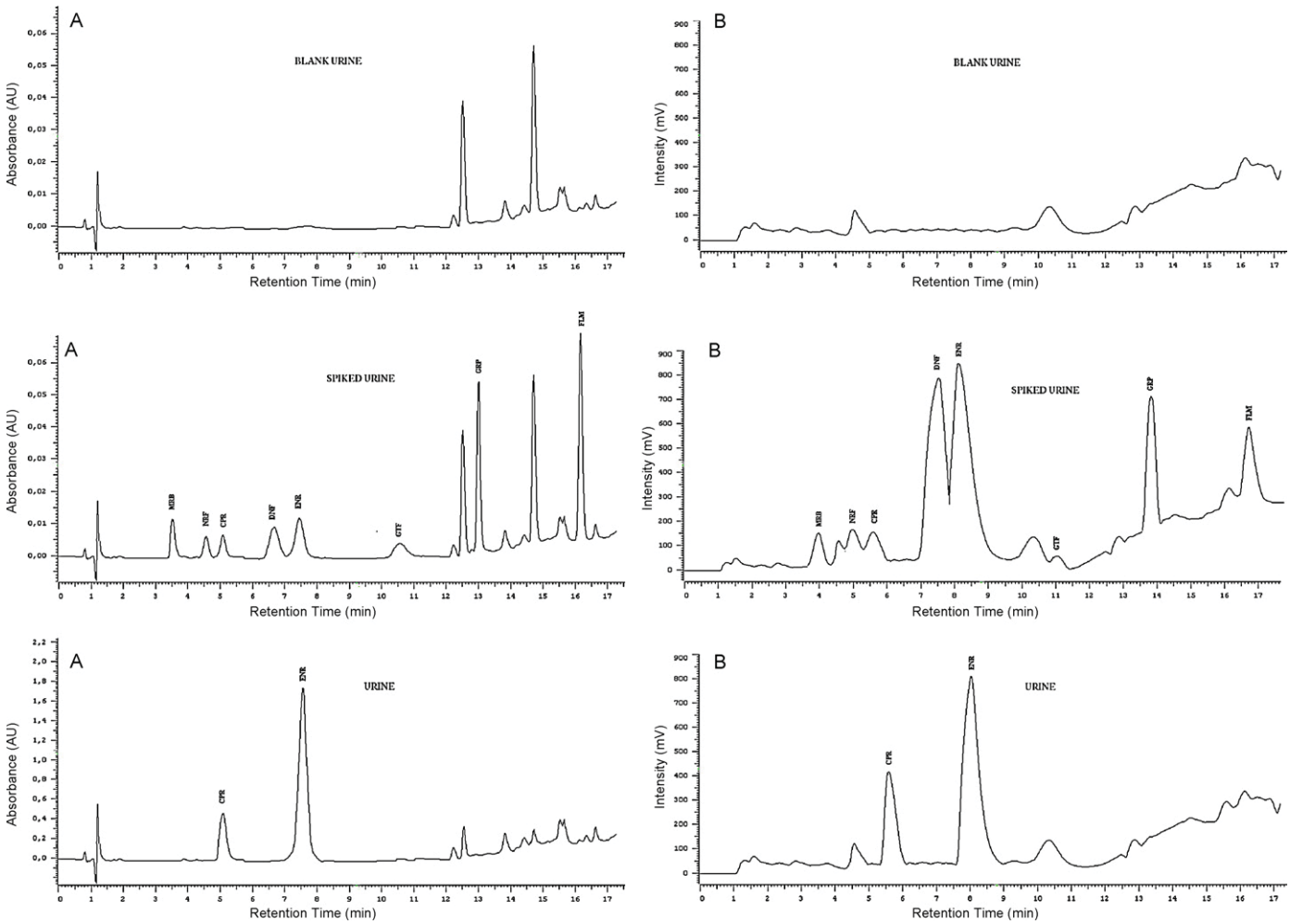


Fig. 2. DAD (a) and FLD (b) chromatograms from blank and spiked (0.5 μg L^{-1}) cow urine samples and from one urine sample obtained after the administration of enrofloxacin submitted to the proposed HF-LPME procedure (for details see text).

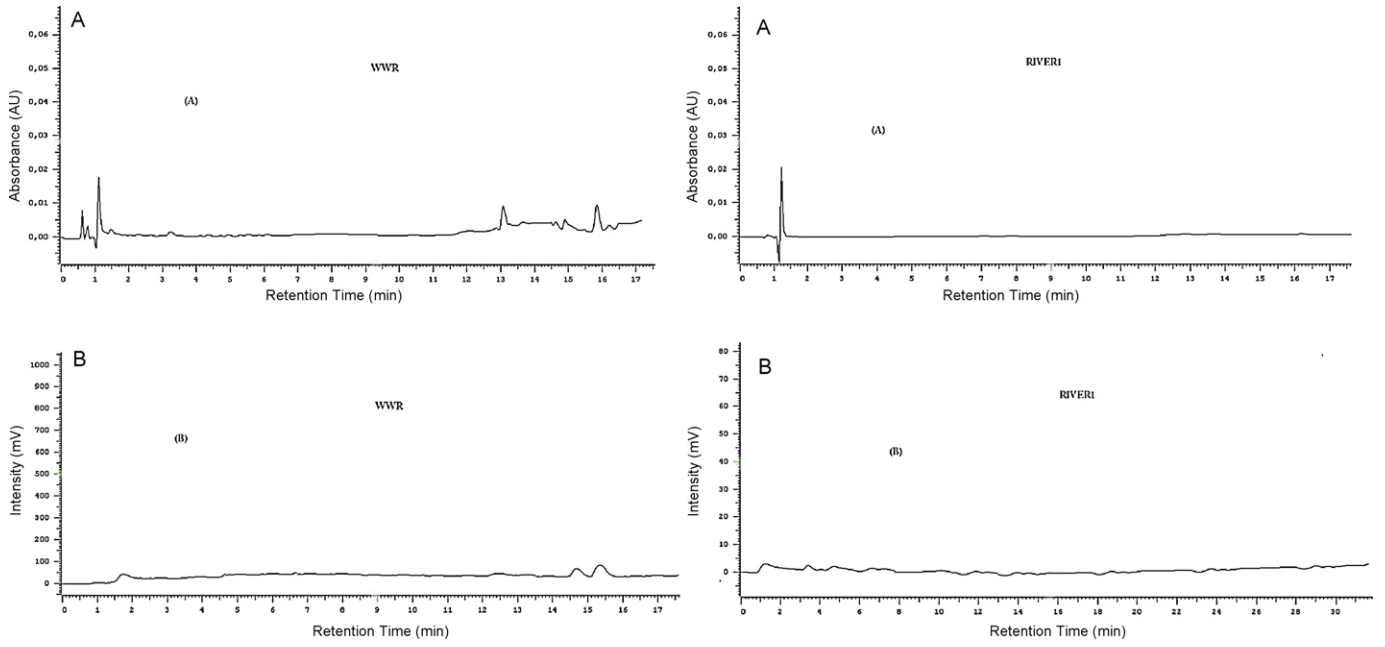


Fig. 3. DAD (a) and FLD (b) chromatograms of blank wastewater (WWR) and river (RIVER1) samples submitted to the proposed HF-LPME procedure.

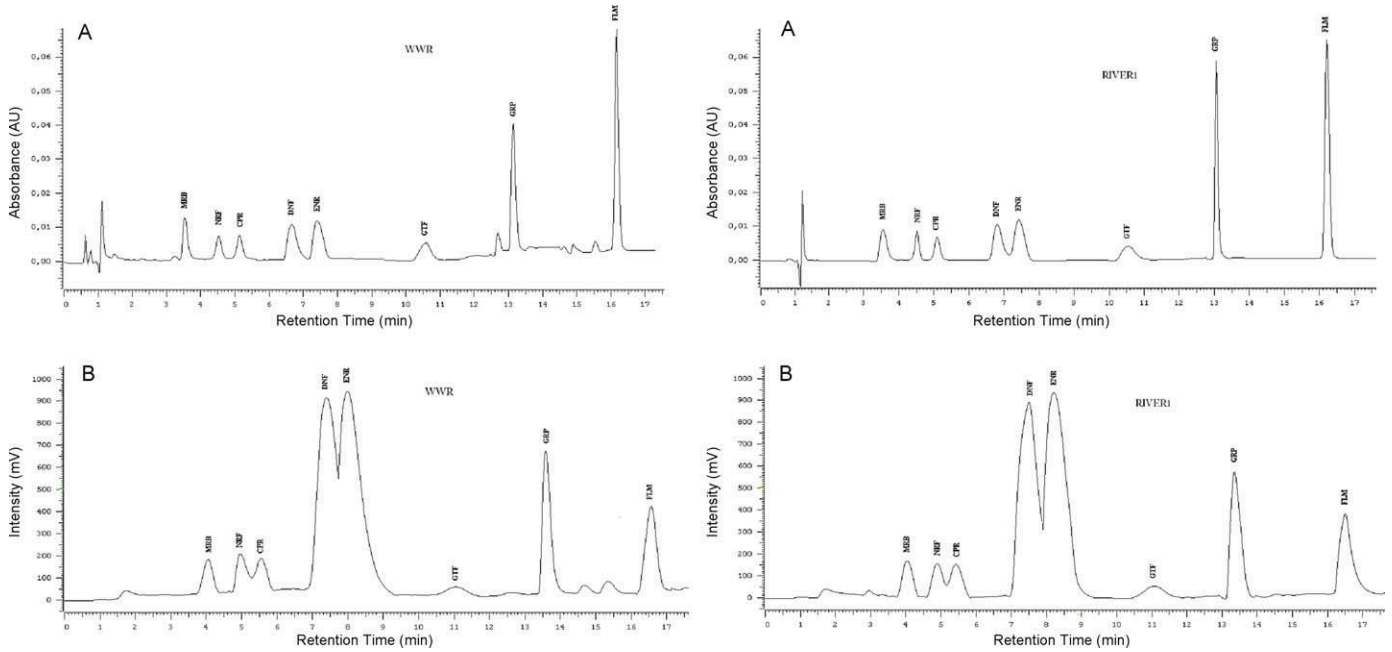


Fig. 4. DAD (a) and FLD (b) chromatograms from spiked (500 ng L^{-1}) wastewater (WWR) and river (RIVER1) samples submitted to the proposed HF-LPME procedure.

obtained; however some peaks appear in the blank samples that not interfere with the corresponding to the analysed fluoroquinolones. Table 6 shows the corresponding recoveries obtained and, as it can be seen, the values obtained were within the range 96–100% for practically all the analysed compounds, only FLQ shows recoveries between 76 and 79%. The data obtained demonstrates that the proposed HF-LPME procedure could be adequate for cow urine samples analysis.

Table 7
Recoveries (%) using the proposed HF-LPME/HPLC method from water spiked samples (average of three determinations ± standard deviation).

Spiked level[a]	Water sample[b]							
	WWR	WW1	WW2	WWT	RIVER1	RIVER2	LAKE	TAP
MRB								
150	86.7 ± 0.5	87.3 ± 1.0	87.1 ± 0.8	88.2 ± 1.1	99.1 ± 0.5	99.6 ± 4.0	100.0 ± 0.7	100.0 ± 1.0
500	90.0 ± 1.0	91.3 ± 0.8	91.1 ± 0.7	92.3 ± 0.7	99.4 ± 0.6	99.8 ± 1.0	100.1 ± 0.5	99.9 ± 0.3
5000	91.2 ± 0.5	91.5 ± 0.7	91.9 ± 0.8	92.7 ± 0.8	100.1 ± 0.5	99.9 ± 1.2	99.9 ± 0.6	100.0 ± 0.5
NRF								
150	96.9 ± 1.5	97.2 ± 0.5	97.9 ± 1.2	98.5 ± 1.1	99.8 ± 0.8	99.6 ± 0.5	99.9 ± 1.0	100.1 ± 0.6
500	99.0 ± 1.6	98.9 ± 1.0	99.2 ± 0.7	100.1 ± 1.0	99.8 ± 0.8	99.9 ± 0.5	100.0 ± 1.3	100.0 ± 0.8
5000	99.1 ± 0.5	99.2 ± 0.6	99.1 ± 0.6	99.5 ± 0.7	99.9 ± 1.1	99.8 ± 0.6	100.0 ± 2.0	100.1 ± 0.5
CPR								
150	96.4 ± 1.0	96.5 ± 0.7	97.2 ± 1.0	98.3 ± 0.7	99.0 ± 0.7	98.9 ± 1.6	100.2 ± 1.2	99.9 ± 0.7
500	99.4 ± 1.1	99.5 ± 1.1	99.6 ± 0.7	99.8 ± 0.7	99.0 ± 0.8	99.2 ± 1.2	99.6 ± 0.7	100.1 ± 1.1
5000	99.5 ± 0.9	99.4 ± 0.6	99.7 ± 0.6	99.9 ± 0.6	100.0 ± 0.7	99.2 ± 1.1	99.9 ± 0.7	100.1 ± 0.5
DNF								
150	98.8 ± 0.6	98.9 ± 1.2	98.9 ± 0.7	99.2 ± 1.3	99.3 ± 0.8	99.6 ± 1.0	99.9 ± 0.8	99.9 ± 0.3
500	99.9 ± 0.6	99.9 ± 1.1	99.8 ± 0.5	100.0 ± 0.6	99.4 ± 0.7	99.3 ± 0.7	99.9 ± 1.5	100.0 ± 0.5
5000	100.0 ± 0.5	99.9 ± 0.5	99.9 ± 0.6	99.9 ± 1.2	100.0 ± 0.7	99.8 ± 0.5	99.9 ± 0.7	100.0 ± 0.7
ENR								
150	96.7 ± 0.6	96.4 ± 1.1	97.6 ± 0.6	97.6 ± 1.2	99.4 ± 1.3	99.7 ± 0.6	100.0 ± 1.1	99.9 ± 1.0
500	98.6 ± 0.7	99.6 ± 0.5	99.8 ± 0.7	99.9 ± 0.8	99.4 ± 0.6	100.0 ± 0.5	99.9 ± 1.2	100.1 ± 0.9
5000	98.9 ± 0.5	99.90 ± 0.6	99.1 ± 0.6	99.5 ± 0.7	99.8 ± 0.7	100.1 ± 0.6	99.95 ± 1.0	99.9 ± 0.6
GTF								
150	98.7 ± 0.7	98.9 ± 0.6	98.9 ± 0.5	99.2 ± 1.3	99.8 ± 0.8	99.8 ± 0.6	99.9 ± 0.7	100.0 ± 0.8
500	99.1 ± 0.7	99.1 ± 1.1	99.5 ± 0.6	99.8 ± 0.6	95.8 ± 0.3	96.6 ± 0.8	99.8 ± 1.2	100.0 ± 0.7
5000	99.3 ± 0.7	99.9 ± 0.5	99.6 ± 1.0	99.8 ± 0.5	99.8 ± 0.5	98.9 ± 0.3	100.1 ± 0.7	99.9 ± 0.7
GRP								
150	98.6 ± 0.6	98.8 ± 1.0	99.0 ± 1.2	99.6 ± 0.6	99.4 ± 0.7	100.0 ± 0.7	100.1 ± 1.3	99.9 ± 0.8
500	99.1 ± 1.0	99.8 ± 0.5	99.6 ± 1.0	99.9 ± 0.7	99.3 ± 0.6	99.0 ± 0.9	99.9 ± 1.0	100.1 ± 0.5
5000	99.3 ± 0.5	99.8 ± 0.5	99.7 ± 0.6	99.9 ± 0.5	99.9 ± 0.6	99.8 ± 0.9	99.9 ± 1.0	100.1 ± 0.6
FLM								
150	91.6 ± 0.7	92.2 ± 0.6	92.6 ± 1.2	95.3 ± 0.8	99.6 ± 0.7	99.8 ± 1.0	99.8 ± 1.1	99.9 ± 0.6
500	93.0 ± 0.3	92.8 ± 0.7	93.9 ± 0.6	95.2 ± 0.8	99.7 ± 0.6	99.7 ± 0.7	99.9 ± 0.8	100.0 ± 0.6
5000	93.5 ± 0.5	93.6 ± 0.5	94.2 ± 1.0	96.9 ± 0.7	100.0 ± 0.5	99.8 ± 1.5	99.9 ± 0.5	100.0 ± 1.1

[a] ng L^{-1}.
[b] Average recovery (%) ± standard deviation (n = 3).

3.4.2. Analysis of urine samples from cows under veterinary treatment

A urine sample from a Jersey cow of approximately 800 kg weight submitted to an enrofloxacin treatment with injectable ALSIR® 5% (Laboratorios Esteve Veterinaria, Spain) at 1 mL/20 kg day doses during five days was collected and submitted to the HF-LPME procedure described in Section 2. Fig. 2 shows the corresponding DAD and FLD chromatograms. Urine contents of 9.4 ± 0.01 and 17.6 ± 0.02 ($g\,mL^{-1}$) for CPR and ENR were measured, respectively. The presence of ciprofloxacin in the urine sample is due to it is the enrofloxacin metabolite.

3.5. Environmental water samples analysis

The different water samples were selected taking into account the maximum variability with respect to provenance and matrix composition. First, the different water samples were submitted to the HF-LPME proposed procedure and analysed. None of the fluoroquinolones were detected in the water analysed samples.

In order to check the suitability of the proposed procedure spiked samples at three concentration levels: 150, 500 and 5000 ng L^{-1}, were analysed. Results obtained are shown in Table 7. As can be seen in all cases excellent recoveries were obtained with values in the 97–100% range, only MRB and FLM show recoveries of 87–92% and 91–96% for the urban wastewater samples that are traditionally complex samples from the analytical point of view.

Figs. 3 and 4 show representative DAD and FLD chromatograms obtained from blank and spiked (500 ng L^{-1}) wastewater (WWR) and river (RIVER1) samples. These samples have been selected as the more complex wastewater (raw wastewater) and surface water. As it can be seen, RIVER1 blank chromatograms show excellent baselines. Spiked WWR and RIVER1 chromatograms only show well defined peaks corresponding to the added substances.

In general, excellent recoveries were obtained with values of practically 100%, only MRB and FLM show slightly lower values for some of the analysed samples, but in any case in the 87–97% range. These recoveries were at least of the same, but frequently better, magnitude order that other previously published methods [16,17,19–21,24,26,30,32]. The excellent recoveries, preconcentrations and clean-up obtained imply a great advantage over other sample treatment procedures which justifies the proposed HF-LPME extraction for its use in environmental water analyses.

4. Conclusions

This study presents a hollow fiber-based liquid phase microextraction (HF-LPME) method combined with an HPLC (DAD–FLD) determination using a highly packed chromatographic column that allows a simple, low-cost, accurate, high sensitive and selective methodology for the determination of eight widely used fluoroquinolones. The proposed extraction procedure has a very low (few μL) organic solvent consumption. The excellent preconcentration and clean-up obtained implies a great advantage over other sample treatment procedures. Additionally, the use of two detectors brings additional selectivity for the method, which is important for the analyses of environmental samples.

Despite the long extraction time used, it is a procedure with little handling and completely unassisted unlike other extraction procedures. Additionally, the cost by sample analysed is practically null compared with other existing extraction alternatives like SPE, mixed-phase cation exchange (MPC) [19,20], magnetic molecularly imprinted polymer (MMIP) [26] or pressurized liquid extraction (PLE) [33–35].

The proposed procedure has been demonstrated adequate for the determination of the analytes in cow urine and environmental water samples, including urban wastewaters that usually require tedious clean-up and preconcentration steps, obtaining, in general, recoveries around 100% for all the analysed compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.01.037

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ANALYTICAL APPLICATIONS OF HOLLOW FIBER LIQUID PHASE MICROEXTRACCION (HF-LPME): A REVIEW

Analytical applications of hollow fiber liquid phase microextraction (HF-LPME): A review

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Abstract

The increasing demand of faster, cheaper, easier and more environmentally-friendly methods has favored the miniaturization of systems for sample preparation. These new procedures have led to lower reagent and materials consumption and waste production. One extraction technique recently introduced is based on the use of hollow fibers as support to liquid membranes which enables the extraction with solvents of different nature from a donor external phase to an acceptor phase inside the lumen of the fiber.

This is an up-to-date comprehensive review on the analytical applications of hollow fiber liquid phase microextraction (HF-LPME) that includes two and three phase configurations, carried-mediated extraction and electromembrane extraction. A brief review on the basic extraction principles for these techniques, describing and discussing the different operation and configuration modes, has been carried out.

Keywords: hollow fiber liquid phase microextraction, HF-LPME, supported liquid membrane, carrier mediated, electromembrane

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INTRODUCTION

Sample preparation is the most time-consuming step in analytical procedures. The sample preparation is constantly evolving due to high consumption of time, sample and reagents (in some cases expensive, toxic or highly polluting).

The interest in the development of new techniques for sample preparation or modification of the existing ones, in order to make them faster, more powerful and versatile has increased in recent years. Liquid-liquid extraction (LLE) is a classical technique widely used in most applications. For aqueous samples, LLE is probably the most accepted and used. It is a versatile sample-preparation technique and it is recommended in many standard analytical methods (Alders 1955, Treybal 1963). However, for trace analysis, large volumes of samples are often required and their handling can be time-consuming and tedious.

Other popular sample preparation procedure, solid-phase extraction (SPE), was introduced commercially in the late 1970s. In SPE the consumption of organic solvents is relatively lower than LLE, and usually requires the evaporation of the eluent after extraction. SPE has been one of the most frequently used techniques for the preparation of samples in the last decade (Thurman 1998). Arthur (1990) introduced a new sample treatment procedure to eliminate or at least reduce the limitations of solid phase extraction, known as solid-phase microextraction (SPME). SPME presented a significant advance in the field of sample preparation and satisfied most of the requirements of a good sample preparation technique, including simplicity of use, automation, low consumption of materials and shorter operation times.

LLE and SPE methods have been used in the analysis of all type of analytes in samples of different nature. Therefore, it is possible to find a large number of publications, both applications and revisions, about this topic. Along with the SPME extraction techniques, they have been frequently used in recent years because of their advantages.

Following the development of SPME, similar researches were conducted to minituarize liquid-liquid extractions into LPME. In 1996, Liu (1996) and Jeannot (1996) proposed a LPME method in which a small drop of a water-immiscible organic solvent was held

on a hole at the end of a PTFE rod. Later, the research group of Lee (He 1997) developed this technique by introducing the concepts of static and dynamic microextraction where the small drop of organic solvent is suspended from the tip of a gas chromatography syringe (single drop microextraction, SDME). This is a simple and low-cost technique and there is a minimal exposure to toxic organic solvents. The main problem of the technique is that the microdrop suspended on the microsyringe needle can be easily dislodged during stirring of the aqueous sample. Furthermore, the technique is not suitable for dirty samples, because particles in the sample affect the extraction by making the drop unstable, and they are potentially detrimental to the analytical instrument (Zhao 2002).

Audunsson (1986) introduced a different concept of liquid microextraction that was further developed by Thordarson (1996) and Pedersen-Bjergaard (1999) based on the use of single, low-cost, disposable, porous hollow fibers made of polypropylene supporting a organic solvent in the pores (liquid membrane). Accordingly, the analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor solution inside the lumen of the hollow fiber. The acceptor phase can be organic, resulting in a two-phase extraction configuration directly compatible with capillary gas chromatography, or it can be an aqueous solution, resulting in a three-phase system compatible with high-performance liquid chromatography, capillary electrophoresis or mass spectrometry. For two and three phase configurations, the migration is pH-dependent in the donor and acceptor phases. In addition to pH gradients, carrier-mediated transport has been reported with LPME extraction (Wieczorek 1997 and Ho 2003), where extraction is promoted by a strong concentration gradient of counter-ions across the supported liquid membrane replacing the polar substances at the interface between the membrane and the acceptor solution.

The small pore size prevents particles present in the donor solution from entering the acceptor and most interfereing substances could not enter in the hollow fiber due to low solubility in the organic phase present in the pores, thus yielding very clean extracts. In the usual configurations hollow fiber is used as disposable material which eliminates the possibility of sample carryover and ensures reproducibility. LPME is a reliable and robust technique which can provide high enrichments and low limits of detection. Some

reviews on hollow fiber-based LPME that describe in detail the different configurations and operation modes, including in some cases mathematical approaches, have been previously reported (Psillakis 2003a, Rasmussen 2004, Lee 2008b, Pedersen-Bjergaard 2008 and Pena-Pereira 2010). Figure 1 shows some typical experimental setups used in HF-LPME.

The presence of charged compounds in aqueous and organic solutions used in liquid extraction procedures led to some authors to propose the use of electrical fields to enhance and to manipulate LLE. In 2006, Pedersen-Bjergaard and Rasmussen demonstrated, for the first time, that an electrical potential produces analytical extraction through a supported liquid membrane (SLM) (Pedersen-Bjergaard 2006). This configuration was termed electromembrane extraction (EME) and the analytes were extracted from an aqueous sample to an aqueous acceptor solution placed inside the lumen of the hollow fiber through an organic solvent immobilized as SLM in the wall of a polypropylene porous hollow fiber. Essentially, it is similar to a HF-LPME where the migration through the SLM is forced by the electrical field generated from two electrodes, one placed out the fiber and the other inside the lumen (electromigration, EMI). In order to ensure an efficient electrokinetic mobility in the EME system, pH must be adjusted to provide total ionization of the analytes in the two aqueous solutions. In the first papers published using EME, basic analytes were analyzed using acid pHs and electrical potential.

The knowledge on EME is currently limited, and only a few papers have emerged in the literature. Since the introduction of EME in 2006, several factors influencing the extraction and enrichment of basic drugs (Gjelstad 2006, 2007a, 2007b and 2009) and acidic drugs (Balchen 2007) have been investigated and optimized. The knowledge about the parameters affecting the extraction efficiency has improved, and a comprehensive theoretical description of the extraction process has been developed.

TWO PHASE HF-LPME

In two phase configurations, the analytes are extracted from an aqueous sample solution (donor phase) through a water-immiscible solvent immobilized in the pores of the

hollow fiber into the same organic solvent (acceptor phase) inside the hollow fiber lumen. The analyte is in its neutral form in the donor phase and the composition of the donor phase is critical to succeed with two phase systems.

The pores and the lumen of the hollow fiber are filled with an organic solvent immiscible with water, so the final extract is an organic phase directly compatible with analytical techniques such as GC, or HPLC and CE after the evaporation of the solvent and reconstitution in an aqueous medium. On the other hand, different ionic liquids have been used as solvents immobilized in the pores of the hollow fiber in order to eliminate organic solvents and make the technique more environmental friendly (Peng 2007 and Basheer 2008a). They can be directly injected for analysis, without previous evaporation of solvent, in liquid chromatographs.

The variation of the analyte concentration into the acceptor phase with the extraction time ($C_a(t)$) can be expressed (Pedersen-Bjergaard 2007a and Pena-Pereira 2010) as:

$$C_a\,(t) = \; C_{eq,a}\,(1 - e^{-kt})$$

where $C_{eq,a}$ is the analyte concentration in the equilibrium into the acceptor phase and *k* the rate constant, and both variables could be defined (Jeannot 1996, 1997) by:

$$C_{eq,a} \; = \frac{K C_d^0 V_a}{V_d + K V_a}$$

$$k = \frac{\beta_a \, A_i}{V_a}\left(1 + \frac{K V_a}{V_d}\right)$$

being K the partition coefficient, C^o_d the initial analyte concentration in the donor phase, V_d and V_a the donor and acceptor phase volumes, β_a the mass transfer coefficient and A_i the interfacial area.

Moreover, enrichment factor (E_f) was defined as the relation between the final analyte concentration in the acceptor phase and the initial one in the donor phase.

THREE PHASE HF-LPME

In three-phase HF-LPME configurations, analytes are extracted from an aqueous solution (donor phase) to another aqueous phase (acceptor phase) present inside the lumen of the hollow fiber through the organic solvent immobilized in the pores of the hollow fiber (organic phase). The organic phase serves as a barrier between the donor phase (sample solution) and the acceptor phase. The migration of the analytes is pH-dependent in both, donor and acceptor phase, so an adjustment of the pH of the donor and acceptor phases is critical to reach a successful extraction.

This extraction mode is limited to basic or acidic analytes with ionizable functionalities, where the analyte is in its neutral form in the donor phase. For extraction of basic compounds, an acidic aqueous solution is used as acceptor phase, whereas an alkaline solution is used to extraction of acidic compounds. Because of this, the analytes become ionized, following the extraction into the acceptor phase, and they are prevented from re-entering the organic solvent in the pores of the hollow fiber. The acceptor phase collected can be directly analysed in a HPLC, CE or MS.

As in two phase LPME, it is possible to work with ionic liquids as solvent immobilized in the pores. However, poor results have been obtained due to the high solubility of ionic liquid in aqueous solutions. (Peng et al. 2007a; Basheer et al. 2008a)

Similary to the two phase configuration, the analyte concentration into the acceptor phase in function of the extraction time ($C_a(t)$) can be expressed (Ma 1998) as

$$C_a(\mathrm{t}) = C_d^0 \frac{\mathrm{V_d}}{\mathrm{V_a}} \left[\frac{\mathrm{k_1 k_3}}{\lambda_2 \lambda_3} + \frac{\mathrm{k_1 k_3}}{\lambda_2(\lambda_2 - \lambda_3)} \mathrm{e}^{-\lambda_2 \mathrm{t}} + \frac{\mathrm{k_1 k_3}}{\lambda_3(\lambda_2 - \lambda_3)} \mathrm{e}^{-\lambda_3 \mathrm{t}} \right]$$

where C^o_d is the initial analyte concentration in the donor phase and k_1 to k_4 the rate constants for the different transfer processes involved, and λ_1 and λ_2 were defined as:

$$\lambda_2 = \frac{1}{2}\left[(\mathrm{k_1 + k_2 + k_3 + k_4}) + \left[(\mathrm{k_1 + k_2 + k_3 + k_4})^2 - 4(\mathrm{k_1 k_3 + k_2 k_3 + k_1 k_4})\right]^{1/2}\right]$$

$$\lambda_3 = \frac{1}{2}\left[(\mathrm{k_1 + k_2 + k_3 + k_4}) - \left[(\mathrm{k_1 + k_2 + k_3 + k_4})^2 - 4(\mathrm{k_1 k_3 + k_2 k_3 + k_1 k_4})\right]^{1/2}\right]$$

k_1 to k_4 are related to the mass transfer coefficients β_a and β_m and the interfacial areas A_{md} and A_{ma} for the liquid membrane – donor phase and liquid membrane – acceptor phase, respectively.

CARRIER MEDIATED HF-LPME

In order to enhance the extraction of hydrophilic drugs, carrier mediated LPME has been reported as an active transport mode for HF-LPME in which a carrier is added to the sample solution (Kramer 2001 and Ho 2003) or is dissolved in the impregnation solvent in the pores of the hollow fiber (Fontas 2005 and Romero 2005). The extraction efficiency is governed by the partitioning of the analyte between the sample matrix and the inmobilised solvent or by the partitioning between the acceptor phase and the inmobilised solvent.

The former form consists in using the carrier dissolved in the membrane phase which separates the donor phase and the acceptor phase. This is based on a supported liquid membrane device with a flowing donor solution and a stagnant acceptor solution. Cationic analytes are extracted with anionic carriers while anionic analytes are extracted with cationic carriers. At the interface between the organic and donor phase, the analyte and the carrier form a neutral ion-pair complex. This way, ionic carriers are associated with counter-ions to maintain electro neutrality in the apolar membrane phase. The complex diffuses thought the membrane and at the interface between the membrane and the acceptor phase, the analyte is exchanged by the corresponding counter-ion.

The latter is via ion-pairing mechanism where a carrier (hydrophobic ion-pair reagent) is dissolved directly into the sample solution to form hydrophobic ion-pair complexes with the analytes. The ion-pair complexes are extracted into the organic liquid membrane immobilised in the pores of the hollow fiber.

In both cases, the analytes are released from the ion-pair complex into the acceptor solution by means or through the contact region, whereas counter ions are present at a very high concentration. These counter-ions form an ion pair with the carrier in the

contact area, so this new ion-pair complex is back-extracted into the donor phase. There, the carrier releases the transporter counter-ion and forms again an ion pair with another analyte molecule, repeating the described cycle. The function of the carrier, then, is to facilitate the transfer of the ionized analyte from the donor to the acceptor phase and the transfer of counter-ions in the opposite direction.

The pH of the sample is adjusted to ensure that the analytes are present in their adequate ionized state in order to form the ion pair, and the pH of the acceptor solution is adjusted typically to low values to ensure that the analyte is in an appropriate form to avoid the formation of new ion pairs that would back-extract it to the donor phase. These low pH values also provide a high concentration of protons to serve as counter ions for the carrier.

ELECTROMEMBRANE HF-LPME

Electromembrane extraction (EME) can decrease the equilibrium time of the extraction step. The equipment for EME is exactly the same described for three-phase extraction, except for the addition of two electrodes and a d.c. power supply. In this configuration, a platinum electrode is placed in the sample solution, and another platinum electrode is located in the acceptor solution inside the lumen of the fiber (Figure 1e). The analytes are ionized both in the sample solution and in the acceptor solution, which promotes electrokinetic migration across the SLM. For basic analytes, the positive electrode is placed in the sample and the negative electrode is located in the acceptor solution (Kjelsen 2008, Middelthon-Bruer 2008 and Gjelstad 2009); this configuration is reversed for acidic compounds (Balchen 2007).

For both EME and LPME, the flux of analytes is controlled by proper selection of pH, by optimization of the SLM, and by strong agitation of the whole system. However, the basic driving force of the system itself, namely the applied voltage, is a fourth parameter that controls the analyte flux and it can be adjusted to control both the extraction rate and the selectivity of the system. The applied voltage is found to be a stronger driving force for analyte movement than a mere pH gradient, and thus, EME provides enhanced extraction speed and increased flexibility compared to conventional HF-LPME. The

technique can also provide excellent clean-up and analyte enrichment from complicated matrix samples. Nevertheless, earlier experiments have shown that the composition of the organic solvent, in addition to the value of the applied voltage, is an important factor to be optimized for a successful extraction (Pedersen-Bjergaard 2007b).

As knowledge about EME is limited, more work must be conducted in order to understand the exact extraction mechanism, to enable optimization of the extraction of a particular type of analyte, and to demonstrate that the technique provides reliable data for a broad range of analytical applications. So far, reported works have shown very rapid extractions from small sample volumes in miniaturized analytical systems. EME is expected to be an interesting option for sample preparation to be integrated with the final chemical analysis (Pedersen-Bjergaard 2006, Gjelstad 2007b and Balchen 2007).

Gjelstad et al (Gjelstad 2007b) described a complex mathematical model for the extraction using electromembranes, which is based on the Nerst-Plank equation and on the transfer of the analytes through the liquid membrane by the electrical field.

APPLICATIONS

Due to editorial restrictions, the complete review on the analytical applications of hollow fiber liquid phase microextraction can be found as supplementary file in the journal page. In this paper only the more recent and relevant analytical contributions are shown.

Two phase HF-LPME

It has been previously described that the analytes are extracted from an aqueous sample into the organic solvent placed inside the lumen of the hollow fiber. As it can be seen in Tables 1a, 1b and 1c the organic solvent is usually of apolar nature, as toluene or hexane. The low volumes used (usually in the 1 – 50 μl range) and the volatile nature of the organic solvent provide suitable extracts for its injection in a gas chromatography system, being the most usual analytical technique used for this microextraction configuration.

In general, the reported gas chromatography applications were carried out without derivatization. However, some papers using derivatization steps have been published: pre-extraction derivatization (Duan 2009), injection-port derivatization (Zhang 2009), simultaneous derivatization-extraction by addition of the derivative agent to the donor solution (Ito 2008, 2009 and Kawaguchi 2008) or into the fiber lumen (Lee 2008a, Es'haghi 2009 and Liu 2009b).

Two phase hollow fiber microextraction is usually carried out with Q3/2 type polypropylene fibers (0.2 μm pore size, 200 μm wall thickness and 600 μm inner diameters). Some authors have used other polypropylene fibers, as the KM type (i.d. 1200 μm, wall thickness 200 μm, pore size 0.2 μm) used by Ouyang (2007) or the Q150/330 type (0.2 μm pore size, 150 μm wall thickness and 600 μm inner diameter) used by Salafranca (2009). More rarely, polyvinylidene difluoride has been applied as an alternative to polypropylene (Cui 2009). Fiber lengths are usually in the 20-300 mm range.

Two phase hollow fiber microextraction is specially recommended for the extraction of apolar substances. The technique has been applied to the determination of different organic compounds as aromatic hydrocarbons, including PAHs, phenols, etc. Also, in the last years, two phase HF-LPME has been extensively applied for the determination of pesticides. As can be seen in Table 1, this extraction method has been applied mainly to organochlorine (OCPs), organophosphorus (OPPs) and organosulfur (OSPs) pesticides, polychlorinated biphenyls (PCBs) and triazines herbicides. In some cases, the proposed methods were applied to the extraction and determination of pesticides of different families (dicarboximide, triazol and OPPs) by Sanagi (2010a) and by 34 pesticides from different families Bolanos (2008).

With respect to clinical and pharmaceutical applications, as it can be seen in Table 1, two phase HF-LPME has been applied to the determination of pharmaceuticals or other substances of clinical interest, as nicotine, in biological fluids, pharmaceutical preparations and environmental samples. Two phase HF-LPME has also been applied to other miscellaneous fields: Lee (2008a) apply this procedure to the determination of

chemical warfare agents and its degradation products such as nerve agent VX, nitrogen mustards blister agents, phosphonic acids and psychotomimetic agents.

In food analysis, HF-LPME has been applied to the determination of the Sudan dyes I-IV by Yu (2008), the determination of active food packaging components by Salafranca (2009) and the determination of mycotoxins by Romero-Gonzalez (2010). Additionally the technique has even begun to be applied as well to the determination of organo-metallic compounds (Jiang 2008 and Duan 2009).

Three phase HF-LPME

As it can be seen in Tables 2.a-c, solvents used as supported liquid membranes in the three phase HF-LPME configurations typically have higher polarity than those employed in two phase HF-LPME, being octanol and dihexyl ether (DHE) the most usual. In some cases, a SLM based on an ionic liquid has been used, as 1-octyl-3-methylimidazolium hexafluorophosphate ([omim]PF6) (Peng 2007) and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF6) (Basheer 2008a).

Respecting the donor and acceptor phases, they are usually prepared with highly different pHs values, usually acidifying one of them with mineral acids (typically HCl) and alkalinizing the other with NaOH or other strong alkalis. In some cases, the extraction is carried out around neutral pH values in the donor phase obtained by the addition of a suitable buffer (Rasmussen 2000, Xie 2009, Malagueno de Santana 2008, Busquets 2009 and Fu 2009) or using the sample pH value without further adjustment (Basheer 2008a).

After the extraction step, the further determination is usually based on a HPLC method (with spectrophotometric or mass spectrometry detection) or capillary electrophoresis. Other techniques have been applied only in a few cases as gas chromatrography (Rasmussen 2000), graphite furnace atomic absorption spectrometry (Jiang 2008), flow injection analysis with chemiluminescence detection (Payán 2009b) or tandem mass spectrometry (Halvorsen 2001b) detection and electrokinetic flow analysis with spectrophotometric detection (Fu 2009).

As in two phase HF-LPME configuration, Q3/2 fiber type is the most widely fiber used. Nevertheless, there are also a significant number of alternatives. Previously described KM type has been applied by Bjorhovde (2003), Pedersen-Bjergaard (2004, 2005) and Baardstu (2007). The 50/280 type (40% porosity, 50 μm wall thickness and 280 μm inner diameter) has been used by Hansson (2009, 2010). Other polypropylene fibers (with their respective pore size/wall thickness/inner diameter) have been used by Gronhaug Halvorsen (2001) (0.4 μm/150 μm/and 330 μm), Liu (2008) (0.2 μm/300 μm/500 μm), Vasskog (2008), Larsson (2009) (0.1 μm/240 μm/30 μm), and Xie (2009) (0.2 μm/70 μm/400 μm).

Tables 2.a and 2.b resume the different applications of three phase HF-LPME. As it can be seen, the technique has been widely applied to the determination of drugs, mainly antidepressant drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and amphetamines (Table 2.a). As two phase HF-LPME, this configuration has also been applied to the determination of organic compounds (typically with phenolic nature) and pesticides (Table 2.b). Three phase HF-LPME has been also used for some miscellaneous applications: determination of organomercuric compounds, food analysis or alkaloid determination, among others (Table 2.b).

Carrier mediated HF-LPME

As it was explained previously, carrier–mediated HF-LPME can be applied in two different ways: adding the carrier to the donor phase in order to form the corresponding ion–par or dissolving the carrier into the liquid membrane. Both of these alternatives can be carried out using two-phase or three-phase configurations. Tables 3.a and 3.b include the four possible combinations of the technique.

Ion–pair agent carriers

Ion–pair agent carriers in two phase configurations have been mainly applied to the extraction of cations and anions which, due to their charge and inorganic nature, have no tendency to get into an organic acceptor phase in the absence of the carrier. As it is depicted in table 3.a, the carriers usually used for these applications are

tetrabutylammonium (TBA) salts or the ammonium salt of pyrrolidine dithiocarbamate (PDTC).

The determination of the inorganic ions is usually based on an instrumental method without a separation step, such as electrothermal atomic absorption spectroscopy for the determination of arsenic (Jiang 2009), nickel and lead (Abulhassani 2010) and selenium and tellurium (Ghasemi 2010); ICP – MS for the determination of arsenic (Pu 2009); ICP - OES for the determination of vanadium (Li 2007) and flow injection analysis with MS/MS detection for the determination of perchlorate (Chen 2009a). In contrast, Saleh (2009) have developed a method for the determination of selenium based on HPLC-UV.

Applications to organic substances have been also reported. Wu (2006) determined acidic pesticides, Kramer (2001) analysed the drug of abuse metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, Hultgren (2009) determined the surfactant dicocodimethylammonium chloride and Cardador (2010) analysed haloacetic acids.

Carrier-mediated three phase HF-LPME based on the addition of an ion–pair agent has been applied to the determination of polar drugs whose high hydrophilic nature avoid them from entering the organic phase. Through a three-step process, the hydrophobic ion-pair complex is formed in the donor phase, then the complex is extracted into the liquid membrane and diffused through it, and finally the analyte pass into the acceptor phase (Ho 2003). This way, polar drugs as morphine, practolol and amphetamine, has been extracted from biological fluids as human urine and plasma (Ho 2003 and Pedersen-Bjergaard 2005).

Carrier dissolved in the liquid membrane

As it can be seen in Table 3.b, the most usual carriers used for this configuration are trioctylphosphine oxide (TOPO), and *N*-methyl-*N*,*N*-dioctyloctan-1-ammonium chloride (Aliquat 336, trademark of Cognis Corp).

Most analytical applications reported appear in the field of biomedical analysis for the determination of sulfonamides as sulfadiazine, sulfamerazine, sulfamethazine, sulfadimethoxine and sulfamethoxazole (Tao 2009a), tetracyclines as tetracycline,

oxytetracycline and doxycycline (Shariati 2009) and estrogenic hormones as 17β-estradiol, estrone and 17α-ethynylestradiol (Zorita 2008). It has also been applied to the determination of pesticides (Piriyapittaya 2008, Hu 2010 and Trtic-Petrovic 2010), aromatic substances as phenols (Hu 2010) and aromatic amines (Tao 2009b).

ELECTROMIGRATION

Literature about HF-LPME using electromigration is scarce (Table 4) and most papers deals with applications related to the determination of basic drugs, like haloperidol, nortriptyline, methadone and loperamide. Gjelstad (2006) and Middelthon-Bruer (2008) offer the widest application spectrum, studying the extraction of twenty and thirty five different basic drugs, respectively. Nevertheless, the technique has been applied to other analytes as peptides (Balchen 2008, 2009, 2010, 2011), acidic drugs as ibuprofen, diclofenac and naproxen (Balchen 2007) and Pb^{2+} ion (Basheer 2008b).

With respect to the experimental conditions, applied voltages are in the 10 – 300 V range, usually obtained with a programmable d.c. power supply. On the other hand, the low voltages employed by Kjelsen (2008) and Eibak (2010) allowed the authors to use a common 9V battery as power supply.

Organic solvents used for the membrane formation usually differ from those typically found in other microextraction techniques. As it can be seen in Table 4, solvents like octanol or heptanol were only used in few cases, being nitrobenzene derivatives or nitrophenyl ethers the most used. Anyway, for electromigration extraction the membrane must be able to conduct the generated electric current, so this rule out the more apolar solvents. On the other hand, no variation in the hollow fiber type is found for these techniques: Q3/2 PP fiber type was used in all reported papers.

Extraction times required for electromigration microextraction are in the 5 – 10 minutes range. These times are markedly shorter than those required for most of the other hollow-fiber microextraction configurations (usually in the 15 – 60 minutes range). Eibak (2010) reduce this time even more, and describe an extraction procedure for

amitriptyline, citalopram, fluoxetine and fluvoxamine from human plasma with one minute duration, achieving detection limits in the 0.4 – 2.43 ng mL^{-1} range.

Finally, some authors have developed alternatives to hollow fiber as liquid membrane supporters for electromigration microextraction. This way, Petersen (2009) have developed a method based on a 1 cm × 1 cm piece of polypropylene membrane (organic solvent supporter) between two aqueous droplets (sample and acceptor solution). Xu (2008) and Lee (2009) employed a polypropylene envelopes with immobilized solvent for the electromigration microextraction of nerve agent degradation agents and chlorophenols, respectively, and Basheer (2010) have developed a method for the simultaneous extraction of acidic and basic drugs combining four sheets of porous polypropylene membrane, heat-sealing three edges in order to form a three-compartment envelope and impregnating its outer skin with the organic solvent (toluene).

CONCLUSIONS

HF-LPME in its different configurations has been widely used as clean-up and preconcentration procedure for a broad type of analytes. It is specially adequate to analyse complex matrices, obtaining very clean samples that can be directly analysed using MS detectors and also allows, in general, good baselines using others detector systems. The more usual hollow fiber used is a polypropylene one, Q3/2 PP from Membrana®. It is a simple and low-cost procedure and, usually, hollow fiber is used as disposable material; this fact eliminates the possibility of sample carryover and ensures reproducibility. The low organic solvents consumptions make the technique environmental friendly according to the new trends to a “Green Chemistry”.

This field of sample preparation will remain attractive because of its ease of implementation, as well as the flexibility it offers to permit imaginative sampling scenarios to be conducted under otherwise most intractable sampling conditions.

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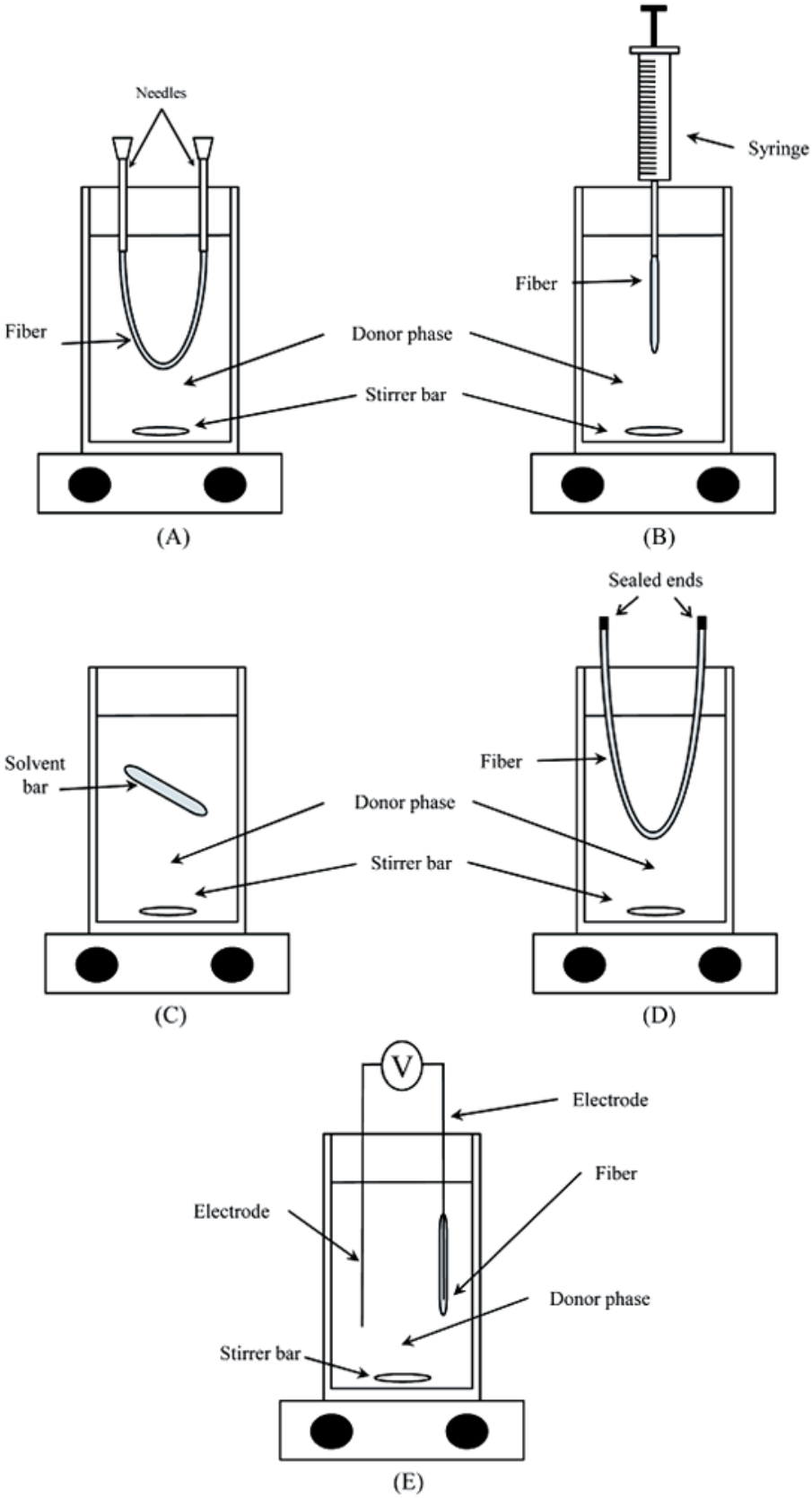

Figure 1. Some typical experimental setups used in HF-LPME. (A) Two needles inserted at both fiber ends (B) One fiber end sealed and a chromatographic srynge placed at the other end (C) Both fiber ends sealed and free fiber stirring (D) Both fiber ends sealed (E) Typical configuration used in electromigration.

TABLE 1 – Applications of two phase HF-LPME

	Matrix	Liquid Membrane	Detection limit	References
Pesticides				
Combination of pesticides	Wine, beer	Octanol	0.01 – 5.61 μg mL^{-1}	Bolanos 2008
Combination of pesticides	Environmental water	Toluene	3 – 7 ng L^{-1}	Sanagi 2010a
OCPs	River water, tomato, strawberry	Octanol	2.7 - 20 ng L^{-1}	Bedendo 2010
OPPs	Lake and ground water	Undecane	15 – 80 ng L^{-1}	Berhanu 2008
OPPs	Vegetable samples	Dodecane	0.099 – 0.128 μg mL^{-1}	Sanagi 2010b
OSPs	Waters, soil, beverages	o-xylene	1.16 – 48.48 μg L^{-1}	Xiong 2008
PCBs	Water	Nonane		Bayen 2009
PCBs	River water	Toluene	13 – 41 ng $mL^{.1}$	Li 2008b
Triazine herbicides	Surface water	Octanol	0.1 – 1 μg L^{-1}	Hu 2009
Miscellaneous Organic Compounds				
Aromatic hydrocarbons	Human urine and plasma	Toluene		Chen 2009b
Aromatic hydrocarbons	Tap water and snow	Dibutyl phthalate /Octanol	0.06 – 0.22 μg L^{-1}	Pusvaskiene 2010
Aromatic hydrocarbons	River and waste water	Octanol	0.005 – 0.03 μg mL^{-1}	Sarafraz-Yazdi 2008
Benzophenones	Human urine	Toluene	0.01 - 0.05 ng mL^{-1}	Ito 2009
Benzophenones	Human urine	Toluene	5 -10 pg mL^{-1}	Kawaguchi 2009a
Chlorophenols	Human urine	Toluene	0.1 – 0.2 ng mL^{-1}	Ito 2008
PAHs	Pine needles	Toluene	0.01 – 0.95 ng g^{-1}	Ratola 2008
Phenols	Human urine	Toluene	0.02 ng mL^{-1}	Kawaguchi 2008
Phenols	Water	Octanol		Liu 2009a
Trihalomethanes	Lake water	Octanol	0.03 – 0.05 μg L^{-1}	Gal 2008
Parabens	Urine	Chlorobenzene	0.01 – 0.20 μg mL^{-1}	Prichodko 2009
Petroleum spill	Spilled oils	Hexane		Li 2009

Pharmaceutical Analysis

Clenbuterol, metoprolol and propranolol	Urine	Methylbenzol/MSTFA	0.08 – 0.10 ng mL^{-1}	Liu 2009b
Diazepam and Prazepam	Human plasma	Octanol	2 ng ml^{-1}	Rasmussen 2000
Diuretics	Human urine	Octanol	0.3 – 6.7 ng mL^{-1}	Tsai 2008
Flunitrazepam	a) Human urine b) Human plasma	a) p-xylene b) p-xylene/Octanol	a) 0.001 ng mL^{-1} b) 0.025 ng mL^{-1}	Cui 2009
Ibuprofen, naproxen, ketoprofen, clofibric acid	Tap water and wastewater	Octanol	0.01 – 0.05 μg L^{-1}	Zhang 2009
Nicotine	Nightshade vegetables, food products	Toluene	2 ng g^{-1}	Shrivas 2010
Phenothiazine drugs	Human urine samples	Toluene	1.4 - 12.2 ng mL^{-1}	Xiao 2010
Progesterone	Human serum	Toluene	0.5 ng mL^{-1}	Kawaguchi 2009b

Others

Active film components	Active package films	Toluene	0.01 – 0.21 μg Kg^{-1}	Salafranca 2009
Chemical warfare agents	Water	Chloroform	0.04 – 0.36 μg L^{-1}	Lee 2008a
Methylmercury	Human hair, sludge	Toluene	0.4 μg L^{-1}	Jiang 2008
Selenoamino acids	Garlic, cabbage, mushroom	Toluene / Chloroform	11 – 23 ng L^{-1}	Duan 2009
Sudan dyes	Strawberry sauce, capsicum oil, salted egg, chilli sauce	Octanol	0.09 – 0.95 μg L^{-1}	Yu 2008
Ochratoxin A, T-2 toxin	Wine, beer	Octanol	0.02 – 0.09 μg L^{-1} (*)	Romero-Gonzalez 2010

(*) *Quantitation limit*

MSTFA = N-methyl-N-(trimethylsilyl) trifluoroacetamide, OCPs = organochlorine pesticides, OPPs = organophosphorus pesticides, OSPs = organosulfur pesticides, PCBs = polychlorinated biphenyls pesticides, *PAHs= Polycyclic aromatic hydrocarbons*

TABLE 2.a - Applications of three phase HF-LPME in clinical and pharmaceutical analysis.

	Matriz	Supported Liquid Membrane	Detection limits	References
Amphetamines				
	Human urine, whole blood, plasma	Dihexyl ether		Gronhaug Halvorsen 2001
	Whole blood and urine	Dihexyl ether	0.4 – 100 ng mL^{-1}	Halvorsen 2001b
	Human urine and plasma	Octanol	5 ng ml^{-1}	Pedersen-Bjergaard 1999
	Human urine and plasma	Octanol	0.7 - 3 ng mL^{-1}	Rasmussen 2000
Antidepressant drugs				
	Breast milk	Silicone oil	50 ng mL^{-1} (*)	Bjorhovde 2003
	Human urine, whole blood, plasma	Dihexyl ether		Gronhaug Halvorsen 2001
	Human plasma	Dihexyl ether	5 – 5.5 ng ml^{-1}	Halvorsen 2001a
	Human plasma	Dihexyl ether	1.25 ng mL^{-1}	Malagueno de Santana 2008
	Human urine and plasma	Octanol	5 ng ml^{-1}	Pedersen-Bjergaard 1999
	Human urine and plasma	Octanol	0.7 - 3 ng mL^{-1}	Rasmussen 2000
	Sewage and wastewater	Dihexyl ether	17 – 618 pg L^{-1}	Vasskog et al. 2008
Antidiabetic drugs				
	Human urine and plasma	Dihexyl ether	0.18 – 2.83 ng mL^{-1}	Al Azzam 2010
	Tap water, human urine and plasma	Dihexyl ether	1.0 μg L^{-1}	Tahmasebi 2009
Basic drugs				
	Human urine and plasma	Plant oils		Pedersen-Bjergaard 2004
	Human plasma	Dodecyl acetate, Octanol		Pedersen-Bjergaard 2005
NSAIDs				
	Sewage treatment plant effluent	Dihexyl ether	0.01 - 0.05 μg L^{-1}	Larsson 2009
	Human urine	Dihexyl ether	1.9 – 52.9 ng mL^{-1}	Payan 2009a
	Human urine, pharmaceuticals	Dihexyl ether	0.03 μg mL^{-1}	Payan 2009b
	Wastewater	Dihexyl ether	0.02 -0.3 μg L^{-1}	Ramos-Payan et al. 2010
	Water	Dihexyl ether	1 ng ml^{-1}	Pedersen-Bjergaard 2000
Others				
Anticonvulsant drugs	Human urine and plasma	Dihexyl ether	0.2 μg L^{-1}	Ebrahimzadeh 2010
Antifungal drugs	Tapwater, human urine and plasma	Dihexyl ether	0.9 – 4.0 μg L^{-1}	Moradi 2010
Antimalarial drugs	Rat plasma	Octanol	4.0 – 4.7 ng mL^{-1} (*)	Magalhaes 2008
Diuretics	Human urine	Octanol	0.5 – 2.0 ng mL^{-1}	Zhang 2008
Nicotine	Human urine		9.7 - 194.5 ng ml^{-1}	Fuh 2010
Pentazocine	Human urine and plasma	Octanol	2 ng ml^{-1}	Saraji 2010
Synthetic strogens	Wastewater	Octanol	0.25 - 0.50 μg L^{-1}	Liu 2008

(*) *Quantitation limit*

TABLE 2.b - Applications of three phase HF-LPME to the determination of aromatic organic compounds, pesticides and other compounds.

	Matrix	Supported Liquid Membrane	Detection limit	Reference
Aromatic organic compounds				
Aliphatic and aromatic hydrocarbons	Storm water	[bmim]PF6	1 – 7 ng L^{-1}	Basheer 2008a
Anilines	Tap, ground, river water	Toluene	0.01 – 0.1 μg L^{-1}	Sarafraz-Yazdi 2009
Aromatic amines	Tap and surface water	Dihexyl ether	0.05 – 0.10 μg L^{-1}	Zhao 2002
Aromatic carboxylic acids, Phenolic compounds	Food simulants	2-octanone, Chloropentane		Rodriguez 2008
Chlorophenols	Ground, river, waste, tap water	[omim][PF6]	0.5 – 1.0 μg L^{-1}	Peng 2007
Dinitrophenols				
Dinitrophenols	Human plasma	Dihexyl ether	0.05 – 0.1 μg mL^{-1}	Hansson 2009
Dinitrophenols	Human plasma	Dihexyl ether	0.05 – 0.1 μg mL^{-1} (*)	Hansson 2010
Heterocyclic amines	Human urine	Octanol	2 - 50 pg g^{-1}	Busquets 2009
Nitrophenols	Water	Octanol	0.01 – 0.16 mg L^{-1}	Sanagi 2010c
Pesticides				
Carbamate pesticides	Vegetables and fruits	Dodecanol	0.004 – 0.01 μg mL^{-1}	Xie 2009
Carbamate pesticidas	Vegetables	Dodecanol	2 μg Kg^{-1}	Fu 2009
Doxepin, Amitriptyline, Clomipramine, Mianserin	Water	Dihexyl ether		Baardstu 2007
Fungicides	Orange juice	2-octanone	0.05 - 0.10 μg L^{-1}	Barahona 2010
Others compounds				
Alkaloids	Corydalis yanhusuo		10.0 – 13.7 μg L^{-1}	Lu 2009
Biogenic amines	Shrimp sauce, tomato ketchup	Dihexyl ether	0.01 - 0.03 μg mL^{-1}	Saaid 2009
Methylmercury	Human hair, sludge	Toluene	0.1 μg L^{-1}	Jiang 2008
Methylmercury, ethylmercury, phenylmercury	Fish and human hair	Bromobenzene	0.03 – 0.14 μg L^{-1}	Li 2008a
Nerve agents degradation products	Water sample	Octanol	0.1 – 500 ng mL^{-1}	Tak 2009
Phytohormones	Coconut juice	Phenetole	0.0009 – 8.8 μg mL^{-1}	Wu 2009

(*) *Quantitation limit*
[bmim]PF6 = 1-butyl-3-methylimidazolium hexafluorophosphate, [omim][PF6] = 1-octyl-3-methylimidazolium hexafluorophosphate

TABLE 3.a - Carrier mediated HF-LPME applications with carrier dissolved in organic solvent

	Matrix	Liquid Membrane	Carrier	Detection limits	Reference
TWO PHASE					
Estrogenic hormones	Tap and sewage water	Dihexyl ether	10% TOPO	1.6 – 10 ng L^{-1}	Zorita 2008
Hg (II)	Sea water	Decaline/Cumene	*N*-benzoyl-*N'*,*N'*-diheptadecyl-thiourea		Fontas 2005
Phenoxy acid herbicides and phenols	Ground water, lake water, seawater	Dihexyl ether	10% TOPO	0.4 – 1.2 μg L^{-1}	Hu 2010
Pesticides	Natural and tap water	Dihexyl ether	TOPO (10%) + TBP (10%)	0.026 – 0.081 μg mL^{-1}	Trtic-Petrovic 2010
THREE PHASE					
Aminophosphonic pesticides	Groundwater	Dihexyl ether	Aliquat-336	0.22 – 3.40 μg L^{-1}	Piriyapittaya 2008
Aromatic amines	Sewage, lake, river, tap, well water	Dihexyl ether	8% TOPO	0.5 – 1.5 μg L^{-1}	Tao 2009b
Cu (II)	Leachate waer	Oleic acid / Dihexyl ether	1,10-dibenzyl-1,10-diaza-18-crown-6	5 μg L^{-1}	Romero 2005
Sulfonamides	Wastewater, duckery, fishery, paddy water	[C8MIM][PF6]	14% TOPO	0.1 – 0.4 μg L^{-1}	Tao 2009[a]
Tetracyclines	Bovine milk, human plasma, water	Octanol	Aliquat-336	0.5 – 1.0 μg L^{-1}	Shariati 2009

[C8MIM][PF6] = 1-Octyl-3-methylimidazolium hexafluorophosphate, TBP =Tributyl phosphate , TOPO = trioctylphosphine oxide

TABLE 3.b - Carrier mediated HF-LPME applications with ion – pair agent carriers

	Matrix	Supported Liquid Membrane	Carrier	Detection limits	Reference
TWO PHASE					
Inorganic ions					
As (III), As (V)	Human hair, tap, pond, river water	Toluene	Ammonium PDTC	0.12 ng mL^{-1}	Jiang 2009
As (III), As (V)	Synthetic, lake and tap water	Nitrobenzene	Ammonium PDTC	0.32 pg mL^{-1}	Pu 2009
ClO_4^-	Surface water	Octanol	Di-*n*-hexyl ammonium acetate	0.5 μg L^{-1}	Chen 2009a
Ni^{2+}, Pb^{2+}	Oyster tissue, tap, subterranean river, underground water	[C8MIM][PF6]	Ammonium PDTC	0.02 – 0.03 μg L^{-1}	Abulhassani 2010
Se (IV)	Human urine and plasma, tap and well water	Octanol	*o*-phenylenediamine	0.02 - 0.1 μg L^{-1}	Saleh 2009
Te (IV), Te (VI), Se (IV), Se (IV)	Environmental water and soil samples	Toluene	Ammonium PDTC	4 - 5 ng L^{-1}	Ghasemi 2010
V (IV), V (V)	Lake and sea water, vehicle exhaust particulates	CCl_4	Ammonium PDTC	71 - 86 pg mL^{-1}	Li 2007
Organic compounds					
11-nor-Δ^9-tetrahydro cannabinol-9-carboxylic acid	Human urine	N,O-bis(trimethylsilyl) trifluoroacetamide/ octane	TBA hydrogen sulphate	1 ng mL^{-1}	Kramer 2001
Dicocodimethylammonium chloride	Tap and process water	Octanol	Octanoate	0.9 μg L^{-1}	Hultgren 2009
Haloacetic acids	Tap and pool water	Decane	TBA hydrogen sulphate	0.3 – 15 μg L^{-1}	Cardador 2010
THREE PHASE					
Polar drugs					
	Human urine and plasma	Octanol	Sodium octanoate		Ho 2003
	Human plasma	Octanol	Octanoic acid		Pedersen-Bjergaard 2005

(*) Quantitation limit
[C8MIM][PF6] = 1-Octyl-3-methylimidazolium hexafluorophosphate, PDTC = Pyrrolidine dithiocarbamate, TBA = Tetrabutylammonium

TABLE 4 – Applications of electromigration HF-LPME

	Matrix	Supported Liquid Membrane	Potential	Reference
Acidic drugs				
	Water	Heptanol	50 V	Balchen 2007
Pb^{2+}				
	Human serum, urine and amniotic fluid and lipstick	Toluene	300 V	Basheer 2008b
Peptides				
	Water	15% di-(2-ethylhexyl) phosphate in 1-octanol	50 V	Balchen 2008
	Human plasma	8% di-(2-ethylhexyl) phosphate in 1-octanol	15 V	Balchen 2009
	Water	10% di-(2-ethylhexyl) phosphate in 1-octanol/di-isobutylketon	50 V	Balchen 2010
	Water	a) Eugenol (hydrophobic and intermediate peptides) b) 10% di-(2-ethylhexyl) phosphate in 1-octanol/di-isobutylketon (hydrophobic and hydrophilic peptides) c) 5% 15-crown-5 ether in 1-octanol (hydrophobic peptides)	25 V	Balchen 2011
Basic drugs				
	Human plasma	1-ethyl-2-nitrobenzene	9 V	Eibak 2010
	Human plasma and urine	2-nitrophenyl octyl ether	150 V	Eskandari 2011
	Water	2-nitrophenyl pentyl ether / 25% di-(2-ethylhexyl) phosphate in 2-nitrophenyl pentyl ether	300 V	Gjelstad 2006
	Water	2-nitrophenyl pentyl ether	300 V	Gjelstad 2007a
	Water	2-nitrophenyl octyl ether	300 V	Gjelstad 2007b
	Human plasma, whole blood	1-ethyl-2-nitrobenzene	10 V	Gjelstad 2009
	Human plasma, urine, breast milk	1-isopropyl-4-nitrobenzene	10 V	Kjelsen 2008
	Water	2-nitrophenyl pentyl ether / 10% di-(2-ethylhexyl) phosphate in 2-nitrophenyl pentyl ether	50 V	Middelthon-Bruer 2008
	Human plasma and urine	2-nitrophenyl octyl ether	300 V	Pedersen-Bjergaard 2006

Conclusiones

Del estudio realizado, que se resume en la presente Memoria, cabe destacar los aspectos que se recogen en las siguientes conclusiones:

1. Se han optimizado diferentes procedimientos de microextracción en fase líquida a través de membranas líquidas soportadas sobre fibras huecas de polipropileno empleando configuraciones en tres fases y microextracción mediante electromembranas para la determinación de diversos principios activos con actividad farmacológica. Los procedimientos se han aplicado satisfactoriamente al análisis de muestras biológicas y medioambientales con excelentes resultados.

2. Se ha puesto a punto un procedimiento rápido de microextracción en fase líquida a través de membranas líquidas soportadas sobre fibra hueca en una configuración de tres fases para la determinación de ibuprofeno y su posterior determinación mediante un sistema FIA con detector de quimioluminiscencia, siendo la primera vez que se combinan ambas técnicas. El procedimiento propuesto permite un límite de cuantificación de 100 µg/L y una reproducibilidad del 1.6%. Se ha aplicado satisfactoriamente a la determinación de ibuprofeno en muestras de orina humana y diferentes preparaciones farmacéuticas obteniendo en ambos casos recuperaciones de prácticamente el 100%. La simplicidad del método lo convierte en una atractiva alternativa a otras técnicas de análisis.

3. Se ha optimizado la separación y extracción de ácido salicílico, diclofenaco e ibuprofeno usando una membrana líquida soportada sobre fibra hueca en una configuración de tres fases, y su posterior determinación mediante cromatografía líquida de alta resolución con detectores de diodo array y fluorescencia, conectados en serie. Las reproducibilidades obtenidas oscilan entre el 1.1% y el 2.3%. Se obtuvieron limites de cuantificación de 41, 176 y 135 µg/L para ácido salicílico, diclofenaco e ibuprofeno, respectivamente, en el caso de DAD, y 23 y 6 µg/L para ácido salicílico e ibuprofeno, respectivamente, en el caso de fluorescencia. El método permite el análisis directo en orina humana, obteniéndose excelentes líneas bases en los correspondientes cromatogramas. Los resultados obtenidos demostraron que también se extraen sus correspondientes metabolitos aunque no pudieron ser determinados por carecer de los correspondientes patrones.

4. Se ha optimizado un procedimiento de separación cromatográfica con detección MS/MS para ácido salicílico, diclofenaco e ibuprofeno tras la microextracción con fibra hueca. Los límites de cuantificación obtenidos varían en el rango 0.1-0.50 µg/L y se obtienen reproducibilidades entre el 1.5% y el 2.1%. El procedimiento ha sido satisfactoriamente aplicado a la determinación directa de los analitos en diversas aguas medioambientales: aguas residuales urbanas, agua de río, agua de lago y agua potable, con recuperaciones en torno al 100%, excepto para ibuprofeno y diclofenaco que, en aguas residuales, muestran recuperaciones del 50 y 70%, respectivamente.

5. Se ha optimizado un procedimiento de microextracción en fibra hueca en una configuración de tres fases, y posterior determinación de siete antiinflamatorios no esteroideos: ibuprofeno, naproxeno, ketoprofeno, diclofenaco, ketorolaco, aceclofenaco y ácido salicílico, empleando un sistema de electroforesis capilar con detector de diodo array. Los límites de cuantificación obtenidos para los analitos estudiados varían entre 0.25-0.86 µg/L, con reproducibilidades entre el 2.1 y 3.2%. El procedimiento se ha aplicado a la determinación de los analitos en aguas residuales y agua de río, obteniéndose excelentes líneas bases y recuperaciones entre el 59 y el 93 %, y cinco de ellos han sido cuantificados o detectados en diferentes aguas residuales urbanas. Además el procedimiento ha sido satisfactoriamente aplicado a la determinación de los analitos en orina humana, obteniéndose recuperaciones superiores al 81%, e incluso se ha puesto de manifiesto la posibilidad de la determinación de algunos de sus metabolitos.

6. Se ha optimizado un procedimiento de microextracción en fibra hueca mediante electromembranas para el análisis de siete antiinflamatorios no esteroideos: ibuprofeno, naproxeno, ketoprofeno, diclofenaco, ketorolaco y ácido salicílico, con posterior determinación mediante cromatografía líquida de alta resolución con detectores de diodo array y fluorescencia, conectados en serie. Los límites de cuantificación para los analitos estudiados varían entre 0.29 y 11.1 µg/L, y entre 0.003 y 3.1 µg/L para DAD y FLD, respectivamente, con reproducibilidades entre el 2.7 y el 5.3%. El procedimiento ha sido aplicado a la determinación de los analitos en agua de residuales y agua de río, con recuperaciones del orden de 100 % para diclofenaco, 75 % para ibuprofeno y del 60 % para el resto de los analitos.

7. Se ha optimizado la extracción y posterior determinación de cuatro sulfonamidas, sulfadiazina, sulfamerazina, sulfametazina, sulfametoxazol y sus correspondientes N^4-acetil metabolitos, sintetizados en nuestro laboratorio. La extracción se lleva a cabo mediante microextracción en fibra hueca en una configuración de tres fases y la determinación cromatográfica posterior se realizó usando detectores de diodo array y fluorescencia, conectados en serie. El procedimiento propuesto permite limites de cuantificación para los analitos estudiados entre 3-50 ng/L y entre 0.9-100 ng/L para DAD y FLD, respectivamente, y la reproducibilidad se encuentra entre 1 y 1.8 %. El procedimiento ha sido aplicado a la determinación de los analitos en diversas aguas medioambientales: aguas residuales urbanas, agua de río, agua de lago y agua potable, con recuperaciones cercanas al 100% en el caso de agua de rio, agua de lago y agua potable, y con recuperaciones entre el 33-90% en aguas residuales, excepto para la sulfametazina, y los metabolitos de la sulfamerazina y sulfametoxazol, cuyas recuperaciones están en torno al 100%. Además, el procedimiento ha sido satisfactoriamente aplicado a la determinación de los analitos y sus correspondientes metabolitos en orina humana, obteniéndose recuperaciones del 100 % en todos los casos.

8. Se ha puesto a punto un procedimiento de microextracción en fibra hueca en configuración de tres fases para la determinación de ocho fluoroquinolonas: marbofloxacina, norfloxacina, danofloxacina, gatifloxacina, grepafloxacina, flumequina, enrofloxacina, y su metabolito, ciprofloxacina. La determinación se llevó a cabo mediante cromatografía líquida de alta resolución con detectores de diodo array y fluorescencia conectados en serie. El procedimiento propuesto permite límites de cuantificación para los analitos estudiados entre 1-50 ng/L, y una reproducibilidad de entre el 1 y el 1.8 %. El procedimiento ha sido aplicado a la determinación de los analitos en diversas aguas mediambientales, con recuperaciones superiores al 90%. Se ha aplicado también a su determinación en muestras de orina bovina, obteniéndose extractos limpios, y recuperaciones en torno al 100% excepto para la flumequina, con recuperaciones del 75%. Se han determinado enrofloxacina y su metabolito, ciprofloxacina, en muestras de orina procedentes de animales a los que se les suministró el fármaco (ALSIR® 5%).

9. El procedimiento de microextracción en fase líquida empleando fibra hueca, se ha convertido en una de las técnicas analíticas más usadas en los últimos años como un procedimiento de tratamiento de muestras, que ofrece simplicidad, bajo coste, y permite una alta selectividad y sensibilidad, y, en muchos casos, se obtienen mejores factores de enriquecimiento en comparación con otras técnicas de extracción. Además, el consumo de disolvente orgánico es mínimo, del orden de los microlitros, acordes con la actual tendencia de una "Química Verde". El pequeño volumen de muestra que se recoge en la fase aceptora hace que no sea necesario evaporar y preconcentrar la muestra previa al análisis. Esta técnica de extracción ofrece excelentes *clean-up* y permite inyectar la muestra directamente en un cromatógrafo obteniendo excelentes líneas bases. Todos los procedimientos que se han desarrollado en esta Tesis, permiten la determinación de los analitos en muestras biológicas y medioambientales, incluyendo aguas residuales urbanas, que por lo general, requieren complejos pretratamientos de muestra previo al análisis y etapas de preconcentración. Por otro lado, se han obtenidos factores de enriquecimiento entre el 32-1000, permitiendo la cuantificación de los analitos a niveles muy bajos en muestras reales.

The most remarkable conclusions derived from this research are listed below:

1. Different liquid phase microextraction procedures based on the use of liquid membranes supported on hollow fibers. Three phase configurations and electromembrane extraction procedures have been optimized for the analysis of pharmacological active substances. All proposed methods were successfully applied to biological and environmental samples allowing excellent results.

2. A rapid hollow fiber-based liquid-phase microextraction method for ibuprofen, using a polypropylene membrane supporting organic solvent followed by a chemiluminescence determination has been optimized in order to propose a rapid and simple determination of ibuprofen which is the first analytical procedure proposed that combines both techniques. The quantitation limit was 0.1 µg/mL and the interday repeatability was 1.6%. The method has been applied to the ibuprofen determination in pharmaceuticals and in real human urine samples, with recoveries of around 100%. The simplicity of the extraction process and analysis procedure, makes the developed method an attractive alternative to other methods.

3. A microextraction method using a polypropylene membrane supporting organic solvent (three-phase hollow fiber-based liquid phase microextraction HF-LPME) was developed for the determination of salicylic acid and two non-steroidal anti-inflamatories drugs: diclofenac and ibuprofen. All the HF-LPME and HPLC parameters were optimized to develop a rapid, simple and sensitive method for the determination of the drugs. Detection was realized by means of a coupled in series diode array and fluorescent detectors in series mode. Quantitation limits with DAD were 41, 176 and 135 µg/L for salicylic acid, diclofenac and ibuprofen, respectively, and with FLD were 23 and 6 µg/L for salicylic acid and ibuprofen, respectively. The interday repeatability was between 1.1 and 2.3%. The method has been successfully applied to their direct determination in human urine and the results obtained demonstrated that it could be also applied to the determination of their corresponding metabolites.

4. A high performance liquid chromatography-mass/mass spectrometry method prior hollow fiber liquid-phase microextraction was developed for the direct analysis of

three acidic pharmaceuticals (salicylic acid, ibuprofen and diclofenac) in raw and treated wastewaters and other environmental waters. Quantitation limits were 0.1, 0.25 and 0.5 µg/L for salicylic acid, diclofenac and ibuprofen, respectively; intermediate precision was in the range of 1.5-2.1%. Extraction effectiveness for the analyzed substances were around 100 %. Recoveries slightly decrease for ibuprofen and diclofenaco in the analysis of wastewaters. The excellent clean-up obtained implies a great advantage over other sample treatment procedures. Raw wastewater sample showed ibuprofen a level that allows its determination and salicylic acid and diclofenac were detected.

5. A capillary electrophoresis determination for some non steroidal anti-inflammatory drugs (ibuprofen, naproxen, ketoprofen, diclofenac, ketorolac, aceclofenac and salicylic acid) using hollow fiber liquid phase microextraction has been developed. The extraction was carried out using a polypropylene membrane in a three phase configuration. Quantitation limits between 0.85 and 2.87 µg/L were obtained, and interday repeatability was in the range 2.1 to 3.2%. The method could be applied to the direct determination of the seven anti-inflammatories in wastewaters, obtaining recoveries between 59-93 %, and five of the analytes were determined or detected in different real urban wastewaters. In addition, the method has been successfully applied to their direct determination in human urine, obtaining recoveries between 81-100 %. The results obtained demonstrated that it could also be applied to the determination of their corresponding metabolites.

6. A rapid electromembrane extraction (EME) combined with a HPLC procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of six non-steroidal anti-inflammatory drugs (NSAIDs): salicylic acid, ketorolac, ketoprofen, naproxen, diclofenac and ibuprofen. The procedure allows low quantitation limits between 0.29-11.1 µg/L and 0,003-3.1 µg/L, by DAD and FLD, respectively; interday repeatability was in the range 2.7 – 5.3%. The direct application of the proposed procedure to urban wastewater samples shows, in general, good results in terms of recovery, approximately 60% and high for all samples, 70% and high for IBU and nearly 100% for DIC.

7. A highly sensitive method by hollow fiber liquid phase microextraction combined with a liquid chromatography procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of four sulfonamides: sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and their main metabolites N^4-acetyl derivatives, synthesized previously in our laboratory. The procedure allows very low quantitation limits in the range 3-100 ng/L and an intermediate precision of 1-1.8%. The proposed method was applied to the determination of the analytes in environmental water (surface, tap and wastewater) and urine samples. In surface and tap water samples, recoveries for all the compounds are within 93 and 101%. The direct application of the proposed HF-LPME procedure to the different wastewater types analyzed show, in general, excellent results with recoveries within 99 and 101% for SMZ, NSMR and NSMX, however the rest of the analyzed compounds show variable recoveries within 33 and 90%. In addition, the procedure has been demonstrated adequate for the determination of the analysis at the usual levels in human urine samples and their corresponding N^4-acetyl metabolites, with recoveries ranged between 99-100 %.

8. A three phase-hollow fiber-based liquid phase microextraction (HF-LPME) combined with a liquid chromatography procedure using diode array (DAD) and fluorescence detection (FLD) has been developed for the determination of eight fluoroquinolones: marbofloxacin, norfloxacin, danofloxacin, gatifloxacin, grepafloxacin, flumequine, enrofloxacin, and its metabolite, ciprofloxacin. The procedure allows very low quantitation limits (1-50 ng/L) and an intermediate precision in the range 1-1.8%. The proposed procedure has been demonstrated adequate for the determination of the analytes in bovine urine and environmental water samples (surface, tap and wastewater). In general, excellent recoveries were obtained with values of practically 100%, only marbofloxacin and flumequine show slightly values for some of the analyzed simples, but in any case, in the range of 87-97% in wastewaters and recoveries between 76-79% for flumequine in cow urine samples. Enrofloxacin and its metabolite, ciprofloxacin, were measured in a real urine sample from a Jersey cow submitted to an enrofloxacin treatment with injectable ALSIR® 5%.

9. The proposed hollow fiber-based liquid phase microextraction (HF-LPME) procedures have been demonstrated to be simple sample pre-treatments that offer simplicity, low cost, high selectivity, sensitivity and in most cases better enrichment compared to most traditional extraction techniques. In addition, the consumption of solvent is significantly reduced by up to several hundred or several thousand times, according to the current trends to a "Green Chemistry". Due to the small volume of extracting solvent, the extracted samples do not require further concentration prior to analysis and thus total analysis time considering decreases in comparison to traditional liquid-liquid extraction (LLE procedures). The excellent clean-up obtained implies a great advantage over other simple treatment procedures and the extracts can be directly injected into the chromatography system allowing excellent baselines. All procedures developed in this Thesis have been demonstrated to be adequate for the determination of the analytes in biological and environmental samples, including urban wastewaters that usually require tedious clean-up and preconcentration steps. In addition, the high enrichments obtained allow the determination at very low levels and could be used for the application in other biological and environmental matrices.

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