UNIVERSIDAD DE JAÉN

ESCUELA POLITÉCNICA SUPERIOR DE LINARES
DEPARTAMENTO DE QUÍMICA FÍSICA Y ANALÍTICA

TESIS DOCTORAL

AUTOMATIZACIÓN Y SIMPLIFICACIÓN DE LAS ETAPAS DE PREPARACIÓN DE MUESTRA PARA LA DETERMINACIÓN DE SUSTANCIAS FARMACOLÓGICAMENTE ACTIVAS EN MATRICES AMBIENTALES, ALIMENTARIAS Y BIOLÓGICAS

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Grado de Doctor por la Universidad de Jaén

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CAPÍTULO II

CAPÍTULO III

OBJETIVOS

OBJETIVOS

El objeto principal de esta Memoria ha consistido en el desarrollo de nuevas metodologías para la determinación simultánea de diferentes tipos de sustancias farmacológicamente activas (antibióticos, antiinflamatorios no esteroideos/analgésicos, β-bloqueadores, reguladores de lípidos, hormonas, antiepilépticos, antidepresivos y antisépticos) en muestras ambientales, alimentos y fluidos biológicos. Para ello se ha profundizado en el estudio de la primera etapa del proceso analítico, las operaciones previas, que se encuentran menos desarrolladas que las etapas de medida/transducción de la señal y adquisición/tratamiento de datos.

Los objetivos más específicos que se han marcado son:

a) Puesta a punto de un sistema continuo para la extracción en fase sólida de sustancias farmacológicamente activas basado en el uso de una columna empaquetada con un sorbente adecuado con el fin de eliminar las interferencias de la matriz de la muestra y aumentar la sensibilidad a través de la preconcentración de los analitos.

b) Simplificación de las etapas de pretratamiento de la muestra previa a la introducción del sistema continuo para la eliminación de algunas sustancias presentes en las matrices de las muestras complejas (suelos, alimentos y fluidos biológicos) que pueden obstruir el sistema continuo, mediante la adición de un disolvente adecuado y centrifugación o uso de un horno de microondas.

c) Simplificación de la etapa de derivatización de las sustancias farmacológicamente activas previa a la determinación mediante cromatografía de gases-espectrometría de masas.

d) Aplicación de las metodologías desarrolladas a la determinación de fármacos en diferentes tipos de muestras ambientales (aguas, suelos, sedimentos y lodos), alimentos (leche, carne y pescado) y fluidos biológicos (sangre y orina).

RESUMEN

RESUMEN

En la presente Tesis Doctoral se ha planteado como objetivo principal la simplificación y automatización de las etapas previas de preparación de muestras para la determinación simultánea de diferentes tipos de sustancias farmacológicamente activas (antibióticos, antiinflamatorios no esteroideos/analgésicos, β-bloqueadores, reguladores de lípidos, hormonas, antiepilépticos, antidepresivos y antisépticos) en muestras ambientales, alimentos y fluidos biológicos. Se ha puesto a punto un sistema continuo para la extracción en fase sólida basado en el uso de una columna empaquetada con 60 mg del sorbente Oasis HLB con objeto de eliminar las interferencias de la matriz de la muestra y aumentar la sensibilidad a través de la preconcentración de los analitos de interés. En el caso de matrices complejas, tales como alimentos, suelos y fluidos biológicos, se han simplificado las etapas de pretratamiento de muestra con objeto de eliminar los componentes de éstas que pueden perturbar a las etapas de extracción en fase sólida, separación cromatográfica o ionización en el detector de espectrometría de masas de los analitos de interés. Esta etapa se ha llevado a cabo mediante la adición de un disolvente adecuado (acetonitrilo o metanol) y centrifugación o extracción en un horno de microondas convencional. También se ha minimizado el consumo de reactivos usados en la etapa de derivatización por sililación de las sustancias farmacológicamente activas, reduciéndose notablemente el tiempo requerido en la reacción cuando se llevaba a cabo en un horno de microondas.

Las diferentes metodologías desarrolladas han permitido la determinación de hasta 22 sustancias farmacológicamente activas (ácido acetilsalicílico, ácido clofíbrico, ácido mefenámico, ácido niflúmico, carbamazepina, cloranfenicol, diclofenaco, estrona, 17β-estradiol, 17α-etinilestradiol, fenilbutazona, florfenicol, flunixino, ibuprofeno, ketoprofeno, metoprolol, naproxeno, paracetamol, propranolol, pirimetamina, tiamfenicol y triclosán) en diferentes tipos de muestras con la suficiente sensibilidad para la cuantificación de estas sustancias a niveles inferiores de ng/l con una buena precisión (desviación estándar relativa inferior a 7 %) y exactitud (recuperaciones próximas al 100 %).

Es importante resaltar la gran variedad de muestras que han sido analizadas mediante los métodos desarrollados en esta Memoria. En la mayoría de las muestras ambientales (aguas, suelos, sedimentos y lodos), alimentos (leche, carne y pescado) y

fluidos biológicos (sangre y orina) se ha encontrado la presencia de algunos antiinflamatorios no esteroideos/analgésicos, antibióticos, hormonas y productos de cuidado personal.

PALABRAS CLAVES: Sustancias farmacológicamente activas; matrices ambientales; alimentos; fluidos biológicos; sistema continuo de extracción en fase sólida; cromatografía de gases-espectrometría de masas.

SUMMARY

The main goal of this doctoral work was to simplify and automate the preliminary sample preparation stages required for the simultaneous determination of various types of pharmacologically active substances including antibiotics, non-steroidal anti-inflammatories/analgesics, β-blockers, lipid regulators, hormones, anti-epileptics, antidepressants and antiseptics in environmental, food and biological matrices. To this end, a continuous solid-phase extraction system based on a column packed with 60 mg of Oasis HLB sorbent was used to preconcentrate the target analytes by extraction without interference from the sample matrix in order to increase the sensitivity of the determination method. The pretreatment steps for complex samples such as foods, soils and biological fluids were simplified in order to remove any components potentially interfering with the solid-phase extraction, chromatographic separation or mass spectrometry ionization detection of the target analytes. This was accomplished by using an appropriate solvent (acetonitrile or methanol) and centrifugation or extraction in a conventional microwave oven. Also, reagent consumption in the derivatization of pharmacologically active substances by silylation was minimized and the process expedited by conducting the reactions in the oven.

The analytical methods used allowed the determination of up to 22 pharmacologically active substances including acetylsalicylic acid, clofibric acid, mefenamic acid, niflumic acid, carbamazepine, cloramphenicol, diclofenac, estrone, 17β-estradiol, 17α-ethinylestradiol, phenylbutazone, florfenicol, flunixin, ibuprofen, ketoprofen, metoprolol, naproxen, paracetamol, propranolol, pyrimethamine, thiamphenicol and triclosan in various types of samples with a sensitivity enabling their

quantitation at sub-nanogram-per-millilitre levels with good precision (relative standard deviations less than 7 %) and a high accuracy (near-quantitative recoveries).

Worth special note was the wide variety of samples successfully analysed in this way. Most of the environmental (water, soil, sediment and sludge), food (milk, meat and fish) and biological fluid (blood and urine) samples were found to contain non-steroidal anti-inflammatories/analgesics, antibiotics, hormones and personal care products.

KEYWORDS: Pharmacologically active substances; environmental matrices; foods; biological fluids; continuous solid-phase extraction system; gas chromatography-mass spectrometry.

INTRODUCCIÓN

1. SUSTANCIAS FARCOLÓGICAMENTE ACTIVAS

En los últimos años, la Química Analítica ha jugado un papel importante en la obtención de información sobre el Medio Ambiente, alimentos y fluidos biológicos. Por ello, uno de los principales objetivos de la misma es desarrollar nuevos métodos analíticos para determinar los contaminantes a niveles de trazas en diferentes tipos de muestras. En este sentido, ha habido en los últimos años un número elevado de publicaciones sobre la determinación de residuos en las aguas (residuales, subterráneas, superficiales o potables), sólidos (sedimentos, suelos o lodos), atmosféricas, alimentos (carne, leche, huevos, verduras, aceites vegetales, etc.) o fluidos biológicos (sangre, plasma, pelo u orina).

Hoy en día, muchas sustancias han sido identificadas como peligrosas para la salud humana y/o el Medio Ambiente, y su uso ha sido prohibido o regulado por las diferentes legislaciones con el fin de reducir la exposición a este tipo de sustancias. Uno de los grupos de contaminantes ampliamente estudiados en las muestras ambientales, en alimentos y en fluidos biológicos son los contaminantes orgánicos persistentes. Diferentes estudios han reportado la presencia de contaminantes tales como hidrocarburos policíclicos aromáticos, bifenilos policlorados o plaguicidas organoclorados en las aguas naturales (Zhang et al., 2007), agua de mar (Sapota, 2004), sedimentos y suelos (Budzinski et al., 1997), atmósfera (Yagoh et al., 2006) y alimentos (Albero et al., 2003; Ferrer y Thurman, 2007; García-Reyes et al., 2007). Por otro lado, las sustancias farmacológicamente activas (SFAs) pertenecen a un grupo de contaminantes que está teniendo mucho interés por parte de los científicos y gobiernos de todo el mundo. Algunos datos preliminares sobre la presencia de SFAs se publicaron

en 1976 en USA y en 1985 en Inglaterra, pero la investigación sistemática se inició en los años 90 cuando los científicos alemanes publicaron resultados obtenidos de estudios de los análisis de aguas de rio (Ternes, 1998). Posteriormente, en diferentes estudios se ha demostrado que la concentración de estas sustancias eran bajas (niveles de ng/l) en muestras de agua de diferentes países como España (Pedrouzo et al., 2007; Reverté et al., 2003), Reino Unido (Thomas y Hilton, 2004), Alemania (Sacher et al., 2001) y USA (Yang y Carlson, 2004; Batt y Aga, 2005). En la última década, la investigación se ha centrado fundamentalmente en la identificación, evolución y los efectos de estos compuestos en muestras procedentes de plantas de tratamiento de aguas residuales debido a que el agua después de su depuración suele ser descargada a los ríos, y estos contaminantes pueden afectar el Medio Ambiente acuático. Por ejemplo, Ternes (1998) encontró distintos fármacos tales como el propanolol, carbamazepina, bezafibrato y diclofenaco en las aguas residuales afluentes y efluentes de distintas estaciones depuradoras de Alemania. Recientemente, la presencia SFAs también se ha estudiado en los lodos de depuradora, ya que los lodos pueden ser utilizados como abono agrícola y estas sustancias pueden contaminar las aguas subterráneas e introducirse en la cadena alimentaria (Kinney et al., 2008).

Una amplia variedad de sustancias farmacológicamente activas, por ejemplo antibióticos, antiinflamatorios, hormonas y otros, pueden contaminar a los alimentos en distintos puntos de la cadena alimentaria. El problema surge cuando residuos de estos fármacos llegan al consumidor a niveles que pueden ser perjudiciales para su salud. Por ejemplo, la presencia de residuos de fármacos en la leche, puede inducir alergias y resistencias bacterianas; a otro nivel puede afectar a los procesos de industrialización de la leche. Y quizá el aspecto que más temor causa, es la incertidumbre de que aún no se sabe cuáles y que efectos tendrán los residuos de multitud de fármacos ingeridos de manera crónica a lo largo de la vida de un consumidor. Un fármaco muy estudiado por sus efectos adversos a la salud humana es el cloranfenicol en los alimentos de origen animal, demostrándose que 1 ppm de éste puede inducir la aparición de anemia aplástica en personas. Por ello es importante detectar los residuos de SFAs en los productos de origen animal por tres razones principales: a) no hacerlo es un procedimiento ilegal, b) es posible que estos productos contengan patógenos además de fármacos puesto que generalmente provienen de una infección, y c) la presencia de fármacos y sus metabolitos activos puede facilitar el desarrollo de reacciones anafilácticas o de otros efectos colaterales. Diferentes trabajos encontrados en la literatura han puesto de

manifiesto la existencia de diferentes residuos de fármacos de diferentes clases terapéuticas en muestras de alimentos de origen animal y fluidos biológicos. Así, Courant et al. (2008) han detectado hormonas esteroideas en leche de origen animal, leche humana, huevos, carne, plasma y orina. En un estudio llevado a cabo por Daesleire et al. (2003) han detectado antiinflamatorios en muestras de leche. También Allmyer et al. (2006a) han encontrado triclosán en plasma y muestras de leche.

El trabajo que se va a desarrollar en esta Memoria se centra en la determinación de sustancias farmacológicamente activas en varias tipos de muestras ambientales, alimentos de origen animal y fluidos biológicos. En la Figura 1 se muestran las principales vías de introducción de estas sustancias en el Medio Ambiente y como pueden introducirse en la cadena alimentaria. El origen de las SFAs puede estar en el tratamiento de personas o de animales. Las SFAs son excretadas por éstos como el compuesto original y/o metabolitos y vertidas a las aguas residuales domésticas o procedentes de granjas. También, a menudo se eliminan de manera inadecuada medicamentos no deseados o caducados directamente en las aguas residuales. Por lo tanto, estas sustancias pueden llegar a las plantas de tratamiento de aguas residuales (E.T.A.R.) a concentraciones elevadas y, si los procesos de depuración no son efectivos para su eliminación de SFAs, pueden permanecer en las aguas superficiales o en los sedimentos durante mucho tiempo.

Debido al impacto de residuos de SFAs en el Medio Ambiente, alimentos y fluidos biológicos, el interés por el desarrollo de metodologías para la determinación de estas sustancias está teniendo un interés elevado en los últimos años. Los métodos deben ser sensibles y selectivos debido a que estos contaminantes están presentes a concentraciones muy bajas (niveles de ng/l) y en muestras complejas. Para lograr este objetivo, los métodos utilizados incluyen etapas de extracción y/o preconcentración y de separación mediante técnicas cromatográficas acopladas con sistemas de detección sensibles.

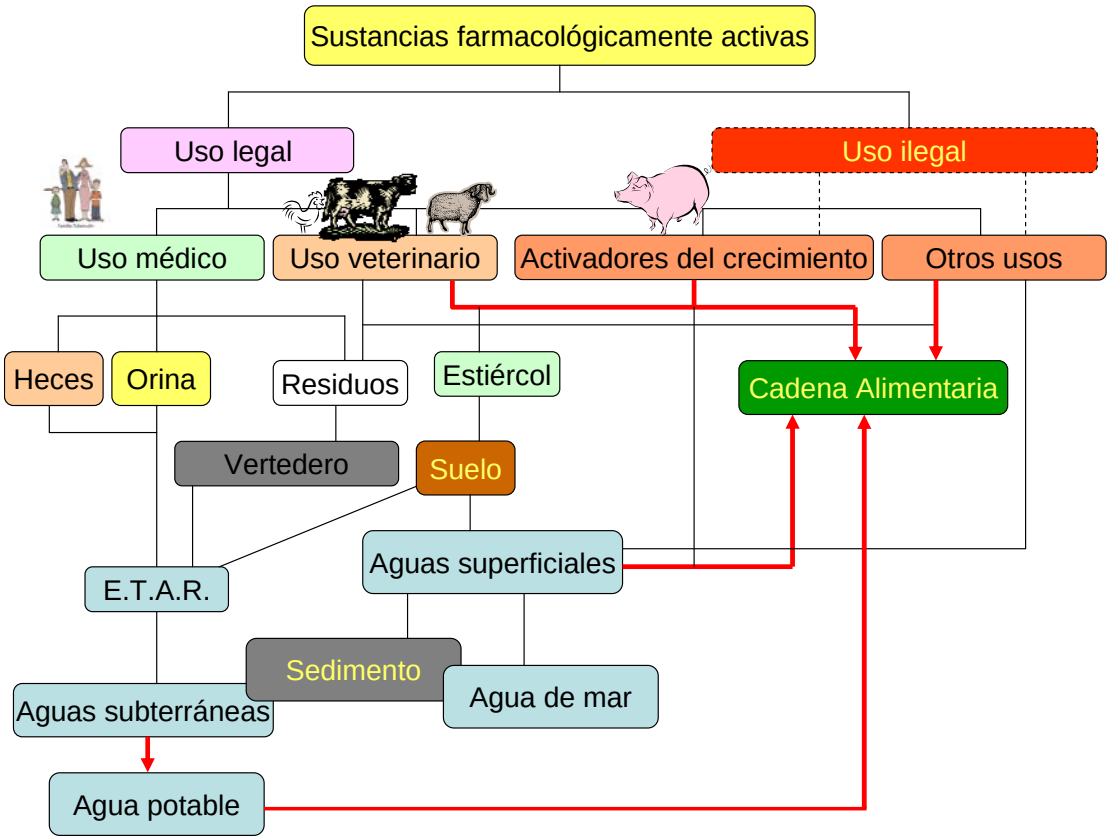


Figura 1: Vías de entrada y distribución de sustancias farmacológicamente activas en el Medio Ambiente y como pueden llegar a la cadena alimentaria.

Como se ha comentado anteriormente, la etapa de preparación de muestra es uno de las etapas más importantes en el desarrollo de nuevos métodos para la determinación de SFAs debido a la complejidad de las muestras analizadas y a la baja concentración que se encuentran estos residuos en las muestras analizadas. Para la extracción de estas sustancias en muestras sólidas o semisólidas se han utilizado diferentes técnicas de extracción tales como la extracción Soxhlet o ultrasonidos. Hoy en día, las técnicas clásicas se están sustituyendo por otras que se basan en el uso de volúmenes reducidos de disolventes y en el uso de altas temperaturas y presiones. Estas técnicas son la extracción asistida por microondas, la extracción de líquido a presión y la extracción en fase sólida SPE. Esta última técnica es la más empleada actualmente para el tratamiento de muestras ambientales y de alimentos. Además, otras técnicas como la microextracción en fase sólida o la microextracción en fase líquida también se están utilizando ampliamente en la actualidad para la extracción de fármacos de agua o fluidos biológicos (Ying, 2007).

Una variedad de técnicas se han utilizado a lo largo de la historia para la determinación de SFAs, de ellas las más utilizadas son la cromatografía de gases (GC) y cromatografía líquida (LC). En el caso de la cromatografía líquida se han utilizado diferentes detectores (fluorescencia, UU-vis, quimioluminiscencia), pero el que más posibilidades ofrece por su información y sensibilidad es el detector de espectrometría de masas (Botsoglou y Fletouris, 2004; Petrovic et al., 2005). En los métodos basados en el uso de la cromatografía de gases, se están empleando fundamentalmente el detector de espectrometría de masas (Maurer, 2004). .

La introducción de la Memoria está dividida en dos apartados. En el primero se van a clasificar a las sustancias farmacológicamente activas, indicándose sus características, aplicaciones, modo de acción, toxicidad y legislación existente sobre el uso de estas sustancias, centrándose principalmente en las SFAs objeto de estudio de esta Memoria en posteriores capítulos. En la segunda parte se describirán las técnicas analíticas más importantes más para la preparación de muestra y determinación de SFAs en diferentes tipos de muestras.

1.1. Clasificación de las sustancias farmacológicamente activas

Las sustancias farmacológicamente activas son cada vez más utilizadas en medicina humana y veterinaria. Su consumo es un aspecto muy importante, y así, por ejemplo en la Unión Europea se utilizan en medicina humana más de 3000 sustancias diferentes (Christen et al, 2010). Los fármacos utilizados en medicina humana más consumidos son los antiinflamatorios no esteroideos/analgésicos, antibióticos, reguladores de lípidos, β-bloqueadores, antiepilépticos, antisépticos y hormonas esteroideas (Christen et al, 2010; Fent et al, 2006). También un gran número de fármacos se utilizan en veterinaria, entre ellos sobresalen los antibióticos y antiinflamatorios no esteroideos. Las cifras de ventas en los distintos países son relativamente altas como se indica en la Tabla 1. Como se puede observar el ácido acetilsalicílico (aspirina) y el paracetamol son los fármacos más consumidos en muchos de los países estudiados (Stuer-Lauridsen et al., 2000; Jones et al., 2002; Calamari et al., 2003; Huschek et al., 2004; Khan y Ongerth, 2004; Carballa et al., 2008). En Europa, alrededor de 350 toneladas de triclosán se producen anualmente para aplicaciones

comerciales (Singer et al., 2002). En los Estados Unidos, el 76 % de cuatrocientos jabones comerciales examinados contenían triclosán (Perencevich et al., 2001). De los fármacos aprobados por la legislación para su uso en ganadería, los antibióticos se encuentran entre los más ampliamente usados que se utilizan además de por su actividad antibacteriana, para mejorar el crecimiento y la eficacia alimenticia en el ganado sano (Levy, 1992).

1.2. Aplicaciones y mecanismos de acción de sustancias farmacológicamente activas

En este apartado se incluye un breve resumen de las aplicaciones y los modos de acción de los distintos tipos de sustancias farmacológicamente activas.

1.2.1. Antiinflamatorios no esteroideos/analgésicos

Los antiinflamatorios no esteroideos (AINEs) son un grupo de compuestos ácidos que pueden presentar diferentes formas químicas, tales como los derivados del ácido arilacético, ácido arilpropiónico, ácido indólico y el ácido antranílico, así como el oxicams. La característica estructural para la mayoría de los AINEs es su grupo ácido, que se implica un intervalo de pK_a entre 3 y 6, por lo cual a pH $\geq$ 7 estos compuestos están presentes principalmente en la forma aniónica. No obstante, la existen otros como el paracetamol o la fenilbutazona que no contienen este grupo ácido. Los AINEs son ampliamente utilizados en medicina humana y veterinaria por su alta capacidad para suprimir o reducir el proceso inflamatorio y los signos clínicos asociados a él, tales como calor, dolor, hinchazón, hiperemia y pérdida de la función (Gowik et al., 1998). En la Tabla 2 se muestran algunas propiedades físico-químicas de los antiinflamatorios no esteroideos van a ser objeto de estudio en esta Memoria.

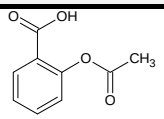
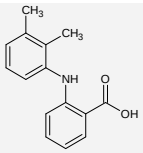
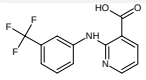
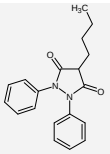
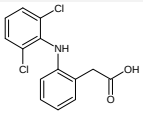
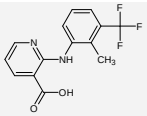
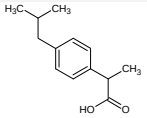
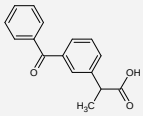
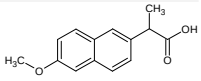
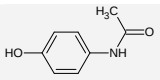
Tabla 1: Consumo de algunas sustancias farmacológicamente activas en el Mundo (t/año).

Compuestos	Alemania 2001	Austria 1997	Dinamarca 1997	Australia 1998	Inglaterra 2000	Italia 2001	Suiza 2004	España 2003	USA 2003
Analgésicos, antipiréticos y antiinflamatorios									
Ácido acetilsalicílico	840	78.4	0.21	20.4			43.8		
Ácido salicílico	71. 7	9.6					5.3		
Paracetamol	620	35.1	0.24	300	390		95.2		
Naproxeno		4.6		22.8	35.1		1.7	42.6	
Ibuprofeno	350	6.7	0.03	14.2	160	1.9	25.0	280	
Diclofenaco	85.8	6.1			26.1		4.5	32.3	
β-Bloqueadores									
Atenolol					28.9	22.7	3.2		
Metoprolol	92.9	2.4					3.2	2.3	
Sotalol								0.70	
Reguladores de lípidos									
Gemfibrazol				20.0			0.40		
Bezafibrato		4.5				7.6	0.76	4,0	
Neuroactivos									
Carbamazepina	87.6	6.3		10.0	40.3		4.4	20.0	35.0
Diazepam			0.21				0.05	0.90	
Antiácidos									
Ranitidina	85.8			33.7	36.3	26.7	1.6		
Cimetidina					35.6		0.06		
Diuréticos									
Furosemida			3.74			6.4	1.0		
Terbutalina			0.46				0.01		
Salbutamol			0.17				0.04		
Varios									
Estradiol			0.12						
17α-etinilestradiol								0.01	
Metformina	520	26.4		90.9	200		51.4		

Los AINEs actúan inhibiendo reversible o irreversiblemente una o ambas de las dos isoformas de la enzima ciclooxigenasa (COX-1 y COX-2), que catalizan la síntesis de prostaglandinas diferentes del ácido araquidónico. Los antiinflamatorios clásicos inhiben tanto la COX-1 y COX-2 en diferentes grados, mientras que algunos antiinflamatorios más novedosos actúan de forma más selectiva sobre la COX-2, la forma inducible responsable de las reacciones inflamatorias. Las diferencias en el tamaño de punto de unión son los responsables de la selectividad de estos fármacos (Gierse et al., 1999; Kurumbail et al., 1996). Las prostaglandinas desempeñan una variedad de funciones fisiológicas dependiendo de la fuente de células y de las moléculas diana. Ellas son conocidas por estar involucradas en distintos procesos, tales como la inflamación y el dolor, la regulación del flujo sanguíneo renal en los procesos de coagulación, y la síntesis de la mucosa gástrica de protección. En el riñón, las prostaglandinas están implicadas en el mantenimiento del equilibrio entre la vasodilatación y la vasoconstricción de los vasos sanguíneos durante el proceso de filtración de la sangre.

Los dos analgésicos que más se consumen son el paracetamol y el ácido acetil salicílico como se ha indicado en la Tabla 1. El modo de acción del paracetamol no está totalmente dilucidado, pero se supone que este fármaco actúa principalmente mediante la inhibición de la ciclooxigenasa del sistema nervioso central y no tiene efectos antiinflamatorios, debido a la falta de inhibición de la ciclooxigenasa periférica involucrada en los procesos inflamatorios. Los efectos adversos del paracetamol se deben principalmente a la formación de metabolitos hepatotóxicos, principalmente la imina N-acetil-p-benzoquinona. El paracetamol también induce la proliferación de cultivos de células de cáncer de mama a través de los receptores de estrógenos sin unirse a ellos (Harnagea-Theophilus et al., 1999).

Tabla 2: Propiedades físico-químicas de algunos antiinflamatorios no esteroides/analgésicos.

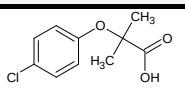
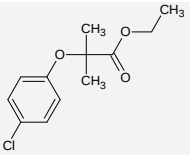
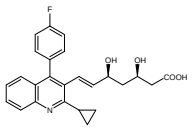
Compuestos	pK_a	$logk_{o/w}$	Número CAS	Masa molecular	Fórmula molecular	Estructura
Ácido acetil salicílico	3.50	1.19	50-78-2	180.16	$C_9H_8O_4$	
Ácido mefenámico	3.75	5.19	61-68-7	241.285	$C_{15}H_{15}NO_2$	
Ácido niflúmico	4.76	–	4394-00-7	282.22	$C_{13}H_9F_3N_2O_2$	
Fenilbutazona	4.40	3.16	853-34-9	308.374	$C_{19}H_{18}N_2O_3$	
Diclofenaco	4.14	4.51	15307-86-5	296.15	$C_{14}H_{11}Cl_2NO_2$	
Flunixino	5.82	4.9	38677-85-9	296.24	$C_{14}H_{11}F_3N_2O_2$	
Ibuprofeno	4.91	3.97	15687-27-1	206.28	$C_{13}H_{18}O_2$	
Ketoprofeno	4.45	3.12	22071-15-4	254.28	$C_{16}H_{14}O_3$	
Naproxeno	4.20	3.20	22204-53-1	230.26	$C_{14}H_{14}O_3$	
Paracetamol	9.38	0.46	103-90-2	151.16	$C_8H_9NO_2$	

1.2.2. Reguladores de lípidos

Hay básicamente dos tipos de medicamentos reguladores de lípidos, las estatinas y los fibratos debido a su elevado uso y su posible toxicidad. En la Tabla 3 se muestran las propiedades físicas y químicas de algunos reguladores de lípidos. Ambos tipos se

utilizan para disminuir la concentración de colesterol (estatinas y fibratos) y triglicéridos (fibratos) en el plasma sanguíneo. Las estatinas actúan como inhibidores de la síntesis del colesterol mediante la inhibición de la 3-coenzima A hydroxymethylglutaril reductasa (HMG-CoA), responsable de la etapa limitante en la síntesis del colesterol, es decir, la conversión de la HMG-CoA en mevalonato (Laufs y Liao, 1998). Como consecuencia de la disminución intracelular de colesterol, la expresión de receptores de LDL en las membranas de los hepatocitos es mayor y por lo tanto, la reabsorción del colesterol LDL en el plasma sanguíneo aumenta. Hay evidencia de que las estatinas afectan a la síntesis de la hormona juvenil en los insectos (Debernard et al., 1994), por ejemplo la fluvastatina suprime por completo su biosíntesis *in vitro* y en el órgano mandibular de la langosta (Li et al., 2003).

Tabla 3: Propiedades físico-químicas de algunos reguladores de lípidos.

Compuestos	**pK_a**	**$logk_{o/w}$**	**Número CAS**	**Masa molecular**	**Fórmula molecular**	**Estructura**
Ácido clofíbrico	3.46	2.60	882-09-7	214.65	$C_{10}H_{11}ClO_3$	
Clofibrato	–	3.62	637-07-0	242.7	$C_{12}H_{15}ClO_3$	
Pitavastatin	–	–	147511-69-1	421.46	$C_{12}H_{15}ClO_3$	

Entre los fibratos, están el ácido clofíbrico que se metaboliza en el cuerpo humano, y la mayoría de los estudios de la presencia de este compuesto en muestras ambientales han reportado niveles de concentración bastantes elevados de éste fármaco y de su metabolito (Petrovic et al., 2005). Los fibratos actúan probablemente activando la enzima lipoproteína lipasa, que es principalmente responsable de la conversión de las lipoproteínas de muy baja densidad (VLDL) a las lipoproteínas de alta densidad (HDL), disminuyendo por lo tanto, la concentración plasmática de triglicéridos (Staels et al., 1998). Los fibratos estimulan la captación celular de ácidos grasos, la conversión a

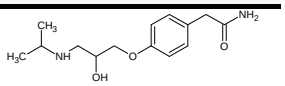
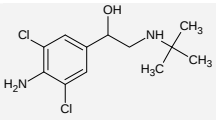
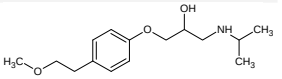
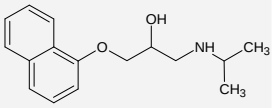
acetil-CoA derivados, y el catabolismo de las vías de la beta-oxidación, lo que combinado con una reducción en la síntesis de ácidos grasos y triglicéridos, se traduce en una disminución en la producción de VLDL (Staels et al., 1998). En un estudio llevado a cabo con ratas se han observado daños hepáticos después de la exposición crónica a los fibratos (Qu et al., 2001) debido posiblemente al estar relacionado con la inhibición de la oxidación mitocondrial (Keller et al., 1992). Cajaraville et al. (2003) han observado una fuerte correlación entre la exposición a los fibratos y el cáncer hepático en roedores. Estos hallazgos aumentan el interés por el impacto ecotoxicológico de esta clase terapéutica de medicamentos.

1.2.3. β-bloqueadores

Los β-bloqueadores se utilizan principalmente para el tratamiento de la hipertensión arterial, insuficiencia cardiaca congestiva, los ritmos anormales del corazón, para aliviar la angina de pecho, y para prevenir infartos cardiacos. Estos fármacos actúan por inhibición competitiva de los receptores β-adrenérgicos. El sistema adrenérgico está involucrado en muchas funciones fisiológicas tales como la regulación de la necesidad de oxígeno del corazón, los mecanismos de vasodilatación de los vasos sanguíneos, y broncodilatación. Además se ha demostrado que el sistema adrenérgico interactúa con el metabolismo de carbohidratos y lípidos, principalmente en respuesta a las necesidades de estrés, tales como el hambre (Jacob et al., 1998).

La mayoría de los β-bloqueadores son compuestos débilmente básicos con pK_a entre 8,7 y 9,7. Estos compuestos contienen un grupo amino secundario y un grupo hidroxilo situado en átomos de carbono adyacentes Tabla 4. Los efectos secundarios de esta clase de fármacos consisten principalmente en la broncoconstricción y la perturbación de la circulación periférica (Hoffman y Lefkowitz, 1998). Debido a su lipofilia se supone que deben atravesar la barrera hematoencefálica y actuar en el sistema nervioso central (Soyka, 1984).

Tabla 4: Propiedades físico-químicas de algunos β-bloqueadores

Compuestos	pK_a	$logk_{o/w}$	Número CAS	Masa molecular	Fórmula molecular	Estructura
Atenolol	9.60	–	29122-68-7	266.34	$C_{14}H_{22}N_2O_3$	
Clembuterol	–	–	37148-27-9	277.19	$C_{12}H_{18}N_2Cl_2O$	
Metoprolol	9.70	1.88	37350-58-6	267.36	$C_{15}H_{25}NO_3$	
Propranolol	9.50	2.60	525-66-6	259.34	$C_{16}H_{21}NO_2$	

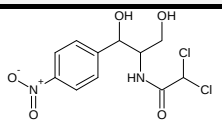
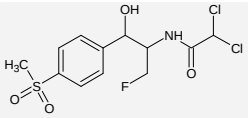
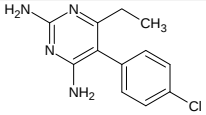
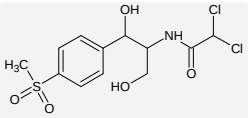
1.2.4. Antibióticos

Los objetivos del uso de los antibióticos son: (1) para el tratamiento de enfermedades en humanos y en animales, (2) como promotores del crecimiento, y (3) para mejorar la eficacia nutricional de los alimentos (Levy, 1992; Lai et al., 2009). Hoy en día, los antibióticos juegan un papel importante en la ganadería moderna y en la acuicultura, y su uso se ha incrementado notablemente en muchos países desarrollados (Sarmah et al., 2006). Estos antibióticos son principalmente administrados a través de los piensos medicamentosos. En la Tabla 5 se muestran algunas de las propiedades físico-químicas de los antibióticos objeto de estudio en esta Memoria.

El mecanismo de acción es idéntico en los tres antibióticos (cloranfenicol, tianfenicol y florfenicol). Las primeras investigaciones sobre el cloranfenicol señalaron que su mecanismo de acción se basa en una potente, aunque reversible, inhibición de la biosíntesis de proteínas en los microorganismos, actuando sobre el centro peptidil-transferasa del ribosoma bacteriana (Weisblum y Davies, 1968). Para llevar a cabo la inhibición de la síntesis bacteriana, los tres análogos estructurales, penetran en el interior de la célula bacteriana, atravesando la membrana de la bacteria mediante difusión facilitada (Kapusnik-Uner et al., 1996). Estos compuestos actúan sobre las células procariotas, y, en menor medida, también actúan sobre las células eucariotas. En concreto, el cloranfenicol actúa sobre la línea roja de mamíferos (médula ósea) (Yunis y Bloomberg, 1964), debido al proceso metabólico que sigue su grupo p-nitro en el hígado, o por la acción de la flora del tracto gastrointestinal, formándose un derivado

nitroso que reacciona fuertemente con el ADN, desencadenando una aplasia medular, principal consecuencia toxicológica derivada del uso de cloranfenicol (Rosenkranz, 1988; Yunis, 1988;). Así pues, su aplicación terapéutica, da lugar a una serie de alteraciones hemáticas. Por ello en muchos países se ha prohibido su uso en animales productores de alimentos con el fin de proteger la salud de los consumidores (Shen et al., 2009).

Tabla 5: Propiedades físico-químicas de algunos antibióticos

Compuestos	**pK_a**	**$logk_{o/w}$**	**Número CAS**	**Masa molecular**	**Fórmula molecular**	**Estructura**
Cloranfenicol	9.61	–	56-75-7	323.131	$C_{11}H_{12}N_2Cl_2O_5$	
Florfenicol	9.03	–	73231-34-2	358.21	$C_{12}H_{14}Cl_2FNO_4S$	
Pirimetamina	7.34	–	58-14-0	248.71	$C_{12}H_{13}ClN_4$	
Tianfenicol	9.76	–	73231-34-2	358.21	$C_{12}H_{15}Cl_2FNO_5S$	

El tianfenicol es un análogo del cloranfenicol en el que se sustituye el grupo nitro en el anillo de benceno con un grupo metilsufónico. El florfenicol, un derivado fluorado de tianfenicol que tiene un átomo de flúor en lugar del grupo hidroxilo situado en la C-3 y que se utiliza exclusivamente en veterinaria, solo provoca la inhibición de la síntesis de proteína mitocondrial tras su administración a dosis elevadas y esto se debe a la acción de su metabolito ácido oxámico sobre el ADN mitocondrial. Sin embargo, y a diferencia de las alteraciones producidas por el cloranfenicol, esta es una discrasia sanguínea dosis-dependiente y transitoria. En contraste con respecto el cloranfenicol, el tianfenicol y florfenicol son menos tóxicos por la ausencia de un grupo nitro, pero sus efectos secundarios se deben que tener en cuenta ya que residuos de estos fármacos pueden suponer un riesgo para la salud de los consumidores (Nagata y Oka, 1996; Campa-Córdova et al., 2006). No obstante los tres fenicoles actúan sobre la línea

mieloide de mamíferos (Paape y Miller, 1990), provocando una alteración de la morfología de los neutrófilos y neutropenia, que parece deberse a la acción depresora de los tres compuestos sobre la flora digestiva de los animales, disminuyendo así la estimulación de sus sistemas de defensa.

1.2.5. Hormonas

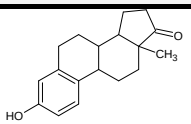
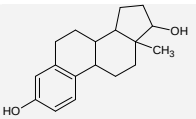
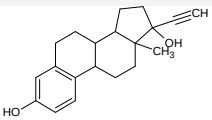
Los fármacos estrogénicos, principalmente xenoestrógenos sintéticos, se utilizan ampliamente en la terapia de reemplazo de estrógenos y de los anticonceptivos orales, en veterinaria para la mejora del crecimiento y en humanos como anticonceptivos. No obstante, las hormonas, tanto de origen animal como sintéticas, se han convertido en un tema de preocupación en las Ciencias Ambientales y Ciencias de la Salud Pública durante las últimas décadas por sus posibles efectos secundarios (Yan et al., 2009).

Se ha estudiado profundamente la acción genómica clásica de las hormonas esteroideas que actúan a través de receptores intracelulares. Se tienen evidencias de que las hormonas esteroideas pueden ejercer la acción no-clásica que se caracteriza por un rápido efecto a corto tiempo. En la mayoría de estos casos, el efecto de la hormona se produce en el nivel de la membrana y no está asociada con la entrada en la célula. Los posibles mecanismos de estas acciones no-clásicas por parte de estas hormonas son: (a) cambios en la fluidez de la membrana, (b) actuación sobre los receptores en las membranas plasmáticas, (e) regulación de los receptores GABA en las membranas plasmáticas, y (d) activación de los receptores esteroideos de factores tales como EGF, IGF-1 y la dopamina.

Las hormonas naturales, como 17β-estradiol y estrona Tabla 6 juegan un papel importante en homeostasis biológica durante el control de las funciones de reproducción en humanos y animales. La hormona sintética 17 α-etinilestradiol se utiliza ampliamente como un anticonceptivo para humanos y/o como principio activo en los preparados farmacéuticos destinados para el control del síndrome de la menopausia y la posmenopausia. Esta sustancia también se utiliza en la terapia del reemplazo fisiológico en condiciones de deficiencia, y en el tratamiento del cáncer de próstata o de mama (Kuster et al., 2004). Por otro lado, algunas hormonas se utilizan ilegalmente como promotores del crecimiento en el ganado vacuno para aumentar el peso, y también son objeto de abuso en el deporte para mejorar el rendimiento de los atletas. Después de que se utilizan, una parte se excreta y el resto permanece en el cuerpo del animal. Por lo cual estos compuestos pueden ser transferidos a las aguas o a los alimentos. Existe en la

bibliografía muchos estudios que indican que la exposición a las hormonas naturales y sintéticas a niveles bajos pueden ser un riesgo potencial para seres humanos y animales (Shao et al., 2005). Así, se ha demostrado que se asocia a muchas enfermedades tales como cáncer de mama y cáncer de útero en seres humanos y el hermafroditismo en la fauna silvestre, y también son conocidos como productos químicos disruptores endocrinos.

Tabla 6: Propiedades físico-químicas de algunas hormonas.

Compuestos	**pK_a**	**$logk_{o/w}$**	**Número CAS**	**Masa molecular**	**Fórmula molecular**	**Estructura**
Estrona	10.20	3.69	53-16-7	270.366	$C_{18}H_{22}O_2$	
17β-estradiol	10.27	4.13	50-28-2	272.38	$C_{18}H_{24}O_2$	
17α-etinilestradiol	10.24	4.25	57-63-6	296.403	$C_{20}H_{24}O_2$	

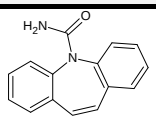
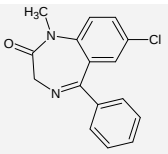
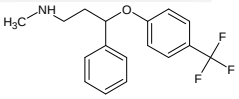
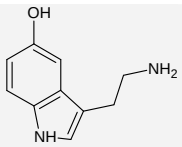
1.2.6. Compuestos neuroactivos

Los fármacos neuroactivos, entre los que se encuentran los antiepilépticos y antidepresivos, se caracterizan porque interactúan sobre el sistema nervioso central. Los antiepilépticos actúan sobre el sistema nervioso central disminuyendo la actividad neuronal en general Tabla 7. Esto se puede lograr mediante el bloqueo de los canales de sodio dependientes de las neuronas excitadoras (por ejemplo la carbamazepina), o mediante la mejora de los efectos inhibidores del neurotransmisor GABA al unirse a un sitio específico en la subunidad gamma de los receptores correspondientes (por ejemplo, diazepam) (Study y Barker, 1981; MacDonald y Olsen, 1994; Rogers et al., 1994).

Entre los fármacos neuroactivos, la fluoxetina es un antidepresivo muy utilizado, que actúa inhibiendo la captación de la serotonina. Este neurotransmisor está implicado en muchos mecanismos hormonales y neuronales, y también es importante en funciones tales como la ingesta de alimentos y el comportamiento sexual. La serotonina

interviene, entre otras, en las funciones endocrinas en organismos acuáticos (Fong et al., 1998; Foran et al., 2004). La fluoxetina y la sertralina y metabolitos de la norfluoxetina y desmetilsertralina son potencialmente bioacumulables porque se han detectado en muestras de pescado en la USA (Brooks et al., 2005).

Tabla 7: Propiedades físico-químicas de algunos compuestos neuroactivos.

Compuestos	**pK_a**	**$logk_{o/w}$**	**Número CAS**	**Masa molecular**	**Fórmula molecular**	**Estructura**
Carbamazepina	7.00	2.47	298-64-4	236.27	$C_{15}H_{12}N_2O$	
Diazepam	3.40	–	439-14-5	284.74	$C_{16}H_{13}ClN_2O$	
Fluoxeteina	–	4.65	54910-89-3	309.33	$C_{17}H_{18}F_3N_2O$	
Serotonina	–	1.39	–	176,21	$C_{10}H_{12}N_2O$	

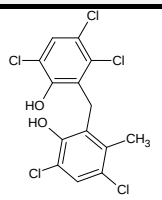
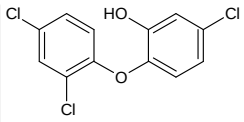
1. 2.7. Antisépticos

El antiséptico más utilizado es el triclosán [5-cloro-2-(2,4-diclorofenoxi) fenol], que es un polvo cristalino, soluble en muchos disolventes orgánicos, con muy baja solubilidad en agua (10 mg/l), y muy lipofílico (log $K_{o/w}$ (octanol-agua) = 4,80) Tabla 8. Fue fabricado por primera vez por Ciba-Geigy Co (Basilea, Swizerland) bajo el nombre comercial DP Irgasan 300 o MP Irgasan (Sanches-Silva et al., 2004). Es un agente sintético de amplio espectro antibacteriano que se ha utilizado ampliamente durante más de 20 años en una variedad de productos de cuidado personal, incluyendo pasta de dientes, enjuagues bucales, desodorantes, jabones, textiles (por ejemplo, calcetines, ropa interior), y utensilios de cocina de plástico (Ye et al., 2008). La similitud de la estructura de triclosán con las hormonas tiroideas, sugiere que este contaminante

químico puede afectar a la acción de estas hormonas que desempeñan un papel muy importante en el desarrollo normal de muchas especies. Por ejemplo puede interferir la metamorfosis de los anfibios (Shi, 2000).

Otra sustancia perteneciente a este grupo es el hexaclorofeno que se utiliza como bactericida y fungicida. Usado antiguamente para el tratamiento tópico de la piel. Sus efectos neurotóxicos se observaron en los años 70, cuando se bañaba a los recién nacidos para evitar infecciones. Se absorbe por la piel y produce edema intramielínico seguido de desmialinización (sobre todo en intoxicaciones severas). El hinchazón del cerebro produce un aumento de la presión intracraneal y también afecta a los fotoreceptores de la retina. Los síntomas son debilidad generalizada, confusión, convulsiones, y en intoxicaciones severas, coma y muerte.

Tabla 8: Propiedades físico-químicas de algunos antisépticos.

Compuestos	pK_a	$logk_{o/w}$	Número CAS	Masa molecular	Fórmula molecular	Estructura
Hexaclorofeno	–	–	70-30-4	406.90	$C_{12}H_6O_2Cl_6$	
Triclosán	7.80	4.80	3380-3-5	289.54	$C_{12}H_7O_2Cl_3$	

1.3. Toxicidad de sustancias farmacológicamente activas

Para que un fármaco produzca sus efectos terapéuticos o tóxicos, debe alcanzar un intervalo preciso de concentraciones en la biofase, es decir, el medio en que interactúa con sus receptores. Debajo de este intervalo, no se observará ningún efecto farmacológico o éste será subterapéutico; por encima, el efecto puede ser excesivo o pueden aparecer otros efectos no deseados. La concentración de un fármaco que se alcanza en su lugar de acción es la consecuencia de los siguientes procesos Figura 2:

a) Absorción, es decir, la entrada del fármaco en el organismo que incluye los procesos de liberación de su forma farmacéutica, disolución y absorción propiamente dicha.
b) Distribución del fármaco desde el lugar de absorción a la circulación sistémica y desde ella hasta los tejidos. Para que el fármaco alcance su lugar de acción, debe atravesar diversas membranas para llegar a la sangre y para pasar de ésta al líquido intersticial y, en su caso, al interior de las células. El paso del fármaco de la sangre a los tejidos depende de la fijación del fármaco a las proteínas del plasma, ya que sólo el fármaco libre difunde libremente a los tejidos.
c) Eliminación del fármaco, sea por metabolismo principalmente hepático o por excreción del fármaco inalterado por la orina, bilis, etc. En algunos casos, este metabolismo puede producir metabolitos activos cuya presencia también deberá tenerse en cuenta por su posible toxicidad.

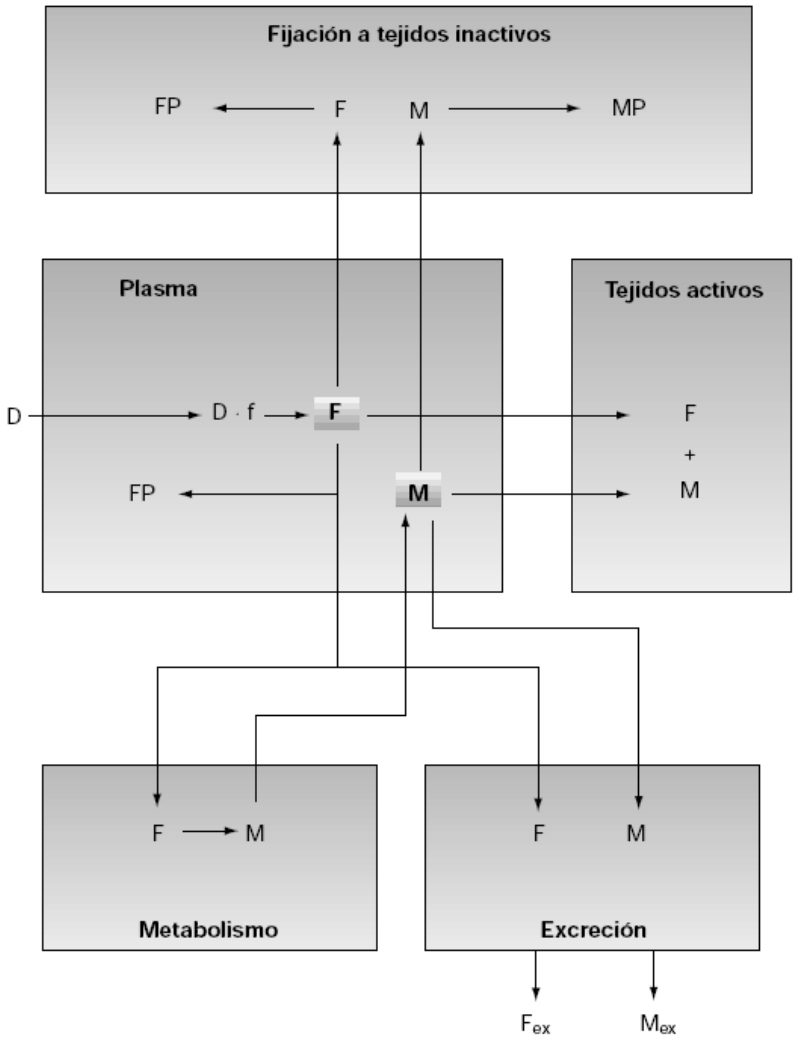


Figura 2: Procesos farmacocinéticos. D = Dosis administrada; D•f = Cantidad de fármaco absorbida; F = Fármaco libre; Fex = Fármaco excretado; FP = Fármaco unido a proteínas; M = Metabolito activo libre; Mex = Metabolito activo excretado; MP = Metabolito activo unido a proteínas.

En general, la literatura muestra que la mayoría de los medicamentos, cuando se detectan, se encuentran en muestras medioambientales a niveles de ppb o ppt (Koutsouba et al., 2003; Bishop y Mitra, 2005). Aunque a estos niveles de concentraciones no suelen inducir efectos adversos, representan un riesgo ecológico. Por otro lado, también se tiene conocimiento que a pesar de que la concentración de los fármacos sea baja si se combinan con otros fármacos pueden provocar efectos aditivos o sinérgicos (Hernando et al., 2004). En este sentido, existe una creciente demanda de información sobre la toxicidad potencial de los residuos de fármacos. Sin embargo, hay una falta de datos relativos a sus efectos sobre la fauna terrestre o acuática. Hasta la fecha, la mayoría de la literatura relacionada con los efectos tóxicos de residuos de

fármacos se centra en los organismos representativos de la cadena alimentaria. La Organización para la Cooperación y el Desarrollo Económicos (OCDE) ha definido las directrices de los métodos estándar de ecotoxicidad (incluyendo algas, bacterias, invertebrados, peces) (OECD Guidelines for the Testing of Chemicals, 2002). Los datos de ecotoxicidad sobre diferentes especies representativas son complicados de interpretar debido a la variabilidad en los procedimientos experimentales y en las condiciones. En la literatura de ecotoxicidad, algunos autores hacen uso de la Directiva 93/67/CEE de la UE para interpretar los datos de toxicidad, que clasifica a las sustancias de acuerdo dosis letal del 50 % de los individuos tratados (valor LC_{50}) (Commission of the European Communities, 1993). Una LC_{50} <1 mg/l implicaría la calificación de "muy tóxico para los organismos acuáticos"; de 1 a 10 mg/l "tóxicos", y de 10 a 100 mg/l "nocivo para los organismos acuáticos". Las sustancias con un LC_{50} > 100 mg/l no se clasificarían. En la Tabla 9 se incluyen las LC_{50} de algunos fármacos que dependen de la especie en la cual se hayan realizado los estudios de toxicidad. A continuación se comentarán los efectos tóxicos más importantes que se han encontrado en las SFAs más importantes.

1.3.1. Antiinflamatorios no esteroideos/analgésicos

Como se ha comentado anteriormente, los antiinflamatorios no esteroideos son ampliamente utilizados en medicina humana y veterinaria, para el tratamiento de procesos inflamatorios. Su rutina o su uso prolongado pueden causar varios efectos secundarios tóxicos, tales como hemorragia gastrointestinal, úlcera intestinal, anemia aplásica, la inhibición de la agregación plaquetaria, y cambios en la función renal. Se ha demostrado que la exposición prolongada a la fenilbutazona induce tumores renales en ratas y tumores de hígado en ratones. Además de su uso con fines terapéuticos, los AINEs se utilizan en la producción animal por sus efectos farmacológicos secundarios, para mejorar algunas características de calidad de las carnes (Vinci et al., 2006). Los daños renales e insuficiencia renal crónica después de un tratamiento antiinflamatorio parecen ser provocados por la falta de prostaglandinas en la vasodilatación.

Tabla 9: Dosis letal (LC_{50} en mg/l) de algunas sustancias farmacológicamente activas.

Grupo terapéutico	Fármaco	LC_{50} (mg/l)			
		Bacterias	**Algas**	**Invertebrados**	**Peces**
Antiinflamatorios/analgésicos	Diclofenaco	13.5–14.4	7.5–200	2–224	–
	Ibuprofeno	12.1–19.1	22–315	9–108	–
	Naproxeno	21.2–35.6	24–320	174	–
	Ketoprofeno	15.6–19.3	–	–	–
Regulador de lípidos	Ácido clofibrico	0.09–0.1	89–200	106–740	2.5–1400
β-Bloqueadores	Metoprolol	–	7.3–320	100–200	–
	Propranolol	–	5.8–114	3.1–17	–
Antibióticos	Cloranfenicol	–	2.7–48	–	–
	Florfenicol	–	0.2–243	–	–
	Tianfenicol	–	35–1550	–	–
Hormonas	17α-Etinilestradiol	–	0.8	0.1	–
Antiepilépticos	Carbamazepina	0.081	0.1–74	0.007–0.013	50–754
Antisépticos	Triclosán	–	–	–	–

Los daños gástricos se cree que son causados por la inhibición de ambas isoformas de la enzima ciclooxigenasa (Wallace, 1997; Wallace et al., 2000). Por el contrario, los daños en el hígado se producen debido a la formación de metabolitos reactivos (por ejemplo, glucurónidos acilo) (Bjorkman, 1998). A pesar del creciente interés en estos fármacos desde el punto de vista medioambiental, las investigaciones sobre sus efectos adversos son muy reducidas. Los estudios actuales realizados en bacterias han revelado la alta toxicidad ("clase muy tóxico") de anti-inflamatorios, tales como el diclofenaco, ibuprofeno, naproxeno o ketoprofeno (Daughton y Ternes, 1999; Farré et al., 2001; Ferrari et al., 2003). También se han encontrado efectos tóxicos del diclofenaco o ibuprofeno sobre invertebrados (Cleuvers, 2003; Ferrari et al., 2003; Halling-Sørensen et al., 1998) y algas (Cleuvers, 2003; Ferrari et al., 2003). Sin embargo, se han observado diferencias respecto a los efectos del diclofenaco y el ibuprofeno sobre diferentes especies de un mismo grupo taxonómico (Halling-Sørensen et al., 1998; Ferrari et al., 2003). Los mismos resultados de toxicidad se han obtenido para el naproxeno sobre algas (Cleuvers, 2003; Ferrari et al., 2003). En la Tabla 9 se muestran los valores de LC_{50} de diferentes anti-inflamatorios no esteroideos/analgésicos encontrados en la bibliografía para algunos grupos taxonómicos.

1.3.2. Reguladores de lípidos

Los efectos tóxicos de los reguladores de lípidos son muy variados sobre la base de los valores reportados de LC_{50} para bacterias (Bound y Voulvoulis, 2004; Ferrari et al., 2003; Henschel et al., 1997) e invertebrados (Henschel et al., 1997; Ferrari et al., 2003; Hernando et al., 2004). Algunos reguladores de lípidos tales como el ácido clofíbrico y gemfibrozilo son muy tóxicos para las bacterias, y son muy poco dañinos para los invertebrados y peces, con la excepción de fenofibrato, que es perjudicial (Henschel et al., 1997; Ferrari et al., 2003; Jos et al., 2003; Koutsouba et al., 2003).

1.3.3. β-bloqueadores

Los β-bloqueadores son excepcionalmente tóxicos y la mayoría de ellos poseen un intervalo terapéutico estrecho, y si se sobrepasa este intervalo puede ser muy perjudicial para la salud. Sus concentraciones inusualmente bajas en la sangre humana, hacen que su análisis sea difícil. Los efectos secundarios de esta clase terapéutica son

principalmente broncoconstricción y perturbación de la circulación periférica (Hoffman y Lefkowitz, 1998).

Se ha encontrado una heterogeneidad en los efectos de los β-bloqueadores para las algas (Cleuvers, 2003) e invertebrados (Halling-Sørensen et al., 1998; Boyd et al., 2003; Cleuvers, 2003). En el caso del propanolol se han observado diferentes efectos tóxicos sobre algas, en contraste con metoprolol, atenolol o betaxolol que no son tóxicos. En la Tabla 9 se muestran los valores de LC_{50} de algunos β-bloqueadores.

1.3.4. Antibióticos

Los antibióticos se consideran como potenciales microcontaminantes debido a que en el Medio Ambiente se encuentran a concentraciones muy bajas (ppb o menos) (Ferreira et al., 2007; Le Bris y Pouliquen, 2004). En algunos casos estos pueden encontrarse en los ambientes acuáticos a mayor concentración, debido a circunstancias externas (por ejemplo, la escorrentía de suelos contaminados, el desbordamiento de los estanques usados en acuicultura, o a efluentes domésticos, industriales o de hospitales) (Ferreira et al., 2007; Sarmah et al., 2006).

Está bien documentados los efectos de resistencia de bacterias debido al amplio (y algunas veces indiscriminado) uso de antibióticos (Daughton y Ternes, 1999). Por ejemplo el cloranfenicol produce efectos tóxicos en el sistema hematopoyético (The Joint FAO/WHO Expert Committee on Food Additives, 1988; IARC, 1990; Young y Alter 1994). El primer efecto que produce es la anemia y con frecuencia se produce una leve reticulocitopenia, acompañada a veces de leucopenia y trombocitopenia, efectos que son en la mayoría de los casos reversibles y depende de la dosis de fármaco administrada. El segundo efecto adverso en la sangre es la anemia aplásica (Festing et al., 2001), que no está relacionada con la dosis administrada y que se desarrolla varias semanas o meses después del tratamiento, y es a menudo irreversible y fatal (Yunis et al., 1988).

El florfenicol no tiene el riesgo de inducir anemia aplásica en humanos. Este fármaco no está sujeto a la acción de la acetiltransferasa, que es utilizado por las enzimas de las bacterias para desarrollar resistencia al cloranfenicol y tianfenicol (Park et al., 2008).

Estudios recientes sobre los efectos de algunos antibióticos sobre diferentes tipos de organismos representativos han propuesto valores reducidos de LC_{50} para la

estreptomicina, flumequina o oxitetraciclina y oxofloxacin en el caso de bacterias; o bien de la tilosina, oxitetraciclina, clortetraciclina o eritromicina para las algas (Halling-Sørensen, 2000; Eguchi et al., 2004; Lalumera et al., 2004). Estudios realizados mediante bioensayos han demostrado que la sulfadimetoxina y oxitetraciclina son tóxicas para los invertebrados; por el contrario, la flumequina y tilosina presentan efectos nocivos o no tóxicos para los invertebrados (Halling-Sørensen et al., 1998; Wollenberg et al., 2000).

1.3.5 Hormonas

La mayoría de los datos de toxicidad sobre las hormonas esteroideas están relacionados con el 17 α-etinilestradiol, el dietilestilbestrol, y el acetato de dietilestilbestrol (Halling-Sørensen et al., 1998). Aunque en los efluentes de las industrias de producción de anticonceptivos orales (17α-etinilestradiol) las concentraciones son bajas (<7 ng/l), se ha demostrado la feminización en los peces machos (Desbrow et al., 1998; Routledge et al., 1998). Por otro lado, la noretindrona produce una alteración endocrina que induce el desarrollo de glándulas mamarias en ratones machos (Repetto et al., 2001). Los estudios realizados por Halling-Sørensen et al. (1998) han demostrado la toxicidad del acetato de 17α-etinilestradiol, dietilestilbestrol y el dietilestilbestrol para las algas, invertebrados y peces.

1.3.6. Antiepilépticos

Los antiepilépticos se encuentran frecuentemente en las aguas superficiales porque los procedimientos de depuración de aguas no son efectivos para la degradación de fármacos. Así por ejemplo la carbamazepina ha sido detectada a concentraciones elevadas (6.3 μg/l) en un estudio realizado en aguas procedentes de depuradoras (Ternes, 1998). El LC_{50} de este compuesto es menor de 1 mg/l para las bacterias, las algas y la mayoría de las especies evaluadas de invertebrados (Cleuvers, 2003; Ferrari et al., 2003); en cambio para peces el LC_{50} es mayor de 100 mg/l (Ferrari et al., 2003; Halling-Sørensen, 2000).

1.3.7. Antisépticos

El triclosán que es ampliamente empleado como un aditivo en muchos productos, debido a su acción bactericida y propiedades antimicrobianas puede sufrir una serie de reacciones de transformación produciéndose unos compuestos más tóxicos y bioacumulables como son los dicloro y triclorofenoles (Morales et al., 2005). Por ello este antiséptico puede plantear una amenaza significativa para los sistemas biológicos y el desarrollo normal de las especies de la fauna y los seres humanos. Los estudios realizados sobre la toxicidad del triclosán han demostrado que puede bioacumularse en los peces a concentraciones subletales (Valters et al., 2005) y actuar como un tóxico en diferentes especies acuáticas en las primeras etapas de la vida de la trucha, crustáceos y algas (Tatarazako et al., 2004). Debido a que el triclosán se ha detectado en leche humana a concentraciones entre 60 y 300 ng/g de peso ha suscitado a que sea necesario un más estudio exhaustivo de la toxicidad de esta sustancia en los seres humanos (Adolfsson-Erici et al., 2002). Unos estudios *in vitro* realizados en ratas han puesto de manifiesto que bajas concentraciones de triclosán puede alterar la homeostasis de los sistemas metabólicos y hormonales (Allmyr et al., 2006b).

1.4. Legislación y Regulación

Diferentes organizaciones internacionales, tales como la Comisión Mixta FAO/OMS, el Codex Alimentarius, la Oficina Internacional de Epizootias (OIE) y la Organización Internacional de Normalización (ISO), con el objetivo de proteger la salud de los consumidores y asegurar la aplicación de prácticas equitativas en el comercio de alimentos de origen animal, han elaborado en conjunto un programa sobre Normas Alimentarias, en el cual están incluidas los programas de controles de residuos de fármacos veterinarios.

Por otro lado, la Organización Mundial de Comercio (OMC) estableció un acuerdo entre los países miembros sobre la Aplicación de Medidas Sanitarias y Fitosanitarias, que se refiere a la aplicación de reglamentaciones en materia de inocuidad de los alimentos y control sanitario de los animales y vegetales. En este acuerdo, si bien es cierto que la OMC autoriza a los países a establecer sus propias

reglamentaciones, estimula a los gobiernos a que armonicen sus medidas, recomendando que se utilicen normas y, directrices internacionales elaboradas por el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) y el Codex Alimentarius cuando existan. Por otro lado, señala que los países pueden aplicar medidas más rigurosas, si hay una justificación científica sobre la base de una evaluación adecuada del riesgo, con el fin de que estas no sean causas de obstáculos innecesarios para el comercio internacional.

Abocados fundamentalmente en el concepto de armonización, en el año 1996 comenzó a funcionar un Comité Internacional de Armonización en Veterinaria (VICH), en el cual participan organizaciones internacionales y gubernamentales y cuyo objetivo principal es armonizar el uso de medicamentos en animales de producción, con el fin de asegurar su eficacia en las especies de destino y la inocuidad de los alimentos provenientes de animales tratados a la población humana.

Para definir un producto libre de residuos químicos los países y organizaciones internacionales establecieron los límites máximos residuales (LMRs) para la mayoría de los plaguicidas y bastantes fármacos que se utilizan en animales de producción de alimentos. El Codex Alimentarius define los LMR como: la concentración máxima (expresada en miligramos por kilo o en microgramos por kilo) de residuos, resultante del uso de un medicamento veterinario y que se recomienda, se permita legalmente o se reconozca como admisible dentro del alimento o en la superficie del mismo (Codex Alimentarius, 2011). Cuando en un alimento se detectan concentraciones residuales superiores a los LMRs, estos se consideran contaminados y dañinos para el consumidor.

El uso generalizado de medicamentos veterinarios en los animales productores de leche puede inducir la presencia de residuos de estas sustancias en la leche. Los antibióticos son en la actualidad los fármacos que más frecuentemente se encuentran en la leche y que tiene efectos indeseables sobre la calidad y propiedades tecnológicas de este alimento y sus derivados, y que puede afectar a las salud humana. De hecho, la leche contaminada puede causar reacciones alérgicas o problemas indirectos en el tratamiento clínico, debido al desarrollo de resistencia bacteriana (Ortelli et al., 2009). Los AINEs son también muy utilizados en veterinaria, para el tratamiento de procesos inflamatorios y los efectos clínicos relacionados, y algunas enfermedades del ganado bovino respiratorio, junto con los antibióticos (Lockwood et al., 2003). Cuando se suministran altas dosis de AINEs por vía oral a los animales de granja en fechas cercanas a su sacrificio, la agregación plaquetaria es inhibida resultando en el sangrado

después de la sacrificio rápido, dando así un aspecto pálido de la carne de cerdo y carne de vacuno, un parámetro de calidad que atrae a muchos consumidores. Además, estos fármacos pueden reducir la lipogénesis (reacción bioquímica por la cual son sintetizados los ácidos grasos y esterificados o unidos con el glicerol para formar triglicéridos o grasas de reserva), y, en consecuencia, las grasas comestibles. Así, la presencia de residuos de AINEs en los alimentos puede suponer un riesgo para la salud de los consumidores, provocando diferentes efectos secundarios dependiendo del fármaco, como ya se ha indicado en un apartado anterior de esta Memoria (Vinci et al., 2006). Por estas razones, la Comisión Europea decidió controlar la presencia de residuos de AINEs como parte de los programas de vigilancia llevada a cabo por los Estados miembros. Para muchos AINEs ha sido establecido por la Comisión Europea un nivel máximo aceptable de residuos tolerable en los alimentos (LMR) a través del Reglamento 2377/90/CEE y sus posteriores modificaciones (Commission of the European Communities, 1990; Commission of the European Communites, 2010). En el Anexo I de este Reglamento se incluye a diferentes AINEs (carprofeno, diclofenaco, ácido tolfenámico, vedaprofeno y flunixino) y los LMR provisionales para las diferentes especies animales y matrices. El uso de diclofenaco está prohibido para los animales productores de leche, y solamente se puede emplear en los animales que van a ser sacrificados para el consumo de su carne. El ketoprofeno, ácido salicílico y salicilatos, así como el ácido acetilsalicílico y acetil salicilatos, figuran en el anexo II del Reglamento, como sustancias permitidas sólo para las especies que no produzcan leche ni huevos para el consumo humano. Como consecuencia de ello, la Comisión Europea recomienda un control riguroso de estas sustancias en animales productores de alimentos, ya que el uso considerable de los AINEs se puede presumir, y los posibles efectos teratogénicos y carcinogénicos causados por algunos de ellos no pueden ser excluidos.

El cloranfenicol como se ha indicado en un apartado anterior puede causar problemas en la sangre del hombre, por lo cual está prohibido su uso en animales productores de alimentos en la UE, USA y Canadá (Tabla 10). En cambio el tianfenicol y el florfenicol, que pueden sustituir al cloranfenicol, si están autorizados para su uso en el tratamiento de ganado. El LMR establecido en los alimentos para la tianfenicol es de 50 μg/kg y para el florfenicol de 200–3000 μg/kg (dependiendo de la especie). (Commission of the European Communites, 2010).

Las hormonas naturales y sintéticas son usadas ilegalmente como promotoras del crecimiento en el ganado vacuno y terneros para aumentar la ganancia de peso de los animales, y también son objeto de abuso en el deporte para mejorar el rendimiento de los atletas (Colborn y Clements, 1992; Holmes et al., 1997). Por lo tanto, estos compuestos han sido prohibidos en la Unión Europea (UE) desde 1988 y también en China en los alimentos de origen animal (Commission of the European Communities, 1996; The Ministry of Agriculture of PR China, 2011). En los Juegos Olímpicos Internacionales, las hormonas naturales y sintéticas y sustancias similares a hormonas también están prohibidas (The World Anti-Doping Code, 2010).

Los β-bloqueadores que se utilizan para el tratamiento de diversos trastornos cardiovasculares como la hipertensión, la angina de pecho y arritmia cardiovascular están prohibidos su uso por el Comité Olímpico Internacional y las Federaciones Internacionales de Deportes (International Olympic Committee, 1995) debido a sus propiedades simpaticomiméticas, al igual que otros estimulantes del sistema nervioso central, y debido a su actividad como agentes anabolizantes.

Finalmente y para los agentes antimicrobianos incorporados en las películas de plástico (compuestas de ácidos orgánicos y ésteres, enzimas y bacteriocinas) usadas para envolver los alimentos no se ha legislado todavía en la Unión Europea (Vermeiren et al., 2002).

Tabla 10: Límites máximos de residuos (LMR) para algunas sustancias farmacológicamente activas.

Sustancias farmacológicamente activas	Animal	LMR	Tejidos diana
Diclofenaco	Bovinos	5 µg/kg 1 µg/kg 5 µg/kg 10 µg/kg 0,1 µg/kg	Músculo Grasa Hígado Riñón Leche
	Porcinos	5 µg/kg 1 µg/kg 5 µg/kg 10 µg/kg	Músculo Piel y grasa Hígado Riñón
Florfenicol	Bovinos, ovinos y caprinos	200 µg/kg 3000 µg/kg 300 µg/kg	Músculo Hígado Riñón
	Porcinos	300 µg/kg 500 µg/kg 2 000 µg/kg 500 µg/kg	Músculo Piel y grasa Hígado Riñón
	Aves de corral	100 µg/kg 200 µg/kg 2500 µg/kg 750 µg/kg	Músculo Piel y grasa Hígado Riñón
	Peces	1000 µg/kg	Músculo
	Todas las demás especies destinadas a la producción de alimentos	100 µg/kg 200 µg/kg 2000 µg/kg 300 µg/kg	Músculo Grasa Hígado Riñón
Flunixino	Bovinos	20 µg/kg 30 µg/kg 300 µg/kg 100 µg/kg	Músculo Grasa Hígado Riñón
	Porcinos	50 µg/kg 10 µg/kg 200 µg/kg 30 µg/kg	Músculo Piel y grasa Hígado Riñón
	Équidos	10 µg/kg 20 µg/kg 100 µg/kg 200 µg/kg	Músculo Grasa Hígado Riñón
Ketoprofeno	Bovinos, porcinos y équidos	No se exige LMR	No procede
Tianfenicol	Todas las especies destinadas a la producción de alimentos	50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg	Músculo Grasa Hígado Riñón Leche
Cloranfenicol	Prohibido	Prohibido	Prohibido

2. DETERMINACIÓN DE SUSTANCIAS FARMACOLÓGICAMENTE ACTIVAS

La determinación de sustancias farmacológicamente activas en muestras ambientales, alimentos y fluidos biológicos requiere de una serie de etapas (muestreo, pretratamiento de muestra, extracción, *clean-up*/preconcentración, detección, cálculo y evaluación de resultados) que se esquematizan en la Figura 3. En este apartado se estudiaran los aspectos más importantes de estas etapas.

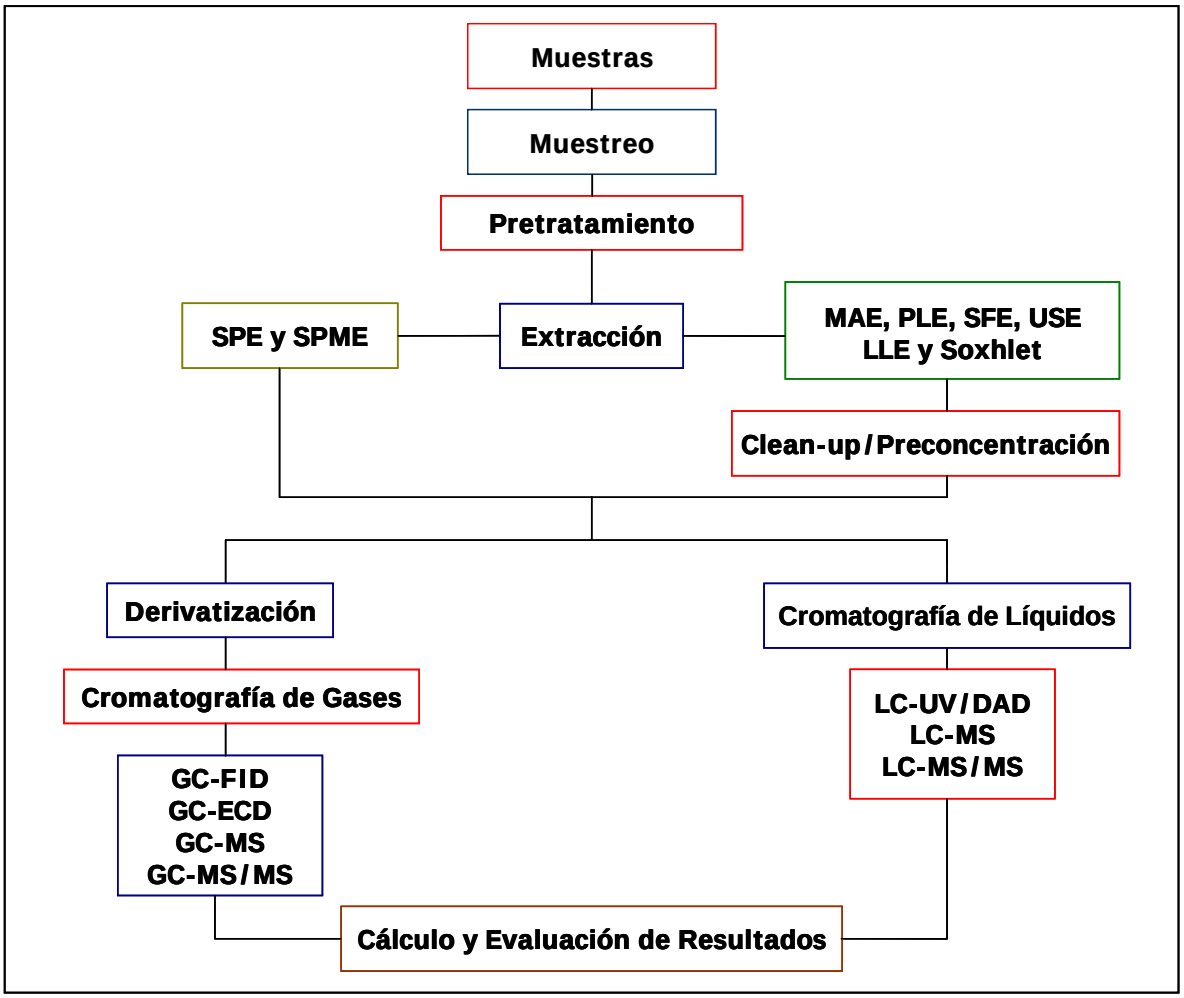


Figura 3: Las principales etapas del proceso analítico para la determinación de sustancias farmacológicamente activas en muestras ambientales, alimentos de origen animal y fluidos biológicos. DAD: detector de diodos en fila; ECD: detector de captura de electrones; FID: detector de ionización de llama; GC: cromatografía de gases; LC: cromatografía de líquidos; LLE: extracción líquido-líquido; MAE: extracción asistida por microondas; MS: espectrometría de masas; MS/MS: espectrometría de masas en tándem; PLE: extracción acelerada con disolventes; SFE: extracción con fluidos supercríticos; SPE: extracción en fase sólida; SPME: microextracción en fase sólida; USE: extracción asistida por ultrasonidos; UV: detector de ultravioleta

2.1. Muestreo, conservación y pretratamiento de muestras

Cuando el analista se enfrenta a la toma de muestra como etapa previa a la resolución de un problema analítico tendrá que basarse en el conocimiento previo ya existente, donde la toma de muestra puede estar ya documentada o descrita en forma de procedimientos normalizados de trabajo. Muchos de los protocolos recomendados en el muestreo se encuentran recogidos en diferentes guías o normas de carácter internacional, tales como las editadas por organizaciones como la Organización Internacional para la Estandarización (ISO) o la Sociedad Americana para Pruebas y Materiales (ASTM), que editan normativas recomendando protocolos para asegurar la calidad de todo el proceso analítico, incluyendo la etapa de toma de muestra. El estado físico de la muestra condicionará en muchos casos la forma de tomar la muestra. La homogeneidad que suele acompañar a las muestras de líquidos y gases permite planificar una toma de muestra para la recogida de pequeñas cantidades o volúmenes, con un riesgo menor de falta de representatividad. En cambio, en el caso de muestra sólidas, su inherente mayor heterogeneidad aconseja llevar a cabo un pretratamiento previo con el fin de disminuir los problemas derivados de dicha heterogeneidad.

Una vez tomada la muestra y en la mayoría de los casos son necesarias medidas preventivas antes de llevar a cabo la preparación de la muestra y determinación de los analitos. Estas etapas previas incluyen la conservación de la muestra, la filtración, el ajuste del pH de las muestras acuosas, secado y homogeneización de muestras sólidas. Aunque estas etapas previas suelen ser poco complejas, si no se realizan correctamente la composición de la muestra original se podría ver seriamente modificada. Incluso el almacenamiento y envío de muestras puede ser una etapa crítica para la posterior preparación de la muestra. En el caso de SFAs, Lee et al. (2003) observaron pérdidas significativas de ácido salicílico, paracetamol y fenofibrato en el almacenamiento de aguas residuales en la oscuridad a 4 ºC. También el almacenamiento de las muestras puede influir en la concentración de aductos como sulfatos y glucoronides (Reddy et al., 2005). Por ello en muchos casos es conveniente analizar las muestras tan pronto como sea posible, o almacenarlas en la oscuridad a -20 ºC si no se puede llevar a cabo el análisis de una manera inmediata.

Una etapa muy común para la conservación de la muestra es el ajuste del pH. Este ajuste del pH se debe de llevar a cabo después de la filtración con el fin de evitar

posibles pérdidas durante esta etapa, debido al aumento en el analito hidrofobicidad. Por ejemplo, algunos medicamentos fibratos tales como las tetraciclinas pueden sufrir epimerización a pH ácido (Beausse, 2004).

La complejidad de las matrices biológicas puede ser muy variada (tejido> leche > sangre> plasma > suero > orina> saliva). En estas matrices y con vistas a la eliminación de proteínas, lípidos y otras sustancias se suelen llevar a cabo distintos tipos de procedimientos, tales como el tratamiento con un ácido o base fuerte a alta temperatura, o, más comúnmente, el uso de un disolvente orgánico (acetonitrilo, metanol, mezcla de disolvente orgánicos, o mezcla de agua-disolventes orgánicos). La mayoría de los métodos bioanalíticos suelen emplear un mínimo de tres partes de disolvente orgánico por una parte de matriz, a continuación se lleva a cabo el mezclado de ambas fases y finalmente se realiza una centrifugación. De esta manera, se separan las proteínas y otras especies interferentes del sobrenadante que contiene a los analitos de interés. En algunos casos se seca este sobrenadante y luego se redisuelve con un disolvente adecuado.

2.2. Preparación de muestra

Una vez obtenida una muestra representativa para su análisis, la siguiente operación dentro del proceso analítico general es la preparación o tratamiento de muestra. Se debe de tratar de disminuir la manipulación de la muestra que conlleve a determinar el analito en ausencia de interferencias. De esta manera, aparte de resultar la determinación más rápida y económica, se minimizan los problemas de contaminación y pérdidas de analito. La extensión del proceso de preparación de muestra puede incluir etapas tales como aislamiento, purificación y preconcentración del analito. En los métodos analíticos donde estén implicadas las técnicas cromatográficas, en muchos casos también es necesaria una etapa de derivatización. A continuación se van a tratar las técnicas de extracción más utilizadas para la determinación de sustancias farmacológicamente activas y finalmente se describirán algunas de las reacciones de derivatización utilizadas para este tipo de estas sustancias.

2.2.1. Extracción

El objetivo de la etapa de extracción es "arrancar el analito de la matriz de la muestra a que está unido. Para ello, la muestra se tiene que poner en contacto con un

extractante (sólido, líquido o fluido supercrítico) en unas determinadas condiciones (pH, temperatura, fuerza iónica) de tal forma que se debiliten completamente las interacciones "analito-matriz de la muestra" a la que vez que se incrementan las interacciones "analito-extractante". En el caso de extracción de sustancias farmacológicamente activas se están utilizando diferentes técnicas analíticas como se indica en la Figura 4. La técnica más utilizada para llevar a cabo la purificación y extracción de SFAs presentes en muestras ambientales, alimentos y fluidos biológicos es la extracción en fase sólida, que ha desplazado a la extracción líquido-líquido empleada tradicionalmente. Especial interés están tomando otras alternativas como la microextracción en fase sólida y la extracción con fluidos supercríticos.

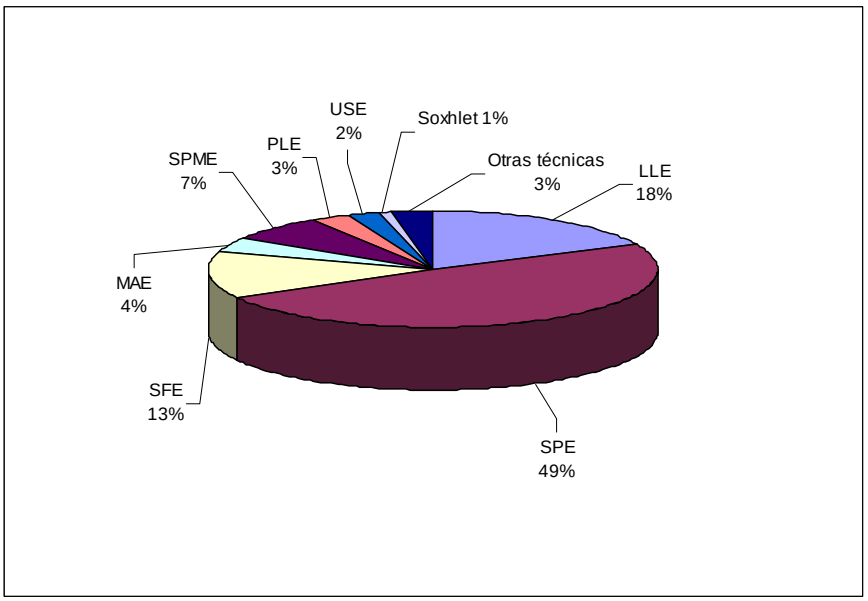


Figura 4: Porcentajes de empleo de distintas técnicas de extracción sustancias farmacológicamente activas en ambientales, alimentos y fluidos biológicos en las publicaciones aparecidas en el período 2005-2011. [Fuente: Scopus; palabras claves: "pharmaceuticals", "environmental samples", "foods animal origin", "biological fluids", "solid-phase extraction" (SPE), "solid-phase microextraction" (SPME), "liquid-liquid extraction" (LLE), "microwave-assisted extraction" (MAE), "supercritical fluid extraction" (SFE), "pressurized liquid extraction" (PLE) or "ultrasonic solvent extraction" (USE)].

La etapa de extracción es esencial en el análisis de muestras ambientales (aguas, suelo, sedimentos y lodos), alimentos de origen animal (leche, músculo, hígado, riñones, etc.) y fluidos biológicos (orina, sangre y plasma) debido a la complejidad de la matriz y a los bajos niveles de los contaminantes que están presentes en dichas muestras. En esta sección se comentarán las principales ventajas e inconvenientes de

cada una de las técnicas más empleadas para la extracción de SFAs en estos tipos de muestras.

2.2.1.1. Extracción y microextracción líquido-líquido

La extracción líquido-líquido (*liquid-liquid extraction*, LLE) es un proceso de transferencia de uno o varios analitos desde una fase líquida (muestra problema) a otra también líquida inmiscible con la primera. Inicialmente, se mezcla un disolvente insoluble (generalmente orgánico) con la muestra (generalmente acuosa) para obtener un sistema de dos fases. La selección del disolvente apropiado, el ajuste del pH de la fase de agua y la adicción de agentes salinos son fundamentales para conseguir un eficacia en la extracción (Falbe y Regitz, 1995; Cammann, 2001). En la Tabla 11 se incluyen algunos ejemplos representativos de la aplicación de la LLE a la determinación de fármacos. Como se puede observar el acetato de etilo es el disolvente más empleado para la LLE de fármacos (Pfeifer et al., 2002; Greiner-Sosanko et al., 2007; Yilmaz et al., 2009; Nguyen et al., 2011).

No obstante la LLE tiene una serie de inconvenientes como son la formación de emulsiones, el manejo de volúmenes elevados de muestra, el empleo de disolventes tóxicos e inflamables, así como los riesgos de pérdidas o contaminaciones durante las distintas etapas. Por lo tanto está técnica esta siendo desplazada por la extracción en fase sólida u otras técnicas de microextracción. Entre estas últimas se encuentra la microextracción líquido-líquido (*liquid-phase microextraction*, LPME) que posee varias ventajas (simplicidad, barata, reducido consumo de disolventes orgánicos, y alta eficacia de enriquecimiento). Desde su descubrimiento en 1996, se han desarrollado diferentes modalidades de LPME, tales como microextracción sobre una sola gota (*single-drop microextraction, SDME)*, microextracción líquida-líquida-líquida (*liquid-liquid-liquid microextraction*, LLLME) y la microextracción líquido-líquido dispersiva (*dispersive liquid-liquid microextraction*, DLLME) (Herrera-Herrera et al., 2010; Rezaee et al., 2006; Xu et al., 2007). Esta última modalidad ha sido aplicada con éxito para la determinación de clembuterol (Melwanki y Fuh, 2008), antisépticos (Guo et al., 2009) y anti-inflamatorios no esteroideos/analgésicos (Zgoła-Grzéskowiak, 2010) en muestras de agua.

Tabla 11: Aplicaciones de la técnica de extracción líquido-líquido para la determinación de sustancias farmacológicamente activas

Fármacos	Muestras	disolvente	Referencia
Antibióticos	Estiércol	Hexano-acetato de etilo	Pfeifer et al., 2002
Medicamentos de uso humano, hormonas, antioxidantes y un plastificante	Aguas	Diclorometano	Soliman et al., 2004
Antiepilépticos	Suero y plasma humanos	Acetato de etilo	Greiner-Sosanko et al., 2007
Cloranfenicol tiamfenicol, florfenicol	Carne e hígado de cerdo y pollo	Hexano	Shen et al., 2009
Metoprolol	Plasma humano	Acetato de etilo y éter dietílico	Yilmaz et al., 2009
Hormones	Fluidos cerebroespinales	Acetato de etilo	Nguyen et al., 2011
Analgésicos/antiinflamatorios Antiepilépticos	Sangre humana	Metanol y acetonitrilo	Sørensen, 2011

2.2.1.2. Extracción en fase sólida

La extracción en fase sólida (*solid-phase extraction*, SPE) es actualmente la técnica más empleada para la preparación de la muestra y ha reemplazado en muchos casos a la extracción líquido-líquido. En ella se hace pasar la muestra a través de una fase sólida (polar, apolar, intercambio iónico, etc.), quedando retenidos algunos de los compuestos y el resto pasan inalterados. Posteriormente, si los analitos de interés han quedado retenidos, éstos podrán ser eluidos con un pequeño volumen de disolvente. Los objetivos que se consiguen con la SPE son: a) preconcentración de trazas, b) limpieza de muestras, c) estabilización de muestras, d) cambio de fase, y e) fraccionamiento de mezclas complejas.

El modo de adsorción del analito en el material de SPE depende de las características de los materiales aplicados. El mecanismo de interacción del soluto con la superficie de la fase estacionaria (RP-C_{18}, poliestireno-divinilbenceno, etc.) se lleva a cabo por fuerzas de Van der Waals, de dispersión o hidrofóbicas. Es un proceso de baja energía (5 Kcal/mol) análogo al que tiene lugar en la extracción líquido-líquido. También pueden darse en menor proporción y dependiendo del sorbente, otros tipos de interacciones como dipolo-dipolo, enlaces de hidrógeno e intercambios iónicos (Dean, 1998; Masqué et al., 1998; Thurman y Mills, 1998). En la Tabla 12 se incluyen algunas características de los materiales sorbentes más utilizados para la SPE de sustancias farmacológicamente activas y a continuación se comentarán.

Tabla 12: Características físicas de sorbentes utilizados en la SPE de sustancias farmacológicamente activas.

Sorbente	Estructura[a]	Superficie específica (m^2/g)	Diámetro de partícula (μm)
Amberlita XAD-1	PS-DVB	100	—
Amberlita XAD-2	PS-DVB	300	20–60
Amberlita XAD-4	PS-DVB	≥ 750	20–60
Amberlita XAD-7	MA-DVB	450	20–60
Amberlita XAD-8	MA-DVB	310	—
Isolute ENV+	PS-DVB-OH	1000–1100	90
LiChrolut EN	PS-DVB hc.	1200	40–120
Oasis HLB	DVB-VP	830	50–65
Oasis MCX	DVB-VP	—	30–60
PLRPS	PS-DVB	550	15–60
Strata X	PS-DVB cm	800	—
Gel de sílice	–Si–OH	—	15–35
Florisil	$MgSiO_3$	—	50–200
Isolute NH_2	$–Si–(CH_2)_3–NH_2$	—	45–65
RP-C_{18}	$–Si–(CH_2)_{17}CH_3$	—	40–63

[a]PS-DVB, poliestireno-divinilbenceno; F-PS-DVB, poliestireno-divinilbenceno con grupo funcional; DVB-VP, divinilbenceno-vinilpirrolidona; MA-DVB, metacrilato-divinilbenceno; hc, *pypercrosslinked*; cm, *chemically modified*.

- ***Sílices modificadas***

Estos sorbentes se pueden clasificar como: a) en fase inversa, formados por sílice enlazada a hidrocarburos alifáticos o aromáticos, tales como octadecilo (C_{18}), octilo, etilo, fenilo y ciclohexilo; b) en fase normal, formados por la sílice enlazada a otros grupos funcionales, tales como el cianopropilo, aminopropilo y diol (Thurman y Mills, 1998). Sus mecanismos de interacción se basan principalmente en las interacciones hidrófobas (fuerzas de Van der Waals) entre los analitos y la fase estacionaria (Fritz, 1999). El uso de estos absorbentes pueden presentar algunos inconvenientes tales como la reducida eficacia en la extracción de compuestos polares, la inestabilidad a pH extremos y la presencia de algunos grupos silanol residuales (Poole, 2003).

En las Tablas incluidas en el siguiente apartado de aplicaciones de las técnicas analíticas se resumen algunos métodos representativos que utilizan C_{18} como sorbente para la SPE de fármacos de distintos tipos de muestras (ambientales, alimentos y fluidos biológicos) (Ternes et al., 2002; Tuerk et al., 2006; Vinci et al., 2006; Bianchi et al., 2007; Togola y Budzinski, 2007; Ye et al., 2008; Rejtharová y Rejthar, 2009; Sun et al., 2009; Samaras et al., 2011). Los disolventes elegidos para la elución de los analitos retenidos en este tipo de sorbente son: acetato de etilo, acetona, acetonitrilo, diclorometano, éter o metanol).

- ***Carbonos activados grafitizados***

El carbón grafitizado es un adsorbente no-específico y no-poroso con una superficie específica de unos 100 m^2/g. Estos adsorbentes son, en ocasiones, los únicos que pueden retener muchos solutos orgánicos altamente polares. Aparentemente, la adsorción no sólo se debe a interacciones hidrofóbicas, sino que en muchas ocasiones se producen interacciones electrónicas específicas con el analito (Yang, 2003). Se piensa que el carbón grafitizado contienen carbono-oxígeno que proporcionan sitios con carga positiva que actúan como intercambiadores de aniones en presencia de muestras acidificadas y permiten la adsorción de analitos extremadamente polares. Una de las desventajas que presentan estos adsorbentes es la excesiva retención de algunos analitos, que en algunos casos pueden ser adsorbidos irreversiblemente. En el caso de sustancias farmacológicamente activas se han utilizado carbón activado para la

extracción de ácido clofibrico e ibuprofeno presentes en fluidos biológicos y diferentes tipos de aguas con rendimientos de extracción entre 80 y 100 % (Neng et al., 2011).

- ***Sorbentes poliméricos***

Los sorbentes más utilizados son los copolímeros de estireno-divinilbenceno (PS-DVB), que tienen una superficie hidrófoba y áreas superficiales comprendidas entre 600 y 1200 m^2/g. Estos materiales superan muchas de las limitaciones de las sílices enlazadas, ya que tienen mayor estabilidad en un intervalo de pH más amplio (1-14) y retienen más eficazmente a los analitos, principalmente polares, porque su superficie hidrófoba contiene un gran número de sitios aromáticos activos lo cual permite interacciones π-π con analitos no saturados. La eficacia de estos polímeros depende de varios parámetros físico-químicos: tamaño de partícula, área superficial, diámetro de poro, volumen de poro, grado de reticulación y distribución del tamaño de partícula (Liska, 2000; Masqué et al., 2001). En la Tabla 12 se incluyen algunas de las características físicas de los sorbentes poliméricos más utilizados para la SPE de fármacos. Igualmente en la Figura 5 se pueden observar las estructuras químicas de algunos de ellos.

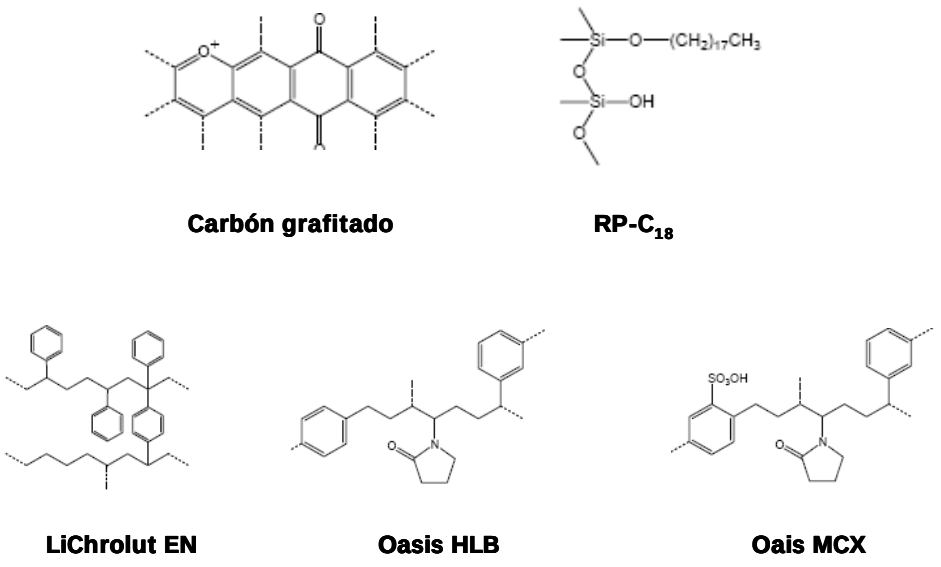


Figura 5: Estructura químicas de los sorbentes poliméricos más utilizados para la SPE de SFAs.

Estos materiales poliméricos están siendo los más utilizados como sorbentes en la SPE de SFAs como se puede observar en las Tablas incluidas en el apartado siguiente dedicado a las diferentes técnicas analíticas para la determinación de SFAs. De todos ellos, el preferido por muchos investigadores es el Oasis HLB (Weigel et al., 2004; Shao et al., 2005; Lin et al., 2005; Babic et al., 2006; Díaz-Cruz et al., 2006; Gómez et al., 2006; Koesukwiwat et al., 2007; Lajeunesse y Gagnon, 2007; Togola y Budzinski, 2007; Raich-Montiu et al., 2007; Zhang y Zhou, 2007; Shen et al., 2009; Vazquez-Roig et al., 2010; Magiera et al., 2011). Otros autores han utilizado el sorbente Oasis MAX con este mismo objetivo (Lee et al., 2005; Kasprzyk-Hordern et al., 2007; Zang et al., 2008).

- ***Polímeros impresos molecularmente***

Una nueva tecnología conduce a la obtención de polímeros sintéticos muy estables, que poseen propiedades de reconocimiento molecular selectivo y que se denominan polímeros impresos molecularmente (*molecularly imprinted polymers*). Los lugares de reconocimiento dentro de la matriz polimérica son complementarios al analito en el perfil y posición de grupos funcionales. Estos sorbentes se preparan sintetizando polímeros altamente entrecruzados en presencia de una molécula plantilla (*template*). Después de separar la molécula, el polímero puede utilizarse como un medio de unión selectivo para la plantilla (analito) o compuestos relacionados estructuralmente (Pichon, 2007). Estos polímeros han sido utilizados para la SPE de cloranfenicol en leche, miel o fluidos biológicos (Boyd et al., 2007; Mohamed et al., 2007). También han sido aplicados este tipo de sorbente para la extracción de antibióticos derivados de la tetraciclina en huevos y tejidos animales (Chen et al., 2009b).

2.2.1.3. Microextracción en fase sólida

La técnica de microextracción en fase sólida (*solid-phase miroextraction*, SPME) se basa en la partición de los analitos contenidos en una matriz y el recubrimiento de una fibra de sílice fundida con un líquido polimérico o un sorbente sólido. El pequeño tamaño de la fibra y su geometría cilíndrica permiten incorporarla en una jeringa. La fibra está unida a un pistón de acero inoxidable cubierto por una aguja protectora, adaptada a un cuerpo de jeringuilla, pero en este caso el émbolo no va a aspirar la

muestra, sino que sólo se va a encargar de hacer salir la fibra al exterior o de introducirla en el interior del dispositivo (Lord y Pawliszyn, 2000). La técnica de SPME surge para eliminar o reducir las limitaciones de SPE, ya que ofrece una rápida transferencia de masa durante la extracción y la desorción, previene las obstrucciones que se pueden producir en la SPE, reduce o elimina el consumo de disolventes y facilita el manejo e introducción directa en los instrumentos analíticos de medida (Figura 6). Tiene limitaciones relacionadas con el coste y la reproducibilidad, principalmente.

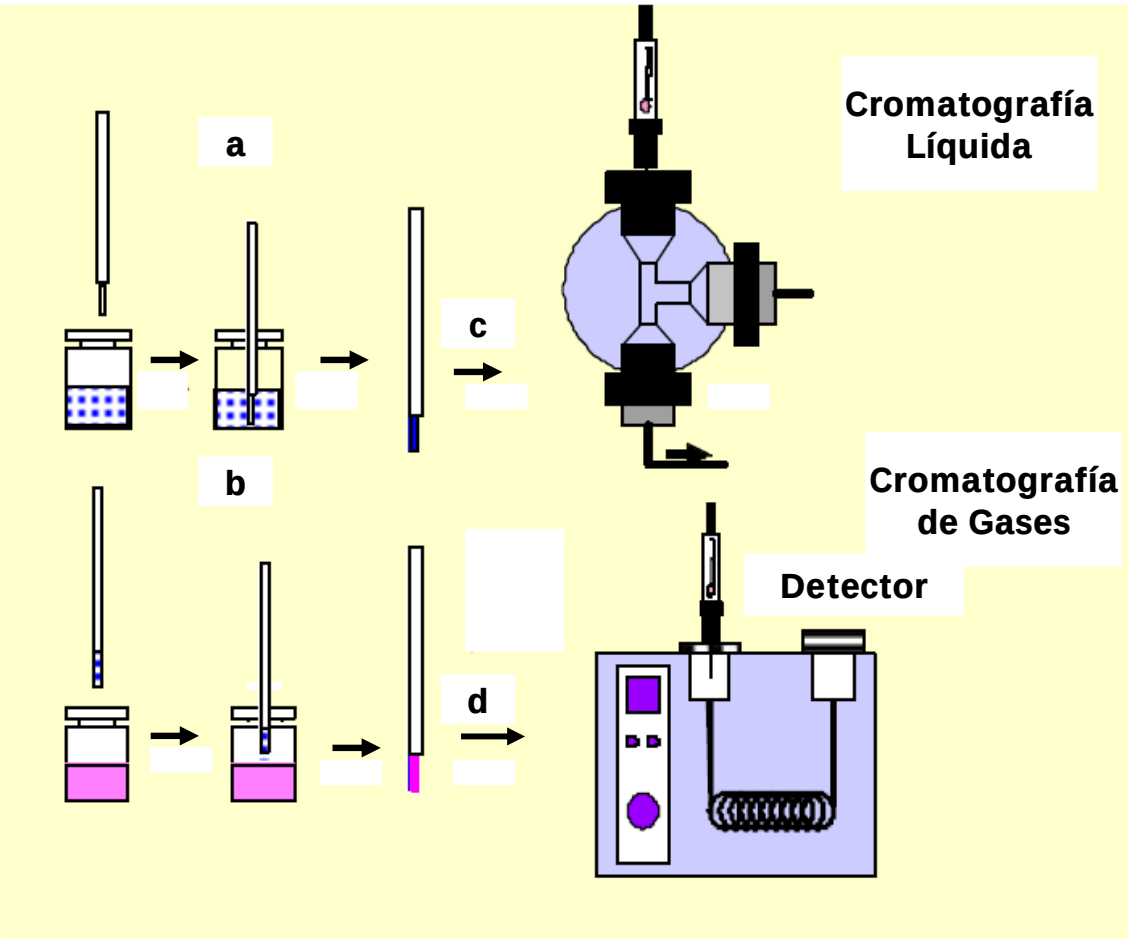


Figura 6: Esquema del proceso de SPME acoplada a CL o a CG; (a) inmersión directa; (b) espacio de cabeza; (c) desorción con disolventes; (d) desorción térmica.

Los materiales sorbentes más utilizados en las fibras de SPME son el polidimetilsiloxano (PDMS), policrilato y divinilbenceno. También algunos investigadores prefieren el uso de otros materiales como el Carbowax o Carboxeno. El proceso de extracción finaliza con la desorción térmica para cromatografía de gases (GC) o con disolventes para cromatografía líquida (LC). Existen tres modos de

extracción en SPME: a) por inmersión directa de la fibra en la muestra líquida, b) por introducción de la fibra en el espacio de cabeza (*headspace,* HS), y c) por protección de la fibra con una membrana semipermeable, impidiendo que compuestos con elevado peso molecular tales como ácidos húmicos o proteínas lleguen a la fibra y puedan dañarla (Risticevic et al., 2009).

En las Tablas incluidas en el apartado se han incluido algunos métodos en los que se utiliza SPME para extracción de algunos tipos de SFAs. Así por ejemplo, Souza et al. (2011) han empleado fibras de polidimetilsiloxano para la adsorción de anfetaminas presentes en fluidos procedentes de la boca. Otros autores han preferido fibras de polimetilsioloxane-divinilbenceno para la SPME de antiinflamatorios y antiepilépticos en aguas de rio (Vera-Candioti, 2008). Jarmalavičiene et al. (2008) han utilizado un nuevo material "*restricted-access media*" como sorbente para la SPME de diferentes tipos de analgésicos y cafeína en plasma de vaca.

2.2.1.4. Extracción en barra

La extracción en barra (*stir-bar sorptive extraction*, SBSE) se basa en el uso de una pequeña barra magnética agitadora recubierta de un polímero que actúa de adsorbente (1 mm de PDMS), comercializada con el nombre de Twister en dos tamaños diferentes (10 mm x 3.2 mm; y 40 mm x 3.2 mm) recomendadas para volúmenes de muestras de 1–50 ml y 100–250 ml, respectivamente. En esta técnica se introduce la barra magnética en una matriz líquida donde se agita durante un tiempo, reteniendo en el material sorbente a los analitos. Finalmente, éstos se desorben con un disolvente o térmicamente en una unidad de deserción (150–300 ºC) para su determinación por GC (Sánchez-Rojas et al., 2009).

La SBSE se ha aplicado con éxito para el análisis de trazas de compuestos volátiles y semi-volátiles en muestras ambientales y en muestras biológicas. Por ejemplo, Silva et al. (2008) han aplicado esta técnica para la extracción de anti-inflamatorios no esteroides/ analgésicos y reguladores de lípidos de muestras de agua. Otros autores han usado la técnica SBSE para la extracción de hormonas y antiepilépticos en muestras biológicas (Almeida y Noguerira, 2006; Queiroz et al., 2008).

2.2.1.5. Extracción asistida por microondas

En los últimos años la extracción asistida por microondas (microwave assisted extraction, MAE) ha aparecido como una clara alternativa a la extracción Soxhlet ya que permite un calentamiento más rápido y eficiente de la muestra. La muestra se extrae aplicando energía de microondas en un disolvente adecuado. Las muestras junto con el disolvente de extracción se irradian con las ondas de microondas durante cortos periodos de tiempo y como consecuencia se produce un aumento de la temperatura que favorece la transferencia de los compuestos retenidos en la matriz al disolvente. Esta técnica de extracción es rápida, ya que los tiempos de irradiación son muy cortos y, además, se pueden extraer varias muestras simultáneamente. Sin embargo, presenta algunos inconvenientes tales como la dificultad de ser automatizada o acoplada a las técnicas de análisis, así como la necesidad de centrifugar o filtrar la muestra después del proceso de extracción.

Los parámetros a tener en cuenta a la hora de optimizar un método basado en la extracción MAE son la composición del agente extractante y su volumen, la temperatura y el tiempo de extracción, así como la naturaleza de la matriz. Esta técnica de extracción depende de la matriz y limita los disolventes que se pueden emplear, ya que conviene que éstos no sean transparentes a la radiación de microondas y que tengan un elevado momento dipolar, sin olvidar la solubilidad de los analitos en los mismos. En general, cuanto mayor sea el momento dipolar del disolvente, mayor será su capacidad de extracción. Así, el hexano, cuyo momento dipolar es prácticamente nulo, es transparente a la radiación de microondas y apenas se calienta cuando se usa dicha radiación. En cambio, metanol, etanol, acetonitrilo, agua y acetona tiene un momento dipolar más alto (Tabla 13) y se calentarán en pocos segundos, aunque algunos compuestos orgánicos presentan una mejor solubilidad en metanol y agua (Letellier y Budzinski, 1999; Eskisson y Björklund, 2000). Como disolvente extractante se suele utilizar mezclas de agua con metanol o acetonitrilo, aunque algunos autores prefieren cloruro de metileno (Rice y Miltra, 2007) o agua (Dobor et al., 2010; Varga et al., 2010) para la extracción de SFAs en lodos y sedimentos consiguiéndose una eficacia en la extracción similar a la que se obtiene con disolventes orgánicos, pero es más barata, segura y ecológica.

Tabla 13: Propiedades dieléctricas de varios disolventes utilizados en el MAE.

	ε´	ε´´	tanδ($\times 10^4$)
Hexano	1.89	0.00019	0.10
Acetato de etilo	6.02	3.2	5312
Acetona	21.1	11.5	5555
Metanol	23.9	15.3	6400
Etanol	24.3	6.1	2500
Acetonitrilo	37.5	2.3	620
Agua	76.6	12.0	1570

La temperatura juega un papel muy importante en el proceso, ya que un aumento de la temperatura se refleja en una mayor capacidad del disolvente para solubilizar los analitos. No obstante un exceso de temperatura puede favorecer la degradación de los mismos. A modo de resumen en la Tabla 14 se incluyen algunos ejemplos de aplicaciones de MAE en la determinación de sustancias farmacológicamente activas en muestras sólidas tales como suelos, sedimentos y lodos. Como se puede observar, en la mayoría de los casos se seca, tamiza (0.5−1 mm) y homogeniza la muestra previa a la extracción con MAE. En algunos casos se controla la temperatura (100−150 ºC) del tratamiento con MAE (Liu et al., 2004a; Matejícek et al., 2007; Forster et al., 2009; Dobor et al., 2010). En otros casos se controla la potencia entre 30 y 800 W durante 2−15 min (Labadie y Budzinski, 2005; Rice y Mitra, 2007; Cueva-Mestanza et al., 2008; Varga et al., 2010).

Tabla 14: Aplicación de la técnica de extracción asistida por microondas para la determinación de sustancias farmacológicamente activas en muestras sólidas.[a]

	Muestras	Pretratamiento de muestra	Condiciones de MAE	Tratamiento del extracto	Determinación	Parámetros analíticos	Referencia
Hormonas	Sedimentos (5g seco)	–	110 ºC, 15 min 25 ml metanol	Centrifugación SPE (gel de sílice, acetato de etilo- hexano)	GC–MS Derivatización con BSTFA+1%TMCS	LOD: 0.2–1 ng/g Rec: 62–133 % RSD: < 24.3 %	Liu et al., 2004a
Hormonas	Partículas flotantes en agua del río (150 mg)	–	30 W, 5min 10 ml metanol:agua Milli-Q (55:45, v/v)	Centrifugación SPE (Oasis HLB, metanol-agua)	GC–MS Derivatización con MSTFA, mercaptoetanol, y NH_4I)	LOD: 0.4–1.9 ng/g Rec: 72–91 %	Labadie y Budzinski, 2005
Hormonas	Sedimentos de río (1 g)	Tamizado (1.0 mm) y homogenizado	100 ºC, 10 min 10 ml agua:metanol (25:75, v/v)	SPE (Oasis WAX, agua-tetrahidrofurano-NH_4OH)	LC–MS/MS	LOD:0.14–0.98 ng/g Rec:83–107 % RSD: 4.9 – 9.6 %.	Matejícek et al., 2007
Cafeína, analgésicos/ ANEs, antiepilépticos, hormonas	Suelo y sedimentos (3 g)	Secado y homogenizado	800 W, Cloruro de metileno: metanol (2:1, v/v)	Centrifugación SPE (columna de gel de sílice; hexano-cloruro de metileno-acetona)	GC–MS Derivación con BSTFA-piridina	Rec: 25–90 %	Rice y Mitra, 2007
Analgésicos/ANEs, RL, β-bloqueadores, antiepilépticos	Sedimentos (2 g)	Secado	500 W, 6 min; 8 ml POLE	Filtración, SPE (Oasis HLB, metanol)	LC–UV	LOD: 4 –167 ng/g Rec: 6–116 % RSD: < 11 %	Cueva-Mestanza et al., 2008
Antibióticos y sus metabolitos	Suelo (10 g)	Secado, tamizado y homogenizado	150 ºC, 15 min, acetonitrilo:agua (1:4, v/v)	–	LC–MS/MS	Rec: 95–101 %	Forster et al., 2009
Analgésicos/ANEs	Lodo (0.5 g)	Secado, tamizado y homogenizado	100 ºC, 30 min 50 ml de agua	Centrifugación SPE (Oasis HLB)	GC-MS Derivatización con hexametildisilazano+ ácido trifluoroacético y hidroxilamina-HCl	LOQ: 15–22 ng/g Rec: 80–105 % RSD: 10–17 %	Dobor et al., 2010

					en piridina)		
Analgésicos/ANEs	Agua de rio, sedimentos (5 g)	Secado, tamizado y homogenizado	600–700 W, 30 min 50 ml de agua destilada	SPE (Oasis HLB)	GC-MS Derivatización con hexametildisilazano+ ácido trifluoroacético y hidroxilamina-HCl en piridina)	LOQ: 2–90 ng/l 2–38 ng/g Rec: 95–103 % RSD: 9–13 %	Varga et al., 2010

[a]ANEs: Antiinflamatorios no esteroideos; BSTFA: N,O-bis-(trimetilsilil)trifluoroacetamida; DAD: detector de diodos en fila; GC: cromatografía de gases; LC: cromatografía de líquidos; LOD: límite de detección; LOQ: límite de cuantificación; MAE: extracción asistida por microondas; MS: espectrometría de masas; MS/MS: espectrometría de masas en tándem; MSTFA: N-metil-N-(trimetilsilil)trifluoroacetamida; POLE: polioxietileno; Rec: recuperación; RL: reguladores de lípidos; RSD: desviación estándar relativa; SPE: extracción en fase sólida; UV: detector de ultravioleta.

2.3. Técnicas analíticas para la determinación de sustancias farmacológicamente activas

Como se puede observar en la Figura 7, donde se recogen las publicaciones analíticas sobre sustancias farmacológicamente activas en muestras ambientales, alimentos y fluidos biológicos durante el período comprendido entre 2005-2011, las técnicas más empleadas para el análisis de SFAs son la cromatografía de líquidos y la cromatografía de gases, seguidas por la electroforesis capilar y las técnicas espectroscópicas, espectrofluorimétricas y quimioluminiscentes. A continuación se tratarán los aspectos más importantes de estas técnicas, distinguiendo entre cromatográficas y no cromatográficas.

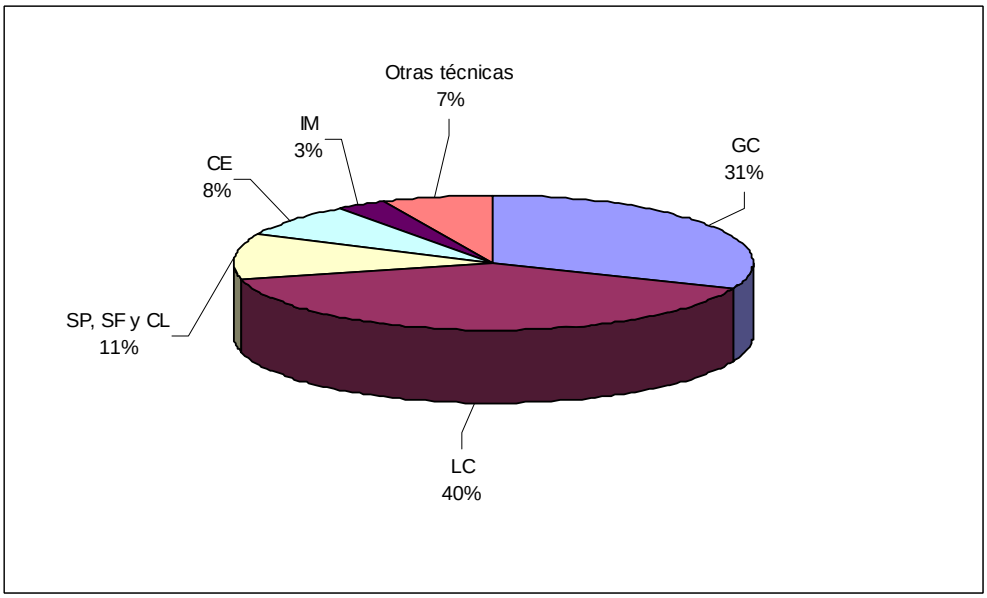


Figura 7: Número de publicaciones aparecidas en el período 2005-2011 relacionadas con la determinación de sustancias farmacológicamente activas. [Fuente: Scopus; palabras claves: "pharmaceuticals", "environmental samples", "foods", biological fluids", "gas chromatography" (GC), "liquid chromatography" (LC), "capillary electrophoresis" (CE), "spectrophotometry" (SP), "spectrofluorimetry" (SF), immunochemical (IM) or "chemiluminescence" (CL)]

2.3.1. Técnicas cromatográficas

Estas técnicas se han convertido en unas herramientas esenciales en la Química Analítica moderna y en las Ciencias relacionadas con la separación de mezclas de

componentes en muestras complejas. De las diferentes modalidades existentes, la cromatografía de gases y cromatografía de líquidos son las más se utilizan para la determinación de SFAs en diferentes tipos de muestras. A continuación se comentarán algunos aspectos importantes relativos a la determinación de estas sustancias mediante GC y LC.

2.3.1.1. Cromatografía de gases

Los primeros métodos basados en la técnica de GC datan de 1970 donde se determinó residuos de cloranfenicol en tejidos animales y penicilinas en leche. Esta técnica ha sido ampliamente utilizada para la determinación de SFAs. No obstante y debido a que estos compuestos, en la mayoría de los casos, son poco volátiles y polares, es necesaria la derivatización previa para su separación por esta técnica.

En cuanto a las columnas utilizadas para la separación cromatográfica de SFAs, las fases más utilizada son las no polares como la de 5 % fenil-metilpolisiloxano (Liu et al., 2004b; Weigel et al., 2004; Lee et al., 2005; Morales et al., 2005; Canosa et al., 2007; Lajeunesse y Gagnon, 2007; Beck et al., 2008; Yilmaz et al., 2009; Magiera et al., 2011; Souza et al., 2011) o 100 % dimetilpolisiloxano (Seo et al., 2005; Rejtharová y Rejthar, 2009) debido a su alta estabilidad térmica y a que son inertes del punto de vista químico.

Las sistemas de detección que normalmente se han utilizado son el detector de captura de electrones (ECD) y el de ionización de llama (FID). Por ejemplo, para la determinación de cloranfenicol y otros fármacos con grupos halogenados se ha utilizado el detector ECD por su alta sensibilidad y selectividad a estos compuestos (Ding et al., 2005; Zhang et al., 2006). El detector de ionización de llama, aunque es menos sensible, lo han utilizado algunos autores para la determinación anti-inflamatorios no esteroideos (Es'haghi, 2009) o parabenos (Farajzadeh et al., 2010) en muestras de agua. En estos métodos utilizan una etapa de preconcentración de microextracción líquido-líquido mejorando notablemente la sensibilidad.

Actualmente la espectrometría de masas es el sistema de detección más utilizado para la determinación de SFAs por GC debido a que además de ofrecer información acerca de su estructura química, permitiendo su identificación inequívoca, se puede aplicar a la determinación cuantitativa de las mismas. Como se puede observar en la Tabla 15, la mayoría de los métodos propuestos para la determinación de estos compuestos en muestras Medio Ambientales, alimentos o fluidos biológicos se basen en

Tabla 15: Métodos para la determinación de sustancias farmacológicamente activas en muestras ambientales, alimentos y fluidos biológicos mediante cromatografía de gases.

Compuestos/Clases terapéuticas [a]	Muestras	Preparación de muestra[b]	Reactivos de derivatización [c]	Determinación [d]	Columna cromatográfica	Parámetros analíticos [e]	Referencia
Cloranfenicol, florfenicol, tianfenicol	Pescado	Centrifugación SPE (Florisil; metanol- éter etílico)	BSA	GC–MS (EI)	HP-5MS	LOD: 5 µg/kg Rec: 61–82 %	Nagata y Oka, 1996
Hormonas	Plasma, orina, pelo, leche materna	SPE (Oasis HLB; metanol)	MSTFA/NH_4I/DTE	GC–MS (EI)	Ultra-2	LOD: 0.2–3 µg/l Rec: 73–104 % RSD: 1.9–14.3 %	Choi et al., 2002
Hormonas	Lodos, sedimentos	Sonicación, centrifugación SPE ($C_{18;}$ acetona), GPC	MSTFA/TMSI/DTE,	GC–MS/MS (EI, trampa de iones)	XTI-5	LOQ: 0.2–4 ng/g Rec: 57–121 %	Ternes et al., 2002
Hormonas, fenoles	Agua de rio y de mar	SPE (gel de sílice; acetato de etilo)	BSTFA+1%TMCS	GC–MS/MS (EI, trampa de iones)	ZB5	LOD: 0.3–3.4 ng/l Rec: 41–114 % RSD: < 20 %	Liu et al., 2004b
ANEs, antiepilépticos, β-bloqueadores, hormonas, RL, antisépticos	Agua superficial	SPE (Oasis HLB; acetato de etilo)	MSTFA	GC–MS/MS (EI, trampa de iones)	HP-5MS	LOQ: 0.05–0.38 ng/l Rec: 70–100 % RSD: 1–21 %	Weigel et al., 2004
ANEs, hormonas, PCPs, fenoles	Aguas residuales	SPE (Oasis MAX; metanol- ácido fórmico)	TBSTFA- 1% TBDMSCI; PFPA	GC–MS (EI)	Restek Rtx-5Sil	LOD: 0.01–0.1 µg/l Rec: 87–110 % RSD: < 7 %	Lee et al., 2005
ANEs, antiepilépticos, RL	Aguas de rio, grifo y residuales	SPE (Oasis HLB; acetona- metanol)	TBA-HSO_4	GC–MS/MS (EI, trampa de iones)	DB-5MS	LOQ: 1.0–8.0 ng/l Rec: 50–108 % RSD:1–10 %	Lin et al., 2005
Triclosán y clorofenoles	Lodos, sedimentos	MAE SPE (Oasis HLB; acetato de etilo)	MTBSTFA	GC–MS/MS (EI, trampa de iones)	HP-5 MS	LOD: 0.4–0.8 ng/g Rec: 78–106 % RSD: 3.6–8.3 %	Morales et al., 2005

Hormonas	Carne	Sonicación SPE ($C_{8;}$ metanol) SPE (NH_2: acetato de etilo-metanol)	MSTFA/NH_4I/DTE	GC–MS	DB-1MS	LOD: 0.1–0.4 µg/kg Rec: 68–106 % RSD: 5–16 %	Seo et al., 2005
Hormonas	Fluidos foliculares de cerdo	MSPD (C_{18} ; éter etílico) SPE (C_{18}; éter etílico)	TFA	GC–MS		LOQ : 0.2–0.8 µg/kg Rec: 81–98 % RSD: < 7 %	Bianchi et al., 2007
Parabenos y triclosán	Polvo	MSPD (C_{18} y Florisil; acetonitrilo)	MTBSTFA	GC–MS/MS (EI, trampa de iones)	HP-5 MS	LOQ: 0.6–2.6 ng/g Rec: 80–114 % RSD: 2.8–13.0 %	Canosa et al., 2007
ANEs, antiepilépticos, PCPs, RL	Aguas residuales	SPE (Oasis- HLB Strata-X; acetato de etilo)	BSTFA +10% TMCS	GC–MS/MS (EI, trampa de iones)	Phenomenex ZB-5ms	LOD: 1–18 ng/l Rec: 72–102 %	Lajeunesse y Gagnon, 2007
Hormonas	Pescado	Sonicación SPE (C_{18}; acetonitrilo)	MSTFA-TMIS-DTE	GC–MS (EI)	HP-5 MS	LOQ: 0.2 µg /kg Rec: 69–100 % RSD: 4.8–10.1 %	Long et al., 2007
ANEs antiepilépticos PCPs, fenoles, herbicidas	Aguas de grifo, pozo, rio y residuales	SBSE (PDMS; acetato de etilo)	MTBSTFA	GC–MS/MS (EI, trampa de iones)	DB-XLB	LOD 1–800 ng/l Rec: 70–130 % RSD: < 20 %	Quintana et al., 2007
ANEs, RL, antiepilépticos, cafeína	Aguas residuales y superficiales	SPE ($C_{18,}$ Oasis HLB; acetona–acetato de etilo)	MSTFA	GC–MS (EI)	HP-5MS	LOD: 0.4 – 2.5 ng/l Rec: 53 – 99 % RSD: <15 %	Togola y Budzinski, 2007
Hormonas	Suelo	PLE SPE (C_{18}; acetone:diclorometano)	MSTFA	GC–MS (EI)	HP-5MS	LOD: 1–20 ng/ml Rec: 79–103 % RSD: ≤ 13 %	Beck et al., 2008
ANEs, EDCs, RL, hormonas, PCPs	Suelo	Sonicación SPE (C_{18}; acetato de etilo)	MTBSTFA	GC–MS (EI)	HP-5MS	LOD: 0.55–9.08 ng/g Rec: 52 –111 %	Xu et al., 2008

Hormonas	Leche, huevos, carne	SPE (C_{18}; acetato de etilo/ H_2SO_4)	MSTFA/TMIS/DTE PFBBr/BSTFA	GC–MS/MS (NCI, triple quadrupolo)	ZB-5MS	LOD: 10 ng/kg	Courant et al., 2008
ANEs	Aguas de grifo, superficiales y residuales	LPME (octanol)	TBA	GC–FID	HYDRODEX β-6TBDM	LOD: 1–2 ng/l Rec: 89–108 % RSD: 3.4–10.2 %	Es'haghi, 2009
Antibióticos	Orina, agua de alimentación, leche, miel	SPE (C_{18}; acetonitrilo-agua MIP (diclorometano-ácido acético-metanol)	BSTFA +1% TMCS	GC–MS (NCI)	HP-1MS	LOD: 0.005–0.2 ng/ml RSD: 2.5–7.0 %	Rejtharová y Rejthar, 2009
Antibióticos	Carne e hígado de cerdo y pollo	Centrifugación LLE (acetato de etilo-amoniaco) SPE(Oasis HLB; metanol)	BSTFA+ 1% TMCS	GC–MS (NCI)	HP-5MS	LOD: 0.1–0.5 µg/kg Rec: 79–106 % RSD: < 17 %	Shen et al., 2009
Metoprolol	Plasma humano	Centrifugación LLE (acetato de etilo-éter dietílico)	MSTFA	GC–MS (EI)	HP-5MS	LOD : 5 ng/ml Rec : 91 % RSD: < 6.4 %	Yilmaz et al., 2009
ANEs, hormonas, PCPs, fenoles	Aguas de ríos, superficiales	SPE (Oasis HLB; metanol, diclorometano)	PFBBr, PFBOCl	GC–MS (NCI)	DB35-MS	LOD: 0.2–2.2 ng/l Rec: 43 –145 %	Zhao et al., 2009
Parabenos	Tomate frito, salsa y preparados farmacéuticos	Extracción con disolvente a presión Centrifugación DLLME (acetona-octanol)	—	GC-FID	SPB-50	LOD: 5–15 ng/ml Rec: 25–72 % RSD: 2–3 %	Farajzadeh et al., 2010
Drogas de abuso	Pelo	Centrifugación SPE (Bond Elut; diclorometano:propanol-amoniaco)	MTBSTFA-TBDMSCI	GC×GC–TOF–MS	DB-5MS BPX50	—	Guthery et al., 2010
ANEs/analgésicos, hormonas,	Aguas de ríos	SPE (Strata-X; acetato de etilo)	Metilación (diazometano)	GC×GC–TOF–MS	TRB-5MS, TRB-50HT	LOD: 0.5–100 ng/l Rec: 41–113 %	Matamoros et al., 2010

antisépticos, antiepilécticos, otros compuestos orgánicos						RSD: < 20 %	
β-bloqueadores, flavonoides, isoflavonas y sus metabolitos	Orina humana	SPE (Oasis HLB; tert-butil metil éter- metanol-ácido fórmico)	MSTFA	GC–MS (EI)	HP-5-MS	LOD: 0.6–9.7 ng/ml Rec: 70–100 % RSD: < 5 %	Magiera et al., 2011
ANEs	Lana	Sonicación	BSTFA +1% TMCS	GC–MS (EI)	ZB-1	—	Richards et al., 2011
ANEs, PCPs, fenoles	Aguas residuales, lodos	Sonicación, centrifugación SPE (C_{18}; acetato de etilo)	BSTFA+1% TMCS	GC–MS (EI)	DB-5MS	LOD: 0.3–33 ng/l Rec: 84–117 % RSD: 1.7– 13 %	Samaras et al., 2011
Anfetaminas	Fluidos bucales	SPME (PDMS)	Formación de alquilcloroformatos	GC–MS (EI)	HP-5MS	LOD : 0.5–2 ng/ml Rec: 7–112 % RSD: < 15 %	Souza et al., 2011

[a] ANEs: Antiinflamatorios no esteroideos; EDCs: compuestos disruptores endocrinos; PCPs: productos de cuidado personal; RL: reguladores de lípidos.

[b] ASE: extracción acelerada con disolventes; DLLME: microextracción líquido-líquido dispersiva; GPC: cromatografía de geles; LLE: extracción líquido-líquido; LPME: microextracción líquido-líquido; MAE: extracción por microondas asistida; MSPD: extracción de la muestras en fase sólida; MIP: polímeros impresos molecularmente; PLE: extracción acelerada con disolventes; PDMS: polidimetilsiloxano; SBSE: Extracción con barra;. SPE: extracción en fase sólida; SPME: microextracción en fase sólida.

[c] BSA: N, O-bis (trimetilsilil)acetamida; BSTFA: N, O-bis (trimetilsilil)trifluoroacetamida; DTE: ditioeritritol; MSTFA: N-metil-N-(trimetilsilil)trifluoroacetamida; MTBSTFA: N-metil-N-(tertbutildimetilsilil)trifluoroacetamida; TMIS: yoduro de trimetilsilano; PFBOCl: cloruro de pentafluorobenzoilo; PFBBr: bromuro de pentafluorobenzilo; PFPA: anhídrido pentafluoropropionico; TBDMSCI: tert-butildimetilclorosilano; TBSTFA: N-t-butildimetilsilil-N-metiltrifluoroacetamida; TBA: tetrabutil amonio; TBA-HSO_4: sulfato ácido de tetrabutilamonio; TFA: anhídrido trifluoroacético; TMCS: trimetilclorosilano; TMSI: trimetilsilisimidazol.

[d] FID: detector de ionización de llama; EI: ionización por impacto electrónico; GC: cromatografía de gases; MS: espectrometría de masas; MS/MS: espectrometría de masas en tándem; NCI: ionización química negativa.

[e] LOD: limite de detección; LOQ: límite de cuantificación; Rec: recuperación; RSD: desviación estándar relativa.

al., 2005; Gómez et al., 2006; Kasprzyk-Hordern et al., 2007; Zhang et al., 2008; Chen et al., 2009a; Sun et al., 2009; Yan et al., 2009; Vazquez-Roig et al., 2010; Gracia-Lor et al., 2011). Por otro lado, Chiuminatto et al. (2010) han utilizado un nuevo método de atomización como es mediante turbo ión spray.

La Directiva 2002/657/EC propone que se necesita un mínimo de tres o cuatro puntos de identificación en el análisis de residuos de fármacos (Comisión of the European Communities, 2002). En este sentido el analizador en tandem cumple con el requisito de las normas Europeas, y es muy utilizado por los investigadores para la identificación de SFAs y sus derivados (Gros et al., 2006). En la Tabla 16 se incluyen algunos ejemplos del uso del analizado de triple cuadrupolo para la identificación de fármacos en diferentes tipos de muestras (ambientales, alimentos y fluidos biológicos). Otros analizadores como el trampa de iones (Vinci et al., 2006; Chen et al., 2009a) o bien el de tiempo de vuelo (Peters et al., 2009; Villar-Pulido et al., 2011) también han sido utilizados pero con menos frecuencia para la determinación de SFAs. Jelic et al. (2009) han utilizado como analizador un híbrido entre triple cuadrupolo y trampa de iones lineal (hybrid triple quadrupole-linear ion trap mass spectrometer, QqLIT-MS) para la determinación un gran número de SFAs, consiguiendo unos límites de detección entre 0.01 y 8.84 ng/g.

Tabla 16: Métodos para la determinación de sustancias farmacológicamente activas en muestras ambientales, alimentos y fluidos biológicos mediante cromatografía de líquidos.

Compuestos/Clases terapéuticas[a]	Muestras	Preparación de muestra[b]	Determinación [c]	Fase estacionaria (FE) Fase móvil (FM)	Parámetros Analíticos [d]	Referencia
Ácido niflúmico y sus metabolitos	Plasma humano	Centrifugación	LC–UV (λ 288 nm)	FE: RP-C_{18} FM: metanol-agua	LOQ: 50 –100 ng/ml Rec : 90–99 % RSD: 2.1 –9.5 %	Jang et al., 2005
Hormonas	Carne, riñones, hígado y leche	Centrifugación SPE (Oasis HLB; metanol-trietilamina)	LC–ESI-MS/MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo-agua; metanol-agua	LOD : 1.0–120 ng/kg Rec : 64–104 % RSD : 2.8–29.1 %	Shao et al., 2005
Hormonas	Aguas, orina	SBSE (polidimetilsiloxano)	LC–DAD (λ 200 nm)	FE: RP-C_{18} FM: acetonitrilo-agua	LOD: 0.3–1.0 µg/l Rec: 11 – 100 % RSD: 2.1–17.1 %	Almeida y Nogueira, 2006
Antibióticos	Aguas residuales	SPE (Oasis HLB; metanol)	LC–DAD (λ 280 nm)	FE: LiChrosphere 100 CN FM: acetonitrilo-disolución acuosa de ácido oxálico	LOQ: 1.5–100 µg/l Rec: 68 – 98 % RSD: 3.3–10.1 %	Babic et al., 2006
Antibióticos	Lodos	PLE SPE (Oasis HLB; metanol, acetona)	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD: 0.001– 0.27 ng/g Rec: 58 – 104 % RSD: 9–17 %	Díaz-Cruz et al., 2006

ANEs/analgésicos, antibioticos, β-bloqueadores, antidepresivos	Aguas residuales del hospital	SPE (Oasis HLB; metanol)	LC–ESI–MS–MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD: 7–47 ng/l Rec: 45–114 % RSD: 0.3– 4.9 %	Gómez et al., 2006
ANEs/analgésicos	Plasma humano	Hidrólisis SPE (Oasis HLB; éter dietílico-metanol)	LC– ESI–MS	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de formato de amonio	LOD: 0.01–0.9 µg/ml Rec: 77–91 % RSD: 5.2–14.1 %	Suenami et al., 2006
Antibióticos	Orina	SPE (C_{18}; metanol-tetrahidrofurano)	LC–DAD (λ = 200–450 nm) LC–MS LC–MS/MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD : 0.03–70 µgl Rec: 61 – 102 % RSD: 5.1–21 %	Tuerk et al., 2006
ANEs	Suero y plasma de animales	Centrifugación SPE (C_{18}; n-hexano-éter dietílico)	LC–ESI–MS–MS (trampa de iones)	FE: Max RP80 FM: acetonitrilo-disolución acuosas de ácido acético	Rec: 72– 101 % RSD : 1.0–21.9 %	Vinci et al., 2006
Antiepilépticos, antibióticos, β-bloqueadores, ANEs/ analgésicos, RL, broncodilatadores, antagonistas del calcio, antidepresivos	Aguas superficiales	SPE (Oasis MCX; metanol, metanol- amoniaco)	LC–ESI–MS–MS (QqQ)	FE: RP-C_{18} FM: metanol-disolución acuosa de ácido acético	LOD: 0.1 – 20 ng/l Rec : 9 –146 % RSD : < 10 %	Kasprzyk-Hordern et al., 2007
Antibióticos	Leche de oveja	SPE (Oasis HLB; metanol)	LC–ESI-MS	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido oxálico	LOD: 0.5–3 ng/ml Rec: 70–106 %	Koesukwiwat et al., 2007
Antibióticos	Aguas superficiales, suelos	MAE SPE (Oasis HLB; acetonitrilo-agua); Derivatización (fluorescamina)	LC-FLD (λex/λem: 405/485 nm)	FE: RP-C_{18} FM: acetonitrilo-tampón acetato de amonio-ácido acético	LOD: 1– 8 ng/l LOD: 1– 6 ng/g Rec: 60–104 % RSD: 2–11 %	Raich-Montiu et al., 2007

ANEs/analgésicos, antibioticos, β-bloqueadores,	Aguas de río y de mar	SPE (Oasis HLB; metanol)	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico; metanol	LOD: 1–288 pg/l Rec: 65–103 % RSD: < 16 %	Zhang y Zhou , 2007
Antibióticos	Leche de vaca	QuEChERS	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: metanol-disolución acuosa de ácido fórmico	LOD : 1– 4 µg/kg Rec : 70–110 % RSD: < 21 %	Aguilera-Luiz et al., 2008
ANEs/analgésicos, antiepilépticos	Aguas de ríos	SPME (polidimetilsiloxano /divinilbenceno)	LC–DAD (λ 200 y 350 nm)	FE: RP-C_{16} FM: acetonitrilo-disolución acuosa de KH_2PO_4	LOD: 0.5– 3.0 µg/l Rec: 72– 123 % RSD: 3.4–8.2 %	Vera-Candioti et al., 2008
Triclosan, parabenos, fenoles	Leche humana	Centrifugación SPE en línea (C_{18}; metano-agua)	LC–MS/MS (QqQ) (fotoionización a presión atmosférica)	FE: RP- C_8 FM: metanol-agua	LOD: 0.1–1.0 ng/ml Rec: 84–119 % RSD: 3.5–16.3 %	Ye et al., 2008
Cloranfenicol, tianfenicol, florfenicol	Carne de pollo	LLE (acetato de etilo) SPE (Oasis MCX; metanol-amoniaco)	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: acetonitrilo- agua	LOD: 0.1–1.0 µg/kg Rec: 95–107 % RSD: < 10.9 %	Zhang et al., 2008
ANEs/analgésicos, antiepilépticos, antibioticos, β-bloqueadores, RL	Lodos, sedimentos	PLE SPE en línea (Oasis HLB; metanol)	LC–ESI–QqLIT–MS/MS	FE: RP-C_{18} FM: acetonitrilo-metanol-agua	LOD: 0.01– 8.84 ng/g Rec: 33–215 % RSD: 1–15 %	Jelíc et al., 2009
Antibióticos	Suelos	MAE SPE (Alúmna; disolución acuosa de ácido acético)	LC–ESI–MS/MS (trampa de iones)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido acético	LOD: 1.4–4.8 ng /g Rec: 86–102 % RSD: 2.7–6.7 %	Chen et al., 2009a
PCPs	Lodos de aguas residuales	PLE	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: metanol-disolución acuosa de ácido acético	LOD: 1.25– 8 µg/kg Rec: 30–108 % RSD: 1–13 %	Nieto et al., 2009

Antibióticos, tranquilizantes, ANEs	Carne, pescado y huevos	Centrifugación SPE (StrataX ; metanol-acetato de etilo; metanol-acetonitrilo	LC–TOF-MS	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	Rec: 70–100 % RSD: 8–20 %	Peters et al., 2009
Hormonas	Aguas de grifo, río, pantano, residuales	SPE (C_{18}; C_8; acetonitrilo) SPE (C_{18} ; acetonitrilo) discos	LC–ESI –MS/MS (QqQ)	FE: RP-C_{18} FM: metanol-acetonitrilo-agua	LOD: 0.5–3.4 ng/l Rec: 76 – 101 % RSD: 0.4–7.4 %	Sun et al., 2009
Hormonas y bisfenol A	Leche de vaca	Centrifugación SPE en línea (C_{30}; acetato amónico)	LC–ESI–MS	FE: RP-C_{18} FM: metanol-agua	LOD: 0.05–0.30 ng/ml Rec: 71–97 % RSD: 8.6–15.0 %	Yan et al., 2009
Antiepilépticos, drogas de abuso	Orina humana	Hidrólisis enzimática SPE en línea (Strata X-CW; acetonitrilo)	LC-MS/MS (QqQ) (ionización mediante turbo ión spray)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD: 0.20 –1.52 µg/l Rec: 94 – 105 % RSD: 0.1–9.3 %	Chiuminatto et al., 2010
Antibióticos y metabolitos	Orina humana	Centrifugación SPE (Bond Elut Plexa; metanol, acetonitrilo, diclorometano)	LC–DAD (λ 230–280 nm) LC–FLD (λex: 235–265 nm; λem: 296–430 nm)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD: 0.01–0.32 µg/ml Rec: 33–102 % RSD. 0.53–11.3 %	Fernández-Torres et al., 2010
ANEs/analgésicos, antiepilépticos, antibioticos, β-bloqueadores, RL, drogas de abuso	Suelos y sedimentos	PLE SPE (SAX; Oasis HLB; metanol)	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: metanol-disolución acuosa de ácido fórmico	LOD: 0.1– 6.8 ng /g Rec:50 – 105 % RSD: 0.7–14.5 %,	Vazquez-Roig et al., 2010

Antibióticos, ANEs/ analgésicos, RL, ansiolíticos, cardiovasculares	Aguas de río y residuales	SPE (Oasis HLB; metanol)	LC–ESI–MS/MS (QqQ)	FE: RP-C_{18} FM: metanol- disolución acuosa de ácido fórmico y acetato amónico	LOD: 0.1–86 pg Rec: 51–142 % RSD: 1–21 %	Gracia-Lor et al., 2011
Antibióticos	Camarones	QuEChERS LLE (ácido tricloroacético) MSPD	LC-ESI-MS (TOF)	FE: RP-C_{18} FM: acetonitrilo-disolución acuosa de ácido fórmico	LOD: 0.06–7 µg/kg Rec: 58–133 % RSD: 5.2–14.9 %	Villar-Pulido et al., 2011

[a] ANEs: antiinflamatorios no esteroideos; EDCs: compuestos disruptores endocrinos; PCPs: productos de cuidado personal; RL: reguladores de lípidos.

[b] QuEChERS: técnica de extracción rápida, fácil, barata, efectiva, robusta y segura (*quick, easy, cheap,effective, rugged, y safe*); MAE: extracción asistida por microondas; MSPD: extracción en fase sólida por dispersión de matriz; extracción acelerada con disolventes; SBSE: extracción en barra; SPE: extracción en fase sólida; SPME: microextracción en fase sólida: LLE: extracción líquido-líquido: PLE: extracción acelerada con disolventes.

[c] ESI: ionización por electrospray; DAD: detector de diodos en fila; FLD: detector de fluorescencia; LC: cromatografía de líquidos; MS: espectrometría de masas; MS/MS: espectrometría de masas en tándem; QqQ: triple cuadrupolo; QqLIT: híbrido triple cuadrupolo y trampa de iones lineal; TOF: espectrometría de masas con tiempo de vuelo; UV: detector de ultravioleta.

[d] LOD: límite de detección; LOQ: límite de cuantificación; Rec: recuperación; RSD: desviación estándar relativa.

2.3.2. Técnicas no cromatográficas

2.3.2.1. Electroforesis capilar

La electroforesis capilar (CE) es una técnica de separación muy atractiva que está teniendo un rápido crecimiento en los últimos años como queda demostrado en el gran número de publicaciones en las que se utiliza. En esta técnica la separación se lleva a cabo en un capilar de sílice fundida, de 25–75 μm de diámetro interno y de 50–100 cm de longitud, rellena de un electrolito de fondo (background electrolite). Las características más importantes de la CE son:

a) Elevada velocidad de separación al emplear voltajes y capilares relativamente cortos.
b) Alta eficacia (el número de platos teóricos varía entre 100000 y 500000). Al utilizar capilares de diámetros muy pequeños se disipa muy bien el calor generado por la aplicación de voltajes elevados, lo cual minimiza el ensanchamiento de las zonas del soluto.
c) Requiere volúmenes de muestra (1–10 ml).
d) Consumo mínimo de reactivos (electrolitos).
e) Gran versatilidad en cuanto a modos de operación y aplicabilidad.
f) Es fácilmente automatizable.

Para conseguir una alta resolución en electroforesis capilar, la anchura de la zona de inyección en la separación debe ser pequeña, por lo cual es necesaria la inyección de un volumen muy pequeño de muestra en los capilares. Debido a este motivo y con los sistemas de detección usuales (UV, por ejemplo) se consiguen límites de detección del orden de mg/l, muy por encima de lo que se requiere en muchos análisis. La solución a este inconveniente se consigue con la preconcentración de los analitos de dos modos: i) preconcentración fuera de línea mediante diferentes técnicas (SPE, SPME, etc.), o bien ii) preconcentración en línea en el propio capilar una vez que la muestra haya sido inyectada. En esta última alternativa, la preconcentración se lleva a cabo mediante la técnicas de inyección de campo amplificado, entre las que se encuentra la isotacoforesis y el hacinamiento de muestra *(sample stacking)*, con o sin eliminación de la matriz.

En el área de determinación de SFAs la CE está teniendo un elevado crecimiento su uso como se puede observar en la Figura 7. En la Tabla 17 se han incluido algunos de

los métodos utilizados para la determinación de SFAs en distintos tipos de muestras, basados en el uso de la técnica de CE. Como se puede observar, para la determinación de estos compuestos se han utilizado varias modalidades, entre las que destaca la electroforesis capilar de zona (Veraart et al., 1998; Beltrán et al., 2004; Macià et al., 2004; Jarmalaviciene et al., 2008; Bailón-Pérez et al., 2008) o cromatografía capilar electrocinética micelar (Nozal et al., 2007). Los sistemas de detección más utilizados para este propósito son los espectrofotométricos UV y diodos en fila (Veraart et al., 1998; Beltrán et al., 2004; Nozal et al., 2007; Sun et al., 2007; Jarmalaviciene et al., 2008; Bailón-Pérez et al., 2008; Gibbons et al., 2011; Maijó et al., 2011; Villar Navarro et al., 2011). En algunos casos también se ha utilizado el detector de fluorescencia para la determinación de antibióticos en sangre y aguas superficiales (Ferdig et al., 2004) o bien el detector de electroquimioluminiscencia para la determinación de β-bloqueadores en orina (Wang et al., 2011). No obstante y como en el caso de las GC y LC, el detector de espectrometría de masas se está imponiendo para la cuantificación de SAFs por su poder de identificación y su sensibilidad (Ahrer et al., 2001; Macià et al., 2004; Servais et al. 2006). En todos los casos incluidos en la Tabla 17 se ha utilizado la ionización mediante electrospray.

Tabla 17: Métodos para la determinación de sustancias farmacológicamente activas en muestras ambientales, alimentos y fluidos biológicos mediante electroforesis capilar

Compuestos/Clases terapéuticas[a]	Muestras	Preparación de muestra [b]	Determinación [c]	Electrolito [d]	Parámetros Analíticos [e]	Referencia
ANEs/analgésicos	Suero y orina	SPE (C_{18}; tampón fosfato-acetonitrilo)	CZE–UV (λ 200 nm)	Tampón acetato sódico (pH 4–5)	LOD: 0.04–0.4 µg/ml RSD: < 15 %	Veraart et al., 1998
ANEs/analgésicos; RL; antieplipticos; antibióticos	Aguas de río	LLE (MTBE- hexano); SPE (LiChrolut EN-C_{18}-Oasis HLB; metanol)	CE–ESI–MS LC-ESI-MS	Tampón acetato amónico (pH 5,1)	LOD: 18–134 µg/l Rec: 63–80 % RSD: 16–30 %	Ahrer et al., 2001
Antibióticos	Sangre y aguas superficiales	Centrifugación SPE (Oasis HLB; metanol–agua)	CE-FD (λ_{exc} 240–400 nm; λ_{em} 435 nm)	Tampón fosfato (pH 7.0–7.5)	LOD: 0.5–50 µg/l Rec: 70–109 % RSD: < 3 %	Ferdig et al., 2004
Antibióticos	Carne de pollo	Centrifugación SPE (C_{18}; ácido trifluoroacético en agua y acetonitrilo)	CZE–DAD (λ 190– 450 nm)	Tampón fosfato (pH 8.2)	Rec: 93–101 %	Beltrán et al., 2004
Naproxeno y RL	Aguas superficiales y residuales	Filtración SPE(C_{18}; metanol) LLE (hexano-MTBE)	CZE–ESI–MS	Tampón acetato amónico disuelto en metanol-agua	LOD: 100 ng/l Rec: 50–89 % RSD: 2–13 %	Macià et al., 2004
Salbutamol	Orina humana	Centrifugación SPE (C_{18})	CE –ESI–MS (Trampa de iones)	Tampón de formato amónico-ácido fórmico y β-ciclodextrina (pH ácido)	LOQ: 20 ng/ml RSD: < 7 %	Servais et al., 2006
ANEs/analgésicos	Orina	LPME (polipropileno hidrofílico)	MEKC–DAD (λ 214 nm)	Na_2HPO_4, SDS y β-ciclodextrina (pH 9.0)	LOD: 1.2–1.7 µg/l Rec: 99 – 104 % RSD: 1.4–3.1 %	Nozal et al., 2007
Antibióticos	Carne de cerdo	Sonicación Centrifugación Filtración (0.22 µm)	CE–DAD (λ 280 nm)	NaH_2PO_4 –$Na_2B_4O_7$– H_3BO_3 (pH 9)	LOD: 0.01–0.04 mg/kg Rec: 72 – 93 % RSD: < 10 %	Sun et al., 2007

Analgésicos, cafeína	Plasma de vaca	Centrifugación SPME	CZE–UV (λ 218 nm)	Tampón borato-fosfato (pH 8.5)	LOD: 0.3–1.9 ng/ml RSD: 0.8–4.5 %	Jarmalavičiene et al., 2008
Antibióticos	Aguas de pozo, de río y residuales	SPE (Oasis HLB; acetonitrilo)	CZE–DAD	Tris(hidroximetil)aminometano (pH 8)	LOD: 0.08–0.80 µg/l Rec: 94 – 99 % RSD: 3.3–7.2 %	Bailón-Pérez et al., 2008
ANEs/analgésicos, antiepilépticos, PCPs	Aguas residuales	SPE (Oasis HLB; metanol-acetona)	CE–UV (λ 200–280 nm)	Tampón borato (pH 8.5–9.5)	LOD: 1.6 –68.7 µg/l	Gibbons et al., 2011
ANEs/analgésicos, RL	Agua de rio	SPE (Oasis HLB; metanol)	CE–DAD (λ 214 nm)	Acetato amónico (pH 9)	LOQ: 0.06–1.0 ng/ml Rec: 82 – 116 % RSD: 0.9–7.1 %	Maijó et al., 2011
ANEs/analgésicos	Agua residuales	LPME	CE–DAD (λ 220–318 nm)	Tampón de acetato (pH 4) y acetonitrilo	LOD: 0.25– 0.86 ng/ml Rec: 60–93 %	Villar Navarro et al., 2011
β-bloqueadores	Orina y preparados farmacéuticos	—	CE–ECL	Tampón fosfato y poli-β-ciclodextrina (pH 10)	LOD: 0.1–0.5 µmol/l Rec: 99–105 % RSD: 0.6–6.6 %	Wang et al., 2011

[a] ANEs: antiinflamatorios no esteroideos; PCPs: productos de cuidado personal; RL: reguladores de lípidos.
[b] LPME: microextracción líquido-líquido; MTBE: metil tert-butil éter; SPE: extracción en fase sólida; SPME: microextracción en fase sólida.
[c] CE: electroforesis capilar; CZE: electroforesis capilar de zona; DAD: detector de diodos en fila; ECL: detector de electroquimioluminiscencia; FD: detector fluorescente, LC: cromatografía de líquidos; MEKC: cromatografía capilar electrocinética micelar; UV: detector de ultravioleta.
[d] SDS: dodecil sulfato sódico.
[e] LOD: límite de detección; LOQ: límite de cuantificación; Rec: recuperación; RSD: desviación estándar relativa.

2.5.2.2. Técnicas espectroscópicas

Desde el pasado se han utilizado metodologías basadas en las medidas mediante técnicas de espectroscopia de absorción molecular o espectrofluorometría para el screening y cuantificación de residuos de SFAs. Estos métodos requieren del manejo de gran cantidad de muestras y disolventes y de laboriosas etapas de extracción, *clean-up* y de evaporación del extracto. Presentan la ventaja de que no requieren de instrumentación especial como es el caso de las técnicas de GC, LC y CE, descritas en apartados anteriores. La mayoría de los métodos basados en técnicas espectroscópicas poseen una adecuada sensibilidad pero adolecen de especificidad, los cuales se sustentan solamente en las características espectrales, que no puede diferenciar entre el analito y las sustancias interferentes, si no se utiliza previamente una técnica de separación previa a la determinación espectroscópica.

En la mayoría de los casos los métodos basados en la determinación espectroscópica se fundamentan en la formación de complejos coloreados entre las SFAs y el reactivo. Así por ejemplo, varios β-bloqueadores se han hecho reaccionar con cobre (II) o cobalto (II) para formar complejos que absorben radiación en el visible (613 nm) (Gölcü et al., 2004). Thanasarakhan et al. (2011) han desarrollado un método para la determinación de antibióticos que utiliza ytrio (III) y un surfactante catiónico para la formación de complejos amarillos que pueden ser medidos a diferentes longitudes de onda dependiendo del fármaco. Este método ha sido aplicado a la determinación de tres fármacos en muestras de miel y leche con límites de detección entre $4.9\ 10^{-6}$ y $7.8\ 10^{-6}$ mol/l y con recuperaciones cercanas al 100 %. También se han utilizado sistemas continuos para llevar a cabo la derivatización de penicilinas y determinación de sus derivados 4,6-dinitrobenzonfuroxan a 510 nm (Evgen'ev et al., 2001). En cuanto a los métodos basados en medidas espectrofluorimétricas, El-Kommos et al. (2003) han puesto a punto un método para la determinación de antibióticos derivados de la quinolona basado en la formación de quelatos metálicos fluorescentes, el cual ha sido aplicado para el análisis de estos fármacos en orina y plasma con límites de detección entre 1.2 y 2.0 ng/ml. También se ha aplicado la espectrofluorimetría para la determinación de ibuprofeno en suero y preparados farmacéuticos, previa la reacción de éste fármaco con la β-ciclodextrina para la formación de un complejo con un límite de cuantificación de 4.7 μg/ml (Hergert y Escandar, 2003).

El uso analítico de la quimioluminiscencia está experimentando un creciente interés debido a que representa una alternativa simple, barata y sensible para cuantificar una gran variedad de compuestos frente a las técnicas anteriormente comentadas. En el caso de fármacos se han propuesto diferentes metodologías quimioluminiscentes que se caracterizan por su sensibilidad, su amplio rango lineal y en muchos casos se consigue automatizar mediante el uso de un sistema continuo (Mervartová et al., 2007). Así por ejemplo, se ha utilizado un sistema de flujo para la reacción del ibuprofeno con sulfito sódico y permanganato potásico en medio ácido y determinación del derivado quimioluministente en muestras de orina humana y en preparados farmacéuticos (Payán et al., 2009).

2.3.2.2. Métodos immunoquímicos

Los métodos inmunoquímicos (IM) son bien conocidos desde hace años por su elevada especificidad y sensibilidad respecto a los métodos clásicos, pero en muchos casos, las aplicaciones comerciales de estos métodos han sido de escasa implantación debido por una parte a su desconocimiento y en otros casos a su elevada complejidad. Actualmente, con el avance de nuevos métodos inmunoquímicos, el análisis de alimentos y muestras Medio Ambientales puede llevarse a cabo en tan solo unos pocos minutos, además, dichos ensayos pueden realizarse *in situ*, sin la necesidad de grandes equipos o incluso ninguno. Estos métodos se basan en la interacción antígeno-anticuerpo, que cuenta con una gran afinidad y especificidad, convirtiéndolos en unas metodologías muy sensibles y selectivas. Estas metodologías se pueden basar en distintas modalidades de IM, pero el más utilizados es el ensayo por inmunoabsorción ligado a enzimas *(enzyme-linked immunosorbent assay, ELISA),* en el cual un antígeno inmovilizado se detecta mediante un anticuerpo enlazado a una enzima capaz de generar un producto detectable como cambio de color o algún otro tipo. En ciertas ocasiones y con el fin de reducir los costos del ensayo, nos encontramos con que existe un anticuerpo primario que reconoce al antígeno y que a su vez es reconocido por un anticuerpo secundario que lleva enlazado la enzima anteriormente mencionada. Los ensayos ELISA han sido utilizados para la determinación de las hormonas 17β-estradiol y 17α-etinilestradiol en distintos tipos de aguas con una excelente sensibilidad (límites de detección entre 0.01–0.05 ng/l) (Hintemann et al., 2006). Este mismo grupo de investigación ha desarrollado uno método basado en ELISA para la determinación de

carbamazepina en aguas superficiales y residuales en el intervalo entre 0.03 y 10 μg/l y con una desviación estándar relativa menor del 30 % (Calisto et al., 2011).

3. AUTOMATIZACIÓN, MINIATURIZACIÓN Y SIMPLIFICACIÓN EN QUÍMICA ANALÍTICA

Ente las tendencias actuales de la Química Analítica se encuentran la automatización, miniaturización y simplificación con tres objetivos claros: reducción la intervención humana, reducción del tamaño y reducción de la envergadura de los procesos, respectivamente. Tal como se muestra gráficamente en la Figura 8, estas tendencias no son independientes, sino que existe una relación sinérgica entre ellas (Valcárcel y Cárdenas, 2000). De esta manera cuando se transforma un método manual en automático casi siempre implica un grado más o menos intenso de miniaturización y simplificación; la simplificación de un proceso exige casi siempre la integración de módulos (miniaturización) y la reducción de la intervención humana (automatización).

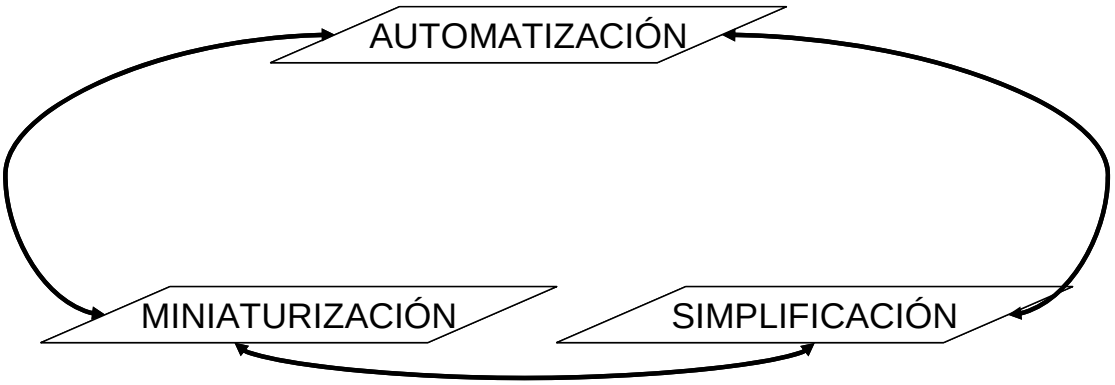


Figura 8: Relaciones sinérgicas mutuas entre tres tendencias de la Química analítica: automatización, miniaturización y simplificación.

La creciente implantación de estas tres tendencias en las herramientas y procesos analíticos está suponiendo y supondrá una "revolución" técnica en la Química Analítica, así como un cambio notable en el establecimiento de los compromisos de calidad implícitos al caracterizar los procesos y resultados mediante las propiedades analíticas.

La automatización en los procesos analíticos surge como consecuencia de la creciente necesidad que existe en áreas como salud, Medio Ambiente o alimentación, de incrementar la capacidad para llevar a cabo un gran número de controles analíticos eficaces que permitan obtener resultados más precisos, específicos y con mayor rendimiento. Los objetivos de la automatización en Química Analítica deben ser coherentes, por una parte, con sus fines y objetivos y, por otra, con las ventajas estratégicas generales que implica la sustitución de la participación humana en los procesos de una organización:

- Mejores decisiones basadas en información fundamentada y eficaz.
- Mejora de la productividad.
- Reducción de riesgos para los empleados y el Medio Ambiente.

Los nueve objetivos que puede perseguir la automatización en Química Analítica se exponen esquemáticamente en la Figura 9.

Por lo tanto, la integración de la automatización en los procesos analíticas ha sido y continúa siendo una tarea muy importante en Química Analítica. El desarrollo de estos métodos surgió de la necesidad de llevar a cabo ensayos analíticos con un menor coste de material y tiempo, a la vez que disminuye la participación humana, con la ventaja que ello implica de mayor comodidad, mejora en la productividad, así como disminución de errores, riesgo para empleados y medio ambiente.

Se considera que el uso de los métodos automáticos de análisis va ligado al concepto de la sustitución de una operación o medida estática realizada manualmente por una operación o medida continua o secuencial. Esta concepción fue introducida por Skeegs en 1957 al describir el primer sistema de análisis segmentado en flujo continuo, y que constituye el primer paso en el desarrollo de los métodos automáticos. En general en los sistemas de flujo se puede realizar una clasificación atendiendo a la forma de introducción de la muestra y a la introducción de burbujas *(flujo segmentado o no segmentado).*

Los sistemas de flujo segmentado se caracterizan porque se introducen las muestras de forma secuencial. Son aspiradas y espaciadas entre sí por medio de burbujas de aire, procurando en todo momento evitar el efecto de transporte, promoviendo la mezcla de muestras y reactivos, y limpiando las paredes del tubo.

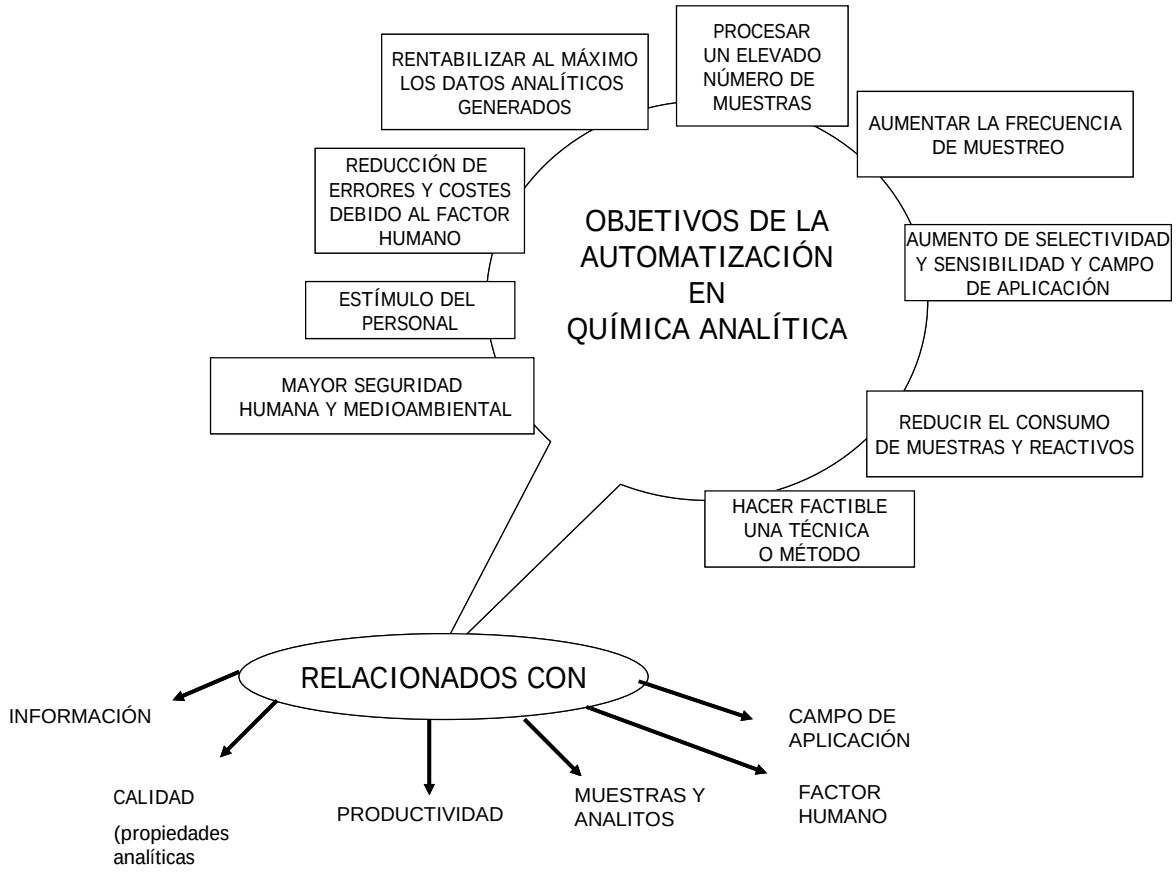


Figura 9: Fines que puede perseguir la sustitución de la intervención humana en Química Analítica y aspectos genéricos con los que están relacionados.

Los sistemas de *flujo no segmentado* se caracterizan por la ausencia de burbujas de aire en el sistema. Las muestras se introducen de forma secuencial en el analizador, mediante inyección o inserción en una corriente de portador. Una diferencia muy importante de estos sistemas con los de flujo segmentado es que en el momento de llevar a cabo la detección, no se ha alcanzado ni el equilibrio físico (homogeneización del flujo) ni químico (desarrollo completo de la reacción analítica). De todos estos sistemas de flujo no segmentado, la técnica más empleada actualmente es el Análisis por Inyección en Flujo (FIA).

Entre las distintas etapas que se pueden automatizar en el proceso analítico, la etapa de preparación de la muestra es en la que más se han realizado esfuerzos los investigadores en los últimos años. Así por ejemplo, se han desarrollado sistemas continuos con objeto de reducir la manipulación humana, el tiempo de análisis y el consumo de reactivos y muestras, además de mejorar la precisión y exactitud en las

medidas. Por ejemplo, se ha desarrollado un sistema continuo para la extracción en fase sólida basado en el uso de una columna de sorbente incorporada en el bucle de una válvula de inyección para la preconcentración y eliminación de interferencias en la determinación de plaguicidas organofosforados (Ballesteros y Parrado, 2004) muestras de aguas. Además se ha propuesto un sistema continuo para la discriminación de N-nitrosaminas aromáticas y alifáticas mediante el uso de dos columnas empaquetadas con dos materiales sorbentes (C_{60} y LiChrolut EN) y que se esquematiza en la Figura 10 (Jurado-Sánchez et al., 2009). El método se aplicó a la determinación de N-nitrosaminas en distintos tipos de aguas (potables, río, pozo, pantano, piscina y residual) por cromatografía de gases-espectrometría de masas.

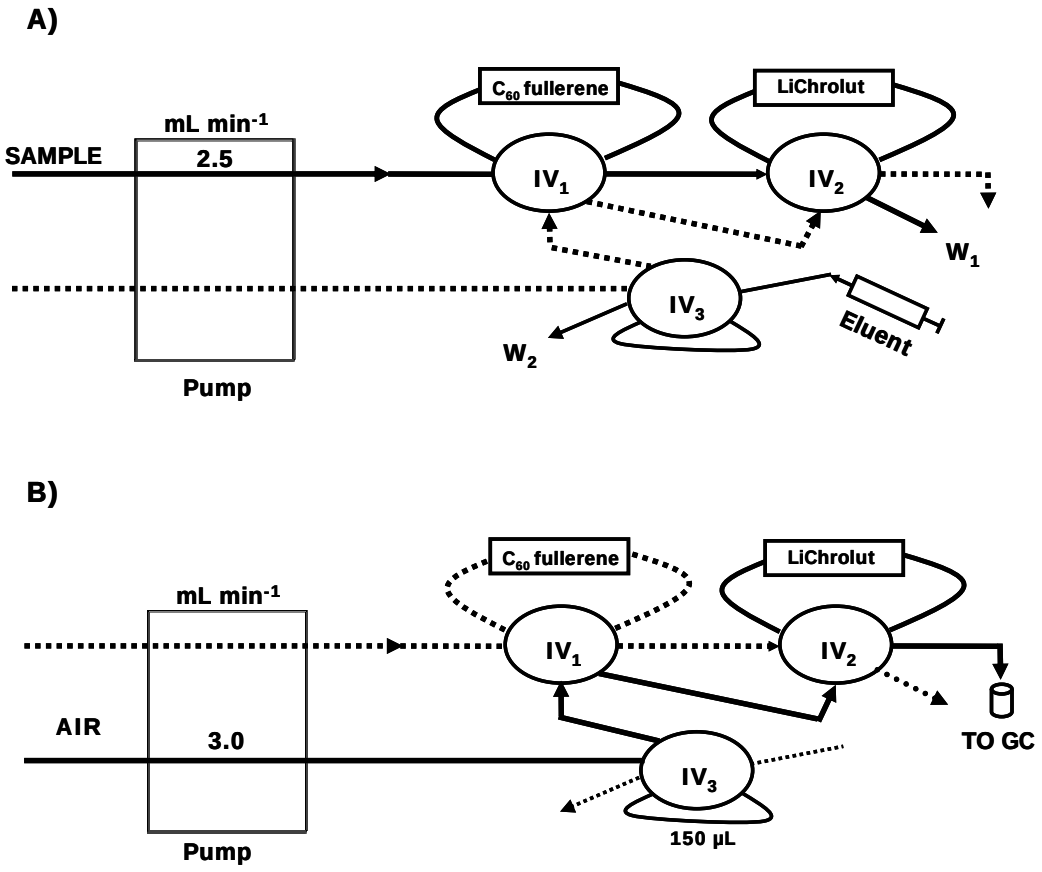


Figura 11: Sistema de flujo continuo para la extracción en fase sólida de N-nitrosaminas

En el caso de la determinación de SFAs se han aplicado el uso de sistemas continuos. Así, Cárdenas et al. (1996) han propuesto un sistema continuo para el tratamiento de fluidos biológicos y determinación de drogas mediante cromatografía de gases-espectrometría de masas. Llorent-Martínez et al. (2007) han desarrollado un optosensor fluorimétrico basado en el uso de un sistema de análisis por inyección

secuencial para la determinación de paracetamol en diferentes preparados farmacéuticos con un límite de detección de 2 μg/ml.

La miniaturización de los procesos analíticos y bioanalíticos se ha convertido en una importante área de investigación y desarrollo durante los últimos 10 años, como una continuación de la tendencia general de reducción de tamaño de los analizadores de laboratorio Analítico. La miniaturización presenta unas ventajas significativas en los ámbitos científicos y tecnológicos reflejados en el Figura 11. Esta tendencia básica también permite a la Química Analítica alcanzar importantes objetivos; los más relevantes de los cuales se esquematizan en dicha Figura. Tal como puede observarse, se clasifican en tres tipos: genéricos (A), básicos (B) y específicos (C). Entre ellos existe una jerarquía de importancia y extensión: A>B>C, es decir, un objetivo específico se enmarca en uno básico que contribuye a alcanzar el general.

Hasta ahora, ha sido una práctica habitual el uso de grandes volúmenes de disolventes orgánicos en el proceso de preparación de la muestra, pero debido a la peligrosidad que conlleva el uso de éstos, se tiende a utilizar técnicas en las que las cantidades de disolventes sean menores que en las habituales. Además es una tendencia significativa en la preparación de muestras conforme con los principios de "Química Verde". Por ello, diferentes técnicas novedosas se han desarrollado con el fin de reducir el tiempo de análisis, disminuir el consumo de muestra y reactivos y para mejorar la calidad y la sensibilidad de los métodos analíticos. En este sentido se encuentran las técnicas de microextracción líquido-liquido (Guo et al., 2009; Zgoła-Grzéskowiak, 2010) y microextracción en fase sólida (Vera-Candioti, 2008; Souza et al., 2011) comentadas en esta Memoria en el apartado de técnicas de extracción, y que están teniendo mucha aceptación entre los investigadores para la determinación de SFAs en muestras líquidas.

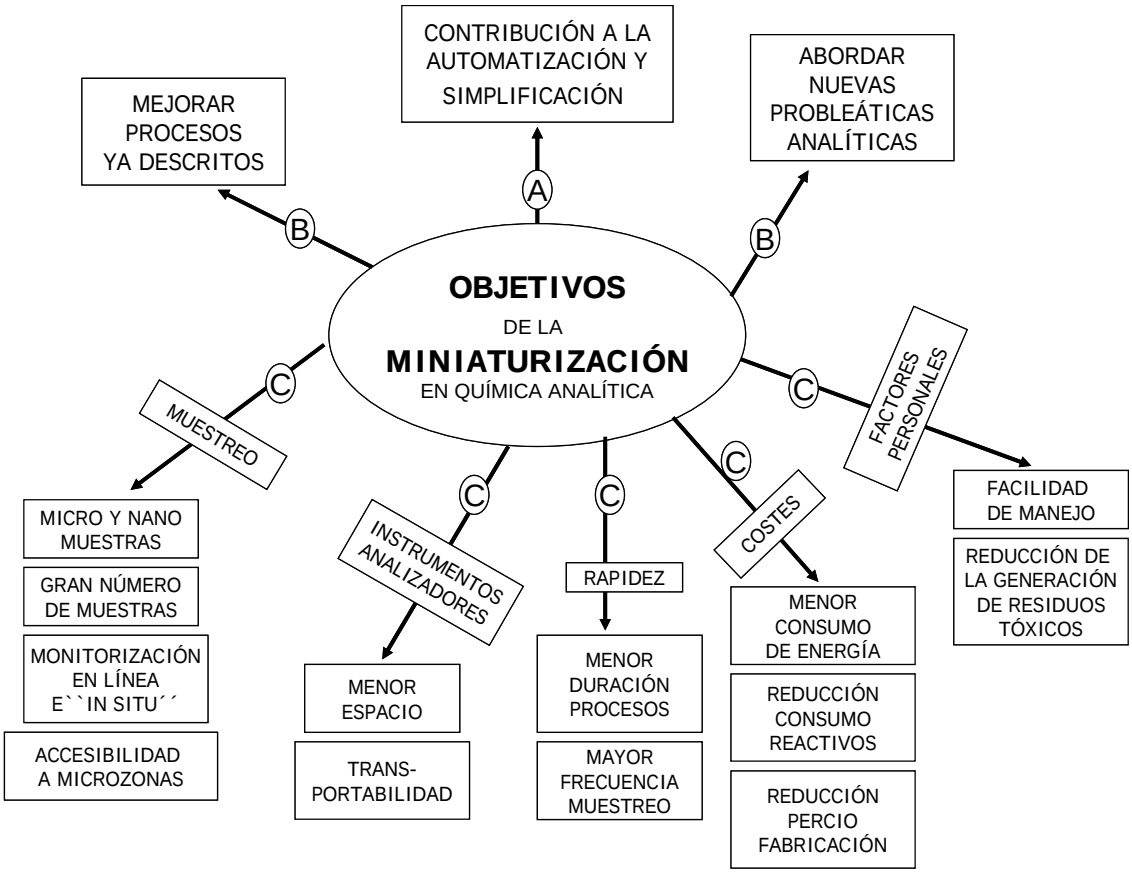


Figura 11: Objetivos genéricos (A), básicos (B) y específicos (C) que pueden alcanzarse al miniaturizar herramientas y procesos analíticos.

REFERENCIAS

REFERENCIAS

Adolfsson-Erici M, Pettersson M, Parkkonen J, Sturve J. 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere, 46: 1485–1489.

Aguilera-Luiz MM, Vidal JLM, Romero-González R, Frenich AG. 2008. Multi-residue determination of veterinary drugs in milk by ultra-high-pressure liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 1205: 10–16.

Ahrer W, Scherwenk E, Buchberger W. 2001. Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. Journal of Chromatography A, 910: 69–78.

Albero B, Sánchez-Brunete C, Tadeo JL. 2003. Determination of organophosphorus pesticides in fruit juices by matrix solid-phase dispersion and gas chromatography. Journal of Agricultural and Food Chemistry, 51: 6915-6921.

Almeida C, Noguerira JMF. 2006. Determination of steroid sex hormones in water and urine matrices by stir bar sorptive extraction and liquid chromatography with diode array detection. Journal of Pharmaceutical and Biomedical Analysis 41: 1303–1311.

Allmyr M, Adolfsson-Erici M, McLachlan MS, Sandbourgh-Englund G. 2006a. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. Science of the Total Environment, 372: 87–93.

Allmyr M, McLachlan MS, Sandborgh-Englund G, Adolfsson-Erici M. 2006b. Determination of triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. Analytical Chemistry, 78: 6542–6546.

Babic S, Ašperger D, Mutavdžic´ D, Horvat AJM, Kaštelan-Macan M. 2006. Solid phase extraction and HPLC determination of veterinary pharmaceuticals in wastewater. Talanta, 70: 732–738.

Bailón-Pérez MI, García-Campaña AM, Cruces-Blanco C, Iruela MdO. 2008. Trace determination of β-lactam antibiotics in environmental aqueous samples using off-line and on-line preconcentration in capillary electrophoresis. Journal of Chromatography A, 1185: 273–280.

Ballesteros E, Parrado MJ. 2004. Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticidas in natural and drinking waters. Journal of Chromatography A, 1029: 267–273.

Batt AL, Aga DS. 2005. Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. Analytical Chemistry, 77: 2940–2947.

Beausse J. 2004. Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. Trends in Analytical Chemistry, 23: 753 – 761.

Beck J, Totsche KU, Kögel-Knabner I. 2008. A rapid and efficient determination of natural estrogens in soils by pressurised liquid extraction and gas chromatography–mass spectrometry. Chemosphere, 71: 954–960.

Beltrán JL, Jiménez-Lozano E, Barrón D, Barbosa J. 2004. Determination of quinolone antimicrobial agents in strongly overlapped peaks from capillary electrophoresis using multivariate calibration methods. Analytica Chimica Acta, 501: 137–141.

Bianchi F, Careri M, Mangia A, Musci M, Santini SE, Basini G. 2007. Porcine follicular fluids: Comparison of solid-phase extraction and matrix solid-phase dispersion for the GC–MS determination of hormones during follicular growth. Journal of Pharmaceutical and Biomedical Analysis, 44: 711–717.

Bishop EJ, Mitra S. 2005. On-line membrane preconcentration for continuous monitoring of trace pharmaceuticals. Journal of Pharmaceutical and Biomedical Analysis, 37: 81–86.

Bjorkman D. 1998. Nonsteroidal anti-inflammatory drug-associated toxicity of the liver, lower gastrointestinal tract, and esophagus. The American Journal of Medicine, 105: 17S–21S.

Blackwell PA, Lutzhoft HCH, Ma HP, Halling-Sorensen B, Boxall ABA, Kay P. 2004. Ultrasonic extraction of veterinary antibiotics from soils and pig slurry with SPE clean-up and LC-UV and fluorescence detection. Talanta, 64:1058–1064.

Botsoglou NA, Fletouris DJ. 2004. Residual antibacterials in food. En Handbook of Food Analysis (Nollet, LML, editor). New York: Marcel Dekker, pp. 931–1035.

Bound JP, Voulvoulis N. 2004. Pharmaceuticals in the aquatic environment—a comparison of risk assessment strategies. Chemosphere, 56: 1143–1155.

Boyd GR, Reemtsma H, Grimm DA, Mitra S. 2003. Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. Science of the Total Environment, 311: 135–149.

Boyd B, Björk H, Billing J, Shimelis O, Axelsson S, Leonora M, Yilmaz E. 2007. Development of an improved method for trace analysis of chloramphenicol using molecularly imprinted polymers. Journal of Chromatography A, 1174: 63–71.

Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environmental Toxicology & Chemistry, 24: 464–469.

Budzinski H, Jones I, Bellocq J, Piérard C, Garrigues P. 1997. Evaluation of sediment contamination by polycyclic aromatic hydrocarbons in the Gironde estuary. Marine Chemistry, 58: 85–97.

Büyüksönmez F, Sekeroglu S. 2005. Presence of pharmaceuticals and personal care products (PPCPs) in biosolids and their degradation during composting. Journal of Residuals Science and Technology, 2: 31-40.

Cajaraville MP, Cancio M, Ibabe A, Orbea A. 2003. Peroxisome proliferation as a biomarker in environmental pollution assessment. Microscopy Research and Technique, 61: 191–202.

Calamari D, Zuccato E, Castiglioni S, Bagnati R, Fanelli R. 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. Environmental Science & Technology, 37: 1241–1248.

Calisto V, Bahlmann A, Scheider RJ, Esteves VI. 2011. Application of an ELISA to the quantification of carbamazepine in ground, surface and wastewaters and validation with LC-MS/MS. Chemosphere, 84: 1708–1715.

Campa-Córdova AI, Luna-González A, Ascencio F, Cortés-Jacinto E, Cáceres-Martínez CJ. 2006. Effects of chloramphenicol, erythromycin, and furazolidone on growth of *Isochrysis galbana* and *Chaetoceros gracilis*. Aquaculture, 260: 145–150.

Cammann, K. 2001. Instrumentelle analytische Chemie : Verfahren, Anwendung und Qualitätssicherung. Heidelberg, Berlin: Spektrum Akademischer Verlag GmbH.

Canosa P, Rodríguez I, Rubí E, Cela R. 2007. Determination of parabens and triclosan in indoor dust using matrix solid-phase dispersion and gas chromatography with tandem mass spectrometry. Analytical Chemistry, 79: 1675-1681.

Carballa M., Omil F., Lema JM. 2008. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. Chemosphere, 72:1118–1123.

Cárdenas S, Gallego M, Valcárcel M, Ventura R, Segura J. 1996. A partially automated pretreatment module for routine analyses for seventeen non-steroid antiinflammatory drugs in race horses using gas chromatography/mass spectrometry. Analytical Chemistry, 68: 118–123.

Chen L, Zeng Q, Wang H, Su R, Xu Y, Zhang X, Yu A, Zhang H, Ding L. 2009a. On-line coupling of dynamic microwave-assisted extraction to solid-phase extraction for the determination of sulfonamide antibiotics in soil. Analytica Chimica Acta, 648: 200–206.

Chen L, Liu J, Zeng Q, Wang H, Yu A, Zhang H, Ding L. 2009b. Preparation of magnetic molecularly imprinted polymer for the separation of tetracycline antibiotics from egg and tissue samples. Journal of Chromatography A, 1216: 3710–3719

Chiuminatto U, Gosetti F, Dossetto P, Mazzucco E, Zampieri D, Robotti E, Gennaro MC, Marengo E. 2010. Automated online solid phase extraction ultra high performance liquid chromatography method coupled with tandem mass spectrometry for determination of forty-two therapeutic drugs and drugs of abuse in human urine. Analytical Chemistry, 82: 5636–5645.

Choi MH, Kim KR, Hong JK, Park SJ, Chung BC. 2002. Determination of non-steroidal estrogens in breast milk, plasma, urine and hair by gas chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry, 16: 2221–2228.

Christen V, Hickmann S, Rechenberg B, Fent K. 2010. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. Aquatic Toxicology, 96: 167–181.

Cleuvers M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters, 142: 185–194.

Codex Alimentarius, 2011. Residuos de Medicamentos Veterinarios en los alimentos. Actualizado hasta la 34ª Reunión de la Comisión del Codex Alimentarius (2011). http://www.codexalimentarius.net/vetdrugs/data/vetdrugs/index.html

Colborn T, Clements C (editores). 1992. Chemically induced alterations in sexual and functional development: the wildlife/human connection, in: Advances in Modern Environmental Toxicology. New York: Princeton Scientific Publishing, pp. 21.

Commission of the European Communities.1990. Reglamento (CEE) No 2377/90 del Consejo de 26 de junio de 1990 por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. Office for Official Publications of the European Communities, Luxemburg , pp.1–113.

Commission of the European Communities, 1993. Directiva 93/67/CEE de la Comisión, de 20 de julio de 1993, por la que se fijan los principios de evaluación del riesgo, para el ser humano y el medio ambiente, de las sustancias notificadas de acuerdo con la Directiva 67/548/CEE del Consejo. Office for Official Publications of the European Communities, Luxemburg, pp. 9–18.

Commission of the European Communities, 1996. Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Office for Official Publications of the European Communities, Luxemburg, pp. 1–14.

Commission of the European Communites. 2002. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Office for Official Publications of the European Communities, Luxemburg, L 221/8–L 221/36.

Commission of the European Communites. 2010. Commision Regulation No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximun residue limitis in foodstuffs of animal origen. Office for Official Publications of the European Communities, Luxemburg, L15/1–L15/71.

Courant F, Antignac JP, Laille J, Monteau F, Andre F, Le Bizec B. 2008. Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples. Journal of Agricultural and Food Chemistry, 56: 3176–3184.

Cueva-Mestanza R, Sosa-Ferrera Z, Torres-Padrón ME, Santana-Rodríguez JJ. 2008. Preconcentration of pharmaceuticals residues in sediment samples using microwave

assisted micellar extraction coupled with solid phase extraction and their determination by HPLC–UV. Journal of Chromatography B, 863: 150–157.

Daeseleire E, Vandeputte R, Van Peteghem C. 1998. Validation of multi-residue methods for the detection of anabolic steroids by GC-MS in muscle tissues and urine samples from cattle. Analyst, 123: 2595–2598.

Daeseleire E, Mortier L, De Ruyck H, Geerts N. 2003. Determination of flunixin and ketoprofen in milk by liquid chromatography tandem mass spectrometry. Analytica Chimica Acta, 488: 25–34.

Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change?. Environmental health perspectives, 107: 907–938.

Debernard S, Rossignol F, Couillaud F. 1994. The HMG-CoA reductase inhibitor fluvastatin inhibits insect juvenile hormone biosynthesis. General and Comparative Endocrinology, 95: 92–98.

Dean JR. 1998. Extraction Methods for Environmental Analysis, Wiley, New York, USA.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environmental Science & Technology, 32:1549–1558.

Díaz-Cruz MS, López de Alda MJ, Barceló D. 2006. Determination of antimicrobials in sludge from infiltration basins at two artificial recharge plants by pressurized liquid extraction–liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1130: 72–82.

Díaz-Cruz MS y Barceló D. 2007. Recent advances in LC-MS residue analysis of veterinary medicines in the terrestrial environment. Trends in Analytical Chemistry, 26: 637–646.

Ding SY, Shen JZ, Zhang SX, Jiang HY, Sun ZW. 2005. Determination of chloramphenicol residue in fish and shrimp tissues by gas chromatography with a microcell electron capture detector. Journal of AOAC International, 88: 57–60.

Dobor J, Varga M, Yao J, Chen H, Palkó G, Záray G. 2010. A new sample preparation method for determination of acidic drugs in sewage sludge applying microwave

assisted solvent extraction followed by gas chromatography–mass spectrometry. Microchemical Journal, 94: 36–41.

Durán-Alvarez JC, Becerril-Bravo E, Silva Castro V, Jiménez B, Gigson R. 2008. The analysis of a group of acid pharmaceuticals, carbamazepine, and potential endocrine disrupting compounds in wastewater irrigated soils by gas chromatography-mass spectrometry. Talanta, 78: 1159–1166.

Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K, Miyamoto K, Yoshimura H. 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere, 57: 1733–1738.

El-Kommos ME, Saleh GA, El-Gizawi SM, Abou-Elwafa MA. 2003. Spectrofluorimetric determination of certain quinolone antibacterials using metal chelation. Talanta, 60: 1033–1050.

Es'haghi Z. 2009. Determination of widely used non-steroidal anti-inflammatory drugs in water samples by in situ derivatization, continuous hollow fiber liquid-phase microextraction and gas chromatography-flame ionization detector. Analytica Chimica Acta, 641: 83–88.

Eskilsson CS, Björklund E. 2000. Analytical-scale microwave-assisted extraction. Journal of Chromatography A, 902: 227–250.

Evans J, Kaake RH, Orr M, Watwood M. 1998. A comparison of ultrasonication and Soxhlet methods for DDT extraction from soil. Journal of Soil Contamination, 7: 589–597.

Evgen'ev MI, Garmonov SY, Shakirova LS. 2001. Flow-injection determination of benzylpenicillin novacaine salt in penicillin preparations with spectrophotometric detection. Journal of Analytical Chemistry, 56: 642–646.

Falbe J. Regitz, M. 1995. CD Römpp Chemie Lexikon. Stuttgart, New York: Georg Thieme Verlag.

Farajzadeh MA, Djozan Dj, Fazeli Bakhtiyari R. 2010. Use of a capillary tube for collecting an extraction solvent lighter than water after dispersive liquid–liquid microextraction and its application in the determination of parabens in different samples by gas chromatography—Flame ionization detection. Talanta, 81: 1360–1367.

Farré M, Ferrer I, Ginebreda A, Figueras M, Olivella L, Tirapu L, Vilanova M, Barceló D. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. Journal of Chromatography A, 938: 187–197.

Fent K, Weston AA, Caminada D. 2006. Ecotoxicology of human pharmaceuticals. Aquatic Toxicology, 76: 122–159.

Ferdig M, Kaleta A, Vo TDT, Buchberger W. 2004. Improved capillary electrophoretic separation of nine (fluoro)quinolones with fluorescence detection for biological and environmental samples. Journal of Chromatography A, 1047: 305–311.

Fernández-Torres R, Consentino MO, Lopez MAB, Mochon MC. 2010. Simultaneous determination of 11 antibiotics and their main metabolites from four different groups by reversed-phase high-performance liquid chromatography–diode array–fluorescence (HPLC–DAD–FLD) in human urine samples. Talanta, 81: 871–880.

Ferrari B, Paxéus N, Giudice RL, Pollio A, Garric J. 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicology and Environmental Safety, 55: 359–370.

Ferreira CSG, Nunes BA, Henriques-Almeida JMDM, Guilhermino L. 2007. Acute toxicity of oxytetracycline and florfenicol to the microalgae *Tetraselmis chuii* and to the crustacean *Artemia parthenogenetica*. Ecotoxicology and Environmental Safety, 67: 452–458.

Ferrer I, Thurman EM. 2007. Multi-residue method for the analysis of 101 pesticides and their degradates in food and water samples by liquid chromatography/time-of-flight mass spectrometry. Journal of Chromatography A, 1175: 24–37.

Ferrer I, Heine CE, Thurman EM. 2004. Combination of LC/TOF-MS and LC/ion trap MS/MS for the identification of diphenhydramine in sediment samples. Analytical Chemistry, 76: 1437–1444.

Festing MFW, Diamanti P, Turton JA. 2001. Strain differences in homatogical response to chloramphenicol succinate in mice: implications for toxicological research. Food and Chemical Toxicology, 39: 375–383.

Fong PP, Huminski PT, D'Urso LM. 1998. Induction and potentiation of parturition in fingernail clams (Sphaerium striatum) by selective serotonin re-uptake inhibitors (SSRIs). Journal of Experimental Zoology, 280: 260–264.

Foran CM, Weston J, Slattery M, Brooks BW, Duane BH. 2004. Reproductive assessment of Japanese Medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. Archives of Environmental Contamination and Toxicology, 46: 511–517.

Forster M, Laabs V, Lamshoft M, Groeneweg J, Zuhlke S, Spiteller M, Krauss M, Kaupenjohann M, Amelung W. 2009. Sequestration of manure-applied sulfadiazine residues in soils. Environmental Science & Technology, 43: 1824–1830.

Fritz JS. 1999. Analytical Solid-Phase Extraction. Wiley-VCH, New York, USA.

García-Reyes JF, Hernando MD, Molina-Díaz A, Fernández-Alba AR. Comprehensive screening of target, non-target and unknown pesticides in food by LC-TOF-MS. Trends in Analytical Chemistry, 2007, 26: 228–241.

Gentili A. 2007. LC-MS methods for analyzing anti-inflammatory drugs in animal-food products. Trends in Analytical Chemistry, 26: 595–608.

Gibbons SE, Wang C, Ma Y. 2011. Determination of pharmaceutical and personal care products in wastewater by capillary electrophoresis with UV detection. Talanta, 84: 1163–1168.

Gibson R, Becerril-Bravo E, Silva-Castro V, Jiménez B. 2007. Determination of acid pharmaceuticals and potential endocrine disrupting compounds in wasterwaters and spring waters by selective elution and analysis by gas chromatography-mass spectrometry. Journal of Chromatography A, 1169: 31–39.

Gierse JK, Koboldt CM,Walker MC, Seibert K, Isakson PC. 1999. Kinetic basis for selective inhibition of cyclo-oxygenases. Biochemical Journal, 339: 607–614.

Gölcü A, Yücesoy C, Serin S. 2004. Spectrophotometric determination of some beta-blockers in dosage forms based on complex formation with Cu(II) and Co(II). II Farmaco, 59: 487–492.

Golet EM, Strehler A, Alder AC, Giger W. 2002. Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. Analytical Chemistry, 74: 5455–5462.

Gómez MJ, Petrovic M, Fernández-Alba AR, Barceló D. 2006. Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography–tandem mass spectrometry analysis in hospital effluent wastewaters. Journal of Chromatography A, 1114: 224–233.

Gowik P, Jűlicher B, Uhlig S. 1998. Multi-residue method for non-steroidal anti-inflammatory drugs in plasma using high-performance liquid chromatography–photodiodearray detection. Method description and comprehensive in-house validation. Journal of Chromatography B, 716: 221–232.

Gracia-Lor E, Sancho JV, Hernández F. 2011. Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1218: 2264–2275.

Greiner-Sosanko E, Lower DR, Virji MA, Krasowski MD. 2007. Simultaneous determination of lamotrigine, zonisamide, and carbamazepine in human plasma by high-performance liquid chromatography. Biomedical Chromatography, 21: 225–228.

Gros M, Petrovic´ M, Barceló D. 2006. Multi-residue analytical methods using LC-tandem MS for the determination of pharmaceuticals in environmental and wastewater samples: a review. Analytical Bioanalytical Chemistry, 386: 941–952.

Guo JH, Li XH, Cao XL, Li Y, Wang XZ, Xu XB. 2009. Determination of triclosan, triclocarban and methyl-triclosan in aqueous samples by dispersive liquid–liquid microextraction combined with rapid liquid chromatography. Journal of Chromatography A, 1216: 3038–3043.

Guthery B, Bassindale T, Bassindale A, Pillinger CT, Morgan GH. 2010. Qualitative drug analysis of hair extracts by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. Journal of Chromatography A, 1217: 4402–4410.

Haller MY, Müller SR, McArdell CS, Alder AC, Suter MJF. 2002. Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography-mass spectrometry. Journal of Chromatography A, 952: 111–120.

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lutzhotf HC, Jorgensen SE. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment. A Review. Chemosphere, 36: 357–393.

Halling-Sørensen B. 2000. Algal toxicity of antibacterial agents used in intensive farming. Chemosphere, 40: 731–739.

Hamscher G, Sczesny S, Höper H, Nau H. 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Analytical Chemistry, 74: 1509–1518.

Hanselman TA, Graetz DA, Wilkie AC. 2003. Manure-borne estrogens as potential environmental contaminants: a review. Environmental Science & Technology, 37: 5471–5478.

Harnagea-Theophilus E, Gadd SL, Knight-Trent, AH, DeGeorge GL, Miller MR. 1999. Acetaminophen-induced proliferation of breast cancer cells involves estrogen receptors. Toxicology and Applied Pharmacology, 155: 273–279.

Heberer T. 2002. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. Journal of Hydrology, 266: 175–189.

Henschel RP, Wenzel A, Diedrich M, Fliedner A. 1997. Environmental hazard assessment of pharmaceuticals. Regulatory Toxicology and Pharmacology, 25: 220–225.

Hergert LA, Escandar GM. 2003. Spectrofluorimetric study of the β-cyclodextrin–ibuprofen complex and determination of ibuprofen in pharmaceutical preparations and serum. Talanta, 60: 235–246.

Hernando MD, Petrovic´ M, Fernández-Alba AR, Barceló D. 2004. Analysis by liquid chromatography—electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid-regulating agents in wastewater samples. Journal of Chromatography A, 1046: 133–140.

Herranz S, Moreno-Bondi MC, Marazuela MD. 2007. Development of a new sample pretreatment procedure based on pressurized liquid extraction for the determination of fluoroquinolone residues in table eggs. Journal of Chromatography A, 1140: 63–70.

Herrera-Herrera, A. V., Asensio-Ramos, M., Hernández-Borger, J., Rodríguez-Delgado, M. A. 2010. Dispersive liquid-liquid microextraction for determination of organic analytes. Trends in Analytical Chemistry, 29: 728–751.

Hintemann T, Schneider C, Scholer HF, Schneider RJ. 2006. Field study using two immunoassays for the determination of estradiol and ethinylestradiol in the aquatic Environment. Water Research, 40: 2287–2294.

Hoffman BB, Lefkowitz RJ. Katecholamine. 1998. Sympathomimetika und Adrenorezeptor-Antagonisten. En Pharmakologische Grundlagen der Arzneimitteltherapie (Goodman G, editor). McGraw Hill Deutsche Ausgabe.

Holmes P, Harrison P, Bergman A, Brandt I, Brouwer B,Keiding N, Randall G, Sharpe R, Skakkebaek N, Ashby J, Barlow S, Dikkerson R, Humfrey C, Smith LM. 1997. Proceedings of European workshop on the impact of endocrine disruptors on human health and wildlife. Weibridge: MRC Institute for Environment and Health.

Huschek G, Hansen PD, Maurer HH, Krengel D, Kayser A. 2004. Environmental risk assessment of medicinal products for human use according to European Commission recommendations. Environmental Toxicology, 19: 226–240.

International Agency for Research on Cancer (IARC). 1990. Monograph, Environmental Protection Agency. Office of Toxic Substances. Chlorinated paraffins. Environmental and Risk Assessment, RM1 Decision Package, Washington DC.

International Olympic Committee. 1995. IOC Medical Code and Explanatory Document; IOC, Lausanne, Switzerland.

Jacob S, Rett K, Henriksen EJ. 1998. Antihypertensive therapy and insulin sensitivity: do we have to redefine the role of beta–blocking agents?. American Journal of Hypertension, 11: 1258–1265.

Jang DJ, Park JS, Ko HR, Jee JP, Kim JK, Kim ST, Kim CK. 2005. Simultaneous determination of niflumic acid and its prodrug, talniflumate in human plasma by high performance liquid chromatography. Biomedical Chromatography, 19: 32–35.

Jarmalavičiene R, Szumski M, Kornyšova O, Kłodzińska E, Westerlund D, Krawczyk S, Mickevičius D, Buszewski B, Maruška A. 2008. Coupling of solid-phase microextraction continuous bed (monolithic) capillaries with capillary zone electrophoresis for direct analysis of drugs in biological fluids. Electrophoresis, 29: 1753–1760.

Jelíc A, Petrovic´ M, Barceló D. 2009. Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. Talanta, 80: 363–371.

Jones OAH, Voulvoulis N, Lester JN. 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Research, 36: 5013–5022.

Jos A, Repetto G, Rios JC, Hazen MJ, Molero ML, del Peso A, Salguero M, Fernández-Freire P, Pérez-Martín JM, Cameán A. 2003. Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. Toxicology in Vitro, 17: 525–532.

Jurado-Sánchez B, Ballesteros E, Gallego M. 2009. Fullerenes for aromatic and non-aromatic N-nitrosamines discrimination. Journal of Chromatography A, 1216: 1200–1205.

Kapusnik-Uner JE, Sande MA, Chambers HF. 1996. Tetracyclines, chloramphenicol, erythromycin, and miscellaneous antibacterial agents. En The pharmacological Basis of Therapeutics. (Goodman & Gilman´s, editors). New York: McGraw-Hill; pp. 1130-1135.

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ. 2007. Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography–positive electrospray ionisation tandem mass spectrometry. Journal of Chromatography A, 1161: 132–145.

Keller BJ,Yamanaka H, Thurman RG. 1992. Inhibition of mitochondrial respiration and oxygen-dependent hepatotoxicity by six structurally dissimilar peroxisomal proliferating agents. Toxicology, 71: 49–61.

Khan SJ, Ongerth JE. 2004. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere, 54: 355–367.

Kinney CA, Furlong ET, Kolpin DW, Burkhardt MR, Zaugg SD, Werner SL, Bossio JP, Benotti MJ. 2008. Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. Environmental Science & Technology, 42:1863–1870.

Koesukwiwat U, Jayanta S, Leepipatpiboon N. 2007. Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in Bovine's milk. Journal of Chromatography A, 1149: 102–111.

Kosjek T, Heath E, Pérez S, Petrovic M, Barceló D. 2009. Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole–time-of-flight mass spectrometry. Journal of Hydrology, 372: 109–117.

Koutsouba V, Hereber Th, Fuhrmann B, Schmidt-Baunmler K, Tsipi D, Hiskia A. 2003. Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography–mass spectrometry. Chemosphere, 51: 69–75.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC, Stallings WC. 1996. Structural basis for selective inhibition of cyclooxygenase-2 by antiinflammatory agents. Nature, 384: 644–648.

Kuster M, López de Alda MJ, Barceló D. 2004. Analysis and distribution of estrogens and progestogens in sewage sludge, soils and sediments. Trends in Analytical Chemistry; 23:790–798.

Labadie P, Budzinski H. 2005. Determination of steroidal hormone profiles along the Jalle d'Eysines river (near Bordeaux, France). Environmental Science & Technology, 39: 5113–5120.

Lai HT, Hou JH, Su CI, Chen CL. 2009. Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae Chlorella pyrenoidosa, Isochrysis galbana, and Tetraselmis chui. Ecotoxicology and Environmental Safety, 72: 329– 334.

Lajeunesse A, Gagnon C. 2007. Determination of acidic pharmaceutical products and carbamazepine in roughly primary-treated wastewater by solid-phase extraction and gas chromatography–tandem mass spectrometry. International Journal of Environmental Analytical Chemistry, 87: 565–578.

Lalumera GM, Calamari D, Galli P, Castiglioni S, Crosa G, Fanelli R. 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere, 54: 661–668.

Laufs U, Liao JK. 1998. Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. The Journal of Biological Chemistry, 273: 24266–24271.

Le Bris H, Pouliquen H. 2004. Experimental study on the bioaccumulation of oxytetracycline and oxolinic acid by the blue mussel (*Mytilus edulis*). An evaluation of its ability to bio-monitor antibiotics in the marine environment. Marine Pollution Bulletin, 48: 434–440.

Lee HB, Sarafin K, Peart TE, Svoboda ML. 2003. Acidic pharmaceuticals in sewage—methodology, stability test, occurrence, and removal from Ontario samples. Water Quality Research Journal of Canada, 38: 667–682.

Lee HB, Peart TE, Svoboda ML. 2005. Determination of endocrine-disrupting phenols, acidic pharmaceuticals, and personal-care products in sewage by solid-phase extraction and gas chromatography–mass spectrometry. Journal of Chromatography A, 1094: 122–129.

Letellier M, Budzinski H. 1999. Microwave assisted extraction of organic compounds. Analusis, 27: 259–271.

Levy SB. 1992. The antibiotic paradox: How miracle drugs are destroying the miracle. New York: Plenum Publication.

Li S,Wagner CA, Friesen JA, Borst DW. 2003. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase in the lobster mandibular organ: regulation by the eyestalk. General and Comparative Endocrinology, 134:147–155.

Lin WC, Chen HC, Ding WH. 2005. Determination of pharmaceutical residues in waters by solid-phase extraction and large-volume on-line derivatization with gas chromatography–mass spectrometry. Journal of Chromatography A, 1065: 279–285.

Lin DL, Wang SM, Wu CH, Chen BG, Liu RH. 2008. Chemical derivatization for the analysis of drugs by GC-MS- A compresive review. Journal of Food and Drug Analysis, 16: 1–10.

Liska I. 2000. Fifty years of solid-phase extraction in water analysis –historical development and overview. Journal of Chromatography A, 885: 3–16.

Liu R, Zhou JL, Wilding A. 2004a. Microwave-assisted extraction followed by gas chromatography–mass spectrometry for the determination of endocrine disrupting chemicals in river sediments. Journal of Chromatography A, 1038: 19–26.

Liu R, Zhou JL, Wilding A. 2004b. Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction–gas chromatography–mass spectrometry. Journal of Chromatography A, 1022: 179–189.

Llorent-Martínez EJ, Šatínsky´ D., Solich P, Ortega-Barrales P, Molina-Díaz A. 2007. Fluorimetric SIA optosensing in pharmaceutical analysis: Determination of paracetamol. Journal of Pharmaceutical and Biomedical Analysis 45: 318–321.

Lockwood PW, Johnson JC, Katz TL. 2003. Clinical efficacy of flunixin, carprofen and ketoprofen as adjuncts to the antibacterial treatment of bovine respiratory disease. Veterinary Record; 152: 392–394.

Löffler, D, Ternes TA. 2003. Determination of acidic pharmaceuticals, antibiotics and ivermectin in river sediment using liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1021, 133–144.

Loke ML, Ingerslev F, Halling-Sorensen B, Tjornelund J. 2000. Stability of tylosin A in manure containing test systems determined by high performance liquid chromatography. Chemosphere, 40: 759–765.

Long Z, Hong L, Jie J. 2007. Determination of β-estradiol residues in fish/shellfish muscle by gas Chromatography-mass spectrometry. Chinese Journal of Analytical Chemistry, 35: 983–987.

López de Alda MJ, Gil A, Paz E, Barceló D. 2002. Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography-electrospray-mass spectrometry. Analyst, 127: 1299–1304.

Lord H, Pawliszyn J. 2000. Evolution of solid-phase microextraction technology. Journal of Chromatography A, 885: 153–193.

MacDonald RL, Olsen RW. 1994. GABA A receptor channels. Annual Reviews of Neuroscience, 17: 569–602.

Macià A, Borrull F, Calull M, Aguilar C. 2004. Determination of some acidic drugs in surface and sewage treatment plant waters by capillary electrophoresis-electrospray ionization-mass spectrometry. Electrophoresis, 25: 3441–3449.

Magiera S, Uhlschmied C, Rainer M, Huck CW, Baranowska I, Bonn GK. 2011. GC–MS method for the simultaneous determination of β-blockers, flavonoids, isoflavones and their metabolites in human urine. Journal of Pharmaceutical and Biomedical Analysis, 56: 93– 102.

Maijó I, Borrull F, Calull M, Aguilar C. 2011. An in-line SPE strategy to enhance sensitivity in CE for the determination of pharmaceutical compounds in river water samples. Electrophoresis, 32: 2114–2122.

Masqué N, Marcé RM, Borrull F. 1998. New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water. Trends in Analytical Chemistry, 17: 384–394.

Masqué N, Marcé RM, Borrull F. 2001. Molecularly imprinted polymers: new tailor-made materials for selective solid-phase extraction. Trends in Analytical Chemistry, 20: 477– 494.

Matamoros V, Jover E, Bayona JM. 2010. Part-per-trillion determination of pharmaceuticals, pesticides, and related organic contaminants in river water by solid-phase extraction followed by comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Analytical Chemistry, 82: 699–706.

Matějíček D, Houserová P, Kubáň V. 2007. Combined isolation and purification procedures prior to the high-performance liquid chromatographic-ion-trap tandem mass spectrometric determination of estrogens and their conjugates in river sediments. Journal of Chromatography A, 1171: 80–89.

Maurer HH. 2004. Screening for drugs in body fluids by GC/MS. En avances in forensic application of mass spectrometry (Yinon J, editor). Boca Raton: CRC Press, pp. 1–61.

Melwanki MB, Fuh MR. 2008. Dispersive liquid–liquid microextraction combined with semi-automated *in*-syringe back extraction as a new approach for the sample preparation of ionizable organic compounds prior to liquid chromatography Journal of Chromatography A, 1198: 1–6.

Mervařtová K, Polášek M, Calatayud JM. 2007. Recent applications of flow-injection and sequential-injection analysis techniques to chemiluminescence determination of pharmaceuticals. Journal of Pharmaceutical and Biomedical Analysis, 45: 367–381.

Mohamed R, Richoz-Payot J, Gremaud E, Tabet JC, Guy PA. 2007. Advantages of molecularly imprinted polymers LC-ESI-MS/MS for the selective extraction and quantification of chloramphenicol in milk-based matrixes. comparison with a classical sample preparation. Analytical Chemistry, 79: 9557–9565.

Morales S, Canosa P, Rodríguez I, Rubí E, Cela R. 2005. Microwave assisted extraction followed by gas chromatography with tandem mass spectrometry for the determination of triclosan and two related chlorophenols in sludge and sediments. Journal of Chromatography A, 1082: 128–135.

Nagata T, Oka H. 1996. Detection of residual chloramphenicol, florfenicol, and thiamphenicol in yellowtail fish muscles by capillary gas chromatography–mass spectrometry. Journal of Agricultural and Food Chemistry, 44: 1280–1284.

Neng NR, Mestre AS, Carvalho AP, Nogueira JMF. 2011. Cork-based activated carbons as supported adsorbent materials for trace level analysis of ibuprofen and clofibric acid in environmental and biological matrices. Journal of Chromatography A, 1218: 6263–6270.

Nguyen HP, Li L, Gatson JW, Maass D, Wigginton JG, Simpkins JW, Schug KA. 2011. Simultaneous quantification of four native estrogen hormones at trace levels in human cerebrospinal fluid using liquid chromatography–tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 54: 830–837.

Nieto A, Borrull F, Marcé RM, Pocurull E. 2009. Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 1216: 5619–5625.

Nozal L, Arce L, Simonet BM, Ríos A, Valcárcel M. 2007. In-line liquid-phase microextraction for selective enrichment and direct electrophoretic analysis of acidic drugs. Electrophoresis, 28: 3284–3289.

OECD Guidelines for the Testing of Chemicals, 2002. http://www.oecd.org/dataoecd/58/60/1946914.pdf.

Ortelli D, Cognard E, Jan P, Edder P. 2009. Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography B, 877: 2363–2374.

Paape MJ, Miller RH. 1990. Effects of florfenicol, chloramphenicol and thiamphenicol on phagocytosis, chemiluminescence and morphology of bovine polymorphonuclear neutrophil leukocytes. Journal of Dairy Science, 73: 1734–1744.

Park BK, Lim JH, Kim MS, Hwang YH, Yun HI. 2008. Pharmacokinetics of florfenicol and its metabolite, florfenicol amine, in dogs. Research in Veterinary Science, 84: 85–89.

Payán MR, López MAB, Fernández-Torres R, Navarro MV, Mochón MC. 2009. Hollow fiber-based liquid-phase microextraction (HF-LPME) of ibuprofen followed by FIA-chemiluminescence determination using the acidic permanganate–sulfite system. Talanta, 79: 911–915.

Pedrouzo M, Reverté S, Borrull F, Pocurull E, Marcé RM. 2007. Pharmaceutical determination in surface and wastewaters using high-performance liquid

chromatography-(electrospray)-mass spectrometry. Journal of Separation Science, 30: 297–303.

Perencevich EN, Wong MT, Harris AD. 2001. National and regional assessment of the antibacterial soap market: A step toward determining the impact of prevalent antibacterial soaps. American Journal of Infection Control, 29: 281–283.

Peters RJB, Bolck YJC, Rutgers P, Stolker AAM, Nielena MWF. 2009. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. Journal of Chromatography A, 1216: 8206–8216.

Petrovic M, Hernando MD, Díaz-Cruz MS, Barceló D. 2005. Liquid chromatography–tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. Journal of Chromatography A, 1067: 1–14.

Pérez-Carrera E, Hansen M, León VM, Björklund E, Krogh KA, Halling-Sørensen B, González-Mazo E. 2010. Multiresidue method for the determination of 32 human and veterinary pharmaceuticals in soil and sediment by pressurized-liquid extraction and LC-MS/MS. Analytical and Bioanalytical Chemistry, 398:1173–1184.

Pfeifer T, Tuerk J, Bester K, Spiteller M. 2002. Determination of selected sulfonamide antibiotics and trimethoprim in manure by electrospray and atmospheric pressure chemical ionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 16: 663–669.

Pichon V. 2007. Selective sample treatment using molecularly imprinted polymers. Journal of Chromatography A, 1152: 41–53.

Poole CF. 2003. New trends in solid-phase extraction. Trends in Analytical Chemistry, 22: 362–373.

Qu B, Li Q-T, Wong, KP, Tan, TMC, Halliwell B. 2001. Mechanism of clofibrate hepatotoxicity: mitochondrial damage and oxidative stress in hepatocytes. Free Radical Biology & Medicine, 31: 659–669.

Queiroz RHC, Bertucci C, Malfará WR, Dreossi SAC, Chaves AR, Valério DAR, Queiroz MEC. 2008. Quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples by stir bar-sorptive extraction and liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis, 48: 428–434.

Quintana JB, Carpinteiro J, Rodríguez I, Lorenzo RA, Carro AM, Cela R. 2004. Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection. Journal of Chromatography A, 1024: 177–185.

Quintana JB, Rodil R, Muniategui-Lorenzo S, López-Mahía P, Prada-Rodríguez D. 2007. Multiresidue analysis of acidic and polar organic contaminants in water samples by stir-bar sorptive extraction–liquid desorption–gas chromatography–mass spectrometry. Journal of Chromatography A, 1174: 27–39.

Raich-Montiu J, Folch J, Compañó R, Granados M, Prat MD. 2007. Analysis of trace levels of sulfonamides in surface water and soil samples by liquid chromatography-fluorescence. Journal of Chromatography A, 1172: 186–193.

Ramsey ED. 1998. Analytical supercritical fluid extraction techniques. Longmont, CO, Springer.

Reddersen K, Heberer T. 2003. Multi-compound methods for the detection of pharmaceutical residues in various waters applying solid phase extraction (SPE) and gas chromatography with mass spectrometric (GC-MS) detection. Journal of Separation Science, 26: 1443–1450.

Reddy S, Iden CR, Brownawell BJ. 2005. Analysis of steroid conjugates in sewage influent and effluent by liquid chromatography—tandem mass spectrometry. Analytical Chemistry, 77: 7032–7038.

Rejtharová M, Rejthar L. 2009. Determination of chloramphenicol in urine, feed water, milk and honey samples using molecular imprinted polymer clean-up. Journal of Chromatography A, 1216: 8246–8253.

Repetto G, Jos A, Hazen MJ, Molero ML, del Peso A, Salguero M, del Castillo P, Rodríguez-Vicente MC, Repetto M. 2001. A test battery for the ecotoxicological evaluation of pentachlorophenol. Toxicology in Vitro, 15: 503–509.

Reverté S, Borrull F, Pocurull E, Marcé R. 2003. Determination of antibiotic compounds in water by solid-phase extraction–high-performance liquid chromatography–(electrospray) mass spectrometry. Journal of Chromatography A, 1010: 225–232.

Rezaee M, Assadi Y, Milani Hosseini MR, Aghaee E, Ahmadi F, Berijani S. 2006. Determination of organic compounds in water using dispersive liquid–liquid microextraction. Journal of Chromatography A, 1116: 1–9.

Rice SL, Mitra S. 2007. Microwave-assisted solvent extraction of solid matrices and subsequent detection of pharmaceuticals and personal care products (PPCPs) using gas chromatography–mass spectrometry. Analytica Chimica Acta, 589: 125–132.

Richards N, Hall S, Scott K, Harrison N. 2011. First detection of an NSAID, flunixin, in sheep's wool using GC–MS. Environmental Pollution, 159: 1446-450.

Risticevic S, Niri VH, Vuckovic D, Pawliszyn J. 2009. Recent developments in solid-phase microextraction. Analytical and Bioanalytical Chemistry, 393: 781–795.

Robards K, Haddad PR, Jackson PE. 1994. Principles and practice of modern chromatographic methods. London: Academis Press.

Rogers CJ, Twyman RE, Macdonald RL. 1994. Benzodiazepine and beta-carboline regulation of single GABAA receptor channels of mouse spinal neurones in culture. Journal of Physiology, 475: 69–82.

Rosenkranz HS. 1988. Chloramphenicol: magic bullet or double-edge sword?. Mutation Research, 196: 1–16.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environmental Science & Technology, 32:1559–1565.

Runnqvist H, Bak SA, Hansen M, Styrishave B, Halling-Sørensen B, Björklund E. 2010. Determination of pharmaceuticals in environmental and biological matrices using pressurised liquid extraction—Are we developing sound extraction methods?. Journal of Chromatography A, 1217: 2447–2470.

Sacher F, Lange FT, Brauch HJ, Blankenhorn I. 2001. Pharmaceuticals in groundwaters Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. Journal of Chromatography A, 938: 199–210.

Samaras VG, Thomaidis NS, Stasinakis AS, Lekkas TD. 2011. An analytical method for the simultaneous trace determination of acidic pharmaceuticals and phenolic endocrine disrupting chemicals in wastewater and sewage sludge by gas chromatography-mass spectrometry. Analytical and Bioanalytical Chemistry, 399: 2549–2561.

Sanches-Silva A, Sendón-García R, López-Hernández J, Paseiro-Losada P. 2005. Determination of triclosan in foodstuffs. Journal of Separation Science, 28: 65–72.

Sanchez-Rojas F, Bosch-Ojeda C, Cano-Pavón JM. 2009. A review of stir bar sorptive extraction. Chromatographia, 69: S79–S94.

Sapota G. 2004. Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in seawater of the southern Baltic Sea. Desalination, 162: 153-157.

Sarmah AK, Meyer MT, Boxall ABA. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere, 65: 725–759.

Seo J, Kim HY, Chung BC, Hong J. 2005. Simultaneous determination of anabolic steroids and synthetic hormones in meat by freezing-lipid filtration, solid-phase extraction and gas chromatography–mass spectrometry. Journal of Chromatography A, 1067: 303–309.

Servais AC, Fillet M, Mol R, Somsen GW, Chiap P, de Jong GJ, Crommen J. 2006. On-line coupling of cyclodextrin mediated nonaqueous capillary electrophoresis to mass spectrometry for the determination of salbutamol enantiomers in urine. Journal of Pharmaceutical and Biomedical Analysis, 40: 752–757.

Shao B, Zhao R, Meng J, Xue Y, Wu G, Hu J, Tu X. 2005. Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta, 548: 41–50.

Shen J, Xia X, Jiang H, Li C, Li J, Li X, Ding S. 2009. Determination of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in poultry and porcine muscle and liver by gas chromatography-negative chemical ionization mass spectrometry. Journal of Chromatography B, 877: 1523–1529.

Shi YB. 2000. Amphibian metamorphosis: from morphology to molecular biology. New York: Wiley-Liss.

Silva ARM, Portugal FCM, Nogueira JMF. 2008. Advances in stir bar sorptive extraction for the determination of acidic pharmaceuticals in environmental water matrices Comparison between polyurethane and polydimethylsiloxane polymeric phases. Journal of Chromatography A, 1209: 10–16.

Singer H, Müller S, Tixier C, Pillonel L. 2002. Triclosan: Occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. Environmental Science & Technology, 36: 4998–5004.

Soliman MA, Pedersen JA, (Mel) Suffet IH. 2004. Rapid gas chromatography-mass spectrometry screening method for human pharmaceuticals, hormones, antioxidants and plasticizers in water. Journal of Chromatography A, 1029: 223–237.

Sørensen LK. 2011. Determination of acidic and neutral therapeutic drugs in human blood by liquid chromatography–electrospray tandem mass spectrometry. Forensic Science International, 206: 119–126.

Souza DZ, Boehl PO, Comiran E, Mariotti KC, Pechansky F, Duarte P CAV, Boni RD, Froehlich PE, Limberger RP. 2011. Determination of amphetamine-type stimulants in oral fluid by solid-phase microextraction and gas chromatography–mass spectrometry. Analytica Chimica Acta, 696: 67–76.

Soyka D. 1984. Beta-blockers in the prophylactic treatment of migraine and tension headache. Nervenheilkunde, 3: 85–89.

Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation, 98:2088–2093.

Study RE, Barker JL. 1981. Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of gamma-aminobutyric acid responses in cultured central neurons. Proceedings of the National Academy of Sciences of the United Stated of American, 78: 7180–7184.

Stuer-Lauridsen F, Birkved M, Hansen LP, Lützhøft HC, Halling-Sorensen B. 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. Chemosphere, 40: 783–793.

Subedi B, Mottaleb MA, Chambliss CK, Usenko S. 2011. Simultaneous analysis of select pharmaceuticals and personal care products in fish tissue using pressurized liquid extraction combined with silica gel cleanup. Journal of Chromatography A, 1218: 6278–6284.

Suenami K, Lim LW, Takeuchi T, Sasajima Y, Sato K, Takekoshi Y, Kanno S. 2006. Rapid and simultaneous determination of nonsteroidal anti-inflammatory drugs in human plasma by LC–MS with solid-phase extraction. Analytical and Bioanalytical Chemistry, 384: 1501–1505.

Sun HW, He P, Lv YK, Liang SX. 2007. Effective separation and simultaneous determination of seven fluoroquinolones by capillary electrophoresis with diode-array detector. Journal of Chromatography B, 852: 145–151.

Sun L, Yong W, Chu X, Lin JM. 2009. Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1216: 5416–5423.

Tatarazako N, Ishibashi H, Teshima K, Kishi K, Arizono K. 2004. Effects of triclosan on various aquatic organisms. Environmental Sciences, 11: 133–140.

Ternes TA. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research, 32: 3245–3260.

Ternes TA, Andersen H, Gilberg D, Bonerz M. 2002. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Analytical Chemistry, 74: 3498–3504.

Thanasarakhan W, Kruanetr S, Deming RL, Liawruangrath B, Wangkarn S, Liawruangrath S. 2011. Sequential injection spectrophotometric determination of tetracycline antibiotics in pharmaceutical preparations and their residues in honey and milk samples using yttrium (III) and cationic surfactant.Talanta, 84: 1401–1409.

The joint FAO/WHO Expert Committee on Food Additives. Residues of some veterinary drugs in animals and foods. Food and Agriculture Organization of the United Nations 1988 FAO Food and Nutrition paper 41.

The Ministry of Agriculture of PR China. 2011. Regulation No. CH11023. China Re-issues Banned Drugs and Substances in Feed and Animal Production. Ministry of Agriculture of PR China, pp. 1–8.

The World Anti-doping Code. 2010. The 2010 WADA Prohibited List International Standard.

Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - a review. Journal of Plant Nutrition and Soil Science, 166: 145–167.

Thomas KV, Hilton MJ. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Marine Pollution Bulletin. 49: 436–444.

Thurman, EM y Mills MS. 1998. Solid phase extraction: principles and pratice. New York: Wiley-Interscience.

Togola A, Budzinski H. 2007. Analytical development for analysis of pharmaceuticals in water samples by SPE and GC–MS. Analytical and Bioanalytical Chemistry, 388:627–635.

Tolls J. 2001. Sorption of veterinary pharmaceuticals in soils: a review. Environmental Science & Technology, 35: 3397–3406.

Toyo'oka T. 1999. Modern derivatización methods for separation sciences. Chichester: John Wily & Sons.

Tuerk J, Reinders M, Dreyer D, Kiffmeyer TK, Schmidt KG, Kuss HM. 2006. Analysis of antibiotics in urine and wipe samples from environmental and biological monitoring—Comparison of HPLC with UV-, single MS- and tandem MS-detection. Journal of Chromatography B, 831: 72–80.

Valcárcel M, Cárdenas S. 2000. Automatización y miniaturización en Química Analítica. Springer-Verlag Ibérica S.A., Barcelona.

Valters K, Li H, Alaee M, D'Sa I, Marsh G, Bergman A, Letcher RJ. 2005. Polybrominated diphenyl ethers and hydroxylated and methoxylated brominated and chlorinated analogues in the plasma of fish from the Detroit River. Environmental Science & Technology, 39:5612–5619.

Varga M, Dobor J, Helenkár A, Jurecska L, Yao J, Záray G. 2010. Investigation of acidic pharmaceuticals in river water and sediment by microwave-assisted extraction and gas chromatography–mass spectrometry. Microchemical Journal, 95: 353–358.

Vazquez-Roig P, Segarra R, Blasco C, Andreu V, Picó Y. 2010. Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1217: 2471–2483.

Veraart, Gooijer JRC, Lingeman H., Velthorst NH, Brinkman UAT. 1998. At-line solid-phase extraction for capillary electrophoresis: application to negatively charged solutes. Journal of Chromatography B, 719: 199–208.

Vera-Candioti L, García MDG, Galerab MM, Goicoecheaa HC. 2008. Chemometric assisted solid-phase microextraction for the determination of anti-inflammatory and antiepileptic drugs in river water by liquid chromatography–diode array detection. Journal of Chromatography A, 1211: 22–32.

Verenitch SS, Lowe CJ, Mazumber A. 2011. Determination of acid drugs and caffeine in municipal wastewater and receiving waters by gas chromatography-ion trap tandem mass spectrometry. Journal of Chromatography A, 1116: 193–203.

Vermeiren L, Devlieghere F, Debevere J. 2002. Effectiveness of some recent antimicrobial packaging concepts. Food Additives and Contaminants, 19: 163–171.

Villar Navarro M, Ramos Payán MR, Fernández-Torres R, Miguel A. Bello-López MA, Callejón Mochón M, Guiraúm Pérez A. 2011. Capillary electrophoresis determination of nonsteroidal anti-inflammatory drugs in wastewater using hollow fiber liquid-phase microextraction. Electrophoresis, 32: 2107–2113.

Villar-Pulido M, Gilbert-López B, García-Reyes JF, Ramos Martos N, Molina-Díaz A. 2011. Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry. Talanta, 85: 1419–1427.

Villaverde-de-Sáa E, González-Mariño I, Quintana JB, Rodil R, Rodríguez I, Cela R. 2010. In-sample acetylation-non-porous membrane-assisted liquid-liquid extraction for the determination of parabens and triclosan in water samples. Analytical and Bioanalytical Chemistry, 397: 2559–2568.

Vinci F, Fabbrocino S, Fiori M, Serpe L, Gallo P. 2006. Determination of fourteen non-steroidal anti-inflammatory drugs in animal serum and plasma by liquid chromatography/mass spectrometry. Rapid Communication in Mass Spectrometry, 20: 3412–3420.

Wallace JL. 1997. Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. Gastroenterology, 112: 1000–1016.

Wallace JL, McKnight W, Reuter BK, Vergnolle N. 2000. NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology, 119: 706–714.

Wang Y, Wu Q, Cheng M, Cai C. 2011. Determination of β-blockers in pharmaceutical and human urine by capillary electrophoresis with electrochemiluminescence detection and studies on the pharmacokinetics. Journal of Chromatography B, 879: 871–877.

Weigel S, Kallenborn R, Hühnerfuss H. 2004. Simultaneous solid-phase extraction of acidic, neutral and basic pharmaceuticals from aqueous samples at ambient (neutral)

pH and their determination by gas chromatography–mass spectrometry. Journal of Chromatography A, 1023: 183–195.

Weisblum B, Davies J. 1968. Antibiotic inhibitors of the bacterial ribosome. Bacteriology Reviews, 32: 493–528.

Wollenberg L, Halling-Sorensen B, Kuska KO. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia Magna*. Chemosphere, 40: 723–730.

Xu L, Basheer C, Lee HK. 2007. Developments in single-drop microextraction. Journal of Chromatography A, 1152: 184–192.

Xu J, Wu L, Chen W, Chang AC. 2008. Simultaneous determination of pharmaceuticals, endocrine disrupting compounds and hormone in soils by gas chromatography–mass spectrometry. 2008. Journal of Chromatography A, 1202: 189–195.

Yagoh H, Murayama H, Suzuki T, Tominaga Y, Shibuya N, Masuda Y. 2006. Simultaneous monitoring method of polycyclic aromatic hydrocarbons and persistent organic pollutants in the atmosphere using activated carbon fiber filter paper. Analytical Sciences, 22: 583–590.

Yamini Y, Asghari-Khiavi M, Bahramifar N. 2002. Effects of different parameters on supercritical fluid extraction of steroid drugs, from spiked matrices and tablets. Talanta, 58: 1003–1010.

Yan W, Li Y, Zhao L, Lina J-M. 2009. Determination of estrogens and bisphenol A in bovine milk by automated on-line C_{30} solid-phase extraction coupled with high-performance liquid chromatography–mass spectrometry. Journal of Chromatography A, 1216: 7539–7545.

Yang RT. 2003. Adsorbents: fundamentals and aplications. New Jersey: John Wiley & Sons Inc.

Yang S, Carlson KH. 2004. Solid-phase extraction–high-performance liquid chromatography–ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. Journal of Chromatography A, 1038: 141–155.

Ye X, Bishop AM, Needham LL, Calafat AM. 2008. Automated on-line column-switching HPLC-MS/MS method with peak focusing for measuring parabens, triclosan, and other environmental phenols in human milk. Analytica Chimica Acta, 622 : 150–156.

Yilmaz B, Arslan S, Akba V. 2009. Gas chromatography–mass spectrometry method for determination of metoprolol in the patients with hypertension. Talanta, 80: 346–351.

Ying, GG. 2007. Analysis of endocrine disrupting chemicals and pharmaceuticals and personal care products in water. En Handbook of Water Analysis (Nollet, LML, editor). Boca Raton: CRC Press, Taylor & Francis Group, pp. 693–727.

Young NS, Alter BP. 1994. Aplastic Anemia: Acquired and Inherited. Saunders WB. Philadephia, pp. 325-339.

Yu Z, Peldszus S, Huch PM. 2007. Optimizing gas chromatographic-mass spectrometric analysis of selected pharmaceuticals and endocrine-disrupting substances in water using factorial experimental design. Journal of Chromatography A, 1148: 65–77.

Yunis AA, Bloomberg GR. 1964. Chloramphenicol toxicity: clinical features and pathogenesis. Progress in Hematology, 4: 138–159.

Yunis AA. 1988. Chloramphenicol: Relation of structure to activity and toxicity. Annual Review of Pharmacology and Toxicology, 28: 83–100.

Zhang S, Sun FY, Li JC, Cheng LL, Shen JZ. 2006. Simultaneous determination of florfenicol and florfenicol amine in fish, shrimp, and swine muscle by gas chromatography with a microcell electron capture detector. Journal of AOAC International, 89: 1437–1441.

Zhang ZL, Zhou JL. 2007. Simultaneous determination of various pharmaceutical compounds in water by solid-phase extraction–liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1154: 205–213.

Zhang S, Zhang Q, Darisaw S, Ehie O, Wang G. 2007. Simultaneous quantification of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. Chemosphere, 66: 1057–1069.

Zhang S, Liu Z, Guo X, Cheng L, Wang Z, Shen J. 2008. Simultaneous determination and confirmation of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in chicken muscle by liquid chromatography–tandem mass spectrometry. Journal of Chromatography B, 875: 399–404.

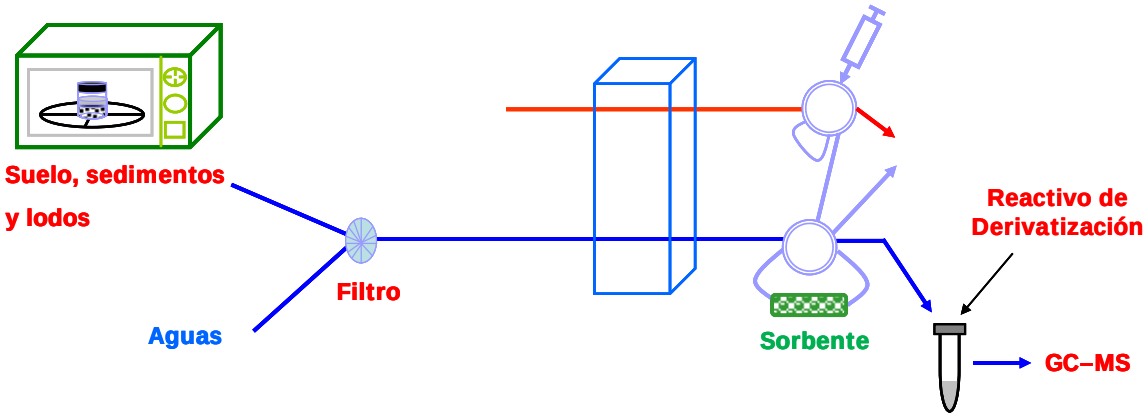
Zhao JL, Ying GG, Wang L, Yang JF, Yang XB, Yang LH, Li X. 2009. Determination of phenolic endocrine disrupting chemicals and acidic pharmaceuticals in surface water

of the Pearl Rivers in South China by gas chromatography–negative chemical ionization–mass spectrometry. Science of the Total Environment, 407: 962–974.

Zgoła-Grzéskowiak A. 2010. Application of DLLME to isolation and concentration of non-steroidal anti-inflammatory drugs in environmental water samples. Chromatographia, 72: 671–678.

CAPÍTULO I

Puesta a Punto de un Sistema Continuo para la Extracción en Fase Sólida para la Determinación por Cromatografía de Gases-Espectrometría de Masas de Sustancias Farmacológicamente Activas en Matrices Medioambientales



Las fuentes más importantes de las sustancias farmacológicamente activas en el Medio Ambiente se encuentran en las depuradoras de aguas residuales del hogar, hospitales, zonas industriales y granjas de cría intensiva de animales o piscifactorías. En la Figura 12 se pueden observar las principales fuentes y vías de contaminación de los medios acuáticos por SFAs.

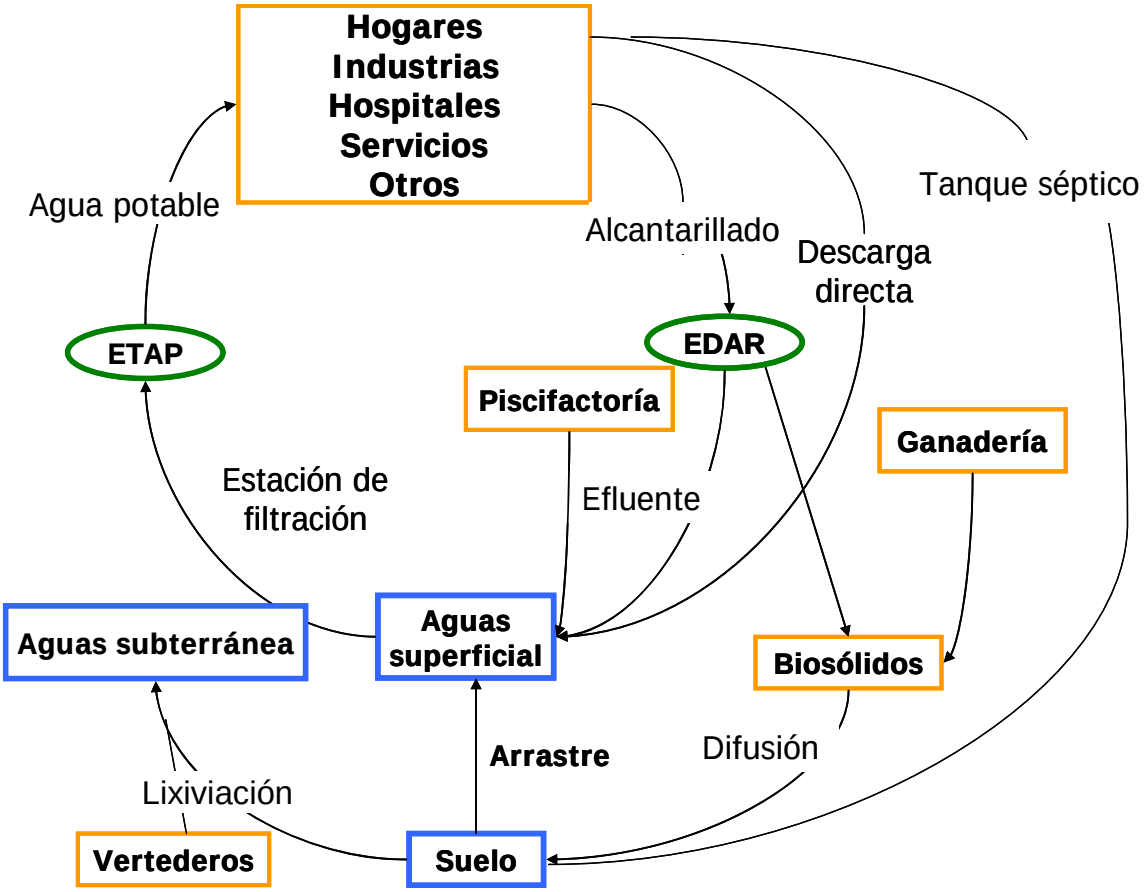


Figura 12: Origen y vías de contaminación de las sustancias farmacológicamente activas en el Medio Ambiente. ETAP: estación de tratamiento de agua potable; EDAR: estación de depuración de aguas residuales.

En este Capítulo se han desarrollado dos metodologías para la determinación de sustancias farmacológicamente activas en muestras ambientales (líquidas o sólidas). En la primera parte de este Capítulo se aborda la puesta a punto de un sistema continuo de extracción en fase sólida para la determinación de ocho fármacos (ácido clofibrico, carbamazepina, diclofenaco, ketoprofeno, ibuprofeno, metoprolol, naproxeno y propranolol), un producto de cuidado personal (triclosán) y tres hormonas (estrona, 17α-etinilestradiol y 17β-estradiol) en distintas muestras de aguas tratadas (grifo y piscina) y no tratadas (río, pozo y residual).

En este sistema se incluye una columna rellena de una cantidad mínima de un sorbente para llevar a cabo la preconcentración de los analitos y permite su elución con un volumen reducido de disolvente (400 µL) para conseguir unos factores de preconcentración elevados. Se han estudiado las diferentes variables que influyen en este método (naturaleza del sorbente, eluyente y pH de la muestra). Para ello se probaron ocho materiales sorbentes (Oasis HLB, LiChrolut EN, XAD-2, XAD-4, RP-C_{18}, Florisil, gel de sílice e Isolute NH_2), consiguiéndose los mejores resultados con el polímero Oasis HLB (divinilbenceno-vinilpirrolidona). La cantidad óptima de este sorbente se demostró que era 60 mg. A continuación se estudiaron diferentes disolventes orgánicos (acetato de etilo, acetonitrilo, acetona, metanol, etanol, 2-propanol y diclorometano) como eluyentes de los analitos retenidos, encontrándose que el acetato de etilo era el que proporcionaba un mayor rendimiento en la elución. Además se optimizó el volumen de eluyente, observándose que los mejores resultados se obtenían para volúmenes superiores de 375 µl de acetato de etilo, para volúmenes inferiores se producía *carry over*, seleccionando un volumen de 400 µl, con objeto de conseguir factores de preconcentración lo más elevados posibles.

La retención de los analitos se puede ver influenciada por el pH de la muestra. Por ello se realizó un estudio de la influencia del pH en el intervalo entre 1,0 y 12,0 (ajustado con disoluciones diluidas de HCl o NaOH). Los mejores resultados para la retención de las diferentes SFAs se obtuvieron en la región de pH cercana a la neutralidad (6,5–7,5). Finalmente se probaron diferentes reactivos de sililación tales como N,O-bis-(trimetilsilil)trifluoroacetamida (BSTFA), N,O-bis-(trimetilsilil)acetamida, y trimetilclorosilano (TMCS), tanto de forma individual como en mezclas. Los mejores resultados en este sentido, se obtuvieron con una mezcla de BSTFA y TMCS. La influencia de la proporción de TMCS (el catalizador) en la mezcla fue examinado en el intervalo de 0,25-15 %, el efecto del tiempo de reacción (1– 40 min), la temperatura (ambiente–90 ºC) y el medio de reacción. Los mejores resultados se consiguieron para un porcentaje del 1 % de TMCS en BSTFA a 70 ºC durante 20 min y en acetato de etilo.

El método desarrollado fue validado, obteniéndose unos resultados analíticos satisfactorios con límites de detección entre 0,01 y 0,06 ng/l para 100 ml de muestra de agua y una buena linealidad (coeficiente de correlación> 0,993). El método proporciona una buena precisión con valores de desviación estándar relativa entre 3.5 y 6.8 %. Además se realizaron estudios de recuperación para los diferentes tipos de muestras de

agua analizadas a tres niveles de concentración (5, 50 y 100 ng/l), consiguiéndose un porcentaje entre 85 y 103 %. Finalmente el método se aplicó a la determinación de las 12 SFAs en diferentes tipos de muestras acuosas (4 de grifo, 3 de río, 2 de pozo, 2 de pantano, 2 de piscina y 2 de residuales). En ninguna de las muestras de agua de grifo se encontraron residuos de SFAs. En cambio las demás muestras analizadas contenían 2 o más analitos, siendo el ibuprofeno el compuesto más frecuentemente encontrado, seguido de metaprolol, diclofenaco, ketoprofeno y dos hormonas (estrona y 17β-estradiol). De todas las muestras analizadas las dos aguas residuales contenían un mayor número de SFAs y a concentraciones más elevadas (16–2800 ng/l). Es significativo resaltar que la hormona sintética 17α-etinilestradiol no se encontró en ninguna de las muestras estudiadas.

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El método propuesto en la segunda parte de este Capítulo se basa en el uso de la técnica de extracción asistida por microondas (MAE) y de un sistema continuo de extracción en fase sólida (SPE) similar al optimizado en la primera parte del Capítulo para la determinación simultánea de 18 SFAs (analgésicos, antibacterianos, antiepilépticos, β-bloqueadores, reguladores de lípidos y anti-inflamatorios no esteroideos), un producto de cuidado personal y 3 hormonas en muestras ambientales sólidas (suelos, sedimentos y lodos). La combinación de la MAE con SPE ha permitido una eliminación eficaz de las diferentes interferencias que contenían la matriz de las muestras y la extracción/preconcentración de los distintos analitos con porcentajes de retención próximos al 100 %. Para la derivatización de los analitos una vez que han sido eluidos del SPE se seleccionó el mismo procedimiento empleado en la primera parte de este Capítulo.

Se estudiaron las principales variables que influían en el rendimiento de la extracción asistida con microondas (naturaleza del disolvente y su volumen, potencia del microondas y tiempo de irradiación). Para el caso de la naturaleza y volumen del disolvente, los mejores rendimientos en la extracción con MAE se obtuvieron con 10 ml de una mezcla metanol-agua (3:2). También se optimizaron la potencia y el tiempo de irradiación, encontrándose que los mejores resultados se conseguían para 500 W y 6 min, respectivamente. Potencias superiores en combinación con largos periodos de tiempo de irradiación provocan la disminución de la señal de algunos analitos (ácido

clorifíbrico, metoprolol y propanol) debido posiblemente a la descomposición de éstos a condiciones extremas. Finalmente se comparó la eficacia de la extracción mediante MAE de los diferentes analitos de interés con la obtenida mediante Soxhlet para los tres tipos de matrices estudiadas (suelos, sedimentos y lodos), consiguiéndose resultados semejantes y próximos al 100 %.

Como se ha comentado anteriormente, el mejor extractante para la MAE es el compuesto por la mezcla metanol-agua en proporción 3:2. Por ello una vez que se ha llevado a cabo esta etapa de MAE para eliminación de la matriz sólida de las muestras, el sobrenadante contenía un alto porcentaje de metanol (60 %). Este alto porcentaje de metanol en el sobrenadante se observó que afecta negativamente a la retención de los analitos en la siguiente etapa de SPE en la columna empaquetada con 60 mg de Oasis HLB. En estudios realizados sobre la composición de la disolución que contenía a los diferentes analitos, se demostró que a partir de una porcentaje de un 5 % en metanol la retención de algunas SFAs disminuía (ácido acetilsalicílico, paracetamol y triclosán), observándose que este efecto negativo en la retención de los demás fármacos era a porcentajes superiores de metanol (10-40 %). Este efecto puede ser atribuido a que en el mecanismo de adsorción está involucrada la partición de los analitos entre una fase polar (agua) y una fase sólida del sorbente polimérico (Oasis HLB) a través de enlaces de hidrógeno o interacciones л-л entre los analitos y la superficie del sorbente. Cuando la fase acuosa contiene una alta proporción de metanol, el alcohol rompe los enlaces anteriormente indicados, provocando que se reduzca notablemente la retención de los analitos en el sorbente de la columna de SPE. Para evitar este inconveniente se optó por evaporar el sobrenadante de la MAE hasta 200 μL con una corriente de nitrógeno y la posterior reconstitución con 10 ml de agua para su introducción en el sistema de SPE.

El método desarrollado posee un amplio intervalo lineal para las 22 SFAs (2.5–20000 ng/kg) con coeficientes de correlación superiores a 0.994. El método es muy sensible, con límites de detección entre 0.8 y 5.1 ng/kg, mejores que los que se han obtenido en otras metodologías incluidas en la bibliografía para suelos, sedimentos y lodos. La precisión del método fue satisfactoria con una desviación estándar relativa menores del 7 %. Finalmente se realizó un estudio de recuperación mediante la adición de 25, 200 y 2000 ng/kg a las tres tipos de muestras estudiadas, consiguiéndose en todos los casos porcentajes de recuperación próximos al 100 %.

El método propuesto se aplicó a la determinación de las 22 SFAs en 8 tipos de muestras (suelos agrícolas, sedimentos de pantano, sedimentos de río y lodos). En el

caso de los dos suelos estudiados se encontraron el mismo número de analitos aunque las concentraciones variaron significativamente de uno a otro, sobre todo en el caso del diclofenaco, ibuprofeno, ketoprofeno, ácido niflúmico, carbamazepina y las tres hormonas. En la mayoría de los sedimentos analizados (2 de río y 2 de pantano) se encontraron residuos de algunos antiinflamatorios, antibióticos y triclosán a concentraciones entre 8 y 350 ng/kg. También se analizaron lodos procedentes de dos plantas de tratamiento de aguas residuales encontrándose una elevada concentración de la mayoría de las SFAs estudiadas (30–3100 ng/kg), sobre todo son significativos los niveles encontrados de ácido salicílico, diclofenaco, ketoprofeno, naproxeno, triclosán y pirimetamina. La presencia de las SFAs procedentes de los tratamientos médicos o veterinarios en las muestras analizadas demuestra su persistencia en las matrices sólidas ambientales.

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Continuous solid-phase extraction and gas chromatography–mass spectrometry determination of pharmaceuticals and hormones in water samples

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Abstract

A semi-automatic flow-based method for the simultaneous determination of 9 pharmaceuticals and 3 hormones in water samples in a single analytical run is proposed. The analytes were retained on a solid-phase extraction sorbent column and 1 μL of the eluate analysed by gas chromatography in combination with electron impact ionization mass spectrometry in the SIM mode. The sorbent used, Oasis HLB, provided near-quantitative recovery of all analytes. The proposed method was validated with quite good analytical results including low limits of detection (0.01–0.06 ng L^{-1} for 100 mL of water) and good linearity ($r^2 > 0.993$) throughout the studied concentration ranges. The method provided good accuracy (recoveries of 85–103%) and precision (between- and within-day RSD values less than 7 %) in the determination of the pharmaceuticals and hormones in tap, river, pond, well, swimming pool and waste water.

Keywords: Pharmaceuticals; Hormones; Solid-phase extraction; Gas chromatography–mass spectrometry; Waters

1. Introduction

Pharmaceuticals, together with their synthetic precursors and transformation products, are continually released into the environment in vast amounts as a result of

their manufacturing and excretion (mainly via urine and faeces), and also of the disposal of unused and expired drugs, whether directly into the domestic sewage system, in landfills or by burial [1]. Steroid hormones, which are largely excreted by humans and animals, may be released into the aquatic environment. Effluents (reclaimed water) from waste water treatment plants are the primary sources of their presence in the environment as a result of conventional treatment processes not being specifically designed to remove these organic contaminants. These pollutants are typically encountered in the aqueous environment at low nanogram per litre levels, which poses a major analytical challenge to their determination [2,3].

Many analytical laboratories determine pharmaceuticals and hormones by using methods that involve some preconcentration or clean-up step, followed by gas or liquid chromatography with one of various possible detectors. The methods for liquid samples (e.g. waste, river, lake, drinking, spring water) usually include liquid–liquid extraction [3], solid-phase microextraction [4,5] or solid-phase extraction. Solid-phase extraction is widely used for sample extraction and analyte enrichment [1]. Materials such as octadecyl (C_{18}) bonded silica [6–8], Oasis HLB (polystyrene–divinylbenzene–N-vinylpyrrolidone terpolymer) [9–18], Oasis MCX (a strong cation-exchange mixed-mode polymer) [19], styrene–methacrylate co-polymers [10] and PRLP-s (cross-linked styrene–divinylbenzene) [20] have been used as sorbents for the extraction of pharmaceuticals and hormones. Oasis HLB, which possesses both hydrophilic and lipophilic retention properties, is now widely used to simultaneously extract neutral and acid compounds [15,16]. This led us to adopt it as the sorbent for the present research. The eluent and its volume were chosen on the grounds of the particular target compounds and SPE material. Common eluents for use with Oasis HLB include methanol, ethyl acetate and acetone [16].

Gas chromatography coupled with mass spectrometry (GC–MS) [2,3,5–7,9–11,13,14,17,18] or tandem mass spectrometry (GC–MS/MS) [8,10,11,15,16,21], and liquid chromatography coupled to mass spectrometry (LC–MS) [19,22] or tandem mass spectrometry (LC–MS/MS) [12,20], are the most widely used choices for determining pharmaceuticals. Recently, the use of liquid chromatography in combination with time-of-flight mass spectrometry proved an effective tool for determining polar pharmaceuticals in hospital effluents [22]. LC–MS/MS has progressed much over the last few decades, so much so that it is now the main choice for measuring ultratrace concentrations of polar pharmaceuticals in environmental samples [23]. With highly

complex samples such as waste water, however, suppression of the electrospray ionization is likely to occur (especially with electrospray ionization, ESI). On the other hand, GC–MS in the selected ion monitoring (SIM) mode and GC–MS/MS have proved highly suitable for environmental analyses of most pharmaceuticals, which are either neutral or moderately acidic [10,20,21,24]. However, the low volatility of some pharmaceuticals, and the presence of various polar groups in others, require the use of a derivatization procedure to obtain more volatile products and improve the sensitivity of the subsequent GC analysis as a result [21]. In this way, the advantages of increasing the sensitivity is sometimes largely offset by the loss of sample during additional manipulation by effect of most existing analytical methods in this context using a derivatization procedure before GC–MS analysis. Silylating reagents such as N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) are the most commonly used in their class for the GC–MS determination of phenolic and acid compounds [1]. BSTFA, with or without trimethylchlorosilane (TMCS), is often used for phenolic compounds [15], as are MTBSTFA and MSTFA for acid pharmaceuticals [1,7,18,25].

Automation in SPE sample preparation has grown dramatically in recent times by virtue of the many advantages of automatic methods over manual methods, which include expeditiousness, higher throughputs, and improved precision and accuracy. For example, continuous-flow solid-phase extraction has been successfully used for isolation and preconcentration in the determination of organophosphorus pesticides [25] and non-aromatic N-nitrosamines [26] in water samples.

The primary aim of this work was to develop a method based on the use of a continuous SPE system for the preconcentration of pharmaceuticals and hormones with a view to their determination at sub-nanogram-per-litre levels in various types of water by GC–MS. In addition, the method should be made selective by removing the sample matrix prior to analysis and simple enough to allow the determination of several analytes in a short time and with minimal manipulation. To this end, we examined various sorbents and eluents in order to adopt the most suitable among them for the determination of the target contaminants at low concentrations. Determining the pharmaceuticals by GC–MS required their prior derivatization for improved chromatographic performance and sensitivity.

Table 1

Therapeutic class, pKa, retention times (tr) and mass values used to detect the pharmaceuticals and hormones

Therapeutic class	Substance	$logK_{o/w}$	pK_a[a]	tr (min)	Detected (*m/z*)[b,c]
Lipid-regulators	Clofibric acid	2.6	na	11.42	128, **143,** 286
Analgesics and	Diclofenac	4.51	4.14	22.34	**214,** 242, 367
anti-inflammatories	Ketoprofen	3.12	4.45	21.08	**282,** 311,73
	Ibuprofen	3.97	4.91	12.03	**160,** 263, 234, 278
	Naproxen	3.2	4.2	19.34	**185**, 243, 302
Anti-epileptics	Carbamazepine	2.47	7	22.01	**193,** 236
and β-blockers	Metoprolol	1.88	9.68	19.15	**72,** 223
	Propranolol	2.60	9.49	20.72	**72,** 215
Antiseptic	Triclosan	4.8	na	20.03	**347**
Hormones	Estrone	3.69	10.20	26.97	218, 257**, 342**
	17β-Estradiol	4.13	10.27	27.35	285, **416**
	17α-Ethinylestradiol	4.25	10.24	28.72	**425,** 440

[a]na= not available.

[b]Base peaks used for quantification are boldfaced.

[c]*m/z* for IS (triphenylphosphate): 77, 325, **326**

2. Experimental

2.1. Chemicals and reagents

All reagents used were analytical-grade or better. The pharmaceuticals and hormones studied included clofibric acid, diclofenac, ketoprofen, ibuprofen, naproxen, carbamazepine, metoprolol, propranolol, triclosan, estrone, 17β-estradiol and 17α-ethinylestradiol, all from Sigma–Aldrich (Madrid, Spain). The most salient properties of the analytes (therapeutic class, pKa and log Ko/w values) are summarized in Table 1. Triphenylphosphate, heptadecanoic acid, hydrochloric acid (reagent-grade, 37% HCl) potassium hydroxide (KOH), inert poly-tetrafluoroethylene white beads (pore diameter 4 Å) and the derivatization agents [N,O-bis-(trimethylsilyl)acetamide, N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane] were purchased from Fluka (Madrid, Spain). All solvents (methanol, ethyl acetate, acetonitrile, acetone, ethanol, n-hexane, dichloromethane, and 2-propanol) were supplied by Merck (Madrid, Spain). Oasis HLB (particle size 50–65 μm) was obtained from Waters (Madrid, Spain). Silica reversed-phase sorbent with octadecyl functional groups (RP-C_{18}, particle size 40–63 μm), Silica Gel (particle size 15–35 μm), Florisil (particle size 16–30 μm), Isolute NH_2 (Aminopropyl, particle size 45–65 μm) and polymeric sorbents including XAD-2 (particle size 20–60 μm) and XAD-4 (particle size 20–60 μm) were purchased from Sigma–Aldrich. LiChrolut EN (particle size 40–120 μm) was obtained from Merck.

Stock standard solutions of the individual pharmaceuticals and hormones at a 1 g L^{-1} concentration each were prepared in methanol and stored at 4º C in the dark. Standard working-strength solutions were prepared by sequential dilution of each standard with water previously purified by passage through a Milli-Q System from Millipore (Bedford, MA) and adjusted to pH 7.

2.2. Instrumentation

The pharmaceuticals and hormones studied were determined by using a Focus GC instrument interfaced to a DSQ II mass spectrometer and controlled by a computer running XCalibur software (Thermo Electron SA, Madrid, Spain). The GC instrument was equipped with a polydimethylsiloxan (95%) cross-linked DB-5 capillary column (Supelco, 30 m × 0.25 mm I.D., 0.25 μm film thickness), using helium (purity > 99.999%) at 1 mL min^{-1} as carrier gas. The oven temperature was held at 70 ºC for 1

min following injection, and then a temperature gradient was applied at a rate of 14 ºC min^{-1} from 70 to 150 ºC. After the first transition, the temperature was increased from 150 to 290 ºC at 6 ºC min^{-1}. The total analysis time for each GC run was 30 min.

The mass spectrometer was used under the following conditions: ion source temperature, 200 ºC; transfer line temperature, 280 ºC; electron impact ionization mode at 70 eV; scan range, *m/z* 60–500. The time for solvent delay was set at 6 min. The mass spectrometer was operated in the selected ion monitoring (SIM) mode; the *m/z* values used for each analyte are listed in Table 1. In all analyses, a volume of 1 μL of organic extract was injected in the split mode (1:20 ratio) and the analytes quantified from their peak areas.

The continuous solid-phase extraction manifold was assembled from a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinyl chloride) pumping tubes, two Rheodyne 5041 injection valves (Cotati, CA, USA) and PTFE (3 mm I.D) and laboratory-made columns of variable length packed with each sorbent material. The Oasis-HLB sorbent columns were conditioned by passing 1 mL of ethyl acetate and 1 mL of purified water. Under these conditions, the column remained active for 3 months.

2.3. Sampling procedure

Water samples from various locations were collected in 1 L opaque PTFE bottles. Samples were then stored at 4 ºC until analysis, which was done within 2 weeks of collection in all cases. The samples were passed through a 0.45 μm membrane filter (mixed cellulose esters, Millipore) if required, and their pH adjusted to 7 with dilute HCl or NaOH, prior to analysis.

2.4. Solid-phase extraction and derivatization

The continuous solid-phase extraction system used for the continuous preconcentration/ elution of pharmaceuticals and hormones in water is depicted in Fig. 1. Sample volumes up to 100 mL (for 60 mg of Oasis-HLB sorbent) or standard solutions containing 0.03–400 ng L^{-1} of each pharmaceutical and hormone at pH 7 were aspirated at 4 mL min^{-1} through the sorbent column, located in the loop of the first injection valve (IV_1). Once all analytes were sorbed and the sample matrix was sent to waste. Simultaneously, the loop of the second valve (IV_2) was filled with the eluent (ethyl acetate containing 500 μg L^{-1} triphenylphosphate as internal standard) by means of a syringe. Any residual water remaining inside the column and the connectors was

flushed by passing an air stream at 4 mL min^{-1} through the carrier line for 2 min. Next, IV_2 was switched to pass the loop contents (400 µL) through the column, in the opposite direction of the sample, in order to elute the analytes. The organic extract was collected in a conical glass insert of 0.5 mL and concentrated to a volume of 35 µL under a stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using an internal standard. Next, a volume of 70 µL of BSTFA + 1% TMCS was added and the vials were heated at 70 °C for 20 min. Finally, Aliquots of 1 µL of the silylated derivatives were analysed by GC–MS in the SIM mode.

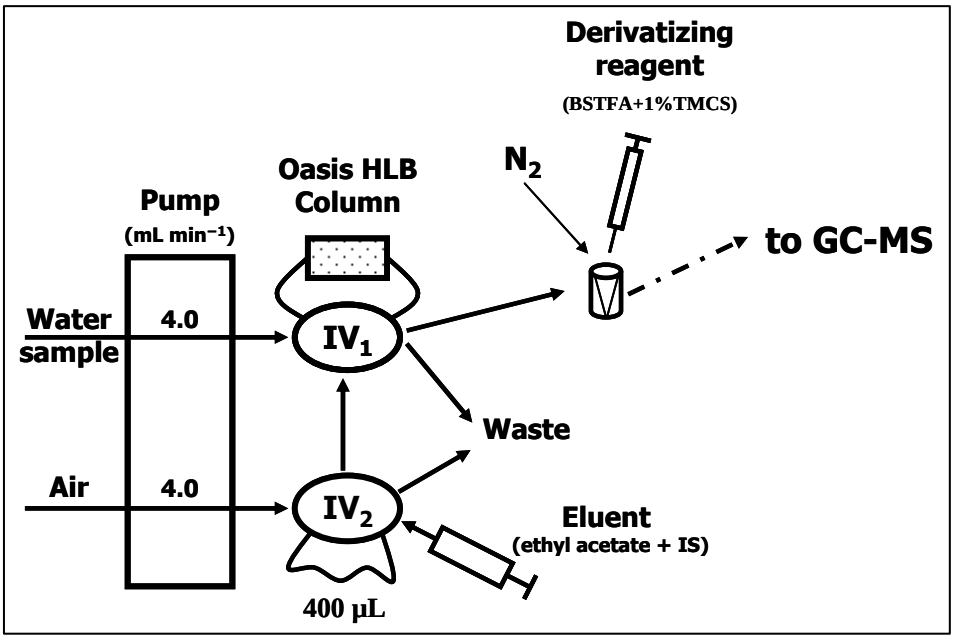

Fig. 1. Flow-injection manifold for the on-line preconcentration of pharmaceuticals and hormones, and their off-line determination by gas chromatography. IV, injection valve; W, waste; IS, internal standard (triphenylphosphate); BSTFA + 1% TMCS, N,O-bis-(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane.

3. Results and discussion

Pharmaceuticals and hormones are currently among the most frequently encountered new classes of environmental pollutants in surface, ground, river, spring, drinking and waste water [1,8,10]. Human and veterinary applications are the main sources of their presence in the environment. Hormones, whether natural or synthetic, are required careful monitoring owing to their endocrine disrupting effects [1]. Acid non-steroidal anti-inflammatory drugs are among the pharmaceuticals most often used to treat human and animal disease [5]. Anti-epileptics are used to treat seizure disorders, relief neuralgia and alleviate a wide variety of mental diseases [27]. Antiseptics and

lipid regulators are common active ingredients of soaps, detergents, perfumes, and skin, hair, and dental care products [28]. Propranolol, metoprolol, sotalol, timolol, oxprenolol, alprenolol, atenolol, pindolol and penbutolol are β-adrenoceptor blocking drugs used in the treatment of various cardiovascular disorders such as hypertension, angina pectoris and cardiac arrhythmia. In this work, we studied nine pharmaceuticals and three hormones representative of their major compound classes. Their most salient properties are shown in Table 1.

3.1. Optimization of the preconcentration–elution process

Solid-phase extraction is the most widely used extraction technique for extracting pharmaceuticals and hormones from water on the grounds of its excellent efficiency [1]. Our research group has developed several systems for the preconcentration and isolation of toxins in environmental and food samples [25,26]. Based on previous experience in this field, in this work we developed a continuous system (Fig. 1) for the preconcentration/isolation of pharmaceuticals and hormones from water samples. In each analytical run, a volume of 100 mL of an aqueous standard solution containing a 100 ng L^{-1} concentration of each analyte was passed through a sorbent column at a flow rate of 4 mL min^{-1}.

Various materials including Amberlites XAD-2 and XAD-4, RP-C_{18}, Oasis HLB, LiChrolut EN, Florisil, Silica gel and Isolute NH_2 were tested as preconcentration sorbents. Sorption tests were done by using a column packed with 70 mg of sorbent material. Fractions of 1 mL of sample were collected in glass vials placed before and after the sorbent column. Both fractions were extracted with 1 mL of ethyl acetate and the resulting extracts concentrated to a volume of 35 μL under a stream of ultrapure N_2. Then, 70 μL of BSTFA + 1% TMCS was added and the vials were heated at 70 °C for 20 min. Finally, 1 μL aliquots of the silylated derivatives thus obtained were injected into the chromatograph for analysis. The sorbent column was rinsed with 1 mL of ethyl acetate and 2 mL of water to remove adsorbed analytes after each sample was processed. The sorption efficiency of the sorbent materials was assessed by comparing the amount of each analyte recovered from the extracts (i.e. unsorbed analyte) with that originally present in the sample, which was taken to be 100 %. As can be seen from Table 2, the best results were obtained with Oasis HLB, with a sorption efficiency close to 100 %. LiChrolut EN and RP-C_{18} exhibited a similar sorption efficiency for several analytes, but much lower values for others. The efficiency of the other materials (Isolute

NH_2, Amberlites XAD-4 and XAD-2, Florisil and Silica gel) was very low or even zero. Oasis HLB was thus adopted for further testing as it proved the most efficient sorbent. Various organic solvents including ethyl acetate, acetonitrile, acetone, methanol, ethanol, 2-propanol and dichloromethane were assayed as solvents. Ethyl acetate was that resulting in the strongest chromatographic peaks by effect of its increased eluting efficiency —the other solvents were approximately 1.4 times less efficient. Therefore, ethyl acetate was selected as eluent for both pharmaceuticals and hormones.

The optimum amount of Oasis HLB sorbent to be used was determined by examining the results obtained with columns containing 20–100 mg of the sorbent. To this end, a series of calibration graphs were run for each analyte and column by passing 100 mL of aqueous standard solutions containing a 5–400 ng L^{-1} concentration of each analyte and then eluting the column with 400 μL of ethyl acetate. Analytical signals increased with increasing amount of sorbent up to 60 mg and then decreased above 65 mg of Oasis HLB owing to the need for a higher volume of eluent to ensure complete elution of the analytes (Fig. S1 in Supplementary Materials). This was confirmed by a second injection (400 μL) of eluent subjected to no preconcentration, which resulted in carry-over to an extent increasing with increase in the amount of sorbent above 65 mg. A working column packed with 60 mg of Oasis HLB was thus adopted for further testing. Retention of the analytes was found to be influenced by the sample pH, the effect of which was studied over a wide range of values (1.0–12.0) that were adjusted with dilute HCl of NaOH prior to preconcentration in the SPE system of Fig. 1. The best preconcentration results were obtained in the neutral region (pH 6.5–8.5). Some authors have studied the co-extraction of matrix components (e.g. humic and fulvic acids) in SPE sorbents and found it to be substantially reduced in neutral media relative to acid media [29]. A sample pH of 7 was thus selected. The ionic strength of the water samples, which was adjusted with potassium nitrate, had no effect on the signals up to 2 M.

The flow rate of sample through the column during the preconcentration step had no effect on the sorption efficiency over the studied range (0.5–5.0 mL min^{-1}). The influence of the elution process was studied by changing the air flow rate from 0.5 to 5.0 mL min^{-1}. All sorbed analytes were completely eluted with a volume of 400 μL throughout this range. A sample flow rate and air flow rate of 4 mL min^{-1} were thus chosen in order to boost sample throughput. An identical air flow rate was used to dry the sorbent column before elution. The effect of the volume of eluent (ethyl acetate) was

studied over the range 50–550 μL, using loops of variable length in the second injection valve (IV_2, Fig. 1). Obviously, as the eluent volume was increased, desorption was more efficient —but analytes were diluted as well (Fig. S2 in Supplementary Materials). Because of these opposing effects, the only way to accurately determine the most suitable eluent volume was by diluting the extracts to a constant volume with the same solvent (ethyl acetate). Therefore, the column eluent (between 50 and 550 μL) was always diluted to 600 μL with ethyl acetate. The desorption efficiency increased with increasing injected volume up to 375 μL, where the analytical signals for all pharmaceuticals and hormones levelled off. The same experiment was repeated with variable eluent volumes between 50 and 550 μL, without dilution to a constant final volume; however, the chromatographic signals increased with increasing volume up to 400 μL and then decreased through increased dilution of desorbed analytes at higher volumes. An injected volume of eluent of 400 μL was thus selected as optimal. The breakthrough volume of the sample was the last variable to be examined. This parameter is crucial in SPE methods because it is directly related to the enrichment factor, and hence to sensitivity. The influence of the breakthrough volume was examined by dissolving an amount of 10 ng of each pharmaceutical and hormone in variable volumes (5–400 mL) of purified water. A sorption efficiency of ca. 100 % was obtained with aqueous volumes up to 200 mL and the Oasis sorbent.

Finally, potential errors made during extraction–elution and derivatization of the analytes were minimized by using an internal standard (IS). To this end, triphenylphosphate or heptadecanoic acid was added to the eluent (ethyl acetate) in order to facilitate the use of relative chromatographic signals (analyte-to-IS peak ratio). Triphenylphosphate provided better results than heptadecanoic acid —the latter was partially retained on the sorbent column. We therefore chose to use triphenylphosphate as IS, and used a solution of this compound at a concentration of 500 μg L^{-1} in ethyl acetate as eluent.

3.2. Optimization of the derivatization reaction

Chemical derivatization is a widespread analytical practice for improving or facilitating the chromatographic separation of low-volatile compounds by gas chromatography. Silylation is the most frequent choice for derivatizing pharmaceutical and hormones [1,2,7,15,18,25]. This led us to assay various silylating reagents including N,O-bis-(trimethylsilyl)acetamide, N,O-bis-(trimethylsilyl)trifluoroacetamide and trimethyl chlorosilane, both individually and in mixtures. To this end, volumes of 50 μL of individual solutions of the reagents were added to 100 μL of a solution containing a 1 μg L^{-1} concentration of each analyte in ethyl acetate. The derivatization reaction was conducted in an air-tight conical glass insert of 0.5 mL that was placed in a water bath at 80 ºC for 30 min. The derivatized compounds thus obtained were determined by GC–MS. The best results in this respect were obtained with a mixture of BSTFA and TMCS. The influence of the proportion of TMCS (the catalyst) in the mixture was examined over the range 0.25–15%, the effect of the reaction time from 1 to 40 min, and that of temperature between room level and 90 ºC (Fig. S3 in Supplementary Materials). The best results in the determination of the analytes at a 1 μg L^{-1} concentration each were obtained by using a proportion of 1% of TMCS, a temperature above 65 ºC —which led us to adopt 70 ºC— and a reaction time of 20 min —after which the reaction was quantitative. We compared the performance of our selected solvent (ethyl acetate) with that of a widely used choice for this purpose, acetonitrile [15], and found both to perform comparably. We therefore chose to stick to ethyl acetate since, as shown in the previous Section, it was also an effective eluent for our solid-phase extraction system.

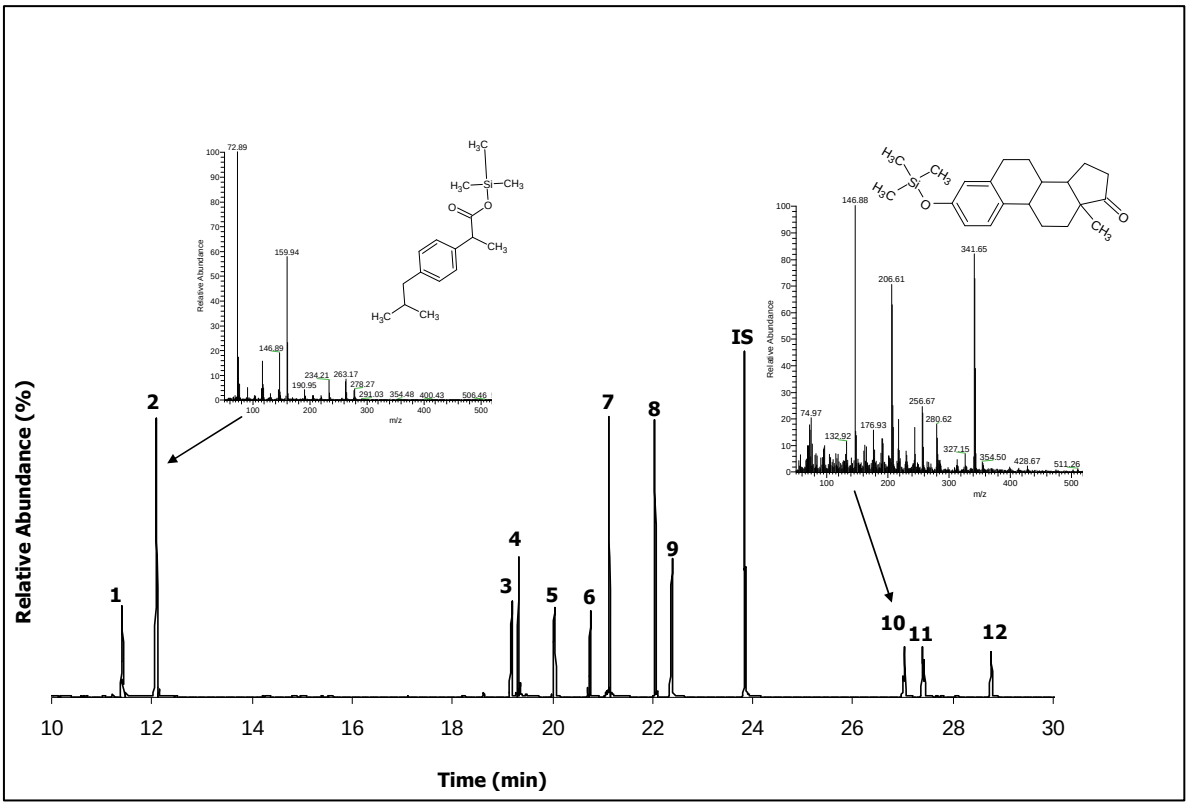

Fig. 2. Typical chromatogram (SIM mode) of pharmaceuticals and hormones as N-trimethylsilyl derivatives (100 ng L^{-1}). 1, clofibric acid; 2, ibuprofen; 3, metoprolol; 4, naproxen; 5, triclosan; 6, propranolol; 7, ketoprofen; 8, carbamazepine; 9, diclofenac; 10, estrone; 11, 17β-estradiol; 12, 17α-ethinylestradiol; IS, internal standard (triphenylphosphate).

Although the optimum amount of reagent to be used in a derivatization reaction has been the subject of much research [15], the way its volume affects detection limits has rarely been considered. In this work, we examined the effect of low volumes of solvent and derivatizing reagent (10–150 μL) in order to achieve detection limits relevant to environmental concentrations. The best results were obtained with a final volume of extract (eluted analytes in ethyl acetate) of 35 μL and one of derivatizing reagent (BSTFA + 1% TMCS) of 70 μL. Therefore, the extract provided by the solid-phase extraction system (400 μL) was concentrated to 35 μL under a stream of ultrapure nitrogen prior to derivatization of the analytes. By way of example, Fig. 2 shows the typical chromatogram of standards as N-trimethylsilyl derivatives (100 ng L^{-1}). As can be seen, the nine pharmaceuticals and three hormones studied were effectively separated with no difficulty in a single chromatographic run in about 30 min. Also, the chromatograms were very clean by effect of the mass spectrometer being used in the SIM mode.

3.3. Calibration and statistical study

The performance and reliability of the proposed off-line SPE-GC-MS system (Fig. 1) were assessed by determining the regression equation, linear range, analyte detectability and relative standard for the 12 analytes (pharmaceuticals and hormones). For this purpose, analytical curves were constructed by analysing 100 mL volumes of aqueous solutions containing variable concentration of the analytes from 0.03 to 400 ng L^{-1}. The regression coefficients thus obtained were greater than 0.993 in all cases (see Table 3). Limits of detection, LODs, (defined as the analyte concentration that provides a chromatographic peak equal to three times the regression standard deviation, Sy/x, divided by the slope of the calibration graph) ranged from 0.01 and 0.06 ng L^{-1} [30]. Similar LODs were obtained when using 12 individual standard solutions containing 0.20 ng L^{-1} of each compound through their mean values and the standard deviations. Within-day and between-day precision, expressed as relative standard deviation (RSD), was evaluated by analysing 11 individual samples spiked with a 5 ng L^{-1} concentration of each analyte on the same and three different days, respectively.

The robustness of the proposed method was checked by performing a recovery test on tap, well, river, pond, swimming pool, and waste water. For this purpose, each type of water was spiked with the analytes at three different concentrations (5, 50 and 100 ng L^{-1}) and subjected to the proposed procedure. Samples were all run in triplicate (n = 3) in order to calculate a standard deviation for each (Table 4). Recoveries ranged from 85 to 103%, which testifies to the applicability of the proposed method to any type of water, however complex its matrix may be (e.g. in waste water). The recoveries provided by existing methods based on SPE with Oasis HLB packed cartridges were much lower; for example, Gómez *et al.* obtained diclofenac, ibuprofen and carbamazepine recoveries of 79, 80 and 81%, respectively, from wastewater [16]. Also, recoveries from tap water with the same SPE sorbent (66% for triclosan, 74% for ibuprofen and 108% for clofibric acid) differed markedly from those provided by the proposed method [10].

Table 3

Analytical figures of merit of the determination of pharmaceuticals and hormones using the proposed method.

Analyte	LR* (ng L^{-1})	r*	LOD* (ng L^{-1})	RSD* within-day (%)	RSD* between-day (%)
Clofibric Acid	0.10-400	0.9989	0.03	4.4	4.9
Diclofenac	0.06-400	0.9985	0.02	3.7	4.3
Ketoprofen	0.03-400	0.9959	0.01	5.2	6.4
Ibuprofen	0.03-400	0.9932	0.01	4.7	5.7
Naproxen	0.06-400	0.9958	0.02	3.5	4.2
Carbamazepine	0.03-400	0.9978	0.01	4.3	5.3
Metoprolol	0.10-400	0.9934	0.03	5.1	6.3
Propranolol	0.06-400	0.9953	0.03	5.9	6.6
Triclosan	0.10-400	0.9965	0.03	3.7	4.2
Estrone	0.15-400	0.9997	0.05	6.1	6.9
17β-Estradiol	0.15-400	0.9998	0.05	4.9	6.1
17α-Ethinylestradiol	0.20-400	0.9978	0.06	5.7	6.8

*r, correlation coefficient (r^2); LR, linear range; LOD, limit of detection; RSD, relative standard deviation (n = 11)

3.4. Application to real samples

The proposed trace enrichment method was successfully applied to the determination of nine pharmaceuticals and three hormones in six types of water samples, namely: tap, swimming pool, well, pond, river and waste. Initially, a volume of 100 mL of each sample was analysed in triplicate, using the above-described procedure. If negative results were obtained, the sample volume was increased to 200 mL (the breakthrough volume for Oasis HLB sorbent) in order to confirm the absence of the analytes at detectable levels. If the concentration of some analyte lay outsider the linear range (Table 3), then the sample concerned was diluted with purified water to bring it within. The results of these tests are shown in Table 5. As can be seen, none of the four tap water samples studied contained any of the analytes. All others, however, contained some pharmaceutical and/or hormone. The analyte most frequently found in the samples was ibuprofen, followed by metaprolol, diclofenac and ketoprofen, and the hormones 17β-estradiol y estrone. On the other hand, the hormone 17α-ethinylestradiol was found in none of the samples. The two wastewater samples (untreated water from

cities with a population of 40 000–60 000) were those containing the greatest numbers of analytes and at the highest concentrations, especially diclofenac, ibuprofen, ketoprofen and propranolol. These results are consistent with those obtained by other authors, who found high concentrations of some pharmaceuticals (diclofenac, ibuprofen, ketoprofen, triclosan, carbamazepine, naproxen) in wastewater by effect some of these substances being among the most frequently prescribed drugs [31,32]. Although only 15–20% of the human therapeutic dose of a pharmaceutical (*e.g.* ibuprofen, metoprolol, propanolol) is excreted as the parent compound in most cases, some drugs have been found at high concentrations in untreated wastewater [14,31]. This statement can also be used to explain the presence of these compounds in environmental water, albeit at much lower concentrations than in wastewater.By way of example, Fig. 3 shows the SIM mode chromatograms for a sample of pond and river water processed with the proposed SPE-GC-MS method. As can be seen, the chromatograms were very clean (only three peaks from the matrix components were detected).

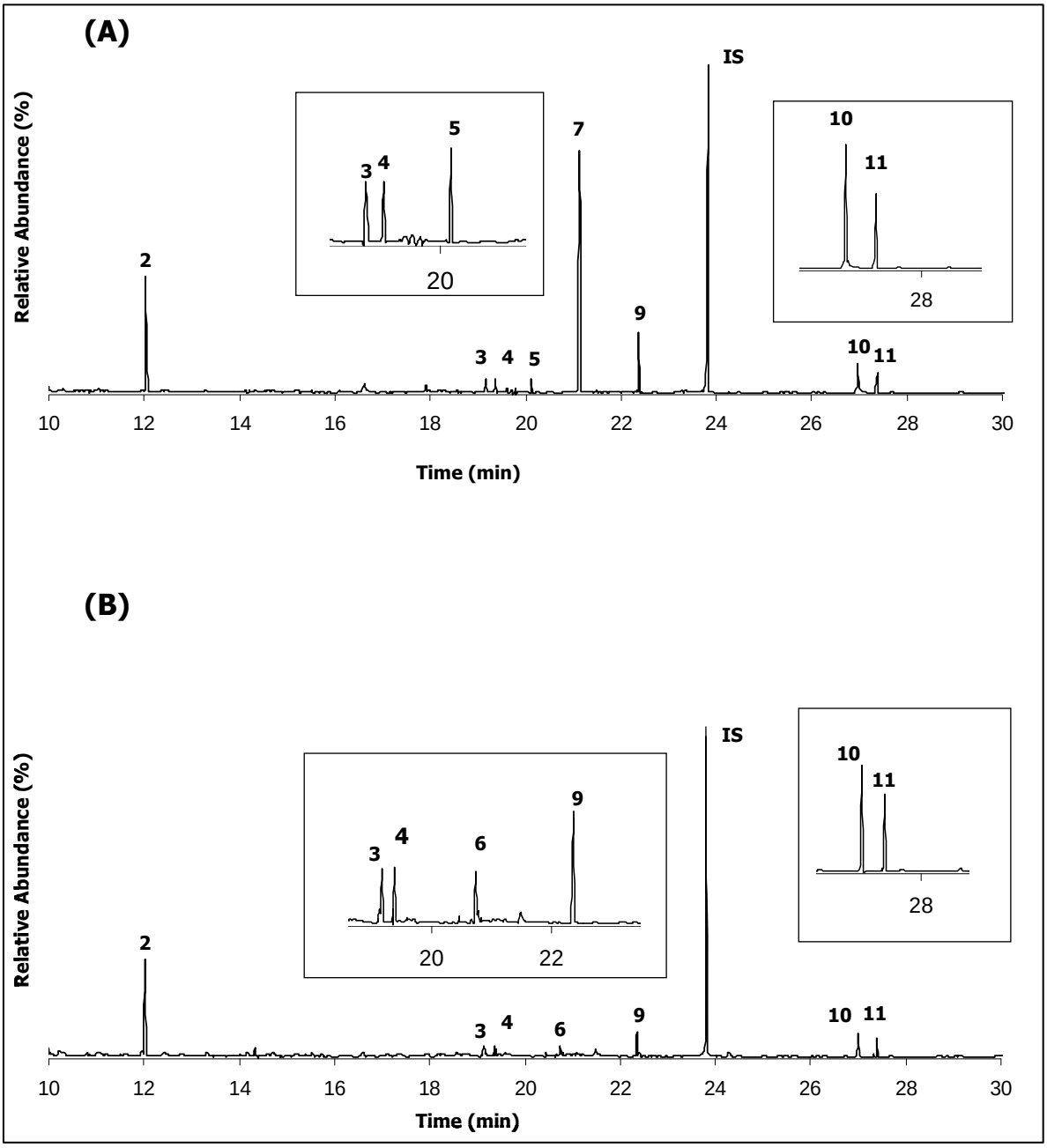

Fig. 3. SIM chromatogram for the pharmaceuticals and hormones as obtained by analyzing 100 mL of pond (A) and river water (B). For peak identification, see Fig. 2.

4. Conclusions

The proposed method combines two current trends in Analytical Chemistry, namely: automation and miniaturization. Automation is introduced in the sample preparation step, using a continuous system to preconcentrate the analytes and remove interferences from the sample matrix; miniaturization is accomplished by using very low volumes of organic solvents and derivatizing reagents. In fact, the procedure affords elution of the analytes retained on the column with only 400 μL of eluent and 70 μL of BSTFA+1% TMCS for derivatization —which takes only 20 min. Existing procedures [2,3,11,17,21] are rather complex and use greater volumes of organic reagents and solvents. The SPE sorbents studied proved effective for the simultaneous extraction of acid, neutral and basic analytes, even at neutral pH. Under these conditions, analytical performance was best with Oasis HLB sorbent. The sensitivity of the proposed method (LODs of 0.01–0.06 ng L^{-1}) surpasses that of alternative GC–MS methods for quantifying the target analytes [2,3,5,14] and affords the determination of trace amounts of pharmaceuticals and hormones in various types of water. Also, the sensitivity of the proposed method surpasses that of other, LC–MS methods for the determination of hormones (estrone, 17β-estradiol and 17α-ethinylestradiol) and pharmaceuticals (clofibric acid, naproxen, ketoprofen, diclofenac, ibuprofen, metoprolol, propranolol and carbamazepine) in natural and treated waters [8,19,22,31]. Unlike such methods, ours requires the prior derivatization of the analytes to improve chromatographic performance. As expected, the wastewater samples were those containing the highest analyte levels (16–2800 ng L^{-1}). This is consistent with the results of previous environmental studies based on which most waste water treatment procedures are ineffective with a view to removing these environmental contaminants [6,7,13,14,16,31-33].

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References

[1] G. G. Ying in Handbook of Water Analysis, (L. M. L. Nollet, Ed.), CRC Press–Taylor & Francis, Boca Raton, FL, 2007, p. 693 (Chapter 25).

[2] Z. Yu, S. Peldszus, P. M. Huck, J. Chromatogr. A 1148 (2007) 65.

[3] M. A. Soliman, J. A. Pedersen, I. H. Suffet, J. Chromatogr. A 1029 (2004) 223.

[4] L. Vera-Candioti, M. D. Gil García, M. Martínez, H. C. Goicoechea, J. Chromatogr. A 1211 (2008) 22.

[5] L. Araujo, J. Wild, N. Villa, N. Camargo, D. Cubillan, A. Prieto, Talanta 75 (2008) 111.

[6] J. Xu, L. Wu, W. Chen, A. C. Chang, J. Chromatogr. A 1202 (2008) 189.

[7] J. Xu, L. Wu, W. Chen, P. Jiang, A. C. S. Chang, J. Health Sci. 55 (2009) 306.

[8] L. Sun, W. Yong, X. Chu, J. Lin, J. Chromatogr. A 1216 (2009) 5416.

[9] I. Rodríguez, J.B. Quintana, J. Carpinteiro, A.M. Carro, R.A. Lorenzo, R. Cela, J. Chromatogr. A 985 (2003) 265.

[10] S. Weigel, R. Kallenborn, H. Hühnerfuss, J. Chromatogr. A 1023 (2004) 183.

[11] J. B. Quintana, J. Carpinteiro, I. Rodríguez, R. A. Lorenzo, A. M. Carro, R. Cela, J. Chromatogr. A 1024 (2004) 177.

[12] M. J. Gómez, M. Petrovic, A. R. Fernández-Alba, D. Barceló, J. Chromatogr. A 1114 (2006) 224.

[13] R. Gibson, E. Becerril-Bravo, V. Silva-Castro, B. Jiménez, J. Chromatogr. A 1169 (2007) 31.

[14] M. J. Gómez, M. J. Martínez Bueno, S. Lacorte, A. R. Fernández-Alba, A. Agüera, Chemosphere 66 (2007) 993.

[15] A. Lajeunesse, C. Gagnon, Intern. J. Environ. Anal. Chem. 87 (2007) 565.

[16] M. J. Gómez, A. Agüera, M. Mezcua, J. Hurtado, F. Mocholí, A. R. Fernández-Alba, Talanta 73 (2007) 314.

[17] J. L. Zhao, G. G. Ying, L. Wang, J. F. Yang, X. B. Yang, L. H. Yang, X. Li, Sci. Total Environ. 407 (2009) 962.

[18] J. C. Durán-Alvarez, E. Becerril-Bravo, V. S. Castro, B. Jiménez, R. Gibson, Talanta 78 (2009) 1159.

[19] B. Kasprzyk-Hordern, R. M. Dinsdale, A. J. Guwy, Anal. Bioanal. Chem. 391 (2008) 1293.

[20] M. Kuster, M. J. Lopez de Alda, M. D. Hernando, M. Petrovic, J. Martín–Alonso, D. Barceló, J. Hydrol. 358 (2008) 112.

[21] S. S. Verenitch, C. J. Lowe, A. Mazumder, J. Chromatogr. A 1116 (2006) 193.

[22] M. J. Gómez, O. Malato, I. Ferrer, A. Agüera, A. R. Fernández-Alba, J. Environ. Monit. 9 (2007) 718.

[23] B. J. Vanderford, R. A. Pearson, D. J. Rexing, S. A. Synder, Anal. Chem. 75 (2003) 6265.

[24] S. S. Morales-Muñoz, J. L. Luque-García, M. J. Ramos, A. Fernández-Alba, M. D. Luque de Castro, Anal. Chim. Acta 552 (2005) 50.

[25] E. Ballesteros, M.J. Parrado, J. Chromatogr. A 1029 (2004) 267.

[26] B. Jurado-Sánchez, E. Ballesteros, M. Gallego, J. Chromatogr. A 1216 (2009) 1200.

[27] Y. Zhang, S.U. Geiben, C. Gal, Chemosphere 73 (2008) 1151.

[28] S. L. Rice, S. Mitra, Anal. Chim. Acta 589 (2007) 125.

[29] V. Pichon, C. Cau Dit Coumes, L. Chen. S. Guenu, M. C. Hennion, J. Chromatogr. A 737 (1996) 25.

[30] N. J. Miller, J. C. Miller; Statictics and Chemometrics for Analytical Chemistry, fifth ed., Prentice Hall, NJ, 2005.

[31] M. D. Hernando, E. Heath, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 385 (2006) 985-991.

[32] H. Kataoka, S. Narimatsu, H. L. Lord, J. Pawliszyn, Anal. Chem. 71 (1999) 4237-4244.

Table 2

Percent sorption of the pharmaceuticals and hormones on various sorbent materials.

Compounds	Oasis-HLB	LiChrolut EN	XAD-4	XAD-2	RP-C18	Florisil	Silica Gel	Isolute NH_2
Clofibric acid	97	85	7	6	10	0	0	19
Diclofenac	100	75	10	0	63	0	0	78
Ketoprofen	100	100	18	0	81	4	5	73
Ibuprofen	94	90	9	0	97	0	6	88
Naproxen	100	96	20	7	85	0	15	76
Carbamazepine	95	100	0	0	43	0	0	0
Metoprolol	100	78	0	0	50	0	88	0
Propranolol	100	70	0	0	64	0	54	0
Triclosan	98	97	8	5	78	0	0	20
Estrone	100	72	9	0	72	0	0	18
17β-Estradiol	100	80	8	0	83	4	0	12
17α-Ethynilestradiol	100	90	7	0	80	2	0	21

Table 4
Average recoveries (± SD, n=3) of pharmaceuticals and hormones spiked to water samples.

Compounds	Water sample									
	Tap		Well		River		Swimming pool		Waste	
	5 ng L^{-1}	100 ng L^{-1}	5 ng L^{-1}	100 ng L^{-1}	5 ng L^{-1}	100 ng L^{-1}	5 ng L^{-1}	100 ng L^{-1}	5 ng L^{-1}	100 ng L^{-1}
Clofibric Acid	89 ± 4	95 ± 4	85 ± 4	103 ± 5	101 ± 5	98 ± 5	89 ± 4	102 ± 4	86 ± 4	101 ± 5
Diclofenac	102 ± 4	99 ± 5	103 ± 5	97 ± 4	99 ± 4	101 ± 5	95 ± 4	85 ± 3	101 ± 4	99 ± 5
Ketoprofen	103 ± 5	95 ± 4	102 ± 4	96 ± 5	88 ± 3	102 ± 5	99 ± 5	103 ± 6	100 ± 5	89 ± 4
Ibuprofen	92 ± 4	101 ± 5	92 ± 4	101 ± 6	102 ± 5	98 ± 5	100 ± 6	92 ± 4	101 ± 4	98 ± 5
Naproxen	100 ± 4	95 ± 4	101 ± 5	89 ± 3	99 ± 4	101 ± 5	101 ± 5	98 ± 4	99 ± 4	102 ± 6
Carbamazepine	95 ± 5	100 ± 4	95± 4	101 ± 5	98 ± 5	88 ± 4	101 ± 4	102 ± 5	89 ± 4	101 ± 6
Metoprolol	87 ± 4	103 ± 6	95 ± 5	102 ± 6	95 ± 5	101 ± 4	85 ± 4	97 ± 5	101 ± 5	98 ± 5
Propranolol	96 ± 5	101 ± 6	102 ± 6	85 ± 5	103 ± 5	93 ± 5	97 ± 6	102 ± 6	100 ± 5	99 ± 5
Triclosan	101 ± 5	98 ± 4	99 ± 4	101 ± 5	87 ± 3	102 ± 5	101 ± 4	99 ± 4	98 ± 4	103 ± 5
Estrone	102 ± 6	90 ± 5	96 ± 5	102 ± 6	103 ± 6	98 ± 5	90 ± 4	101 ± 5	96 ± 5	103 ± 6
17β-Estradiol	91 ± 4	102 ± 5	88 ± 4	90 ± 4	102 ± 6	92 ± 4	102 ± 5	89 ± 4	103 ± 5	99 ± 5
17α-Ethynylestradiol	102 ± 6	85 ± 4	101 ± 6	93 ± 5	100 ± 5	101 ± 6	89 ± 5	101 ± 6	89 ± 4	102 ± 5

Table 5
Results (ng L^{-1}) obtained in the analysis of water samples by the proposed enrichment method.

Sample	Clofibric acid	Diclofenac	Ketoprofen	Ibuprofen	Naproxen	Carbamazepine	Metoprolol	Propranolol	Ticlosan	Estrone	17β-Estradiol	17α-Ethynyl-estradiol
Tap 1	–	–	–	–	–	–	–	–	–	–	–	–
Tap 2	–	–	–	–	–	–	–	–	–	–	–	–
Tap 3	–	–	–	–	–	–	–	–	–	–	–	–
Tap 4	–	–	–	–	–	–	–	–	–	–	–	–
Swimming pool 1	11.7 ± 0.6	–	–	12.1 ± 0.6	–	–	–	–	–	45.1 ± 3.0	–	–
Swimming pool 2	–	15.3 ± 0.8	–	24.5 ± 1.4	–	–	19.4 ± 1.1	–	–	9.8 ± 0.6	3.8 ± 0.2	–
Well 1	–	32.4 ± 1.5	–	24.6 ± 1.2	13.8 ± 0.6	–	–	–	–	17.5 ± 1.2	24.5 ± 1.4	–
Well 2	–	25.6 ± 1.1	91.4 ± 6.4	16.3 ± 0.9	–	–	–	–	–	–	11.2 ± 0.6	–
Pond 1	–	37.5 ± 1.8	1.5 ± 0.1	96.6 ± 5.0	–	–	12.7 ± 0.7	–	–	26.4 ± 1.7	14.5 ± 0.8	–
Pond 2	–	49.1 ± 2.2	89.2 ± 6.0	44.0 ± 2.2	10.3 ± 0.5	–	13.4 ± 0.8	–	23.4 ± 1.0	54.5 ± 3.5	34.0 ± 2.0	–
River 1	–	40.2 ± 1.7	9.0 ± 0.6	36.6 ± 2.0	14.5 ± 0.7	–	14.6 ± 0.8	–	–	48.6 ± 3.0	28.5 ± 1.6	–
River 2	–	18.9 ± 0.8	–	39.0 ± 2.2	9.2 ± 0.5	–	15.2 ± 0.9	14.5 ± 1.0	–	44.2 ± 2.8	31.0 ± 1.8	–
River 3	–	73.5 ± 3.3	88.3 ± 5.7	84.2 ± 4.5	–	–	7.5 ± 0.4	–	–	–	33.1 ± 1.8	–
Waste 1	15.9 ± 0.9	2780 ± 125	410 ± 25	1895 ± 100	34.9 ± 1.5	66.3 ± 3.4	30.5 ± 1.8	335 ± 20	145 ± 7.0	90.0 ± 6.0	–	–
Waste 2	–	240 ± 10	1440 ± 80	1555 ± 80	56.5 ± 2.9	47.0 ± 2.3	25.0 ± 1.5	465 ± 30	220 ± 10	89.3 ± 5.7	32.8 ± 1.9	–

Continuous solid-phase extraction and gas chromatography–mass spectrometry determination of pharmaceuticals and hormones in water samples: Supplemental Information

Journal of Chromatography A, 1217 (2010) 2956–2963

Optimization of variables affecting elution and derivatization

Figures illustrating the optimization of the amount of Oasis HLB sorbent (Figure S1), eluent volume (Figure S2) and derivatization temperature (Figure S3). The results for 4 representative analytes (ibuprofen, diclofenac, metoprolol and estrone) are shown –all others behaved similarly.

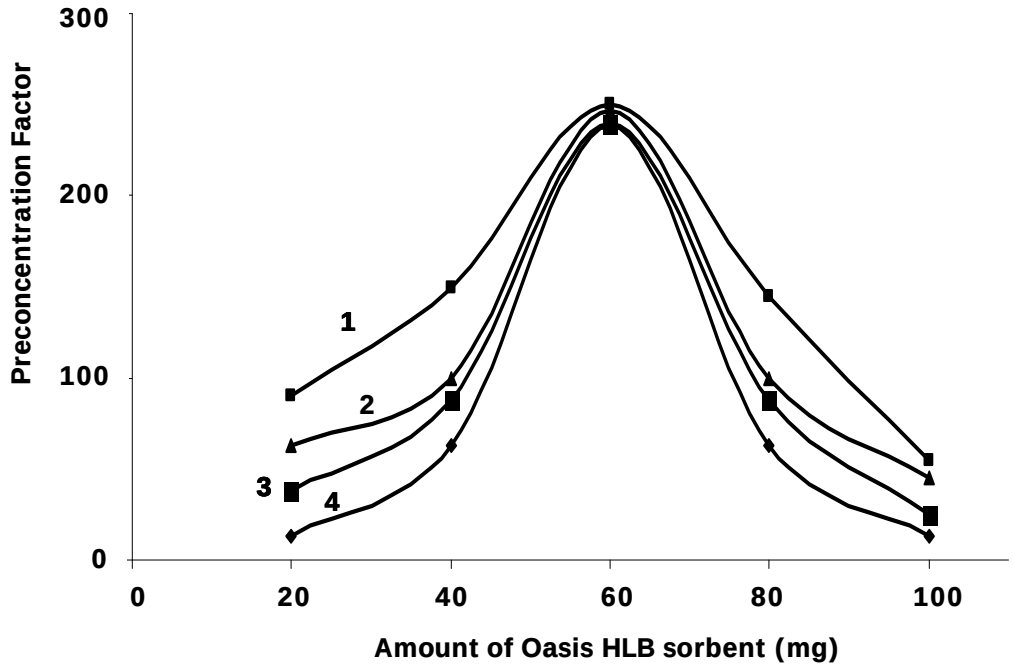

Figure S1. Influence of the amount of Oasis HLB sorbent used on retention of pharmaceuticals and hormones. 1. Ibuprofen; 2. diclofenac; 3. metoprolol; 4. estrone.

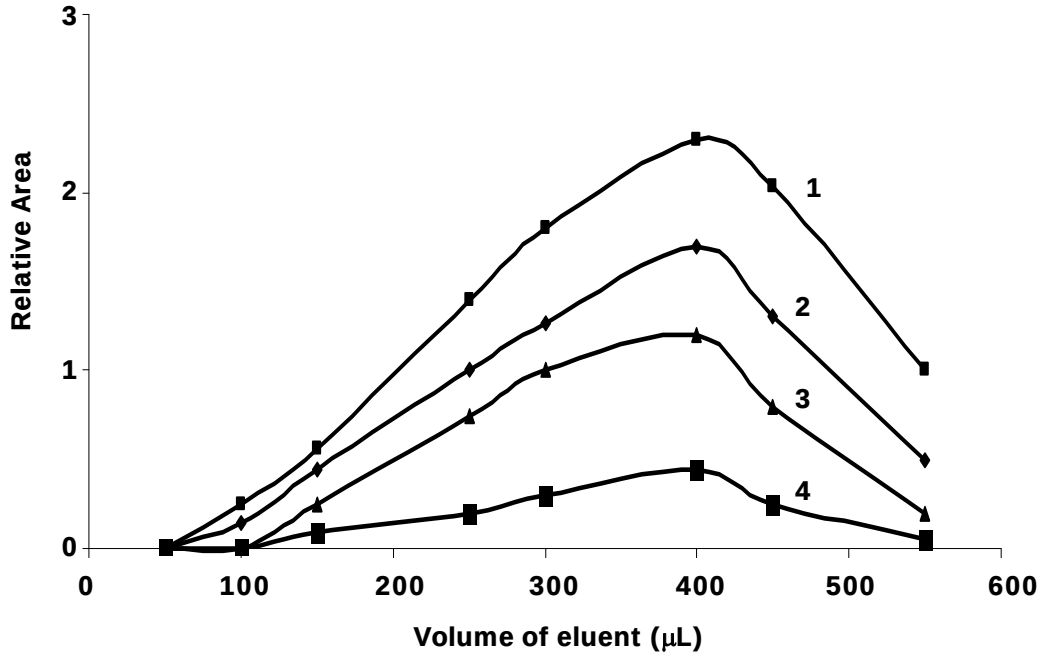

Figure S2. Influence of the volume of ethyl acetate on elution of sorbed analytes. For identification of analytes, se Fig. 1.

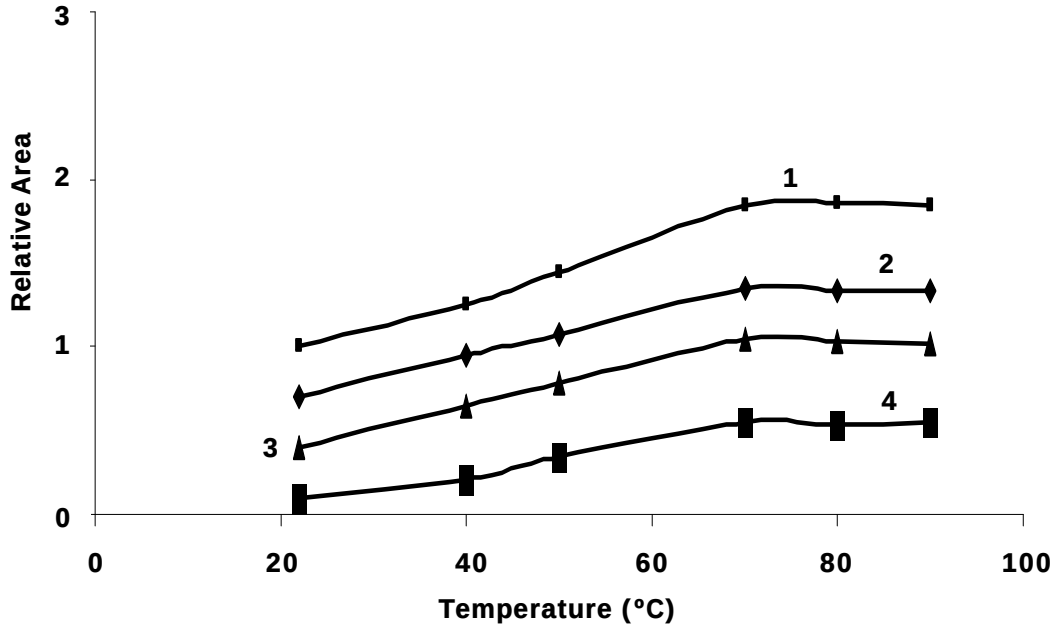

Figure S3. Influence of temperature on the sylilation reaction of pharmaceuticals and hormones. For identification of analytes, see Fig. 1.

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Combined microwave-assisted extraction and continuous solid-phase extraction prior to gas chromatography-mass spectrometry determination of pharmaceuticals, personal care products and hormones in soils, sediments and sludge

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ABSTRACT

This paper reports a sensitive analytical method based on microwave-assisted extraction and continuous solid-phase extraction (SPE), followed by gas chromatography-mass spectrometry (GC–MS), for the simultaneous determination of residues of 18 pharmaceuticals (analgesics, antibacterials, anti-epileptics, β-blockers, lipid regulators and non-steroidal anti-inflammatories), one personal care product and 3 hormones in soils, sediments and sludge. The analytes are extracted with 3:2 methanol/water under the action of microwave energy and the resulting extract is passed through a SPE column to clean up the sample matrix and preconcentrate the analytes. Then, the analytes, trapped on Oasis-HLB sorbent, are eluted with ethyl acetate, silylated and determined by GC–MS. The proposed method provides a linear response over the concentration range 2.5–20 000 ng/kg with correlation coefficients higher than 0.994 in al cases. Also, it features low limits of detection (0.8–5.1 ng/kg), good precision (within- and between-day relative standard deviation less than 7%) and recoveries ranging from 91 to 101%. The method was successfully applied to agricultural soils, river and pond sediments, and sewage sludge. All samples contained some target analyte and sludge contained most —some at considerably high concentrations.

Keywords: Pharmaceuticals · Personal care products · Hormones · Soil · Microwave-assisted extraction · Continuous solid-phase extraction · Gas chromatography-mass spectrometry

1. Introduction

Tons of pharmaceutical substances are used in human medicine for diagnosis, treatment or prevention every year. For example, animal and fish farming rely heavily on drugs mostly administered as food additives to prevent disease, promote growth or fight parasites. Human and veterinary drugs are continuously being released into the environment mainly as a result of manufacturing processes, disposal of unused or expired products and excreta. Residual pharmaceuticals detected in environmental compartments include antibiotics, analgesics, anti-inflammatories, lipid regulators, β-blockers, antiepileptics, contraceptives, steroids and related hormones (Henderson and Coats, 2009). Because of their physical and chemical properties, many of these substances, or their bioactive metabolites, end up in soils and sediments, where they can accumulate and induce adverse effects on terrestrial or aquatic organisms. Although the environmental concentrations of pharmaceutical residues are generally at trace levels (ng/L to low μg/L) they can suffice to induce toxic effects in some cases (Ying, 2007). Such effects include increasingly widespread bacterial resistance resulting from extensive use of antibiotics in animal and fish farming, and the growing practice of adding manure and sewage sludge to agricultural fields, which is of particular concern. Steroid estrogens, which include natural and synthetic ones, are most potent endocrine disrupting compounds and their estrogenic effects have been observed in laboratory studies at very low concentrations. They enter the environment predominantly through sewage discharge after they have been excreted (Peng et al., 2006). Soils, sewage sludge and sediments have scarcely been studied in this respect relative to aquatic media (Díaz-Cruz et al., 2003). There is thus a perceived need for analytical methods affording the determination of pharmaceuticals in these environmental samples, which pose special problems owing to their high complexity and usually very low contents of the target compounds.

There are various available methodologies for determining pharmaceuticals in solid environmental samples. In recent years, target species have usually been extracted from sediment, soil and sludge by liquid partitioning under sonication (Chenxi et al., 2008; Peng et al., 2006; Ternes et al., 2002; Xu et al., 2008), Soxhlet extraction (Peng et al., 2006), pressurized liquid extraction (Jelic et al., 2009; Ternes et al., 2005; Vazquez-Roig et al., 2010) or microwave-assisted extraction (MAE) (Antonic and Heath, 2007; Dobor et al., 2010; Hibberd et al., 2009; Liu et al., 2004; Matejicek et al., 2007; Morales et al., 2005; Varga et al., 2010). The last technique is greener than the others; thus, it uses substantially smaller amounts of solvents, which reduces sample consumption and waste production and shortens extraction times, thereby reducing overall energy input and costs (Madej, 2009; Tobiszewski et al., 2009). The principle behind MAE is the direct effect of microwaves on molecules via ionic conduction and dipole rotation (Sanchez-Prado et al., 2010). One novel use of microwave-assisted extraction is in combination with micellar media as

extractants, which has been used to extract various compound types from environmental samples (Cueva-Mestanza et al., 2008a, 2008b). Solid-phase extraction (SPE) has been introduced as a clean-up step in preparation procedures to minimize interferences with matrix components and preconcentrate the target analytes (Jelic et al., 2009; Vazquez-Roig et al., 2010). The combination of microwave-assisted extraction and SPE for clean-up has become the most widely used choice to prepare solid samples for instrumental analysis (Antonic and Heath , 2007; Cueva-Mestanza et al., 2008a, 2008b; Dobor et al., 2010; Hibberd et al., 2009; Jelic et al., 2009; Liu et al., 2004; Matejicek et al., 2007; Morales et al., 2005; Varga et al., 2010; Vazquez-Roig et al., 2010). Recently, MAE and SPE were on-line coupled to determine antibiotics in soil samples (Chen et al., 2009). Most of the previous methods use a hydrophilic–lipophilic balanced reversed-phase (Oasis-HLB) sorbent (Cueva Mestanza et al., 2008a, 2008b; Dobor et al., 2010; Hibberd et al., 2009; Jelic et al., 2009; Matejicek et al., 2007; Morales et al., 2005; Varga et al., 2010; Vazquez-Roig et al., 2010; Yang JF et al., 2010), which has proved suitable for multi-residue determinations using an appropriate eluent (solvent) at neutral pH (Azzouz et al., 2010). Other sorbents including silica gel (Liu et al., 2004), Strata X (Antonic and Heath, 2007; Vazquez-Roig et al., 2010) and alumina (Chen et al., 2009) have been used for the SPE of pharmaceuticals from soils, sediments and sludge.

Interest in the determination of pharmaceutical residues by liquid chromatography-mass spectrometry (Jelic et al., 2009) or liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Chen et al., 2009; Chenxi et al., 2008; Matejicek et al., 2007; Pailler et al., 2009; Ternes et al., 2005; Vazquez-Roig et al., 2010) and liquid chromatography with UV detection (Cueva-Mestanza et al., 2008a, 2008b) has grown substantially over the last decade. By virtue of their low cost, low limits of detection, superior resolution and widespread availability of the required equipment, gas chromatography–mass spectrometry (GC–MS) (Antonic and Heath, 2007; Azzouz et al., 2010, 2011; Dobor et al., 2010; Liu et al., 2004; Peng et al., 2006; Varga et al., 2010; Xu et al., 2008; Zhao et al., 2009) and gas chromatography–tandem mass spectrometry (Hibberd et al., 2009; Morales et al., 2005; Ternes et al., 2002) remain the preferred tools for identifying and quantifying pharmaceuticals in environmental matrices, however. The low volatility of some pharmaceuticals and the presence of various polar groups in others requires their conversion into volatile derivatives prior to their GC determination.

In previous work, we developed an analytical method for the determination of pharmaceuticals in natural waters or milk samples by continuous SPE and GC–MS (Azzouz et al., 2010, 2011). In this work, we focused on the development of a reliable method based on the combination of MAE with continuous SPE to remove interferences from the sample matrix and extract/preconcentrate 18 pharmaceuticals, one personal care product and 3 hormones from soils, sediments and sludge. The influence of operational variables on performance in the MAE and

continuous SPE steps was systematically evaluated. Analytes were silylated and selectively determined by GC–MS, and the overall performance of the proposed method was assessed in terms of linearity, sensitivity, precision and matrix effects.

2. Experimental

2.1. Standards and reagents

The compounds studied (*viz.* acetylsalicylic acid, carbamazepine, chloramphenicol, clofibric acid, diclofenac, florfenicol, flunixin, ibuprofen, ketoprofen, mefenamic acid, metoprolol, naproxen, niflumic acid, paracetamol, phenylbutazone, propranolol, pyrimethamine, triclosan and thiamphenicol, 17α-ethinylestradiol, 17β-estradiol and estrone), the internal standard (triphenylphosphate) and the derivatizing reagents [*N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS)] were all purchased from Sigma-Aldrich (Madrid, Spain) in the highest available purity. Chromatographic grade solvents (acetone, acetonitrile, ethyl acetate, methanol and *n*-hexane) were supplied by Merck (Madrid, Spain). Oasis-HLB (particle size 50–65 μm) was obtained from Waters (Madrid, Spain). Ultrapure water was obtained by using a Milli-Q purification system from Millipore (Bedford, MA, USA).

Individual stock solutions containing an analyte concentration of 1 g/L (Table 1) were prepared in methanol and stored in glass-stopped bottles at 4 ºC in the dark until use. For elution in the continuous SPE system, ethyl acetate containing 500 μg/L internal standard (IS) was used.

2.2. Equipment

GC–MS analyses were carried out on a Focus GC instrument interfaced to a DSQ II mass spectrometer controlled via a computer running X Calibur Software (Thermo Electron SA, Madrid, Spain). The target analytes were separated by using a DB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with 5% phenylmethylpolysiloxane (Supelco, Madrid, Spain), and helium (99.999%) at a constant flow rate of 1 mL/min was used as carrier gas. The column temperature programme was as follows: 70 °C for 1 min, ramp to 150 °C at 14 °C/min, and ramp to 290 °C at 6 °C/min. The injection port and transfer line temperatures were maintained at 270 and 280 °C, respectively. The ion source temperature for the electron impact ionization mode (70 eV) was 200 ºC. The mass spectrometer was operated in the selected ion monitoring mode (SIM); the *m/z* values used for each pharmaceutical and hormone are listed in Table 1. The MS instrument was used in the full scan mode (60–500 amu) for identification. The time for solvent

delay was set to 8 min. Sample injection (1 μL) was performed in the split mode (1:20), and analyte quantitation was based on peak areas.

The continuous solid-phase extraction system used consisted of a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinyl chloride) tubes, two Rheodyne (Cotati, CA, USA) 5041 injection valves and a PTFE laboratory-made sorption column (5 cm × 3 mm i.d.) containing 60 mg of Oasis-HLB sorbent. The column was conditioned with 1 mL of ethyl acetate and 1 mL of purified water between samples. Under these conditions, it remained serviceable for at least 400 analyses with no appreciable change in its properties. Millex-LG filter units (hydrophilic, PTFE, pore size 0.20 μm, diameter 25 mm, filtration area 3.9 cm^2) were obtained from Millipore Ibérica, S.A.

2.3. Samples

Sewage sludge samples from two wastewater treatment plants (sample 1 from a city of 50000 inhabitants and sample 2 from one of 500000) were collected in pre-cleaned 500 mL amber glass bottles. River and pond sediment samples were collected at a sediment depth of 5 cm and a distance of approximately 0.5 m from the bank. For soil samples, two agricultural fields of Andalusia (Spain) (sample 1 from Jaén, soil irrigated with well water; sample 2 from Sevilla, soil irrigated with pond water) were used to select a 1 m^2 sampling area from which three subsamples were collected at random. All samples were placed in stoppered lyophilization vials and freeze-dried on a Telstar LyoQuest apparatus (Madrid, Spain) at –55 º C under vacuum (0.05 bar) for 72 h. Then, the samples were passed through a 2 mm ∅ sieve, and homogenized. Finally the freeze-dried samples were stored in stoppered vials at –20 ºC until analysis.

The soil and sediment samples that were found to contain no pharmaceuticals or hormone in a preliminary test were used as blanks, and also to optimize and validate the proposed method. These samples were also freeze-dried and homogenized as described above.

2.4. Microwave-assisted extraction

An amount of 1 g of each freeze-dried sample was weighed into a 50 mL glass bottle and supplied with 10 mL of extractant (3:2 methanol/water). Then, the bottle was tightly sealed and placed in an household microwave oven, in front of the magnetron, for extraction at 500 W for 6 min. Finally, the resulting supernatant being passed through a 0.20 μm Millex-LG filter and evaporated under a gentle N_2 stream to final volume of 200 μL.

Table 1
Therapeutic class, pk_a, $logK_{o/w}$, retention time and mass values used to determination of the pharmaceuticals, personal care products and hormones.

Therapeutic Class	Compounds	pKa	$logK_{o/w}$	Retention Time (min)	*m/z*[b]		
					$M^{+\cdot}$	$[M-15]^+$	Additional ions
Analgesics	Acetylsalicylic acid	3.5	1.19	10.5	252	237	120, **195**, 210
	Paracetamol	9.38	0.46	11.4	295	**280**	116, 206
Non-steroidal anti-inflammatories	Diclofenac	4.14	4.51	22.2	367	352	**214**, 242
	Flunixin	5.82	4.90	19.8	368	**353**	251, 263
	Ibuprofen	4.91	3.97	12.0	278	263	**160**, 234
	Ketoprofen	4.45	3.12	21.0	326	311	73, **282**
	Mefenamic acid	3.73	5.19	20.8	313	298	208, **223**
	Naproxen	4.20	3.20	19.2	302	287	**185**, 243
	Niflumic acid	4.76	na[a]	19.0	354	339	**236**, 263
	Phenylbutazone	4.40	3.16	23.9	308[c]	–[c]	77,**183**, 252,
Lipid regulator	Clofibric acid	3.46	2.60	11.3	286	271	128, **143**
Personal care product	Triclosan	7.80	4.80	19.9	362	**347**	200, 310
Anti-epileptic	Carbamazepine	7.00	2.47	22.0	308	293	**193**, 250
β-Blockers	Metoprolol	9.70	1.88	19.1	339	324	72, **223**
	Propranolol	9.50	2.60	20.6	331	316	72, **215**
Antibacterials	Chloramphenicol	9.61	na[a]	24.6	466	451	208, **225**, 242
	Florfenicol	9.03	na[a]	26.1	429	414	**257**, 360
	Pyrimethamine	7.34	na[a]	21.2	392	**377**	171, 281
	Thiamphenicol	9.76	na[a]	27.9	499	484	242, 257, **330**
Hormones	Estrone	10.20	3.69	26.8	**342**	327	218, 257
	17β-estradiol	10.27	4.13	27.3	**416**	401	285, 326
	17α-ethinylestradiol	10.24	4.25	28.6	440	**425**	232, 300

[a] na, not available.
[b] The peaks used for quantification are boldfaced; m/z for IS (triphenylphosphate): 77, 170, 325, **326.**
[c] The phenylbutazone is determined as non-derivatized.

2.5. Continuous solid-phase extraction/clean-up and derivatisation

Figure 1 depicts the continuous SPE system used to determine the target pharmaceuticals, personal care products and hormones in soil, sediment and sludge samples. MAE extracts (200 μL) were reconstituted with 10 mL of purified water at pH 7 adjusted with dilute HCl or NaOH. Then, each reconstituted sample or a standard aqueous solution containing a 2.5–20000 ng/kg concentration of each analyte at pH 7 was aspirated through the sorbent column (located in the loop of the injection valve, IV_1) at 4 mL/min. All analytes were sorbed as a result and the sample matrix was sent to waste; simultaneously, the loop of the second valve (IV_2) was filled with eluent (ethyl acetate containing 500 μg/L triphenylphosphate as IS) by means of a syringe. Any residual water remaining inside the column and connectors was flushed with an air stream (4 mL/min) for 2 min. Next, IV_2 was switched to pass the loop contents (400 μL) through the column, in the opposite direction of the sample, in order to elute the pharmaceuticals, personal care products and hormones. The organic extract was collected in a conical glass insert of 0.5 mL and concentrated to a final volume of 35 μL under a stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using an internal standard. Next, a volume of 70 μL of BSTFA + 1% TMCS was added and the vials (tightly closed) were heated at 70 ºC for 20 min. Finally, 1 μL aliquots of the silylated derivatives (except phenylbutazone, which is determined as non-derivatized) were determined by GC–MS in the SIM mode.

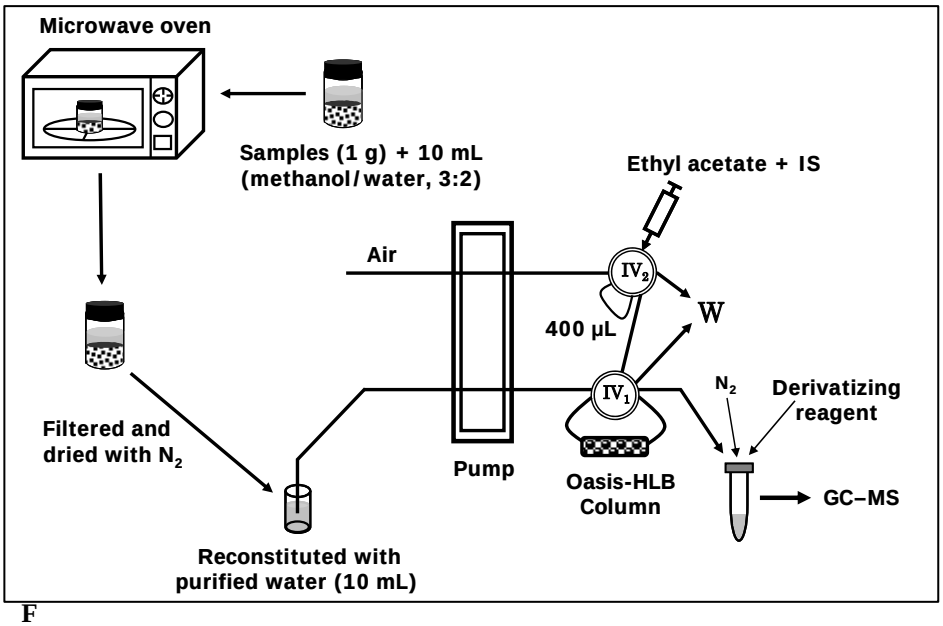

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Fig. 1. Experimental set-up for the microwave-assisted extraction and continuous solid-phase extraction of pharmaceuticals, personal care products and hormones from soils, sediments and sludge. IV: injection valve, W: waste.

3. Results and discussion

3.1. Optimization of microwave-assisted extraction

The main variables influencing MAE performance included (*a*) the nature of the solvent and its volume, (*b*) the microwave power and (*c*) the irradiation time (Madej, 2009); their effect was examined as follows: an amount of 1 g of freeze-dried soil blank sample was spiked with a 500 ng/kg concentration of each pharmaceutical, personal care product and hormone, and mixed with the solvent. After extraction in a household microwave oven, the supernatant was filtered, evaporated under a gentle N_2 stream to a final volume of 200 µL and reconstituted with 10 mL of purified water at pH 7. Then, the reconstituted sample was introduced into the continuous SPE system (Fig. 1), the analytes being retained on the sorbent column and subsequently eluted with ethyl acetate for derivatization. Finally, the silylated derivatives were determined by GC–MS as described under Experimental.

Ensuring efficient microwave-assisted extraction of the analytes from the samples required the use of an appropriate solvent, which was selected among the six tested (acetone, acetonitrile, ethyl acetate, *n*-hexane, methanol and water). To this end, a volume of 10 mL of each was used for MAE at 500 W for 5 min. The best results were

obtained with methanol and water, but differed between analytes owing to their differences in polarity. This led us to test methanol/water mixtures of which one in a 3:2 ratio was found to provide optimal results —similarly to previous reports for MAE of pharmaceuticals from sediments (Matejicek et al., 2007). The influence of the solvent volume was examined over the range 1–25 mL. No provision for analyte dilution in the mixture was made since the supernatant was always evaporated to 200 μL and then reconstituted with 10 mL of purified water for continuous SPE. A solvent volume of 8 mL or greater resulted in maximal extraction of the analytes, thus 10 mL was adopted for further testing.

Once the optimum solvent for MAE was selected, the effects of the irradiation time and microwave power were examined. The choice of these parameters depends on the nature of the solvent and its relative permittivity, as well as on the nature of the sample matrix and the properties of the target analytes. We used 6 different irradiation times (1, 2, 4, 6, 8 and 10 min) in combination with a variable microwave power from 50 to 750 W. Short irradiation times were found to require an increased power to maximize extraction of the analytes from soil. However, high microwave powers in combination with long times diminished the signals for some analytes (viz. clofibric acid, metoprolol and propranolol), possibly because they decomposed under these extreme conditions (Camel, 2000; Madej, 2009). This led us to select a microwave power of 500 W and an irradiation time of 6 min, which maximized extraction of all analytes while preventing their decomposition. To prove the efficiency of MAE, a Soxhlet extraction procedure similar to that used by Antonic and Heath (2000) has been used. The extract of Soxhlet was evaporated to 200 μL under a gentle N_2 stream and reconstituted with 10 mL of purified water for insertion into the SPE system described in the Continuous solid-phase extraction/clean-up and derivatization Section. Three representative samples (sediment, soil and sludge) were extracted by Soxhlet extraction and by the MAE procedure optimized above. The efficiency extraction for the three samples analysed ranged from 95 to 100% for MAE and 90 to 95% for Soxhlet extraction.

3.2. Optimization of continuous solid-phase extraction and derivatization

In previous work (Azzouz et al., 2010), we developed a flow system for the simultaneous preconcentration of 12 pharmaceuticals and hormones in water samples with Oasis-HLB material as sorbent, ethyl acetate as eluent and BSTFA + 1% TMCS as derivatizing reagent. This system was initially adopted to determine the 22 analytes studied (18 pharmaceuticals, one personal care product and 3 hormones) in soil,

sediment and sludge samples; however, the complexity of the sample matrices and the large number of analytes they might contain led us to examine the effects of chemical and flow-related variables influencing the preconcentration–elution process and derivatization (silylation) reaction. For this purpose, an amount of 1 g of a blank soil sample was spiked with a 500 ng/kg concentration of each pharmaceutical and hormone and subjected to MAE as described under Experimental. The resulting supernatant was filtered, evaporated under a gentle N_2 stream to final volume of 200 μL and reconstituted with 10 mL of purified water for insertion into the SPE system. We initially examined the retention of the 22 analytes on columns containing an amount of 60 mg of Amberlite XAD-2 or XAD-4, Oasis-HLB, LiChrolut EN or RP-C_{18} and their elution with acetone, acetonitrile, ethyl acetate or methanol. The best preconcentration results for all analytes were obtained with Oasis-HLB and their optimal elution was accomplished with ethyl acetate. We then examined the influence of pH on SPE of the analytes and found the neutral region (pH 6.5–7.5) to provide the best extraction yields. Also, co-extraction of matrix components and analytes was significantly reduced at pH 7 relative to pH 2 or 4 (Gómez et al., 2006). pH 7 was thus selected, and adjusted with dilute HCl or NaOH, for further testing. We also optimized other variables affecting continuous SPE performance such as the flow rate of sample and air, and the volume of eluent. A flow rate of 4 mL/min for both air and the sample, and an eluent volume of 400 μL, were chosen. The influence of the sample breakthrough volume was studied on an SPE column packed with 60 mg of Oasis-HLB sorbent and a sorption efficiency of ca. 100 % was obtained with aqueous volumes up to 200 mL.

As stated above, the best solvent for MAE was a 3:2 methanol/water mixture. However, the supernatant —containing the analytes— obtained after MAE contained a high volume of methanol (~60%), which required examining its effect on retention of the analytes onto a sorbent column packed with 60 mg of Oasis-HLB, using a system similar to that of Fig. 1. For this purpose various standard solutions containing a 500 ng/L concentration of each analyte in various mixtures of methanol and water containing 0 to 50% (v/v) alcohol were aspirated through the sorbent column. As can be seen from Fig. 2 for 11 representative analytes, their behaviour varied. Thus, methanol had no adverse effect on retention of the two analgesics (acetylsalicylic acid and paracetamol) or triclosan up to 5%, but affected retention of propranolol, carbamazepine, diclofenac and chloramphenicol above 10 %, all other analytes allowing a higher content of organic solvent (20–40%) to be used. The higher was the proportion of methanol in the aqueous sample, the lower was retention of the pharmaceuticals, personal care products and

hormones on the sorbent column. This can be ascribed to the mechanism of analyte sorption, which involved partitioning of the analytes from a polar phase (water) into a non-polar phase such as a polymeric sorbent (Oasis-HLB) via hydrogen bonding and л–л interactions between the analytes and the underlying sorbent surface. When the aqueous sample contained a high proportion of methanol, the alcohol broke bonds and effectively dissolved the pharmaceuticals and hormones, thereby dramatically decreasing their sorption on the column. In order to avoid this effect of methanol on retention of the analytes, the MAE supernatant was evaporated to 200 μL under a gentle N_2 stream and reconstituted with 10 mL of purified water (~1.2% of methanol in the final solution) for insertion into the SPE system.

Finally, we examined the influence of the different variables affecting derivatization of the 21 analytes with BSTFA and TMCS (except phenylbutazone, which is determined as non-derivatized). The silylating solution selected for this purpose was BSTFA containing 1 % TMCS, which provided the highest derivatization yields. The optimum temperature and time for the derivatization reaction were found to be 70 ºC and 20 min, respectively, and the optimum volume of silylating reagent was 70 μL, which ensured quantitative derivatization of a volume of eluate of 35 μL from the SPE system —originally 400 μL, but concentrated under a stream of ultrapure N_2 as described under Experimental.

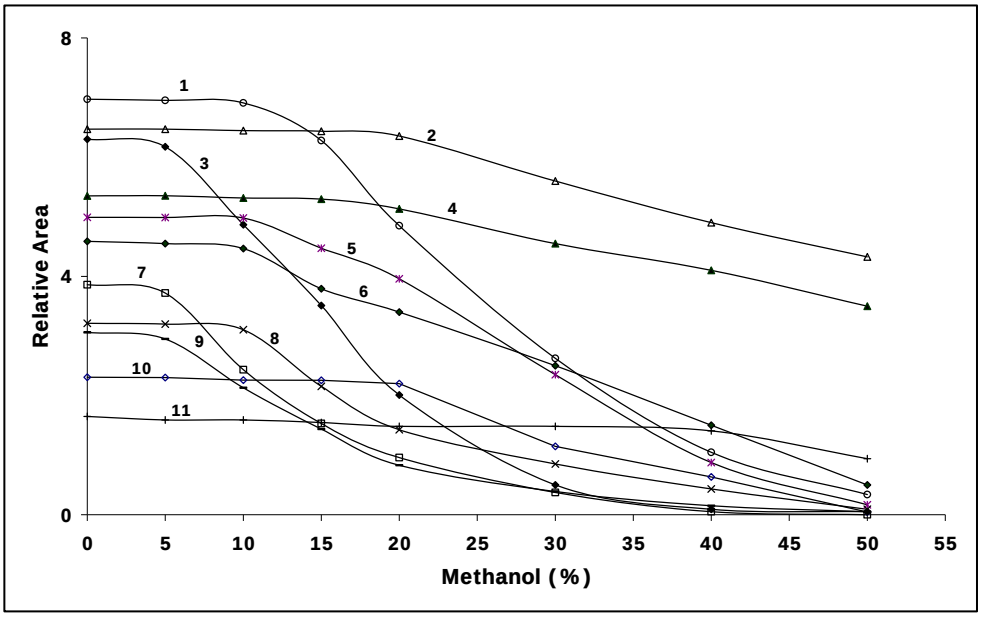

Fig. 2. Influence of the methanol content in the eluent (v/v) on retention of the analytes on the sorbent column. 1: chloramphenicol; 2: mefenamic acid; 3: paracetamol; 4: ibuprofen; 5: carbamazepine; 6: diclofenac; 7: triclosan; 8: propranolol; 9: acetylsalicylic acid; 10: clofibric acid; 11: 17β-estradiol.

3.3. Analytical performance

The freeze-dried soil blank used for off-line MAE–SPE–GC–MS (Fig. 1) was spiked with the 18 pharmaceuticals, one personal care product and three hormones as follows: volumes of 1 mL of standard solutions containing the 22 analytes in amounts from 2.5 pg to 20 ng in methanol were spiked to 1-g amounts of soil blank and the methanol was evaporated for about ~4 min. The samples were then allowed to stand for at least 1 h to facilitate sorption similarly as in nature (Hibberd et al., 2009). Finally, each soil sample was extracted with 10 mL of 3:2 methanol/water and processed as described under Experimental. This addition procedure caused analytes in the soil to be largely present in the deposited state, even though some analyte–matrix interactions were possible; as a result, the recoveries of the analytes in the spiked soil ranged from 94 to 99% relative to aqueous standards directly introduced into the continuous SPE system. Based on these results, the soil blank was used to run calibration graphs for the 22 analytes. The internal standard was triphenylphosphate, which was spiked at a 500 μg/L concentration to the eluent. Calibration was performed by plotting analyte-to-IS peak area ratios against analyte concentrations. The figures of merit of the proposed method are shown in Table 2. As can be seen, the correlation coefficients were all higher than 0.994 (12 points per calibration). The limits of detection (LODs, calculated as three times the standard deviation, SD, of background noise divided by the slope of each calibration graph) ranged from 0.8 to 5.1 ng/kg. The precision of proposed method, as relative standard deviation (RSD), was calculated by measuring 11 uncontaminated soil samples spiked with 25, 200 and 2000 ng/kg concentrations of each pharmaceutical, personal care product and hormone, following the addition procedure described at the top of this section. A comparative study of within-day and between-day precision was conducted —the latter over seven days—, at the previous three concentration levels. The RSD obtained were found to be satisfactory with RSD lower than 6 % (within-day precision) or 7% (between-day precision). This precision is better than those of existing methods such as one for the determination of eight pharmaceuticals in sediment samples using microwave assisted micellar extraction and LC–UV (RSD, 6–11%) (Cueva-Mestanza et al., 2008a) or for the determination of thirteen hormones using MAE, SPE and LC–MS/MS (RSD, 4.9–9.6%) (Matejicek et al., 2007). Vazquez-Roig et al. (2010) have recently developed a method for the determination of pharmaceuticals in soil and sediment using pressurized liquid extraction and LC–MS/MS, which in some cases

improves the precision obtained with our method, with within-day and between-day precision from 0.7 to 7.9% and from 1.6 to 14.5%, respectively.

In order to validate the proposed method, a recovery study was conducted to analyse agricultural soil, river sediment and sewage sludge samples fortified with 25, 200 and 2000 ng/kg concentrations of each analyte in triplicate (n = 3), following the procedure described at the top of this section. Since all samples contained some analyte, recoveries were calculated by subtracting the previously quantified endogenous compounds from their total contents. All compounds were accurately identified and their average recoveries acceptable (91–101%) in all samples (Table 2). Therefore, matrix interferences were completely suppressed during microwave-assisted extraction and continuous solid-phase extraction.

3.4. Analyses of soil, sediment and sludge samples

Prior to analysis, soil, sediment and sludge samples subjected to no freeze-drying were characterized for pH, moisture and total organic carbon (Table 3). pH was determined by using the AOAC Official Method 994.16 (AOAC, 2000). To this end, an amount of 5 g of solid sample was mixed with doubly distilled water and stirred for measurement of the pH in the supernatant. Moisture was determined by weighing, drying to a constant weight at 105 ºC and weighing of the dry sample (Dane and Topp, 2002). Finally, total organic carbon was determined by wet oxidation with sulphuric potassium dichromate and titration of excess dichromate with $Fe(NH_4)_2(SO_4)_2$ (Hesse, 1972).

Table 2
Analytical figures of merit of the proposed MAE–SPE–GC–MS method for determination of pharmaceuticals, personal care products and hormones in soils, sediments and sludge.

Compounds	LOD (ng/kg)	Linear range (ng/kg)	r[a]	RSD (%) (n=11)[b]		Recovery (%)[c]		
				Within-day	Between-day	Agricultural soil	River sediment	Sewage sludge
Acetylsalicylic acid	3.0	10–20 000	0.995	4.2	4.8	101 ± 5	99 ± 5	97 ± 5
Paracetamol	1.0	3.1–20 000	0.996	4.8	5.4	94 ± 4	95 ± 5	93 ± 4
Diclofenac	2.0	6.5–20 000	0.998	5.2	5.6	93 ± 5	101 ± 6	98 ± 6
Flunixin	1.1	3.4–20 000	0.995	4.3	5.8	95 ± 6	95 ± 5	96 ± 5
Ibuprofen	1.1	3.4–20 000	0.999	4.5	5.8	95 ± 5	99 ± 5	96 ± 5
Ketoprofen	1.0	3.1–20 000	0.999	5.2	6.0	100 ± 6	91 ± 5	97 ± 6
Mefenamic acid	0.9	3.0–20 000	0.999	5.0	5.3	97 ± 5	94 ± 5	99 ± 5
Naproxen	2.1	7.0–20 000	0.997	5.5	6.4	101 ± 6	92 ± 5	94 ± 5
Niflumic acid	1.3	4.1–20 000	0.996	4.8	6.3	95 ± 5	98 ± 5	93 ± 4
Phenylbutazone	4.5	15–20 000	0.994	5.1	6.5	98 ± 6	96 ± 5	100 ± 6
Clofibric acid	3.1	10–20 000	0.998	4.9	6.3	94 ± 5	100 ± 5	97 ± 5
Triclosan	3.0	10–20 000	0.999	5.5	5.9	92 ± 5	98 ± 6	96 ± 6
Carbamazepine	1.2	4.0–20 000	0.999	4.2	6.0	101 ± 4	95 ± 4	91 ± 4
Metoprolol	2.9	9.5–20 000	0.999	5.1	6.4	92 ± 5	101 ± 6	99 ± 5
Propranolol	3.0	10–20 000	0.998	4.8	5.2	98 ± 5	99 ± 5	95 ± 5
Chloramphenicol	0.8	2.5–20 000	0.995	5.2	6.0	96 ± 5	97 ± 5	93 ± 5
Florfenicol	1.1	3.4–20 000	0.999	5.3	6.4	101 ± 6	100 ± 6	94 ± 5
Pyrimethamine	4.7	15–20 000	0.996	4.9	6.2	100 ± 5	95 ± 5	98 ± 5
Thiamphenicol	1.0	3.1–20 0000	0.994	6.0	6.9	99 ± 7	96 ± 6	100 ± 6
Estrone	4.7	15–20 000	0.995	4.9	5.4	92 ± 4	96 ± 5	94 ± 5
17β-estradiol	5.0	16–20 000	0.997	5.1	5.7	94 ± 5	97 ± 5	96 ± 5
17α-ethinylestradiol	5.1	17–20 000	0.996	5.3	6.2	96 ± 5	98 ± 6	97 ± 5

[a] r, correlation coefficient.
[b] Relative standard deviation (n = 11) for a soil sample spiked with 25 ng/kg of each pharmaceutical.
[c] Percent recovery (± SD, n = 3) for un 1 g of samples spiked with 25 ng/kg of each pharmaceutical

The proposed method was used to determine the 18 pharmaceuticals, one personal care product and 3 hormones in sediment, soil and sludge samples from various locations. To this end, freeze-dried samples of the three types were analysed in triplicate, following the analytical procedure described under Experimental. Table 3 shows the concentrations of the target analytes detected in the contaminated soil, sediment and sludge samples. As can be seen, the agricultural soil irrigated with well water (soil 1) and that irrigated with pond water (soil 2) contained exactly the same analytes —by exception, the latter additionally contained metoprolol—; however, the concentrations of some analytes differed markedly between the two [particularly those of diclofenac, ibuprofen, ketoprofen, niflumic acid, carbamazepine and the three hormones (estrone, 17β-estradiol and 17α-ethinylestradiol)]. The concentrations of these analytes (9–460 ng/kg) were lower than those previously found by other authors in similar samples (viz. 0.55–9.08 ng/g (Xu et al., 2008), 1.43–6.57 ng/g (Vazquez-Roig et al., 2010) or 0.52–1.99 ng/g (Vazquez-Roig et al., 2011). By way of example, Fig. 3A shows the chromatograms for agricultural soil 2.

Table 3 shows the results for two sediment samples collected from two different rivers in Andalusia and another two obtained from two ponds in the same region. All four samples contained flunixin, ibuprofen, ketoprofen, naproxen, triclosan, carbamazepine and pyrimethamine at concentrations from 15 to 190 ng/kg. Also, most of the sediment samples contained niflumic acid, chloramphenicol, 17β-estradiol and 17α-ethinylestradiol (Fig. 3B). On the other hand, diclofenac was only present, at very low concentrations (8.5–9.5 ng/kg), in the river sediment samples. The levels of the non-steroidal anti-inflammatories diclofenac and naproxen determined with the proposed method were lower than those reported by Varga et al. (2010) in natural sediments (2–38 ng/g). The level encountered by proposed method for metoprolol, propranolol, carbamazepine, ibuprofen, clofibric acid and diclofenac (9.0–320 ng/kg) were lower than those found for these farmaceuticals in sediment samples from L'Albufera (Valencia, Spain; 0.62–35.83 ng/g) (Vazquez-Roig et al., 2011). The presence of the three hormones (estrone, 17β-estradiol and 17α-ethinylestradiol) in these types of samples has frequently been studied. Pond and river sediments were found to contain 17β-estradiol and 17α-ethinylestradiol at levels from 44 to 210 ng/kg, but no estrone. These hormone concentrations are lower than those previously determined by GC–MS/MS (0.9–1.5 ng/g) (Ternes et al., 2002). Peng et al. (2006) have not found any of these hormones in sediments samples.

Table 3
Pharmaceutical, personal care product and hormone residues found in soil, sediment and sludge samples (±SD, ng/kg, n=3), and physical-chemical characterization of each sample.

Parameters	Agricultural soil 1	Agricultural soil 2	Pond sediment 1	Pond Sediment 2	River sediment 1	River sediment 2	Sewage sludge 1	Sewage sludge 2
Water content (%)	0.13	0.15	2.2	2.4	1.5	1.7	6.5	6.6
pH	6.7	8.2	7.6	7.5	7.8	8.0	6.8	6.7
C org (% dry wt)	3.3	1.6	2.1	1.9	1.7	2.6	12.8	12.5
Compounds								
Acetylsalicylic acid	nd[a]	nd	nd	nd	nd	nd	nd	590 ± 25
Paracetamol	nd	nd	nd	nd	nd	nd	80 ± 4	nd
Diclofenac	9.0 ± 0.5	90 ± 4	8.5 ± 0.4	9.5 ± 0.5	nd	nd	95 ± 5	770 ± 40
Flunixin	180 ± 10	140 ± 10	190 ± 10	100 ± 5	55 ± 3	45 ± 3	290 ± 20	200 ± 10
Ibuprofen	98 ± 5	190 ± 10	71 ± 4	30 ± 2	56 ± 3	21 ± 1	120 ± 7	90 ± 5
Ketoprofen	460 ± 30	200 ± 10	85 ± 5	40 ± 2	21 ± 1	60 ± 3	75 ± 4	560 ± 30
Mefenamic acid	nd	nd	nd	nd	nd	nd	nd	nd
Naproxen	220 ± 10	150 ± 10	82 ± 5	25 ± 1	45 ± 3	15 ± 1	280 ± 20	850 ± 50
Niflumic acid	430 ± 20	240 ± 10	360 ± 20	160 ± 10	nd	67 ± 3	nd	370 ± 20
Phenylbutazone	nd	nd	nd	nd	nd	nd	nd	nd
Clofibric acid	nd	nd	nd	nd	nd	nd	nd	nd
Triclosan	270 ± 20	310 ± 20	170 ± 10	56 ± 3	49 ± 3	140 ± 10	260 ± 20	3100 ± 200
Carbamazepine	40 ± 2	260 ± 20	32 ± 2	24 ± 1	35 ± 2	77 ± 4	52 ± 2	190 ± 10
Metoprolol	nd	320 ± 20	nd	nd	nd	nd	180 ± 10	340 ± 20
Propranolol	nd	nd	nd	nd	nd	nd	150 ± 10	160 ± 10
Chloramphenicol	nd	nd	15 ± 1	20 ± 1	30 ± 2	nd	nd	310 ± 20
Florfenicol	nd	nd	nd	nd	nd	nd	30 ± 2	nd
Pyrimethamine	190 ± 10	120 ± 7	17 ± 1	34 ± 2	55 ± 3	35 ± 2	130 ± 6	2300 ± 100
Thiamphenicol	nd	nd	nd	nd	nd	nd	nd	nd
Estrone	16 ± 0.8	51 ± 3	nd	nd	nd	nd	nd	nd
17β-estradiol	190 ± 10	90 ± 6	100 ± 6	nd	90 ± 6	nd	nd	nd
17α-ethinylestradiol	220 ± 10	350 ± 20	96 ± 6	44 ± 3	210 ± 10	nd	250 ± 10	450 ± 30

[a] nd: not detected.

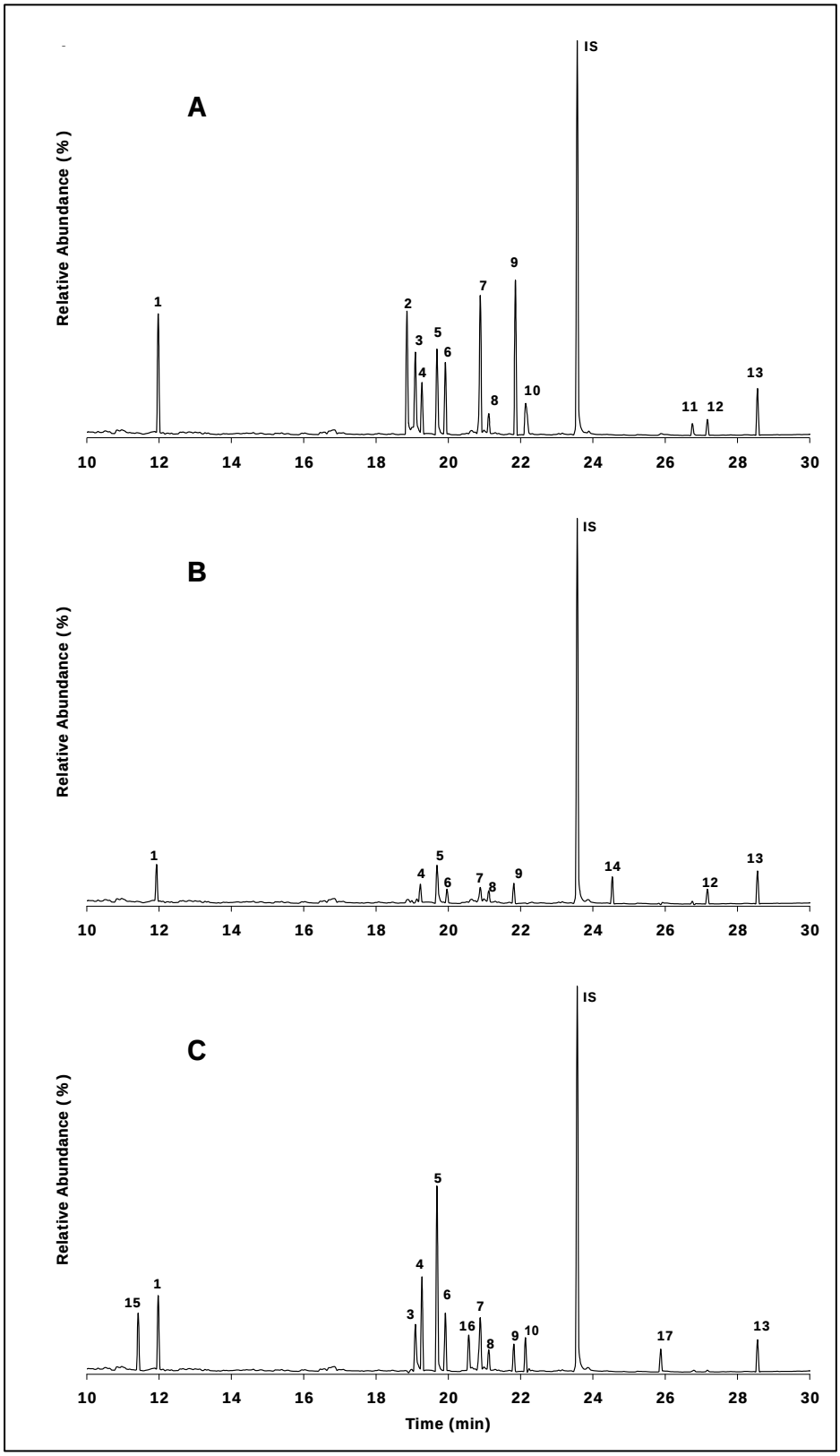

Fig. 3. GC–MS chromatograms in the SIM mode obtained in the analysis of agricultural soil 2 (A), river sediment 1 (B), and sewage sludge 1 (C). 1: ibuprofen; 2: niflumic acid; 3: metoprolol; 4. naproxen; 5: flunixin; 6: triclosan; 7: ketoprofen; 8: pyrimethamine; 9: carbamazepine; 10: diclofenac; 11: estrone; 12: 17β-estradiol; 13: 17α-ethinylestradiol; 14: chloramphenicol; 15: paracetamol; 16: propranolol; 17: florfenicol; IS: internal standard.

We also analysed sewage sludge from two wastewater treatment plants serving a population of 50000 (sewage sludge 1) and 500000 (sewage sludge 2), respectively. As can be seen from Table 3, the analyte concentrations in these samples were much higher than those found in the other sample types (soil and sediments) by effect of the former coming from the processing of wastewater containing large residual amounts of the pharmaceuticals (Hernando et al., 2006). Also, they contained some analytes not present in the other samples (viz. paracetamol, acetylsalicylic acid, propranolol and florfenicol). Some pharmaceuticals such as acetylsalicylic acid, diclofenac, ketoprofen, naproxen, triclosan, metoprolol, pyrimethamine and the hormone 17α-ethinylestradiol were detected at high concentrations. Other authors also found high levels of pharmaceuticals in these types of samples [e.g. Jelic et al. (2009 detected up to 34 pharmaceuticals including many of ours at concentrations from 1.2 to 74.9 ng/g, and Dobor et al. (2010) reported concentrations of 10–140 ng/g for ibuprofen, naproxen, ketoprofen and diclofenac in sewage sludge].

4. Conclusions

In this work, we developed and validated of a new method for the simultaneous determination of 22 analytes (pharmaceuticals, personal care products and hormones) in soils, sediments and sludge. The analytes were isolated from solid samples by using MAE followed by continuous SPE clean-up for analysis by GC–MS. This ensures efficient recovery of the analytes (91–101%) and effective, expeditious removal of matrix interferents. The method is quite sensitive and precise. Thus, its limits of detection (0.8–5.1 ng/kg) are better than those of existing methods such as one for the determination of 17 pharmaceuticals in soil and sediments (LC-MS/MS, 0.1–6.8 ng/g) (Vazquez-Roig et al., 2010), and others for four hormones in river sediments (GC–MS, 0.2–0.4 ng/g) (Liu et al., 2004), thirteen hormones in river sediments (LC–MS/MS, 0.14–0.98 ng/g) (Matejicek et al., 2007), four non-steroidal anti-inflammatory drugs in river sediments (GC–MS, 0.03–0.08 μg/g) (Antonic and Heath, 2007) and eight pharmaceuticals in sediment samples (LC–UV, 4–167 ng/g) (Cueva Mestanza et al., 2008a).

The usefulness of the proposed method for routine multi-residue analyses was demonstrated by applying it to agricultural soils, river and pond sediments, and sewage sludge. Based on the results, the sludge samples contained a large number and variety of analytes, which is unsurprising since they consisted of processed wastewater highly contaminated with most of the analytes. All samples contained the non-steroidal anti-inflammatories flunixin, ibuprofen and ketoprofen, the personal care product triclosan, the anti-epileptic carbamazepine and the antibacterial pyrimethamine, all of which are frequently used in the treatment of human and animal diseases — and are quite persistent in soil, sediment and sludge.

Acknowledgements

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References

Antonic J, Heath E. Determination of NSAIDs in river sediment samples. Anal Bioanal Chem 2007; 387:1337–42.

AOAC Official Method 994.16, in AOAC Official Methods of Analysis. Chap. 2. Gaithersburg, MD: AOAC; 2000. p. 40–44.

Azzouz A, Souhail B, Ballesteros E. Continuous solid-phase extraction and gas chromatography-mass spectrometry determination of pharmaceuticals and hormones in water samples. J Chromatogr A 2010; 1217:2956–63.

Azzouz A, Jurado-Sánchez B, Souhail B, Ballesteros E. Simultaneous determination of 20 pharmacologically active substances in cow's milk, goat's milk, and human breast milk by gas chromatography-mass spectrometry. J Agric Food Chem 2011; 59:5125–32.

Camel V. Microwave-assisted solvent extraction of environmental samples. Trends Anal Chem 2000; 19:229–48.

Chen L, Zeng Q, Wang H, Su R, Xu Y, Zhang X, Yu A, Zhang H, Ding L. On-line coupling of dynamic microwave-assisted extraction to solid-phase extraction for the determination of sulfonamide antibiotics in soil. Anal Chim Acta 2009; 648:200–6.

Chenxi W, Spongberg AL, Witter JD. Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. Chemosphere 2008; 73:511–18.

Cueva-Mestanza R, Sosa-Ferrera Z, Torres-Padrón ME, Santana-Rodríguez JJ. Preconcentration of pharmaceuticals residues in sediment samples using microwave assisted micellar extraction coupled with solid phase extraction and their determination by HPLC–UV. J Chromatogr B 2008a; 863:150–57.

Cueva-Mestanza R, Torres-Padrón ME, Sosa-Ferrera Z, Santana-Rodríguez JJ. Microwave-assisted micellar extraction coupled with solid-phase extraction for preconcentration of pharmaceuticals in molluscs prior to determination by HPLC. Biomed Chromatogr 2008b; 22:1115–22.

Dane JH, Topp GC, editors. Methods of Soil Analysis. Part 4. Physical methods, Soil Science. Madison: Society of America, Inc.; 2002. p. 422–26.

Díaz-Cruz MS, López de Alda MJ, Barceló D. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trends Anal Chem 2003; 22:340–51.

Dobor J, Varga M, Yao J, Chen H, Palkó G, Záray G. A new sample preparation method for determination of acidic drugs in sewage sludge applying microwave assisted solvent extraction followed by gas chromatography-mass spectrometry. Microchem J 2010; 94:36–41.

Gómez MJ, Petrovic M, Fernández-Alba AR, Barceló D. Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters, J Chromatogr A, 2006; 1114:224–33.

Henderson KL, Coats JR, editors. Veterinary Pharmaceuticals in the Environment. Washington DC: American Chemical Society; 2009.

Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 2006; 69:334–42.

Hesse PR, A Textbook of Soil Chemical Analysis. New York: Chemical Publishing Company; 1972.

Hibberd A, Maskaoui K, Zhang Z, Zhou JL. An improved method for the simultaneous analysis of phenolic and steroidal estrogens in water and sediment. Talanta 2009; 77:1315–21.

Jelic A, Petrovic M, Barceló D. Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. Talanta 2009; 80:363–71.

Liu R, Zhou JL, Wilding A, Microwave-assisted extraction followed by gas chromatography-mass spectrometry for the determination of endocrine disrupting chemicals in river sediments. J Chromatogr A 2004; 1038:19–26.

Madej K. Microwave-assisted and cloud-point extraction in determination of drugs and other bioactive compounds. Trends Anal Chem 2009; 28:436–46.

Matejicek D, Houserová P, Kubán V. Combined isolation and purification procedures prior to the high-performance liquid chromatographic-ion-trap tandem mass spectrometric determination of estrogens and their conjugates in river sediments. J Chromatogr A 2007;1171: 80–9.

Morales S, Canosa P, Rodríguez I, Rubí E, Cela R. Microwave assisted extraction followed by gas chromatography with tandem mass spectrometry for the determination of triclosan and two related chlorophenols in sludge and sediments. J Chromatogr A 2005; 1082:128–35.

Pailler JY, Krein A, Pfister L, Hoffmann L, Guignard C. Solid phase extraction coupled to liquid chromatography-mass spectrometry analysis of sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in Luxembourg. Sci Total Environ 2009; 407:3436-43.

Peng X, Wang Z, Yang C, Chen F, Mai B. Simultaneous determination of endocrine-disrupting phenols and steroid estrogens in sediment by gas chromatography-mass spectrometry. J Chromatogr A 2006; 1116:51–6.

Sanchez-Prado L, Garcia-Jares C, Llompart M. Microwave-assisted extraction: Application to the determination of emerging pollutants in solid samples. J Chromatogr A 2010; 1217:2390–414.

Ternes TA, Andersen H, Gilberg D, Bonerz M. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal Chem 2002; 74:3498–504.

Ternes TA, Bonerz M, Herrmann N, Löffler D, Keller E, Lacida BB, Alder AC. Determination of pharmaceuticals, iodinated contrast media and musk fragrances in sludge by LC/tandem MS and GC/MS. J Chromatogr A 2005; 1067:213–23.

Tobiszewski M, Mechlinska A, Zygmunt B, Namiesnik J. Green analytical chemistry in sample preparation for determination of trace organic pollutants. Trends Anal Chem 2009; 28: 943–51.

Varga M, Dobor J, Helenkár A, Jurecska L, Yao J, Záray G. Investigation of acidic pharmaceuticals in river water and sediment by microwave-assisted extraction and gas chromatography-mass spectrometry. Microchem J 2010; 95:353–58.

Vazquez-Roig P, Segarra R, Blasco C, Andreu V, Pico Y. Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. J Chromatogr A 2010; 1217:2471–83.

Vazquez-Roig P, Andreu V, Onghena M, Blasco C, Picó Y. Assessment of the occurrence and distribution of pharmaceuticals in a Mediterranean wetland (L'Albufera, Valencia, Spain) by LC–MS/MS. Anal Bioanal Chem 2011; 400:1287–1301.

Xu J, Wu L, Chen W, Chang AC. Simultaneous determination of pharmaceuticals, endocrine disrupting compounds and hormone in soils by gas chromatography-mass spectrometry. J Chromatogr A 2008; 1202:189–95.

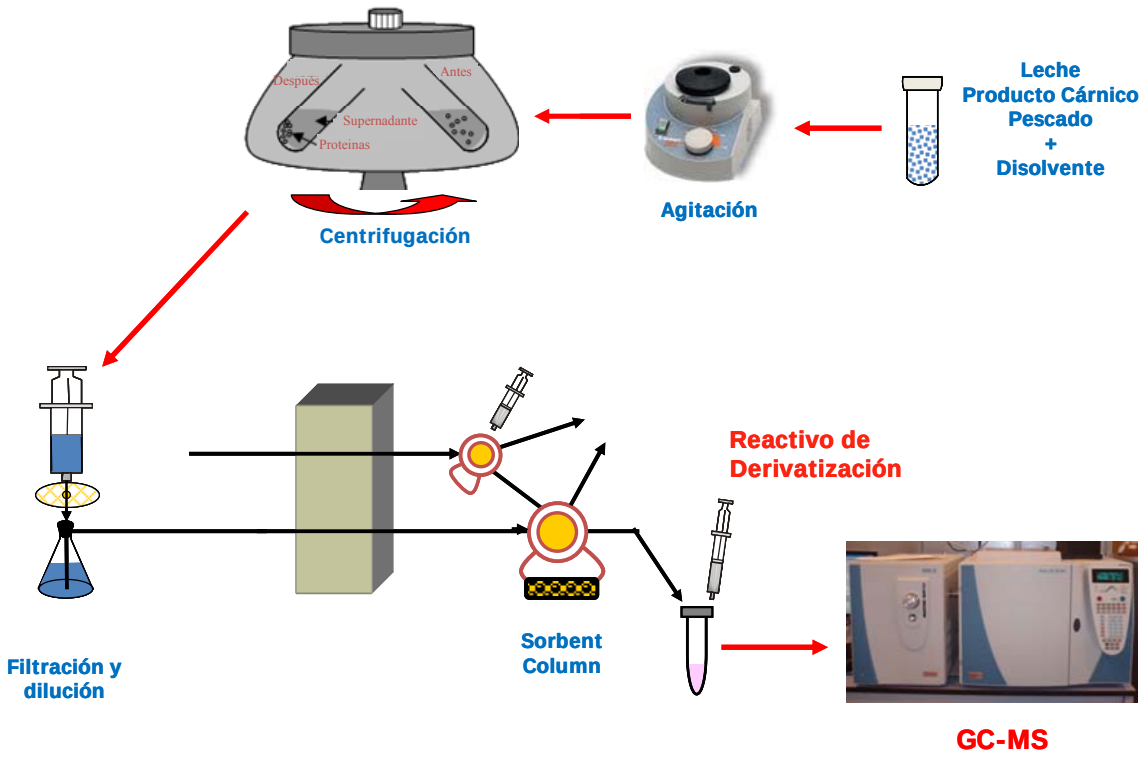
Yang JF, Ying GG, Zhao JL, Tao R, Su HC, Chen F. Simultaneous determination of four classes of antibiotics in sediments of the Pearl Rivers using RRLC–MS/MS. Sci Total Environ 2010; 408:3424-32.

Ying GG. Analysis of endocrine disrupting chemicals and pharmaceuticals and personal care products in water. In: Nollet LML, editor. Handbook of Water Analysis. Boca Raton, FL: CRC Press-Taylor & Francis; 2007. p. 693–727.

Zhao JL, , Ying GG, Wang L, Yang JF, Yang XB, Yang LH, Li X. Determination of phenolic endocrine disrupting chemicals and acidic pharmaceuticals in surface water of the Pearl Rivers in South China by gas chromatography–negative chemical ionization–mass spectrometry. Sci Total Environ 2009; 407:962-74.

CAPÍTULO II

Desarrollo de Metodologías para la Determinación de Sustancias Farmacológicamente Activas en Alimentos de Origen Animal



Para evitar brotes de enfermedades en animales criados en ganadería intensiva o en piscifactorías es necesario el tratamiento de estos animales con SFAs. Una porción de las SFAs administradas permanece en el cuerpo del animal, pero una fracción significativa se excreta y contamina al Medio Ambiente, que a su vez pueden contaminar a los alimentos. Para proteger la salud de los consumidores y asegurar una alta calidad de los productos alimenticios, la Comisión del Codex Alimentarius de la Organización para la Alimentación y la Agricultura (FAO), la Organización Mundial de la Salud (OMS) y la Comunidad Europea han establecido límites máximos de residuos (LMR) de una variedad de productos farmacéuticos en los alimentos de origen animal. En este Capítulo de la Memoria se han desarrollado dos metodologías para la determinación de residuos de fármacos en alimentos de origen animal teniendo en cuenta dos premisas: la baja concentración a la que se encuentran estos residuos en estas muestras pero que pueden ser perjudiciales para la salud del consumidor, y la complejidad de las matrices a las que nos enfrentamos (leche, productos cárnicos y pescado).

El objetivo de primera parte de este Capítulo ha consistido en la puesta a punto de un método fiable para la determinación simultánea de residuos de antibióticos, antiinflamatorios no esteroideos, β-bloqueadores, reguladores de lípidos, hormonas, antiepilépticos, antidepresivos y antisépticos en leche de origen animal y leche materna mediante el uso de un sistema continuo de SPE y cromatografía de gases acoplada a un espectrómetro de masas.

Como es sabido, la leche es un fluido biológico complejo que contiene un número elevado de macromoléculas tales como proteínas, lípidos y otras sustancias minerales, las cuales pueden dañar a la columna de SPE y perjudicar a la ionización de los analitos en el espectrómetro de masas. Como resultado, los procedimientos de preparación de muestras de leche para su posterior determinación de residuos de fármacos requieren de una etapa previa de precipitación de proteínas con un disolvente adecuado, centrifugación de la mezcla, y la filtración del sobrenadante resultante. En este trabajo, se ha estudiado la eficacia de diversos disolventes (acetonitrilo, metanol, etanol, acetona) y ácido tricloroacético para la precipitación de proteínas de la leche. Del estudio se concluyó que el acetonitrilo era el disolvente más eficaz para la precipitación de proteínas en estos tipos de muestras, seleccionando 5 ml de este disolvente para la precipitación de estas sustancias en 5 g de leche. También se estudiaron la influencia de las diferentes variables relacionadas con la centrifugación (velocidad, temperatura y

tiempo) sobre la eficacia de la precipitación y separación de las proteínas previa a la introducción en el sistema continuo de SPE. Se seleccionaron como óptimas una velocidad de centrifugación a 3500 rpm durante 10 min, manteniendo la temperatura a 4 ºC.

Por otra parte se estudió como influía la presencia de acetonitrilo en las muestras que se pasaban a través del sistema continuo para la SPE de los diferentes analitos. Como ocurría en el Capítulo anterior, en aquel caso con el metanol, también el acetonitrilo afectaba negativamente a la retención de las diferentes SFAs estudiadas. Se observó que a partir de un 15 %, el rendimiento de la adsorción de los diferentes analitos disminuía. Este efecto puede explicarse de igual manera que se explicó en el método desarrollado para la determinación de residuos de fármacos en suelos, sedimentos y lodos del Capítulo II de esta Memoria. Por ello se solucionó el problema evaporando la disolución de acetonitrilo hasta 100 µl y redisolviendo después el extracto con 5 ml de agua purificada. A continuación se comprobó que el sistema continuo de SPE era eficaz para la retención de los diferentes SFAs en una columna de 60 mg de Oasis HLB. Finalmente los analitos se eluyeron con 400 µL de acetato de etilo, evaporándose éste hasta 35 µL con una corriente de nitrógeno previa a la derivatización con la mezcla BSTFA+1 % de TMCS a 70 ºC.

A continuación se realizó un estudio de estabilidad de los analitos en las muestras debido a que éstas se congelaban hasta su análisis. Para ello se añadieron 100 ng/kg de cada una de las SFAs a 1 kg de leche y se dividió la mezcla en distinta porciones. Una se analizó inmediatamente y las demás se congelaron a -20 ºC, analizándose por triplicado un porción cada dos días durante un mes. No se encontró divergencia entre los resultados obtenidos de los diferentes análisis, por lo cual se concluyó que por lo menos durante un mes los analitos permanecían estables en las muestras a las condiciones de conservación anteriormente comentadas. El método propuesto proporciona una respuesta lineal en el intervalo entre 0,6 y 5000 ng/kg con una sensibilidad muy aceptable (limites de detección entre 0,2 y 1,2 ng/kg). El análisis repetitivo de la misma muestra en el mismo día o en diferentes días ha puesto de manifiesto que el método es preciso con desviaciones estándares relativas entre 3,5 y 7,2 % o entre 4,3 y 7,8 %, respectivamente. También los estudios de recuperación demostraron que el método tiene una exactitud adecuada con porcentajes de recuperación entre 95 a 102 % para los diferentes tipos de muestras de leche estudiadas.

Finalmente el método fue aplicado al análisis de muestras de leche de vaca, cabra y mujer. En la mayoría de las muestras analizadas se encontró alguna de las SFAs estudiadas. En el caso de la leche de vaca (cruda, entera, semidesnatada o desnatada) se encontraron en muchas de las muestras dos hormonas (estrona y 17β-estradiol), varios antiinflamatorios (ketoprofeno, fenilbutazona, diclofenaco, flunixino y ácido niflúmico) y dos antibióticos (florfenicol y piremetamina). En las muestras de leche de cabra (entera y semidesnatada) se encontró en una de ellas ácido niflúmico y flunixino. También se analizaron 3 muestras de leche humana encontrándose en todas ellas alguna de las hormonas estudiadas y algunos antiinflamatorios (ibuprofeno y naproxeno) y el triclosán a niveles entre 0.035 y 1.9 μg/kg. Por último se analizó leche en polvo de vaca, en la cual no se detectó la presencia de ninguna de las SFAs estudiadas en este trabajo.

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En la segunda parte de este Capítulo se describe el método desarrollado para la determinación de diferentes tipos de SFAs en productos cárnicos y pescado. Como en el caso anterior, la matriz es bastante compleja y como se ha encontrado en la bibliografía requiere de una etapa de pretratamiento con objeto de eliminar el alto contenido que tiene de proteínas y lípidos que pudieran interferir a la SPE de los diferentes analitos en el sistema continuo descrito en el Capítulo II de esta Memoria. Por ello se pensó en utilizar una etapa de precipitación de estas sustancias interferentes con un disolvente adecuado y posterior centrifugación para conseguir un sobrenadante libre de proteínas y lípidos. Para ello se estudiaron varios disolventes (acetato de etilo, agua, acetonitrilo, metanol, y etanol) y el ácido tricloroacético. Se seleccionó la mezcla acetonitrilo-agua (3:2, v:v) por ser la que proporcionaba un rendimiento superior en la precipitación y posterior eliminación de proteínas y lípidos en los alimentos estudiados. Para 2 g de producto cárnico o pescado se encontró que se conseguía la eliminación total de proteínas y lípidos cuando se utilizaba un volumen de 10 ml de la mezcla acetonitrilo-agua. También se examinaron los efectos de la velocidad, tiempo y temperatura en la etapa de de centrifugación, seleccionándose como óptimas 4000 rpm, 10 min y 4 ºC, respectivamente. Para la eliminación del elevado porcentaje de acetonitrilo en el sobrenadante, se optó como en la primera parte de este Capítulo por la evaporación con una corriente de nitrógeno hasta un volumen de 200 μl y redisolución con 5 ml de agua

purificada a pH 7. Para la etapas de SPE y derivatización se utilizaron las mismas condicionas empleadas en el método anterior incluido en este Memoria.

El método propuesto es sensible con límites de detección reducidos (0,4–2,7 ng/kg para 2 g de muestra) y una buena linealidad (coeficientes de correlación> 0,995). La precisión es aceptable con desviaciones estándares relativas menores de 7 %. Además se obtuvieron unos porcentajes de recuperación excelentes (92–101 %). Con objeto de poder demostrar su aplicabilidad, se analizaron diferentes tipos de productos cárnicos (músculo, hígado y riñón) de distintos tipos de animales (cordero, vaca, cerdo y pollo) y pescados (salmón, lubina y lenguado). La carne de pollo fue la única muestra que no contenía residuos de las SFAs a los niveles de sensibilidad permitidos por el método. En cambio, las demás muestras contenían la hormona 17β-estradiol y en la mayoría de ellas había la presencia de estrona, florfenicol y piremetamina a niveles por debajo del límite máximo de residuo permitido en este tipo de alimentos. También se detectaron residuos de otros fármacos tales como fenilbutazona (en carne de cordero y vaca, e hígado de pollo) y flunixino (en carne de vaca).

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Simultaneous Determination of 20 Pharmacologically Active Substances in Cow´s, Goat´s Milk and Human Breast Milk by Gas Chromatography-Mass Spectrometry

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ABSTRACT

This paper reports a systematic approach to the development of a method that combines continuous solid-phase extraction and gas chromatography–mass spectrometry for the simultaneous determination of 20 pharmacologically active substances including antibacterials (chloramphenicol, florfenicol, pyrimethamine, thiamphenicol), non-steroideal anti-inflammatories (diclofenac, flunixin, ibuprofen, ketoprofen, naproxen, mefenamic acid, niflumic acid, phenylbutazone), antiseptic (triclosan), anti-epileptic (carbamazepine), lipid regulator (clofibric acid), β-blockers (metoprolol, propranolol) and hormones (17α–ethinylestradiol, estrone, 17β–estradiol) in milk samples. The sample preparation procedure involves deproteination of the milk, followed by sample enrichment and cleanup by continuous solid-phase extraction. The proposed method provides a linear response over the range 0.6–5000 ng/kg and features limits of detection from 0.2 to 1.2 ng/kg depending on the particular analyte. The method was successfully applied to the determination of pharmacologically active substance residues in food samples including whole, raw, half-skim, skim and powdered milk from different sources (cow, goat and human breast).

KEYWORDS: Pharmacologically active substances, milk, continuous solid-phase extraction, gas chromatography–mass spectrometry.

INTRODUCTION

Antibacterials, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulators, hormones, anti-epileptics and antiseptics are the most widely used medical and veterinary drugs at present. As a result, it is very common for milk to contain residues of pharmacologically active substances (PAS) which have undesirable effects on the quality and technological properties of dairy products, and also, more important, on human health. In fact, contaminated milk can cause allergic reactions or indirect problems through bacterial resistance to clinical treatments. Human milk is also frequently contaminated with PAS by effect of many women using some personal care products or requiring medication while breast feeding. Accumulation of some drugs in milk can pose a risk on infants exceeding the benefits of breast feeding.[1] In order to protect consumer health and ensure a high product quality, the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO), the World Health Organization (WHO)[2] and the European Community[3] have set maximum residue limits (MRLs) for some drugs in milk —primarily cow and, occasionally, goat milk. Carefully controlling the presence of PAS residues is especially important in milk and dairy products owing to their ubiquitous presence in human nutrition.

Detecting trace levels of PAS in such a complex matrix as milk requires sample preparation and cleanup. The sample preparation procedures usually employed for this purpose include homogenization, extraction/cleanup, enrichment and, where needed, derivatization of the analytes. Protein precipitation is also required to prevent emulsification during extraction. Sample preparation procedures such as liquid–liquid extraction,[4–6] solid-phase microextraction[7,8] and solid-phase extraction (SPE) are common choices for simultaneous extraction and cleanup. The last has gained increasing popularity over liquid–liquid extraction by virtue of its ease of operation and environmental friendliness. Sorbents including C_{18}, C_{30}, SCX and ion-exchange resins have been used in the analysis of PAS residues in various matrices such as milk,[9–14] animal tissues, plasma and urine.[14–17] Others polymeric sorbents such as Evolute™ ABN,[18] Amberlite XAD-2,[19] and Strata-X[20] have been used for antibiotics, and non-steroidal anti-inflammatory drugs in cow milk, other food samples and urine. Recently, polymeric SPE adsorbents with a dual nature (e.g. hydrophilic–lipophilic balance in Oasis-HLB) have enabled simultaneous sample cleanup and analyte enrichment in

biological, environmental and food matrices..[21] Oasis-HLB SPE cartridges have been successfully used to determine chloramphenicol, thiamphenicol and florfenicol in poultry and porcine muscle and liver, and in seafood;[22,23] as well as antibacterial, non-steroidal anti-inflammatories, anti-epileptics, β-blockers and hormones in milk, plasma, urine, hair and water samples.[21,24–27]

A variety of analytical techniques have been used to determine PAS residues in foods. The most common choices for this purpose are gas chromatography (GC) and liquid chromatography.[28] Thus, liquid chromatography–mass spectrometry[5,8–11,13–16,18,22,26,27] and high resolution liquid chromatography accurate mass time-of-flight mass spectrometry[20,29] have been used to determine various types of PAS residues in some types of food. The prevalent analytical methods for determining these residues in milk, muscle tissue, urine and water, based on gas chromatography–mass spectrometry (GC–MS), provide a high sensitivity, specificity and good chromatographic resolution.[12,14,17,19,21,23–25,30] However, the low volatility of some PAS and the presence of various polar groups in others require the use of an appropriate derivatization procedure to obtain more volatile products.[21]

Continuous flow systems are useful tools for the simplification and automation of analytical processes with a view to enabling the implementation of reliable separation techniques with increased sensitivity and selectivity. Thus, continuous solid-phase extraction has been successfully applied to the determination of pharmaceuticals in water,[21] amines in water and beer samples,[31,32] and organophosphorus pesticides in water.[33] The advantages of continuous solid-phase extraction over traditional SPE include: (i) reduced sample, sorbent and eluent consumption; (ii) increased precision; (iii) improved preconcentration by effect of the analytes being efficiently eluted with small volumes (100–300 μl) of organic solvent; and (iv) a high throughput derived from the ability to operate in the array mode.

The aim of this work was to develop a reliable multi-residue method for the simultaneous quantitative determination of antibacterials, non-steroid anti-inflammatory drugs, hormones and other pharmacologically active substances in milk by using a continuous SPE system to preconcentrate PAS residues and gas chromatography–mass spectrometry for their determination. The proposed method improves on an earlier one developed by our group to determine pharmaceuticals and hormones in water samples;[21] the new method affords application to a more complex matrix such as milk and spans a much broader range of analytes potentially present in animal and human samples. Milk

proteins, which can interfere with the determination of PAS, are precipitated by addition of acetonitrile and centrifugation prior to solid-phase extraction. Also, PAS are converted into their silyl derivatives with a mixture of *N*,*O*-*bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) in order to improve volatility and thus sensitivity in the subsequent GC–MS analysis.

MATERIALS AND METHODS

Gas Chromatographic-Mass Spectrometry. GC–MS analyses were performed on a Focus GC instrument (Thermo Electron SA, Madrid, Spain) interfaced to a DSQ II mass spectrometer (single quadrupole) controlled via a computer running XCalibur software. The GC instrument was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm) coated with 5% phenylmethylpolysiloxane (Supelco, Madrid, Spain). The carrier gas was helium (purity 6.0) and circulated at a flow rate of 1 mL/min. The oven temperature was held at 70 ºC for 1 min following injection, and then raised from 70 to 150 ºC at 14 ºC/min. After the first transition, the temperature was raised from 150 to 290 ºC at 6 ºC/min. The injection port and transfer line temperatures were maintained at 270 and 280 ºC, respectively. A 10 μL syringe was washed 3 times with methanol before and after each injection, and rinsed with 8 μL of sample solution before 1 μL of sample was injected in the split mode (1:20).

The mass spectrometer was used under the following conditions: ion source temperature, 200 ºC; transfer line temperature, 280 ºC; electron impact ionization mode at 70 eV. The time for solvent delay was set at 4 min. Samples were analysed in the selected ion monitoring (SIM) mode. Quantitation was based on the peak areas of the analytes relative to the internal standard (IS). Scans spanned the *m/z* range from 60 to 500. Specific ions were selected for each compound, the most abundant being used as quantitation ion and three others as qualifier ions (**Table 1**). As prescribed by Commission Decision 2002/657/EC33, which was adhered to in its entirety, the two most abundant fragments were used as diagnostic ions.[34] The Commission Criteria include the specification that the signal-to-noise ratio for each diagnostic ion should be equal to or greater than 3. The relative retention times for the analytes differed by less than 0.5% from that for the calibration standard. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most abundant ion, corresponded to those of samples fortified at comparable concentrations within the tolerances specified by the Commission Decision.[34]

Chemicals and Reagents. All reagents used were analytical-grade or better. The pharmacologically active substances used were purchased from Sigma–Aldrich (Madrid, Spain). Triphenylphosphate (IS) and the derivatization agents (BSTFA and TMCS) were purchased from Fluka (Madrid, Spain). All solvents (methanol, ethyl acetate, acetonitrile, acetone, ethanol, *n*-hexane, dichloromethane and 2-propanol) were supplied by Merck (Madrid, Spain). Oasis-HLB in particle size 50–65 μm was obtained from Waters (Madrid, Spain). Millex-LG filter units (hydrophilic, PTFE, pore size 0.20 μm, diameter 25 mm, filtration area 3.9 cm^2) were obtained from Millipore Ibérica. Water is purified by passage through a Milli-Q system (Millipore Ibérica, Madrid, Spain).

Standards. Stock standard solutions of the individual antibacterials, non-steroid anti-inflammatories, hormones and other pharmacologically active substances at a 1 mg/mL concentration each were prepared in methanol and stored at 4 ºC in the dark. Working-standard solutions were prepared on a daily basis by dilution of the individual stock standard solutions in purified water and adjustment to pH 7 with dilute NaOH as required. Freshly made solutions of ethyl acetate containing a 500 μg/L concentration of triphenylphosphate and prepared on a daily basis were used as eluents for continuous SPE.

Milk Samples. Cow and goat milk samples (whole, half-skim, skim and powdered) were purchased at local markets in Spain or Morocco. Raw cow milk samples were obtained from various farms using standard commercial breeding protocols. Human milk from lactating women who volunteered for the study with permission of the bioethics committee was also used. All samples were frozen at −20 ºC in the dark until analysis. The powdered infant milk sample studied (half-skim cow milk) was purchased a local chemist's shop and stored in a dry, cool place.

Table 1. Analytical Figures of Merit of the Proposed Solid-Phase Extraction Method for the Determination of Pharmacologically Active Substances in Milk

compound	retention time (min)	linear range (ng/kg)	r[a]	detection limit (ng/kg)	sensitivity [signal/(ng/kg]	Precision (RSD ,%) [b]		$M^{+\cdot}$ (*m/z*)	$[M-15]^{+}$ (*m/z*)	Additional ions[c] (*m/z*)
						within-day	between-day			
clofibric acid	11.3	1.8–5000	0.995	0.6	1.075	5.1	5.5	286	271	128, **143**
ibuprofen	11.9	0.6–5000	0.994	0.2	3.185	4.9	5.3	278	263	**160**, 234
niflumic acid	18.8	0.6–5000	0.997	0.2	3.190	4.6	4.8	354	339	**236**, 263
metoprolol	19.1	1.9–5000	0,996	0.6	1.095	6.2	6.6	339	324	72, **223**
naproxen	19.3	1.2–5000	0.994	0.4	1.590	6.7	7.0	302	287	**185**, 243
flunixin	19.8	0.6–5000	0.995	0.2	3.065	4.5	5.7	368	353	251, **263**
triclosan	19.9	1.9–5000	0.998	0.6	1.030	6.7	7.4	362	**347**[c]	200, 310
propranolol	20.6	1.8–5000	0.999	0.6	1.075	6.1	6.5	331	316	72, **215**
mefenamic acid	20.9	0.7–5000	0.996	0.2	3.220	5.8	6.5	313	298	208, **223**
ketoprofen	21.0	1.2–5000	0.997	0.4	1.610	7.1	7.5	326	311	73, **282**
pyrimethamine	21.3	3.1–5000	0.997	1.0	0.615	6.9	7.0	392	**377**[c]	171, 281
carbamazepine	22.0	0.6–5000	0.998	0.2	3.230	5.8	6.4	308	293	**193**, 250
diclofenac	22.3	0.7–5000	0.994	0.2	3.180	5.9	6.2	367	352	**214**, 242
phenylbutazone	24.0	3.2–5000	0.996	1.0	0.610	7.6	7.8	308	–	77, **183**, 252
chloramphenicol	24.5	0.6–5000	0.995	0.2	3.245	5.1	5.5	466	451	208, **225,** 242
florfenicol	26.2	0.6–5000	0.999	0.2	3.195	4.5	5.0	429	414	**257,** 360
estrone	26.9	3.2–5000	0.998	1.0	0.625	7.0	7.5	**342**[c]	327	218, 257
17β–estradiol	27.3	4.0–5000	0.997	1.2	0.515	5.3	5.7	**416**[c]	401	285, 326
thiamphenicol	27.9	0.6–5000	0.997	0.2	3.185	7.2	7.6	499	484	242, 257, **330**
17α–ethinylestradiol	28.7	3.4–5000	0.995	1.1	0.570	5.9	6.4	440	**425**[c]	232, 300

Sample Pretreatment. About 5 g of milk sample was weighed into a 50 mL round polypropylene centrifuge tube and mixed with 5 mL of acetonitrile in a vortex mixer (REAX Control, Heidolph, Kelheim, Germany) for 30 s. This was followed by centrifugation on a Centrofriger BL-II apparatus (JP Selecta, Barcelona, Spain) at 3500 rpm 4 ºC for 10 min. Then, the supernatant, which has previously been separated from the precipitate material, was evaporated under a stream of ultrahigh-purity N_2 to a final volume of approximately 100 µL and diluted to 5 mL with purified water at pH 7 adjusted with dilute NaOH as required. For powdered milk, an amount of 1 g was dissolved in 5 mL of purified water as per the manufacturer's instructions and the solution processed like all other samples. The pretreated, sample was thus made ready for continuous solid-phase extraction.

The continuous solid-phase extraction manifold used was assembled from a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinyl chloride) pumping tubes, two Rheodyne 5041 injection valves (Cotati, CA, USA) and a laboratory-made PTFE sorption column containing 60 mg of Oasis-HLB sorbent (5 cm x 3 mm I.D.). The sorbent column was conditioned with 1 mL of ethyl acetate and 1 mL of purified water. Under these conditions, the column remained effective for at least 1–2 months with no change in its properties. A laboratory-made polytetrafluoroethylene (PTFE) filter furnished with a paper disk (4-cm^2 filtration area) was also employed.

The continuous solid-phase extraction unit employed for the preconcentration of pharmaceutically actives substances is depicted in **Figure 1**. In the preconcentration step, 5 mL of pretreated sample was filtered to prevent suspended particles from reaching the continuous unit and passed at 4 mL/min through the sorbent column, located in the loop of injection valve IV_1. Retention of PAS residues was instantaneous and the sample matrix was immediately sent to waste. In the drying step, IV_1 was switched to the injected position and the sorbent column dried for 2 min with an air stream at 4 mL/min inserted via the carrier line of the second valve (IV_2); simultaneously, the loop of IV_2 was filled with eluent (ethyl acetate containing 500 µg/L triphenylphosphate as IS) by means of a syringe. In the elution step, IV_2 was switched to have the loop contents (400 μL of eluent) injected into the same air stream used in the drying step in order to elute PAS residues —in the opposite direction of sample aspiration. The organic extract was collected in a conical glass insert of 0.5 mL and concentrated to a volume of 35 µL under a stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using an internal standard. Next,

a volume of 70 µL of BSTFA + 1% TMCS was added and the vials were heated at 70 ºC for 20 min. Finally, 1 µL aliquots of the silylated derivatives were analysed by GC–MS in the SIM mode.

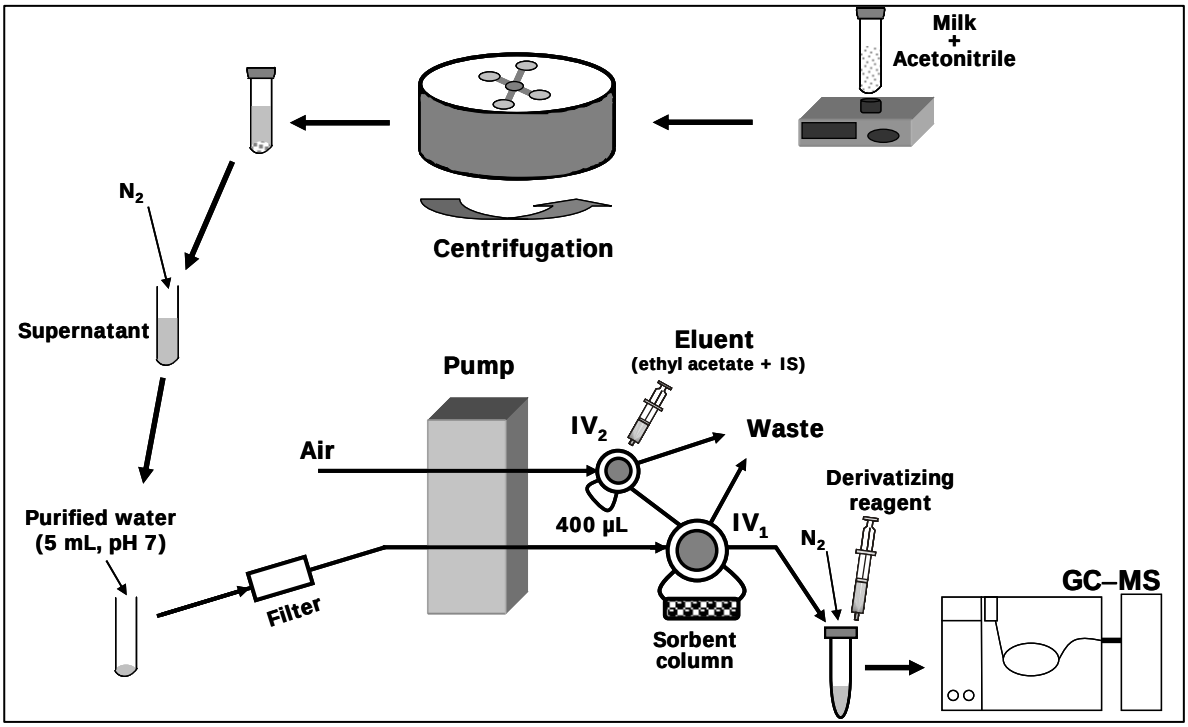

Figure 1. Experimental setup for the removal of protein from milk and the continuous preconcentration of pharmacologically active substances in milk samples. Abbreviations: IV, injection valve; IS: internal standard; GC–MS, gas chromatograph–mass spectrometer.

RESULTS AND DISCUSSION

In previous work, we developed a continuous system consisting of a solid-phase extraction module for the preconcentration of pharmaceuticals and hormones from environmental water samples and the determination of derivatized (silylated) analytes by GC−MS.[21] We studied various SPE sorbents including Oasis-HLB, LiChrolut EN, XAD-2, XAD-4, RP-C18, Florisil, Silica Gel and Isolute NH_2, and found Oasis-HLB to exhibit the highest efficiency in retaining the analytes (94–100 %). A study of the influence of pH on the SPE efficiency revealed that the best extraction results for all analytes were obtained in the neutral pH region (6.5–7.5), so pH 7 was adopted. We also examined the effects of other factors influencing analyte derivatization, retention and elution. The results are shown in **Table 2**. In this work, we expanded the number of

analytes. This entailed checking that the operational conditions chosen for the previous determination[21] fell within the optimum ranges for the new analytes as well. To this end, we used the above-described system to preconcentrate the twenty analytes from milk samples. The new analytes were found to respond similarly to the previous ones to the optimum operating conditions. The optimum amount of Oasis HLB sorbent to be used was optimized by using columns containing between 20 and 100 mg of sorbent. To this end, a calibration test was conducted for each analyte and column by passing 5 mL of aqueous standard solutions containing a 0.1–8 μg/L concentration of each analyte and then eluting the column with 400 μL of ethyl acetate. The resulting analytical signals increased with increasing amount of sorbent up to 60 mg and then decreased above 65 mg of Oasis HLB owing to the need for a higher volume of eluent to ensure the complete elution. Quantitative retention and the absence of carry-over were confirmed by subjecting an aqueous solution containing a 2000 ng/L concentration of each pharmacologically active substance to SPE as described under Materials and Methods. This elution was followed by a second one (IV_2 in **Figure 1**) with the same eluent (400 μL of ethyl acetate). The absence of carry-over was confirmed by the presence of none of the analytes in the second eluate, which indicates that the eluent volume used ensured complete elution. However, because the milk samples contained potentially interfering proteins, we examined the effects of the matrix in the pretreated samples to be passed through the SPE system. Below is described the influence of the variables affecting protein removal from the milk by precipitation and centrifugation.

Table 2. Variables Influencing the Milk Sample Pretreatment, Sorption/Elution Process and Derivatization of Pharmacologically Active Substances

variable	studied range	optimum range (selected value)
protein precipitation and centrifugation[a]		
Volume of acetonitrile (mL)	1–10	4–10 (5)
centrifugation rate (rpm)	1500–5000	2500–5000 (3500)
centrifugation time (min)	1–30	5–30 (10)
centrifugation temperature (ºC)	0–30	0–10 (4)
continuous solid-phase extraction		
sample pH	1–12	6.5–7.5 (7)
amount of Oasis-HLB (mg)	20–100	55–65 (60)
volume of ethyl acetate (μL)	50–500	350–450 (400)
flow rate of sample (mL/min)	1–5	3.5–4.5 (4)
breakthrough volume (mL)	1–500	1–200 (50)
flow rate of air (mL/min)	1–5	3.5– 4.5 (4)
derivatization (silylation)		
percentage of TMCS (catalyst) in BSTFA[b]	0.25–15	0.75–15 (1)
reaction time	1–30	15–25 (20)
temperature	20–90	65–75 (70)

[a] To 5 g of sample. [b]BSTFA: *N*,*O*-*bis*-(trimethylsilyl)trifluoroacetamide; TMCS: trimethylchlorosilane

Optimization of Variables Affecting Sample Pretreatment. One immediate requirement for the target multi-compound analysis was to develop a generic sample preparation method suitable for extracting pharmaceuticals from various types of matrices of animal and human origin. Milk is a complex biological fluid containing a number of macromolecules such as proteins and lipids the presence of which can damage an SPE column and suppress ionization in the mass spectrometer. As a result, sample preparation procedures for the determination of pharmaceutical residues in milk often involve protein precipitation with an appropriate solvent, centrifugation of the mixture and filtration of the resulting supernatant.[7–9 11–14,18,20] In this work, we studied the efficiency of various solvents (acetonitrile, methanol, ethanol, acetone) and trichloroacetic acid in precipitating milk proteins. For this purpose, an amount of *ca.* 5 g of milk was mixed with a volume of 5 ml of each organic solvent or a aqueous solution of 30% m/v trichloroacetic acid and centrifuged at 3500 rpm at 4 ºC for 10 min. Acetonitrile, which caused more than 95% of the protein content to precipitate, proved the most efficient solvent; also, it facilitated separation of the precipitate by filtration,

which is consistent with previous results.[11–13,18] The optimum volume of acetonitrile for addition to 5 g of milk was determined by changing it over the range 1–10 mL; protein precipitation was found to peak at 4.5 mL acetonitrile, so a solvent volume of 5 mL was adopted as optimal. We also examined the effects of centrifugation-related variables (rate, temperature and time) over the respective ranges shown in Table 2. Centrifugation at 3500 rpm at 4 ºC for 10 min resulted in optimal separation of precipitated milk proteins from the target species. Also, most of the fat initially present in the milk samples was removed by effect of the low centrifugation temperature used in the protein precipitation step (4 ºC).[35]

Once the precipitation and centrifugation conditions were optimized, the filtered supernatant matrix was checked to be compatible with the continuous SPE system. To this end, we examined the effect of the presence of acetonitrile on retention of the 20 studied PAS by the SPE system. This involved preparing aqueous solutions (50 mL) containing a 10 ng/L concentration of each PAS at pH 7 with dilute NaOH and a variable proportion of acetonitrile from 0 to 50%. The analytes present in the extract after elution were derivatized as described under Analytical Method and determined by GC–MS. The results were compared with those obtained in the absence of acetonitrile. The solvent had no effect on retention of PAS in proportions up to 15 %. This can be ascribed to the peculiar sorption mechanism, which involves the partitioning of moderately polar organic compounds from a polar phase (water) into a polymeric sorbent (Oasis-HLB) via a polar interaction such as hydrogen bonding between the hydroxyl group in the PAS and the underlying sorbent surface. When the aqueous sample contains a high concentration of acetonitrile, the solvent breaks the bonds and effectively solubilizes PAS, thereby dramatically reducing sorption of analytes. Therefore, the supernatant was evaporated under a stream of ultrahigh-purity N_2 to a final volume of 100 μL and diluted with 5 mL of purified water (pH 7) and introduced into continuous SPE system.

Method Validation. All PAS studied exhibited a good gas chromatographic behavior. Control samples from milk previously checked to contain no residual PAS were prepared as described above. One control sample per calibration standard level was used. Calibration curves for PAS standards were obtained by processing an amount of 5 g of uncontaminated cow milk sample (whole 1 in **Table 4**, see **Figure 2A**) that was aliquoted into 50 mL polypropylene tubes containing variable concentrations of the

analytes over the range 0.6−5000 ng/kg. After spiking, the samples were vortexed and allowed to stand for 15 min before pretreatment and SPE extraction as described under Materials and Methods. Curves were constructed by plotting analyte-to-IS peak area ratios against analyte concentrations. The linear range, limit of detection (LOD), and precision for the determination of the 20 PAS studied by GC–MS in the SIM mode, and the *m/z* values used for GC–MS confirmation, are shown in **Table 1**. Correlations coefficients were greater than 0.994 in all instances (12 points per calibration). Limits of detection were calculated as three times the standard deviation (SD) of background noise divided by the slope of each calibration graph. The precision of the proposed method, as relative standard deviation (RSD), was calculated by measuring 11 uncontaminated milk samples spiked with a 10, 200 or 2000 ng/kg concentration of each PAS. A comparative study of within-day and between-day precision was conducted —the latter over seven days— at three analyte concentration levels (10, 200 or 2000 ng/kg); the former parameter was found to range from 3.5 to 7.2 % and the latter from 4.3 to 7.8 %.

Because no certified reference material for milk containing the studied analytes was available, the proposed method was validated by analysing various types of milk (raw, whole, half-skim, skim and powdered) from different sources (cow, goat and human breast) that were spiked with a 10, 50 and 2000 ng/kg concentration of a standard mixture of the analytes before pretreatment. Each sample was analysed in triplicate (n = 3) in order to calculate a standard deviation. Some samples contained only a few PAS, which allowed recoveries to be calculated by subtracting the previously quantified endogenous compounds from the total contents. As can be seen in **Table 3**, recoveries ranged from 91 to 104 % for 10 and 50 ng/kg of analytes added. When high concentration of analytes (2000 ng/kg) is spiked to milk samples, recoveries ranged from 95 to 102 %, which testifies to the applicability of the proposed method to any type of milk sample and also that matrix interferences are reduced or completely suppressed by the sample pretreatment and cleanup step in the SPE module.

Table 3. Percent Recovery (± SD, n = 3) of Pharmacologically Active Substances Added to Milk Samples

compound	Cow								goat (ng/kg)		powdered (ng/kg)		human (ng/kg)	
	whole (ng/kg)		half-skim (ng/kg)		skim (ng/kg)		raw (ng/kg)							
	10	50	10	50	10	50	10	50	10	50	10	50	10	50
clofibric acid	104 (6)	103 (5)	104 (5)	99 (5)	101 (6)	91 (4)	98 (4)	91 (4)	101 (5)	99 (4)	98 (5)	104 (6)	100 (5)	104 (5)
ibuprofen	103 (5)	96 (4)	101 (5)	104 (6)	98 (4)	94 (4)	103 (5)	96 (5)	103 (4)	95 (4)	104 (5)	98 (5)	103 (5)	98 (5)
niflumic acid	99 (5)	100 (4)	92 (4)	95 (4)	99 (5)	98 (5)	104 (5)	99 (4)	102 (5)	101 (5)	98 (4)	103 (5)	98 (5)	104 (5)
metoprolol	101 (7)	99 (6)	97 (7)	101 (6)	104 (6)	103 (5)	95 (5)	101 (6)	91 (5)	104 (7)	103 (6)	100 (6)	93 (5)	100 (6)
naproxen	98 (6)	102 (7)	104 (7)	98 (6)	101 (7)	99 (6)	97 (6)	103 (6)	92 (6)	93 (6)	96 (7)	104 (7)	101 (6)	103 (6)
flunixin	102 (5)	95 (5)	99 (5)	104 (6)	98 (5)	104 (5)	101 (5)	104 (5)	96 (4)	94 (4)	97 (4)	103 (6)	96 (5)	104 (5)
triclosan	96 (6)	103 (7)	102 (6)	99 (7)	95 (5)	104 (6)	100 (7)	92 (6)	102 (6)	92 (6)	98 (7)	97 (5)	98 (6)	101 (7)
propranolol	102 (6)	101 (6)	95 (6)	98 (6)	102 (5)	101 (7)	99 (6)	102 (6)	99 (5)	103 (7)	102 (6)	92 (5)	103 (5)	92 (5)
mefenamic acid	104 (6)	95 (5)	104 (5)	103 (6)	97 (4)	92 (4)	102 (6)	99 (6)	97 (5)	94 (5)	103 (5)	101 (6)	103 (6)	99 (6)
ketoprofen	104 (7)	97 (7)	102 (6)	94 (6)	93 (6)	102 (7)	98 (7)	101 (7)	101 (7)	93 (6)	102 (7)	99 (7)	92 (6)	103 (7)
pyrimethamine	96 (6)	98 (6)	101 (7)	102 (7)	99 (7)	95 (6)	103 (7)	96 (7)	100 (6)	92 (6)	102 (7)	98 (6)	104 (7)	98 (7)
carbamazepine	94 (5)	104 (6)	104 (6)	101 (6)	98 (5)	101 (6)	98 (6)	91 (5)	94 (5)	102 (5)	93 (6)	103 (6)	93 (5)	101 (6)
diclofenac	104 (6)	100 (6)	93 (5)	92 (5)	101 (6)	102 (6)	101 (6)	104 (6)	92 (5)	99 (6)	97 (6)	96 (6)	101 (7)	96 (6)
phenylbutazone	97 (7)	99 (7)	101 (6)	104 (8)	103 (8)	97 (7)	100 (6)	92 (6)	91 (6)	103 (7)	104 (8)	100 (7)	101 (7)	100 (7)
chloramphenicol	94 (5)	103 (6)	99 (5)	100 (5)	92 (4)	103 (5)	103 (5)	101 (6)	99 (5)	103 (6)	91 (4)	99 (5)	93 (4)	101 (5)
florfenicol	103 (5)	101 (5)	94 (4)	92 (4)	101 (4)	95 (4)	102 (5)	94 (4)	102 (5)	92 (4)	98 (4)	94 (5)	98 (4)	104 (5)
estrone	91 (6)	94 (6)	101 (7)	102 (7)	100 (7)	93 (6)	99 (7)	97 (7)	92 (6)	104 (8)	98 (6)	102 (7)	98 (6)	102 (7)
17β-estradiol	103 (6)	104 (5)	96 (5)	100 (6)	94 (5)	104 (5)	96 (5)	104 (6)	93 (4)	100 (5)	101 (6)	93 (5)	103 (6)	93 (5)
thiamphenicol	98 (7)	101 (7)	92 (6)	94 (7)	103 (7)	95 (6)	95 (7)	103 (7)	102 (7)	97 (7)	93 (6)	101 (7)	99 (7)	100 (7)
17α-ethinylestradiol	91 (5)	94 (6)	104 (6)	97 (6)	101 (6)	93 (5)	104 (6)	100 (6)	104 (7)	99 (6)	95 (6)	104 (6)	102 (6)	98 (6)

Analysis of Milk Samples. The proposed method was applied to the determination of twenty pharmacologically active substances in seven types of milk (whole, semi skim, skim and raw cow milk; whole and half-skim goat milk; human milk; and powdered milk) from Spain and Morocco. Samples were analysed in triplicate by using the analytical procedure described under Materials and Methods. If the concentration of any analyte lay outside the linear range (**Table 1**), then the sample concerned was diluted with purified water to bring it within the range. Previously, the effect of diluting the milk samples with water prior to analysis was examined by using an amount of 500 g of uncontaminated (whole) cow milk that was supplied with a 4500 ng/kg concentration of each PAS and split into 50 g portions which were diluted with 25, 50, 75 or 100 ml of purified water for analysis with the proposed method. The analyte concentrations thus found were similar to those in the undiluted sample — taking into account the dilution of each sample.

Preliminary freeze-thaw stability tests were conducted with a view to assessing the stability of the analytes in milk at a storage temperature of –20 ºC, which is similar to those used by other authors.[8,13,18,36] An amount of 1 kg of whole cow milk spiked with a 100 ng/kg concentration of each analyte was split into 50 g portions and frozen at –20 ºC; by exception, one portion was analysed in triplicate as described under Material and Methods on the same day. All other portions were subjected to the same analytical procedure in triplicate every 2 days for 1 month following thawing one hour before preparation. Freezing the samples under these conditions was found to suppress any adverse effect of the matrix on analyte stability; in fact, the results were always similar, within the error range for the method (RSD < 8%), to those for the unfrozen sample. Similarly results were obtained with whole cow milk spiked with 2000 ng/kg concentration of each analyte.

Table 4. Pharmacologically Active Substances Detected in the Bovine Milk Samples (± SD, $n = 3$)

milk sample[a]	pharmacologically active substance	concentration found (μg/kg)
raw 1	ketoprofen	0.15 ± 0.01
	estrone	0.075 ± 0.005
	17β-estradiol	3.1 ± 0.2
raw 2	pyrimethamine	0.15 ± 0.01
	phenylbutazone	0.020 ± 0.001
	estrone	0.080 ± 0.006
	17β-estradiol	3.2 ± 0.2
raw 3	phenylbutazone	0.13 ± 0.01
	estrone	0.025 ± 0.001
	17β-estradiol	0.085 ± 0.005
whole 1	none	
whole 2	niflumic acid	0.87 ± 0.04
	mefenamic acid	0.14 ± 0.01
	ketoprofen	0.11 ± 0.01
	diclofenac	0.070 ± 0.004
	phenylbutazone	0.075 ± 0.005
	florfenicol	1.2 ± 0.1
	estrone	0.035 ± 0.002
	17β-estradiol	1.1 ± 0.1
	17α-ethinylestradiol	0.020 ± 0.001
whole 3	niflumic acid	0.18 ± 0.01
	naproxen	0.35 ± 0.02
	flunixin	0.080 ± 0.004
	ketoprofen	1.1 ± 0.1
	pyrimethamine	0.21 ± 0.01
	diclofenac	0.045 ± 0.003
	phenylbutazone	0.060 ± 0.004
	florfenicol	0.10 ± 0.01
	estrone	0.13 ± 0.01
	17β-estradiol	1.2 ± 0.1
	17α-ethinylestradiol	0.035 ± 0.002
whole 4[b]	none	
whole 5[b]	17β-estradiol	5.4 ± 0.3
	17α-ethinylestradiol	0.11± 0.01
whole 6[b]	estrone	3.5 ± 0.2
semi skim 1	none	
semi skim 2	niflumic acid	0.075 ± 0.003
	triclosan	0.35 ± 0.02
	phenylbutazone	0.21 ± 0.01
	estrone	1.2 ± 0.1
	17β-estradiol	2.3 ± 0.1
skim 1	niflumic acid	0.015 ± 0.001
	naproxen	0.14 ± 0.01
	flunixin	0.065 ± 0.003
	ketoprofen	0.15 ± 0.01
	pyrimethamine	0.070 ± 0.005
	diclofenac	0.025 ± 0.001
	phenylbutazone	0.065 ± 0.005
	estrone	0.045 ± 0.003
	17β-estradiol	1.1 ± 0.1
skim 2	niflumic acid	0.085 ± 0.004
	naproxen	0.35 ± 0.02
	ketoprofen	0.060 ± 0.004
	pyrimethamine	0.17 ± 0.01
	diclofenac	0.090 ± 0.005
	florfenicol	0.025 ± 0.001

	estrone	0.060 ± 0.004
	17β-estradiol	1,2 ± 0.1
	17α-ethinylestradiol	0.025 ± 0.001

[a] From Spain; [b] From Morocco

Table 5. Pharmacologically Active Substances in Human, Goat and Powdered Milk Samples (± SD, $n = 3$)

milk sample	pharmacologically active substance	Concentration found (μg/kg)
human breast 1	triclosan	0.16 ± 0.01
	17α-ethinylestradiol	0.035 ± 0.002
human breast 2	ibuprofen	0.37 ± 0.02
	triclosan	0.25 ± 0.02
	estrone	0.055 ± 0.004
	17β-estradiol	0.54 ± 0.03
	17α-ethinylestradiol	0.045 ±0.003
human breast 3	naproxen	1.9 ± 0.1
	estrone	0.17 ± 0.01
	17β-estradiol	0.49 ± 0.03
goat (whole)	none	
goat (half-skim)	niflumic acid	0.085 ± 0.005
	flunixin	0.095 ± 0.006
powdered 1–2	none	

The results for the three raw cow milk samples (**Table 4**) indicate the presence of the two natural hormones studied (estrone and 17β-estradiol) in amounts from 0.02 and 3.2 μg/kg. These samples additionally contained other PAS (ketoprofen, pyrimethamine and phenylbutazone) at levels below 0.15 μg/kg. Their contents in ketoprofen were lower than those reported by Daeseleire *et al.*[11] One analyte frequently found in raw cow milk,[11,16] flunixin, was not detected in our samples. All whole milk samples except no. 1 (Spain) and no. 4 (Morocco) contained several PAS. Worth special note are samples 2 and 3, which contained up to 12 analytes including non-steroidal anti-inflammatories (niflumic, naproxen, flunixin, mefanamic acid, ketoprofen, diclofenac and phenylbutazone), hormones (estrone, 17β-estradiol and 17α-ethinylestradiol) and antibacterials (pyrimethamine and florfenicol). **Figure 2B** shows the chromatogram for sample 3, which contained 11 PAS. On the other hand, the whole milk samples from Morocco only contained hormones among the studied PAS. The half-skim and skim milk samples contained much lower levels of PAS than the whole milk samples; by exception, hormones were detected at similar levels in the three types of milk, which is consistent with previous results of Courant *et al.*[37] Only diclofenac among the analytes present in the cow milk samples has a maximum residue limit (MRL) for milk;[3] in any case, its content was always below such an MRL (0.1 μg/kg). Some samples contained florfenicol, use of which on animals producing milk for human consumption is banned by EU Regulations.[3] One other legally restricted compound is thiamphenicol (MRL 50 μg/kg), which, however, was found in none of the samples.

The goat milk samples (whole and semi skim) were found to contain niflumic acid and flunixin at concentrations of 0.085 and 0.095 μg/kg, respectively (**Table 5**). On the other hand, neither sample of powdered milk for infant feeding contained any of the PAS studied.

As stated above, the samples included human milk from lactating women who volunteered for the study. As can be seen from **Table 5**, the antiseptic triclosan, which is widely used in a variety of personal care products, was detected in two samples; its concentrations (0.16 and 0.25 μg/kg) are within the range reported by other authors for this type of milk (<0.018–0.95 μg/kg).[36] Ibuprofen and naproxen were also detected in two samples (no. 2 and 3); these analytes are among the most commonly used medical anti-inflammatories. Naturally occurring hormones, dietary and synthetic hormones were found in the three human milk samples. The 17β-estradiol concentration in them was lower than that reported by Choi *et al.* (7.9–18.5 μg/kg) for human milk.[24] By way of example, **Figure 2C** shows the chromatogram obtained in the analysis of human milk sample 2 with the proposed method. As can be seen, it exhibited no peaks for the matrix, but only for the five PAS and the internal standard.

In summary, the proposed SPE–GC–MS method allows the simultaneous determination of different types of pharmacologically active substances (antibacterials, non-steroidal anti-inflammatory, hormones, antiseptics, lipid reguladors, β-blokers and anti-epileptics) in milk samples. The method features good linearity, accuracy and precision. Its limits of detection (0.2–1.2 ng/kg) are better than those provided by existing methods for the determination of PAS in milk samples (LOD, 0.59–2.69 μg/L,[12] 0.46–2.86 μg/L,[18] 0.5–50 μg/L[29]). Also, recoveries with the proposed method ranged from 91 to 104%, which indicates that matrix interferences were reduced or completely suppressed during the precipitation of milk proteins and clean-up in the SPE module, and also that derivatization of the PAS with the BSTFA+1% TMCS mixture was quantitative. Therefore, our method is highly suitable for monitoring PAS residues in milk from different sources (cow, goat, lactating women).

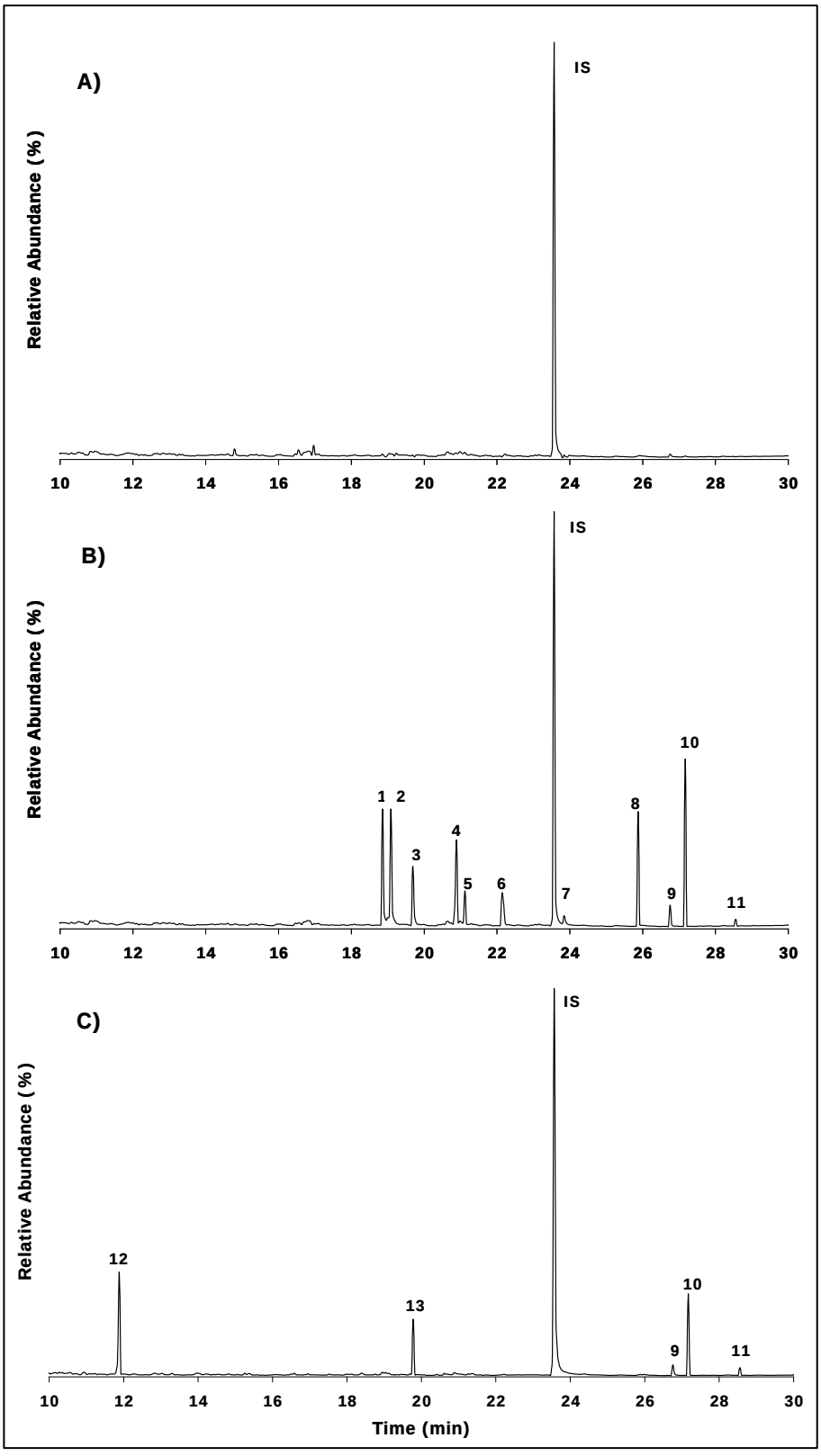

Figure 2. GC–MS chromatograms in the SIM mode for whole cow milk sample 1 (A) and 3 (B), and human milk sample 2 (C) (see Tables 4 and 5). Peaks: (1) niflumic acid; (2) naproxen; (3) flunixin; (4) ketoprofen; (5) pyrimethamine; (6) diclofenac; (7) phenylbutazone; (8) florfenicol; (9) estrone; (10) 17β-estradiol; (11) 17α-ethinylestradiol; (12) ibuprofen; (13) triclosan; (IS) internal standard.

ABBREVIATIONS USED

BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; IS, internal standard; IV, injection valve; MRLs, maximum residue limits; PAS, pharmacologically active substances; RSD, relative standard deviation; SD, standard deviation; SIM, selected ion monitoring; SPE, solid-phase extraction; TMCS, trimethylchlorosilane.

SAFETY

All products were handled with care, using efficiently ventilated hoods, wearing latex gloves and avoiding inhalation or skin contact since pharmaceuticals are usually toxic.

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REFERENCES

(1) Katritzky, A. R.; Dobchev, D. A.; Hür, E.; Fara, D. C.; Karelson, M. QSAR treatment of drugs transfer into human breast milk. *Bioorg. Med. Chem.* **2005**, *1* , 1623–1632

(2) Codex Alimentarius Commission. Codex Veterinary Drug Residues in Food Online Database. Last update on March 10, 2011. http://www.codexalimentarius.net/vetdrugs/data/index.html?lang=en

(3) Council regulation (EEC) No. 37/2010 of 20 December 2009 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal* **2010**, *L15*, 1–72.

(4) Perez, N. ; Gutierrez, R. ; Noa, M. ; Diaz, G.; Luna, H.; Escobar, I.; Munive, Z. Liquid chromatographic determination of multiple sulfonamides, nitrofurans, and chloramphenicol residues in pasteurizad milk. *J. AOAC Int.* **2002**, *85*, 20–24.

(5) Greiner-Sosanko, E.; Lower, D. R.; Virji, M. A.; Krasowski, M. D. Simultaneous determination of lamotrigine, zonisamide, and carbamazepine in human plasma by high–performance liquid chromatography. *Biomed Chromatogr.* **2007**, *21*, 225–228.

(6) De Brabander, H.F.; Noppe, H.; Verheyden, K.; Vanden Bussche, J.; Wille, K.; Okerman, L.; Vanhaecke, L.; Reybroeck, W.; Ooghe, S.; Croubels, S. Residue analysis : Future trends from a historical perspective. *J. Chromatogr. A* **2009**, *1216*, 7964–7976.

(7) Wen, Y.; Zhang, M.; Zhao, Q.; Feng, Y. Q. Monitoring of five sulfonamide antibacterial residues in milk by in-tube solid-phase microextraction coupled to high-performance liquid chromatography. *J. Agric. Food Chem.* **2005**, *53*, 8468–8473.

(8) Huang, J. F.; Zhang, H. J.; Feng, Y. Q. Chloramphenicol extraction from honey, milk, and eggs using polymer monolith microextraction followed by liquid chromatography–mass spectrometry determination. *J. Agric. Food Chem.* **2006**, *54*, 9279–9286.

(9) Moors, M.; Massart, D. L. Evaluation of solid-phase extraction of basic drugs from human milk. *J. Pharm. Biomed. Anal.* **1991**, *9*, 129–139.

(10) Shimoyama, R.; Ohkubo, T.; Sugawara, K. Monitoring of carbamazepine and carbamazepine 10,11-epoxide in breast milk and plasma by high–performance liquid chromatography. *Ann. Clin. Biochem.* **2000**, *37*, 210–215.

(11) Daeseleire, E.; Mortier, L.; De Ruyck, H.; Geerts, N. Determination of flunixin and ketoprofen in milk by liquid chromatography–tandem mass spectrometry. *Anal. Chim. Acta* **2003**, *488*, 25–34.

(12) Dowling, G.; Gallo, P.; Fabbrocino, S.; Serpe, L.; Regan, L. Determination of ibuprofen, ketoprofen, diclofenac and phenylbutazone in bovine milk by gas chromatography–tandem mass spectrometry. *Food Addit. Contam.* **2008**, *25*, 1497–1508.

(13) Yan, W.; Li, Y.; Zhao, L.; Lin, J. M. Determination of estrogens and bisphenol A in bovine milk by automated on–line C_{30} solid–phase extraction coupled with high-performance liquid chromatography–mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 7539–7545.

(14) Rejtharová, M.; Rejthar, L. Determination of chloramphenicol in urine, feed water, milk and honey samples using imprinted polymer clean-up. *J. Chromatogr. A* **2009**, *1216*, 8246–8253.

(15) Ito, Y.; Oka, H.; Ikai, Y.; Matsumoto, H.; Miyazaki, Y.; Nagase, H. Application of ion–exchange cartridge clean-up in food analysis V. Simultaneous determination of sulphonamide antibacterials in animal liver and kidney using high performance liquid chromatography with ultraviolet and mass spectrometric detection. *J. Chromatogr. A* **2000**, *898*, 95–102.

(16) Boner, P.L.; Liu, D.D.W.; Feely, W.F.; Wisocky, M. J.; Wu, J. Determination and confirmation of 5-hydroxyflunixin in raw bovine milk using liquid chromatography tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3753–3759.

(17) Kubala-Drincic, H.; Bazulic, D.; Sapunar-Postruznik, J.; Grubelic, M.; Stuhne, G. Matrix solid-phase dispersion extraction and gas chromatography determination of chloramphenicol in muscle tissue. *J. Agric. Food Chem.* **2003**, *51*, 871–875.

(18) Dowling, G.; Gallo, P.; Malone, E.; Regan, L. Rapid confirmatory analysis of non–steroidal anti–inflammatory drugs in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 8117–8131.

(19) Cardenas, S.; Gallego, M.; Valcárcel, M.; Ventura, R.; Segura, J. A partially automated pretreatment module for routine analyses for seventeen non-steroid antiinflammatory drugs in race horses using gas chromatography–mass spectrometry. *Anal. Chem.* **1996**, *68*, 118–123.

(20) Peters, R. J. B.; Bolck, Y. J. C.; Rutgers, P.; Stolker, A. A. M.; Nielen, M. W. F. Multi-residue screening of veterinary drugs in eggs, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 8206–8216.

(21) Azzouz, A.; Souhail, B.; Ballesteros, E. Continuous solid-phase extraction and gas chromatography–mass spectrometry determination of pharmaceuticals and hormones in water samples. *J. Chromatogr. A* **2010**, *1217*, 2956–2963.

(22) Gikas, E.; Kormall, P.; Tsipi, D.; Tsarbopoulos, A. Development of a rapid and sensitive SPE–LC–ESI MS/MS method for the determination of chloramphenicol in seafood. *J. Agric. Food Chem*. **2004**, *52*, 1025–1030.

(23) Shen, J.; Xia, X.; Jiang, H.; Li, C.; Li, J.; Li, X.; Ding, S. Determination of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in poultry and porcine muscle and liver by gas chromatography–negative chemical ionization mass spectrometry. *J. Chromatogr. B* **2009**, *877*, 1523–1529.

(24) Choi, M.H.; Kim, K.R.; Hong, J. K..; Park, S.J.; Chung, B.C. Determination of non-steroidal estrogens in breast milk, plasma, urine and hair by gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2221–2228.

(25) Weigel, S.; Kallenborn, R.; Hühnerfuss, H. Simultaneous solid-phase extraction of acidic, neutral and basic pharmaceuticals from aqueous samples at ambient (neutral) pH and their determination by gas chromatography–mass spectrometry. *J. Chromatogr. A* **2004**, *1023*, 183–195.

(26) Shao, B.; Zhao, R.; Meng, J.; Xue, Y.; Wu, G.; Hu, J.; Tu, X. Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography–tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *548*, 41–50.

(27) Koesukwiwat, U.; Jayanta, S.; Leepipatpiboon, N. Solid-phase extraction for multiresidue determination of sulfonamide, tetracyclines, and pyrimethamine in bovine's milk. *J. Chromatogr. A* **2007**, *1149*, 102–111.

(28) Botsoglou, N. A.; Fletouris, D. J. Residual antibacterials in food. In *Handbook of Food Analysis*, edition 2; Nollet, L. M. L., Ed.; Marcel Dekker, Inc.: New York, 2004; Vol. 2, pp 931–1035.

(29) Ortelli, D.; Cognard, E.; Jan, P.; Edder, P. Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. *J. Chromatogr. B* **2009**, *877*, 2363–2374.

(30) Nagata, T.; Oka, H. Detection of residual chloramphenicol, florfenicol, and thiamphenicol in yellowtail fish muscle by capillary gas chromatography–mass spectrometry. *J. Agric. Food Chem*. **1996**, *44*, 1280–1284.

(31) Jurado-Sánchez, B.; Ballesteros, E.; Gallego, M. Gas chromatographic determination of *N*-nitrosamines in beverages following automatic solid-phase extraction. *J. Agric. Food Chem.* **2007**, *55*, 9758–9763.

(32) Jurado-Sánchez, B.; Ballesteros, E.; Gallego M. Continuous solid-phase extraction method for the determination of amines in human urine following on-line microwave-assisted acid hydrolysis. *Anal. Bioanal. Chem*. **2010**, *396*, 1929–1937.

(33) Ballesteros, E.; Parrado, M. J. Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticides in natural and drinking waters. *J. Chromatogr. A* **2004**, *1029*, 267–273.

(34) Council Directive (EEC) No. 96/23 of 12 August 2002 concerning the performance of analytical methods and the interpretation of results. *Official Journal* **2002,** *L221*, 8−36.

(35) Seo, J.; Kim, H. Y.; Chung, B. C.; Hong, J. *J. Chromatogr. A* **2005**, *1067*, 303−309.

(36) Allmyr, M.; Adolfsson-Erici, M.; McLachlan, M. S.; Sandbourgh-Englund, G. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *Sci. Total Environ.* **2006**, 372, 87–93.

(37) Courant, F.; Antignac, J. P.; Laille, J. ; Monteau, F. ; Andre, F.; Le Bizec, B. Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples. *J. Agric. Food Chem.* **2008**, 56, 3176–3184.

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Determination of residual pharmaceuticals in edible animal tissues by continuous solid-phase extraction and gas chromatography–mass spectrometry

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ABSTRACT

A sensitive, reliable method using continuous solid-phase extraction and gas chromatography-mass spectrometry was developed for the simultaneous determination of twenty pharmaceuticals including antibacterials, anti-epileptics, antiseptics, β-blockers, lipid regulators, hormones and non-steroidal anti-inflammatories at trace levels in edible animal tissues. The procedure involves deproteination and delipidation of samples by precipitation/centrifugation/filtration, followed by sample enrichment and cleanup by continuous solid-phase extraction. The proposed method was validated with quite good analytical results including low limits of detections (0.4–2.7 ng kg^{-1} for 2 g of sample) and good linearity ($r^2 > 0.995$) throughout the studied concentration ranges. In addition, the method is quite accurate (recoveries ranged from 92 to 101%) and precise (within-day and between-day RSD values were less than 7%), which allows the determination of residual pharmaceuticals in tissues from agricultural farm and fish hatchery animals (pig, veal, lamb and chicken muscle, kidney and liver; and salmon, sea bass and sole flesh). The analytes most frequently found in the studied samples were the hormones estrone and 17β-estradiol, and the antibacterials florfenicol and pyrimethamine.

Keywords: Pharmaceutical residues; Edible animal tissues; Continuous solid-phase extraction; Gas chromatography–mass spectrometry.

1. Introduction

The usually high density of animals grown in agricultural facilities and fish hatcheries can increase the potential for disease outbreak. This raises the need for medications to be given therapeutically to treat existing infections, or prophylactically, to minimize the impact of an outbreak spreading across an animal population. A portion of administered pharmaceuticals remains in the animal's body, but a significant fraction is discharged into the environment by excretion. The ensuing risks of pharmaceutical residues (PRs) reaching edible products and the potential health hazards associated to their consumption have become a public safety issue [1]. Veterinary and human pharmaceuticals can also reach animal foods by effect of animals eating or drinking contaminated feed or water. Residues may include the unaltered parent compound and its metabolites and/or conjugates, and may have direct toxic effects on consumers (e.g. allergic reactions in hypersensitive individuals or the development of resistant bacterial strains in response to some antibacterials) [2]. The Codex Alimentarius Commission of the Food and Agriculture Organization, the World Health Organization [3] and the European Community [4] have set maximum residue limits (MRLs) for a variety of pharmaceuticals in foodstuffs of animal origin [5].

The extremely low (parts-per-billion) levels at which pharmaceutical residues can be encountered in so highly complex biological matrices as milk, meat or eggs pose a major analytical challenge that can only be met by using effective procedures for the selective extraction of analytes, removal of coextractants, and sensitive, specific detection of the analytes. The sample preparation procedures typically used with pharmaceutical residues include homogenization and/or enzymatic digestion of the homogenate, extraction/cleanup, enrichment, and, where needed, derivatization of the analyte [1]. Liquid–liquid extraction [6,7], solid-phase extraction (SPE) [8,9] and solid-phase microextraction [10] are common choices for extraction and cleanup in this context. Emulsification during extraction of the sample is usually avoided by prior protein precipitation or delipidation. Proteins in meat, egg and milk samples can be precipitated and fat delipidated by adding methanol [11,12], acetonitrile [13,14], hydrochloric acid [15,16] or trichloroacetic acid [17], for example. In some cases, deproteination can be accomplished simply by heating the sample in the presence of a buffer [18], and delipidation by freezing–lipid filtration [11]. Finally, SPE of pharmaceutical residues usually relies on sorbent materials such as octadecyl bonded

silica (RP-C_{18}) [7,12,19], Oasis-HLB (polystyrene–divinylbenzene–*N*-vinypyrrolidone terpolymer) [8,9,20,21], Oasis MCX (a strong cation-exchange mixed-mode polymer) [22] or amino-propyl-NH_2 [11,12,18,23] .

Immunoassays are often used in control analyses of pharmaceutical residues in meat products. The analyte (antigen) binds highly specifically to the antibodies raised against it [24]. The high sensitivity of immunoassays facilitates the rapid screening of large numbers of samples for individual pharmaceuticals, which has led to its gradually superseding multi-residue techniques [1]. Pharmaceutical residues present in various animal meat products, and their derivatives, have been determined by liquid chromatography with fluorescence or electrochemical detection [25,26]. This methodology is simple and rapid, but has limited sensitivity. In combination with mass spectrometry, however, liquid chromatography allows PRs in various types of foods to be determined with good sensitivity and accuracy [6,10,14]. Recently, new approaches using the potential of liquid chromatography coupled with tandem mass spectrometry (MS–MS) [8,12,15,16,22,23] or time-of-flight mass spectrometry [13,14] have been developed for multiclass residue screening. The prevalent instrumental methods for determining PRs in meat samples, which are based on gas chromatography–mass spectrometry (GC–MS), provide increased sensitivity, specificity and chromatographic resolution, but require derivatization of the analytes prior to their determination [7,9,11,21,27,28]. Gas chromatography–tandem mass spectrometry has also been used for the determination of hormones in kidney and meat, and milk and egg [18,29]; and that of non-steroidal anti-inflammatories at the microgram-per-kilogram level in milk [30].

The aim of this work was to develop a reliable multi-residue method for the simultaneous determination of selected pharmaceuticals (antibacterials, non-steroidal anti-inflammatory, hormones, antiseptics, β-blockers, lipid regulators and anti-epileptics) in tissues from agricultural farm and fish hatchery animals (viz. pig, veal, lamb and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh). The proposed method, which improves on an earlier one developed by our group to determine pharmaceuticals in water samples [21], involves the removal of protein and lipids —which can interfere with the determination of PRs— from the sample matrix by precipitation/centrifugation/filtration. Following cleanup of the resulting supernatant, the analytes are preconcentrated by continuous solid-phase extraction on an Oasis-HLB

column for conversion into their silyl derivatives to improve sensitivity and resolution in their GC–MS measurements.

2. Experimental

2.1. Standards and reagents

All products were handled with care, using latex gloves, a respiratory protection device and fume hoods. Stock solutions of the 20 pharmaceuticals studied, which were supplied in the highest available purity by Sigma–Aldrich (Madrid, Spain), were prepared at a 1 g L^{-1} concentration in methanol and stored at 4 °C in the dark. Triphenylphosphate and the derivatizing reagents [*N*,*O*-*bis*(trimethylsilyl)trifluoro-acetamide (BSTFA) and trimethylchlorosilane (TMCS)] were purchased from Fluka (Madrid, Spain). Oasis-HLB (particle size 50–65 μm) was obtained from Waters (Madrid, Spain). Solvents (acetonitrile, methanol and ethyl acetate) were obtained from Merck (Darmstadt, Germany). Millex-LG filter units (hydrophilic, PTFE, pore size 0.20 μm, diameter 25 mm, filtration area 3.9 cm^2) were supplied by Millipore Ibérica, S.A. (Madrid, Spain). The standard solutions used to prepare spiked samples were obtained by diluting the stocks in purified water from a Milli-Q System (Millipore, Bedford, MA, USA) and adjusted to pH 7.

2.2. Instruments and apparatus

GC–MS analyses were performed on a Focus GC instrument (Thermo Electron SA, Madrid, Spain) interfaced to a DSQ II mass spectrometer controlled via a computer running XCalibur software. The chromatograph was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) coated with 5% phenylmethylpolysiloxane (Supelco, Madrid, Spain). Helium (purity 6.0) at a flow rate of 1 mL/min was employed as the carrier gas. For pharmaceutical determinations, the column temperature was initially kept at 70°C for 1 min, raised to 150 °C at 14 °C min^{-1}, and then to 290 °C at 6 °C min^{-1}. The injection port and transfer line temperatures were kept at 270 and 280 °C, respectively. The ion source temperature for the 70 eV electron impact ionization mode was 200 °C.

Table 1. Analytical figures of merit of the proposed method for determination of residual pharmaceuticals in edible animal tissues.

Therapeutic class	Compounds	Retention time (min)	LOD (ng kg^{-1})	Linear range (ng kg^{-1})	r[a]	RSD (%) (n=11) Within-day	RSD (%) (n=11) Between-day	m/z[b,c]
Non steroidal anti-inflammatories	Diclofenac	22.3	1.1	3.3-10000	0.996	5.1	5.8	**214**, 242, 367
	Flunixin	19.7	0.5	1.6-10000	0.996	5.0	5.6	251, 263, **353**
	Ibuprofen	11.9	0.5	1.5-10000	0.995	4.8	5.3	**160**, 234, 263, 278
	Ketoprofen	21.0	0.5	1.5-10000	0.999	5.8	6.7	73, **282**, 311
	Mefenamic acid	20.9	0.4	1.3-10000	0.996	4.9	5.5	208, **223**, 313
	Naproxen	19.3	1.0	3.2-10000	0.996	5.4	6.0	**185**, 243, 302
	Niflumic acid	18.6	0.6	1.8-10000	0.996	4.5	5.1	**236**, 263, 353
	Phenylbutazone	24.0	2.3	7.4-10000	0.997	4.9	5.4	**183**, 252, 308
Hormones	Estrone	26.9	2.4	7.9-10000	0.998	5.4	6.3	218, 257, **342**
	17β-estradiol	27.3	2.6	8.3-10000	0.998	5.6	6.4	285, **416**
	17α-ethinylestradiol	28.7	2.7	8.7-10000	0.996	6.2	6.7	**425**, 440
Antibacterials	Chloramphenicol	24.5	0.4	1.3-10000	0.996	5.0	5.5	208, **225**
	Florfenicol	26.2	0.5	1.6-10000	0.998	5.8	6.5	**257**, 414
	Pyrimethamine	21.3	2.5	8.0-10000	0.998	5.5	5.9	**377**, 392, 394
	Thiamphenicol	27.9	0.5	1.5-10000	0.998	6.3	6.9	242, 257, **330**
Anti-epileptics and β-blokers	Carbamazepine	22.0	0.6	1.9-10000	0.997	4.1	5.0	**193**, 236
	Metoprolol	19.0	1.4	4.5-10000	0.996	5.3	6.2	72, **223**
	Propranolol	20.6	1.6	5.2-10000	0.998	5.1	6.1	72, **215**
Antiseptic and lipid regulators	Clofibric acid	11.3	1.5	5.0-10000	0.995	5.2	6.2	128, **143**, 286
	Triclosan	19.9	1.5	4.8-10000	0.997	4.6	5.3	200**, 347**

[a] r, correlation coefficient.
[b] The base peaks under for quantification are boldfaced.
[c] *m/z* for IS (triphenylphosphate): 77, 325, **326**.

The mass spectrometer was operated in the selected ion monitoring (SIM) mode. The MS instrument was set in full scan mode (70–500 amu) for identification, and the quantification fragments (*m*/z) for each pharmaceutical (SIM mode) were selected according to abundance (highest sensitivity) and specific criteria. The *m/z* values used for each analyte are listed in Table 1. The time for solvent delay was set to 8 min. In all analyses, a volume of 1 μL of the silylated derivatives was injected in the split mode (1:20 ratio) and the resulting peak area was used as analytical signal for quantification.

The flow system comprised a peristaltic pump (Gilson Minipuls-3, Villiers-le-Bel, France), two Rheodyne 5041 injection valves (Cotati, CA, US), poly(vinylchloride) pumping tubes, PTFE tubing of 0.5 mm ID and standard connectors. The sorbent column was prepared by packing a PTFE column with 60 mg of Oasis-HLB sorbent material as described elsewhere [21].

2.3. Sampling

Tissues from agricultural farm and fish hatchery animals (viz. pig, veal, lamb and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh) were purchased at a local supermarket. Samples were all received in frozen form and stored at –20 ºC until analysis.

2.4. Sample pretreatment

Figure 1 depicts the procedure used to prepare the samples and determine the residual pharmaceuticals. Tissues were homogenized in an A320R1 grinder from Moulinex (Barcelona, Spain). A portion of about 2 g of tissue homogenate was then weighed into a 50 mL round polypropylene centrifuge tube and mixed with 4 mL of water and 6 mL of acetonitrile in a vortex mixer (REAX Control, Heidolph, Kelheim, Germany) for 30 s. This was followed by centrifugation on a Centrofriger BL-II apparatus (JP Selecta, Barcelona, Spain) at 4000 rpm for 10 min (4 ºC). Next, the supernatant was passed through a 0.20 μm Millex-LG filter. The filtered supernatant was carefully evaporated under a stream of ultrahigh-purity N_2 to a final volume of 200 μL and redissolved to 5 mL with purified water a pH 7. The pretreated sample was thus made ready for continuous solid-phase extraction.

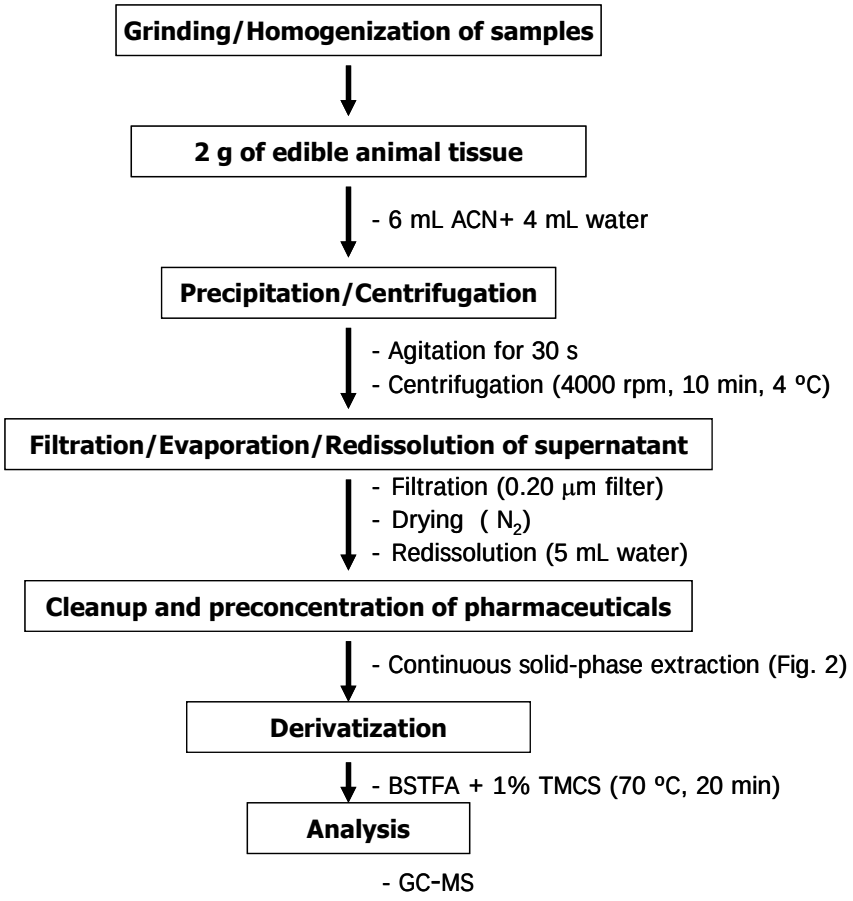

Fig. 1. Flow chart of the procedure for determining residual pharmaceuticals in edible animal tissues.

2.5. Cleanup and continuous solid-phase extraction

Figure 2 shows the continuous system used for the solid-phase extraction of pharmaceutical residues from animal tissues. A volume of 5 mL of pretreated sample or standard aqueous solution containing a 1.3–10 000 ng kg^{-1} concentration of each analyte at pH 7 was continuously passed at 4 mL min^{-1} through the sorbent column placed in the loop of injection valve IV_1. All pharmaceuticals were sorbed and the sample matrix was sent to waste. Simultaneously, the loop of IV_2 was filled with eluent containing the IS (500 μg L^{-1} triphenylphosphate in ethyl acetate) by means of a syringe. Any residual water remaining inside the column or connectors was flushed by passing an air stream at 4 mL min^{-1} through the carrier line for 2 min. Next, IV_2 was switched to have the loop contents (400 μL) injected into the same air stream used in the drying step in order to elute PRs —in the opposite direction of sample aspiration. The organic extract was collected in a conical glass insert of 0.5 mL and concentrated to a volume of 35 μL

under a stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using an internal standard. Next, a volume of 70 µL of BSTFA + 1% TMCS was added and the vials were heated at 70 ºC for 20 min. Finally, 1 µL aliquots of the silylated derivatives were analysed by GC–MS in the SIM mode. The sorbent column was conditioned with 1 mL of methanol and 5 mL of purified water between samples. Under these conditions, the column remained serviceable for about 2 months.

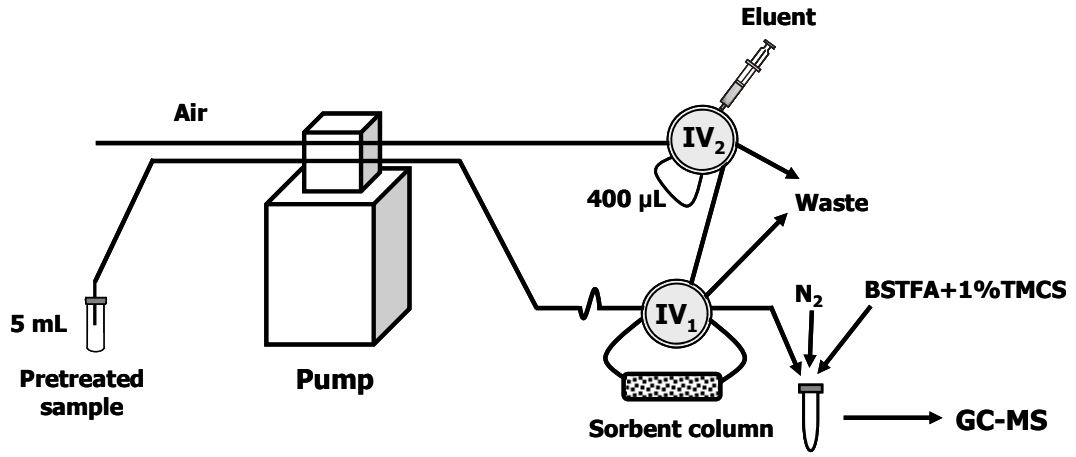

Fig. 2. Continuous flow system fort the cleanup and preconcentration of pharmaceutical residues in edible animal tissues. *IV*, injection valve.

3. Results and discussion

In previous work, we developed a method for the determination of pharmaceuticals in environmental water samples [21]. The method was optimized by examining the performance of various sorbents (viz. Oasis–HLB, LiChrolut EN, XAD-2, XAD-4, RP-C18, Florisil, Silica Gel and Isolute NH_2) in the preconcentration and solid-phase extraction of the pharmaceuticals and Oasis–HLB selected on the grounds that it retained all analytes virtually completely. Also, various organic solvents including ethyl acetate, acetonitrile, acetone, methanol, ethanol, 2-propanol and dichloromethane were examined as eluents and ethyl acetate was found to be the most efficient in eluting the pharmaceuticals.

In this work, we analysed a greater number of compounds and examined the effect of all variables potentially influencing retention and elution of the 20 pharmaceuticals studied, in addition to those having some effect on their derivatization with a 99:1 BSTFA–TMCS mixture. To reach the best operational conditions, we used a

univariate method, where one variable is varied by maintaining constant the rest. The optimum range and selected values for each variable are given in Table 2. The high complexity of the matrix of edible animal tissues required the removal of proteins and lipids in order to avoid their interference with the GC–MS determination of the pharmaceuticals [11]. Below are described the effects of the variables influencing the removal of proteins and lipids by precipitation and centrifugation.

Table 2. Variables affecting the meat and fish tissues pretreatment, continuous solid-phase extraction and derivatization processes of pharmaceutical residues.

Variable	Optimum range (selected value)
Sample pretreatment (precipitation and centrifugation)[a]	
Volume of water–acetonitrile mixture (3:2, v/v, mL)	9–20 (10)
Centrifugation rate (rpm)	3500–5000 (4000)
Centrifugation time (min)	5–30 (10)
Centrifugation Temperature (ºC)	0–10 (4)
Continuous solid-phase extraction[b]	
Sample pH	6.5–7.5 (7)
Amount of sorbent (Oasis-HLB, mg)	55–65 (60)
Volume of eluent (ethyl acetate, μL)	350–450 (400)
Sample flow rate (mL min^{-1})	3.5–4.5 (4)
Air flow rate (mL min^{-1})	3.5– 4.5 (4)
Breakthrough volume (mL)	1–200 (5)
Percentage of acetonitrile (%)	0–15
Derivatization (silylation of pharmaceuticals)[b]	
Percentage of TMCS in BSTFA[c]	1–15 (1)
Reaction time (min)	15–25 (20)
Temperature of reaction (ºC)	65–75 (70)

[a] To 2 g of samples.
[b] These variables have been previously optimized [21].
[b] TMS: trimethylchlorosilane; BSTFA: *N,O*-bis-(trimethylsilyl)trifluoacetamide.

3.1. Variables influencing sample pretreatment

One preliminary step to be taken towards the intended multicomponent analyses was the development of a generic sample preparation method suitable for extracting the analytes from various types of food matrices of animal origin. In fact, a large amount of lipids (phospholipids, triacylglycerolipids, phosphocholine lipids and cholesterol,

mainly) and protein is co-extracted with the target compounds owing to their high solubility in the organic solvents used to extract pharmaceuticals from animal tissues. Also, lipids and proteins are easily adsorbed in different parts of a gas chromatograph such as the injection port and column, which detracts from chromatographic performance. In addition, pharmaceutical residues in meat and fish tissues may be present as conjugates (e.g. modified by glucuronide or acetyl groups) [2]. A number of preparation methods including the removal of lipids and proteins are available for the determination of pharmaceuticals in meat and fish [1,14,31,32]. Many authors prefer acetonitrile over methanol or ethyl acetate since the latter two extract too many matrix compounds and complicate further cleanup as a result. Mol et al. [32] tested a number of solvents and their combinations, and found the best choice to depend on the particular matrix; they selected the water/acetonitrile mixture. In this work, we assessed the efficiency of various solvents (water, acetonitrile, methanol, ethanol and ethyl acetate) and trichloroacetic acid in removing proteins and lipids from meat and fish tissues. For this purpose, an amount of *ca.* 2 g of animal tissue was mixed with a volume of 10 mL of each solvent or solvent mixture (water-methanol, water-acetonitrile and water-ethanol), or a solution of 30% m/v trichloroacetic acid, and centrifuged at 4 ºC at 4000 rpm for 10 min. A 3:2 (v/v) acetonitrile–water mixture caused more than 95% of the protein and lipid content to be removed and proved the most efficient solvent; also, it facilitated separation of the precipitate, which is consistent with previous results [14]. The optimum volume of acetonitrile–water mixture for addition to meat and fish tissues was established by examining its effects over the range 1−20 mL; protein and lipid precipitation was found to peak at 9 mL, so a solvent volume of 10 mL (6 mL acetonitrile + 4 mL purified water) was adopted as optimal. We also examined the effects of the centrifugation rate, temperature and time used with the sample–solvent mixture to facilitate separation of the protein–lipid precipitate from the other components of the sample matrix. The effects of the centrifugation rate, temperature and time were examined over the ranges 1500–5000 rpm, 0–20 ºC and 1–30 min, respectively As can be in Figure 3 for six representative analytes, the signals increase the up to 2500 rpm of centrifugation rate, after which it stays constant. For the other analytes the behaviour is similar. We therefore selected as centrifugation rate 4000 rpm. In the case of the temperature and time of centrifugation, 4 °C and 10, respectively, have been selected as intermediate values in their optimum ranges (Table 2).

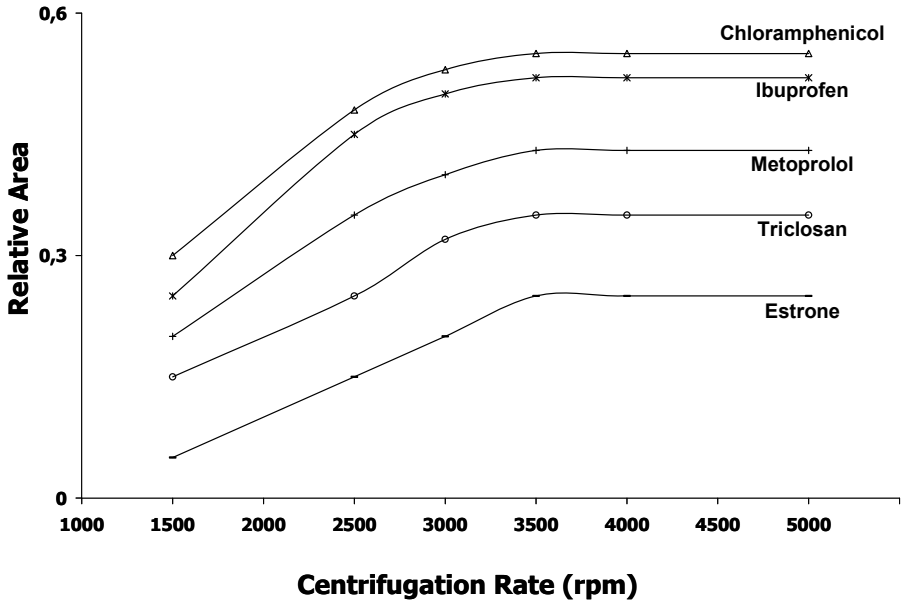

Fig. 3. Effect of centrifugation rate for separating proteins and lipids from the sample matrix.

Once the operating conditions for separating proteins and lipids from the sample matrix by precipitation and centrifugation were established, we checked that the filtered supernatant was compatible with the continuous solid-phase extraction of the target pharmaceuticals. To this end, we examined the effect of acetonitrile on retention of the 20 pharmaceuticals by the sorbent column in the continuous SPE system by preparing aqueous solutions containing a 50 ng L^{-1} concentration of each compound and a variable proportion of acetonitrile from 0 to 50% in a volume of 10 mL. The pharmaceuticals present in the eluate were derivatized as described under Analytical Procedure and determined by GC–MS for comparison with the results obtained in the absence of acetonitrile. Based on them, acetonitrile in proportions below 15 % had no effect on retention. Higher proportions, however, resulted in diminished sorption of the analytes. This was the likely consequence of the special sorption mechanism involved, by which moderately polar organic compounds partitioned between a polar phase (water) and a polymeric sorbent (Oasis-HLB) via polar interactions such as hydrogen bonding between the hydroxyl group in the pharmaceuticals and the underlying sorbent surface. In the presence of a high proportion of acetonitrile in the aqueous sample, the solvent probably broke bonds and helped dissolve the pharmaceuticals, thereby dramatically reducing their adsorption. This entailed lowering the proportion of solvent in the supernatant remaining after the sample treatment, which contained about 40% of acetonitrile, by evaporation to a final volume of 200 μL under a stream of ultrapure N_2

and redissolution in 5 mL of purified water at pH 7 for introduction into the continuous SPE system.

3.2. Analytical performance

The performance and reliability of the proposed method were assessed by determining the regression equation, linear range, analyte detectability and precision for the 20 pharmaceuticals. For this purpose, an amount of 2 g of uncontaminated muscle (chicken) tissue was fortified with 50–200 μL of standard solutions containing all pharmaceuticals at concentrations over the range 1.3–10000 ng kg^{-1}. The fortified samples were pretreated and extracted as described in the Experimental Section (Fig. 1). Chromatographic resolution and efficiency were assessed from plots of analyte-to-IS peak area ratio against analyte concentration. The figures of merit of the proposed method are listed in Table 1. As can be seen, the correlation coefficient was higher than 0.995 (12 points per calibration) in all instances. The limit of detection (LOD, defined as the analyte concentration giving a chromatographic peak equal to three times the corresponding regression standard deviation, *Sy/x*, divided by the slope of the calibration graph) ranged from 0.4 to 2.7 ng kg^{-1}. The precision, calculated as the relative standard deviation for 11 chicken muscle samples spiked with a 50 ng kg^{-1} concentration of each analyte ranged from 4.1 to 6.3% (within-day) and from 5.1 to 6.9% (between-day).

The proposed method for animal tissues was validated in terms of recovery. For this purpose, various types of tissues (muscle, liver, kidney and fish) spiked with a 25 or 100 ng kg^{-1} concentration of a standard mixture of the analytes (before sample pretreatment) were analysed in triplicate (n = 3). The fact that most of the samples contained some residual pharmaceutical allowed recoveries to be calculated by subtracting the previously quantified endogenous compounds from the total contents. The average recoveries thus obtained are listed in Table 3. As can be seen, all analytes were accurately identified; also, the average recoveries (92–101%) for all matrices were quite acceptable. Therefore, the precipitation/centrifugation step, or the cleanup step in the SPE system, efficiently reduced or even completely suppressed matrix interferences.

Table 3. Percent recovery (± SD, n=3) of pharmaceuticals added to animal tissue samples.

Compounds	Chicken muscle (ng kg^{-1})		Veal liver (ng kg^{-1})		Pig kidney (ng kg^{-1})		Sole flesh (ng kg^{-1})	
	25	100	25	100	25	100	25	100
Diclofenac	101 ± 6	95 ± 5	101 ± 6	99 ± 6	94 ± 5	97 ± 6	99 ± 6	100 ± 6
Flunixin	100 ± 5	96 ± 5	100 ± 6	101 ± 6	96 ± 5	98 ± 5	92 ± 5	97 ± 6
Ibuprofen	96 ± 5	101 ± 5	96 ± 5	98 ± 5	100 ± 6	94 ± 5	95 ± 5	96 ± 5
Ketoprofen	99 ± 6	100 ± 7	101 ± 6	96 ± 6	93 ± 5	100 ± 6	97 ± 6	98 ± 6
Mefenamic acid	101 ± 6	95 ± 5	100 ± 5	98 ± 5	95 ± 5	94 ± 5	101 ± 6	99 ± 5
Naproxen	100 ± 6	99 ± 6	95 ± 5	98 ± 6	101 ± 6	93 ± 6	97 ± 6	92 ± 5
Niflumic acid	97 ± 5	101 ± 5	101 ± 5	99 ± 5	92 ± 5	98 ± 5	100 ± 5	99 ± 5
Phenylbutazone	96 ± 5	93 ± 5	92 ± 5	101 ± 5	101 ± 6	97 ± 5	95 ± 5	93 ± 5
Estrone	92 ± 5	98 ± 6	99 ± 6	101 ± 6	96 ± 6	95 ± 6	100 ± 6	97 ± 6
17β-estradiol	99 ± 7	100 ± 6	96 ± 6	94 ± 6	99 ± 6	101 ± 7	95 ± 6	98 ± 6
17α-ethinylestradiol	93 ± 6	97 ± 6	99 ± 7	100 ± 7	95 ± 6	92 ± 6	101 ± 7	100 ± 6
Chloramphenicol	100 ± 6	101 ± 6	97 ± 6	98 ± 6	100 ± 6	93 ± 5	95 ± 6	98 ± 5
Florfenicol	101 ± 6	97 ± 5	97 ± 7	92 ± 6	98 ± 6	100 ± 7	94 ± 6	94 ± 6
Pyrimethamine	98 ± 6	96 ± 6	99 ± 6	101 ± 6	95 ± 6	96 ± 6	101 ± 6	96 ± 6
Thiamphenicol	94 ± 6	97 ± 7	100 ± 7	92 ± 6	96 ± 6	92 ± 6	95 ± 6	101 ± 7
Carbamazepine	101 ± 5	95 ± 5	101 ± 5	99 ± 5	98 ± 5	97 ± 5	93 ± 5	92 ± 5
Metoprolol	100 ± 6	101 ± 6	94 ± 5	96 ± 5	101 ± 6	97 ± 6	97 ± 6	101 ± 6
Propranolol	97 ± 6	101 ± 6	95 ± 5	100 ± 6	97 ± 6	93 ± 6	101 ± 6	94 ± 5
Clofibric acid	98 ± 6	96 ± 5	101 ± 6	99 ± 6	101 ± 5	92 ± 6	98 ± 6	95 ± 5
Triclosan	99 ± 5	94 ± 5	101 ± 6	101 ± 5	98 ± 5	100 ± 5	97 ± 5	95 ± 5

3.3. Analysis of edible animal tissues

The proposed method was successfully applied to the determination of 20 pharmaceuticals in tissues from agricultural farm and fish hatchery animals (viz. lamb, veal, pig and chicken muscle, liver and kidney; and salmon, sea bass and sole flesh). Samples were analysed in triplicate, following the analytical procedure described in the Experimental Section. If the concentration of any analyte fell outside the linear range (Table 2), then the SPE extract from the sample concerned was diluted with eluent (500 μg L^{-1} triphenylphosphate in ethyl acetate) after derivatization of the analytes with BSTFA + 1% TMCS. The results of these tests are shown in Table 4. As can be seen, the hormones estrone and 17β-estradiol were present in all samples except chicken muscle, at concentrations from 0.51 to 6.7 μg kg^{-1}. These concentrations are consistent with others previously found in muscle, kidney and liver tissues [29]. As regards non-steroidal anti-inflammatories, flunixin was detected at concentrations from 1.5 to 3.5 μg kg^{-1} in veal muscle and pig kidney, and phenylbutazone was detected at concentration from 1.2 to 7.8 μg kg^{-1} in lamb and veal muscle and liver chicken. The antibacterials florfenicol and pyrimethamine were found in all samples except chicken muscle; their concentrations were relatively high (0.59–28.2 μg kg^{-1}), but always below their MRLs (from 100 μg kg^{-1} for poultry muscle to 3000 μg kg^{-1} for bovine, ovine and caprine liver) [4]. Other authors detected florfenicol a 260 μg kg^{-1} levels, and pyrimethamine at lower concentrations (0.6–14.0 μg kg^{-1}), in porcine muscle [20]. No other pharmaceuticals were detected. By way of example, Fig. 4 shows the SIM mode chromatograms for samples of veal muscle, chicken liver and sole flesh processed with the proposed SPE-GC–MS method. As can be seen, the chromatogram contained few significant peaks due to the sample matrix, which facilitated identification of the analytes and testifies to the efficiency of the cleanup treatment.

Table 4
Pharmaceuticals detected in edible animal tissues (± SD, μg kg^{-1}, n=3).

Compounds	Muscle[a]	Muscle[b]	Muscle[c]	Muscle[d]	Liver[a]	Liver[b]	Liver[c]	Liver[d]	Kidney[a]	Kidney[b]	Kidney[c]	Flesh[e]	Flesh[f]	Flesh[g]
Diclofenac	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Flunixin	–	1.5 ± 0.1	–	–	–	–	–	–	–	–	3.5 ± 0.2	–	–	–
Ibuprofen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ketoprofen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mefenamic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Naproxen	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Niflumic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phenylbutazone	7.8 ± 0.4	2.8 ± 0.2	–	–	–	–	–	1.2 ± 0.1	–	–	–	–	–	–
Estrone	0.65 ± 0.04	0.99 ± 0.07	0.51 ± 0.03	–	–	1.9± 0.1	0.85 ± 0.05	0.64 ± 0.04	4.5 ± 0.3	–	–	1.3 ± 0.1	0.78 ± 0.05	0.52 ± 0.03
17β-estradiol	1.5 ± 0.1	1.3 ± 0.1	0.82 ± 0.05	–	1.2 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	3.7 ± 0.2	6.7 ± 0.4	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.81 ± 0.05
17α-ethinylestradiol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chloramphenicol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Florfenicol	28.2 ± 1.8	0.59 ± 0.04	18.2 ± 1.1	–	–	3.5 ± 0.2	–	1.5 ± 0.1	27.5 ± 1.8	–	–	3.4 ± 0.2	1.9 ± 0.1	0.60 ± 0.04
Pyrimethamine	14.0 ± 0.8	0.83 ± 0.05	–	–	5.3 ± 0.3	–	3.2 ± 0.2	–	–	1.4 ± 0.1	1.7 ± 0.1	–	–	–
Thiamphenicol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbamazepine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Metoprolol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propranolol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Clofibric acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Triclosan	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Lamb.
[b] Veal.
[c] Pig.
[d] Chicken.
[e] Sea bass.
[f] Sole.
[g] Salmon.

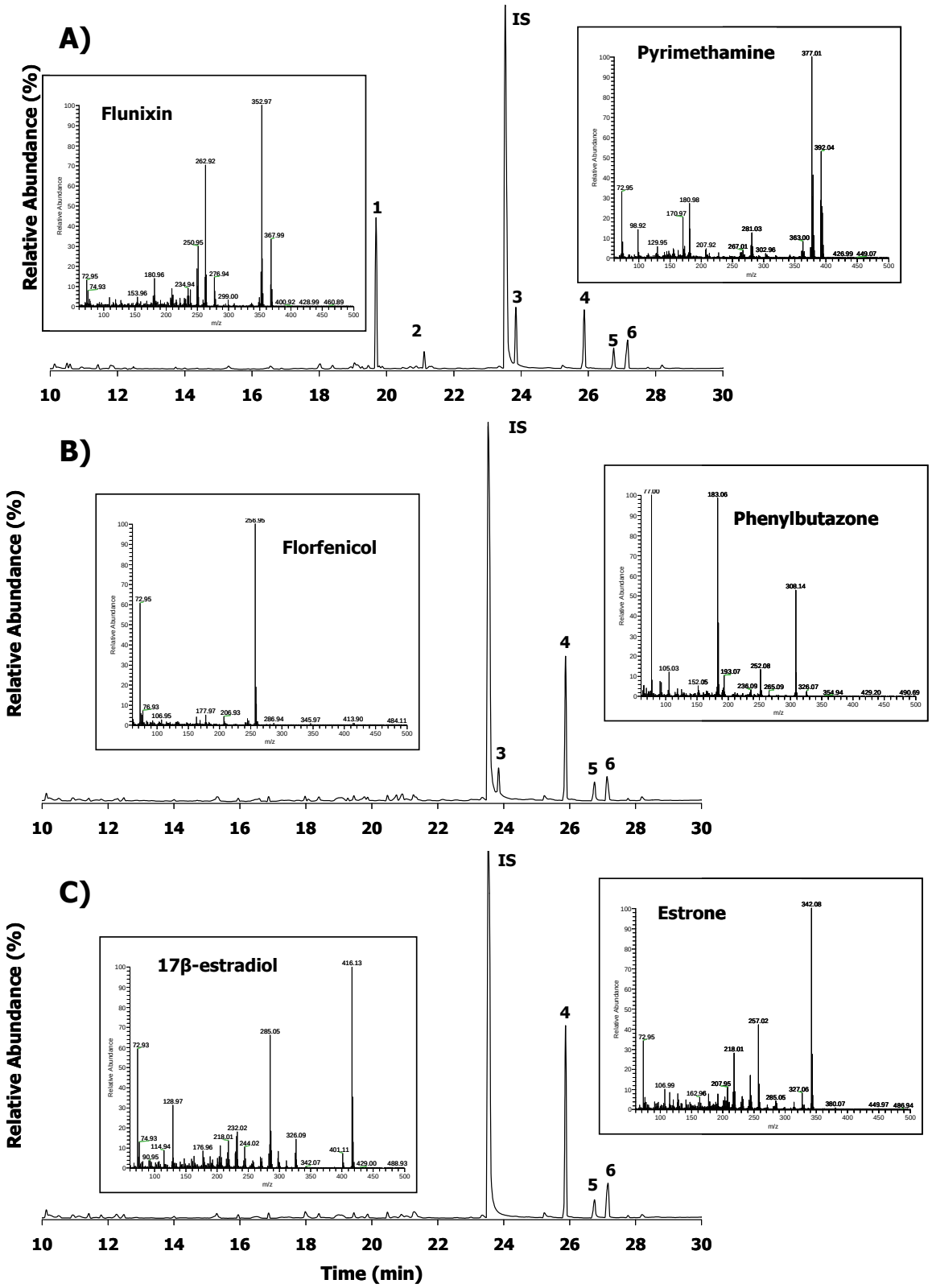

Fig. 4. GC–MS chromatograms obtained in the SIM mode in the analysis of 2 g of veal muscle (A), chicken liver (B) and sole flesh (C) (see Table 4). Peaks: *1*, flunixin; *2*, pyrimethamine; *3*, phenylbutazone; *4*, florfenicol; *5*, estrone; *6*, 17β-estradiol; *IS*, internal standard (triphenylphosphate).

4. Conclusions

A method for the simultaneous determination of 20 pharmaceuticals (antibacterials, non-steroidal anti-inflammatories, antiseptics, anti-epileptics, lipid regulators, β-blockers and hormones) in agricultural farm and fish hatchery animals was developed. Precipitation/centrifugation/filtration of on a mixture of animal tissue with acetonitrile–water and subsequent continuous solid-phase extraction provide a reliable procedure for removing co-extracting interferences (proteins and lipids, mainly) from complex matrices and facilitate preconcentration of the pharmaceuticals. The method is quite sensitive, accurate and precise. Thus, its LODs (0.4–2.7 ng kg^{-1}) are better than those for other methods such as those for the determination of ten hormones in meat (0.1–0.4 μg kg^{-1}) [11], flunixin in bovine tissues (0.1–0.2 μg kg^{-1}) [16], and four antibacterials in farmed aquatic species (0.1–1.0 μg kg^{-1}) [6] or chicken muscle (0.1–1.0 μg kg^{-1}) [22]. Pharmaceutical recoveries from various samples of edible animal tissues ranged from 92 to 101 %. By contrast, previously reported recoveries for some pharmaceuticals varied over wider ranges (e.g. 77.1–98.3% for flunixin in edible bovine tissues [16], and 80.9–105.5% for antibacterials in poultry and porcine muscle [20] or 72.4–107.4 % in meat, kidney and liver tissues [23]). The proposed method is therefore applicable to a variety of animal tissues. Nearly all samples were found to contain the hormones estrone and 17β-estradiol, and the antibacterials florfenicol and pyrimethamine, all at concentrations below their maximum allowed levels.

Acknowledgements

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References

[1] N. A. Botsouglou, D.J. Fletouris, in L.M.L. Nollet (Ed.), Handbook of Food Analysis, Marcel Dekker, New York, NY, 2004, p. 931 (Chapter 26).

[2] S.B. Tunipssed, W.C. Andersen, in: Y. Picó (Ed.), Comprehensive analytical Chemistry. Food Contaminants and Residue Analysis, Elsevier, Amsterdam, 2008, p. 307 (Chapter 10).

[3] Codex Alimentarius Commission. Codex Veterinary Drug Residues in Food Online Database. http://www.codexalimentarius.net/vetdrugs/data/index.html (accessed 31-January-2011)

[4] Council regulation No. 37/2010/EEC. Laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. L15 (2010) 1

[5] J.D.G. McEvoy, Anal. Chim. Acta 473 (2002) 3.

[6] J.M. Van de Riet, R.A. Potter, M. Christie-Fougere, G. Burns, J. AOAC Int. 86 (2003) 510.

[7] M.R. Futh, S.Y. Huang, T.Y. Lin, Talanta 64 (2004) 408.

[8] U. Koesukwiwat, S. Jayanta, N. Leepipatpiboon, J. Chromatogr. A 1140 (2007) 147.

[9] Á. Sebök, A. Vasanits-Zsigrai, A. Helenkár, G. Záray, I. Molnár-Perl, J. Chromatogr. A 1216 (2009) 2288.

[10] J.F. Huang, H.J. Zhang, Y.Q. Feng, J. Agric. Food Chem. 54 (2006) 9279.

[11] J. Seo, H.Y. Kim, B.C. Chung, J. Hong, J. Chromatogr. A 1067 (2005) 303.

[12] C. Blasco, C.V. Poucke, C.V. Peteghem, J. Chromatogr. A 1154 (2007) 230.

[13] D. Ortelli, E. Cognard, P. Jan, P. Edder, J. Chromatogr. B 877 (2009) 2363.

[14] R.J.B. Peters, V.J.C. Bolck, P. Rutgers, A.A.M. Stolker, M.W.F. Nielen, J. Chromatogr. A 1216 (2009) 8206.

[15] P.L. Boner, D.D.W. Liu, W.F. Feely, M.J. Wisocky, J. Wu, J. Agric. Food Chem. 51 (2003) 3753.

[16] P.L. Boner, D.D.W. Liu, W.F. Feely, R.A. Robinson, J. Wu, J. Agric. Food Chem. 51 (2003) 7555.

[17] S. Souverain, S. Rudaz, J.L. Veuthey, J. Pharm. Biomed. Anal. 35 (2004) 913.

[18] S. Impens, K. De Wasch, M. Cornelis, H.F. De Brabander, J. Chromatogr. A 970 (2002) 235.

[19] A.P. Pfenning, J.E. Roybal, H.S. Rupp, S.B. Turnipseed, S.A. Gonzales, J.A. Hurlbut, J. AOAC Int. 83 (2000) 26.

[20] J. Shen, X. Xia, H. Jiang, C. Li, J. Li, X. Li, S. Ding, J. Chromatogr. B 877 (2009) 1523.

[21] A. Azzouz, B. Souhail, E. Ballesteros, J. Chromatogr. A 1217 (2010) 2956.

[22] S. Zhang, Z. Liu, X. Guo, L. Cheng, Z. Wang, J. Shen, J. Chromatogr. B 875 (2008) 399.

[23] B. Shao, R. Zhao, J. Meng, Y. Xue, G. Wu, J. Hu, X. Tu, Anal. Chim. Acta 548 (2005) 41.

[24] G.A. Korsrud, J.M. Naylor, C.D. Salisbury, J.D. MacNeil, J. Agric. Food Chem. 35 (1987) 556.

[25] T. Nagata, M. Saeki, J. Liq. Chromatogr. 15 (1992) 2045.

[26] H. Yamada, K. Yoshizawa, T. Hayase, J. Chromatogr. B 775 (2002) 209.

[27] T. Nagata, H. Oka, J. Agric. Food Chem. 44 (1996) 1280.

[28] E. Daeseleire, R. Vandeputte, C. Van Peteghem, Analyst 123 (1998) 2595.

[29] F. Courant, J.P. Antignac, J. Laille, F. Monteau, F. Andre, B. Le Bizec, J. Agric. Food Chem. 56 (2008) 3176.

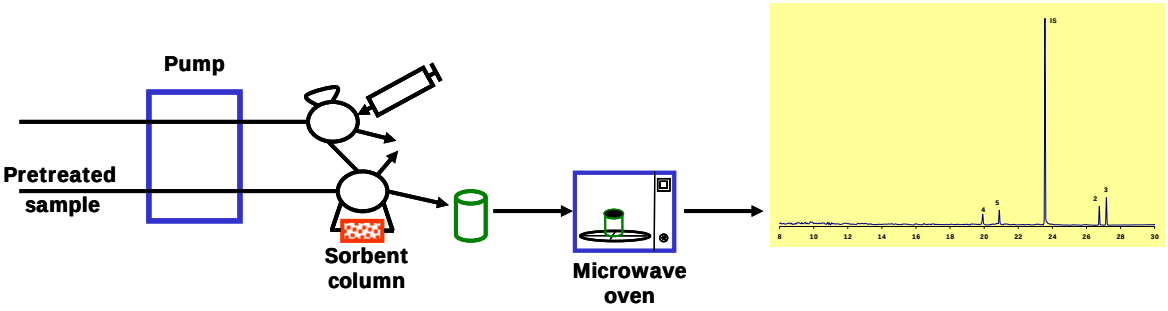
[30] G. Dowling, P. Gallo, S. Fabbrocino, L. Serpe, L. Regan, Food Addit. Contam. 25 (2008) 1497.

[31] A.A.M. Stolker, U.A.T. Brinkman, J. Chromatogr. A 1067 (2005) 15.

[32] H.G.J. Mol, P. Plaza-Bolaños, P. Zomer, T.C. de Rijk, A.A.M. Stolker, P.P.J. Mulder, Anal. Chem. 80 (2008) 9450.

CAPÍTULO III

Método para la Determinación por Cromatografía de Gases-Espectrometría de Masas de Sustancias Farmacológicamente Activas en Fluidos Biológicos mediante el uso de un Sistema Continuo de Extracción en Fase Solida y Derivatización Asistida por Microondas



El control de residuos de SFAs en fluidos biológicos es muy interesante no solo desde el punto de vista del estudio de la efectividad de los tratamientos terapéuticos, sino que también para evitar el riesgo de consumir alimentos de origen animal o agua contaminados con estas sustancias. El objetivo de este Capítulo ha consistido en el desarrollo de un método para la determinación simultánea de diferentes tipos de SFAs en muestras biológicas humanas y animales (orina y sangre) mediante el uso de un sistema continuo de extracción en fase sólida para la preconcentración de este tipo de analitos y eliminación de interferencias de la matriz de la muestra, empleado previamente para determinar residuos de fármacos en muestras de aguas y alimentos. Como novedad principal de este método es el empleo de un horno de microondas convencional para acelerar la reacción de derivatización de los analitos antes de su determinación por GC-MS.

Como en el caso de los alimentos, las muestras de algunos fluidos biológicos contienen una matriz compleja. En el caso de la orina se realizaron unos estudios preliminares de la influencia de la matriz de la muestra sobre la SPE de las SFAs en la columna de sorbente incluida en el bucle de inyección del sistema continuo. No se observó ninguna influencia sobre la eficacia de la extracción de estas sustancias, ni ningún efecto sobre el sistema continuo. Por lo cual se optó por introducir las muestras de orina directamente en el sistema continuo.

En cambio, la sangre contiene una matriz mucho más compleja que la orina, con un 45 % de fracción celular y un 65 % de plasma (mezcla de proteínas, aminoácidos, carbohidratos, lípidos, hormonas, enzimas, compuestos inorgánicos y otras sustancias). De todos estos componentes, son sin duda las proteínas (concentración media en sangre entre 60 y 80 g/l) las que pueden afectar a la eficacia de la SPE de SFAs además de que pueden bloquear el paso de la muestra a través de la columna sorbente del sistema continuo. Por ello es necesaria una etapa de eliminación de estas proteínas previa a la introducción en el sistema continuo de SPE. Se optó por la precipitación de las proteínas con un disolvente adecuado, seguida de una etapa de centrifugación y de otra de filtración previas a la introducción del sobrenadante en el sistema continuo. Para ello se evaluaron diversos disolventes (metanol, acetonitrilo y diclorometano), mezclas de disolventes (agua-acetonitrilo y metanol-acetonitrilo) y ácido tricloroacético para la precipitación de las proteínas de la sangre. Los mejores resultados de precipitación de las proteínas y su posterior separación del sobrenadante se consiguieron cuando se utilizaban 2 ml de acetonitrilo como disolvente para un volumen de 1 ml de sangre.

También se estudiaron las variables que podían afectar a la centrifugación (velocidad, tiempo y temperatura). Los mejores resultados se consiguieron para una velocidad de centrifugación de 4000 rpm durante 10 min y a 4 ºC. Como se ha indicado en los Capítulos anteriores, la presencia de acetonitrilo en el sobrenadante afecta muy negativamente a la extracción de las SFAs en la etapa de SPE en el sistema continuo. Como en los casos anteriores se ha optado por la evaporación del sobrenadante con una corriente de nitrógeno hasta un volumen de 200 µl y su posterior la redisolución de éste con 5 ml de agua purificada a pH 7 antes de su introducción en el sistema continuo de SPE.

Una novedad incluida en este trabajo con respecto a las metodologías convencionales es el uso del horno de microondas para facilitar la reacción de derivatización de las SFAs. En este caso se han estudiado las diferentes variables (medio de reacción, tiempo, reactivo de sililación y potencia de radiación de microondas) que pueden afectar a la etapa de derivatización de los fármacos. Los mejores resultados se obtuvieron cuando se utilizaba la mezcla BSTFA + 1 % TMCS como reactivos para la derivatización de las distintas SFAs en acetato de etilo, a 350 W de potencia del microondas y durante 3 min. Por lo cual se reduce notablemente el tiempo de tratamiento de muestra, con respecto a los procedimientos expuestos en los Capítulos I y II de esta Memoria, donde se necesitaban 20 min para llevar a cabo la reacción de derivatización a 70 ºC.

El método propuesto presenta una sensibilidad elevada con límites de detección entre 0,2 a 1,3 ng/l para muestras de orina y entre 0,8 y 5,6 ng/l para muestras de sangre. La precisión es satisfactoria con desviaciones estándar relativas menores del 7 %, calculadas para muestras de orina o sangre que contenían las diferentes SFAs estudiadas. Por otra parte, se realizó un estudio de recuperación, obteniéndose porcentajes de recuperación entre el 85 y el 102 %. Por último se estudio la estabilidad de estas SFAs en las muestras de sangre y orina cuando se conservaban congeladas (–20 ºC). No se observó ningún efecto adverso de la matriz sobre la estabilidad de los analitos en el periodo de 30 días cuando se conservaban las muestras a las condiciones anteriormente indicadas.

Con estas premisas el método fue aplicado a diferentes tipos de fluidos biológicos (orina y sangre) de personas y animales (vaca, cerdo y cordero). De todas las muestras analizadas de sangre solo una recogida de una persona sana estaba libre de residuos de los fármacos estudiados. Las demás muestras de sangre, tanto humanas

como de animales, contenían alguna hormona (estrona y/o 17β-estradiol) y varios antiinflamatorios/analgésicos (ácido acetilsalicílico, ibuprofeno, ketoprofeno y fenilbutazona) o algún antibiótico (florfenicol y pirimetamina) a concentraciones entre 0.34 y 3.8 μg/l. Las muestras de sangre humana contenían también triclosán, un antiséptico utilizado en pasta de dientes (0.25–0.89 μg/l). En el caso de las muestra de orina, también contenían la mayoría de los analitos encontrados en las muestras de sangre, pero a concentraciones más reducidas (0.16–3.3 μg/l). El método propuesto presenta unas significativas ventajas con respecto a las metodologías desarrolladas hasta el momento, que han estado centradas en la determinación SFAs de uno o dos tipos de clases terapéuticas, y en orina o sangre de personas o animales por separado.

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Gas chromatography-mass spectrometry determination of pharmacologically active substances in urine and blood samples by use of a continuous solid-phase extraction system and microwave-assisted derivatization

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ABSTRACT

A sensitive method based on gas chromatography–mass spectrometry was used to determine 22 pharmacologically active substances (frequently used in the treatment of human and animal's diseases) including analgesics, antibacterials, anti-epileptics, antiseptics, β-blockers, hormones, lipid regulators and non-steroidal anti-inflammatories in blood and urine samples. Samples were subjected to continuous solid-phase extraction in a sorbent column (Oasis HLB), and then the target analytes were eluted with ethyl acetate and derivatized in a household microwave oven at 350 W for 3 min. Finally, these products were determined in a gas chromatograph-mass spectrometer equipped with a DB-5 fused silica capillary column. The analyte detection limits thus obtained ranged from 0.2 to 1.3 ng L^{-1} for urine samples and 0.8 to 5.6 ng L^{-1} for blood samples. Recoveries from both blood and urine ranged from 85 to 102%, and within-day and between-day relative standard deviations were all less than 7.5%. The proposed method offers advantages in reduction of the exposure danger to toxic solvents used in conventional sample pretreatment, simplicity of the extraction processes, rapidity, and sensitivity enhancement. The method was successfully used to quantify pharmacologically active substances in human and animal (lamb, veal and pig) blood

and urine. The hormones estrone and 17β-estradiol were detected in virtually all samples, and so were other analytes such as acetylsalicylic acid, ibuprofen, ketoprofen and triclosan in human samples, and florfenicol, pyrimethamine and phenylbutazone in animal samples.

Keywords: Pharmacologically active substances; Biological fluids; Continuous solid-phase extraction; Microwave-assisted derivatization; Gas chromatography–mass spectrometry

Introduction

Controlling residual amounts of pharmacologically active substances (PAS) in biological fluids is extremely important with a view to assessing the effectiveness of medical therapies or the risks of consuming of food or water contaminated with such substances [1]. Non-opioid analgesics and non-steroidal anti-inflamamatories, which are used mainly for the treatment of osteoarthritis, rheumatoid arthritis and other painful musculoskeletal illnesses [2,3], are among the most widely used PAS in over-the-counter preparations. The most common side-effect of some of PAS is gastric or intestinal ulceration, which is occasionally accompanied by anaemia due to the resulting blood loss. In addition, these substances can cause disturbances in platelet function, prolong pregnancy or spontaneous labour, and alter renal function [1]. Chloramphenicol, florfenicol and thiamphenicol are three broad-spectrum antibacterials commonly used as chemotherapy agents to control some diseases in veterinary and aquacultural practice [4]. Chloramphenicol is highly effective on animals because it inhibits a variety of aerobic and anaerobic microorganisms [5]; by contrast, it is toxic to human bone marrow and has been associated to blood disorders such as aplastic anaemia [1]. β-Blockers are mainly used to treat hypertension, congestive heart failure and abnormal heart rhythms, as well as to relieve angina and prevent cardiac infarctions (heart attacks) in humans [1]. However, β-blockers have been misused by some athletes to relieve performance anxiety by controlling hand tremor, lowering heart rate and reducing blood pressure [6]. This has led to their banning by the World Anti-Doping Agency (WADA) in some sports such as archery and shooting [7]. Some hormones such as 17α-ethinylestradiol, a semi-synthetic estrogen used as a contraceptive, may have a positive impact on public health [8]. Other PAS such as triclosan, used in personal care products, may be a potentially toxic environmental contaminant since *in vitro* studies on rat and human material have shown low concentrations of this substance to disturb metabolic systems and hormone homeostasis [9].

Quantifying trace amounts of small molecules (< 500 amu) in biological samples is rather challenging. Most biological matrices contain a wide variety of abundant species that can interfere with the determination of the target analytes. Determining PAS, especially in biological fluids (urine and whole blood), is difficult owing to their low concentrations and those of their metabolites relative to the typically high levels of endogenous compounds in the matrix. Therefore, detecting trace levels of these

substances requires sample preparation and cleanup. A number of authors have used extraction, sample clean-up and derivatization procedures to facilitate the determination of PAS in various types of biological samples. Such procedures are based on solid-phase extraction (SPE) [4,10–19], solid-phase microextraction [20,21], liquid–liquid extraction [13,22–25], stir bar sorptive extraction [26,27] and supercritical fluid extraction in combination with solid phase extraction [10,28]. Solid-phase extraction is probably the most popular technique in this context by virtue of its expeditiousness, reproducibility and low cost. This technique is usually performed using a small column or cartridge containing an appropriate packing. During the last years a series of different polymer-based materials for the SPE of either acidic, neutral and basic compounds out of different sample matrices have been developed [29]. Oasis-HLB is seemingly the most commonly used sorbent for the solid-phase extraction of PAS from biological samples [11,13,14,19,30]. Oasis HLB is a macroporous copolymer consisting of two monomer components, the lipophilic divinylbenzene and the hydrophilic *n*-vinylpyrrolidone.

Determinations of PAS in biological fluids have used various detection techniques (especially gas chromatography and liquid chromatography). In fact, liquid chromatography (LC) has been used in combination with mass spectrometry [12–16,18,20,24,25], UV-visible and diode array detectors [3,13,15,22,26,27] to determine PAS in different types of biological fluids, and so has gas chromatography, mostly in conjunction with mass spectrometers detector (GC–MS) [4,11,17,19,23,28].

The aim of this work was to develop an accurate, sensitive method for the simultaneous determination of different types of PAS (antibacterials, analgesics, non-steroidal anti-inflammatories, anti-epileptics, antiseptics, β-blockers, lipid regulators, and hormones) in human and animal biological samples (urine and whole blood) by using a continuous SPE system for preconcentration/clean-up previously employed by our group to determine PAS in water and food samples [30,31]. As a major novelty, a microwave oven was used here to expedite derivatization of the target analytes prior to their GC–MS determination.

2. Experimental

2.1. Instruments and apparatus

All analyses were carried out on a Focus gas chromatograph coupled to a DSQ II mass spectrometer equipped with an AI/AS 3000 autosampler (Thermo Electron SA, Madrid, Spain) and controlled by a computer running XCalibur software (Thermo Electron SA, Madrid, Spain). The transfer line was kept at 280 ºC. The mass spectrometer worked in the electron impact mode (70 eV) by scanning from 60 to 500 amu to obtain full spectra of the target analytes or by selected ion-monitoring (SIM) for the quantification of the analytes. For each silyl derivative, $M^{+\cdot}$, $[M-15]^+$, and other additional ions were monitored which are included in Table 1, where $M^{+\cdot}$ is the molecular mass and $[M-15]^+$ is the molecular mass minor 15 corresponding to the loss of a CH_3 of the $Si(CH_3)_3$ group (Figures S1-S5 of Supplementary Information). The chromatograph was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) coated with 5% phenylmethylpolysiloxane (Supelco, Madrid, Spain). Helium (purity 6.0) at 1 mL min^{-1} was employed as the carrier gas. The injection port was maintained at 280 ºC, and all injections were done in the split mode (1:20 ratio). The time for solvent delay was set at 4 min. The oven temperature was held at 70 ºC for 1 min following injection and then raised from 70 to 150 ºC at 14 ºC min^{-1}. After the first transition, the temperature was raised from 150 to 290 ºC at 6 ºC min^{-1}. The total GC run time was ~ 30 min.

The continuous SPE system was assembled from a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) fitted with poly(vinylchloride) tubes and two Rheodyne (Cotati, CA) 5041 injection valves. A PTFE laboratory-made sorbent column packed with 60 mg of Oasis-HLB as described elsewhere was also employed [30]. The sorbent column was conditioned with 1 mL of ethyl acetate and 1 mL of purified water, which rendered it serviceable for at least 2 month.

2.2. Chemicals and materials

Pharmacologically active substances (Figure 1) were all purchased from Sigma–Aldrich (Madrid, Spain) in the highest available purity. Triphenylphosphate and the derivatizing reagents [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS)] were obtained from Fluka (Madrid, Spain). All solvents (acetonitrile, methanol, dichloromethane and ethyl acetate), sodium hydroxide and inert poly-tetrafluoroethylene white beads (pore diameter 4 Å) were obtained from Merck (Darmstadt, Germany). Oasis-HLB in particle size 50−65 μm was obtained from Waters (Madrid, Spain). Millex-LG filter units (hydrophilic, PTFE, pore size 0.20 μm, diameter 25 mm, filtration area = 3.9 cm^2) were obtained from Millipore Ibérica S. A. (Madrid, Spain). Ultrapure water was obtained using a Milli-Q purification system from Millipore Ibérica S.A.

Stock standard solutions of the individual PAS at 1 g L^{-1} concentration each were prepared in methanol and stored at 4 ºC in the dark. Working-standard solutions were prepared on a daily basis by dilution of the individual stock standard solutions in purified water and adjustment to pH 7 with dilute NaOH as required. Freshly made solutions of ethyl acetate containing a 500 μg L^{-1} concentration of triphenylphosphate (internal standard, IS) and prepared on a daily basis were used as eluents for continuous SPE.

2.3. Biological samples

All biological samples were obtained in accordance with the guidelines of the bioethics committee. Human urine samples from healthy volunteers were collected in sterilized polyethylene bottles. Cow, pig and lamb urine samples were supplied by local stockbreeders not practicing intensive breeding, using sterilized polyethylene bottles to ensure the absence of contamination. When the time between sample collection and analysis was to exceeded 8 h, the samples were stored at −20 °C for up to 60 days to avoid degradation. Frozen samples were allowed to thaw at room temperature prior to analysis. Urine samples were gently mixed and directly transferred into vials for analysis in triplicate (n = 3).

Human whole blood samples were collected from healthy volunteers at various hospitals. Animal (veal, lamp and pig) blood samples were obtained by jugular venipuncture. All blood samples were collected in a blood-pack unit including sodium citrate (3.8%) as an anticoagulant, in a 1:16 ratio to whole blood [13]. Also, all were immediately frozen and stored at –20°C until use.

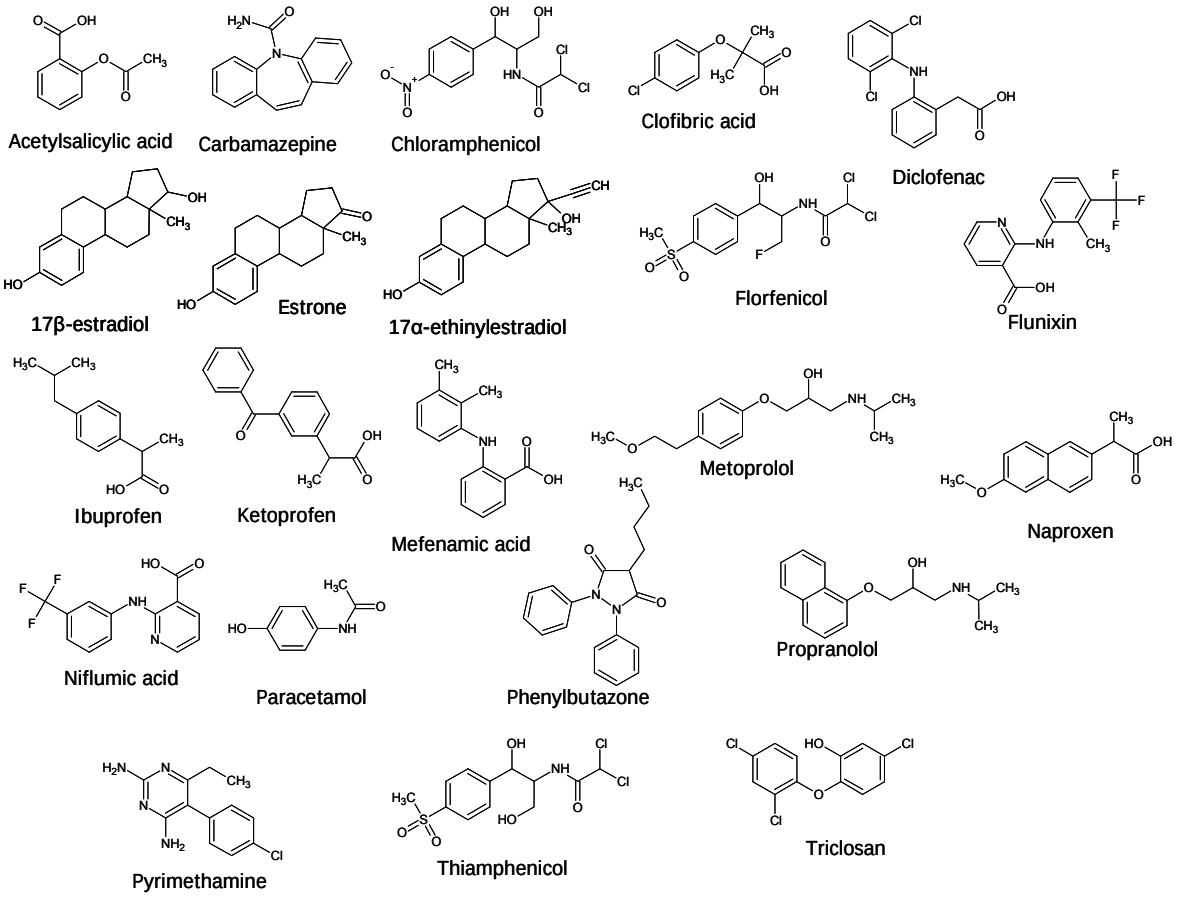

Fig. 1. Chemical structures of pharmacologically active substances.

2.4. Sample pretreatment

Previous study on the influence of pH on the SPE efficiency revealed that the best extraction results for all analytes were obtained in the neutral pH region (6.5–7.5), so pH 7 was adopted [30,31]. Therefore, urine samples were simply adjusted to pH 7 with

dilute NaOH (0.5 M) and passed through a 0.20 μm Millex-LG filter. For blood samples, a volume of 1 mL of each was placed in a 15 mL round polypropylene centrifuge tube and mixed with 2 mL of acetonitirile in a REAX Control vortex mixer from Heidolph (Kelheim, Germany) for 2 min. Then, the mixture was centrifuged on a Centrofriger BL-II apparatus from JP Selecta (Barcelona, Spain) at 4000 rpm and 4 ºC for 10 min, the supernatant being passed through a 0.20 μm Millex-LG filter and carefully evaporated to dryness under a stream of ultra-pure N_2 to a final volume of 200 μL and redissolved to 5 mL with purified water at pH 7.

2.5. Continuous solid-phase extraction and microwave-assisted derivatization

The continuous SPE unit used for the cleanup/preconcentration of pharmacologically active substances from pretreated samples is depicted in Fig. 2. In the preconcentration step, a volume of 5 mL of pretreated sample was continuously passed through the sorbent column, located in the loop of injection valve IV_1, at 4 mL min^{-1}. PAS were immediately retained, and the sample matrix was sent to waste. Next, IV_1 was switched and the sorbent column dried for 2 min with an air stream at 4 mL/min; simultaneously, the loop of IV_2 (400 μL) was filled with eluent (ethyl acetate containing 500 ng L^{-1} triphenylphosphate as internal standard) by means of a syringe. In the elution step, IV_2 was switched to pass 400 μL of eluent, carried through the column by the air stream in the opposite direction of sample aspiration. The organic extract was collected in a 0.5 mL air-tight conical glass insert and evaporated to a volume of ~35 μL under a gentle stream of ultrapure N_2. Potential errors in measuring the final extract volume were avoided by using the internal standard. Next, 70 μl of mixture BSTFA+1%TMCS (derivatising agent) were added to sample extract of 35 μl. After that the vial was tightly sealed and the analytes were derivatised using a household microwave oven out for 3 min at 350 W. Finally, 1 μL aliquot of silylated derivatives was analysed by GC–MS in the SIM mode.

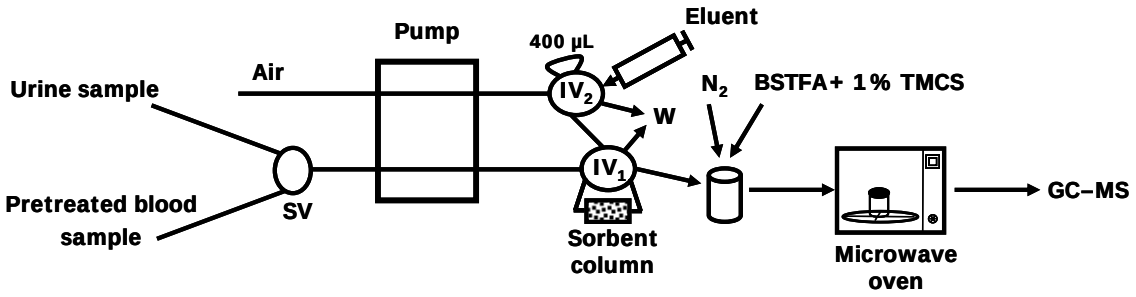

Fig. 2. Continuous solid-phase extraction system for the cleanup/preconcentration of pharmacologically active substances in urine and blood samples. IV, injection valve; SV, selection valve; W, waste; GC–MS, gas chromatograph with mass spectrometric detector; BSTFA-TMCS, N,O-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (derivatizing reagents).

3. Results and discussion

3.1. Variables affecting sample pretreatment

Appropriate sample preparation is an important prerequisite for chromatography of biosamples. The sample pretreatment to be used depends on the particular type of sample. Thus, whole blood and tissue (homogenates) require deproteination and filtration/centrifugation prior to SPE, whereas urine usually requires no more than dilution and/or centrifugation —and some SPE procedures allow urine to be used untreated [15,32]. We checked whether direct introduction of the urine samples into our previously developed continuous SPE system for determining PAS in water and milk samples [30,31] would pose any problem. To this end, a volume of 5 mL of uncontaminated urine sample was supplied with a 500 ng L^{-1} concentration of each PAS, adjusted to pH 7, passed through a 0.20 µm Millex-LG filter and analysed as described in Section 2.5. An identical procedure was applied to 5 mL of a solution of purified water at pH 7 that was also supplied with each PAS at a 500 ng L^{-1} concentration. The results for both types of samples were very similar, so we chose not to pretreat the urine samples and simply filter them in order to prevent any solid particles from reaching the continuous SPE system.

Blood proteins are no doubt the components most likely to affect the solid-phase extraction of pharmacologically active substances at concentrations of 60–80 g L^{-1} from

blood by effect of their blocking the sorbent column. This entails their prior removal by precipitation with a solvent such as acetone, acetonitrile [22], a mixture of methanol and acetonitrile [18] or dichloromethane [20], or their denaturation with a strong acid (trichloroacetic, hydrochloric or sulphuric acids) [9,16]. In this work, we assessed the efficiency of various solvents including 1% trichloroacetic acid, methanol, acetonitrile and dichoromethane, and of solvent mixtures (water–acetonitrile and methanol–acetonitrile), in precipitating proteins from whole blood. For this purpose, 1 mL of uncontaminated blood sample spiked with a 500 ng L^{-1} of each analyte was mixed with 2 mL of solvent and centrifuged at 5000 rpm for 10 min, after which the supernatant was filtered and evaporated to dryness under an N_2 stream to a final volume of 200 μL and redissolved to 5 mL with purified water at pH 7. Finally, the pretreated sample was introduced into the continuous SPE system [30,31] and processed as described in Section 2.5. The highest extraction efficiency for all PAS (~95%) was obtained with acetonitrile, which facilitated precipitation of proteins and their subsequent separation by centrifugation. We thus chose it to precipitate proteins from blood. The optimum volume of acetonitrile to be added to 1 mL of blood was determined by changing it over the range 1–10 mL; protein precipitation was found to peak at 1.5 mL acetonitrile, so a solvent volume of 2 mL was adopted as optimal. We also examined the effects of centrifugation-related variables such as rate, time and temperature over the ranges 1500–5000 rpm, 1–15 min and 0–25 ºC, respectively. Centrifugation at 4000 rpm at 4 ºC for 10 min resulted in optimal separation of precipitated blood proteins from the target species.

As previously shown by our group with the SPE of PAS in milk, the presence of acetonitrile at concentrations above 15% in the aqueous solution seriously impairs retention of the analytes on the sorbent column [30]. This led us to evaporate the supernatant from the centrifugation of the blood samples under a stream of ultra-pure N_2 to a final volume of 200 μL and dilute it with 5 mL of purified water at pH 7 prior to introduction into the continuous SPE system.

3.2. Optimization of microwave-assisted derivatization

Pharmacologically active substances have three polar groups: amino, hydroxyl and carboxyl (Fig. 1). Pharmacologically active substances require derivatization prior to GC–MS analysis and silylation is the most frequent choice for this purpose

[4,11,17,19,23,30,31]. As shown in several studies, a mixture of BSTFA and TMCS can simultaneously silylate amino, alcohol and carboxyl groups in PAS in a single step [23,30,31,33]. However, the derivatization reaction takes a long time (more than 20 min) at 60–70 °C [23,30,31,33]. Combining a silylating reagent with microwave-assisted derivatization (MAD) can efficiently reduce analysis times. Microwave heating avoids the time for energy transfer required in traditional heating, thus resulting in a rapid rise in temperature and hence in fast completion of reactions. In this work, we optimized the microwave-assisted derivatization of PAS by examining the influence of four major variables (reaction solvent, derivatizing reagent, microwave power output and irradiation time). To this end, volumes of 100 μL of individual solutions of the derivatizing reagents [*N*,*O*-bis-(trimethylsilyl)acetamide, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlororosilane, both individually and in mixtures] were added to 50 μL of a solution containing a 1 μg L^{-1} concentration of each analyte in ethyl acetate. The derivatization reaction was conducted in an air-tight 0.5 mL conical glass insert that was placed in the microwave oven at 250 W for 5 min. The derivatized PAS thus obtained were determined by GC–MS. The best results in this respect were obtained with a 99:1 mixture of BSTFA and TMCS.

Tests were conducted with ethyl acetate and acetonitrile, two solvents commonly used for the silylation of PAS [34]. A volume of 50 μL of a solution containing a 1 μg L^{-1} concentration of each PAS in each solvent was supplied with 100 μL of 99:1 BSTFA–TMCS mixture and placed in an air-tight conical glass insert of 0.5 mL for derivatization in a microwave oven at 250 W for 5 min. Although the two solvents provided similar results, ethyl acetate was selected because it surpassed acetonitrile in its ability to elute the sorbent column in the continuous SPE system [30]. Detection limits were found to depend on the volume of solvent used. In this work, we examined the effect of low volumes of solvents and derivatizing reagent (10–150 μL) in order to achieve detection limits relevant to environmental concentrations. The best results were obtained with a final volume of sample extract of 35 μL and one of derivatizing (BSTFA + 1% TMCS) of 70 μL.

The MAD conditions were also optimized, via the microwave power output and irradiation time. Thus, a volume of 5 mL of an aqueous sample containing a 500 μg L^{-1} concentration of each PAS was introduced into the continuous system as described in Section 2.5, the organic extract being collected in an air-tight 0.5 mL conical glass

insert, evaporated to a volume of ~35 μL under a gentle stream of ultrapure N_2 and supplied with 70 μL of 99:1 BSTFA–TMCS mixture [34]. The vial containing the analytes was then tightly sealed and placed in a household microwave oven at variable power (70–500 W) for an also variable time (1−10 min). The highest derivatization efficiency was obtained by irradiation at 350 W for 3-4 min. It was observed that when applied a power under 350 W, low yields in the derivatization were achieved; for values greater than 400 W, the signal of the analytes decreases possibly due to the degradation of these compounds, as occurs with a power of 350 W for times higher of 4 min. Therefore, a power of irradiation of 350 W for 3 min was selected for subsequent work. By way of example, Fig. 3 shows the typical chromatogram of standard as N-trimethylsily derivatives (1 μg L^{-1}). As can be seen, the 22 PAS studied were effectively separated with no difficulty in a single chromatographic run in about 30 min.

3.3. Analytical performance

Linear range, analyte detectability, and precision of the proposed method were studied under optimal experimental conditions (see Table 1). Calibration curves were constructed by using uncontaminated urine samples (5 mL, pooled human urine sample) and uncontaminated whole blood (1 mL, pooled sample of human blood) spiked with 0.6–5000 or 2.8–15000 ng L^{-1} of each analytes, respectively, and processed as described in Sections 2.4 and 2.5. The equations for the standard curves were obtained by plotting the analyte to internal standard peak area ratios against the amount of PAS. Regression coefficients were over 0.995 in all cases. Limits of detection (LODs) were determined as the analyte concentration that provides a chromatographic peak equal to 3 times the regression standard deviation, Sy/x, divided by the slope of each calibration graph, ranged between 0.2 and 1.3 ng L^{-1} or between 0.8 and 5.6 ng L^{-1} for urine or blood samples, respectively. As can be observed in Table 1, analytes with lower LODs are carbamazepine, chloramphenicol, diclofenac, florfenicol, flunixin, ibuprofen, mefanamic acid, niflumic acid, paracetamol and thiamphenicol. In contrast, PAS with higher LODs are the three hormones and phenylbutazone. The latter compound is determined as non-derivatized as demonstrated in the mass spectrum included in Fig. S6 of Supplementary Information. In the fragmentation pattern of phenylbutazone is observed that the peak *m/z* 308 corresponds to the atomic mass of this compound without derivatization.

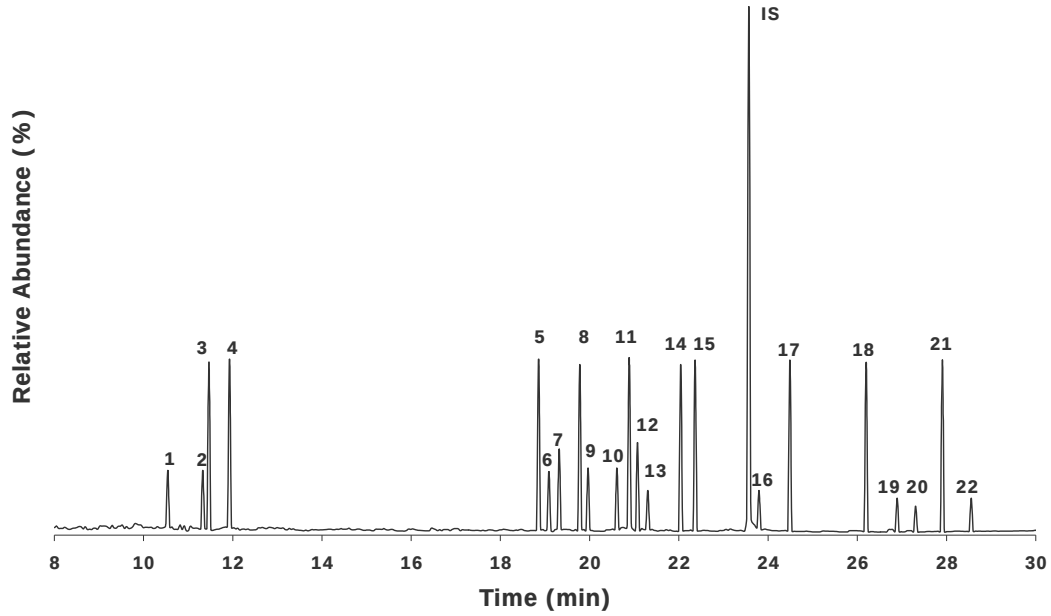

Fig. 3. Typical chromatogram (SIM mode) of pharmacologically active substances as N-trimethylsilyl derivatives (1 μg L-1). 1- acetylsalicylic acid; 2- clofibric acid; 3- paracetamol; 4- ibuprofen; 5 - niflumic acid; 6 - metoprolol; 7 - naproxen: 8 - flunixin; 9 - triclosan; 10 - propranolol; 11 - mefenamic acid; 12 - ketoprofen; 13 -pyrimethamine; 14 - carbamazepine; 15 - diclofenac; 16 - phenylbutazone; 17 -chloramphenicol; 18 - florfenicol; 19 - estrone; 20 - 17β-estradiol; 21 - thiamphenicol; 22 - 17α-ethinylestradiol; IS - triphenylphosphate (internal standard).

The precision of the proposed method, as relative standard deviation (RSD), was calculated by measuring 11 uncontaminated urine samples spiked with 10, 100 and 1000 ng L^{-1}, or 11 uncontaminated blood samples spiked with 50, 500, and 5000 ng $L^{-1}$of each target compounds. A comparative study of within-day and between-day precision was conducted [35]. For the study of between-day precision, three pooled samples of urine (120 mL) or blood (25 mL) were taken to which were added the 22 PAS at the three concentration levels listed above. The pooled samples were split into portions of 15 mL (urine) or 3 mL (blood), and a portion was analyzed on the first day in triplicate. The others portions were frozen at –20 °C, and were subjected to the same analytical procedure in triplicate every day for 6 days following thawing 1 h before preparation. The within-day precision was found to range from 3.9 to 6.8% and the between-day from 4.5 to 7.4%. Also, freeze-thaw stability test were conducted with a view to assessing the stability of the analytes in urine and blood samples at a storage temperature of –20ºC. For this purpose, three pooled samples of urine (120 mL) or

blood (25 mL) were spiked with all analytes at the three concentration levels listed above. The pooled samples was split into 15 mL portions and frozen at –20ºC; by exception, one portion was analyzed in triplicate as described under Experimental on the same day. All other portions were subjected to the same analytical procedure in triplicate every 5 days for 1 month following thawing 1 h before preparation. Freezing the samples under these conditions was found to suppress any adverse effect of the matrix on analyte stability; in fact, the results were always similar, within the error range for the method (RSD < 7.5%), to those for the unfrozen sample.

Because no certified reference material for urine or blood containing the studied analytes was available, the proposed method was validated by analysing uncontaminated urine samples spiked with 10, 100 and 1000 ng L^{-1}, and uncontaminated blood samples spiked with 50, 500, and 5000 ng $L^{-1}$of each target compounds and analyzed in triplicate. The average recoveries ranged from 85 to 102% (Table 2), which testifies to the applicability of the proposed method in these complex matrices.

3.4. Analysis of urine and blood real samples

The proposed method was applied to the determination of 22 pharmacologically active substances including analgesics, antibacterials, anti-epileptics, antiseptics, β-blockers, hormones, lipid regulators and non-steroidal anti-inflammatories in real urine and blood samples from humans and animals (lamb, cow and pig). Samples were analysed in triplicate, following the pretreatment and analytical procedures described under Experimental.

As can be seen from Table 3, the hormones estrone and/or 17β-estradiol were detected in eight blood samples, at concentrations from 1.9 to 4.8 μg L^{-1} and 0.67 to 5.9 μg L^{-1}, respectively. These 17β-estradiol levels are higher than those previously reported by other authors for human blood [11]. The human blood samples contained PAS commonly used as analgesics (acetylsalicylic acid), non-steroidal anti-inflammatories (ibuprofen and ketoprofen) and antiseptics (triclosan). The antibacterials florfenicol and pyrimethamine were additionally found in some animal blood samples. Phenylbutazone was detected in a cow blood sample, at a concentration similar to those reported by Cárdenas et al. [32] for race horses.

Like the blood samples, most urine samples contained the hormones estrone and 17β-estradiol, albeit at lower concentrations (0.42–3.3 μg L^{-1}). This was also the case with the other PAS, which were found at concentrations of 0.16–1.8 μg L^{-1} in urine and 0.25–3.8 μg L^{-1} in blood. By way of example, Fig. 4 shows the SIM mode chromatograms for samples of cow blood and human urine processed with the proposed method.

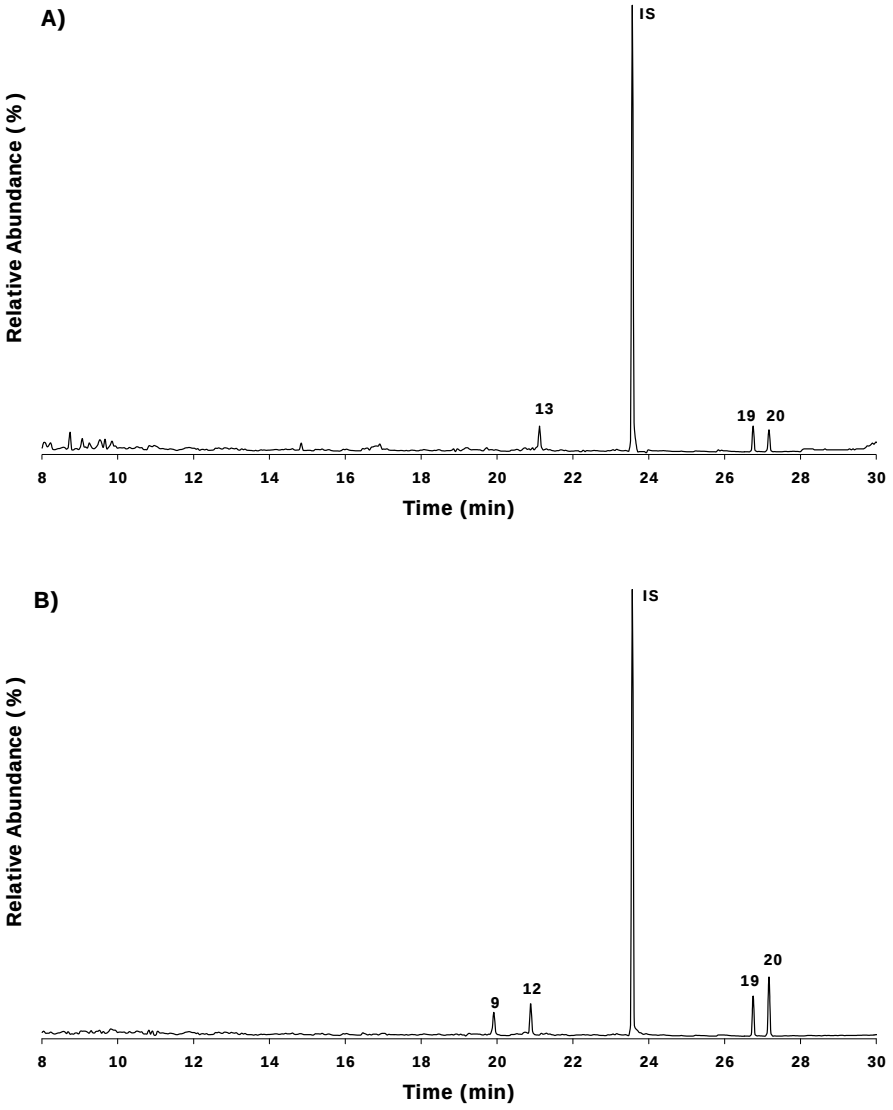

Fig. 4. Chromatograms in SIM mode for pharmaceutical active substances in the analysis of 1 mL of cow blood 1 (A) and 5 mL of human urine 1 (B) (see Table 3). For peak identification, see Fig. 3.

4. Conclusions

The objectives of this work were acceptably fulfilled. In fact, the proposed method is rapid, sensitive and selective in the determination of PAS in blood and urine samples. The method has several salient advantages, namely: (*a*) urine samples require no treatment other than filtering and pH adjustment prior to introduction into the SPE system; (*b*) the SPE step is highly efficient, which significantly increases the sensitivity and selectivity of the determination, with limits of detection of 0.2–1.3 ng L^{-1} in urine and 0.8–5.6 ng L^{-1} in blood; (*c*) using a household microwave oven allows PAS to be silylated in 3 min as opposed to more than 20 min with conventional methods such as heating at 60–70 °C [23,30,31,34]; (d) the proposed method affords the determination of a wide range of PAS belonging to a number of therapeutic classes, while most of the methodologies that had been developed so far focused on one or two types of therapeutic classes, and (e) the method allows PAS to be determined in both animal and human biological fluids, while the methods found in the literature are concerned with the determination of PAS in these samples from human or animal samples separately..

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References

[1] K.C. Osterhoudt, T.M. Penning, in: L.L. Brunton, B.C. Chabner, B.C. Knollmann (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, Vol. 1, 12 ed., McGraw-Hill Medical, New York, 2010.

[2] F.A. Aly, S.A. Al-Tamimi, A.A. Alwarthan, Anal. Chim. Acta 416 (2000) 87.

[3] P. Gowik, B. Jülicher, S. Uhlig, J. Chromatogr. B 716 (1998) 221.

[4] T. Nagata, H. Oka, J. Agric. Food Chem. 44 (1996) 1280.

[5] Z.Y. Huang, M.Y. Sun, S. Li and G.L. Huang, Aquacult. Res. 37 (2006) 1540.

[6] M. Lu, L. Zhang, B. Qiu, Q. Feng, S. Xia, G. Chen, J. Chromatogr. A 1193 (2008) 156.

[7] The World Anti-Doping Agency International Standard, WADA, Lausanne, 2006.

[8] E.M. Ghoneim, H.S. El-Desoky, M.M. Ghoneim, J. Pharm. Biom. Anal. 40 (2006) 255.

[9] M. Allmyr, M.S. McLachlan, G. Sandborgh-Englund, M. Adolfsson-Eric, Anal. Chem. 78 (2006) 6542.

[10] K. Hartonen, M.L. Riekkola, J. Chromatogr. B 676 (1996) 45.

[11] M.H. Choi, K.R. Kim, J.K. Hong, S.J. Park, B.C. Chung, Rapid Commun. Mass Spectrom. 16 (2002) 2221.

[12] H.H. Maurer, O. Tenberken, C. Kratzsch, A.A. Weber, F.T. Peters, J. Chromatogr. A 1058 (2004) 169.

[13] M. Sultan, G. Stecher, W.M. Stöggl, R. Bakry, P. Zaborski, C.W. Huck, N.M. El Kousy, G.K. Bonn, Curr. Med. Chem.12 (2005) 573.

[14] K. Suenami, L.W. Lim, T. Takeuchi, Y. Sasajima, K. Sato, Y. Takekoshi, S. Kanno, Anal. Bioanal. Chem. 384 (2006) 1501.

[15] J. Tuerk, M. Reinders, D. Dreyer, T.K. Kiffmeyer, K.G. Schmidt, H.M. Kuss, J. Chromatogr. B 831 (2006) 72.

[16] F. Vinci, S. Fabbrocino, M. Fiori, L. Serpe, P. Gallo, Rapid Commun. Mass Spectrom. 20 (2006) 3412.

[17] H. Budzinski, M.H. Devier, P. Labadie, A. Togola, Anal. Bioanal. Chem. 386 (2006) 1429.

[18] L. Kristoffersen, E.L. Øiestad, M.S. Opdal, M. Krogh, E. Lundanes, A.S. Christophersen, J. Chromatogr. B 850 (2007) 147.

[19] S. Magiera, C. Uhlschmied, M. Rainer, Ch.W. Huck, I. Baranowska, G.K. Bonn, J. Pharm. Biom. Anal. 56 (2011) 93.

[20] X. Hu, J. Pan, Y. Hu, G. Li, J. Chromatogr. A 1216 (2009) 190.

[21] A. Aresta, D. Bianchi, C.D. Calvano, C.G. Zambonin, J. Pharm. Biom. Anal. 53 (2010) 440.

[22] V.V. Pavan Kumar, M.C.A. Vinu, A.V. Ramani, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 125.

[23] B. Yilmaz, S. Arslan, V. Akba, Talanta 80 (2009) 346.

[24] H.P. Nguyen, L. Li, J.W. Gatson, D. Maass, J.G. Wigginton, J.W. Simpkins, K.A. Schug, J. Pharm. Biom. Anal. 54 (2011) 830.

[25] L. K.Sørensen, Forensic Sci. Int. 206 (2011) 119.

[26] C. Almeida, J.M.F. Noguerira, J. Pharm. Biom. Anal. 41 (2006) 1303.

[27] R.H. Costa Queiroz, C. Bertucci, W.R. Malfará, S.A. Carvalho Dreossi, A. Rodrigues Chaves, D.A. Rodrigues Valério, M.E. Costa Queiroz, J. Pharm. Biom. Anal. 48 (2008) 428.

[28] G. Meissner, K. Hartonen, M.L. Riekkola, Fresenius J. Anal. Chem. 360 (1998) 618.

[29] C.W. Huch, G.K. Bonn, J. Chromatogr. A 885(2000) 51.

[30] A. Azzouz, B. Jurado-Sánchez, B. Souhail, E. Ballesteros, J. Agric. Food Chem. 59 (2011) 5125.

[31] A. Azzouz, B. Souhail, E. Ballesteros, J. Chromatogr. A 1217 (2010) 2956.

[32] S. Cárdenas, M. Gallego, M. Valcárcel, R. Ventura, J. Segura, Anal. Chem. 68 (1996) 118.

[33] A. Lajeunesse, C. Gagnon, Int. J. Environ. Anal. Chem. 87 (2007) 565.

[34] L.W. Chung, K.L. Lin, T.C.C. Yang, M.R. Lee, J. Chromatogr. A 1216 (2009) 4083.

[35] Validation of Analytical Procedures: Text and Methodology, Q2(R1), International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human USE, November 2005. http://www.ich.org

Table 1

Analytical figures of merit of the proposed method for the determination of pharmacologically active substances in urine[a] and blood[b] samples.

Compounds	Linear range (ng L^{-1})		Sensitivity [signal (ng L^{-1})]		LOD (ng L^{-1})		RSD (%)				m/z[c]		
							Within-day		Between-day				
	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	$M^{+\cdot}$	$[M-15]^+$	Additional ions
Acetylsalicylic acid	2.0–5000	9.9–15000	1.075	0.230	0.6	3.0	5.4	5.7	5.7	6.3	252	237	120, **195**, 210
Carbamazepine	0.6–5000	2.8–15000	3.230	0.625	0.2	0.8	4.7	5.6	5.8	5.8	308	293	**193**, 250
Chloramphenicol	0.6–5000	2.9–15000	3.245	0.660	0.2	0.9	4.1	4.8	4.6	5.2	466	451	208, **225**, 242
Clofibric acid	1.7–5000	8.8–15000	1.080	0.230	0.6	2.6	4.5	4.7	5.0	4.9	286	271	128, **143**
Diclofenac	0.7–5000	3.4–15000	3.195	0.645	0.2	1.1	4.8	4.3	5.0	5.3	367	352	**214**, 242
17α-ethinylestradiol	3.5–5000	17.0–15000	0.540	0.110	1.2	5.2	5.5	6.0	5.9	6.5	440	**425**	232, 300
17β-estradiol	3.8–5000	19.3–15000	0.515	0.100	1.3	5.6	4.5	4.8	5.5	5.3	**416**	401	285, 326
Estrone	3.3–5000	16.5–15000	0.570	0.125	1.1	5.1	5.2	5.8	5.7	6.5	**342**	327	218, 257
Florfenicol	0.7–5000	3.4–15000	3.190	0.655	0.2	1.1	6.2	6.4	6.8	6.6	429	414	**257**, 360
Flunixin	0.6–5000	3.3–15000	3.050	0.605	0.2	1.0	4.7	4.2	5.0	4.7	368	**353**	251, 263
Ibuprofen	0.6–5000	3.3–15000	3.095	0.630	0.2	1.0	3.9	4.3	4.5	5.5	278	263	**160**, 234
Ketoprofen	1.1–5000	6.0–15000	1.625	0.340	0.4	1.9	4.3	4.5	5.2	5.7	326	311	73, **282**
Mefenamic acid	0.6–5000	3.1–15000	3.210	0.665	0.2	0.9	5.4	4.6	6.0	5.6	313	298	208, **223**
Metoprolol	1.8–5000	9.1–15000	1.115	0.215	0.6	2.9	4.8	4.3	5.5	5.6	339	324	72, **223**
Naproxen	1.2–5000	6.6–15000	1.580	0.325	0.4	2.0	5.3	4.9	6.2	6.1	302	287	**185**, 243
Niflumic acid	0.6–5000	3.1–15000	3.150	0.645	0.2	1.0	4.6	4.2	4.9	5.0	354	339	**236**, 263
Paracetamol	0.6–5000	3.3–15000	3.215	0.660	0.2	1.0	5.3	5.6	5.9	6.0	295	**280**	116, 206
Phenylbutazone	3.1–5000	16.1–15000	0.605	0.130	1.1	4.7	4.9	5.3	5.4	5.6	308[d]	–[d]	77**,** **183**, 252
Propranolol	1.7–5000	8.8–15000	1.100	0.225	0.6	2.8	4.0	4.9	4.6	5.7	331	316	72, **215**
Pyrimethamine	2.9–5000	14.6–15000	0.655	0.140	0.9	4.2	6.5	5.9	7.0	7.3	392	**377**	171, 281
Thiamphenicol	0.6–5000	3.0–15000	3.205	0.635	0.2	0.9	6.0	6.8	6.6	7.4	499	484	242, 257, **330**
Triclosan	1.9–5000	9.3–15000	1.050	0.205	0.6	2.9	4.4	4.6	5.1	5.5	362	**347**	200, 310

[a] Volume of sample: 5 mL.
[b] Volume of sample: 1 mL.
[c] The peaks used for quantification are boldfaced; m/z for IS (triphenylphosphate): 77, 170, 325, **326.**
[d] The phenylbutazone is determined as non-derivatized.

Table 2

Percent recovery (± SD, n = 3) of pharmacologically active substances added to urine and blood samples.

Compounds	Urine ($ng\ L^{-1}$)			Blood ($ng\ L^{-1}$)		
	10	100	1000	50	500	5000
Acetylsalicylic acid	87 ± 4	94 ± 5	101 ± 6	92 ± 5	98 ± 6	99 ± 6
Carbamazepine	101 ± 5	97 ± 5	99 ± 6	97 ± 6	98 ± 6	97 ± 5
Chloramphenicol	101 ± 5	102 ± 5	96 ± 4	86 ± 5	100 ± 5	95 ± 5
Clofibric acid	90 ± 5	91 ± 5	99 ± 4	94 ± 5	96 ± 5	101 ± 5
Diclofenac	99 ± 5	90 ± 6	96 ± 5	100 ± 5	94 ± 5	101 ± 5
17α-ethinylestradiol	99 ± 6	101 ± 5	97 ± 6	102 ± 6	92 ± 5	100 ± 6
17β-estradiol	97 ± 5	100 ± 6	94 ± 5	101 ± 5	96 ± 5	93 ± 4
Estrone	99 ± 5	102 ± 5	101 ± 6	95 ± 6	94 ± 5	101 ± 6
Florfenicol	88 ± 5	92 ± 6	102 ± 6	98 ± 6	91 ± 5	92 ± 5
Flunixin	99 ± 5	96 ± 4	101 ± 5	93 ± 4	98 ± 5	95 ± 4
Ibuprofen	89 ± 4	97 ± 5	102 ± 4	95 ± 4	94 ± 4	98 ± 4
Ketoprofen	100 ± 5	93 ± 4	99 ± 5	91 ± 4	94 ± 5	101 ± 5
Mefenamic acid	97 ± 5	95 ± 5	102 ± 6	99 ± 5	97 ± 5	100 ± 5
Metoprolol	91 ± 5	100 ± 5	97 ± 4	88 ± 4	95 ± 4	100 ± 5
Naproxen	96 ± 5	89 ± 5	97 ± 6	94 ± 5	100 ± 5	99 ± 5
Niflumic acid	97 ± 4	101 ± 5	100 ± 5	86 ± 5	101 ± 4	98 ± 5
Paracetamol	95 ± 6	95 ± 5	98 ± 6	88 ± 5	90 ± 5	102 ± 6
Phenylbutazone	100 ± 5	89 ± 4	95 ± 5	88 ± 5	97 ± 6	92 ± 5
Propranolol	91 ± 4	100 ± 5	96 ± 4	100 ± 5	101 ± 5	97 ± 4
Pyrimethamine	96 ± 5	102 ± 6	98 ± 6	89 ± 5	93 ± 5	98 ± 6
Thiamphenicol	102 ± 6	94 ± 6	93 ± 5	99 ± 7	90 ± 6	91 ± 6
Triclosan	95 ± 5	91 ± 4	96 ± 4	85 ± 4	101 ± 5	98 ± 5

Table 3

Determination of pharmacologically active substances in blood and urine samples by proposed methoda.

Sample[b]	Compound	Concentration found (μg L^{-1})	Sample[c]	Compound	Concentration found (μg L^{-1})
Human blood 1	Acetylsalicylic acid	1.2 ± 0.1	Human urine 1	17β-estradiol Estrone	2.8 ± 0.2
	17β-estradiol	1.6 ± 0.1		Ketoprofen	1.6 ± 0.1
	Estrone	2.8 ± 0.2		Triclosan	0.51 ± 0.03
	Triclosan	0.25 ± 0.01			0.41 ± 0.02
Human blood 2	Estrone	2.0 ± 0.1	Human urine 2	Acetylsalicylic acid	0.78 ± 0.04
	Ibuprofen	0.69 ± 0.03		17β-estradiol Estrone	0.87 ± 0.05
	Ketoprofen	0.34 ± 0.02			1.5 ± 0.1
	Triclosan	0.89 ± 0.05			
Human blood 3	–	–	Human urine 3	Estrone Ketoprofen	2.6 ± 0.1
				Triclosan	0.8 ± 0.1
					0.24 ± 0.01
Lamb blood 1	17β-estradiol Florfenicol	5.9 ± 0.3	Human urine 4	Acetylsalicylic acid	0.30 ± 0.01
		0.51 ± 0.03		Estrone	2.9 ± 0.2
				Ibuprofen	0.41 ± 0.02
				Triclosan	0.16 ± 0.01
Lamb blood 2	17β-estradiol	1.9 ± 0.1	Human urine 5	–	–
	Estrone	2.8 ± 0.2			
Cow blood 1	17β-estradiol	4.5 ± 0.3	Lamb urine 1	17β-estradiol	3.3 ± 0.2
	Estrone	4.8 ± 0.3		Estrone	0.78 ± 0.04
	Pyrimethamine	3.8 ± 0.2			
Cow blood 2	17β-estradiol	1.3 ± 0.1	Lamb urine 2	17β-estradiol	0.42 ± 0.02
	Phenylbutazone	3.3 ± 0.2		Florfenicol	0.16 ± 0.01
Pig blood 1	17β-estradiol Florfenicol	0.79 ± 0.04	Cow urine	17β-estradiol Estrone	0.52 ± 0.03
		1.9 ± 0.2		Pyrimethamine	1.7 ± 0.1
					1.8 ± 0.1
Pig blood 2	17β-estradiol	0.67 ± 0.04	Pig urine	17β-estradiol	0.47 ± 0.03
	Estrone	1.9 ± 0.1		Estrone	0.23 ± 0.01
	Pyrimethamine	2.7 ± 0.2			

[a] To 1 mL of blood samples or 5 mL of urine samples (± SD, n = 3).
[b] Human blood: 1, woman (25 years); 2, man (45 years); 3, man (30 years).
[c] Human urine: 1, woman (25 years); 2, woman (40 years); 3, man (26 years); 4, woman (50 years); 5, man (20 years).

SUPLEMENTARY INFORMATION

Gas chromatography-mass spectrometry determination of pharmacologically active substances in urine and blood samples by use of a continuous solid-phase extraction system and microwave-assisted derivatization

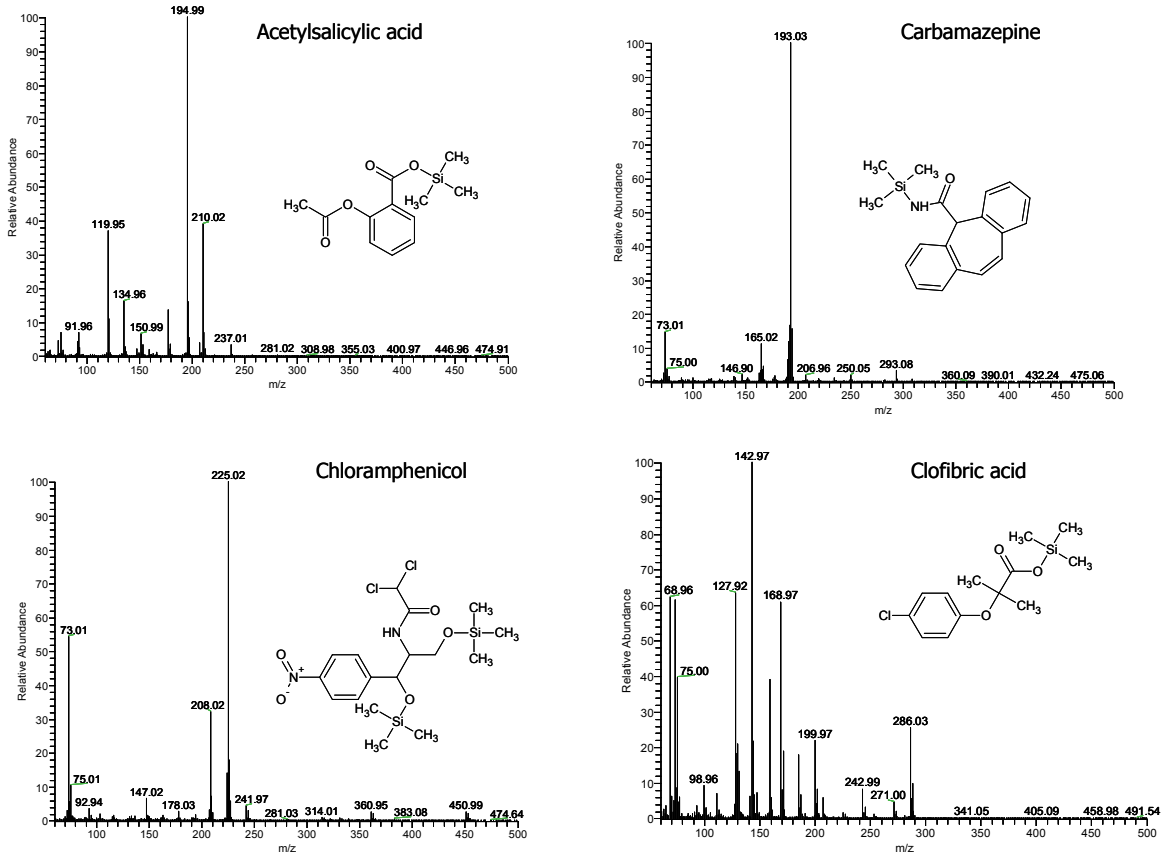

Figure S1. GC-MS (EI) mass spectra recorder of acethyl salycilic, carbamazepine, chloramphenicol and clofibric acid as N-trimethylsilyl derivatives.

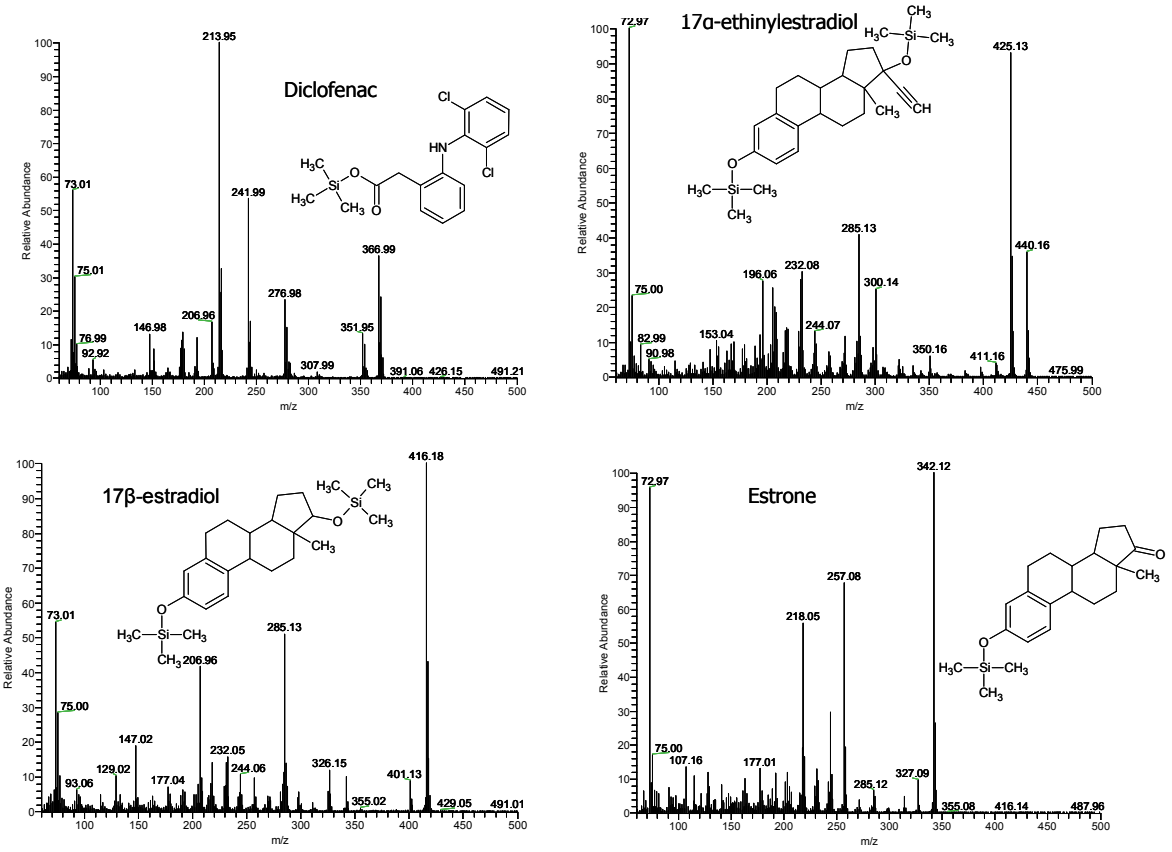

Figure S2. GC-MS (EI) mass spectra recorder of diclofenac, 17α-ethinylestradiol, 17β-estradiol, and estrone as N-trimethylsilyl derivatives.

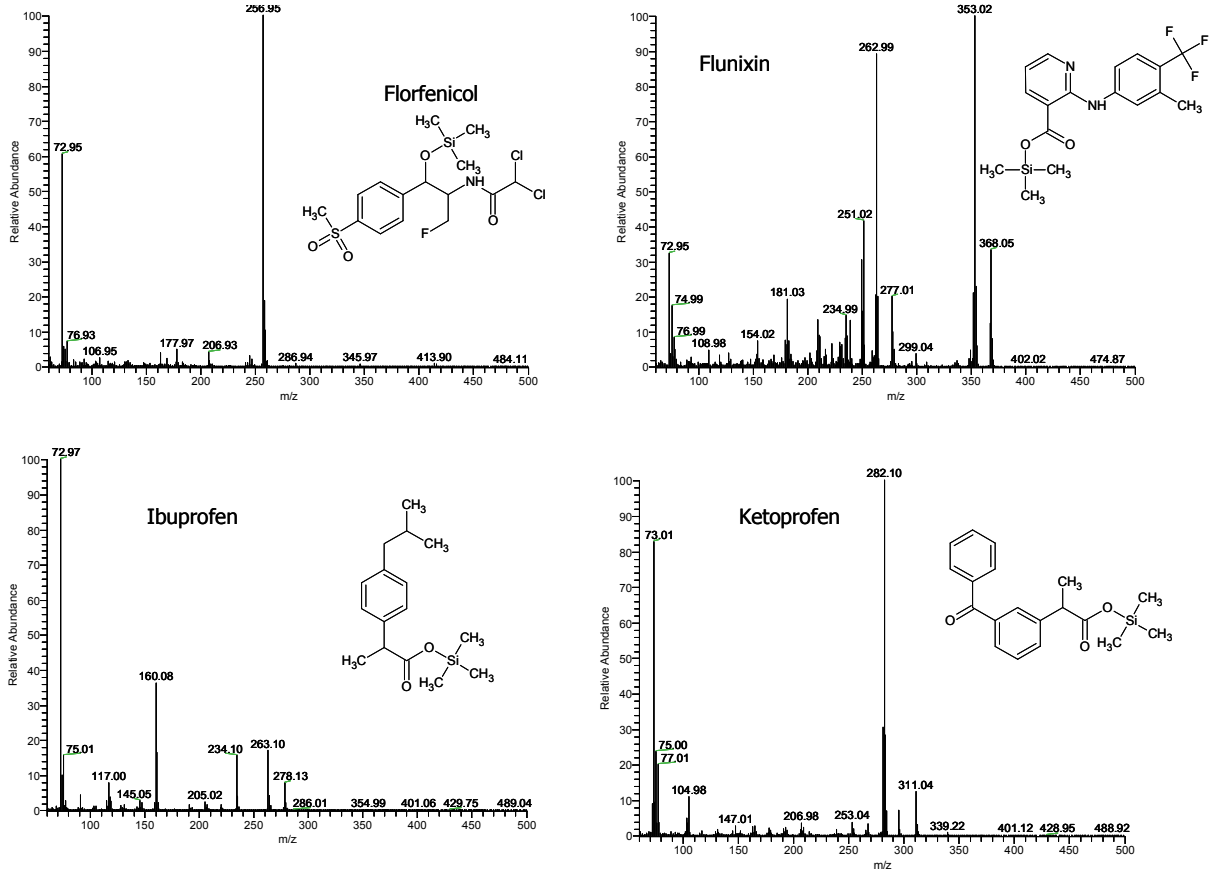

Figure S3. GC-MS (EI) mass spectra recorder of florfenicol, flunixin, ibuprofen and ketoprofen as N-trimethylsilyl derivatives.

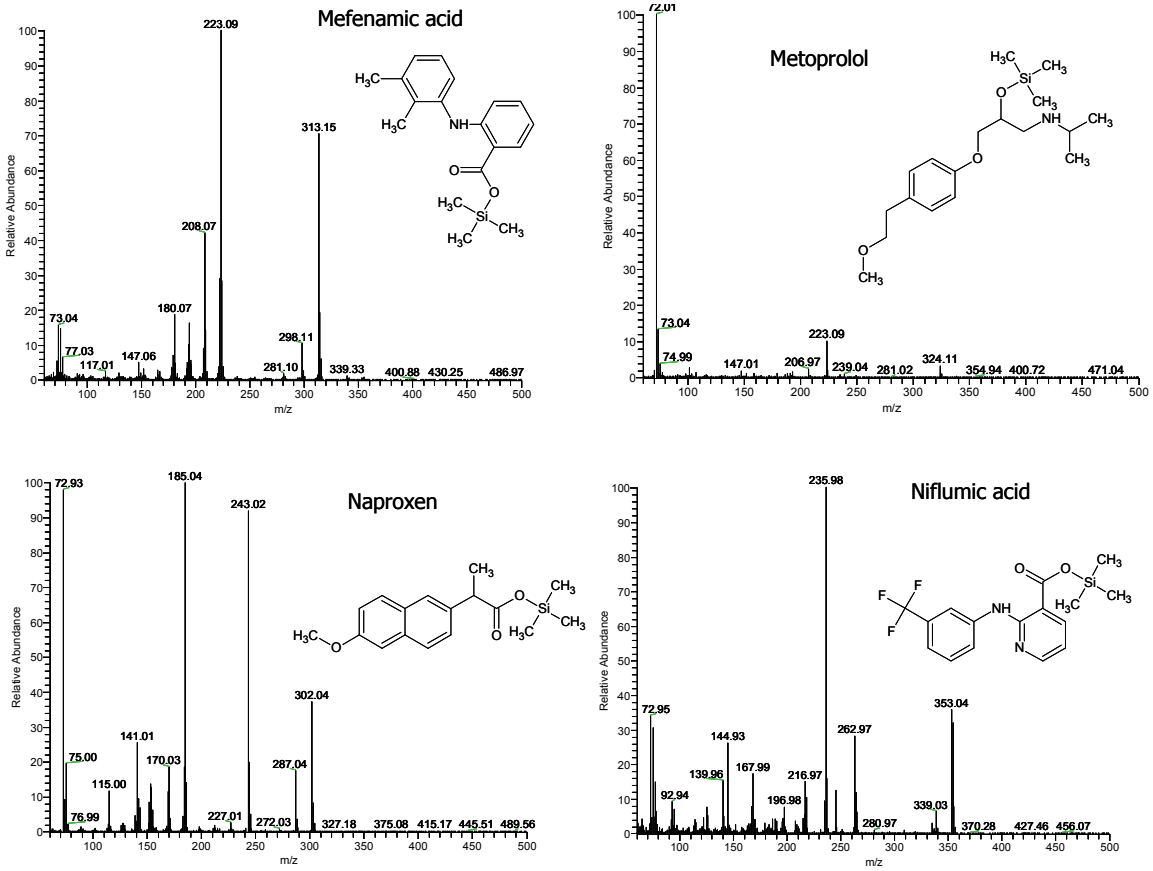

Figure S4. GC-MS (EI) mass spectra recorder of mefenamic acid, metoprolol, naproxen and niflumic acid as N-trimethylsilyl derivatives.

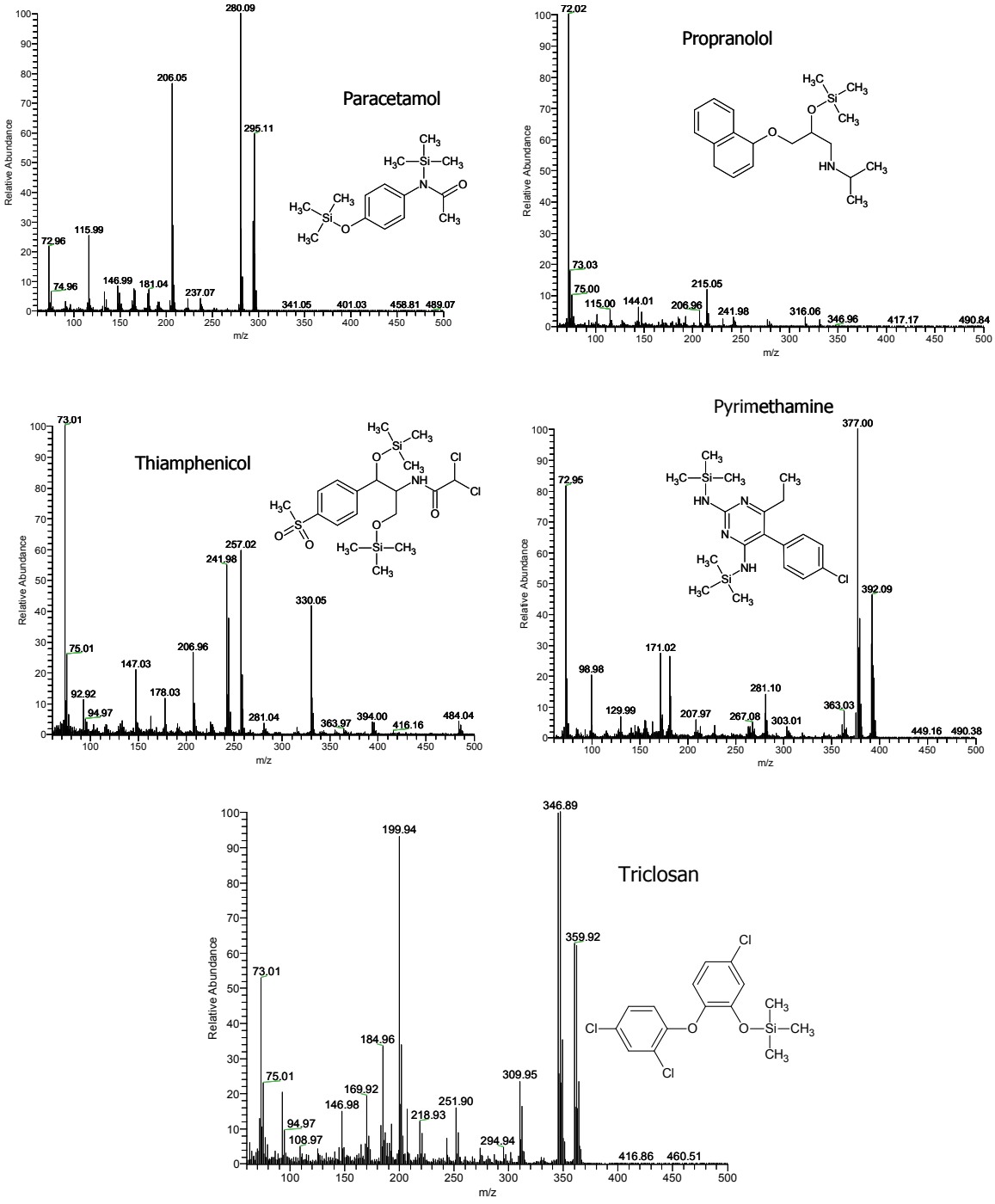

Figure S5. GC-MS (EI) mass spectra recorder of paracetamol, propranolol, pyrimethamine, thiamphenicol and triclosan as N-trimethylsilyl derivatives.

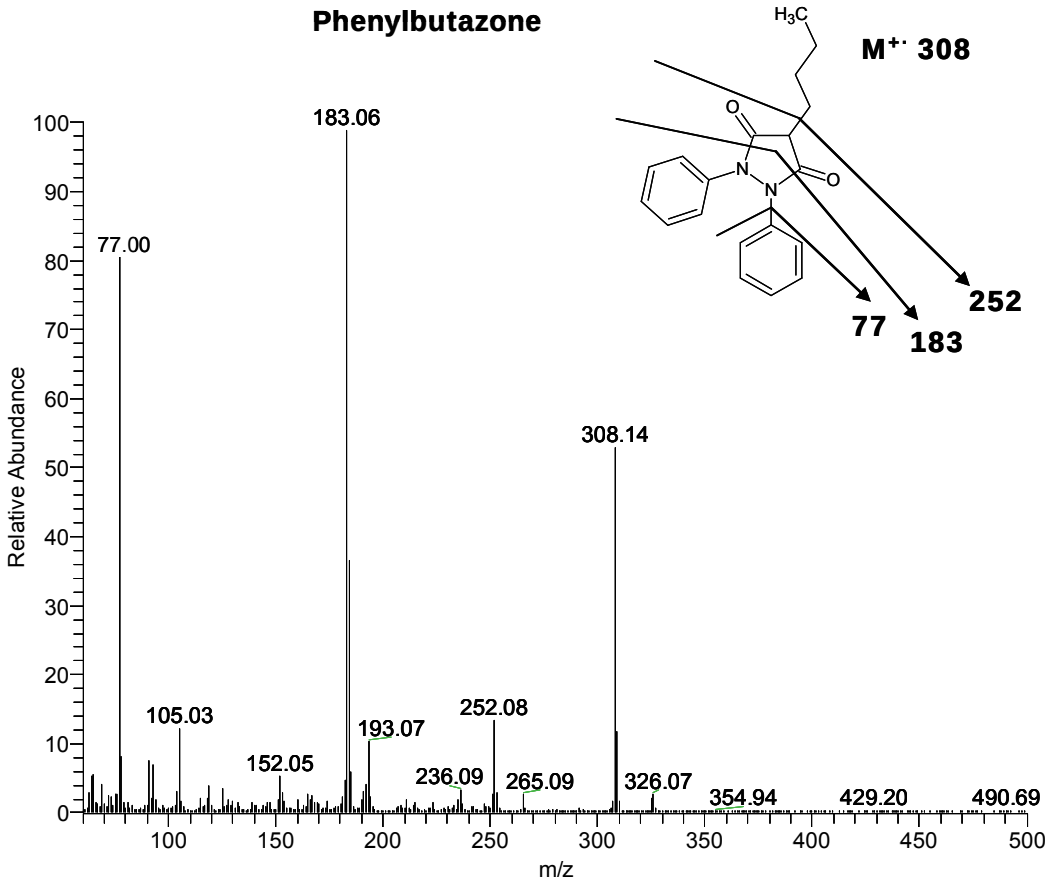

Figure S6. GC-MS (EI) mass spectra recorder of phenylbutazone

CONCLUSIONES

CONCLUSIONES

A tenor de los resultados obtenidos durante el trabajo de investigación realizado en la presente Memoria, se pueden establecer las siguientes conclusiones generales:

1. Se ha desarrollado un sistema continuo de extracción en fase sólida compuesto por una columna rellena de 60 mg del sorbente Oasis HLB en la que se consigue la retención cuantitativa de hasta 22 productos farmacéuticos, hormonas y productos de cuidado personal, siendo desechada la matriz de la muestra. El sistema permite además la elución de los analitos mediante un volumen reducido de eluyente (400 μL de acetato de etilo) con objeto de obtener factores de preconcentración elevados. De esta manera, ha sido posible determinar los analitos objeto de estudio de esta Memoria a niveles inferiores del límite máximo permitido en muestras ambientales, alimentos y fluidos biológicos.

2. Se han simplificado las etapas de pretratamiento de muestra mediante el uso de disolventes especiales seguida de una centrifugación o extracción en un horno de microondas convencional. Estas estrategias permitieron la eliminación cuantitativa de componentes de las matrices de las muestras que pueden perturbar a las etapas de extracción en fase sólida, separación cromatográfica o ionización en el detector de espectrometría de masas de los analitos de interés.

3. Se ha minimizado el consumo de reactivos usados en la etapa de derivatización por sililación de las sustancias farmacológicamente activas mediante la mezcla de BSTFA + 1 % de TMCS. Se ha reducido notablemente el tiempo requerido para conseguir el máximo rendimiento en la reacción de derivatización mediante el empleo de un horno de microondas convencional.

4. La combinación de las técnicas de cromatografía de gases con la espectrometría de masas ha permitido la determinación simultánea de hasta 22 sustancias farmacológicamente activas pertenecientes a diferentes clases terapeúticas (antibióticos, antiinflamatorios no esteroideos/analgésicos, β-bloqueadores, reguladores de lípidos, hormonas, antiepilépticos, antidepresivos y antisépticos)

en menos de 30 minutos con límites de detección de inferiores a la mayoría de las metodologías propuestas en la bibliografía y con desviaciones estándares relativas inferiores al 7 % en todos los casos.

5. Los estudios llevados a cabo utilizando las metodologías desarrolladas han demostrado la presencia de productos farmacéuticos, hormonas y productos de cuidado personal en muestras ambientales (aguas, suelos, sedimentos y lodos), alimentos (leche y carne) y fluidos biológicos (sangre y orina). La presencia de estos contaminantes en estas muestras puede tener efectos negativos y, por esta razón, es importante que estas sustancias se tengan en cuenta en la elaboración de normativas más restrictivas de su uso, sobre todo los más tóxicos.

6. Los resultados obtenidos de los análisis mediante los métodos propuestos en esta Memoria son comparables con los conseguidos en otras metodologías encontradas en la bibliografía. Es destacable la presencia de cuatro antiinflamatorios no esteroideos/analgésicos (ibuprofeno, diclofenaco, ketoprofeno y naproxeno) y las tres hormonas (estrona, 17α-etinilestradiol y 17β-estradiol) en la mayoría de las muestras ambientales analizadas. En el caso de los alimentos analizados, en la mayoría de las muestras analizadas de leche y carne de distinto origen o pescado se han detectado fenilbutazona, florfenicol y las hormonas estrona y 17β-estradiol. Estas dos hormonas también se han encontrado en muchas de las muestras de orina y sangre analizadas, además de algunos antibióticos, antiinflamatorios/analgésicos y el producto de cuidado personal triclosán.

ANEXO

PRODUCCIÓN CIENTÍFICA FRUTO DE ESTA MEMORIA

ARTÍCULOS

Continuous solid-phase extraction and gas chromatography–mass spectrometry determination of pharmaceuticals and hormones in water samples.
A. Azzouz, B. Souhail, E. Ballesteros.
Journal of Chromatography A, 1217 (2010) 2956–2963.

Simultaneous determination of 20 pharmacologically active substances in cow's milk, goat's milk, and human breast milk by gas chromatography–mass spectrometry.
A. Azzouz, B.Jurado-Sánchez, B. Souhail, E. Ballesteros.
Journal of Agricultural and Food Chemistry, 59 (2011) 5125–5132.

Determination of residual pharmaceuticals in edible animal tissues by continuous solid-phase extraction and gas chromatography–mass spectrometry.
A. Azzouz, B. Souhail, E. Ballesteros.
Talanta, 84 (2011) 820–828.

Combined microwave-assisted extraction and continuous solid-phase extraction prior to gas chromatography–mass spectrometry determination of pharmaceuticals, personal care products and hormones in soils, sediments and sludge.
A. Azzouz, E. Ballesteros.
Science of the Total Environment, 419 (2012) 208–215.

Gas chromatography–mass spectrometry determination of pharmacologically active substances in urine and blood samples by use of a continuous solid-phase extraction system and microwave-assisted derivatization.
A. Azzouz, E. Ballesteros.
Journal of Chromatography B, xxx (2012) xxx– xxx.
doi:10.1016/j.jchromb.2012.02.013

COMUNICACIONES A CONGRESOS

Congreso Flow Analysis XI, Mallorca, 2009

Continuous solid-phase extraction and determination of pharmaceutical products in water samples by gas chromatography-mass spectrometry.

A. Azzouz, B. Jurado-Sánchez, B. Souhail, E. Ballesteros.

I Reunión Científica Hispano-Marroquí sobre Seguridad Alimentaria. Tetuán Marruecos, 2009.

Extraction sur phase solide continue et détermination par GC-MS des produits pharmaceutiques et des hormones dans les Eaux.

A. Azzouz, B. Souhail, E. Ballesteros.

Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica, Córdoba, 2010.

Study of various sorbents for the continuous solid-phase extraction of pharmaceuticals from environmental water samples.

A. Azzouz, E. Ballesteros.

VII Reunión de la Sociedad Española de Seguridad Alimentaria Avances en la Seguridad de los Alimentos. Granada, 2010.

Determinación de residuos de productos farmacéuticos en diferentes tipos de muestras de leche mediante cromatografía de gases/espectrometría de masas.

E. Ballesteros, A. Azzouz.

V Reunión de la Sociedad Española de Espectrometría de Masas. (V RSEEM). Málaga, 2011.

Combined microwave assisted extraction and continuous solid-phase extraction prior to gas chromatography-mass spectrometry determination of pharmaceuticals in soils and sediments.

A. Azzouz, E. Ballesteros.

I Congreso "Investigación en Agricultura para el Desarrollo. Madrid, 2011.

Desarrollo de nuevas metodologías para la determinación de residuos de productos farmacéuticos en alimentos procedentes de España y Marruecos.

A. Azzouz, E. Ballesteros.

Semana de la Ciencia 2011, Ciencia y Tecnología Resolviendo Problemas de la Sociedad. Escuela Politécnica Superior de linares, Universidad de Jaén, 2011.

Presencia de fármacos en alimentos de origen animal.

A. Azzouz, E. Ballesteros.