HOLLOW LIQUID-PHASE MICROEXTRACTION IN THE DETERMINATION OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

Ester SAGRISTÀ i PUIG

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Tesi Doctoral

HOLLOW FIBER LIQUID-PHASE MICROEXTRACTION IN THE DETERMINATION OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

Ester Sagristà i Puig

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Programa de doctorat en Ciències Experimentals i Sostenibilitat

Dirigida per: Dra. Victòria Salvadó Martín i Dra. Manuela Hidalgo Muñoz

Memòria presentada per optar al títol de doctora per la Universitat de Girona

La Dra. Victòria Salvadó Martín i la Dra. Manuela Hidalgo Muñoz, catedràtiques del Departament de Química de la Universitat de Girona,

CERTIFIQUEM:

Que aquest treball, titulat *“**Hollow fiber liquid-phase microextraction in the determination of pharmaceuticals and personal care products**”* ha estat realitzat sota la nostra direcció per Ester Sagristà Puig per a l’obtenció del títol de Doctora i que compleix els requeriments per poder optar a Menció Internacional.

I perquè així consti, signem la present certificació.

Dra Victòria Salvadó Martín   Dra Manuela Hidalgo Muñoz

Girona, 11 de juny de 2012

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E. Sagristà, J.M. Cortés, E. Larsson, V. Salvadó, M. Hidalgo, J.Å. Jönsson, Comparison of two extraction methods for the determination of selective serotonin reuptake inhibitors in sewage sludge by hollow fiber liquid-phase microextraction. Journal of Separation Science, accepted.

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RESUM

L'augment de població i de les activitats industrials i agrícoles ha provocat que tones de substàncies biològicament actives siguin alliberades contínuament al medi ambient. Aquesta emissió de substàncies es produeix a través de la descàrrega d'aigües residuals als medis aquàtics o de biosòlids produïts en les plantes de tractament d'aigües residuals en l'agricultura (degut a la seva incompleta eliminació de les aigües o per la seva adsorció en els fangs primaris i secundaris). Dintre d'aquestes substàncies s'hi troben els fàrmacs i productes d'higiene personal que són contaminants emergents no regulats. La presència d'aquestes substàncies en el medi ambient, tot i trobar-se a concentracions baixes, ha generat una gran preocupació perquè es desconeix com actuen i quins són els mecanismes implicats en la seva transformació i/o transport. A la vegada, també són desconeguts els possibles efectes negatius en la salut de les persones i la fauna que poden derivar-se pel contacte amb aquestes substàncies. Per tant, la presència d'aquestes substàncies en aigües residuals, medis aquàtics i fangs és un tema de preocupació.

Un dels grans reptes de la química analítica és dissenyar mètodes selectius i sensibles per a la determinació de contaminants emergents, i els seus productes de transformació en matrius complexes. Tot i la millora de les tècniques instrumentals, la complexitat de les matrius i el fet de que aquests compostos es detectin a nivells traça fa que sigui necessari aplicar tècniques de purificació i enriquiment. L'extracció en fase sòlida és la tècnica més aplicada. No obstant, el seu principal inconvenient és la baixa selectiva i, per tant, la co-elució de possibles interferents que poden afectar la detecció i quantificació dels anàlits escollits. Una alternativa més ecològica per a la determinació de contaminats orgànics neutres, àcids i bàsics és la microextracció en fase líquida amb fibra buida. Aquesta tècnica requereix poc consum de dissolvents orgànics, poca manipulació i és una tècnica de baix cost.

La recerca presentada en aquesta tesi es centra en el desenvolupament de nous mètodes analítics basats en l'ús de la microextracció en fase líquida amb fibra buida

(HF-LPME) i la cromatografia líquida per a la determinació d'alguns fàrmacs i productes d'higiene personal en aigües residuals, medis aquàtics i biosòlids.

Dels productes d'higiene personal, el triclosan i el triclocarban són dos agents antimicrobians utilitzats arreu del món. El metil-triclosan és un producte de degradació del triclosan que es genera a les plantes de tractament d'aigües residuals, concretament en els processos biològics on el triclosan es metila. Tot i que aquests compostos han sigut àmpliament estudiats, només hi ha un estudi on s'hagin determinat alhora els tres anàlits amb un únic mètode d'anàlisi. La tècnica aplicada és la cromatografia líquida amb detecció ultraviolada (HPLC-DAD) però la fase mòbil utilitzada és complexa. Aquesta limitació és deguda a que el triclocarban no es pot analitzar per cromatografia de gasos mentre que el metil-triclosan no es pot analitzar per cromatografia líquida acoblada a espectrometria de masses (LC-MS). En aquesta tesi s'ha desenvolupat un mètode senzill, sensible i selectiu per a la determinació simultània del triclosan, el triclocarban i el metil-triclosan en mostres aquoses. El mètode dissenyat s'ha basat en l'ús de la HF-LPME i la HPLC-DAD. Per la HF-LPME s'ha utilitzat en mode de dues fases per extreure els anàlits de la mostra aquosa a un dissolvent orgànic (di-*n*-hexil èter:decà, 1:1). Alguns paràmetres que afecten la microextracció en fase líquida amb fibra buida es van optimitzar per tal d'obtenir els majors factors d'enriquiment possibles (compresos entre 430 i 707) i els menors límits de detecció. Els límits de detecció obtinguts (entre 5 i 10 ng L^{-1}) es poden comparar amb els valors obtinguts en altres estudis amb espectrometria de masses. Aquests compostos són hidrofòbics i tendeixen a estar retinguts a la matèria particulada present en les aigües, amb el mètode desenvolupat només la part lliure en l'aigua és determinada. Finalment, s'ha avaluat la recuperació del mètode (superior al 74%) i s'ha demostrat la seva aplicació per a la determinació del triclosan, triclocarban i metil-triclosan en aigües residuals de sortida, superficials i potables.

La següent part de la tesi va consistir en aplicar l'anterior mètode de HF-LPME com a tècnica de purificació i enriquiment per a la determinació dels mateixos compostos en biosòlids i sòl fertilitzat amb fang. L'extracció líquida pressuritzada combinada amb la

HF-LPME i LC-MS/MS pel triclosan i triclocarban, i la HPLC-DAD pel metil-triclosan han estat aplicades per la seva quantificació en mostres sòlides. D'acord amb el nostre coneixement, aquesta és la primera vegada que aquests tres compostos són extrets conjuntament en sòls. Alguns paràmetres de l'extracció líquida pressuritzada van ser optimitzats (metanol com a extractant i 60 ºC). Es va observar una gran influència de la matèria orgànica present en la mostra a causa d'algun tipus d'interacció dels anàlits que impedeix la seva extracció mitjançant la microextracció en fase líquida amb fibra buida. Per dur a terme la quantificació dels anàlits en biosòlids es va utilitzar el mètode de l'addició estàndard i es va reduir la quantitat de mostra (0.2 g). Les recuperacions obtingudes van ser 42, 84 i 95% pel metil-triclosan, triclocarban i triclosan, respectivament. Pel sòl fertilitzat amb fang amb un contingut de l'1% en matèria orgànica es va desenvolupar un mètode més senzill amb més quantitat de matriu (0.5 g) i sense addició estàndard i es van obtenir recuperacions superiors al 80% per tots els anàlits. Com era d'esperar, es van obtenir límits de detecció menors pel sòl fertilitzat amb fang (1.2 - 2.1 ng g^{-1}) i superiors pels biosòlids (29 - 51 ng g^{-1}).

D'altra banda, s'ha aplicat la microextracció en fase líquida amb configuració de fibra buida en un sistema de tres fases amb transport facilitat mitjançant gradient de pH per a la determinació de fàrmacs àcids i bàsics en diferents tipus de mostres. Primer, es va desenvolupar un mètode per determinar alguns antiinflamatoris (naproxèn, diclofenac i ibuprofèn) i l'àcid clofíbric en mostres aquoses amb les tècniques HF-LPME i HPLC-DAD. Quan s'utilitza la detecció ultraviolada és necessari aplicar tècniques de preconcentració amb un elevat factor d'enriquiment. La majoria de mètodes utilitzen l'extracció en fase sòlida amb cartutxos Oasis HLB per a l'extracció dels anàlits. A causa de la seva baixa selectivitat es va estudiar la tècnica HF-LPME. Es van avaluar diferents paràmetres que afecten el sistema HF-LPME com el dissolvent orgànic, el temps d'extracció o l'addició de salt. Sota les millors condicions (mostres aquoses a pH 1.5 i una dissolució aquosa com a fase receptora a pH 13) es van obtenir elevats factors d'enriquiment (fins a 11,740). Per tant, s'ha aconseguit obtenir un mètode molt sensible amb límits de detecció semblants als reportats a la bibliografia amb espectrometria de masses (0.5 - 10 ng L^{-1}) però en aquest cas l'equipament és més

barat. El mètode desenvolupat es va comparar amb l'extracció en fase sòlida, i les recuperacions en mostres reals van ser similars (> 60%) però els cromatogrames van ser més nets amb la HF-LPME, per tant, el mètode és més selectiu.

El sistema va ser aplicat a mostres reals per avaluar alguns agents desinfectants (àcid peracètic, clor i radiació UV) en la seva eliminació en estudis de laboratori i en plantes de tractament d'aigües residuals. En el laboratori, els resultats han demostrat que no hi ha degradació dels anàlits sota els efectes dels agents oxidants (àcid peracètic i clor). Quan es va aplicar radiació UV es va observar fotodegradació de tots els anàlits, especialment pel diclofenac i l'àcid clofíbric que es van degradar en 10 i 60 minuts en aigua. Les constants de degradació ajustades a una equació cinètica de primer ordre es van calcular en aigua i aigua residual i es van obtenir valors inferiors en aigües residuals (0.003, 0.007, 0.028 and 0.18 min^{-1} per l'ibuprofèn, naproxèn, àcid clofíbric i diclofenac, respectivament). En la planta de tractament, l'eliminació dels compostos amb tractament UV, i àcid peracetic combinat amb radiació UV es va produir a nivells compresos entre el 35 i el 53%. En aigües d'entrada, les concentracions mitjanes han sigut 3,547 ng L^{-1} pel ibuprofèn, 2,088 ng L^{-1} pel naproxèn, 275 ng L^{-1} per l'àcid clofíbric i 123 ng L^{-1} pel diclofenac.

La última part d'aquesta tesi ha estat centrada en l'avaluació de la microextracció en fase líquida amb configuració de fibra buida com a tècnica d'extracció, purificació i enriquiment per a la determinació de fàrmacs àcids en fangs (mètode HF-LPME directe). S'ha dissenyat i aplicat un nou mètode senzill, selectiu, amb poc consum de dissolvent orgànic i baix cost utilitzant HF-LPME i LC-MS. El procediment consisteix en deixar en contacte durant tota la nit el fang amb aigua i tot seguit la suspensió de fang s'utilitza com a fase de càrrega (fortificada o no) en el procés d'extracció amb HF-LPME. Aquest sistema d'extracció es basa en equilibris i l'aplicació d'un modificat mètode d'addició estàndard en el sistema de HF-LPME. Un cop finalitzat el procés de HF-LPME, la fase receptora s'extreu i s'analitza per LC-MS. Amb les millors condicions del sistema d'extracció (HF-LPME) es van obtenir factors d'enriquiment de fins a 3,254. Aplicant balanços de matèria entre les diferents fases (fang-aigua-fase orgànica-fase

receptora) es va aconseguir una equació lineal. Aquesta relaciona la quantitat amb què es fortifica la suspensió de fangs (corresponent a l'eix de les x) i la concentració de la fase receptora (eix de les y), mentre que la quantitat d'anàlit en el fang correspon a l'ordenada a l'origen tenint en consideració el pendent de la recta. Per verificar la linealitat del sistema, es van utilitzar diferents quantitats de fang humit (0.5, 1 i 1.5 g) i per cada una d'elles les dades es van ajustar a una regressió lineal amb coeficients de determinació superiors a 0.88. Els límits de detecció obtinguts van ser 1.1 – 1.9 ng g^{-1} pel ketoprofèn, 1.2 - 1.9 ng g^{-1} pel naproxèn, per 3.4 - 5.6 ng g^{-1} l'ibuprofèn i 2.8 – 3.1 ng g^{-1} pel diclofenac. Per les diferents quantitats de fangs es obtenir concentracions semblants per cada anàlit amb valors de 29, 138, 39 and 122 ng g^{-1} pel ketoprofèn, naproxèn, diclofenac i ibuprofèn, respectivament.

Es va aplicar el mateix mètode però amb diferents condicions per a la determinació d'inhibidors selectius de recaptació de seròtina amb caràcter bàsic (citalopram, fluoxetina, paroxetina i sertralina) i un metabòlit (norfluoxetina) en fangs (mètode HF-LPME directe). En aquest estudi es van utilitzar una suspensió de fangs bàsica a pH 12 i una dissolució a pH 2 com a fase receptora (fosfat amoni) en el sistema HF-LPME per tal de crear un gradient de pH entre les fases i facilitar el transport. El temps d'extracció va ser de 6 hores, amb valors de preconcentració entre 221 – 995 en aigua Milli-Q, i per diferents quantitats de fangs els coeficients de determinació de les rectes obtingudes van ser de 0.94. Segons el nostre coneixement, només un altre estudi ha aplicat HF-LPME per a l'extracció de cinc inhibidors selectius de recaptació de seròtina en fangs. Tot i així, en el seu sistema la membrana de fibra buida no va ser directament immersa en una suspensió aquosa de fang perquè prèviament el fang va ser extret.

També s'ha desenvolupat un mètode en el que s'ha utilitzat l'extracció pressuritzada amb aigua calenta (PHWE) com a tècnica d'extracció i la HF-LPME com a tècnica de purificació i enriquiment. Aquest procediment s'ha comparat amb el mètode directe de HF-LPME per a la determinació de compostos bàsics. La PHWE és una tècnica d'extracció ecològica que utilitza aigua (en el cas d'aquesta tesi àcid fosfòric a pH 2) a alta temperatura (120 ºC) i pressió per tal d'extreure contaminants orgànics d'una

mostra sòlida amb la mateixa eficàcia que quan s'utilitza un dissolvent orgànic. Després de l'extracció, l'extracte es va sotmetre al procés HF-LPME i la fase receptora es va analitzar amb LC-MS. El sistema PHWE es va optimitzar i es van obtenir altes recuperacions (> 72%). Amb la HF-LPME els factors d'enriquiment van ser 1,244 – 2,068. Per tal d'evitar efectes matrius en el sistema HF-LPME i en el LC-MS es va aplicar el mètode d'addició estàndard en els extractes de la PHWE. Comparant els mètodes de HF-LPME directe i el PHWE-HF-LPME, els límits de detecció van ser de pocs ng g^{-1} i les concentracions del citralopram, paroxetina i fluoxetina van similars amb valors de 530, 40 i 200 ng g^{-1}, respectivament. Els resultats van demostrar que els dos mètodes eren selectius i requerien poca quantitat de dissolvent orgànic i poca manipulació.

RESUMEN

El incremento de población y de las actividades industriales y agrícolas ha provocado que toneladas de sustancias biológicamente activas sean introducidas de manera continuada en el medio ambiente. Esta emisión se produce mediante la descarga de aguas residuales en los medios acuáticos o mediante lodos originados en las plantas de tratamiento de aguas residuales en la agricultura (debido a su incompleta eliminación en las aguas o por su adsorción en los lodos primarios o secundarios). De entre estas sustancias, los fármacos y productos de higiene personal son contaminantes emergentes no regulados. La presencia de estas sustancias en el medio ambiento, aunque se encuentren a concentraciones bajas, ha generado una gran preocupación porque se desconocen cómo actúan y cuáles son los mecanismos implicados en su transformación y transporte. A la vez, también son desconocidos los posibles efectos negativos en la salud de las personas y la fauna que pueden derivarse por el contacto con estas sustancias. Por lo tanto, su presencia en aguas residuales, medios acuáticos y lodos es un tema de preocupación.

Uno de los grandes retos de la química analítica es diseñar métodos selectivos y sensibles para la determinación de contaminantes emergentes y sus productos de transformación en matrices complejas. A pesar de la mejora de las técnicas instrumentales, la complejidad de las matrices y el hecho de que los analitos son detectados a niveles traza hace que sea necesario aplicar técnicas de purificación y enriquecimiento. La extracción en fase sólida es la técnica más aplicada. No obstante, su principal inconveniente es su baja selectividad y, por lo tanto, la co-elución de posibles interferentes que puedan afectar la detección y cuantificación de los analitos escogidos. Una alternativa más ecológica para la determinación de contaminantes orgánicos neutros, ácidos y básicos es la microextracción en fase líquida con fibra vacía. Esta técnica requiere poco consumo de disolvente orgánico, poca manipulación y es una técnica de bajo coste.

La investigación presentada en esta tesis se centra en el desarrollo de nuevos métodos analíticos basados en el uso de la microextracción en fase líquida con fibra vacía (HF-

LPME) i cromatografía líquida para la determinación de algunos fármacos y productos de higiene personal en aguas residuales, medios acuáticos y biosólidos.

De los productos de higiene personal, el triclosán y el triclocarbán son agentes antimicrobianos utilizados en todo el mundo. El metil-triclosán es un producto de degradación del triclosán generado en las plantas de tratamiento de aguas residuales, concretamente en los procesos biológicos donde se produce la metilación del triclosán. Aunque estos compuestos han sido ampliamente estudiados, sólo hay un estudio donde se han determinado a la vez los tres analitos con un único método de análisis. La técnica aplicada es la cromatografía líquida con detección ultraviolada (HPLC-DAD) pero en este estudio se utiliza una compleja fase móvil. Esta limitación es debida a que el triclocarbán no se puede analizar por cromatografía de gases mientras que el metil-triclosán no se puede analizar por cromatografía líquida acoplada a espectrometría de masas (LC-MS). En esta tesis se ha desarrollado un método simple, sensible y selectivo para la determinación simultánea del triclosán, triclocarbán y el metil-triclosán en muestras acuosas. El método diseñado se basa en el uso de la HF-LPME y HPLC-DAD. Para la HF-LPME se basa en el uso de la técnica HF-LPME en modo de dos fases para extraer loa analitos de la muestra acuosa a un disolvente orgánico (di-n-hexyl éter: decano, 1:1). Algunos parámetros que afectan la microextracción en fase líquida con fibra vacía se optimizaron para obtener los mayores factores de enriquecimiento (entre 430 y 707), y los menores límites de detección. Los límites de detección obtenidos (entre 5 - 10 ng L^{-1}) pueden ser comparados con los valores obtenidos en otros estudios en aguas mediante espectrometría de masas. Estos compuestos son hidrofóbicos y tienden a ser retenidos en la materia particulada presente en las aguas, con el método desarrollado sólo la parte libre en el agua es determinada. Finalmente, se ha avaluado la recuperación del método (superior al 74%) y se ha demostrado su aplicación para la determinación del triclosán, triclocarbán y el metil-triclosán en aguas residuales de salida, superficiales y potables.

La siguiente parte de la tesis consistió en aplicar el método anterior (HF-LPME) como técnica de purificación y enriquecimiento para la determinación de los mismos

compuesto en biosólidos y suelo fertilizado con lodo. La extracción líquida presurizada combinada con HF-LPME y LC-MS/MS para el triclosán y el triclocarbán, y HPLC-DAD para el metil-triclosán han sido aplicadas para su cuantificación en muestras sólidas. De acuerdo con nuestro conocimiento, esta es la primera vez que estos tres compuestos son extraídos conjuntamente en sólidos. Algunos parámetros de la extracción líquida presurizada fueron optimizados (metanol como extractante y 60 º C). Se observó una gran influencia de la materia orgánica presente en la muestra a causa de algún tipo de interacción con los analitos que impide su extracción mediante la microextracción en fase líquida con fibra vacía. Para poder cuantificar los analitos en biosólidos se utilizó el método de adición estándar y se redujo la cantidad de muestra. Las recuperaciones fueron del 42, 84 y 95% para el metil-triclosán, triclocarbán y triclosán, respectivamente. Para suelo fertilizado con lodo con un contenido del 1% en materia orgánica se desarrollo un método más simple con 0.5 g y sin adición estándar y se obtuvieron recuperaciones superiores al 80% para todos los analitos. Como era de esperar se obtuvieron límites de detección menores para suelo fertilizado con lodo (1.2 – 2.1 ng g^{-1}) y superiores para los biosólidos (29 – 51 ng g^{-1}).

Por otro lado, se ha aplicado la microextracción en fase líquida con fibra vacía con un sistema de tres fases con transporte facilitado mediante gradiente de pH se ha aplicado para la determinación de fármacos ácidos y básicos en diferentes tipos de muestras. Primero, se desarrolló un método para algunos antiinflamatorios (naproxeno, diclofenaco e ibuprofeno) y ácido clofíbrico en muestras acuosas con HF-LPME y HPLC-DAD. Cuando se utiliza la detección ultraviolada es necesario aplicar técnicas con una elevada pre-concentración. La mayoría de los métodos descritos utilizan la extracción en fase sólida con cartuchos Oasis HLB para la extracción de los analitos. Debido a su baja selectividad se estudió la HF-LPME. Después de evaluar diferentes parámetros que afectan el sistema HF-LPME como el disolvente orgánico, el tiempo de extracción o la adición de sales. Con las mejoras condiciones (muestras acuosas a pH 1.5 y una disolución acuosa a pH 13 como fase receptora) se obtuvieron elevados factores de enriquecimiento (hasta 11,740). Por lo tanto, se ha desarrollado un método muy sensible con límites de detección parecidos a los de la bibliografía

mediante espectrometría de masas (0.5 – 10 ng L^{-1}) pero en este caso el equipamiento es más barato. El método desarrollado se comparó con la extracción en fase sólida, y las recuperaciones en muestras reales fueron similares (> 60%) pero los cromatogramas fueron más limpios con HF-LPME demostrando su selectividad.

El sistema se aplicó a muestras reales para evaluar algunos agentes desinfectantes (ácido peracético, cloro y radiación UV) para su eliminación en estudios de laboratorio y en plantas de tratamiento de aguas residuales. En el laboratorio, los resultados han demostrado que no hay degradación bajo los efectos de los agentes oxidantes (ácido peracético y cloro). Cuando se aplicó radiación UV se observó fotodegradación de todos los analitos, especialmente para el diclofenaco y el ácido clofíbrico que se degradan en 10 y 60 minutos en agua. Las constantes de degradación ajustadas a una ecuación cinética de primer orden se calcularon en agua pura y agua residual con valores inferiores en las aguas reales (0.003, 0.007, 0.028 y 0.18 min^{-1} para el ibuprofeno, el naproxeno, el ácido clofíbrico y el diclofenaco, respectivamente). En la planta de tratamiento, la eliminación de los compuestos con tratamiento UV, y ácido peracético combinado con radiación UV se ha producido entre niveles entre el 35 y el 53%. En aguas residuales de entrada, las concentraciones medianas han sido 3,545 ng L^{-1} para el ibuprofeno, 2,088 ng L^{-1} para el naproxeno, 275 ng L^{-1} para el ácido clofíbrico y 123 ng L^{-1} para el diclofenaco.

La última parte de esta tesis se ha centrado en la evaluación de la microextracción en fase con fibra vacía como técnica de extracción, purificación y enriquecimiento para la determinación de fármacos ácidos en lodos (método HF-LPME directo). Un nuevo método simple, selectivo, con poco consumo de disolvente orgánico y bajo coste se ha diseñado y aplicado utilizando HF-LPME y LC-MS. El procedimiento consiste en dejar en contacto durante toda la noche el lodo con el agua para obtener una suspensión de lodos (fortificada o no) que se utiliza como fase de carga en el proceso de extracción con HF-LPME. Este sistema de extracción se basa en equilibrios y la aplicación de un método modificado de adición estándar en el sistema de HF-LPME. Una vez finalizado el proceso de extracción, la fase receptora se extrajo y analizó por LC-MS. Con las

mejores condiciones del sistema de extracción (HF-LPME) se obtuvieron factores de enriquecimiento de hasta 3,254. Aplicando balances de materia entre las diferentes fases (lodo-agua-fase orgánica-fase receptora) se consiguió obtener una ecuación lineal. Esta relaciona la cantidad con qué se fortifica la suspensión de lodos (correspondiente al eje de las x) y la concentración de la fase receptora (eje de las y), mientras que la cantidad de analito en el lodo corresponde a la ordenada de origen teniendo en cuenta el pendiente. Para verificar la linealidad el sistema, se utilizaron diferentes cantidades de lodo húmedo (0.5, 1 y 1.5 g) y por cada una de ellas los datos se ajustaron a una regresión lineal con coeficientes de determinación superiores a 0.88. Los límites de detección obtenidos fueron 1.1 – 1.9 para el ketoprofeno, 1.2 - 1.9 ng g^{-1} para el naproxeno, 3.4 - 5.6 ng g^{-1} para el ibuprofeno y 2.8 – 3.1 ng g^{-1} para el diclofenaco. Para las diferentes cantidades de lodos se obtuvieron concentraciones parecidas por cada analito con valores de 29, 138, 39 y 122 ng g^{-1} para el ketoprofeno, el naproxeno, el diclofenaco y el ibuprofeno, respectivamente.

El mismo método pero con diferentes condiciones se aplicó para la determinación de inhibidores selectivos de recaptación de serótina con carácter básico (citalopram, fluoxetina, paroxetina y sertralina) y un metabólito (norfluoxetina) en lodos (método HF-LPME directo). En este estudio se utilizó una suspensión de lodos a pH 12 y una disolución a pH 2 como fase receptora (fosfato amónico) con el fin de crear un gradiente de pH entre las fases y facilitar el transporte. El tiempo de extracción fue de 6 horas, con valores de preconcentración entre 221 – 996 en agua purificada, y por diferentes cantidades de lodos los coeficientes de determinación de las rectas obtenidas fueron de 0.94. De acuerdo con nuestro conocimiento, solamente otro estudio ha aplicado HF-LPME para la extracción de cinco inhibidores selectivos de recaptación de serótina en lodos. Pero en su sistema la membrana de fibra vació no fue directamente sumergida en una suspensión acuosa de lodo porque previamente el lodo fue extraído.

Finalmente, se ha desarrollado un método combinando la extracción líquida presurizada con agua caliente (PHWE) como técnica de extracción y la HF-LPME como

técnica de purificación y enriquecimiento para la determinación de compuestos básicos. Éste se comparó con el método directo de HF-LPME para la determinación de los mismos analitos. La PHWE es una técnica de extracción ecológica que utiliza agua (en el caso de esta tesis ácido fosfórico a pH 2) a alta temperatura (120 ºC) y presión con tal de extraer contaminantes orgánicos de una muestra sólida con la misma eficacia que cuando se utiliza un disolvente orgánico. Después de la extracción, el extracto se sometió al proceso HF-LPME y la fase receptora se analizó con LC-MS. El sistema PHWE se optimizó y se obtuvieron altos valores de recuperación (> 72%), por otro lado con la HF-LPME los factores de preconcentración fueron 1,244 – 2,068. Para evitar efectos matrices en el sistema HF-LPME y en el LC-MS se aplicó el método de adición estándar en los extractos de la PHWE. En los métodos de HF-LPME directo y el PHWE-HF-LPME, los límites de detección fueron de pocos ng g^{-1} y las concentraciones del citalopram, paroxetine y fluoxetina fueron similares con valores de 530, 40 y 200 ng g^{-1}, respectivamente. Los resultados demostraron que los dos métodos eran selectivos y requerían poca cantidad de disolvente orgánico y poca manipulación.

SUMMARY

As a result of human development, extensive amounts of pharmaceuticals and personal care products (organic emerging pollutants) are introduced into the environment mainly by wastewater treatment plants discharges either effluent wastewater (due to the incomplete removal during wastewater treatment) reaching into the aquatic environment or sewage sludge which is spread onto agricultural land. These compounds are not regulated and it is unknown their negative effects on humans and wildlife, thus their presence in environmental waters, threat to drinking water sources, or sewage sludge is a concerning issue.

One of the main challenges of analytical chemistry is to develop selective and sensitive methods for the detection and quantitation of pharmaceuticals and personal care products and their transformation products in complex matrices. Despite the great instrumental advances achieved so far, matrix complexity and the fact that these compounds must frequently be monitored at very low concentrations, makes sample enrichment and clean-up indispensable steps. Solid-phase extraction is the most commonly used technique for the clean-up and preconcentration of emerging pollutants. However, their determination in complex samples as untreated wastewater, some environmental waters and solid samples is hampered by co-extracted organic interferences. Hollow fiber liquid-phase microextraction is an alternative environmentally friendly technique which can be applied for the determination of acidic, neutral and basic organic pollutants requiring low amount of organic solvents and low manipulation.

The research presented in this thesis is focused on the development of new methods based on the use of hollow fiber liquid-phase microextraction (HF-LPME) technique and liquid chromatography for the determination of some of the most consume pharmaceuticals and personal care products in wastewaters, environmental waters and sewage sludge.

As personal care products, triclosan and triclocarban are broad antimicrobials applied around world. Methyl-triclosan is the methylated product of triclosan generated in wastewater treatment plants. In the literature several reports have studied these analytes, however only one report have determined the three organic compounds by a unique method by liquid chromatography-DAD detection (HPLC-DAD) using a complex mobile phase. Triclocarban cannot be analyzed by gas chromatography-mass spectrometry while methyl-triclosan is quantified with this technique and no with liquid chromatography-mass spectrometry (LC-MS). In this thesis, a simple, sensitive and selective method has been employed for the simultaneous determination of neutral personal care products (triclosan, triclocarban and methyl-triclosan) in aqueous samples by HF-LPME and HPLC-DAD using as mobile phases acetonitrile and reagent water. The developed method is based on the use of two-phase HF-LPME extracting the analytes from the aqueous samples to an organic solvent (di-*n*-hexyl ether:decane (1:1)). The HF-LPME parameters were optimized to obtain the highest enrichment factors (430 - 707) and lowest limits of detection (5 – 10 ng L^{-1}), whose can be compared to those reported for triclosan, triclocarban and methyl-triclosan in waters using mass spectrometry. These compounds are hydrophobic compounds whose tend to adsorb to particulate matter, with the method developed only the free-water content in water was determined. Finally, the method was evaluated spiking different type of water samples demonstrating that the method was suitable for the selected analytes, recoveries were higher than 74% in effluent wastewater, surface water and drinking for all the analytes.

The following part of this thesis was the application of the previously developed HF-LPME method as clean-up and enrichment technique for the determination of the same analytes in solid samples (biosolids and sludge amended soil). Pressurized liquid extraction combined to HF-LPME and LC-MS/MS for triclosan and triclosan, and HPLC-DAD for methyl-triclosan was applied for their quantitation in sludge amended soil and biosolids. To our knowledge is the first time that these three compounds are extracted together from soils. Pressurized liquid extraction was optimized and under the optimized conditions (methanol as extractant and 60 ºC) a great influence of sample

organic content was observed due to some kind of interaction with the analytes not allowing the extraction of them by hollow fiber liquid-phase microextraction. Hence, standard addition and a low sample size (0.2 g) were used for the quantitation of the analytes on biosolids with recoveries of 42, 84 and 95% for M-TCS, TCC and TCS, respectively. For sludge amended soil with 1% organic matter a simple method with 0.5 g and without standard addition was used with recoveries higher than 80% for all the analytes. As was expected, lower limits of detection were obtained for soil (1.2 - 2.1 ng g^{-1}) than for sludge (29 - 51 ng g^{-1}).

On the other hand, three-phase HF-LPME by pH gradient has been applied for the determination of acidic and basic pharmaceuticals in different sample matrices. First of all, a method for the determination of anti-inflammatory drugs (naproxen, diclofenac and ibuprofen) and clofibric acid in aqueous matrices has been developed using HF-LPME and HPLC-DAD. When ultraviolet detection is used selective techniques with high preconcentration are required, however most of the methods of literature used solid-phase extraction with Oasis HLB cartridges for the extraction of analytes but the selectivity of this technique is low. After evaluation of different parameters affecting the HF-LPME system such as membrane solvent, extraction time or salt addition, and under the best conditions using aqueous donor samples at pH 1.5 and an aqueous acceptor solution at pH 13, very high enrichment factors up to 11,740 were obtained allowing having a very sensitive method with limits of detection similar to those reported in the literature by mass spectrometry quantitation (0.5 - 10 ng L^{-1}) but in this case the equipment required was cheaper. Moreover, the selectivity of the system was very high as could be observed on the chromatogram. The developed method was compared with solid-phase extraction, and recoveries in real samples were similar (> 60%) but cleaner chromatograms were obtained by HF-LPME.

The system was employed to real samples for the evaluation of some disinfectants agents (peracetic acid, chlorine and UV radiation) for their elimination in laboratory studies and in a wastewater treatment plant. In laboratory, results have shown that no degradation occurs under oxidant agents (PAA and chlorine) while under UV radiation

photodegradation of all analytes was observed, especially for diclofenac and clofibric acid whose were degraded in 10 and 60 min in reagent water. Degradation rate constants fitted to pseudo-first order kinetic were calculated in reagent water and wastewater observing lower values in wastewater than in reagent water which were 0.003, 0.007, 0.028 and 0.18 min^{-1} for ibuprofen, naproxen, clofibric acid and diclofenac. In a WWTP, removal efficiencies of UV treatment or PAA/UV treatment were similar with values between 35 and 53%. In the WWTP, the highest average concentrations in the influent wastewater correspond to ibuprofen (3,547 ng L^{-1}) followed by naproxen (2,088 ng L^{-1}), clofibric acid (275 ng L^{-1}) and diclofenac (123 ng L^{-1}).

The last part of this thesis has been focused on the evaluation of three-phase HF-LPME by pH gradient as extraction, clean-up and enrichment technique for determining acidic pharmaceuticals in sewage sludge (direct HF-LPME). A novelty, simple, selective, free organic solvent and low cost method was performed using HF-LPME combined with LC-ESI-MS. After leaving sludge and water in contact overnight, slurry sludge samples (spiked o no) were used as donor phase in the HF-LPME process which was based on equilibriums and a modified standard addition on HF-LPME system. After the extraction (4 hours), the acceptor phase was analyzed by LC-MS. Under the best HF-LPME conditions enrichment factors were up to 3,254. Employing mass balances between the difference phases (sludge-water-organic phase-acceptor phase), an equation was obtained and fitted like a regression line having the amount spiked to slurry samples as x-axis and concentration in acceptor phase as y-axis, while the amount of analyte in the sludge was related to the intercept taking into account the slope. Several amounts of wet sludge were tested for evaluating the linearity of the system (0.5, 1 and 1.5 g), obtaining one regression line for each sample size with coefficients of determination higher than 0.88. Method limits of detection were between 1.1 - 1.9 ng g^{-1} for ketoprofen, 1.2 - 1.9 ng g^{-1} for naproxen, 3.4 - 5.6 ng g^{-1} for ibuprofen and 2.8 – 3.1 ng g^{-1} for diclofenac. Similar concentrations for each analyte with different sample sizes were obtained with values of 29, 138, 39 and 122 ng g^{-1} for ketoprofen, naproxen, diclofenac and ibuprofen, respectively.

The same method but with different HF-LPME conditions was applied for the determination of basic selective serotonin reuptake inhibitors (citalopram, fluoxetine, paroxetine and sertraline) and one metabolite (norfluoxetine) in sewage sludge (direct HF-LPME). In this case, a basic slurry sludge solution at pH 12 and an acceptor solution at pH 2 (ammonium phosphate) were used in order to create a pH gradient. Extraction time was about 6 hours, preconcentration rates were 221 – 995 in reagent water and for different sludge amounts obtained regression lines had coefficients of determination higher than 0.94 for modified standard addition. To our knowledge, only another study has employed HF-LPME for the extraction of five SSRIs from sewage sludge. However, HF-LPME was not performed directly from the aqueous sludge suspension because previously to HF-LPME the analytes were transferred to reagent water and the sludge was removed.

Another approach based on the use of pressurized hot water extraction as extraction technique and HF-LPME as clean-up and enrichment was developed and compared to direct HF-LPME for basic compounds. Pressurized hot water extraction (PHWE) is a green solvent extraction technique which employs water (in this case was phosphoric acid at pH 2) at high temperature (in this case was 120 ºC) and pressure for the extraction of organic pollutants from solid sample with the same efficiency as organic solvents. After the extraction, the extract was submitted to HF-LPME and the acceptor phase was analyzed by LC-MS. High recoveries (> 72%) were obtained by pressurized hot water extraction after its optimization, on the other hand enrichment factors of HF-LPME system were 1,244 - 2,068. Standard addition on PHWE extracts was used in order to compensate matrix effects on HF-LPME and LC-MS. In direct HF-LPME and PHWE-HF-LPME methods, limits of detection were of few ng g^{-1} and similar concentrations were found for citalopram, paroxetine and fluoxetine with values of 530, 40 and 200 ng g^{-1}, respectively. The results showed that both methods which are environmental friendly, selective and require very few manipulation can be applied.

CHAPTER 1:

Introduction

Because of human and economic development, tons of biologically active chemicals are introduced into the environment. For decades, chemical contamination has been focused on priority pollutants which are hydrophobic, persistent in the environment and carcinogenic. Priority pollutants such as heavy metals, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins or pesticides are regulated and they have been identified and studied in order to determine their presence and distribution in the environment [1-3].

Currently, this interest has changed to new contaminants called emerging pollutants due to the increasing use of chemicals in industry, agriculture, livestock, domestic consumption and personal care as well as increased consumption of drugs. Emerging pollutants are not regulated but they are usually present in the environment at low levels (several ng L^{-1} to µg L^{-1}); as a consequence, there is increasing concern about their presence in the environment and their risk, owing to [4-11]:

- Their toxicity.
- Their incomplete removal in wastewater treatment plants (WWTPs).
- The constant input of the compounds from society into the environment, which means they have to be considered pseudo-persistent although their half life could be low.
- The formation of more toxic by-products.
- The long-term effects of the parent compounds, metabolites and by-products on humans and wildlife due to their continuous introduction into the environment.
- The synergistic effect between compounds which increases the risk for both the ecosystem and humans.

Therefore, it is interesting to determine at which levels emerging pollutants are present in the environment in order to evaluate the possible effects on humans and wildlife. The identification and quantitation of emerging pollutants is not an easy task because of the matrix complexity of the samples and the requirement of sensitive analytical methods which are able to determine them at trace levels. Furthermore, due

to the lack of legislation governing their determination there are no standard methods proposed and improved procedures are required. In this context, this thesis has focused on the development of new, environmentally friendly, methodologies based on hollow fiber liquid-phase microextraction (HF-LPME) for the detection and determination of some non-regulated organic pollutants in complex environmental samples such as wastewaters and solid samples.

1.1. ANALYTES

Emerging pollutants include products used in high quantities for human and veterinary applications; some classes of emerging pollutants are pharmaceuticals, personal care products, hormones, brominated flame retardants, synthetic musk compounds or alkylphenols [1].

Three types of organic compounds have been studied in this thesis: 1) pharmaceuticals which include non-steroidal anti-inflammatory drugs, antiepileptics, lipid regulators and antidepressants with acidic, basic and neutral properties, 2) bactericides present in personal care products and, 3) endocrine disruptors such as hormones and industrial products which are phenolic derivates. In Table 1.1 the selected compounds, with some relevant physical-chemical properties, are presented.

Table 1.1. Molecular weight, pKa, LogK_{ow} and water solubility of the selected analytes.

	Structure	Mw	pKa	Log K_{ow}	Water solubility (mg L^{-1})	Ref
Pharmaceuticals						
Non-steroidal anti-inflammatory drugs (NSAIDs)						
Naproxen		230.26	4.15	3.18	15.9	[12,13]
Ketoprofen		254.28	4.45	3.12	51	[12,13]
Ibuprofen		206.3	4.91	3.97	21	[12,13]
Diclofenac		296.15	4.15	4.51	2.37	[12,13]
Antiepileptic						
Carbamazepine		236.27	<2, 13.9	2.45	13.9	[12,14]

Table 1.1. Molecular weight, pKa, LogK_{ow} and water solubility of the selected analytes (continued).

	Structure	Mw	pKa	Log K_{ow}	Water solubility (mg L^{-1})	Ref
Lipid regulator						
Clofibric acid		214.6	2.84	2.84		[15]
Selective Serotonin reuptake inhibitors (SSRIs)						
Citalopram		324.16	9.59	3.74	31.1	[16]
Paroxetine		329.14	10.32	3.95	35.3	[16]
Norfluoxetine		295.12	9.05	4.07		[17]

Table 1.1. Molecular weight, pKa, LogK_{ow} and water solubility of the selected analytes (continued).

	Structure	Mw	pKa	Log K_{ow}	Water solubility (mg L^{-1})	Ref
Fluoxetine		309.13	10.05	4.05	60.3	[16]
Sertraline		305.07	9.47	5.29	3.52	[16]
Endocrine disruptors						
Hormone						
Estradiol		272.4	10.4	4.01	13	[18,19]
Industrial products						
4-nonylphenol		220.35	7.2	5.76		[20,21]

Table 1.1. Molecular weight, pKa, LogK_{ow} and water solubility of the selected analytes (continued).

	Structure	Mw	pKa	Log K_{ow}	Water solubility (mg L^{-1})	Ref
Bisphenol A		228.3	8.9	3.32		[22]
Personal care products						
Triclosan		289.5	7.9	4.8	4.62, 12	[23,24,25]
Triclocarban		315.6	12.77	4.9	0.65	[23,24,26]
Methyl-triclosan		303.5	12.77	5.2		[23,24,26]

1.1.1. PHARMACEUTICALS

Pharmaceuticals are therapeutic drugs used for the diagnosis, cure, mitigation, treatment or prevention of diseases in humans and animals. Pharmaceuticals are produced in large quantities worldwide (hundred of metric tons per year) with more than 3,000 active substances in the market [27,28]. Pharmaceuticals are classified in families according to their purpose: non-steroidal anti-inflammatory drugs, antidepressants, antiepileptics, β-blockers, antibiotics, sedatives or contraceptives [28].

After consumption, pharmaceuticals are not completely metabolized by the human body and they are excreted as the parent compounds, water soluble conjugates or as metabolites to soils, sediments or, the most important destination, wastewater, which is treated in WWTPs. Moreover, it is known that metabolites can be more biologically active and toxic than the parent compounds which makes their determination important [10,29].

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most used over the counter pharmaceuticals for human and veterinary applications worldwide. In addition, NSAIDs are one of the most frequently detected groups in aquatic environments at concentration levels of μg L^{-1} in surface water, drinking water and wastewater [5].

NSAIDs have analgesic, antipyretic and anti-inflammatory properties, reducing inflammation and pain by the inhibition of cyclooxygenases (COX-1 and COX-2) which are enzymes that catalyze the synthesis of prostaglandins. NSAIDs have been associated with cardiac abnormalities, disruption of the heat shock response in rainbow trout or teratogenicity in zebrafish embryos. Some of the members of this group are ketoprofen (KTP), naproxen (NPX), ibuprofen (IBP) and diclofenac (DCF). For example, DCF has been demonstrated to bioaccumulate and hinder the stimulation of prostaglandin synthesis in the kidney at concentrations of 0.5 - 50 μg L^{-1}. Finally, it has

also been reported that the toxicity of NSAIDs can be additive because they act in the same way [27,30,31].

The selected compounds, being an aromatic carboxylic acid group, are highly soluble in water, stable and non-volatile, and, moreover, they have a moderate tendency to adsorb onto solids. Some properties of KTP, NPX, IBP and DCF are presented in Table 1.1 [13].

Antiepileptic

Carbamazepine (CRB) is one of the most used antiepileptic drugs for nervous system and related illnesses, such as epilepsy, bipolar disorder or acute mania because it reduces brain excitation. Another application is for the treatment of schizophrenia because of its mood-stabilizing properties [32]. Chemically, CRB is a neutral compound with relatively low solubility in water. CRB has frequently been detected in wastewater and surface water [33].

Lipid regulators

Clofibric acid (CLF) is the active metabolite of the blood regulators clofibrate, etofyllin clofibrate and etofibrate which are used for reducing triglycerides and decreasing the risk of cardiovascular events [32]. CLF is a refractory compound with a low degradability and high mobility in water. It has been detected in wastewater, surface water and ground water [33].

Antidepressants

Selective serotonin reuptake inhibitor (SSRI) antidepressants are the most consumed and prescribed psychiatric drugs worldwide. SSRIs are used to treat psychiatric illnesses such as depression, compulsive-obsessive disorder or panic disorder. SSRIs inhibit the reuptake of the neurotransmitter serotonin, thus increasing the serotonin level at presynaptic neuronal membranes and that enzyme is related to functions such as food intake and hormonal and neuronal mechanism [16,28]. Four SSRIs have been studied in this thesis: citalopram (CTP), fluoxetine (FLX), paroxetine (PRX) and sertraline (SRT). Like other pharmaceuticals, psychiatric drugs are not completely

metabolized by the human body and they are excreted as the parent compounds or as metabolites to wastewater. This is the case of norfluoxetine (NFX), one N-desmethyl product of FLX, although it is less potent than the parent compound itself [2,16,28].

SSRIs have been detected in fish species and it is known that some possible effects in fish are hormonal changes, alteration of their behavior or increment of the mortality of some species. These compounds are the most commonly detected pharmaceuticals in surface waters and WWTP effluents at trace levels and they have also been detected in sediments at ng g^{-1} levels [16,27,28]. Although the environmental concentration of SSRIs is not enough to cause negative effects, organisms are exposed to other compounds that can have additive effects.

1.1.2. Personal care products

Personal care product (PCP) ingredients are a group of chemicals for topical use present in disinfectants, fragrances, insect repellents or sunscreens and consumer products. Large amounts of PCPs are used and they enter into the environment unaltered or as metabolites; these compounds are environmentally persistent, bioactive and bioaccumulative [2,34,25].

Two disinfectants have been studied in this thesis. Triclosan (TCS), also called Irgasan DP 300 or Irgare MP, and triclocarban (TCC) are two of the most consumed and detected biocide compounds in wastewater. Moreover, they have also been detected in sludge and sediments due to their lipophilic character [2,8,36,37]. They are broad spectrum antimicrobial agents extensively used in a wide variety of PCPs such as toothpaste, soap, shampoo or deodorant and consumer products such as biocides in baby toys, carpets, sportswear or plastic kitchenware [7,24,35,38,40]. In Europe, around 350 tons $year^{-1}$ of TCS are produced while in the United States between 227 and 454 tons $years^{-1}$ of TCC are consumed [37,38].

TCS is a chlorinated phenoxyphenol which has been used for more than 25 years as an antimicrobial active compound in consumer care products with a maximum concentration of 0.3% (w/w), according to European Union directive 76/768/CEE and

subsequent amendments [41,42]. It is known that TCS can act as an endocrine disruptor in some organisms and it is toxic for some aquatic organisms such as algae species [26]. Although concentrations found in environmental studies are lower than the values required for their negative effects, chronic exposure of organisms is environmentally relevant. Furthermore, *in vitro* studies indicate that low levels may disturb metabolic thyroid hormone homeostasis in rats [43]. Also, the toxicity of TCS increases in the presence of linear alkyl benzene sulphonates (LAS) which are also present in wastewater [44]. Moreover, TCS is a lipophilic compound which can photodegrade and biodegrade into species that are more persistent and toxic such as dioxins or methyl-triclosan (M-TCS) [7,8,24,37,45-47]. M-TCS is one of the non-polar metabolites of TCS which is environmentally persistent, more lipophilic than TCS and bioaccumulative [6,46,47]. M-TCS is a biodegradation by-product of TCS that is formed in biological processes applied in WWTPs.

Furthermore, TCC is a common antimicrobial agent which has been used since 1957. TCC is a toxic, persistent and recalcitrant compound and it has been classified as a potential endocrine disruptor that amplifies the expression of testosterone. Moreover, this compound has been proved to bioaccumulate and some of its degradation products are carcinogenic [8,24,26].

TCS, TCC and M-TCS are relatively hydrophobic compounds at neutral pH; however TCS can desprotonate, increasing its solubility in water because of its hydroxyl group [18].

1.1.3. ENDOCRINE DISRUPTORS

Endocrine disruptors are agents that interfere with the normal function of the endocrine system. These chemicals can antagonize the effects of the hormones, modify the synthesis and the metabolism of the hormones and modify hormone receptor levels. Some of the effects are infertility, alteration of the thyroid, sexual underdevelopment, hyperactivity or attention deficit disorder. Endocrine disruptors can be classified into different groups; among them [1,4,9,48]:

- Natural hormones such as fitoestrogens.
- Synthetic hormones such as estrogens (estradiol or estrone), which can be considered pharmaceuticals.
- Industrial and household chemicals such as 4-nonylphenol, bisphenol A, isophorone or benzophenone.
- Pharmaceuticals with hormonal effects such as NSAIDs, antidepressants, antibiotics or analgesic drugs.
- PCPs such as disinfectants (TCS), fragrances or UV screens.

As can be seen, some pharmaceuticals and personal care products (PPCPs) can be considered endocrine disruptors. In this thesis, three phenolic compounds have been studied: one natural and synthetic hormone (estradiol) and two industrial chemicals (4-nonylphenol and bisphenol A).

Estradiol

Estradiol (EST) is an endogenous estrogen steroid which can be considered one of the most potent estrogens used. EST regulates the metabolism and reproductive function of organisms and, because of that, it is used as the active ingredient in birth control pills and in drugs associated with menopause [32]. Hence, it can be considered a pollutant due to its high consumption, bioaccumulation and persistence, and its low biodegradability [49,50].

4-nonylphenol

4-nonylphenol (NP) is the main alkylphenol used in the production of non-ionic surfactants (alkylphenol ethoxylates) for detergents and PCPs such as shampoos, with a production of 73,500 tons per year in Europe (2002). In addition, it is used as a stabilizer for ethylcellulose resin, oil-soluble phenol resin and esters [48,51-53]. Moreover, this compound can be formed in WWTPs during biological treatment when the wastewater contains alkylphenol ethoxylates. Interest in NP is due to its high production and toxicity which is related to its low degradation and high bioaccumulation. Related to its toxicity, for example, in fish the fertility of the females

was reduced after 4 weeks of exposition to NP [49,52,54]. This alkylphenol has been detected in several environmental waters at trace levels and is considered a priority pollutant in waters by the EU Water Framework Directive (2000/60/CE).

Bisphenol A

Bisphenol A (BPA) is widely used in households and industry and is one of the most produced chemicals worldwide. BPA is a plasticizer manufactured in high quantities and it is used as a monomer in the synthesis of epoxy resins, polycarbonate plastics, flame-retardants and unsaturated polyester-styrene resins which can be used as additives in dental fillings and as antioxidants in plastics such as water containers [18, 55,56]. As a consequence, it can enter into the environment through either the final products or the manufacturing processes and it is one of the most detected compounds in the environment. It has been detected in wastewater, surface water and drinking water [18,49,56].

1.2. FATE OF EMERGING POLLUTANTS IN THE ENVIRONMENT

Emerging pollutants are introduced into the environment by different sources which can be summarized as (Figure 1.1) [9-11, 29,48,57]:

- Effluents from WWTPs to rivers and sea.
- Sewage sludge from WWTPs to agricultural land.
- Surface water.
- Landfill sites.
- Direct discharge into waters.

Figure 1.1. Origins and fate of pharmaceuticals and personal care products in the environment [modified from USEPA, 2007] [58].

Nowadays, due to the high consumption of PPCPs and industrial products by humans, one of the most important routes of these compounds to the environment is WWTPs, either through effluent wastewater or sewage sludge.

Pharmaceuticals and hormones are introduced into the environment from human and veterinary applications. After their intake, they are excreted in urine and feces to surface water and wastewater, in addition unwanted or expired pharmaceuticals can be disposed of into waters. Furthermore, personal care products and industrial products are directly discharged into wastewater after industrial or domestic use; for example, personal care products are discharged into wastewater after washing clothes or skin.

In WWTPs, wastewater is treated and it is known that carbon, nitrogen, phosphate, pathogens, particulate matter and metals ions are effectively removed, but for organic

micropollutants conventional treatment processes are not effective. Hence, pharmaceuticals and their metabolites, personal care products, surfactants, flame retardants or industrial additives are not completely removed in WWTPs and emerging pollutants are detected in effluent wastewater at levels of several µg L^{-1}. Effluent wastewater ends up in rivers, groundwater or lakes and, as a consequence, emerging pollutants are introduced into the aquatic environment as illustrated in Figure 1.1 [55]. In aquatic environments, aquatic organisms are exposed to organic pollutants and they are introduced into the ecosystem. Moreover, treated wastewater can be used for irrigation and PPCPs can reach terrestrial environments.

The EU Water Framework Directive (2000/60/CE) establishes a framework for the protection and prevention of water pollution, improvement of aquatic ecosystems, mitigation of the effects of floods and droughts, and the promotion of sustainable water usage. In this directive, a list of priority dangerous substances was established and in Directive 2008/105/CE maximum concentration levels of those substances were regulated. PPCPs were not considered, only NP was regarded as a contaminant [59-61].

Another way to introduce emerging pollutants into the environment is through sewage sludge generated in WWTPs from physical, chemical and biological treatment [57]. Sewage sludge, also called biosolid when the sludge is treated for land application, is the main solid produced in WWTPs. Due to the high production of sludge, millions of tons per year, it would be interesting to find an application that would enable sewage sludge to be recycled and re-used. Biosolids are an important source of nitrogen, phosphorus, organic matter as organic acids, metals and other nutrients, and for this reason the European Union promotes its use as a fertilizer for agricultural land as the best environmental option (Figure 1.2) [62-63].

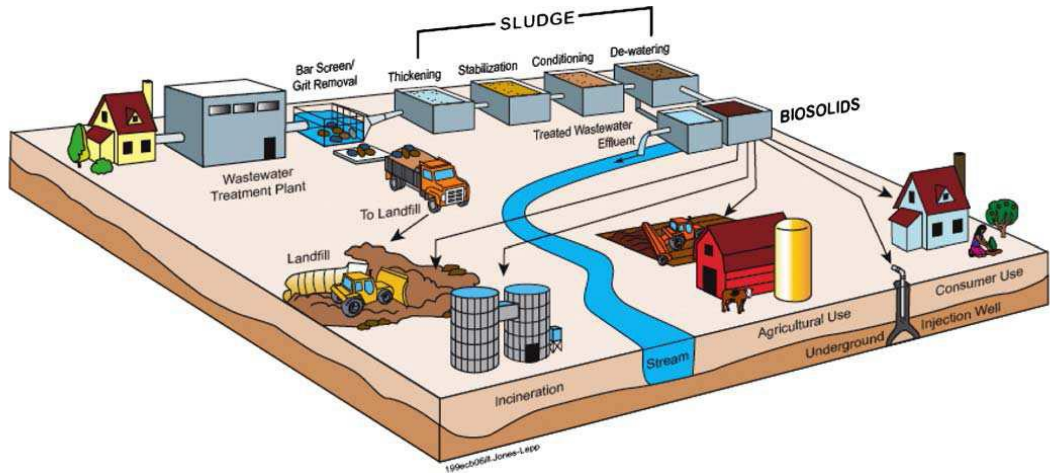

Figure 1.2. Production and distribution of biosolids [64].

However, sewage sludge may be a potential route for pharmaceuticals to reach the environment because, in WWTPs, organic pollutants which have lipophilic properties are adsorbed onto sludge. Several papers have reported the presence of PPCPs in sewage sludge at trace levels (ng g^{-1}).

Unfortunately, there is little information about the effects on soil organisms caused by the presence of emerging pollutants in agriculture. For this reason, some European countries, such as The Netherlands or Germany, have declined the use of sewage sludge for agriculture, thus increasing the percentage of sludge incinerated [38,65]. However, in other countries, such as Spain, the amount of sludge used as fertilizer has increased over recent years. In 2006 the main disposal option for sewage sludge was agricultural land (64%), followed by landfill application (17%) and incineration (11%) [66].

Directive 86/278/ECC regulates the application of sewage sludge on agricultural land but emerging pollutants are not considered. In 2000, the EU published a third draft of a future sludge directive where concentration limit values for some organic contaminants such as organohalogens, surfactants, phthalates, PAHs, PCBs, NP and dioxins were considered [38,66,67]. Moreover, some countries have set limits for some of these organic contaminants but there is no agreement on which contaminants have to be regulated. In any case, there is a lack of concentration limits for pharmaceuticals and personal care products.

Therefore, it is interesting to be aware of the occurrence and fate of emerging pollutants in WWTP processes (raw wastewater, effluent wastewater or sewage sludge) and environmental water in order to evaluate the possible negative effects to aquatic and terrestrial organisms, and the possible migration of the compounds to surface and groundwater. Therefore, it is necessary to develop analytical methods which can detect and quantify the analytes at low levels in different and complex environmental samples.

1.3. DETERMINATION OF ORGANIC POLLUTANTS

The main objective of an analytical process is to obtain partial or global information (chemical, biochemical or biological) about a varied kind of materials or systems. In an analytical process, the analysis of samples does not only require an analytical determination. Sampling, a pre-treatment of the sample, extraction and preconcentration of the analytes from the sample may be necessary prior to the analytical measure for the preparation and clean-up of the samples and enrichment of the analytes. Next, the main steps of the analytical process are outlined and the significance of analytical separation techniques in sample preparation is emphasized [69-70].

Sampling

The main objective of sampling is to obtain a representative portion of a total mass which is treated in the laboratory for the detection and quantitation of the analytes. Collected samples can be treated or stored. When the samples are stored, liquid samples can be frozen or refrigerated under darkness conditions, while solid samples are usually frozen at -18ºC.

Pre-treatment of the sample

Usually samples are heterogeneous and they need to be treated before the extraction in order to obtain a representative portion.

For liquid samples, the most common treatment is filtration of the sample in order to remove particulate matter.

For solid samples, the pre-treatment is quite different. Usually two steps may be applied [71]:

- Drying: water content can be an interference in the extraction process of some analytes because reactions such as hydrolysis can occur. Moreover, for the extraction of organic pollutants, an organic solvent immiscible in water can be used and therefore a dry sample is preferred. Water content can be eliminated by air-drying, heating in an oven or lyophilization.
- Homogenization by grounding and sieving the sample: it is important that the particles of the sample have similar small sizes in order to reduce possible errors and in this way more surface area can be exposed to the extraction.

Extraction and preconcentration

In recent years, there has been an increasing interest in the development of fast, precise, accurate and sensitive methodologies for the determination of analytes in different matrices such as biological or environmental samples. The analysis of complex matrices and the detection or quantitation of the analytes present at very low levels in the samples are two of the main analytical challenges. Sample preparation is usually necessary in order to extract, to isolate and to concentrate the analytes of interest from complex matrices such as environmental waters or sewage sludge. Therefore, sample preparation must include clean-up procedures for the elimination of possible interferences on the instrumental analysis. Also, extraction and preconcentration techniques must bring the analytes to an adequate concentration level for their detection and the compounds of interest must be in a suitable organic solvent or sorbent for the analytical determination [72-75].

Chromatographic analysis

Finally, the extracts from the clean-up and preconcentration step have to be analyzed for the separation, detection and quantitation of the analytes.

1.4. EXTRACTION TECHNIQUES FOR LIQUID SAMPLES

Several extraction and preconcentration techniques have been developed for the extraction of organic compounds from aqueous samples. Conventional sample preparation techniques are liquid-liquid extraction (LLE) and solid-phase extraction (SPE), but these techniques have some drawbacks, such as time-consuming procedures or the use of large amounts of organic solvent. For these reasons, in the last decade, attention has focused on the development of miniaturized extraction techniques such as solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and liquid-phase microextraction (LPME) which are techniques which are easy to use, require low organic solvent consumption and do not require multistage operations.

1.4.1. LIQUID-LIQUID EXTRACTION

LLE is the traditional extraction technique for preconcentration and clean-up which is based on the transfer of analytes from an aqueous sample to an organic solvent water immiscible. LLE has been extensively used for many applications but it has several disadvantages such as the high amount of organic solvent used, emulsion formation or precipitation or the possible loss of analytes during the process [70,72]. Despite the high number of drawbacks LLE is still applied in the determination of organic pollutants such as IBP, CLF and TCS which have been extracted by 100 mL of dichloromethane [76].

1.4.2. SOLID-PHASE EXTRACTION

SPE is the most used technique for the extraction, preconcentration and clean-up of organic compounds in liquid samples [77,78]. SPE consists of the sorption of the analytes onto a solid sorbent phase and then re-extraction to a liquid phase by means of a solvent. The main advantages are simplicity, the wide range of sorbent types that can be used depending on the chemical properties of the analytes, easy automation, using a smaller amount of organic solvent than in LLE, and the high concentration factors achieved [78-81]. Some drawbacks are the poor selectivity of the sorbents which is a problem for complex matrices, time consuming steps, the significant

volumes of solvent, intensive sample handling, the extra steps necessary for the concentration of the extract and the high cost.

Extraction process

The extraction and preconcentration of the analytes by SPE is based on several steps as can be seen in Figure 1.3 [78,82]:

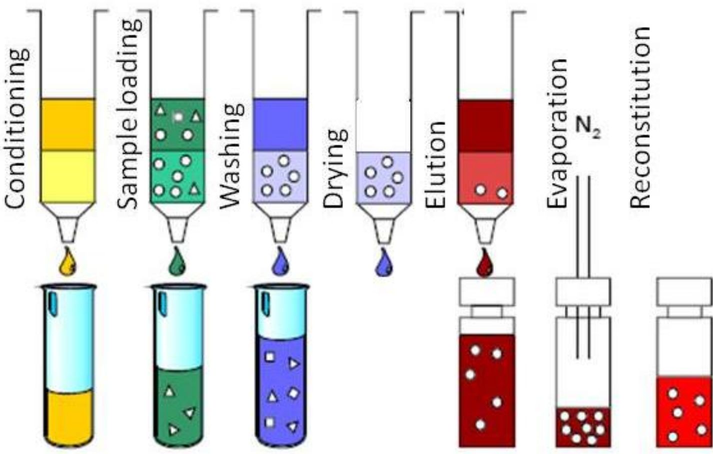

Figure 1.3. Steps required in SPE [83].

Prior to adsorption of the analytes onto the sorbent, it is necessary to condition it. Next, the sample is passed through the cartridge at a constant flow rate. During the process, the analytes from the sample are retained in the sorbent by different kinds of interactions. Thirdly, a washing step is applied for the elimination of unwanted matrix material adsorbed onto the sorbent without removal of the analytes. Then, a drying step followed by the elution of the target compounds by an organic solvent for organic pollutants with high affinity for the analytes is carried out. Finally, evaporation of the eluting solvent and reconstitution in an adequate solvent for the instrumental determination can be required.

SPE mechanism

In SPE, different extraction modes can be applied for the clean-up and enrichment of the samples depending on the polarity and functional groups of the sorbent and analytes [78,81,84]. Ion exchange mode is used for ionic analytes or analytes that can be converted to ionic form. In this mode, compounds are retained in a sorbent

containing anion-exchange groups or cation-exchange groups such as silica-based modified with NH_2 groups. In normal phases, a polar sorbent is used for the extraction of polar compounds (aldehydes or alcohols) from a non-polar sample such as oil and the elution is carried out by a polar solvent which disrupts the polar interactions between functional groups and the sorbent. Some possible sorbents are nonbonded phases such as silica, alumina or magnesium silicate. In reversed phases, relatively non-polar compounds are separated from a polar phase such as water by a hydrophobic sorbent such as silica-based modified with C_{18} or C_8 groups. The compounds retained are eluted by an organic solvent. Due to the nature of the compounds studied, reversed phase mode has been chosen for the analysis in this thesis.

In addition, different functional groups in the same sorbent can be used inducing different types of interactions in a mixed mode. In this thesis, the following modified sorbents have been used in reversed phase mode: ISOLUTE ENV+ (polystyrene-divinylbenzene based polymer with a hydroxyl group), Strata X (polystyrene-divinylbenzene based polymer with a pyrrolidone group), Oasis HLB (N-vinylpyrrlidone-divinylbenzene) and Speedisk H_2O-Philic DVB.

In the literature, one of the most common sorbents applied is Oasis HLB which has been used in the determination of all target compounds in several matrices; different multi-residue methods have been developed with Oasis HLB for the determination of pharmaceuticals and personal care products in environmental waters [85-89]. Nevertheless, other approaches with different types of sorbent have been applied for the analysis of a wide range of PPCPs using the same method. For example, Strata X, which has similar properties to Oasis HLB, has been selected for the determination of NSAIDs, antibiotics or EDCs [88,89]. Silica modified, such as ISOLUTE C18 cartridges, has also been applied to the quantitation of biocides such as TCS and M-TCS [90]. Reversed phase combined with ion exchange has been reported as a good mode for the clean-up and preconcentration of several analytes. For acidic analytes such as

NSAIDs, salicylic acid or indomethacin and neutral analytes, Oasis MAX has been applied [91]; while for antidepressants, Oasis MCX has been used [85].

1.4.3. Solid-phase microextraction

SPME is based on the extraction of analytes from an aqueous or gaseous sample to a solid phase (sorbent) consisting of a coated fiber that contains a thin layer of solid polymeric material. The main advantage of this technique is the simplicity. On the other hand, coated fibers are expensive, have a short lifetime, pose a possible contamination risk between analyses and have low capacity [72-73]. Some applications of this technique are the ones reported by Canosa et al. [92] who developed a SPME method for the determination of TCS and M-TCS in aqueous samples and Lamas et al. [93] who determined SSRIs in environmental waters by SPME followed by GC-MS.

1.4.4. Stir-bar sorptive extraction

SBSE is based on the SPME principle but, in SBSE, a magnetic stir bar coated with a polydimethylsiloxane polymer (sorbent) is placed in the sample solution and stirred. Hence, analytes are extracted from the sample to the fiber by sorption. After the extraction process ends, the analytes are recovered by thermal desorption for gas chromatography or by a solvent for liquid chromatography [79]. The main advantages of this technique are simplicity and low cost while the main disadvantages are low capacity and low breakthrough of the analytes [94,95]. Quintana et al. [96] determined some NSAIDs and TCS in environmental waters; Ferreira et al. [97] quantified TCS and M-TCS and Pedrouzo et al. [98] determined TCC, TCS and UV filters in waters and compared the method to SPE showing similar recoveries. Other applications extracted SSRIs, sulfonamides or EDCs by SBSE [88].

1.4.5. Liquid-phase microextraction

LPME is based on the miniaturization of the LLE technique. In LPME, analytes are transferred from an aqueous sample containing the analytes into a small amount of a water immiscible solvent over time. As in LLE and SPME, the analytes are extracted until the system attains equilibrium and also the partition of the analytes is related to

the octanol-water coefficient. Through LPME, excellent clean-up is achieved, low consumption of organic solvent is needed, and no carry-over problems are possible because only a single extraction can be carried out. Moreover, it is simple, inexpensive, no further preparation is required and a wide range of pH can be used in the extraction procedure. In addition, LPME can be performed with large sample volumes, with small volumes of acceptor solution (μL), and therefore high enrichment factors can be obtained [99].

LPME can be divided into three main categories (Figure 1.4): single-drop microextraction (SDME), hollow-fiber microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME). This thesis has focused on the applicability of HF-LPME as an extracting, clean-up and enrichment technique for some pharmaceuticals, personal care products and endocrine disruptors in water and sludge [73,100].

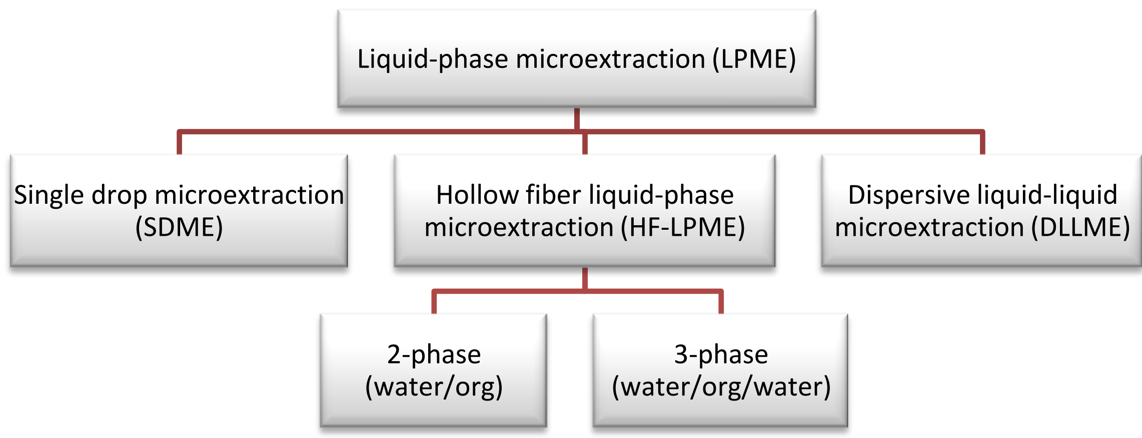

Figure 1.4. Types of liquid phase microextraction.

Single-drop microextraction

SDME is based on the use of a microdrop of an organic solvent as acceptor phase which is suspended from the tip of a microsyringe. The microdrop is immersed in an aqueous solution or exposed to the headspace of the sample. The main problems are the poor robustness of the technique due to the possible instability of the droplets from the needle tip of the microsyringe during the extraction process [72,99].

Dispersive liquid-liquid microextraction

This technique was introduced by Rezaee and is based on the use of an extracting solvent (organic water immiscible solvent) and a disperser solvent soluble with the extracting and the aqueous phase such as acetone, methanol, ethanol or acetonitrile. The extracting solvent and the disperser are injected quickly into the sample (aqueous phase) and a cloudy solution consisting of droplets is formed in the aqueous solution. As a result, there is a large interface between the extraction solvent and therefore equilibrium conditions are achieved in a few seconds. Finally, the phases are separated by centrifugation [73,101]. This technique has been widely applied for the extraction of relatively hydrophobic compounds such as biocides [102-104].

1.4.5.1. Hollow fiber liquid-phase microextraction

In 1999, Pedersen-Bjergaard and Rasmussen converted the basic principle of supported liquid membrane into simple and inexpensive extraction units for liquid-liquid-liquid microextraction using commercial polypropylene hollow fiber as membrane. In HF-LPME, the organic solvent immiscible in water is placed in the pores of a hollow fiber in rod configuration or in a U-shape [72,99,105,106].

In the HF-LPME procedure, the pores of a piece of hollow fiber are impregnated with organic solvent and the lumen of the hollow fiber is filled with a µL volume of an acceptor solution that can be the same organic solvent (2-phase system) or an aqueous solution (3-phase system) as seen in Figure 1.5 [72,99,107]. Finally, the hollow fiber is placed in an aqueous solution (sample), also called donor solution, for the extraction of the target analytes and after the extraction, the acceptor solution is analyzed. In HF-LPME, large sample volumes and small volumes of the acceptor solution are used. Therefore, high enrichment factors can be obtained without any further steps and this is related to extraction efficiency, acceptor phase volume and the sample volume of the system. In addition to the advantages of LPME, the small pore size of the hollow fiber excludes macromolecules and particles from being transported to the acceptor phase. Also, molecules that are not soluble in the organic solvent are not extracted [72,73,100,105,108].

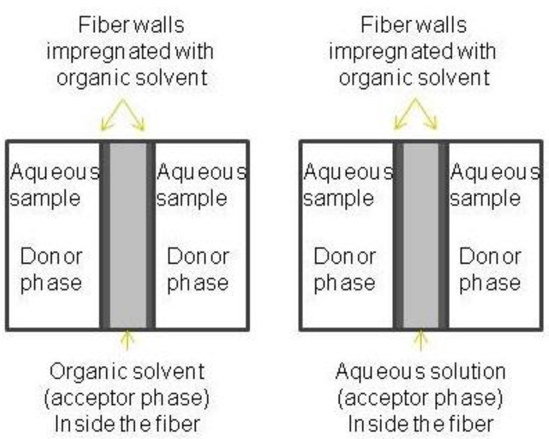

Figure 1.5. Two-phase and three-phase HF-LPME system [72].

The performance of HF-LPME is characterized by two parameters: the enrichment factor and the extraction efficiency. Extraction efficiency (*E*) measures the amount of the analyte in the sample recovered in the acceptor side and is defined as Eq. (1.1) [109].

$$E = \frac{m_{\mathrm{Ae}}}{m_{\mathrm{Di}}} \times 100\% \qquad (1.1)$$

Where m_{Ae} is the amount of a compound in the acceptor solution at equilibrium and m_{Di} is the amount of a compound present in the donor solution.

Enrichment factor (*Ee*) reflects how many times the concentration of the analyte in the acceptor phase is increased compared to the initial sample concentration and it is defined as Eq. (1.2):

$$Ee = \frac{C_{\mathrm{Ae}}}{C_{\mathrm{Di}}} \qquad (1.2)$$

Where C_{Ae} is the concentration of a compound in the acceptor solution at equilibrium and C_{Di} is the concentration of a compound in the donor solution at the beginning of the extraction.

1.4.5.1.1.Two-phase HF-LPME

Principle

Neutral analytes are extracted from an aqueous sample into an organic solvent (acceptor phase) placed in the pores and in the lumen of the hollow fiber. The hollow fiber membrane is used as a barrier between the aqueous and the organic phase. This process can be described by the following equation [72,73,110]:

$$A_{sample} \leftrightarrow A_{org} \quad (1.3)$$

Where A_{sample} is the target analyte in the sample solution and A_{org} is the analyte in the organic phase.

The acceptor phase can be analyzed by gas chromatography, normal phase liquid chromatography, or the organic phase can be evaporated and reconstituted in an aqueous solution or an organic solvent miscible with water in order to be determined by reversed phase liquid chromatography or capillary electrophoresis.

Transport mechanism

In 2-phase HF-LPME, analytes are transferred from the sample phase to the acceptor phase by passive diffusion which is correlated to the octanol-water partition coefficient. For this reason, neutral hydrophobic compounds are extracted by this system because of their high solubility in the organic phase. Usually, organic compounds extracted by a 2–phase system have LogK_{ow} higher than 4 and the affinity to the organic solvent is really important [73,111].

This technique has been applied previously in the determination of TCS in environmental waters with recoveries higher than 83% and a method limit of detection of 20 ng L^{-1} [112]. Zorita et al. [113] developed a method for the preconcentration and clean-up of steroid hormones by 2-phase HF-LPME, but in this case tri-n-octylphosphine oxide was added to the organic solvent in order to increase the extraction of alcohols, enrichment factors were in the 1500 - 3400 range with extraction efficiencies of 45 - 98%. Other types of contaminants are PAHs, phenols or

polybrominated biphenyls which have been quantified in environmental samples by HF-LPME combined with GC with high recoveries [108]. Triazine herbicides have also been extracted from 3 mL water samples with enrichment factors between 42 and 208 [110]. In order to extract some analytes, an ion carrier can be added to the organic solvent. Hultgren et al. [114] extracted and preconcentrated a quaternary ammonium surfactant from industrial waters with octanoate as ion carrier and an enrichment factor of 400 for 250 mL in reagent water and 158 in process waters, indicating a matrix effect on the extraction process.

1.4.5.1.2.Three-phase HF-LPME

Principle

Three-phase HF-LPME systems are used for the extraction of ionizable/polar compounds in aqueous samples. In this mode, the analytes are extracted from an aqueous sample (donor sample) to an aqueous solution (acceptor solution) placed in the lumen of the hollow fiber. Both aqueous phases are separated by the hollow fiber membrane which contains an organic solvent immiscible with water in the pores (organic phase). This process can be described by the following equation [72,73,107,110]:

$$A_{sample} \leftrightarrow A_{org} \leftrightarrow A_{acceptor} \qquad (1.4)$$

Where A_{sample} is the target analyte in the sample solution, A_{org} is the analyte in the organic phase and $A_{acceptor}$ is the analyte in the acceptor solution.

In this system, good selectivity and clean-up are achieved and the acceptor solution is suitable for liquid chromatography or capillary electrophoresis analysis.

Transport mechanism

The analytes to be extracted must be soluble in an organic solvent and for this reason uncharged species are required. In order to have non-ionic analytes, a pH adjustment of the sample or the addition of an ion pairing or complexing agent in the sample or in the organic membrane is necessary. Then, the analytes are back-extracted from the

organic phase into the acceptor phase where they are trapped as ions by pH adjustment or by adding a suitable stripping agent. Therefore, a pH gradient or a facilitated transport can be carried out [105,107,111].

This thesis has focused on the extraction by pH adjustment of basic and acidic organic compounds which can be protonated and desprotoned; in other words, the transport of the analytes is achieved by a pH gradient between the sample and acceptor solution. The general mechanism for ionic compounds by pH gradient is illustrated in Figure 1.6. In this way, hydrophobic ionic analytes are back-extracted into the aqueous acceptor phase.

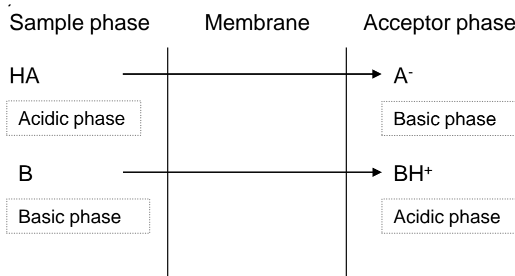

Figure 1.6. Transport mechanism depending of the pH for acidic and basic organic compounds.

Different processes take place: partition of the analytes between the sample and the organic solvent, diffusion through the membrane and ionization into the acceptor solution. All these parameters are related to the transport of the compounds, which depends on several factors, such as the chemical properties of the analytes, the type of organic solvent and the thickness of the membrane, which affect the diffusivity of the analytes through the membrane.

HF-LPME by pH gradient has been applied to the determination of some compounds like SSRIs, NSAIDs or pesticides in environmental waters and solid samples with high enrichment factors and recoveries [115-120]. Payan et al. [121] determined fluoroquinolones in biological and environmental matrices by 3-phase HF-LPME with enrichment in the 50 - 900 range. Another type of compounds are heterocyclic aromatic amines; Shah et al. [122] extracted them from human urine with extraction efficiencies of higher than 68% and enrichment factors of about 300. The high

enrichment of the analytes by 3-phase HF-LPME has been demonstrated by Ho et al. [123]. In their method, enrichments of about 25,000 were obtained in the analysis of five antidepressants from 1 L of the sample with limits of detection of few pg L^{-1}. Kou et al. [124] applied HF-LPME to the determination of haloacetic acids in drinking water with low extraction efficiencies; however high enrichment factors were obtained (300 – 3,300) and limits of detection were comparable to those achievable by USEPA standard method. Furthermore, this technique has been applied to different matrices like urine, plasma or environmental waters producing very clean extracts [99].

1.4.5.1.3.Critical parameters

In HF-LPME some parameters can be optimized in to order to achieve the best clean-up and enrichment of the analytes (Figure 1.7) [109,120].

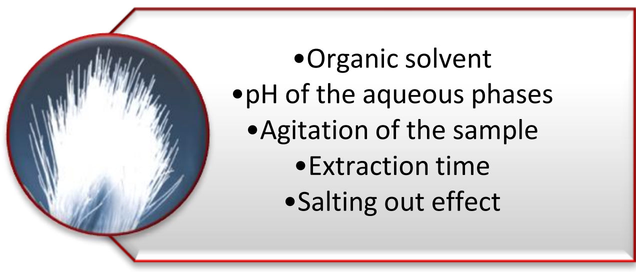

Figure 1.7. Principal parameters that effect HF-LPME.

Organic solvent

The organic solvent employed for the extraction procedure is one of the most important parameters to be considered in both systems. Organic solvents with different polarity or a mixture of organic solvents can be used in order to increase the solubility and affinity of the compounds in the organic phase. Moreover, organic solvent properties have to be taken into account; it has to be water immiscible, easily and strongly immobilized in the pores of the HF, and have low volatility.

Recently, ionic liquids have been shown to be an interesting *green* alternative to organic solvents. Several studies have reported their use as an organic solvent. Peng et al. [125] extracted chlorophenols in environmental waters with limits of detection in the 0.5 – 10 g L^{-1} range and Tao et al. [126] extracted sulfonamides with enrichment factors of between 58 - 135 using ionic liquids.

pH of the aqueous phases

In the three-phase mode, the pH adjustment of the donor and acceptor solution is critical for acidic and basic compounds, while for the two-phase system only the pH adjustment of the donor phase can be evaluated. In both systems, the analytes present in the donor solution have to be uncharged and for this reason the pH of the sample donor solution has to be 2 units lower than the pKa value for acidic compounds and 2 units higher than the pKa value for basic compounds.

In three-phase HF-LPME, the pH of the aqueous acceptor solution has to be higher than the pKa of acidic compounds while for basic compounds it has to be lower than the pKa of the analyte. In this case, the use of a high capacity buffering solution is recommended because some compounds can be co-extracted and modify the pH of the acceptor solution.

Agitation of the sample

Agitation of the donor solution has an influence on the analytical transport of the analyte into the organic solvent. When stirring is applied, diffusion of analytes from the sample to membrane increases. Although an increase of stirring speed enhances mass transfer, high speed can generate the formation of air bubbles which attach to the membrane and promote the loss of the organic solvent impregnated in the membrane.

Extraction time

In HF-LPME, the transport of the analytes from the donor phase to the acceptor phase depends on time. When time increases, extraction efficiency increases until equilibrium is reached and no further amount of analyte is extracted in the acceptor phase. It is important to note that for long extraction times stability can decrease because the organic solvent present in the pores may be lost.

Salting out effect

The addition of salt decreases the solubility of hydrophobic compounds in the aqueous phase and the transfer of analytes to the organic solvent increases. Therefore, extraction and enrichment are enhanced.

1.5. EXTRACTION TECHNIQUES FOR SOLID SAMPLES

As in liquid samples, solid samples need to be treated prior to analytical determination. First, analytes are extracted from a solid phase to an organic or aqueous phase but usually a high number of matrix compounds are co-extracted and it is necessary to apply a clean-up and enrichment step.

Several extraction techniques can be applied for the determination of organic pollutants in solid samples; soxhlet and ultrasonic solvent extraction (USE) are conventional techniques usually applied for this purpose. Other techniques which require less organic solvent are supercritical fluid extraction (SFE), microwave assisted extraction (MAE), pressurized liquid extraction (PLE) or pressurized hot water extraction (PHWE). For the clean-up and pre-concentration, the techniques used are the same as in liquid samples, which have been described in section 1.4. In Figure 1.8 a scheme of an analytical process for solid samples is shown. Next, extraction techniques are described.

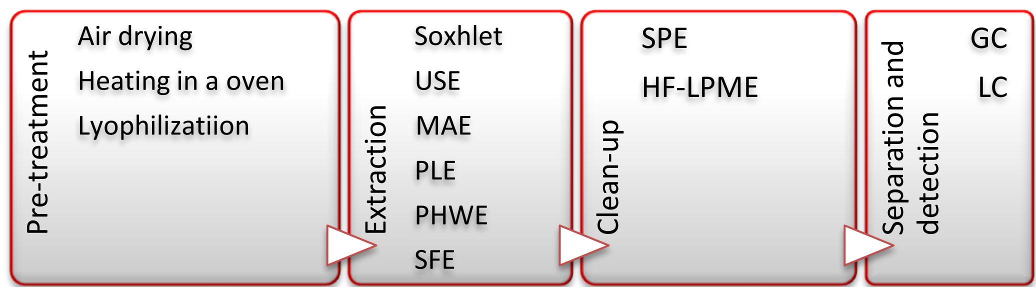

Figure 1.8. Analytical process for a solid sample.

Soxhlet

In soxhlet extraction the sample is continuously in contact with fresh solvent from a distillation flask; in this way equilibrium transfer is improved. In soxhlet extraction,

long extraction times are needed, large amount of solvent are used and it is labor intensive. Also, thermal degradation of the analytes can occur due to the continuous exposure of the analytes to elevated temperatures [69,70].

Supercritical fluid extraction

SFE uses a compound, such as carbon dioxide, which is not toxic and non-flammable in its supercritical state at elevated pressure and temperature. However polar and ionic compounds are difficult to extract because carbon dioxide has a low dielectric constant, the extraction depends on the sample matrix, an additional clean-up step is necessary and the cost of the extraction procedure is high [127,128].

Microwave assisted extraction

MAE is based on the extraction of the organic compounds from solid samples to a liquid extractant with the help of microwave energy [70,129,130]. The main advantages of this technique are low organic solvent consumption, low extraction time and the possible control of temperature, pressure and power. However, the extract needs to be filtrated and the choice of organic solvent is limited. A high number of matrix compounds are co-extracted, so after the extraction a clean-up step should be applied [69].

Published analytical methods have detected and quantified different kind of PPCPs in dried sediments or sludge such as NSAIDs, lipid regulators, antiepileptics or analgesic compounds by MAE [131,132]. Langford et al. [133] reported a wide range of recoveries for CTP, DCF, FLX, IBP, TCS and TCC in solid samples, from 8% for CTP to 461% for IBP depending on the sludge or sediment. NSAIDs have been extracted effectively by MAE with recoveries between 80 - 105% [131].

Ultrasound solvent extraction

USE is another extraction technique where sound waves of high frequency are applied to help the extraction of the analytes. Easy, few and cheap equipment is necessary and a low extraction time is required because the vibration of the molecules enhances the

extraction. Furthermore, in comparison to other techniques it is not necessary to work at high temperatures and this fact is a potential advantage when the analytes are thermolabile [134,135].

USE has been applied in the extraction of a wide range of PPCPs, such as biocides, NSAIDs, antibiotics like fluoroquinolones or sulfonamides in solid samples such as sludge or sediments with high recoveries [57,88]. Ternes et al. developed a method for the determination of acidic and neutral pharmaceuticals and iodinated contrast media in sludge by USE; three types of analytes were extracted by USE and then three different clean-up and preconcentration procedures were applied using SPE [136].

1.5.1. PRESSURIZED LIQUID EXTRACTION

PLE is one of the most common techniques for the extraction of organic compounds in solid matrices. In PLE, an extractant (an organic solvent) is applied on solid samples at high temperature (50 - 200ºC) and high pressure (1500 - 2000 psi) in order to obtain extractions in a short time. Thus, the analytes are transferred from the solid sample to the organic solvent. The main advantages are short extraction times, low volumes of organic solvent and that no further filtration is required. On the other hand, it is quite expensive and matrix dependent [71,75,128].

In PLE, the sample is placed into the cell and mixed with an inert material in order to increase the dispersion of the sample and the surface area, improving the contact between the solid sample and the organic solvent. Different materials can be used: sand, aluminum oxide, sodium sulfate, diatomaceous earth or Hydromatrix.

Then, the cell is loaded into the oven and the extraction process starts. First, the cell is preheated, the solvent fills the cell and then the static valve closes, allowing the pressurization of the cell at the temperature chosen. After the static time, the organic solvent is collected and the cell is purged with nitrogen gas. If more than one cycle is applied, the process is repeated (Figure 1.9) [71,137].

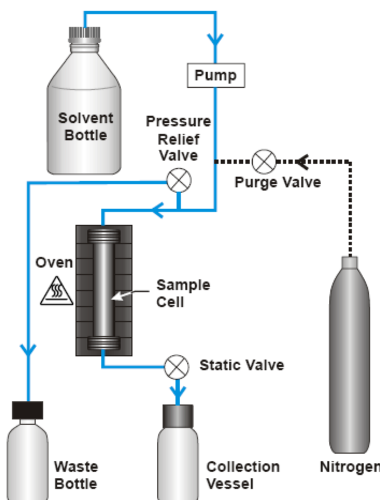

Figure 1.9. Scheme of PLE process [138].

PLE is the most used technique applied for the extraction of PPCPs in different kind of solid samples; Runnqvist et al. [137] have reviewed PLE applications in environmental and biological samples where antidepressants, NSAIDs, antibiotics, estrogens or biocides have been extracted using different organic solvents [71]. PLE has been combined with SPE in order to decrease ion suppression in liquid chromatography in the analysis of several pharmaceuticals and biocides. For example, Jelić et al. [139] developed a multi-residue method for the determination of a wide range of compounds by PLE and SPE. Recovery yields were high except for KTP, NPX and IBP, with values lower than 41% in sludge. Radjenović et al. [140] also reported a method for the determination of some pharmaceuticals in sewage sludge. However, in both studies standard addition was used in order to compensate the ion suppression.

Critical parameters

Several parameters need to be optimized in PLE in order to obtain the best recovery of the analytes: extraction solvent, temperature, pressure, static extraction time, number of cycles and amount of sample [69,71].

- Extraction solvent

Organic solvent, as in other techniques, is one of the most important parameters. In order to extract the analyte from the solid sample, the polarity of the compound and

the solvent need to be similar. For this reason, one solvent or a mixture of solvents can be applied; a mixture of organic solvents or water can also be used.

- Temperature

In order to increase the extraction of the analytes, high temperatures are employed because the viscosity of the solvent is reduced and the capacity of the solvent to solubilize the analytes is higher, thus increasing the recovery. Despite extraction improvement when temperature increases; analytes can degrade, more unwanted matrix components are co-extracted and low selectivity is achieved, so a compromise temperature is required.

- Pressure

In PLE, pressure is not a critical parameter but it is necessary to work at high pressure for two reasons. Firstly, high pressure is necessary in order to keep organic solvent in a liquid state when the system works above its boiling point. Secondly, at high pressure the solvent viscosity decreases, increasing the contact with the sample and the extraction efficiency because the matrix-analyte interactions can break easily.

- Static extraction time

Static time is the time that the sample is exposed to the solvent at high temperature and pressure. It is related to the contact time between the sample and the solvent which affects the extraction of the analytes. When extraction time increases, the penetration of the solvent into the sample also increases and better recoveries can be achieved.

- Number of cycles

The number of cycles is the number of times that fresh solvent enters into the cell where the sample is placed. When more than one cycle is used, the extraction volume is divided by the number of cycles and the total volume does not increase.

- Amount of sample

Finally, the last parameter of PLE is the amount of sample used in the extraction procedure which is related to the limit of detection of the whole procedure. Sample

weight depends on two factors: the matrix and the volume of the cell. When complex matrices are used, a low amount of sample must be used because less interferences are co-extracted. Finally, depending on the capacity of the cell, the amount of sample varies.

Pressurized hot water extraction

A different mode of PLE technique for the extraction of organic pollutants in solid samples is PHWE, which is considered a green solvent extraction technique that avoids the use of organic solvent. PHWE is based on the use of water as an extracting solvent at elevated temperatures and under pressure to keep it in a liquid state. In this way, the polarity of water can be reduced close to the polarity of alcohols, and organic compounds can be dissolved in the aqueous solvent. Furthermore, the aqueous extract of the PHWE technique can be used for clean-up techniques such as SPE or HF-LPME, thus avoiding evaporation steps for organic solvents. This technique has the same principle as PLE and the same equipment is required for the extraction, so the same critical parameters need to be optimized. The only difference with PLE is that, instead of organic solvent, an aqueous phase is applied, and for this reason the pH must be evaluated [141-143]. Some applications are: the determination of polycyclic aromatic hydrocarbons in sediments [141], N-nitrosamines and aliphatic primary amines in sewage sludge [143-144] and NSAIDs in sewage sludge combined with HF-LPME [145]; with recoveries for almost all the compounds higher than 80%.

In the study carried out in this thesis, PLE and PHWE have been tested for the extraction of pharmaceuticals and personal care products in sewage sludge before HF-LPME.

1.6. CHROMATOGRAPHIC ANALYSIS

The most applied methods for the separation and determination of pharmaceuticals, personal care products and endocrine disruptors in environmental samples are gas chromatography (GC) and liquid chromatography (LC) combined with mass spectrometry (MS) [146].

In GC the analyte is partitioned between a stationary phase and a gaseous phase; this technique is applied to non-polar, volatile and thermostable compounds. Due to the low volatility and polarity of the substances studied, derivatization would be necessary prior to GC analysis. Derivatization is time-consuming and sometimes irreproducible, and for this reason LC has been applied for the determination of the analytes studied in this thesis [1].

In LC a liquid mobile phase and a stationary phase placed in a column are used for the separation of the analytes. The sample is transported to the column by the mobile phase where the analytes are retained and eluted after some time [69]. To date, the most common detector used for LC has been an ultraviolet-visible detector with a single wavelength (UV) or a diode array (DAD); in the latter, more than one wavelength can be monitored during the analysis.

Nowadays, LC-MS or LC-MS/MS are the most used techniques for the identification and detection of organic pollutants in environmental samples. In MS/MS, a mass transition can be monitored, thus improving the selectivity and the sensibility of the method developed while, in MS, only a selected ion can be monitored for each compound. When LC-MS and LC-MS-MS are used an interface is required. Electrospray ionization (ESI) is commonly used for polar compounds while atmospheric pressure chemical ionization (APCI) is used for medium-polarity and low-polarity compounds [1,147,148]. In mass spectrometry, it is usual to observe ion suppression due to matrix effects. For this reason a standard addition method or an internal standard with structurally similar unlabeled compounds or isotopically-labeled standards can be applied [147,148].

Due to the polarity of the compounds analyzed, a reversed phase with octadecyl C_{18}-bonded or octyl C_8 bonded silica is the most commonly used stationary phase for the determination of pharmaceuticals and personal care products. As mobile phases, acetonitrile and methanol are used as organic solvents. For acidic and basic compounds, in order to reproduce retention time, a buffer or acidification of the aqueous phase is usually applied. Different buffers and acids can be applied, but in LC-

MS volatile buffers such as ammonium acetate, ammonium formate, acetic acid or formic acid are required, while for DAD detection, phosphoric acid and phosphate can be used as additives [1,148].

In this thesis, HPLC-UV and LC-MS in reversed phase have been applied for the analytical determination of pharmaceuticals, personal care products and endocrine disruptors studied in environmental waters and sewage sludge.

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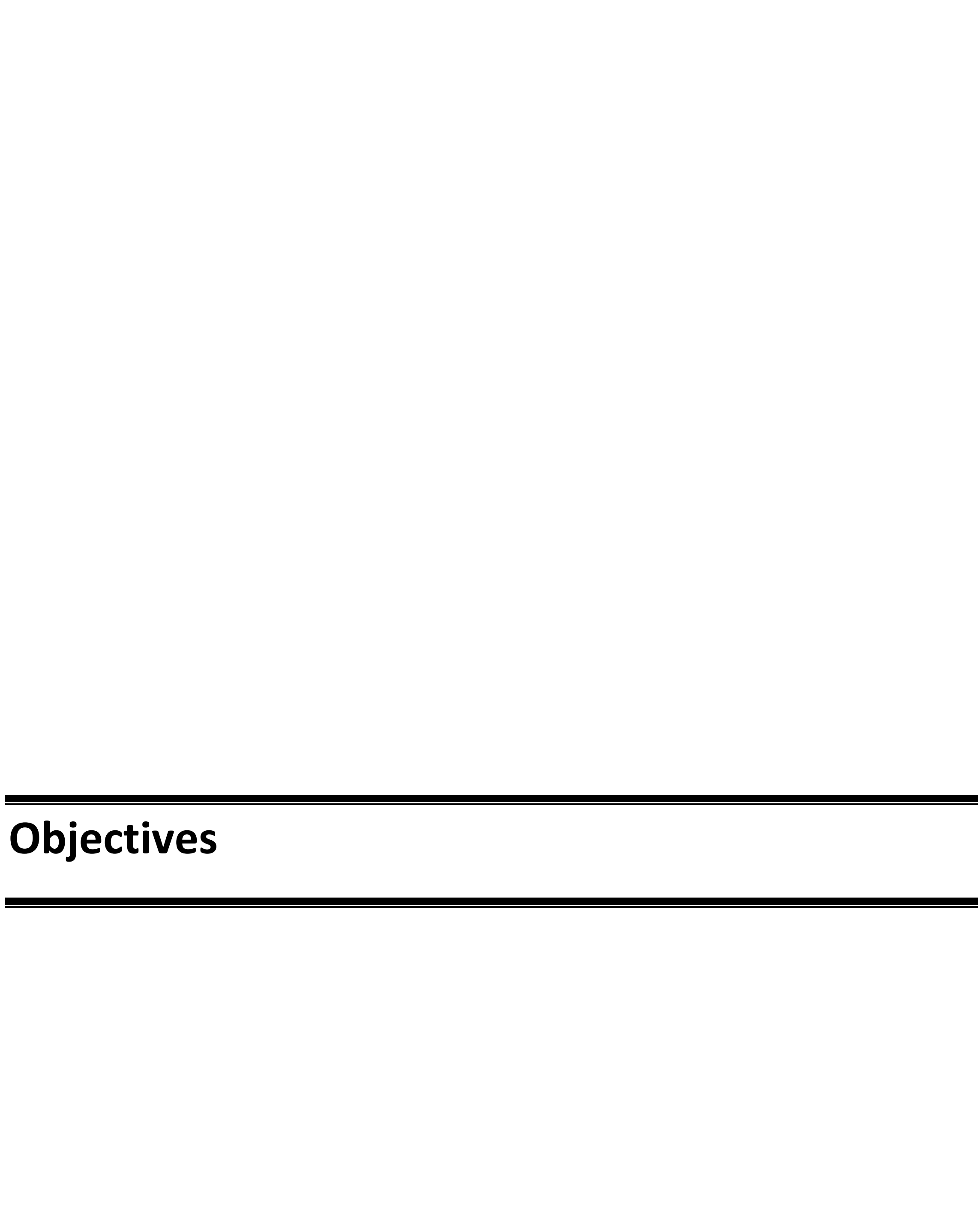

Objectives

Distribution and fate of pharmaceuticals and personal care products onto the environment is a topic of increasing interest due to their growing consumption and continuous introduction. The main objective of this thesis was the development of new analytical methodologies low cost, environmentally friendly and with low manipulation based on hollow fiber liquid-phase microextraction technique for the determination of organic emerging pollutants in environmental waters and sewage sludge. In the light of the aforementioned the aims of this study were the followings:

1. To develop and apply a hollow fiber liquid-phase microextraction combined with HPLC-DAD method for the simultaneous determination of triclosan, triclocarban and methyl-triclosan in water samples.

2. To develop a method using pressurized liquid extraction, hollow fiber liquid-phase microextraction and LC-MS/MS or HPLC-DAD for triclosan, triclocarban and methyl-triclosan determination in biosolids and sludge-amended soil.

3. To develop a hollow fiber liquid-phase microextraction method for the detection and quantitation of acidic pharmaceuticals (clofibric acid, naproxen, diclofenac and ibuprofen), carbamazepine and phenolic compounds (bisphenol A, 4-nonylphenol and triclosan) in environmental waters. To apply the methodology developed to the analysis of different water samples and to evaluate the elimination of acidic pharmaceuticals by disinfectant agents: peracetic acid, chlorine and UV radiation.

4. To design a direct hollow fiber liquid-phase microextraction method using LC-MS for the determination of some anti-inflammatory drugs in sewage sludge.

5. To develop and compare a direct hollow fiber liquid-phase microextraction method and a pressurized hot water extraction combined with hollow fiber liquid-phase microextraction method for the determination of selective serotonin reuptakes in sewage sludge.

CHAPTER 2:

Determination of triclosan, triclocarban and methyl-triclosan in aqueous samples by hollow fiber liquid-phase microextraction prior to liquid chromatography

2.1. INTRODUCTION

The continuous introduction at trace levels of biocides used in personal care products, such as triclosan (TCS) and triclocarban (TCC), and their possible transformation by-products, i.e., methyl-triclosan (M-TCS), in waters has made it necessary to develop new analytical methods for their simultaneous determination.

Due to their domestic use, TCS and TCC can be found in sewage entering wastewater treatment plants (WWTPs). Although about 90% of these compounds are eliminated in WWTPs, they are still present in the effluent wastewater and so reach the surface waters [1-4]. Several studies have reported the occurrence of TCS and TCC in influent and effluent wastewaters and surface water from ng L^{-1} to µg L^{-1} levels [1,5-16]. For example, Singer et al. [5] detected TCS in effluent wastewater at a concentration of 42 ng L^{-1} even though removal rates in WWTPs were 79% and 15% due to biodegradation and sorption of TCS in sludge. Kumar et al. [17] monitored TCS and TCC in influent, effluent and sludge and found TCS and TCC to be largely eliminated in WWTPs with concentrations from 2 - 38 µg L^{-1} and 1.6 – 20 µg L^{-1} in the influent to 0.152 to 4.76 µg L^{-1} and 0.157 to 1.37 µg L^{-1} in the effluent. In this study the fraction bound to particulate matter was found to be 13 - 67% (influent) and 5 - 19% (effluent) for TCS whereas for TCC it was 27 - 82% (influent) and 40 – 72% (effluent), and this is related to their $LogK_{ow}$ (Table 1.1).

On the other hand, M-TCS has been detected at ng L^{-1} levels with higher concentrations in effluent than in influent wastewater as has been shown in different studies. This can be explained by the fact that TCS can undergo biological methylation leading to the formation of M-TCS [3,8].

2.1.1. EXTRACTION TECHNIQUES

The determination of the selected biocides in environmental waters requires the development of extraction and clean-up methodologies [18]. Different sample preparation techniques have been applied for the extraction and enrichment of the target compounds, as can be seen in Table 2.1.

Table 2.1. Comparison of methods for TCS, TCC and M-TCS reported in aqueous samples.

Analyte	*Matrix*	*Extraction technique*	*Analytical determination*	*Recovery (%)*	*MLOD (ng L^{-1})*	*Ref.*
TCS **TCC**	Wastewater	LLE	LC-MS/MS	>90		[17]
TCS	Wastewater	LLE	GC-MS	138	17	[19]
TCS	Wastewater and surface water	SPE	GC-MS	89-104	3[a]	[1]
TCS **M-TCS**	Wastewater and surface water	SPE	GC-MS	87-98	12-21	[20]
TCS	Wastewater	SPE	GC-MS	93	10	[21]
TCS	Influent and effluent wastewater	XAD-Bag-samples	GC-MS	76	20-13	[22]
TCS **TCC**	Wastewater and surface water	SPE	UHPLC-MS-MS	85-92 39-69	3-10 20	[23]
TCC	Surface water, drinking water and wastewater	SPE	LC-ESI-MS	95	0.06-1.9	[4]
TCS **TCC**	Influent and effluent wastewater	SPE	LC-MS	74-90	10-30	[7]
TCS **TCC**	Wastewater and surface water	SPE	LC-MS/MS	69-100 92-136	0.23-50 0.008-0.05	[9]
TCS **TCC**	Surface water	SPE	LC-MS/MS	64 17	1[a] 0.25[a]	[15]
TCS	Effluent wastewater and surface waters	SPE	LC-MS/MS	91-104	4-10[a]	[24]
TCS	Wastewater	SPE	GC-MS	93	3.6	[26]
TCS **M-TCS**	Wastewater and surface water	SPME	GC-MS	100-104 99-107	2[a]	[27]
TCS	Wastewater and surface water	HS-SPME	GC-MS	87-101	6.5	[28]
TCS	Wastewater	SBSE/LD	LC-DAD	78	100	[29]
TCS **M-TCS**	Wastewater and drinking water	SBSE/TD	GC-MS	78-95	1.39 0.54	[30]
TCS **TCC**	Wastewater and surface water	SBSE/LD	UHPLC-MS-MS	84-89 44-50	2.5, 5	[31]
TCC	Wastewater	SBSE/LD	LC-MS/MS	92-96		[32]
TCS	Wastewater, surface water and drinking water	SBSE/TD	GC-MS	70-92	29	[33]
TCC	Wastewater and surface water	SBSE/LD	LC-MS/MS	50-60	10-20	[34]

MLOD: Method limit of detection
[a]Method limit of quantitation

Table 2.1. Comparison of methods for TCS, TCC and M-TCS reported in aqueous samples (Continued).

Analyte	*Matrix*	*Extraction technique*	*Analytical determination*	*Recovery (%)*	*MDL (ng L^{-1})*	*Ref.*
TCS **TCC**	Wastewater and drinking water	DLLME	HPLC-MS-MS	70-72 72-73	40-580	[35]
TCS **TCC** **M-TCS**	Wastewater and surface water	DLLME	HPLC-UV	77-96 64-103 76-121	134 42.1 236	[36]
TCS **M-TCS**	Wastewater, surface water and drinking water	DLLME	GC-MS	100	2 [a] 5 [a]	[37]
TCS	Wastewater and drinking water	USAEME	GC-MS	86-94	5.84	[38]
TCS	Drinking water and reservoir water	HF-LPME	GC-MS	84-114	20	[39]
TCS	Wastewater	MALLE	GC-MS	93	1.1	[41]

[a]MLOQ

Although liquid-liquid extraction (LLE) is not very common nowadays, it has still been applied in the determination of TCS and TCC using dichloromethane or toluene as extraction solvents with recoveries of higher than 90% [17,19].

Solid-phase extraction (SPE) is the most common extraction technique. Different types of cartridges such as silica bonded phase (Sep-Pak C_{18} [1] and ISOLUTE C_{18} [20]) or modified polymeric sorbents with polar groups like Oasis MAX [21], Oasis MCX [22], Bond Elut Plexa [23] or Oasis HLB [4,7-9,15,22,24,25] have been applied for the determination of TCS with similar results (about 90% recovery in almost all cases). Samaras et al. [26] compared the influence of two different sorbents (Oasis HLB and Sep-Pak C_{18}) on the recovery of TCS at different sample pHs, and both cartridges gave similar results at pH 2.5 and 5.3 with values of around 65%; but at pH 7 Sep-Pak C_{18} showed lower recoveries due to the weak acidic nature of TCS (17%). Yu et al. [22] also compared Oasis HLB to Oasis MCX, obtaining better results with the cation-exchanger. In both systems the sample had an acidic pH, so TCS was not charged and therefore ionic interactions were not supposed to be the main interaction mechanism between the sorbent and the TCS. Another kind of SPE mode applied was non-polar XAD

polymeric resins used as bag-SPE, which sorbed the target analytes from aqueous matrices with recoveries of about 75% [22].

For TCC, almost all the previous works applied SPE with Oasis HLB with a surrogate standard, reporting recoveries higher than 75% [4,7,9]. When no correction was carried out recoveries were lower, with values of 17% and 39 - 69% [15,23]. Pedrouzo et al. [23] compared Oasis HLB and Bond Elut Plexa, with better recoveries for the latter, but its application to wastewater was limited because of the low sample percolation flow rate. In almost all the studies where Oasis HLB cartridges were used, the lowest detection limits for all the target compounds were reported [7,9,15,23,24]. For M-TCS, ISOLUTE C_{18} cartridges were applied with recovery yields in the 87 - 98% range [20].

Canosa et al. [27] applied solid-phase microextraction (SPME) for the determination of TCS, M-TCS and related compounds in wastewater and surface water with recoveries of about 100% for TCS and 80% for M-TCS. Different fibers such as PA, PDMS, PDMS-DVB and CW-PDMS were tested with similar results. Headspace extraction mode was also evaluated at 100 ºC with better results for M-TCS, while for TCS better yields were obtained by direct immersion of the fiber. The effect of the sample pH was not significant and salt addition did not improve the yield of the extraction [27]. Regueiro et al. [28] also developed a method based on SPME for the analysis of parabens, triclosan and related chlorophenols in wastewater and although in MilliQ water results were better when direct immersions were applied, headspace was finally chosen for the determination of the analytes in complex matrices with recoveries of higher than 87% and method limits of detection (MLODs) in the low ng L^{-1}.

Stir-bar sorptive extraction (SBSE) technique with PDMS coated stir bars was used by Silva et al. [29] for the determination of TCS by HPLC-DAD in different type of matrices; recovery yield in real samples was 78.5% and MLOD was 100 ng L^{-1}. No effect of the pH and NaCl addition on the extraction of TCS was observed. Ferreira et al. [30] developed a method for the quantitation of TCS, M-TCS and parabens in water by SBSE in combination with GC; showing an improvement on the extraction efficiency for TCS

after in-situ derivatization. Pedrouzo et al. [31] also evaluated the efficiency of SBSE as an extraction technique for TCS and TCC with recovery values of around 87% and 44 - 50%, respectively. For TCC, Klein et al. [32] reported recovery values in the 92 - 96% range. In almost all the cases the addition of NaCl did not improve the recovery of the analytes, except in the method reported by Quintana et al. [33] who found an improvement in the recovery of TCS when ionic strength was increased. The effect of sample pH was also studied, and for TCS and TCC high pHs decreased the extraction efficiency of the SBSE technique; while at low pHs only TCC had a lower yield than at neutral pH [31]. In all three studies a non-polar PDMS coated stir bar was used. Bratkowska et al. [34] prepared a poly(vinylpyrrolidone-divinylbenzene)-coated stir bar, which is more polar, for the analysis of some pharmaceuticals and personal care products (PPCPs), including TCC, but recoveries were in the 50 – 60% range.

Finally, liquid-phase microextraction is another preconcentration and clean-up technique applied prior to chromatographic analysis. Dispersive liquid-liquid microextraction (DLLME) mode was used by Zhao et al. [35] for the determination of TCC and TCS in wastewater and drinking water. Methanol was selected as a disperser and an ionic liquid as extracting solvent with recoveries between 70 and 100% for both compounds. Guo et al. [36], using HPLC-DAD, had chosen $C_6H_4Cl_2$ and THF for the formation of the cloudy solution for TCS, TCC and M-TCS with recovery yields in the 64 – 121% range. In both methods the detection limits achieved were in the 40 and 580 ng L^{-1} range. Montes et al. [37] applied DLLME with CH_3CCl_3/MeOH as extracting/disperser and in-situ derivatization for the analysis of TCS and M-TCS with recoveries of 100%. With a sample volume of 10 mL and a final volume of 40 µL, the limits of quantitation obtained were 2 and 5 ng L^{-1} with enrichment factors of 256 and 231 for TCS and M-TCS, respectively. Another type of sample preparation technique is ultrasound-assisted emulsification-microextraction (USAEME) which is based on the formation of emulsions of an organic extractant in an aqueous sample by ultrasound radiation and subsequent separation of phases by centrifugation. Regueiro et al. [38] developed an in-situ derivatization method where 100 µL of 1,1,1-trichloroethane as

organic phase and 10 mL of aqueous water containing surrogate standards were used for the determination of TCS with a MLOD of 5.84 ng L^{-1}.

The 2-phase HF-LPME method with in-situ derivatization followed by GC-MS has also been developed for the determination of TCS in drinking water and reservoir water; toluene, heptane and *n*-dodecane as extracting solvents were compared. However, for toluene and heptane partial evaporation was observed, so *n*-dodecane was chosen; with recoveries higher than 83% in the analysis of environmental waters and MLOD of 20 ng L^{-1} [39]. Membrane-assisted liquid-liquid extraction using a non-porous membrane bag as interface between the sample and the organic solvent [40] has been applied for the determination of TCS using chloroform as the acceptor phase and in-situ derivatization. The extraction efficiency in reagent water was 89% but in real samples, such as wastewater, an influence of the matrix was observed, so a surrogate internal standard was applied in order to compensate for that. The method limit of detection was 1 ng L^{-1} [41].

In this study, due to the non-polar character of TCS, TCC and M-TCS, two-phase HF-LPME technique was chosen for the extraction, clean-up and enrichment of the analytes. As has been explained in the introduction, in two-phase mode the extraction process takes place via a porous hydrophobic membrane which is situated between an aqueous phase (donor phase) and an organic phase. The analyte is extracted from the aqueous sample to an organic solvent immiscible in water that fills the pores and the lumen of the hollow fiber membrane [42-45]. In this way, the non-polar compounds present in the sample pass through the membrane to a small volume of organic phase. At the end of the extraction, the organic phase is removed and used for GC or normal phase HLPC [42]. Moreover, after HF-LPME an extra step can be added for the evaporation of the organic solvent and its reconstitution in an appropriate phase for the analysis by HPLC.

2.1.2. CHROMATOGRAPHIC ANALYSIS

Different chromatographic methodologies have been reported for the quantitation of biocides in water samples, including GC-MS, LC-MS and HPLC-UV (Table 2.1). GC has

been widely used for the determination of TCS and M-TCS, but no method for the determination of TCC by GC has been reported [1,19,25,27,33,39]. Although TCS has been analyzed without derivatization by Kantiani et al. [20] and Coogan et al. [8], due to the high polarity of TCS, derivatization is required prior to GC analysis in order to improve the chromatographic behavior of the analyte; reducing its polarity and volatilization. Different derivatization agents can be selected for phenolic compounds such as diazomethane [19] and pentafluoropropionic acid anhydridre (PFPA) [21] for the esterification, or bis(trimethylsilyl)trifluoroacetamide (BSTFA) [26], *N*-Methyl-*N*-trifluoroacetamide (MSTFA) [25] and *N*-*t*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) for the silylation [27,33]. In-situ derivatization can be performed with acetic acid as was reported by Zhao et al. [35] who added acetic acid with sodium hydroxide for the phenol acetylation of TCS in the sample before HF-LPME.

On the other hand, LC-MS has been applied for the analysis of TCS and TCC. For LC-MS/MS analysis, Gonzàlez-Mariño et al. [9] studied different mobile phase additives at different concentrations: 0 - 20 mmol L^{-1} ammonium acetate, 0 - 0.2% formic acid and 0 – 2% acetic acid using MeOH as organic phase. For TCC no effect was observed because it was protonated in all the cases due to its high pKa (12.8). For TCS ion suppression was observed, especially when organic acids were used in the mobile phase because TCS was in its neutral form. For this reason, ammonium acetate was selected as additive. Halden et al. [4] study was focused on the development of a sensitive and selective method for the determination of TCC by LC-ESI-MS/MS, using a mixture of acetonitrile, water and acetic acid (10 mM) as mobile phase but TCC/acetic adducts were generated.

No method has been reported for the simultaneous determination of the three analytes with MS detection. Coogan et al. [8] monitored the three analytes in water samples in order to study algal bioaccumulation but two chromatographic methods were applied; TCS and M-TCS were determined by GC-MS while TCC analysis was

carried out by LC-ESI-MS using a C_{18} column and acetonitrile and water with 5 mmol L^{-1} ammonium acetate as a mobile phase.

Although HPLC-UV is the lesser applied technique, according to the literature, UV detection is the only type of detection affordable for the determination of TCS, TCC and M-TCS together. The paper by Guo et al. [36] is the only report where TCS, TCC and M-TCS are determined together. A UHPLC method was developed using a bridge ethylene hybrid (BEH) C18 column (50 mm x 2.1 mm, 1.7 µm), a buffer solution containing boric acid, potassium chloride and sodium hydroxide in water as mobile phase A, and acetonitrile as mobile phase B. UV detection was carried out at 283 nm. Al-Rajab et al. [46] also determined TCS, M-TCS and TCC by HPLC-UV at 274 nm but using two methods; in the first one an isocratic elution was applied for TCS and M-TCS while for TCC a gradient was used. In both cases, methanol and water were used as mobile phases. Ying et al. [47] analyzed TCS and TCC by liquid chromatography with UV detection at a wavelength of 265 nm for TCC and 205 nm for TCS. In almost all the cases a C_{18} column was used.

The aim of this study is to develop a 2-phase HF-LPME method followed by liquid chromatography with UV and MS detection for the determination of TCS, TCC and M-TCS in water samples. This new method has been validated and compared to SPE for the analysis of complex matrices and applied to the analysis of real environmental samples.

2.2. EXPERIMENTAL

2.2.1. CHEMICALS AND STANDARDS

All chemicals and solvents were of analytical reagent grade. Methanol (MeOH) HPLC-grade, acetonitrile (ACN) HPLC-grade, acetone, n-hexane and ammonium acetate were purchased from Carlo Erba (Milan, Italy). Dodecanol, cumene and sulfuric acid were obtained from Merck-Schuchardt (Hohenbrunn, Germany) and TCC, M-TCS, DHE, dibutyl ether, hexylbenzene, amylbenzene, undecane, anethol, 3-chlorotoluene, decane, n-dodecane, cis and trans- decahydronaphtalene (decaline), toluene and n-

octanol from Sigma Aldrich (Steinheim, Germany). TCS and isooctane were obtained from Fluka (Barcelona, Spain). Sodium hydroxide, sodium thiosulfate and sodium chloride were provided by Panreac (Barcelona, Spain). Water was purified by a MilliQ system (Millipore Iberica S.A., Barcelona, Spain).

Individual stock solutions were prepared in MeOH with a concentration of 1,000 mg L^{-1} for TCS, TCC and M-TCS. Standards and working solutions were prepared in MeOH for the validation of the chromatographic method (in the range of mg L^{-1}) and in reagent water for the assessment of the whole analytical procedure (in the range of μg L^{-1}). The stock solutions and standards were stored at 4 ºC.

2.2.2. SAMPLES

Drinking water samples were from a laboratory at the University of Girona and surface water samples from the Muga River in Figueres (northeast Spain). Wastewater samples were collected from three WWTPs located in the North-East of Spain: Blanes (WWTP1), Castell-Platja d'Aro (WWTP2) and Palamós (WWTP3). All water samples were collected in 1 L pre-cleaned brown glass bottles and kept refrigerated during transportation; the samples were kept in the darkness at 4 ºC until analysis. Surface water and wastewater were filtered through a 0.7 µm glass fiber filter (Whatman, Maidstone, UK) prior to the extraction and 100 mg of sodium thiosulfate was added to drinking water samples in order to avoid possible degradations because TCS can produce chlorophenols in the presence of chlorine [10].

2.2.3. HF-LPME

HF-LPME was performed by using polypropylene membranes with a thickness of 200 µm, 0.2 µm pore size and an internal diameter of 300 µm (Azko Nobel, Wuppertal, Germany). The sample volume used for extraction was 500 mL.

Fibers were cut into 5 cm long pieces. One of the ends was sealed and the other was attached to a needle syringe (Hamilton, Bonaduz, Switzerland), as can be seen in Figure 2.1. Next, pores and lumen were impregnated with the organic solvent by using a 250 µL syringe (Hamilton, Bonaduz, Switzerland) and the excess of solvent was

removed by immersion of the fiber in reagent water. Then, the fiber was immersed into the sample, protected from light exposure with aluminum foil and the solution was magnetically stirred. After extraction, the organic solvent was withdrawn with a syringe, evaporated under a nitrogen stream and the residue reconstituted in 0.2 mL of MeOH.

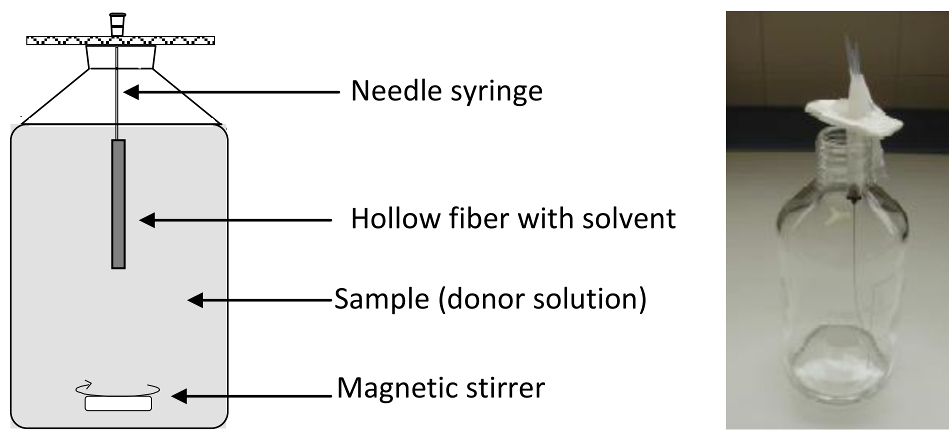

Figure 2.1. Scheme of 2-phase HF-LPME system.

In order to compare the results from the different experiments, extraction efficiency (E) parameter was used. This is defined as in Eq. (2.1):

$$E = \frac{m_F}{m_D} \times 100\% \quad (2.1)$$

where m_F is the amount of analyte in the MeOH solution and m_D is the amount of analyte added to the donor solution.

2.2.4.SPE

The results obtained from the developed HF-LPME methodology were compared to those obtained by using SPE, the most conventional technique for the extraction of analytes from influent wastewater. The conditioning of the Oasis HLB cartridge from Waters (Milford, MA, USA) was performed with 2 mL of MeOH:acetone (1:1), 2 mL of MeOH and 6 mL of reagent water. Then, 300 mL of influent wastewater was passed through the cartridge at about 5 mL min^{-1} using a vacuum pump (Gilson, Williers Le Bel, France). The loaded cartridge was rinsed either with 1 mL of MeOH:water (5:95,

v/v) or 1 mL of n-hexane at a flow rate of 1 mL min^{-1}. Next, the cartridge was dried under vacuum and the analytes were eluted with 4 mL of MeOH:acetone (1:1). Finally, the extract was evaporated to dryness under a gentle stream of nitrogen and the residue dissolved in 0.5 mL of MeOH.

2.2.5. HPLC-DAD

HPLC analysis were performed using an Agilent Technologies 1200 series separations module with a Bin Bump SL, a degasser and ALS SL injector and an Agilent Technologies 1290 infinity DAD SL. Separation of the analytes was carried out on a Kinetex C_{18} column (2.6 μm, 50 x 2.1mm) supplied by Phenomenex (Macclesfield, United Kingdom). An injection volume of 5 µL was used for all the analyses. Cellulose nitrate and nylon membrane filters with 0.22 µm pore size were used for the preparation of the mobile phases. The mobile phase used contained ACN as eluent A and reagent water as eluent B at a flow rate of 0.5 mL min^{-1}. The elution started with 30% of eluent A held for 3 min, ramped to 40% at 32 min, ramped to 60% at 33 min, held for 3 min and ramped to 95% in 6 min. DAD detection was carried out at 224 and 265 nm which correspond to the maximum absorbance of TCC and TCS, and M-TCS, respectively.

For confirmation purposes, some of the samples were analyzed by LC coupled to electrospray ionization-tandem mass spectrometry (ESI-MS) for the determination of TCS and TCC. Briefly, chromatographic separation was performed using an Agilent Technologies 1290 Infinity series separation module with a Bin Bump SL, a degasser, ALS SL injector and a thermostatic column compartment. Separation of the analytes was carried out on a Synergi Fusion-RP 100A C_{18} column (2.5 μm, 50 x 2 mm) supplied by Phenomenex (Macclesfield, United Kingdom) at 30 ºC. The mobile phase used contained MeOH as eluent A and reagent water containing 10 mmol L^{-1} ammonium acetate as eluent B. Mass spectrometry was performed on an Agilent Technologies 6430 triple quadrupole mass spectrometer fitted with an ESI source and controlled by a Mass Hunter workstation. Analyses were performed in negative ionization mode under the following MS/MS conditions: N_2 flow rate of 10 mL min^{-1}, nebulizer pressure

of 45 psi and a source temperature of 350 ºC. Optimum multiple reaction monitoring conditions are summarized in Table 2.2.

Table 2.2. Multiple reaction monitoring conditions for TCS and TCC.

Analyte	*Precursor ion (m/z)*	*Product (m/z)*	*Fragmentor (V)*	*Collision energy (V)*
TCS	287	35	80	4
TCC	313	160	100	5

2.3. RESULTS AND DISCUSSION

2.3.1. HPLC-DAD

Several columns and mobile phases were tested for the separation of TCS, TCC and M-TCS by HPLC-DAD. Chromatographic separation of TCS and TCC was only achieved with a Kinetex C_{18} column (2.6 μm, 50 x 2.1mm); other C_{18} columns were tested but in no cases were the two analytes separated. In addition, different mobile phases and different additives were tested. ACN and MeOH as organic solvents and the effect of the addition of potassium phosphate monobasic 20 mmol L^{-1} and ammonium acetate 5 mmol L^{-1} to the aqueous phase were evaluated. The best chromatographic separation was observed with ACN, while no improvement was observed when potassium phosphate monobasic and ammonium acetate were added. For these reasons, ACN and reagent water were used as mobile phases.

For the quantitation of the analytes, a spectrum for each compound was performed. For TCS and M-TCS the highest absorption was observed at 224 nm while for TCC it was at 264 nm. Therefore, those wavelengths were used in order to obtain the best sensitivity. In Table 2.3, figure of merits of the chromatographic system are shown.

Table 2.3. ILODs, ILOQs, intra-day precision, inter-day precision, coefficients of determination and range studied of HPLC-DAD method (N=5).

Analyte	*ILOD (μg L^{-1})*	*ILOQ (μg L^{-1})*	*Intra-day precision (%)*	*Inter-day precision (%)*	*r^2*	*Range (mg L^{-1})*
TCS	4	1.3	4.2	5.0	0.999	ILOQ - 10
TCC	2	7	2.4	3.2	0.996	ILOQ - 10
M-TCS	1	3.3	1.4	1.9	0.999	ILOQ - 10

2.3.2. HF-LPME OPTIMIZATION

In HF-LPME, several parameters need to be considered in order to achieve optimum extraction efficiency, hence the influence of the organic solvent, fiber length, stirring rate, extraction time, volume and pH of the donor phase, the salting-out effect and humic acid effect were evaluated in reagent water.

Starting conditions for the HF-LPME system were a fiber length of 5 cm, a stirring rate of 300 rpm, an extraction time of 5 hours and a sample volume of 500 mL at pH 6 without salt addition.

2.3.2.1. Organic solvent

The organic solvent is one of the most important factors that affect the extraction. Compounds need to have affinity and to be soluble in the organic solvent. Also, the volatility and the viscosity of the solvent are factors that can modify the extraction efficiency. In addition, the time required for the further evaporation of the solvent prior to HPLC analysis has to be taken into account [48,49].

Several organic solvents were tested for the extraction of TCS, M-TCS and TCC. Toluene, n-octanol, dibutyl ether, isooctane and 3-chlorotoluene were applied for HF-LPME but after the extraction no organic solvent was recovered. Hexylbenzene, anethol, n-dodecane with 4% dodecanol and DHE were somewhat effective but a long time for the evaporation under nitrogen stream before reconstitution in MeOH was required. In Figure 2.2 the results obtained with the most suitable solvents tested are presented. Extraction efficiencies in the 6 - 13% range were achieved for TCS and M-TCS using n-dodecane, decaline, decane, cumene, DHE:decane (1:1) and amylbenzene, with better results for decaline. On the other hand, TCC was only extracted with DHE:decane (1:1) and amylbenzene but this last solvent required a long time for evaporation. Also, undecane was tested but no extraction was achieved for TCC. Therefore, DHE:decane (1:1) was chosen for further optimization and application to the analysis of real samples.

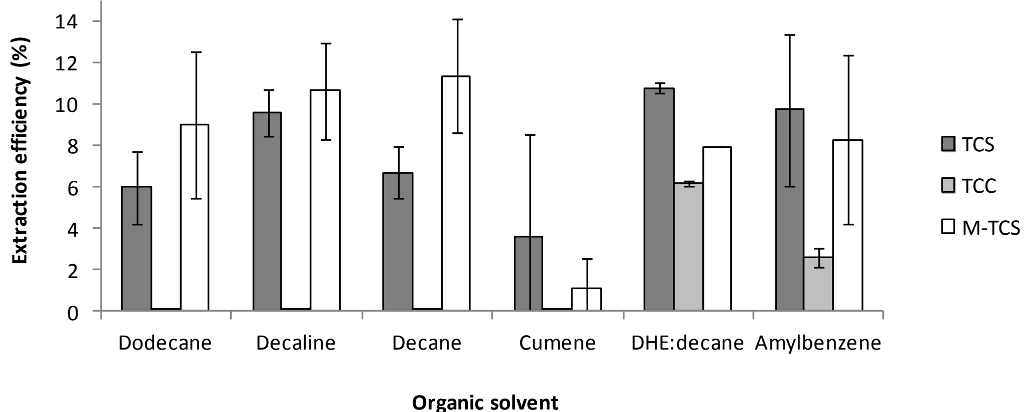

Figure 2.2. Organic solvent effect on the extraction efficiency of target compounds at concentration of 5 µg L^{-1} in reagent water and 5 hours extraction time (N=3).

2.3.2.2. Fiber length and stirring rate

Another variable studied was the length of the fiber. Three different lengths (3 cm, 5 cm and 7 cm) were evaluated with no observed differences in the extraction results between membranes. However, high relative standard deviations (35 – 45%) were obtained with 3 cm long fibers while the lowest relative standard deviation values (3 – 4%) were observed for 7 cm, but the use of a longer fiber might lead to a decrease in the stability of the system over prolonged times. Therefore, a fiber of 5 cm was chosen for further experiments with relative standard deviations values in the 11 - 15% range.

The effect of the stirring rate was studied using magnetic stirring at 200 rpm, 300 rpm and 400 rpm. Similar results were achieved for the three conditions tested; only a slight increase of 10% was observed for M-TCS from 200 to 300 rpm. A stirring rate of 300 rpm was chosen in order to avoid losses of solvent from the fiber over long operating times.

2.3.2.3. Extraction time

Extraction time is an important parameter to be optimized because mass transfer is a time-dependent process. Figure 2.3 shows the effect of the extraction time of the compounds studied on the extraction efficiency of the HF-LPME system. As can be seen, the extraction efficiency was still increasing after 24 hours operating. At this time no equilibrium conditions were achieved but, since long extraction times can produce losses of organic solvent, the extraction time was set at 24 hours.

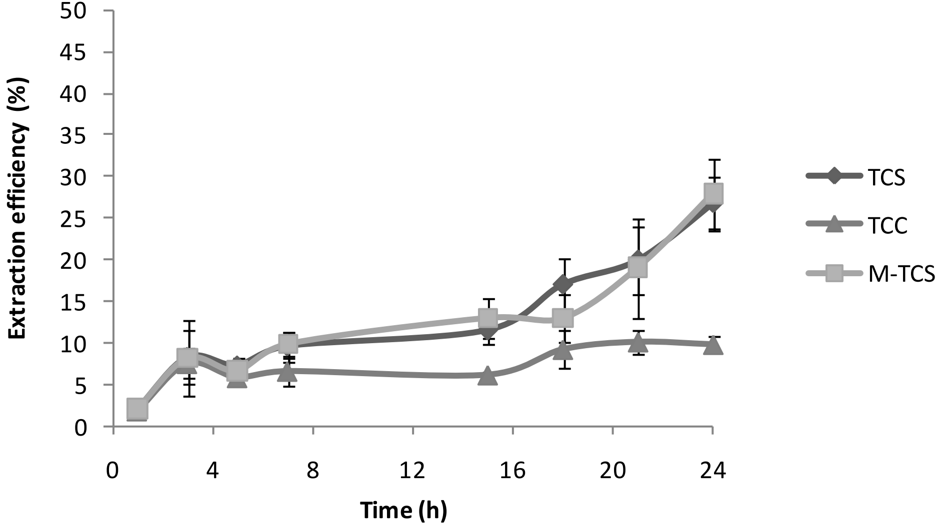

Figure 2.3. Extraction time profiles on the extraction efficiency of target compounds at a concentration of 5 µg L^{-1} in reagent water (N=3).

2.3.2.4. Volume and pH of the sample

Sample volume also plays an important role in the application of the proposed methodology for environmental samples. It can affect both the enrichment factor and the extraction efficiency. Three sample volumes were evaluated (100 mL, 500 mL and 1000 mL). When the volume was increased from 100 mL to 500 mL an increase in the concentration of analytes in the acceptor solution was observed with values between 2.7 and 1.7 times higher. From 500 mL and 1000 mL no differences were observed. Therefore, a volume of 500 mL was used for subsequent experiments.

Also, the influence of sample pH was studied in reagent water. Three different pH values were tested: pH 2 (adjusted with sulfuric acid), pH 6 (pH of reagent water) and pH 8.5 (adjusted with sodium hydroxide 5 mol L^{-1}). Figure 2.4 shows the results obtained; as can be seen, no difference between pH 2 and 6 can be seen, while for pH 8.5 a slight decrease in the extraction efficiency was observed. This can be explained by the pKa of the compounds: at high pHs the target compounds are partially charged and their affinity for the organic phase decreases. Therefore, an adjustment of the pH was not required in further experiments. Pedrouzo et al. [23] also observed a similar tendency for TCC and TCS when SBSE is used and Bratkowska et al. [34] observed a decrease in the extraction efficiency for TCC when the pH increases.

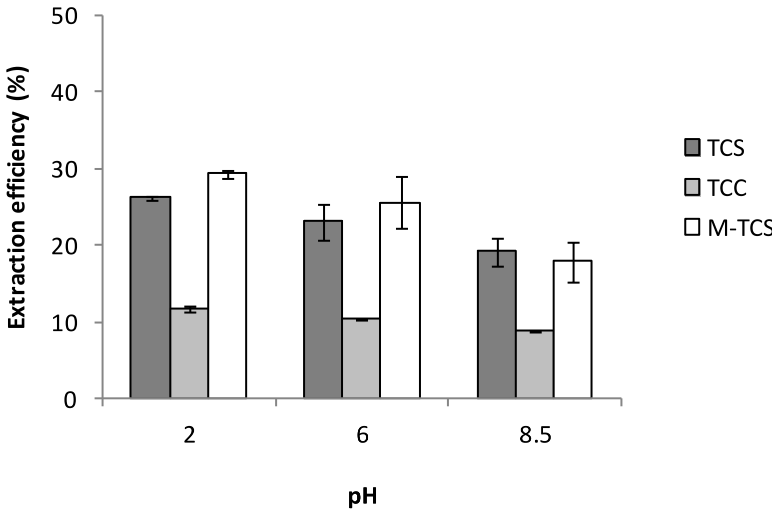

Figure 2.4. Effect of the sample pH on the extraction efficiency of target compounds at a concentration of 5 µg L^{-1} in reagent water (N=3).

2.3.2.5. Salting-out effect

It is well-known that the addition of salt may cause a decrease in the solubility of organic compounds in aqueous solutions because of the increase in ionic strength and partition coefficients in organic solvent. In this way, an enhancement of the extraction efficiency of the analytes from the aqueous solution to the organic solvent can be achieved. This effect is especially important for hydrophobic analytes like TCS, TCC and M-TCS.

In this study three different amounts of NaCl (10 g, 25 g and 50 g) were added to the sample in order to evaluate its influence on the extraction efficiency. As can be seen in Figure 2.5, there is no enhancement in the extraction of TCS and M-TCS when 10 and 25 g of NaCl were added, while the addition of higher amounts of NaCl (50 g) caused a decrease in the extraction efficiency. On the other hand, a positive effect was observed for TCC, the most hydrophobic compound; as can be seen in Figure 2.5 the extraction efficiency increases when ionic strength increases. Therefore, the addition of 25 g of NaCl was chosen as a compromise value for the application of the method to real samples analysis.

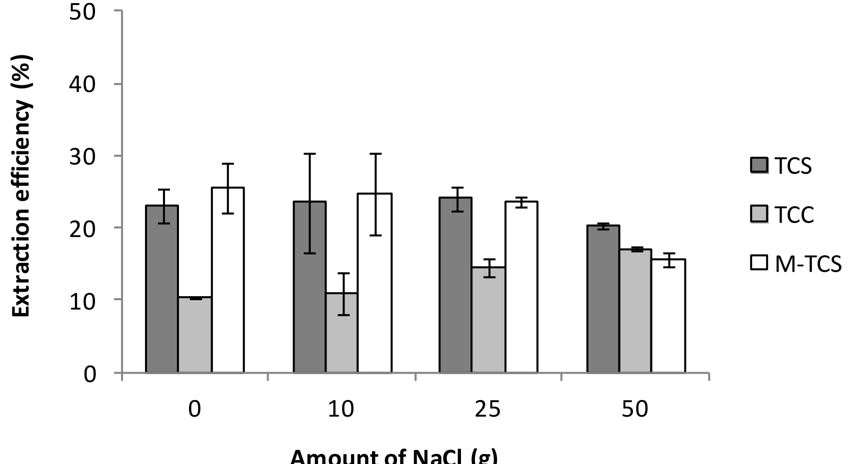

Figure 2.5. Influence of salt addition on the extraction efficiency of target compounds at a concentration of 5 µg L^{-1} in reagent water (N=3).

2.3.2.6. <u>Matrix effect: presence of humic acids</u>

The presence of organic matter in waters can also have an effect on the extraction efficiency and enrichment factor. Some organic molecules can be co-extracted and, furthermore, the functional groups of the chemical compounds constituting the organic matter can interact with organic pollutants and reduce the presence of freely dissolved analytes in water. Therefore, a decrease in the extraction efficiency can be expected [50]. The influence of three different concentrations of humic acids in reagent water was tested. As can be seen in Figure 2.6, the extraction efficiency decreases for all three analytes to values below 10% when humic acid is added at a concentration of 50 mg L^{-1}. This effect is probably not due to the extraction of humic acids, since high molecular mass compounds are excluded by the microporous membrane but is more likely due to the interaction of organic matter with the target compounds which reduces the free fraction of free analytes that can pass through the membrane.

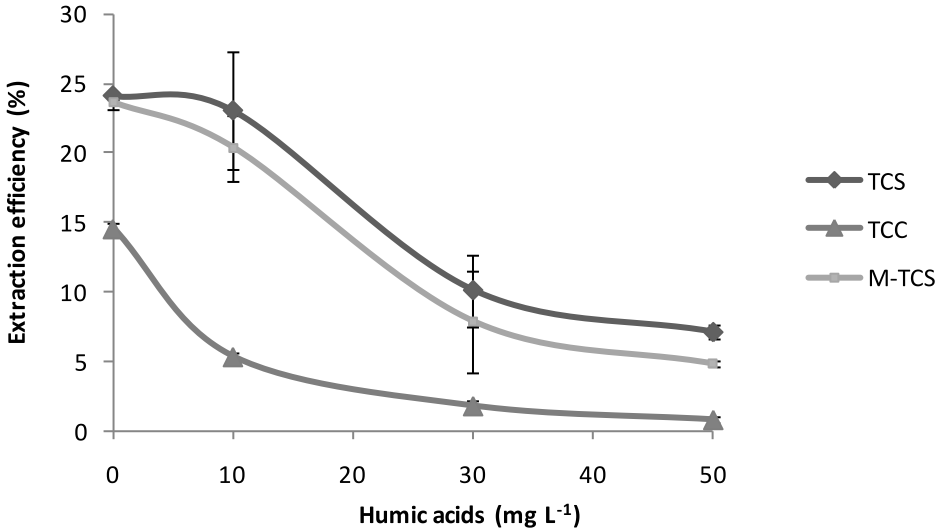

Figure 2.6. Influence of humic acids on the extraction efficiency of target compounds at concentration of 5 µg L^{-1} in reagent water (N=3).

2.3.2.7.Analytical performance

In order to evaluate the HF-LPME method for the determination of TCS, TCC and M-TCS; linearity, sensitivity, inter-day precision, intra-day precision, enrichment factor and extraction efficiency were established under optimized conditions (Table 2.4). Linear regression lines were obtained with coefficients of determination higher than 0.995 in the range between the limit of quantitation and 50 µg L^{-1} for all the analytes. Method limits of detection (MLODs) and method limits of quantitation (MLOQs) were calculated as 3 and 10 times the signal-to-noise ratios, respectively. Low limits of detection of 8 ng L^{-1}, 5 ng L^{-1} and 10 ng L^{-1} were achieved for TCS, TCC and M-TCS, respectively. These values are of the same order as the ones reported in the literature by SPE and lower than the ones reported by liquid-phase microextraction (Table 2.1).

To evaluate the intra-day precision and inter-day precision, five spiked samples at 5 µg L^{-1} concentration were subjected to the entire analytical procedure. Values in the range from 6 to 12% and from 9 to 14% were obtained for intra-day precision and inter-day precision, respectively. The enrichment factor values obtained were 430, 646 and 707 for TCC, M-TCS and TCS, respectively.

Table 2.4. MLODs, MLOQs, intra-day precision, inter-day precision, average enrichment factor (Ee) and extraction efficiency (E) for reagent water spiked at 5 µg L^{-1} for HF-LPME method (N=5).

Analyte	*MLOD (ng L^{-1})*	*MLOQ (ng L^{-1})*	*Intra-day precision (%)*	*Inter-day precision (%)*	*Ee*	*E (%)*
TCS	8	27	5.5	8.9	707	24.1
TCC	5	17	6.5	14.2	430	14.6
M-TCS	10	33	11.6	12	646	23.6

2.3.3. Application to real waters

2.3.3.1. Recovery on spiked waters

Recovery assays were performed on four different types of waters: effluent wastewater, influent wastewater, surface water and drinking water. Recoveries were evaluated at two concentration levels (0.5 and 5 µg L^{-1}) for all the analytes. The results obtained are collected in Table 2.5. As can be seen, a recovery for TCS of about 80% was obtained in all the samples while TCC and M-TCS have similar values in effluent wastewater, surface water and drinking water in the 75 - 99% range. For influent wastewater low recoveries were achieved for TCC and M-TCS. This can be explained by the presence of organic matter or even small particles to which analytes can interact, thus decreasing the freely dissolved content of the target compounds. In addition, it has been reported that TCC sorbs strongly to wastewater particulate matter [51]. Figure 2.7 shows a chromatogram for influent wastewater spiked at 5 µg L^{-1}; as can be seen, all the analytes were clearly identified.

Table 2.5. Influence of various sample matrices on the recoveries of the target analytes at 0.5 and 5 µg L^{-1} by HF-LPME (N=5).

Water type	*Spiked level (µg L^{-1})*	*TCS*	*TCC*	*M-TCS*
Influent wastewater	0.5	80 ± 10	15 ± 4	49 ± 10
	5	90 ± 5	21 ± 3	44 ± 6
Effluent wastewater	0.5	82 ± 11	86 ± 9	91 ± 8
	5	84 ± 12	75 ± 12	99 ± 12
Surface water	0.5	77 ± 9	82 ± 7	80 ± 10
	5	88 ± 8	97 ± 12	90 ± 12
Drinking water	0.5	75 ± 10	84 ± 12	84 ± 15
	5	85 ± 12	93 ± 9	91 ± 10

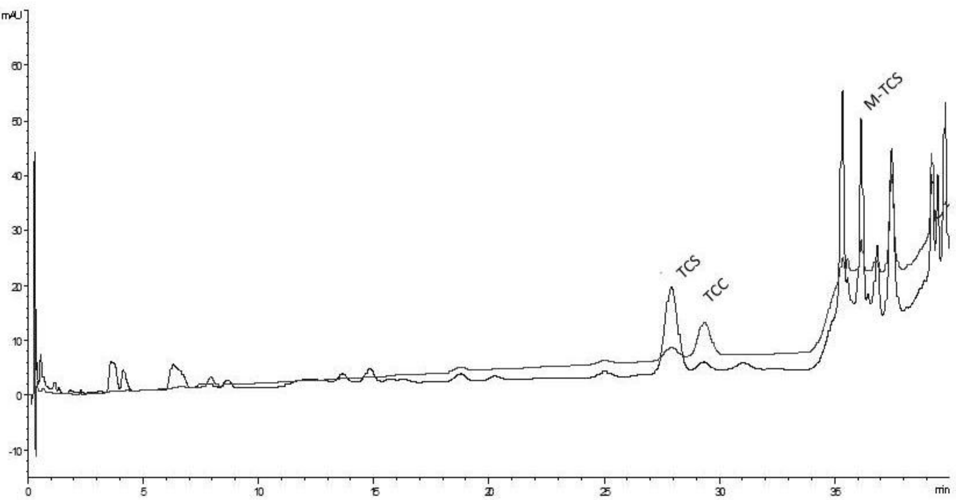

Figure 2.7. Chromatogram obtained by HPLC-DAD of TCS, TCC and M-TCS for influent wastewater spiked at 5 µg L^{-1}.

In order to evaluate the possible interference of the particulate matter present in the aqueous phase, influent wastewater was filtered by filters with different pore sizes. Figure 2.8 shows recoveries obtained for filtered and unfiltered influent wastewater. When water was not filtered recoveries were lower; this can be explained by the bounding of the analytes to particulate matter, which is consistent with a previous study in which retention of TCS and TCC to particulate matter was reported [17]. For water filtered with glass microfiber filters of 0.7 µm pore size and cellulose nitrate membrane filters of 0.22 µm pore size similar results were obtained. For this reason, for the application of the method to real samples before HF-LPME all the samples were filtered with glass microfiber filters of 0.7 µm pore size.

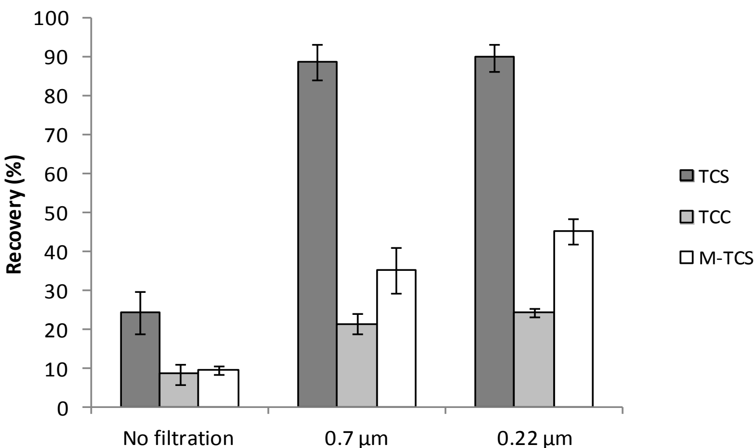

Figure 2.8. Effect of the filtration prior HF-LPME on the recovery of influent wastewater spiked at 5 µg L^{-1} (N=3).

Finally, HF-LPME recoveries for influent wastewater were compared to the results obtained from two SPE procedures commonly referred to in the literature, using Oasis HLB as the sorbent. As can be observed in Figure 2.9, better recoveries were achieved with SPE when the cartridge was rinsed with MeOH:H_2O (5:95). Apparently, when *n*-hexane is used more interferences have to be removed and cleaner chromatograms have to be obtained, but, due to the low polarity of biocides analytes, they could be lost when *n*-hexane was applied. When comparing HF-LPME and SPE, higher recoveries were obtained with SPE. This can be explained by considering that analytes associated with organic matter or small particles are also retained in the sorbent and could be released in subsequent elution, whereas only the free fraction of the analytes are extracted by HF-LPME [17]. However, the selectivity of the SPE method was lower than with HF-LPME method because dirty chromatograms were obtained.

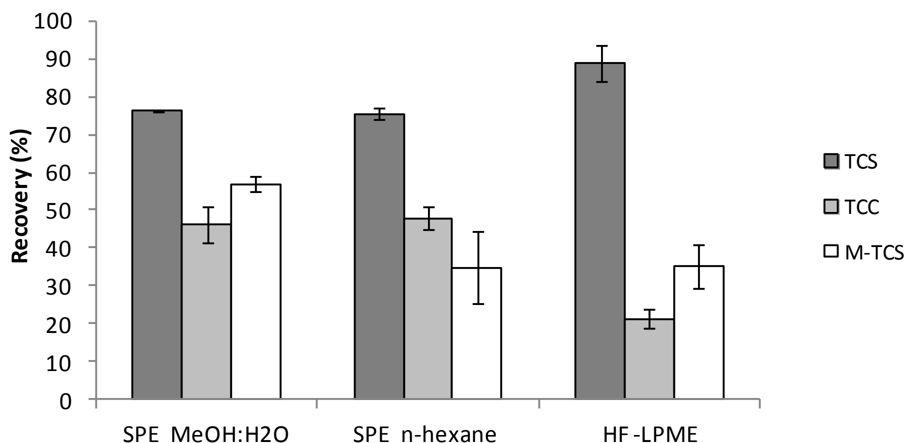

Figure 2.9. Recoveries for influent wastewater spiked at 5 µg L^{-1} applying SPE and HF-LPME (N=3).

The recoveries obtained using the HF-LPME method developed in this chapter (Table 2.5) can be compared to some of the ones previously reported in literature. Pedrouzo et al. [31] achieved recoveries in the 84 - 89% range for TCS and 44 – 50% for TCC in surface water and influent and effluent wastewater applying SBSE; while, when using SPE, recoveries were in the range of 85 – 92% and 39 – 69% for TCS and TCC, respectively. González-Mariño et al. [9] developed a method based on SPE-LC-MS/MS for the determination of TCS and TCC with recoveries at around 95% and 80% for TCS and TCC, respectively. Canosa et al. [27] developed a method for the determination of TCS and M-TCS by SPME in wastewaters with recoveries at around 100%. Finally, Zhao

et al. [39], by using HF-LPME, obtained recoveries between 84 and 114% for TCS in environmental waters. In almost all the studies, low recoveries for TCC in environmental samples were achieved when no correction was applied. In the HF-LPME method developed in this study, recoveries of higher than 75% were achieved, except for triclocarban and methyl-triclosan in effluent wastewater. All these results indicate that the developed methodology could be applied for the determination of TCS, TCS and M-TCS at trace levels in most water samples.

2.3.3.2.<u>*Application to monitoring of wastewater*</u>

Samples were collected from three different municipal WWTPs in Blanes (WWTP1), Castell-Platja d'Aro (WWTP2) and Palamós (WWTP3) located in the North-East of Spain. WWTP1 treats the sewage of a population of 109,985, WWTP2 of 175,000 and WWTP3 of 165,450. The wastewater treatment processes in all the WWTPs consistsed of physical treatment with a screen and a primary clarifier, then the wastewater undergoes biological treatment (aerobic) followed by a secondary clarifier. For WWTP2 and WWTP3 tertiary treatment was applied. In WWTP2 the tertiary treatment applied was ultraviolet disinfection whereas in WWTP3 the tertiary treatment was a chemical treatment with chlorine disinfection. The treated water was discharged in different places; to river, agricultural land, the Mediterranean Sea or aquifers.

Effluent wastewater, influent wastewater, surface water and drinking water were analyzed by the proposed HF-LPME methodology. As can be seen in Table 2.6, TCC was not detected or it is present below its MLOQs in all the samples, whereas M-TCS was quantified in only two effluent samples. TCS was the most frequently detected analyte with minimum and maximum concentrations of 120 and 883 ng L^{-1} in influent wastewater. In effluent wastewater a wide range of concentrations (<MLOD - 323 ng L^{-1}) were observed for TCS and in surface water it was detected with concentrations of 23 and 51 ng L^{-1}. In drinking water none of the target compounds were observed. These results are consistent with the concentration levels reported in other studies [15,20,23,27]. Rodil et al. [52] detected TCS in influent and effluent wastewater at median concentrations of 16 and 57 ng L^{-1} and it was not detected in drinking water.

Hua et al. [24] reported concentrations of TCS in effluent wastewater of about 60 ng L^{-1} of TCS and in surface water between 4 and 8 ng L^{-1}, while Heidler et al. [7] detected concentration ranges of 0.8 - 23.9 µg L^{-1} for TCS and 1.3 - 20.5 µg L^{-1} for TCC in influent wastewater, and in effluent wastewater concentrations were in the range of <0.01 – 0.84 µg L^{-1} and 0.01 - 1.78 µg L^{-1} for TCS and TCC, respectively. Differences in the concentration levels of influent wastewater can be explained by the season in which the sampling took place or the population of the WWTP, while concentrations in effluent wastewater can differ because of the treatment applied at the WWTP.

After biological treatment and the secondary clarifier (WWTP1 effluent, and WWTP2 and WWTP3 secondary wastewater), removal rates for TCS were between 0 and 77%. Although several studies have reported high elimination yields, in our study lower values were obtained. M-TCS was detected after biological treatment while, in influent wastewater, it was detected in only one sample, and below its MLOQ. This can be explained by the methylation of TCS under biological treatment [3]. When comparing tertiary treatments, chlorination was more effective for the elimination of TCS than UV chlorination, with removal values of >37% and 10 - 33%, respectively. Some studies have observed that TCS reacts with free chlorine inducing chlorinated triclosan derivates [10,13] whereas, under UV radiation, Mezcua et al. [54] reported photodegradation of TCS into dioxins in wastewater and Hua et al. [24] observed a removal of about 22% when UV radiation was applied. Moreover, phototransformation can be related to the low levels of TCS quantified in surface water.

Table 2.6. Concentration (ng L^{-1}) of the personal care products in environmental samples.

Analyte	*WWTP1*		*WWTP2*			*WWTP3*			
	Influent	*Effluent after biological*	*Influent*	*Secondary*	*Effluent after UV*	*Influent*	*Secondary*	*Effluent after chlorine*	*Surface water*
April 2011									
TCS			494		308				51
TCC			n.d.		n.d.				n.d.
M-TCS			34		n.d.				n.d.
May 2011									
TCS	883	195	134	95	85	120	153	76	23
TCC	n.d.	n.d.	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	n.d.
M-TCS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October 2011									
TCS	417	307	485	471	323	536	219	<MLOD	
TCC	n.d.	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	n.d.	
M-TCS	n.d.	64	<MLOQ	77	70	n.d.	n.d.	n.d.	

n.d.: not detected

2.4. CONCLUSIONS

In this study, a simple and sensitive method for the simultaneous determination of TCS, TCC and M-TCS in different environmental water samples and drinking water has been developed based on HF-LPME combined with HPLC-DAD. For the target analytes, large enrichment factors were achieved with values higher than 400 with low limits of detection of 5 ng L^{-1}, 8 ng L^{-1} and 10 ng L^{-1} for TCC, TCS and M-TCS, respectively. Recovery values obtained for drinking water, surface water and effluent wastewater and acceptable reproducibility also indicate that the method is suitable for the determination of the analytes in these kinds of samples.

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CHAPTER 3:

Hollow fiber liquid-phase microextraction as clean-up in the determination of triclosan, triclocarban and methyl-triclosan in biosolids and soil-amended sludge

3.1. INTRODUCTION

Due to the high consumption of pharmaceuticals and personal care products it is interesting to study their fate and occurrence through wastewater treatment plants (WWTPs). In WWTPs several treatments are applied for the elimination of contaminants; usually primary clarifiers and biological treatments (with activated sludge) are used, during these processes the waste stream is separated into two components, liquid effluents and sewage sludge, which is also treated.

Treated sewage sludge (biosolids) has to be re-used or eliminated. The spread of this sludge, as a fertilizer and soil conditioner, onto agricultural land is an attractive management option. It is an ecological and sustainable option due to its high content of organic matter and nutrients such as phosphor or potassium which can enhance the physical properties of soil as well as the plant yield [1-3]. The recycling of sewage sludge in agriculture has been regulated by Directive 86/278/ECC. The directive addresses both pathogen reduction and the potential for the accumulation of persistent pollutants in soil. However, severe concerns about the presence of non-legislated contaminants such as organic chemicals in soils may hinder a more confident and widespread application because of their potential indirect effects on human health through their effects and resistance in the microbial environment [2-4].

Data on pharmaceuticals and personal care products in biosolids applied to land is limited, therefore it is important to develop analytical methods for the determination of organic pollutants in order to understand the behavior of these compounds in WWTPs, and to contribute to assessing the consequences and their significance for the environment when sewage sludge is recycled to farmland as a fertilizer. Until now, attention has been focused on effluent wastewater, but more recently, interest in biosolids has increased.

Triclosan (TCS) and triclocarban (TCC) are partially removed in WWTPs and they are released into the environment either by effluent wastewater or by land application of biosolids due to their tendency to adsorb to sewage sludge. In Chapter 2 a method based on hollow fiber liquid-phase microextraction (HF-LPME) for the quantitation of

TCS, TCC and methyl-triclosan (M-TCS) in wastewater and environmental waters was developed and applied for the monitoring of these compounds in WWTPs. In the following study, a method for the determination of TCS, TCC and M-TCS in soils and biosolid samples has been developed.

3.1.1. OCCURRENCE IN SLUDGE AND SOILS

Regarding TCS and TCC fate in activated sludge processes, data has shown that they are significantly removed (58 - 96%) in biological reactors by sorption onto sludge or biodegradation [5-10].

Triclocarban seems to have resistance to biodegradation and biotransformation, and tends to partition to activated sludge owing to its hydrophobicity and low water solubility [11,12]. Heidler et al. [12] evaluated a mass balance of TCC during conventional sewage treatment, and results showed that 3% ended up in effluent wastewater, 21% of TCC was transformed under biological treatment, and 76% was accumulated in sludge. As a consequence, the worrying for its presence in biosolids has increased, especially when considering that a high half-life (70 days) in soils has been estimated under aerobic treatment; whereas under anaerobic conditions very low degradation after 70 days was observed [13]. A similar half-life was calculated by other reports with values of 108 days [1]. Hence, the presence of triclocarban in biosolids can be expected.

For triclosan, biodegradation seems to be the main process for its removal in WWTPs [14]. Stasinakis et al. [15] determined a percentage of biodegradation for TCS of 98% and Singer et al. [16] determined that 79% of TCS was degraded in biological treatment and only 15% was sorbed onto sludge. Bester et al. [6] observed that 65% of TCS was degraded and 30% was adsorbed. Chen et al. [17] found, under aerobic conditions, calculated degradation yields of 49% and sorption yields of 10%. These differences can be explained by several factors, such as the presence of organic carbon, temperature, pH and ionic strength, which can influence or modify the sorption of the analytes onto sludge [18]. Nevertheless, TCS is partially removed by sorption. Furthermore, since the pH of the environmental samples is around 8, and taking into account the pKa and

logK_{ow} values, the protoned form in waters led the sorption of TCS onto particles [16]. Regarding its stability in sludge treatment, Ying et al. [13] determined a half-life of 18 days under aerobic conditions whereas, under anaerobic conditions, no degradation was observed within 70 days. Although rapid biodegradation under aerobic conditions is observed in sludge, high concentrations of TCS have been found in biosolids [14].

On the other hand, TCS and TCC can be removed by sorption onto primary sludge, thus avoiding exposure to activated sludge tanks and, as a consequence, to biological treatment [8,12,15,19]. Furthermore, TCS biodegrades to M-TCS, which is more toxic than TCS, and different authors have investigated its generation and fate in WWTPs. Chen et al. [17] evaluated the transformation of TCS to M-TCS under aerobic and anaerobic conditions, observing the formation of M-TCS in both cases (16%).

As has been explained, the evaluation of the fate of pollutants in sludge-amended soil is of great concern. Lozano et al. [4] studied the degradation of TCS in soils over time after sludge application. In their study, sludge was added to soil and, according to their results, a significant reduction of TCS was observed in 7 - 9 months (78.2%) and in 16 - 21 months (96.1%). Furthermore, Kinney et al. [20] studied the transfer of emerging organic pollutants, including TCS, from different types of soil to earthworms. Soils, sludge-amended soils and manure-amended soils were evaluated, and it was observed that earthworms from sludge-amended soils accumulated TCS.

A complete understanding of the occurrence and fate of TCS, TCC and M-TCS in biosolids and sludge-amended soil for agricultural land is necessary but for this purpose reliable analytical methods for their detection and quantitation are required.

3.1.2. ANALYTICAL METHODS

Several studies have been carried out for the determination of TCS, TCC and M-TCS in sludge, soils and sediments. Due to the high content of organic matter and the presence of the analytes at trace levels in biosolids, extraction techniques such as pressurized liquid extraction (PLE), ultrasonic solvent extraction (USE) or microwave assisted extraction (MAE) combined with clean-up and enrichment techniques such as

solid-phase extraction (SPE) are applied. In Table 3.1. some of the analytical methods reported in the literature for the determination of TCS, TCC and M-TCS in solid samples are summarized.

Table 3.1. Comparison of methods for TCS, TCC and M-TCS reported in solid samples.

Analyte	*Matrix*	*Extraction technique (clean-up)*	*Analysis*	*MLOD (ng g^{-1})*	*Recovery (%)*	*Ref.*
TCS	Sludge	Soxhlet(SPE-GPC)	GC-MS	4	94	[6]
TCS **TCC**	Sludge Soils	PLE	LC-MS/MS	0.05-0.1 0.58-3.08	97-98 96-99	[1]
TCS **TCC**	Sludge	PLE	LC-MS/MS	8 1.25	77 103	[21]
TCS	Sludge	PLE	LC-MS	1000	78	[8]
TCS	Sludge and sediment	PLE	GC-MS	5[a]	100	[16]
TCS	Sediment	PLE (SPE)	LC-MS/MS	3	110	[22]
TCS **TCC**	Sludge and sediment	PLE	LC-MS/MS	50 20		[18]
TCS **TCC**	Sludge	PLE(SPE)	LC-MS/MS	5 0.5	98	[23]
TCS	Sludge	PLE (SPE)	LC-MS/MS		<55	[25]
TCS/TCC	Sludge	PLE(SPE)	LC-MS/MS		>70	[9]
TCS	Biosolids	PLE (SPE)	LC-MS	1	101	[4]
TCS **M-TCS**	Sludge and sediments	Matrix solid-phase dispersion	GC-MS	7 6	99-113 86-109	[24]
TCS	Biosolid	USE(SPE)	GC-MS	5	74	[5]
TCS	Natural soil	USE (SPE)	GC-MS	0.4[a]	96-115	[26]
TCS	Sludge	USE (SPE)	GC-MS	15	93	[27]
TCS **TCC**	Sludge	USE(SPE)	LC-MS/MS	487 183	105 96	[2]
TCS	Biosolids	USE (SPE)	LC-MS/MS	17	53-68	[29]
TCS	Sediment	MAE (SPE)	GC-MS		90	[30]
TCS	Sludge and sediment	MAE (LLE-SPE)	GC-MS	0.4-0.8	82-100	[31]
TCS **M-TCS**	Sludge and sediments	SBSE	GC-MS	0.16 0.37	3 3	[32]

[a]Method limit of quantitation

Usually, the most used technique is PLE combined with SPE in reversed mode, thus making the evaporation of the PLE extract and reconstitution in reagent water for the

subsequent clean-up necessary. However, PLE has also been used without further clean-up [1,8,16,21]. For PLE, one possible extraction solvent is a mixture of MeOH:water in order to evaporate less volume of the PLE extract. Barron et al. [22] used PLE for the determination of TCS in sludge and soil followed by SPE and LC-MS/MS. As extractant, a mixture of MeOH:water (50:50) was used and after the extraction the methanol was reduced to less than < 5%. However, for sludge, recoveries and method limit of detection (MLOD) were not calculated whereas, for soils, recoveries of 110% were reported and MLOD was 3 ng g^{-1}.

Langford et al. [18] also applied PLE for the extraction of TCS and TCC from sludge and sediments using MeOH as the extraction solvent at 70 ºC. After PLE, evaporation was carried out, and PLE extracts with high amounts of fat and other organic matter were reduced to 8 - 9 mL, whereas for the ones with less organic matter content, the extract was reduced to 1 – 2 mL. In both experiments the evaporated extracts were diluted with water and insoluble components were removed by centrifugation. Mean recoveries in the three types of sludges were: 5, 73 and 225% for TCC and 40% for TCS, with relative standard deviations between 3 and 113%. In sediments, TCC recoveries were between 30 - 734% and TCS recovery was 28%. MLODs were 2 and 20 ng g^{-1} for TCC, and 10 and 50 ng g^{-1} for TCS in sludge and sediment, respectively. In this method the clean-up is also avoided but poor results were observed.

For the quantitation of TCS and TCC in sludge and sediments, other organic solvents such as acetone: MeOH (95:5) [9] or acetone [8] have been used as extractants in PLE, but the most used is dichloromethane. Singer et al. [16] determined TCS by applying PLE followed by percolation through a silica column and GC-MS analysis with a 100% recovery and a method limit of quantitation (MLOQ) value of 5 ng g^{-1}. Chu et al. [23] determined TCS and TCC in biosolids by PLE, SPE as the clean-up technique and LC-MS/MS with isotope dilution with obtained recoveries of 98% for TCS and TCC and low limits of detection of 5 and 0.5 ng g^{-1} for TCS and TCC, respectively. Dichloromethane has also been demonstrated to be effective in the matrix solid-phase dispersion technique in the simultaneous extraction and clean-up of TCS and M-TCS from sludge

and sediments with recoveries higher than 86% in a simple and effective method with low limits of quantitation (6 ng g^{-1} for M-TCS and 7 ng g^{-1} for TCS) [24].

Another study compared USE and PLE without clean-up for the extraction of several pharmaceuticals and personal care products in sludge, including TCS. Better results were obtained by PLE with MeOH at pH 4 at 100ºC. Although it seems a simple method because clean-up was not applied, low recoveries were obtained (50%) [25].

Several studies have reported the extraction of pharmaceuticals and personal care products in sewage sludge or sediments by USE followed by SPE [5,26]. Samaras et al. [27] determined TCS in wastewater and sludge by applying USE with a mixture of MeOH:water at 50 ºC for 30 minutes. After collecting the supernatant, the extract was reconstituted in water and SPE was carried out. In their study different amounts of sludge and different proportions of MeOH:water were tested. In addition, when the percentage of MeOH was higher than 50%, extraction improved with mean recoveries of 93%.

Yu et al. [28] developed a method for the determination of endocrine disruptors including TCS by USE followed by evaporation, reconstitution in water, SPE and GC-MS. Different extractant solvents were tested (acetone, ethyl acetate, methanol and acidified methanol), with better results with MeOH. Recovery was 90% and MLOD was 2.1 ng g^{-1}.

McClellan et al. [2] applied EPA method 1694 for the determination of TCS and TCC in biosolids which consists of USE with acetonitrile, centrifugation, rotary evaporation and SPE with Oasis HLB followed by LC-MS/MS. In their method, 1 g of sludge was treated obtaining recoveries higher than 96%, however their MLODs were quite high in comparison with other reports (487 and 183 ng g^{-1} for TCS and TCC, respectively).

Another less applied technique is microwave assisted extraction (MAE), which has been used for the determination of TCS. A simple method for the analysis of sediments from a lake was developed by Rice et al. [30] using methylene chloride:methanol as the extractant, followed by a clean-up step with a silica microcolumn with a recovery

of 90%. A more complex method was developed using an acetone:MeOH (1:1) mixture and after MAE the extract was dissolved in 0.2 M NaOH and washed with *n*-hexane followed by SPE and GC-MS. Although high manipulation was required, recoveries of between 81 and 100% in different kinds of sludge and river sediment were obtained. MLODs were between 9.4 and 0.8 ng g^{-1} [31].

A new approach has been developed by Ferreira et al. [32] for the determination of TCS and M-TCS in soils, sediments and sludge. The method was based on the use of the stir bar sorptive extraction (SBSE) technique as the extraction, clean-up and pre-concentration, followed by gas chromatography. In this method, sludge was mixed with 5 mL of an aqueous solution and submitted to SBSE, but very low recoveries were obtained (3%). MLODs were 160 and 370 ng g^{-1} for TCS and M-TCS, respectively.

To our knowledge no method for the determination of the three analytes in solid samples has been reported and no method using HF-LPME as a clean-up technique has been applied. The main goal of this study was to develop a method for the determination of TCS, TCC and M-TCS in sewage sludge and sludge-amended soil by PLE followed by HF-LPME, combined with liquid chromatography coupled to tandem mass spectrometer (LC-ESI-MS/MS) for TCS and TCC, and high performance liquid chromatography with UV detection (HPLC-DAD) for M-TCS.

3.2. EXPERIMENTAL

3.2.1. CHEMICALS AND STANDARDS

All chemicals and solvents were of analytical reagent grade. Methanol (MeOH) HPLC-grade, acetonitrile (ACN) HPLC-grade, acetone, n-hexane, dichloromethane (DCM) and ammonium acetate were purchased from Carlo Erba (Milan, Italy). Sulfuric acid and ethyl acetate were obtained from Merck-Schuchardt (Hohenbrunn, Germany) and TCC, M-TCS, decane and di-*n*-hexyl ether (DHE) from Sigma Aldrich (Steinheim, Germany). TCS was obtained from Fluka (Barcelona, Spain). Sea sand, sodium hydroxide and sodium chloride (NaCl) were provided by Panreac (Barcelona, Spain). Water was purified by a MilliQ system (Millipore Iberica S.A., Barcelona, Spain).

Individual stock solutions were prepared in MeOH with a concentration of 1000 mg L^{-1} for TCS, TCC and M-TCS. Standards and calibration solutions were prepared in MeOH for the validation of the chromatographic method (in the range of mg L^{-1}). The stock solutions and standards were stored in darkness at 4 ºC.

3.2.2. SAMPLES

Samples were collected in January 2011 at Castell-Platja d'Aro, Girona, North-East Spain. This WWTP treats the sewage of a population of 165,450 and the wastewater treatment processes consist of a physical treatment with a screen and a primary clarifier, followed by a biological treatment (aerobic), a secondary clarifier and ultraviolet disinfection. Primary and biological sludge undergoes aerobic biological treatment and dewatering to produce the final biosolid which has been used in this study.

For the soil samples, sludge-amended with loamy sandy soil with 1% of organic content was collected at Mas Badia experimental agricultural centre located in la Tallada d'Empordà in North-East Spain in January 2012.

Biosolids and soils were collected in glass bottles and kept refrigerated during transportation to the laboratory, then the biosolid and soil were dried by lyophilization, sieved and frozen for storage until analysis. To develop the extraction process and to determine the recoveries, sludge and soils were spiked at 20,000 ng g^{-1} with a standard solution in methanol at the appropriate concentration inside the cell with sea sand, and the solvent was left to evaporate overnight. Surrogate standards were used for final verification of recovery results. Concentration in samples was referred to dry weight.

3.2.3. PLE

Analytes were extracted by PLE using a Dionex ASE 150 instrument (Sunnyvale, CA, USA). For organic solvent optimization, 0.5 g of dried biosolid was transferred into a 33 cm^3 stainless steel extraction cell containing 20 g of sea sand. After PLE extraction, rotary evaporation was carried out and reconstitution in 100 mL of reagent water was

applied before HF-LPME. This process was used for all the extraction solvents except for MeOH:H_2O (1:1). For optimization of other PLE parameters using MeOH as extractant, an 11 cm^3 stainless steel extraction cell containing 10 g of sea sand was used and PLE extracts were diluted to 100 mL with reagent water prior to HF-LPME without evaporation.

The final PLE method for sludge was based on the use of 0.2 g of dried biosolid which was transferred into an 11 cm^3 stainless steel extraction cell containing 10 g of sea sand. The PLE extraction conditions of TCS, TCC and M-TCS were as follows: MeOH as extraction solvent, 60 ºC as extraction temperature, 1500 psi, 3 min preheating time, 5 min static extraction time, 1 cycle, 60% of flush volume and 60 s of nitrogen purge time. The final extract volume obtained from sewage sludge was about 35 mL and the extract was transferred into a 50 mL volumetric flask with MeOH. Aliquots of 5 mL of the solutions were transferred to 100 mL volumetric flasks spiked with different concentrations of analytes for standard addition procedure prior to HF-LPME.

For sludge-amended soil, 0.5 g were transferred into an 11 cm^3 stainless steel extraction cell containing 10 g of sea sand and the extraction of the selected analytes was carried out under the same conditions as for the sludge. After, the extract was diluted to 100 mL with reagent water and the solution was submitted to HF-LPME.

3.2.4. HF-LPME CLEAN-UP/ENRICHMENT

HF-LPME was based on the method developed in Chapter 2 of this thesis. The clean-up was performed by using polypropylene membranes with a thickness of 200 µm (0.2 µm pore size) and an internal diameter of 300 µm (Azko Nobel, Wuppertal, Germany). Briefly, fibers were cut into 5 cm long pieces and one of the ends was sealed and the other was attached to a needle syringe (Hamilton, Bonaduz, Switzerland). Next, pores and lumen were filled with a mixture of DHE:decane (1:1) as organic solvent by using a 250 µL syringe (Hamilton, Bonaduz, Switzerland) and the excess of solvent was removed by immersion of the fiber in reagent water. Then, the fiber was immersed into the extract, collected in amber glass bottles with 5 g of NaCl and the solution was magnetically stirred for 24 h. After extraction, the organic solvent was withdrawn with

a syringe, evaporated under a nitrogen stream and the residue reconstituted in 0.7 mL MeOH.

3.2.5. LIQUID CHROMATOGRAPHIC ANALYSIS

For TCS and TCC, analyses were carried out by LC coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS). Chromatographic separation was performed using an Agilent Technologies 1290 Infinity series separations module with a Bin Bump SL, a degasser, ALS SL injector and a thermostated column compartment. Separation of the analytes was carried out on a Synergi Fusion-RP 100A C_{18} column (2.5 μm, 50 x 2 mm) supplied by Phenomenex (Macclesfield, United Kingdom) at 30 ºC. The mobile phase used contained MeOH as eluent A and reagent water containing 10 mmol L^{-1} ammonium acetate as eluent B. An injection volume of 5 μL was used for all the analyses. Mass spectrometry was performed on an Agilent Technologies 6430 triple-quadrupole mass spectrometer fitted with an ESI source and controlled by a Mass Hunter workstation. Analyses were performed in negative ionization mode under the following MS/MS conditions: N_2 flow rate of 10 mL min^{-1}, nebulizer pressure of 45 psi and a source temperature of 350 ºC. The optimum multiple reaction monitoring conditions obtained by automatically injecting standard solutions and comparing areas are summarized in Table 3.2.

Table 3.2. Multiple reaction monitoring conditions of MS/MS for TCS and TCC.

Analyte	*Precursor ion (m/z)*	*Product (m/z)*	*Fragmentor (V)*	*Collision energy (V)*
TCS	287	35	80	4
$^{13}C_{13}$-TCS	299	35	80	4
TCC	313	160	100	5
$^{13}C_{12}$-TCC	319	160	100	5

For M-TCS, HPLC analysis were performed using an Agilent Technologies 1200 series separations module with a Bin Bump SL, a degasser and ALS SL injector and an Agilent Technologies 1290 infinity DAD SL. Separation of the analytes was carried out on a Kinetex C_{18} column (2.6 μm, 50 x 2.1mm) supplied by Phenomenex (Macclesfield, United Kingdom). An injection volume of 5 μL was used for all the analyses. The mobile phase used contained ACN as eluent A and reagent water as eluent B at a flow rate of

0.5 mL min^{-1}. The elution started with 40% of eluent A ramped to 60% in 5 min and held for 5 min. DAD detection was carried out at 224 nm, which corresponds with the maximum absorbance of M-TCS.

3.3. RESULTS AND DISCUSSION

3.3.1. LIQUID CHROMATOGRAPHIC ANALYSIS

For LC-MS analysis only TCS and TCC could be measured because M-TCS is too lipophilic for efficient ionization using either ESI or APCI sources [24]. Different mobile phases were tested: 0.1% formic acid and 10 mM ammonium acetate. When formic acid was used less sensitivity was observed for TCS whereas for ammonium acetate higher signals were observed. González-Mariño et al. [33] observed the same effect; this was related to TCS pKa because when an acidic modifier was added the negative form of TCS decreases and signal was lower. On the other hand, Chu et al. [23] reported that when 10 mM ammonium acetate was used ion suppression was increased. In order to obtain reproducible retention times 10 mM ammonium acetate was selected.

Figures of merit for the chromatographic method are shown in Table 3.3, instrumental limit of detection (ILOD) and instrumental limit of quantitation (ILOQ) were calculated as 3 and 10 times the signal-to-noise ratios, respectively. ILODs were 0.7, 1 and 1 µg L^{-1} for TCC, TCS and M-TCS. The precision was evaluated by the intra-day and inter-day precision with five repeated injections of 0.5 mg L^{-1} in MeOH during the same day and on five successive days, respectively. Values were lower than 10%.

Table 3.3. ILODs, ILOQs, intra-day precision, inter-day precision, coefficients of determination (r^2) and range studied of LC-MS method for TCS and TCC, and HPLC-DAD for M-TCS (N=5).

Analyte	*ILOD (µg L^{-1})*	*ILOQ (µg L^{-1})*	*Intra-day precision (%)*	*Inter-day precision (%)*	*r^2*	*Range (mg L^{-1})*
TCS	1	3.3	5.4	9.6	0.991	ILOQ–5
TCC	0.7	2.3	1.3	9.4	0.990	ILOQ–5
M-TCS	1	3.3	1.4	1.9	0.999	ILOQ-10

3.3.2. PLE DEVELOPMENT

Starting conditions for the PLE method development were: a pressure of 1500 psi, a purge time of 60 s, a static time of 5 min, a temperature of 60 ºC, 1 cycle, a flush volume of 60% and 0.5 g of biosolid. In order to compare the results from different experiments, apparent recoveries which correspond to the concentrations determined in spiked samples without subtracting the signal of the analyte of non-spiked samples were used.

3.3.2.1. Organic solvent

There have been several studies evaluating the effect of extracting solvents on the recovery of biocides. The extraction of TCS, TCC and M-TCS was evaluated by dichloromethane, acetone, MeOH:acetone (1:1) and MeOH and, after the PLE extraction, rotary evaporation to dryness was carried out, except for MeOH:H_2O (1:1). As can be seen in Figure 3.1, low apparent recoveries were obtained for TCC and M-TCS by all organic solvents. For TCS, better recoveries were obtained with MeOH:H_20, but for the rest of the analytes recoveries were lower; this was anticipated because TCC and M-TCS are more hydrophobic and the mixture containing water is the most polar. These results differ from the ones obtained by Chu et al. [23]. In their study DCM at 60 ºC was used as the extracting solvent and recoveries were 79.7 and 110.5% for TCS and TCC; however our results did not show the same efficiency. Nieto et al. [21] developed a method for the determination of TCS and TCC among others by PLE and LC-MS/MS. Different solvents were evaluated, and for TCS recoveries of about 100% were obtained when MeOH, ACN or a mixture of MeOH:DCM (1:1) were used. For TCC, maximum recoveries were obtained when MeOH and ACN were used, with values of 54%.

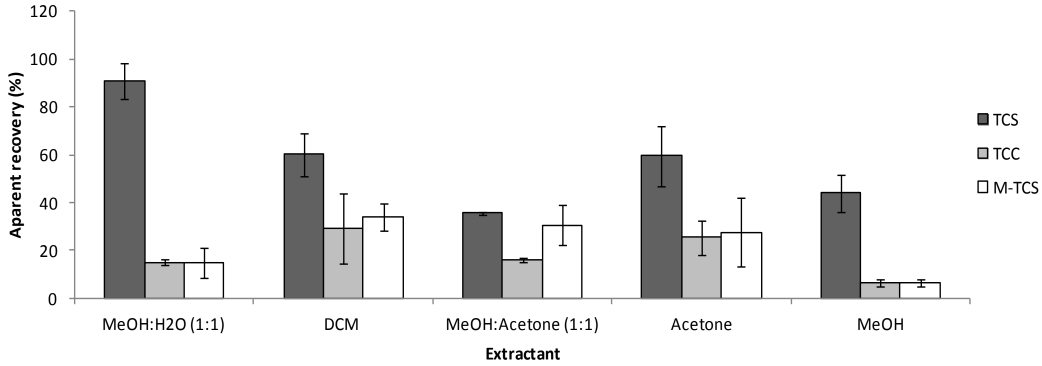

Figure 3.1. Effect of extracting solvents on the PLE and HF-LPME method for spiked sludge. PLE conditions: MeOH as extractant, 60 ºC temperature, 1 cycle and a flush volume of 60% (N=3).

The contradictory results lead us to suspect that rotary evaporation could affect analyte recoveries. To evaluate this step, MeOH spiked at 10 µg L^{-1} was subjected to rotary evaporation and the HF-LPME procedure. MeOH was selected because when partial or no evaporation was applied, a percentage of organic solvent was present in the donor phase of the HF-LPME system and MeOH is miscible with water.

Figure 3.2 shows the effect when no evaporation was applied, when 90% of the solvent was evaporated and when the extract was dried. As can be seen, apparent recovery was higher for all analytes when no evaporation was applied, with values between 45 and 110%. Low recoveries can be explained by the formation of colloidal fraction water-insoluble when reconstitution with reagent water was applied and TCS and TCC tend to interact with organic matter or fat from sludge which can retain the analytes [27,34]. Our results are consistent with Langford et al. [18] who also observed an insoluble fraction when reagent water was added to the evaporated extract and a centrifugation step was required prior to clean-up. In addition, the low solubility of the analytes in water makes the reconstitution of the analytes after evaporation difficult [11]. Due to all these factors, the rotary evaporation step was omitted, MeOH was chosen as extracting solvent and 11 mL extraction cells were used in order to reduce the percentage of MeOH in subsequent experiments.

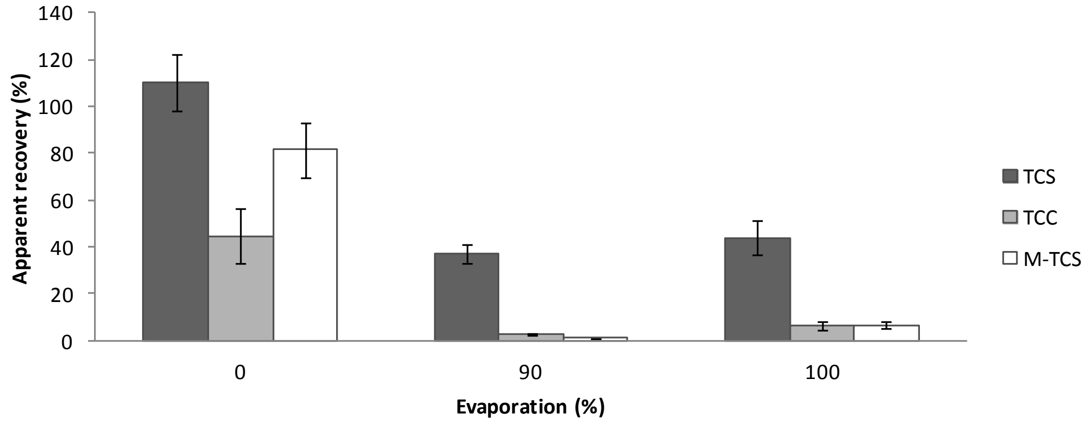

Figure 3.2. Effect of evaporation on the PLE and HF-LPME method for spiked sludge. PLE conditions: MeOH as extractant, 60 ºC temperature, 1 cycle and a flush volume of 60% (N=3).

3.3.2.2. Temperature

Temperature is an important parameter in PLE; high temperatures increase the extraction because the diffusivity of the analytes is enhanced. Three temperatures were tested: 60, 80 and 100 ºC (Figure 3.3). It was observed that when the temperature increased the recovery decreased and higher recoveries were obtained with the lowest temperature (60 ºC). Barron et al. [22] evaluated the effect of temperature in the extraction of TCS and reported a decrease in the extraction for higher temperatures. This can be due to degradation of the analytes or to extraction of matrix components which can interfere in the subsequent clean-up step. Nieto et al. [21] observed no effect of the temperature for TCS, whereas for TCC at 100 ºC recoveries were 77% and at 125 ºC were 65% when using an extraction time of 5 min. Therefore, 60 ºC was used as the optimum temperature in subsequent experiments.

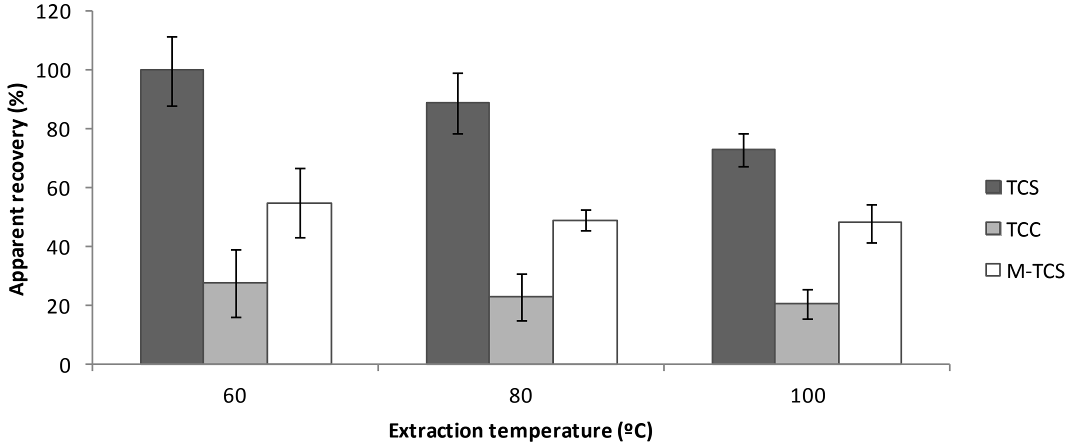

Figure 3.3. Effect of temperature on the PLE and HF-LPME method for spiked sludge. PLE conditions: MeOH as extractant, 1 cycle and a flush volume of 60% (N=3).

3.3.2.3. Number of cycles and flush volume

The extraction of TCS, TCC and M-TCS under 60 ºC and MeOH was tested over 1, 2 and 3 cycles and the best recoveries were achieved with one cycle. When more than one cycle is used fresh solvent is introduced into the sample, therefore more substances can be extracted and have a negative influence on the clean-up of the extract. Finally, when the flush volume was tested with 30, 60 and 90%, better apparent recoveries were obtained with 60%. Therefore, 1 cycle and 60% of flush volume were chosen as optimum values.

3.3.3. HF-LPME CLEAN-UP/ENRICHMENT

3.3.3.1. Effect of methanol in HF-LPME

The HF-LPME method applied for the determination of TCS, TCC and M-TCS is based on the study presented in Chapter 2 of this thesis with some modifications. The most important parameter modified was the donor volume; in this study a volume of 100 mL was applied instead of 500 mL. Taking into account that the volume was reduced five times, the amount of NaCl added to the donor phase was also decreased five times. The final conditions for the clean-up and preconcentration step of TCS, TCC and M-TCS in sludge extracts were: DHE:decane (1:1) as the organic solvent, 24 h extraction time, 5 cm fiber length, 100 mL of donor volume and 5 g of NaCl.

In the PLE process MeOH was selected as the extractant solvent and no evaporation was applied, thus forcing the presence of MeOH in the donor phase of the HF-LPME system. For this reason, the possible decrease of the extraction efficiency due to the presence of MeOH in the donor phase was evaluated. As can be seen in Figure 3.4, a significant negative effect for a MeOH content lower than 15% in the donor phase of extraction performance was not observed. When more than 15% was added an important decrease of the concentration in the acceptor phase was noted. Although the presence of MeOH decreases the efficiency of the extraction, calibration curves could be obtained when up to 35% of MeOH was used with coefficients of determination higher than 0.993 in the 1 – 50 μg L^{-1} range.

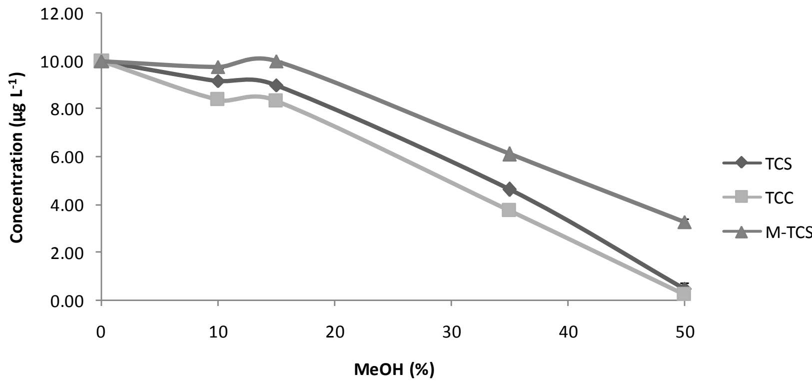

Figure 3.4. Effect of the presence of MeOH in the sample solution of the HF-LPME in reagent water with different percentages of MeOH spiked at 10 μg L^{-1} (N=3).

3.3.3.2. Donor phase pH

The influence of the pH on the interaction of the analytes with the organic matter was tested by modifying the pH of the donor phase obtained from the PLE extracts. The extracts diluted with reagent water were acidified at pH 2 with sulfuric acid, not adjusted (pH 6) and basified at pH 10 with 5 M sodium hydroxide (Figure 3.5). At low pH the analytes seemed to interact more with the matrix and lower recoveries were observed; at high pH more interferences were extracted as was observed in the HPLC-DAD analysis. Moreover, at pH 10 the recovery of TCS was lower because it is charged and is less soluble in the organic phase (DHE:decane, 1:1); whereas for TCC similar results were obtained at pH 10 and neutral pH. Hence, no adjustment of the pH was carried out.

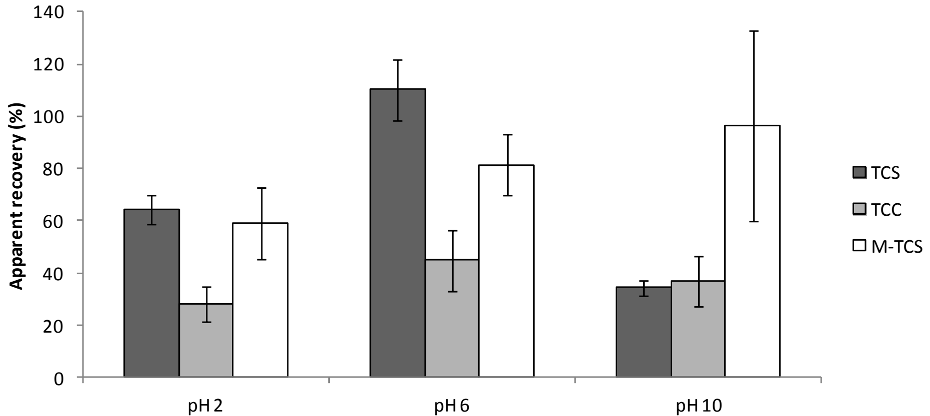

Figure 3.5. Effect of donor pH on HF-LPME for spiked sludge (N=3).

3.3.3.3.Sample size

Low apparent recoveries were obtained for biosolids, especially for TCC and for this reason matrix effects were evaluated. When determination is accomplished by LC-MS/MS, ion suppression can be observed due to matrix effects. In order to compensate for this problem, internal standards or the standard addition method can be used. In our study, standard addition prior to HF-LPME was carried out. After diluting the PLE extract to 50 mL, 5 mL were taken and diluted to 100 mL and four solutions were spiked at 4 levels (0, 10, 20 and 30 µg L^{-1}).

Co-extracted organic interferences from the matrix can also have a negative effect on the clean-up and enrichment step due to the interaction of the analytes with organic matter from the PLE extract. High sample sizes could decrease LODs for analytes but could also cause more matrix effects. Biosolids have a high content of organic matter, for this reason two sample amounts were evaluated (0.2 and 0.5 g). To calculate these recoveries, the signal of the non-spiked sample was subtracted. As can be seen in Figure 3.6, lower recoveries were obtained for 0.5 g; a higher content of sludge colloidal fraction appeared when the extract was mixed with reagent water whereas, with 0.2 g, this colloidal fraction was not observed. Based on the results obtained, 0.2 g was chosen as the optimum amount of biosolid. This is consistent with previous studies: Samaras et al. [27] tested three sample sizes (0.5, 1 and 3 g) and for 3 g lower TCS recoveries and worse peak shapes were obtained while for 0.5 and 1 g no differences were observed. Yu et al. [28] observed that, for TCS, when 0.1 and 0.7 g

were used recoveries were lower than 60% whereas, with 0.04 g, better results were achieved. Figure 3.7 shows the chromatograms obtained by LC-MS/MS for TCS and TCC and HPLC-DAD for M-TCS for 0.2 g of biosolids spiked at 20,000 ng g^{-1}.

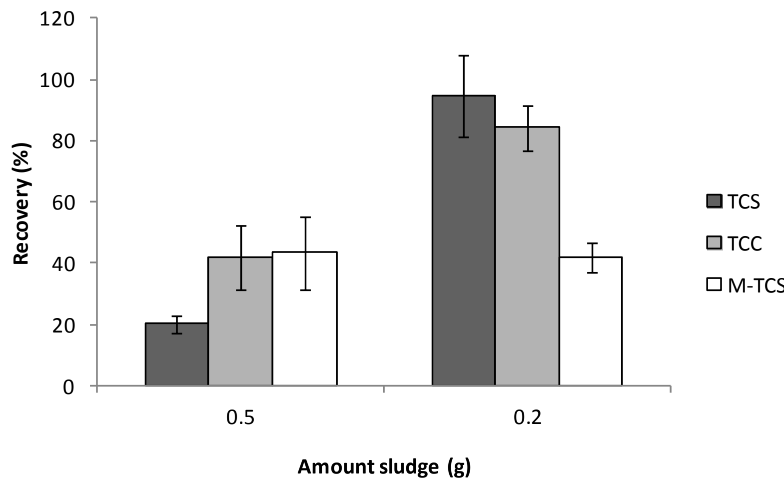

Figure 3.6. Effect of sample size on spiked sludge. PLE conditions: MeOH as extractant, 60 ºC, 1 cycle and a flush volume of 60% (N=3).

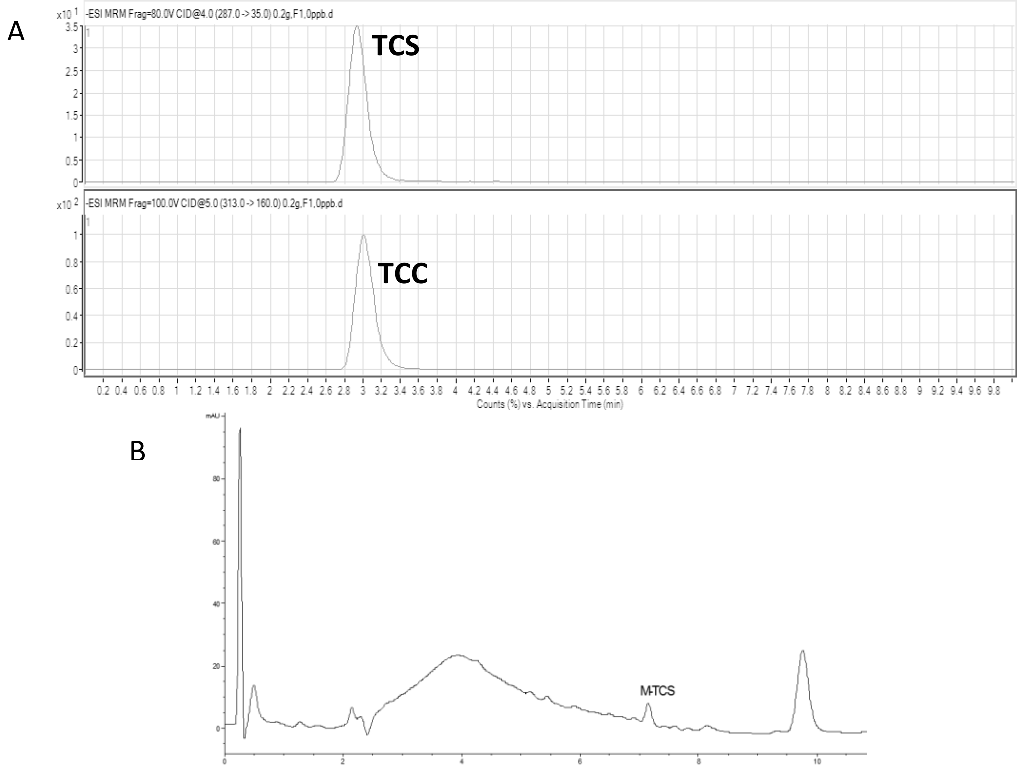

Figure 3.7. Chromatograms obtained by LC-MS/MS (A) and HPLC-DAD (B) from 0.2 g of biosolids spiked at 20,000 ng g^{-1}.

3.3.4. ANALYTICAL PERFORMANCE

Recoveries and precision of the method for biosolids with a high organic matter content were evaluated with spiked sludge under optimum PLE conditions (Table 3.4). Coefficients of determination of standard addition curves were higher than 0.997. Recovery yields were 84% and 95% for TCS and TCC, respectively. These values were verified with isotopic labeled standards ($^{13}C_{13}$-TCS and $^{13}C_{12}$-TCC) giving similar recovery values (91% for both compounds). For M-TCS, which was determined by HPLC-DAD, lower recoveries (42%) were obtained. Method limits of detection and quantitation were calculated by taking into account instrumental limits of detection, enrichment factors and the consecutive dilutions applied to the PLE extract. MLODs were 34, 29 and 51 ng g^{-1} for TCS, TCC and M-TCS, respectively. Inter-day precision was calculated on three different days and relative standard deviation values were between 8 and 15%. In non-spiked biosolids, TCS and TCC were determined at high concentrations with values of 4,361 and 4,764 ng g^{-1}, respectively.

Table 3.4. Recovery (%), method inter-day precision as relative standard deviations for biosolid spiked at 20,000 ng g^{-1} and, MLODs, MLODs and enrichment factor of HF-LPME system (N=3).

Analyte	*Recovery (%)*	*Inter-day precision (%)*	*MLOD (ng g^{-1})*	*MLOQ (ng g^{-1})*	*Ee*
TCS **$^{13}C_{13}$-TCS**	95 ± 14 91 ± 10	14.6	34	113	146
TCC **$^{13}C_{12}$-TCC**	84 ± 7 91 ± 5	8.0	29	98	119
M-TCS	42 ± 4	9.4	51	172	97

Initially, the procedure described was designed to deal with sludge samples but due to the low organic content of soils, a simpler method was applied to sludge-amended soil. The same PLE conditions were applied to 0.5 g of soil after PLE and the extract was diluted to 100 mL with reagent water and submitted to HF-LPME. Under these conditions, the recoveries obtained in soil ranged from 80 to 97% in sludge-amended soil for TCS, TCC and M-TCS. These values are similar to the surrogate standard recoveries; for $^{13}C_{13}$-TCS and $^{13}C_{12}$-TCC they were 102 and 84% (Table 3.5). MLODs and MLOQs are presented in Table 3.5. MLODs were between 1.2 and 2.1 ng g^{-1} and the

inter-day precision was lower than 11%. In the light of the results, methods could be applied to real samples. In sludge-amended soil, no analytes were detected.

Table 3.5. Recovery (%), method inter-day precision as recoveries standard deviations for sludge amended soil spiked at 20,000 ng g^{-1} and, MLODs and MLODS (N=3).

Analyte	Recovery (%)	Inter-day precision (%)	MLOD (ng g^{-1})	MLOQ (ng g^{-1})
TCS **$^{13}C_{13}$-TCS**	97 ± 10 102 ± 3	10.8	1.3	4.3
TCC **$^{13}C_{12}$-TCC**	80 ± 8 84 ± 3	10.6	1.2	4
M-TCS	85.2 ± 0.5	0.6	2.1	7

3.3.5. APPLICATION TO REAL SAMPLES

The method developed was applied to the determination of TCS, TCC and M-TCS in treated sludge and sludge-amended soil (Table 3.6). These high concentrations in biosolids are in line with their high consumption and their $LogK_{ow,}$ which indicates high partitioning. However, the methylated degradation product of TCS (M-TCS) was detected in sludge samples below its MLOQ.

Table 3.6. Concentration (ng g^{-1}) of the personal care products in solid samples.

Analyte	***Concentration (ng g^{-1})***	
	Sludge	***Sludge amended soil***
TCS	4,361	n.d.
TCC	4,764	n.d.
M-TCS	<MLOQ	n.d.

A wide range of concentrations have been reported in biosolids. High concentrations for TCS have been determined by Barron et al. [22], Samaras et al. [27] or Kinney et al. [20], with values of 24,600 ng g^{-1}, 17,900 ng g^{-1} and 10,500 ng g^{-1}, respectively. Lozano et al. [4] also calculated high concentrations of between 12,100 and 18,800 ng g^{-1} for TCS; no difference between seasons was observed, but high concentrations from 2005/06 to 2007 were observed. Furthermore, McClellan et al. [2] evaluated the presence of different pharmaceuticals and personal care products in biosolids,

observing that the most abundant compounds were TCS and TCC with mean concentrations of 12,600 and 36,000 ng g^{-1}, respectively.

Yu et al. [28] and Singer et al. [16] quantified lower TCS concentrations with values of between 272 and 1,965 ng g^{-1}, and Bester et al. [6] of between 400 and 8,800 ng g^{-1}. Nieto et al. [21] also observed similar concentrations in three different sewage sludges with concentrations in 1,300 – 1,490 ng g^{-1} range for TCS; whereas Cha et al. [1] measured concentrations from 90 to 7,060 ng g^{-1} for TCS and from 4,890 to 9,280 ng g^{-1} for TCC in biosolids. Kumar et al. [9] quantified concentrations for TCS and TCC of between 515 and 1,611 ng g^{-1}, and 466 and 1,425 ng g^{-1}, respectively. Chu et al. [23] observed concentrations in the range of 620 - 11,550 ng g^{-1} for TCS and 2,170-5,490 ng g^{-1} for TCC in sludge and biosolids in Canada. This difference can be related to the sludge treatment: aerobic and anaerobic digesters.

Under anaerobic conditions, Heidler et al. [8] obtained concentrations for TCS in the range of 20,000 - 50,000 ng g^{-1}. Ying et al. [5] reported TCS concentrations ranging from 90 to 16,790 ng g^{-1} in nineteen biosolids from Australia with mean concentrations of 5,580 ng g^{-1}, thus observing that aerobic digestion is more effective for the elimination of TCS. This is in agreement with McAvoy et al. [14] who reported TCS concentrations of between 530 and 1,500 ng g^{-1} under aerobic digestion and 15,600 ng g^{-1} for anaerobic digestion. Heidler et al. [7], when comparing different types of sludge for the elimination of TCS, obtained lower concentrations in aerobic sludge: 410 - 46,000, 580 - 610 and 1,100 - 17,500 ng g^{-1} in anaerobically, aerobically and undigested sludge, respectively. Levels of TCC were also studied, giving the following ranges: 4,700 - 63,000, 16,400 - 19,600 and 21,600 - 43,200 ng g^{-1} in anaerobically, aerobically and undigested sludge, respectively [7]. These results are consistent with the low degradation of TCC. In our study, an aerobic digester was applied, therefore, concentrations are consistent with those reported.

Finally, González-Mariño et al. [24] studied TCS and its methylated product and reported concentrations in the range of 460 – 2,640 ng g^{-1} for TCS and 51 - 191 ng g^{-1} for M-TCS in secondary sludge, and 345 – 2,620 ng g^{-1} and 37 – 115 ng g^{-1} for TCS and

M-TCS in primary sludge. In final sewage, concentrations were low: 272 ng g^{-1} and 15 ng g^{-1}. Sánchez-Brunete et al. [35] reported concentrations from 4 to 311 ng g^{-1} for M-TCS and 54 – 2,987 ng g^{-1} for TCS in sludge from Madrid and in sludge-amended soil values were 1 ng g^{-1} for both compounds. Cha et al. [1] also analyzed sludge-amended soil with average concentrations of 3.6 and 11.6 ng g^{-1} for TCC, and 0.39 and 0.16 ng g^{-1} for TCS.

3.4. CONCLUSIONS

Two methods for the determination of triclosan, triclocarban and methyl-triclosan in sewage and sludge-amended soil have been developed. The method was based on the use of PLE followed by two-phase HF-LPME combined with LC-ESI-MS/MS for TCS and TCC, and HPLC-DAD for M-TCS. A simple method using 0.5 g was used for soil with a low organic content, whereas standard addition was necessary for quantifying the selected analytes in 0.2 g of biosolids. Recoveries were higher than 80% for all the analytes and all sample matrices, except for M-TCS in sludge with recoveries of 42%. MLODs were 34, 29 and 51 ng g^{-1} in biosolids and 6, 1.5 and 23 ng g^{-1} for TCS, TCC and M-TCS, respectively. Methods were applied successfully to biosolids and sludge-amended soil. The three analytes were quantified in biosolids whereas they were not detected in sludge-amended soil.

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CHAPTER 4:

Determination of acidic pharmaceuticals in water samples by hollow fiber liquid-phase microextraction prior to liquid chromatography and evaluation of their removal in a tertiary treatment

4.1. INTRODUCTION

In recent years, the occurrence of organic pollutants in the environment has become an issue of major concern [1–4]. Recent studies have shown that pharmaceuticals are inadequately removed at wastewater treatment plants (WWTPs) as their elimination was not a primary objective in their design [5–9]. Acidic pharmaceuticals such as some non-steroidal anti-inflammatory drugs (naproxen, diclofenac and ibuprofen) and clofibric acid; neutral pharmaceuticals such as carbamazepine and phenolic endocrine disruptors such as triclosan, estradiol, bisphenol A and 4-nonylphenol (NP) are among the compounds detected in influent and effluent wastewater at trace levels [9–20]. Moreover, estradiol has been included as a priority drinking water contaminant and estradiol (EST), diclofenac (DCF) and ibuprofen (IBP) were proposed as priority pollutants for the revision of the EU water framework directive (2000). Gómez et al. [21] studied the elimination of carbamazepine (CRB), diclofenac, bisphenol A (BPA), triclosan (TCS) and ibuprofen in WWTP with removal values of 20, 59, 81, 88 and 95% and determined concentration ranges of 0.12 - 0.31, 0.2 - 3.6, 0.72 - 3.4, 0.39 - 4.2 and 34 - 168 μg L^{-1} in influent, and 0.11 - 0.23, 0.14 - 2.2, 0.14 - 0.98, 0.08 - 0.4 and 0.24 - 28 μg L^{-1} in effluent wastewater, respectively. As a consequence, wastewater discharges are one of the most important sources of spreading pharmaceuticals into the environment. Therefore, it is interesting to develop improved methodologies for studying the occurrence and fate of organic pollutants in wastewater and to investigate the efficiency of disinfectant treatments for the removal of pharmaceuticals in WWTPs [6,16].

In this study, a method for the determination of pharmaceuticals in several aqueous matrices has been developed. This method has been applied for the evaluation of the removal of the selected analytes by different disinfectant agents in laboratory studies and in a WWTP.

4.1.1. DETERMINATION OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS

To date, several extraction procedures followed by GC or LC have been developed for the determination of phenolic endocrine disruptors and acidic pharmaceuticals in aqueous samples [8,16,21–23].

The most common technique for the enrichment and clean-up of the analytes is solid-phase extraction (SPE) because of the wide range of sorbents available and its high extraction efficiency. Different sorbents have been used for the determination of pharmaceuticals and personal care products. Strata X combined with LC-MS/MS has been applied for the determination of several pharmaceuticals including clofibric acid (CLF), IBP and naproxen (NPX) with recoveries of higher than 73% [24]. Another type of sorbent is Supelco LC-18 which has been used for the quantitation of acidic pharmaceuticals by SPE combined with GC-MS following derivatization (methylation) [25].

However, Oasis HLB is the preferred sorbent because it can extract both polar and hydrophobic compounds. Santos et al. [26] applied Oasis HLB combined with HPLC using both UV and fluorescence detectors for the quantitation of acidic (DCF, IBP and NPX) and neutral compounds (CRB). In their study, the effect of sample pH was evaluated, obtaining similar results at neutral pH and at pH 2 with recoveries of 78, 89 and 98% for DCF, IBP and NPX, respectively. Method limits of detection (MLODs) were 0.28, 0.25 and 0.02 μg L^{-1} in influent and 0.14, 0.12 and 0.01 μg L^{-1} in effluent wastewater for DCF, IBP and NPX, respectively. High enrichment factors (Ee) of 1,000 and 2,000 were obtained for influent and effluent wastewater, respectively. This method was applied to study the occurrence of NSAIDs in four WWTPs in Spain with higher concentration levels for IBP; with maximum levels of 604 μg L^{-1} in influent and 55 μg L^{-1} in effluent wastewater. Lower values were determined for DCF; in all samples concentrations were below LOD [27].

Gracia-Lor et al. [28] developed a multi-residue method for determining 66 pharmaceuticals including DCF, IBP and NPX using SPE combined with LC-MS/MS. In their method Oasis HLB and Oasis MCX were compared at pH 2 resulting in lower

recoveries for the cation exchanger sorbent. Moreover, with Oasis HLB different pHs were evaluated, obtaining better results at pH 7. Recoveries of 97, 84 and 120% were obtained in influent wastewater, and method limits of quantitation (MLOQs) were 30, 53 and 247 ng L^{-1} for NPX, DCF and IBP, respectively. For influent wastewater, similar recoveries were obtained but high MLOQs were observed with values of 49, 137 and 642 ng L^{-1} for NPX, DCF and IBP. The maximum concentration level was 39.8 µg L^{-1} for IBP in influent wastewater whereas NPX and DCF had similar concentrations levels (1.49 and 3.58 µg L^{-1}). In the effluent, minimum concentration was below MLOQ for IBP and for NPX and DCF concentrations were 0.74 and 0.72 µg L^{-1}, respectively. Camacho et al. [29] also compared both cartridges for EST, CLF, NPX, DCF and IBP with better results with Oasis HLB and acidifying the sample at pH 2. In their study several elution solvents were tested (acetone, ethyl acetate and methanol), higher recoveries were obtained when ethyl acetate was used (>79% in influent and effluent wastewater, and >88% in surface water).

Oasis HLB was also used in combination with GC-MS for the determination of non-steroidal anti-inflammatory drugs (NSAIDs) after derivatization. Oëllers et al. [30] developed an SPE-GC-MS method for the determination of neutral and acidic pharmaceuticals including NSAIDs, CLF and CRB using diazomethane as derivatizing agent with recoveries of about 50% for CRB and around 90% for the acidic compounds. MLODs were in the 0.3 to 4.5 ng L^{-1} range. Sebök et al. [31] also applied SPE-GC-MS and compared different derivatization agents, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and hexamethyldisilazane catalyzed by trifluoroacetic acid. The same efficiency was observed for the three agents with recoveries of higher than 84%, with hexamethyldisilazane being the most cost-effective. Gómez et al. [21] applied the same techniques and recovered more than 78% of the target compounds with MLODs of 23, 17, 100, 7 and 30 ng L^{-1} for IBP, TCS, DCF, BPA and CRB, respectively.

Other multi-residue methods for the determination of the selected analytes by SPE and GC-MS have been developed [32,33]. Lee et al. [32] used Oasis MAX and a

sequential elution with methanol (for phenols) and 2% formic acid in methanol (for acidic pharmaceuticals). The derivatization of phenols and acidic pharmaceuticals was performed with pentafluoropropionic acid anhydride and *N*-*t*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% tertiary-butyldimethylsilyl chloride, respectively. Although the recoveries were high (75 -115%) and the limits of detection were 0.1 μg L^{-1} for NP, 0.001 μg L^{-1} for EST and 0.01 μg L^{-1} for other compounds, the method is time consuming and needs high manipulation.

Another extraction technique applied is stir bar sorptive extraction (SBSE). A multi-residue method based on SBSE with gas chromatography has been developed for the determination of IBP, DCF, NPX, CLF, NP, BPA, TCS and CRB among other compounds [23]. The influence in the extraction process of different parameters, such as sample volume and pH were evaluated. Another factor was salt addition; an increase of extraction efficiency for hydrophilic compounds was achieved when ionic strength increased except for 4-nonylphenol for which a negative effect was observed. MLODs were in the 1 - 228 ng L^{-1} range with extraction efficiencies of lower than 10% for BPA and CRB, of 107% for IBP, 21% for CLF, 31% for NPX, 53% for TCS and 89% for DCF. Using internal standards the recoveries were higher than 70% for all the compounds except for NP for which an internal standard was not available. Solid bar microextraction technique which is based on the use of a piece of hollow fiber filled with sorbent material (LiChrosorb RP-8) followed by MeOH elution and HPLC-UV have been applied for the determination of CRB, DCF and IBP. However, low recoveries (< 50%) and high LODs (0.7 and 0.9 μg L^{-1}) were achieved [34].

As explained in Chapter 1, an alternative technique for the extraction of organic pollutants in water is hollow fiber liquid-phase microextraction (HF-LPME) and several authors have reported the use of this technique for the determination of acidic organic pollutants. Payán et al. [35] applied a three-phase HF-LPME with HPLC-MS/MS for the determination of salicylic acid, ibuprofen and diclofenac in wastewaters. Using the following conditions: sample pH of 2, di-*n*-hexyl ether as the organic solvent and an alkaline acceptor solution at pH 12.5, method limits of detection were 0.5 μg L^{-1}.

In the method developed by Quintana et al. [36] for determining acidic pharmaceuticals in wastewater, including CLF, NPX, IBP and DCF; 1-octanol was selected as the organic solvent and they optimized different parameters such as acceptor solution and sample volume, pH and ionic strength. No positive effect when increasing ionic strength was observed. Equilibrium time was achieved after 45 minutes with a sample volume of 22 mL; enrichment factors were between 70 and 234. Moreover, they observed that filtration of the sample was required prior to the analysis. MLOQs were 0.5, 10, 14 and 25 ng L^{-1} for CLF, NPX, IBP and DCF, respectively.

Larsson et al. [37] applied a continuous flow HF-LPME procedure for the determination of four NSAIDs using di-*n*-hexyl ether (DHE) as the organic solvent and ammonium carbonate at pH 8.9 as the acceptor phase. With a sample volume of 1 L, enrichment factors between 720 - 940 were achieved and MLODs were 0.01 and 0.05 µg L^{-1}.

For BPA, a preconcentration method based on three-phase liquid membrane extraction was developed by Liu et al. [38]. In their method, BPA was first extracted by dichloromethane and the analyte was back-extracted to a phosphate solution at pH 12.

Two-phase HF-LPME has also been applied for the determination of some analytes studied in this chapter. Estradiol has been determined from aqueous samples by two-phase HF-LPME followed by GC-MS [39] using DHE containing 10% of TOPO with high enrichment factors (about 3,400). Furthermore, BPA and NP were determined in reservoir and drinking water by two-phase HF-LPME with recoveries of higher than 88% and enrichment factors of 148 – 105 [40].

Dynamic two-phase HF-LPME followed by GC-MS has also been applied for the determination of CLF, NPX and IBP in wastewater with recoveries of higher than 97% and MLODs from 10 to 20 ng L^{-1} [41]. Lower MLODs were obtained with an in-situ derivatization continuous two-phase HF-LPME combined with GC-FID for the determination of NPX and IBP with values of 2 and 1 ng L^{-1}, respectively. In this method, the acceptor phase contained *n*-octanol and tetrabutylammonium sulfate as the ion-pair derivatization agent [42]. In both methods, similar enrichment factors were obtained with values up to 272.

In this study, three-phase HF-LPME was chosen as the extraction and clean-up technique for the determination of the selected analytes present in environmental waters in order to improve the sensitivity.

Transport mechanism of three-phase HF-LPME for acidic compounds

Taking into account the polar and acidic character of the analytes, a pH gradient between the acceptor phase and the donor phase has been applied. In the acidic sample phase the analytes are protonated and they can pass through the membrane to the organic phase in uncharged form. Finally, they can be re-extracted in a basic acceptor aqueous phase because the analytes are trapped as ions (Figure 4.1). As the analytes end up in an aqueous solution, LC can be applied for the analysis.

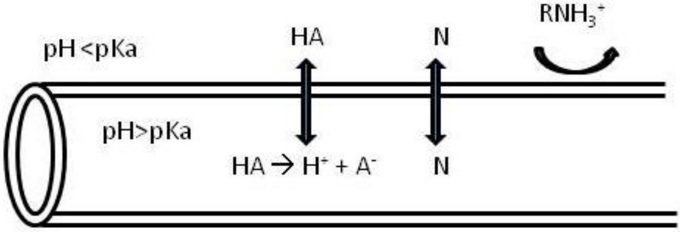

Figure 4.1. Diagram of the HF-LPME mechanism for acid compounds.

4.1.2. NSAIDs IN THE WASTEWATER TREATMENT PROCESS

Wastewater treatment in sewage plants is designed for the elimination of organic matter and microorganisms. Mechanical and biological treatments are commonly used followed or not by tertiary treatments such as chlorine, polishing ponds or UV radiation. Different removal efficiencies have been reported for the selected analytes in wastewater treatment plants (WWTPs). DCF has been found at levels of 2.51 μg L^{-1} with a removal of 17%, whereas, in other studies, removal rates of 69% have been achieved [1]. Removal efficiencies for CLF and NSAIDs are in 8 - 90% range; this wide variation in recoveries can be explained by the influence of several factors such as water pH and TOC, and compound properties such as pKa, adsorption coefficient and photolability [8,9,12,15,27].

Our study has focused on the effect of UV radiation, peracetic acid and chlorination which are used in WWTPs as disinfectants in the removal of the selected compounds. Due to its capacity to deactivate bacteria, viruses and protozoan cysts, chlorine disinfection is commonly used around the world in the tertiary treatment of urban wastewaters to safeguard human health by preventing the spread of pathogens into the environment [43,44]. However, chlorine and its compounds react with the organic matter present in wastewater to form toxic disinfection by-products (DBPs) such as trihalomethanes or haloacetics with mutagenic and/or carcinogenic activity, which are potentially harmful to humans and aquatic organisms. Consequently, the toxicity of the effluent to be either discharged or reused is increased [44–48]. For this reason, it is highly desirable to find alternative disinfectants to chlorine in order to be effective against microbial contamination of wastewater without generating DBPs.

Peracetic acid or peroxyacetic acid (PAA) is a strong oxidant and disinfectant which has greater oxidation potential than chlorine or chlorine dioxide [49,50]. Furthermore, it has been found to be effective against bacteria and viruses present in urban wastewater and not to generate toxic degradation products. Recent studies evaluating the formation of DBPs have not found significant amounts of genotoxic DBP when moderate doses of PAA are used [44,48,51–54]. However, a few studies suggest that DBPs are formed from PAA but that the quantity is smaller and the spectrum less broad than when chlorine and ozone are used. Moreover, PAA offers the advantages of low cost, simple operation and ease of start-up [55]. Commercially available PAA contains acetic acid (CH_3COOH), hydrogen peroxide (H_2O_2), peracetic acid and water [50,56,57]. However, although several studies have been carried out to investigate disinfection using PAA, few have evaluated its capacity to degrade selected pharmaceuticals.

Other oxidant agents such as ferrate, ozone or hydroxide peroxide have been proposed as alternatives to chlorine for disinfection in WWTPs. Lee et al. [58,59] evaluated the degradation of several pharmaceuticals by chlorine, ferrate^{IV} and ozone, observing a low removal of IBP when ozone and hydroxyl radicals were applied and no

removal for the other treatments. Therefore, it seems that degradation of IBP is only achieved in the presence of hydroxyl radicals. Ozone has also been reported to be more effective than ferrate^{IV} for the removal of EST, BPA, TCS, CRB and DCF; however ozone was less stable and the elimination when using both treatments was comparable.

On the other hand, UV disinfection is an important physical procedure that efficiently eliminates enteric bacteria, viruses, bacterial spores and parasitic cysts and avoids the production of DBPs and other chemical residues. Several studies investigating the photodegradation of pharmaceuticals have found that UV treatments are ineffective for a variety of pharmaceuticals in water. However, other studies have reported more encouraging results. Kim et al. [10] showed that a UV process was able to degrade some compounds, such as NPX and DCF, up to 40% and 90% in ten minutes. Canonica et al. [60] have evaluated the extent of photodegradation of four pharmaceuticals in a UV disinfection treatment for drinking water which gave a 27% reduction of diclofenac. UV combined with H_2O_2 was found to effectively degrade carbamazepine whereas using UV alone did not [61]. A slight increase in the removal rate for NPX has been reported when UV radiation is combined with an oxidant [62,63]. The results obtained in these studies suggest that it is interesting to investigate a synergic effect on combining the application of PAA/UV radiation in the treatment of wastewater.

The objectives of the present study were: 1) To develop a sensitive and selective method using three-phase HF-LPME followed by HPLC-DAD for the determination of NPX, DCF, IBP, CLF, CRB, TCS, EST, BPA and NP in environmental waters and drinking water and to compare its performance with SPE; 2) To test the efficiency of disinfection reagents (PAA, NaClO and UV radiation) in the removal of pharmaceuticals from reagent water and wastewater in the laboratory and to evaluate the efficiency of a disinfection system consisting of an oxidant (PAA) and UV radiation in a WWTP for the elimination of pharmaceutical compounds.

4.2. EXPERIMENTAL

4.2.1. CHEMICALS AND STANDARDS

All the chemicals and solvents were of analytical reagent grade. Methanol (MeOH) HPLC-grade, acetonitrile (ACN) HPLC-grade, *n*-hexane and acetone were obtained from Carlo Erba (Milan, Italy). Dodecanol, cumene, ethyl acetate and sulfuric acid were obtained from Merck-Schuchardt (Hohenbrunn, Germany) and CLF, CRB, NPX, DCF, IBP, EST, NP, di-*n*-hexyl ether (DHE), decane, *n*-dodecane, cis + trans decahydronaphtalene (decaline), toluene and *n*-octanol from Sigma Aldrich (Steinheim, Germany). Orto-phosphoric acid (H_3PO_4) (85%), potassium phosphate monobasic (KH_2PO_4), TCS, humic acids and isooctane were obtained from Fluka (Barcelona, Spain). Sodium hydroxide (NaOH), sodium hypochlorite solution (NaClO) and sodium thiosulfate ($Na_2S_2O_3$) and sodium chloride (NaCl) were provided by Panreac (Barcelona, Spain). Water was purified by a MilliQ system (Millipore Iberica S.A., Barcelona, Spain). Peracetic acid (PAA) equilibrium mixture containing 15% of PAA, 13 - 16% of H_2O_2 and 21 – 26% of CH_3COOH was purchased from Solvay (Italy).

Individual stock solutions were prepared in MeOH with a concentration of 500 mg L^1 for CLF, NPX, DCF, IBP, EST, NP, BPA, TCS and CRB. Standards and calibration solutions were prepared in MeOH for the validation of the chromatographic method (in the range of mg L^{-1}) and in 0.1 mol L^{-1} NaOH for the assessment of the whole analytical procedure (in the range of μg L^{-1}). The stock solutions and standards were stored at 4 ºC.

4.2.2. WATER SAMPLES FOR METHOD DEVELOPMENT

Drinking water was sampled from a laboratory at the University of Girona and surface water samples from the river Matamors in Banyoles (NE Spain). Wastewater samples for method development were collected from Castell-Platja d'Aro WWTP, Girona, north-east Spain. All water samples were collected in 1 L pre-cleaned brown glass bottles and kept refrigerated during transportation; the samples were stored in darkness at 4 ºC until analysis. River water and wastewater were filtered through a 0.7 µm glass microfiber filter (Whatman, Maidstone, UK) prior to the extraction and 100 mg of $Na_2S_2O_3$ was added to drinking water samples in order to avoid possible

degradations. For recovery evaluation, samples were spiked at concentrations of 200 μg L^{-1} for NPX and IBP, and 20 μg L^{-1} for CLF and DCF.

4.2.3. EXPERIMENTS AND SAMPLING FOR THE EVALUATION OF A TERTIARY SYSTEM IN WWTP

4.2.3.1. Laboratory experiments

The efficiency of different disinfectant agents (NaClO, PAA and UV radiation) for the elimination of pharmaceuticals was tested in the laboratory using reagent water spiked at 0.2 mg L^{-1} and wastewater samples collected after the secondary/clarification treatment spiked at 2 mg L^{-1}. The effect of NaClO and PAA oxidants was tested individually with different doses (1, 5 and 10 mg L^{-1}) each over a 24 h period. UV experiments to evaluate the photodegradation of pharmaceuticals were performed with an 8 W germicidal lamp emitting ultraviolet light (Sylvania, United Kingdom) with 250 mL of sample in an oval recipient of 21 cm x 16 cm (80 W m^{-2}). Finally, the combination of oxidants and UV radiation was tested by adding a dose of 5 mg L^{-1} of either PAA or NaClO to reagent water or secondary clarifier wastewater for 150 minutes and applying UV radiation. All the experiments were carried out in the dark, and samples were taken and analyzed immediately to avoid degradation.

4.2.3.2. WWTP operational process

Field experiments were performed in a conventional WWTP situated at El Port de la Selva (Spain), treating 2,625 m^3 day^{-1} of urban wastewater for a population equivalent of 10,500. The WWTP process (Figure 4.2) consists of a primary clarifier, followed by an activated sludge reactor, a secondary clarifier and a tertiary treatment. Before tertiary treatment, hydraulic residence time depends on the season: in winter it is about 22 h while in summer it is between 27 and 32 h. The tertiary treatment studied involved the addition of peracetic acid and/or UV radiation with a hydraulic residence time of 5 min. The effluent is irradiated with a Trojan UV Swift SC (London, Ontario, Canada) consisting of 16 low pressure UV lamps (1.32 W m^{-2}). The physico-chemical parameters of the influent and effluent are presented in Table 4.1. Maximum and minimum flows were 7.2 and 25.1 m^3 $month^{-1}$ in November 2008 and July 2009, respectively. These

values reflect the increase in population in the summer months as the area is a popular tourist destination.

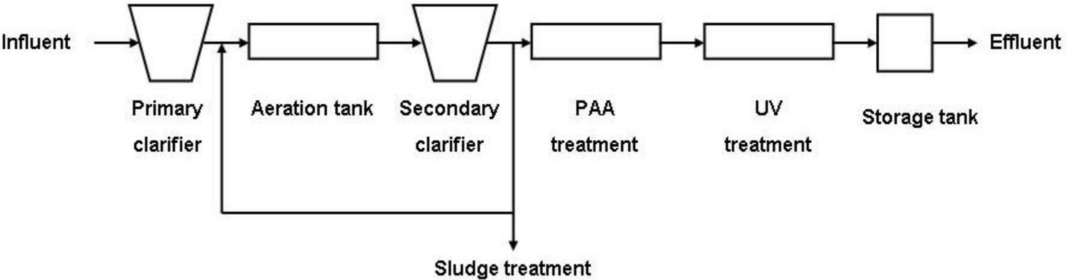

Figure 4.2. Diagram of El Port de la Selva WWTP.

Table 4.1. Physico-chemical characteristics of the influent and effluent of El Port de la Selva WWTP.

	Influent		***Effluent***	
	Average	***Range of values***	***Average***	***Range of values***
pH	7.2	(7–7.3)	7.1	(7–7.3)
Conductivity (ds m^{-1})	2.0	(1.4–2.9)	1.9	(1–4)
Total solids (mg L^{-1})	143.0	(64–244)	6.1	(3–8)
BOD_5 (mg L^{-1} O_2)	146.8	(57–194)	4.2	(3–13)
COD (mg L^{-1} O_2)	309.0	(110–567)	51.1	(35–71)
Total nitrogen (mg L^{-1} N)	43.5	(29.1–57.8)	12.5	(4.8–23.9)
Total phosphorus (mg L^{-1} P)	14.7	(6.1–26.8)	4.5	(2–7.3)

As can be seen in Figure 4.3, three different disinfection treatments were evaluated as tertiary treatments in the WWTP: 1) PAA at different doses (1, 3 and 5 mg L^{-1}) for 3 hours; 2) UV radiation for 5 minutes and 3) PAA for 3 hours followed by UV radiation for 5 minutes.

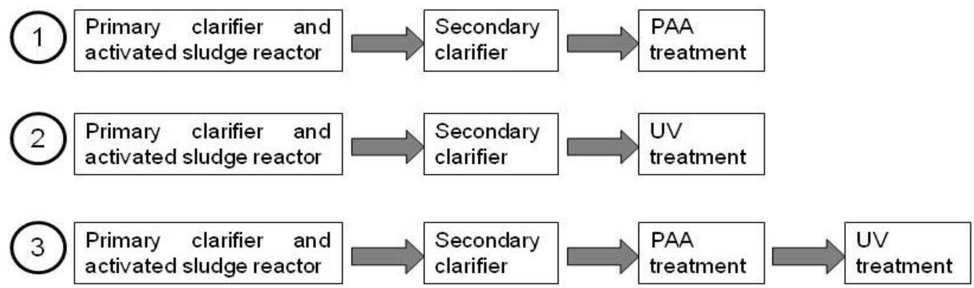

Figure 4.3. Diagram of the experiments performed at the WWTP.

Secondary clarifier and effluent wastewater sampling was performed in campaigns between November 2008 and July 2009 while influent wastewater samples were taken

from fall 2008 to spring 2009. Samples were collected in 1 L glass bottles and kept refrigerated during transportation. Filtering took place within 48 hours of collection using 0.7 µm glass fibre membrane filter. Storage was at 4 ºC in the darkness until analysis. In order to remove the excess of oxidants and to stop the oxidation reaction, $Na_2S_2O_3$ was added to the samples. The addition of this compound was not found to produce significant differences in the results.

4.2.4. HF-LPME

HF-LPME was performed by using polypropylene membranes with a thickness of 200 µm (0.2 µm pore size) and an internal diameter of 300 µm (Azko Nobel, Wuppertal, Germany). Fibers were cut into 20 cm long pieces and the ends were attached to needle syringes (Hamilton, Bonaduz, Switzerland) in a U-shape configuration. Next, the pores of the fibers were impregnated with the organic solvent by using a 250 µL syringe (Hamilton, Bonaduz, Switzerland) and the excess of solvent was removed by immersion of the fiber in reagent water. Then, the lumen was filled with 250 µL of the acceptor phase (0.1 mol L^{-1} NaOH) and the fiber was immersed into 1 L of the sample acidified to pH 1.5 with sulfuric acid (Figure 4.4), protected from light exposure with aluminum foil and the solution was magnetically stirred. After extraction, the acceptor was withdrawn with a syringe and directly analyzed by HPLC.

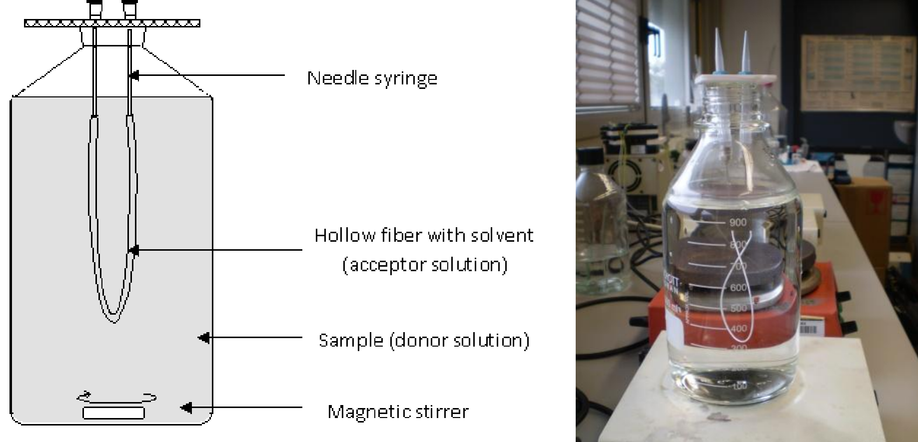

Figure 4.4. Diagram of the HF-LPME system.

4.2.5. SPE

For SPE, four different cartridges were evaluated with standard solutions in reagent water: Oasis HLB (60 mg, Waters, Mildford, USA), Strata-X (200 mg, Phenomenex, Macclesfield, United Kingdom), ISOLUTE ENV+ (100 mg, Biotage, Sweden) and Speedisk H_2O-Philic DVB (Baker, Deventer, Holland).

The conditioning of the cartridges was performed according to a previous study with some modifications [26]. First, 3 mL of ethyl acetate, 3 mL of MeOH and 3 mL of reagent water at a flow rate of 3 mL min^{-1} were passed through the cartridge without allowing the cartridge to dry out. Then, 500 mL of standard solutions or samples previously spiked and acidified at pH 1.5 with sulfuric acid, was passed through the cartridge at about 5 mL min^{-1} using a vacuum pump (Gilson, Williers Le Bel, France). The loaded cartridge was rinsed with 3 mL of MeOH:water (5:95, v/v) and 3 mL of *n*-hexane at a flow rate of 1 mL min^{-1}. Next, the cartridge was dried under vacuum and the analytes were eluted with three aliquots of 1 mL ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen and the residue dissolved in 0.5 mL of MeOH.

4.2.6. HPLC-DAD

HPLC analysis for the optimization of the HF-LPME system was carried out by a SpectraSystem HPLC (Thermo Finnigan, San Jose, USA) with a SN4000 connector, a SCM1000 degasser, a P2000 binary pump and an UV6000LP diode array detector and a Rheodyne Series 7725i manual injector valve with a 20 µL injection loop equipped with a Luna C_8 column (150 x 4.60mm, 5µm) and C_{18} guard cartridge (4 mm x 3 mm) obtained from Phenomenex (Macclesfield, United Kingdom). Chromatographic separation was carried out using a gradient of mobile phases: A (ACN with 0.1% H_3PO_4) and B (reagent water with 0.1% H_3PO_4). The elution started in an isocratic mode for 5 min at 35% of A, followed by a linear increase of mobile phase B from 35% to 42% in 7 min, then to 60% in 8 min and to 75% in 15 min with a flow rate of 1 mL min^{-1}.

HPLC analysis for the final HF-LPME and SPE method were performed using an Agilent Technologies 1200 series separations module with a Bin Bump SL, a degasser, ALS SL

injector and an Agilent Technologies 1290 Infinity DAD SL equipped with a Luna C_{18} column (50 mm x 2 mm, 2.5 µm) and C_{18} guard cartridge (4 mm x 2 mm) supplied by Phenomenex (Macclesfield, United Kingdom). An injection volume of 5 µL was used for all the analyses. Cellulose nitrate and nylon membrane filters with 0.22 µm pore size and 47 mm diameter were used for the preparation of the mobile phases. The mobile phase used contained ACN as eluent A and 20 mmol L^{-1} KH_2PO_4 as eluent B at a flow rate of 0.5 mL min^{-1}. The elution started with 25% of eluent A; at 3 min it was 65%, at 6 min it was 35% and back to initial conditions in 1 min. The reequilibration time was 2 min. UV detection was carried out at 224 nm.

4.3. RESULTS AND DISCUSSION

4.3.1. HPLC-DAD

For chromatographic analysis by HPLC-DAD of final method, linearity was assessed by using standard solutions in MeOH ranging from the instrumental limit of quantitation to 50 mg L^{-1}. Calibration curves were constructed for each compound with r^2 >0.99. Instrumental limits of detection (ILODs) and quantitation (ILOQs) were calculated as 3 and 10 times the background noise with values of 0.5, 5, 10 and 50 µg L^{-1}; and 1.7, 17, 33 and 170 µg L^{-1} for NPX, CLF, IBP and DCF, respectively. The intra-day and inter-day precision (N=5) for each compound were in the range 1 - 2.5% and 4 – 8.4%.

4.3.2. HF-LPME OPTIMIZATION

In order to achieve the best HF-LPME conditions several parameters were optimized. Starting conditions were as follows: aqueous sample at pH 1.5, 4 h extraction time, 0.01 mol L^{-1} NaOH as acceptor phase, stirring rate of 600 rpm, no NaCl addition and 20 cm fiber length.

4.3.2.1. Solvent selection

The organic solvent used to fill in the pores of the hollow fiber plays a critical role for the good performance of the extraction system. The organic solvent should have high affinity for the fiber material (in order to be properly immobilized), low water solubility and volatility and high capability to dissolve the analytes. In Figure 4.5 the results

obtained for the organic solvents tested are presented. Although 1-octanol has been used in several studies as the extraction solvent [36,41], in our study the poorest results were obtained with this solvent, whereas the best enrichment factors for most of the target compounds were obtained when using DHE or *n*-dodecane with 4% of dodecanol as the organic solvent, with a higher extraction efficiency for NPX with DHE. TCS was recovered with decaline (30%), DHE:decane (1:1) (13%) and DHE (4%) whereas BPA was only transported to the acceptor phase using DHE:decane (1:1). In order to obtain lower limits of detection, DHE was selected for subsequent experiments.

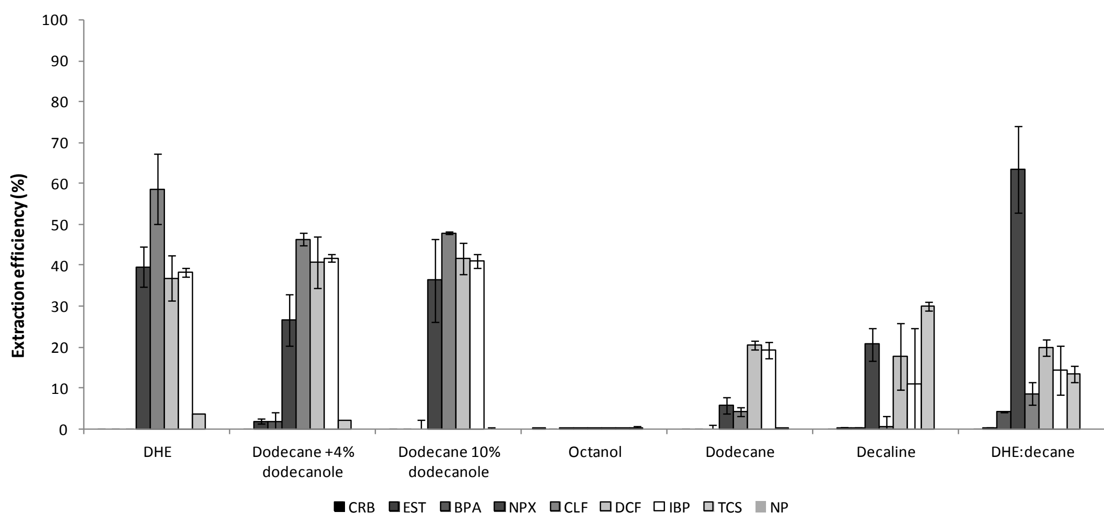

Figure 4.5. Organic solvent effect of pharmaceuticals for reagent water spiked at concentrations of 10 μg L^{-1} and 4 hours extraction time (N=3).

4.3.2.2. Extraction time

Since the extraction time is an important parameter affecting extraction by the HF-LPME system, the influence of the extraction time on the enrichment of the target compounds was studied. Figure 4.6 shows the effect of the extraction time on extraction efficiency. As expected, the amount of analyte extracted increases with time until a constant value is attained, indicating equilibrium conditions for CLF, NPX, DCF and IBP. However, for CRB, EST, BPA, TCS and NP no improvement was observed after a prolonged time. In this study, in order to improve the enrichment of these compounds, a 14 h extraction time was chosen for subsequent experiments. Although it may seem to be a long time, samples were usually handled overnight and many samples can be treated simultaneously.

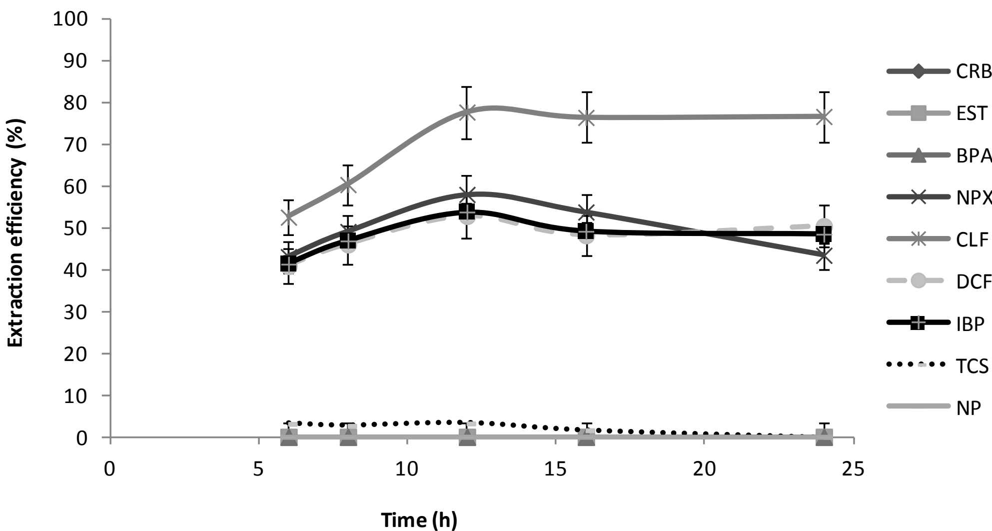

Figure 4.6. Extraction time profiles for reagent water spiked at concentrations of 1 µg L^{-1} (N=3).

4.3.2.3.Acceptor phase pH

When performing a three-phase HF-LPME with pH gradient it is important to have the analytes in their ionic form in the acceptor solution in order to avoid re-extraction of analytes from the acceptor phase to the organic solvent. For this reason, two pHs (12 and 13) were studied with 0.01 mol L^{-1} NaOH and 0.1 mol L^{-1} NaOH. The results (Figure 4.7) demonstrated that no differences in extraction efficiency were observed for CRB, EST, BPA, TCS and NP. On the other hand, for CLF, NPX, DCF and IBP, extraction efficiency increased at pH 13 about two times, therefore 0.1 mol L^{-1} NaOH was fixed as the acceptor phase.

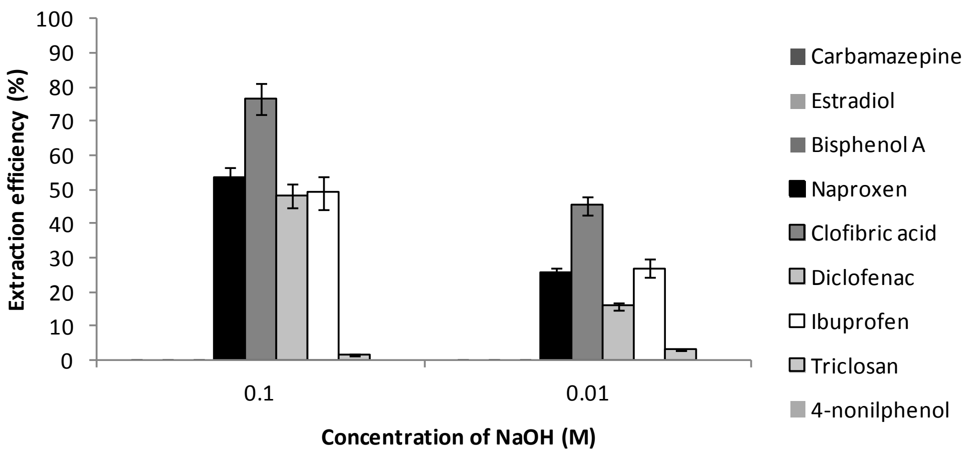

Figure 4.7. . Effect of concentration of NaOH on extraction efficiency for reagent water spiked at a concentration of 1 µg L^{-1} (N=3).

4.3.2.4.Effect of stirring rate

It is expected that sample stirring improves mass transfer of the analytes through the membrane, eventually increasing extraction. Two stirring rates were tested (600 and 1200 rpm) with a higher area for CLF at 1200 rpm while for NPX; DCF and IBP no differences were observed (Figure 4.8). Hence, 1200 rpm was chosen for further optimization. As in previous experiments, no or little extraction was observed for the other compounds.

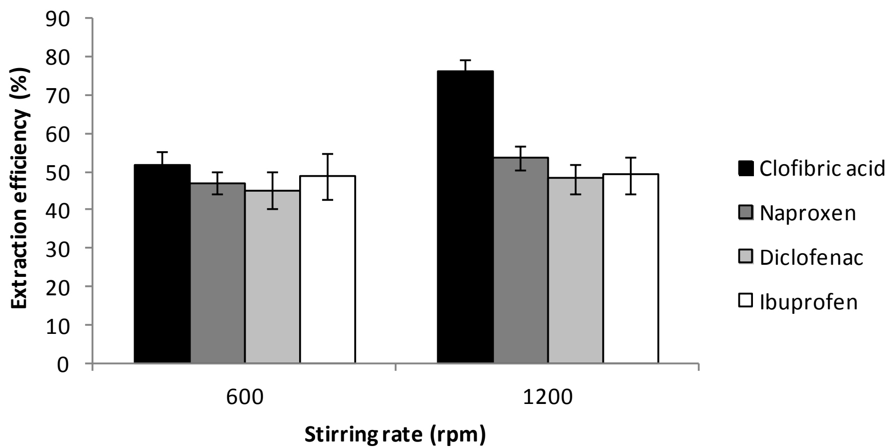

Figure 4.8. . Effect of stirring rate on extraction efficiency for reagent water spiked at a concentration of 1 µg L^{-1} (N=3).

4.3.2.5.Effect of salt addition

The use of the salting-out effect to improve extraction efficiency was investigated by adding NaCl to the donor phase in a concentration range of 0 to 100 g L^{-1}. Generally, the addition of salt can decrease the solubility of the analytes in the samples, thus enhancing their partitioning into the organic solvent. In our case, as can be observed in Figure 4.9, salt addition had a positive effect on CLF extraction while for DCF extraction efficiency decreased when NaCl concentration increased. For NPX and IBP the effect of salt addition was negligible. These results can be related to the hydrophilicity of the acidic compounds; a positive effect was observed for CLF, which is the least hydrophobic analyte, while a negative effect was observed for DCF, which is the most hydrophobic compound. Although a positive effect for hydrophobic compounds was expected to be observed, our results show the opposite. The same tendency was

observed by Quintana et al. [23] for SBSE. Thus, in further experiments salt was not added to the samples and only CLF, NPX, DCF and IBP were extracted with HF-LPME.

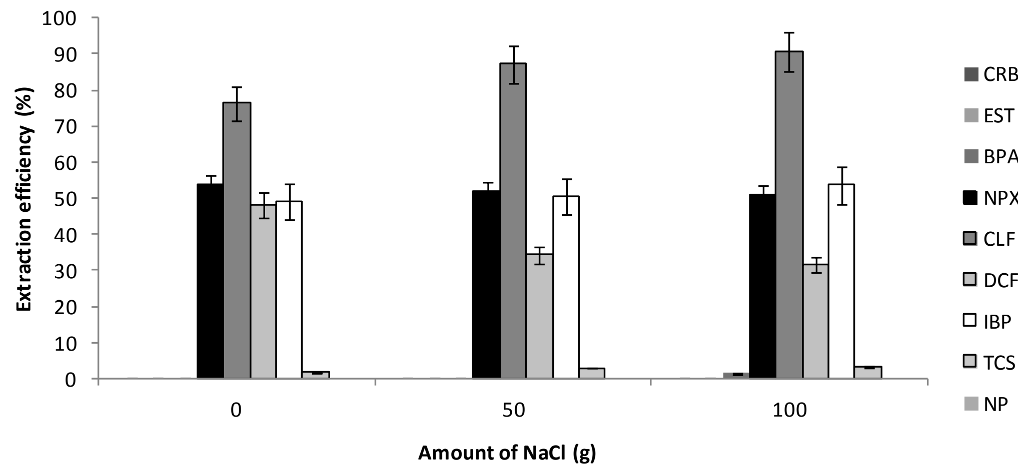

Figure 4.9. Effect of NaCl addition on extraction efficiency for reagent water spiked at a concentration of 1 µg L^{-1} (N=3).

4.3.2.6. Matrix effect: presence of humic acids

Organic matter has polar functional groups (hydroxylic and carboxylic acid groups) which can interact with organic compounds and reduce the presence of freely dissolved organic compounds in water. In addition, depending on the sample pH, organic matter can be co-extracted, which affects the chromatographic analysis and decreases the enrichment factors of the analytes.

Some experiments were performed to assess the effect of humic acids, as a model of the organic matter present in environmental water, on the HF-LPME procedure for the target pharmaceuticals. Figure 4.10 shows that the addition of humic acids at a concentration of higher than 5 mg L^{-1} causes a decrease in the extraction efficiency of the compounds. This effect is probably not due to the extraction of humic acids since high molecular mass compounds are excluded by the microporous membrane, but is more likely due to the interaction of organic matter with the target analytes.

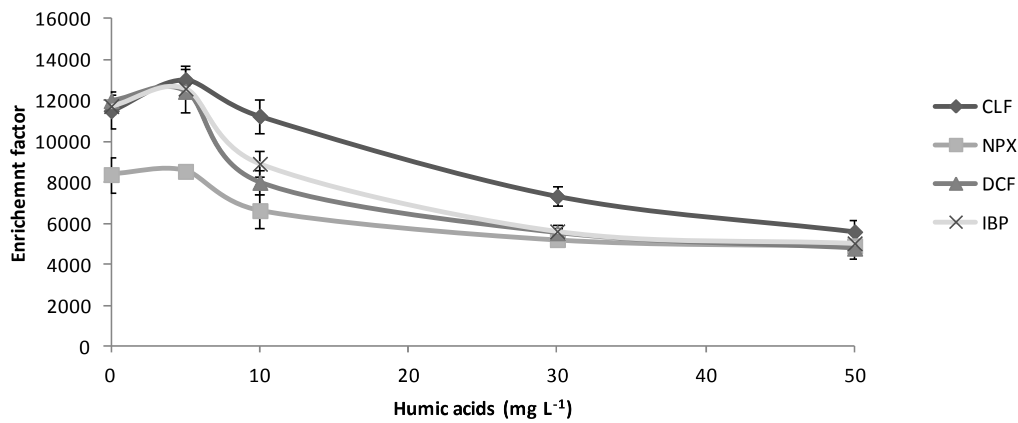

Figure 4.10. Humic acids concentration effect on the enrichment factor of pharmaceuticals for reagent water spiked at a concentration of 1 µg L^{-1} (N=3).

4.3.2.7. <u>Analytical performance</u>

Analytical figures of merit concerning linearity, method limits of detection and method limits of quantitation are listed in Table 4.2. All the compounds exhibited good linearity with coefficients of determination (r^2) higher than 0.9984. Method limit of detection (MLOD) and method limit of quantitation (MLOQ) were calculated as 3 and 10 times the signal-to-noise ratios, respectively. For MLOD very low values between 0.5 and 10 ng L^{-1} were achieved. The intra-day precision and inter-day precision studies were performed with an aqueous sample spiked at 0.1 µg L^{-1} of each compound (N=5). Relative standard deviations were from 9 to 11% and from 5 to 12% intra-day precision and inter-day precision, respectively. In reagent water high enrichment factors were achieved with values between 10,397 and 11,740, which are higher than most values reported in the literature and close to those (15,000) obtained by Wen et al. [64] for the determination of IBP in wastewater by two-step liquid-liquid-liquid microextraction.

Table 4.2. Linearity, MLODs and MLOQs of HF-LPME for the extraction of pharmaceuticals in reagent water by HPLC-DAD (N=3).

Analyte	*Linear range (µg L^{-1})*	*r^2*	*MLOD (ng L^{-1})*	*MLOQ (ng L^{-1})*	*Ee*
CFL	0.033-50	0.9997	10	33	11,510
NPX	0.0017-50	0.9984	0.5	1.7	10,397
DCF	0.017-50	0.9996	5	17	11,590
IBP	0.017-50	0.9995	5	17	11,740

4.3.3. SPE

4.3.3.1. Evaluation of different sorbents

Four SPE commercial sorbents were evaluated using reagent water spiked with the target compounds. As can be seen in Figure 4.11, better recoveries were obtained using Oasis HLB and Strata X cartridges with recoveries of between 84 – 95% and 61 – 93%, respectively. Both sorbents have similar properties, therefore these results are consistent; moreover, for CLF, similar results to the ones obtained by Nebot et al. [24] were achieved, although in this study IBP presented lower recoveries. Gros et al. [65] also compared Oasis HLB and ISOLUTE ENV+, showing the same tendency. In light of the results, Oasis HLB was selected for the subsequent series of experiments.

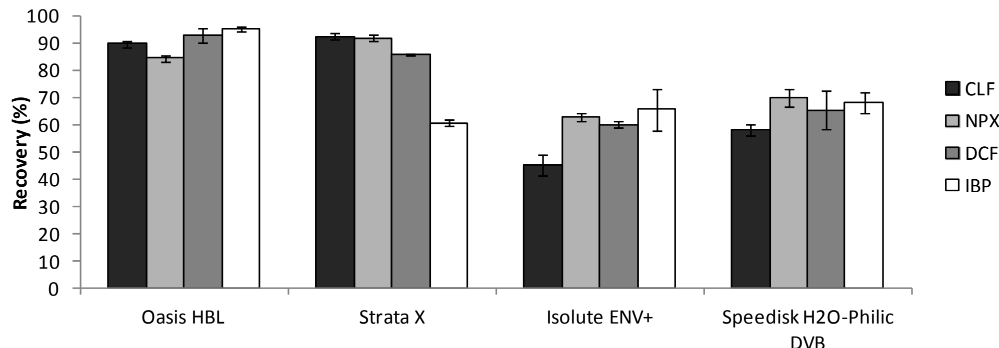

Figure 4.11. Average recoveries of pharmaceuticals for different SPE cartridges for reagent water spiked at a concentration level of 2 µg L^{-1} (N=3).

4.3.3.2. pH of sample solution

Once the sorbent was selected, recoveries in real samples with the pH adjusted to 1.5 with sulfuric acid were compared to the ones obtained without any pH adjustment (Figure 4.12). It was observed that, for almost all the compounds, an important increase in the recovery was obtained for all kinds of samples when they were acidified with values of around 90% for NPX and IBP and higher than 70% for CLF; only for DCF were no differences observed when the pH was adjusted. In other studies no influence of sample pH was reported [24,28]. However, Camacho et al. [29] obtained higher recoveries at pH 2 for DCF, IBP and CLF by Oasis HLB, which is in agreement with our results. Therefore, for further experiments the pH of the samples was adjusted to pH 1.5.

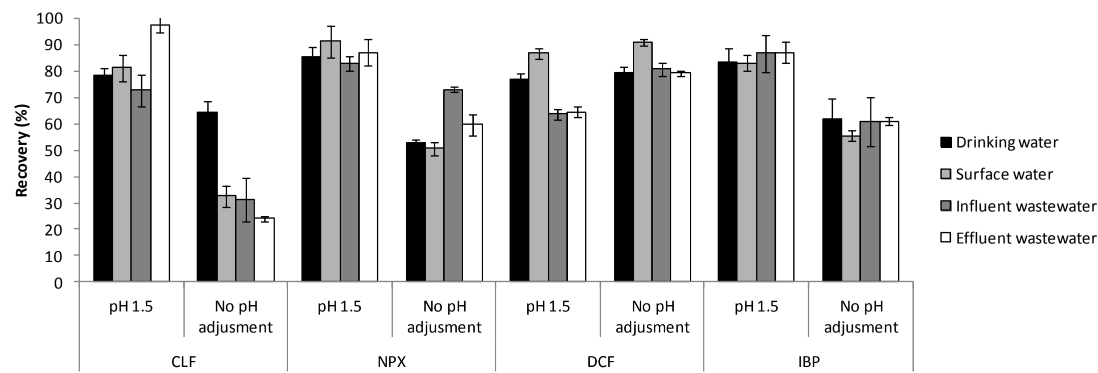

Figure 4.12. Effect of the pH sample pH on the recoveries of pharmaceuticals for real samples spiked at a concentration level of 20 µg L^{-1} (N=3).

4.3.4. SPE AND HF-LPME COMPARISON

Finally, recoveries obtained by HF-LPME and SPE methods were compared (Table 4.3). In drinking water, IBP recoveries wee about 85% for both methods, while for the other compounds higher values were obtained by HF-LPME (84% for DCF, 94% for NPX and 98% for CLF). For surface water, similar values were obtained in the two procedures developed, while for wastewater similar or lower recoveries were obtained by HF-LPME because only the free content was determined. However, as shown in Figure 4.13, cleaner chromatograms were obtained by HF-LPME, hence HF-LPME is a suitable method for the determination of CLF, NPX, IBP and DCF in environmental waters, with recoveries of around 90%, and influent and effluent wastewater, with values between 60 and 84%.

Table 4.3. Influence of various sample matrices on the recoveries of pharmaceuticals by SPE and HF-LPME (N=3).

Sample	*Recovery (%)*							
	HF-LPME				*SPE Oasis HLB*			
	CLF	*NPX*	*DCF*	*IBP*	*CLF*	*NPX*	*DCF*	*IBP*
Drinking water	98.1	93.5	83.7	85.5	78.3	85.2	76.9	83.5
Surface water	91.4	90.6	95.4	98.0	81.3	91.1	86.7	82.9
Influent wastewater	61.4	76.7	62.6	60.3	97.5	87.0	64.5	87.0
Effluent wastewater	73.2	84.0	70.1	68.3	72.7	82.9	63.8	86.8

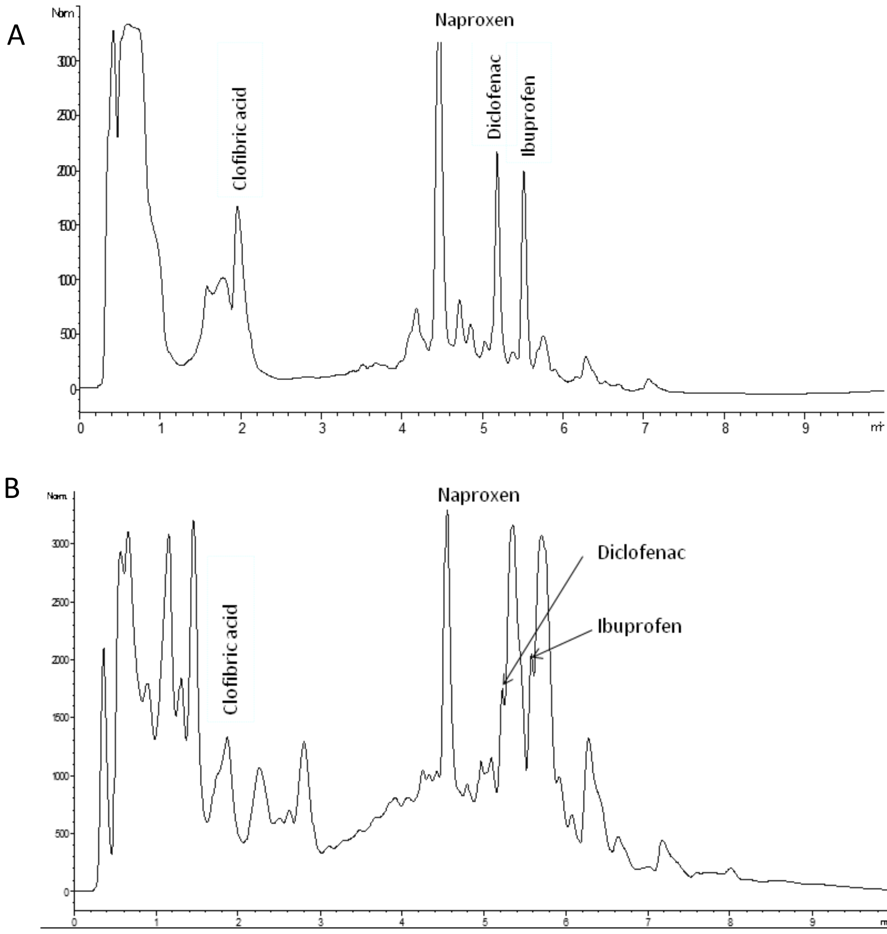

Figure 4.13. Chromatograms of the (A) hollow fiber extract and (B) SPE extract of an effluent of Castell-Platja d'Aro WWTP.

The recoveries obtained by both methods are quite similar to those reported in the literature (Table 4.4). As can be seen in Table 4.4, the most applied clean-up technique is SPE followed by HPLC-MS or GC-MS with similar recoveries to those reported in this study. Furthermore, HF-LPME methods found in the literature for the determination of NSAIDs and CLF showed similar recoveries, however lower MLODs were obtained in the proposed method. In almost all the reports detection limits were similar to our results, but in our case analyses were carried out by HLPC-UV, as HF-LPME has been proven to be an efficient clean-up technique due to its selectivity and the high enrichment factors achieved.

Table 4.4. Comparison of methods for CLF, NPX, IBP and DCF reported in aqueous samples.

Analyte	*Matrix*	*Extraction technique*	*Analytical determination*	*Recovery (%)*	*MDL (ng L^{-1})*	*Ref.*
CLF	Wastewater	LLE	GC-MS	91	8.3	[22]
IBP				100	6.7	
CLF	Wastewater and surface water	SPE	LC-ESI-MS/MS	30-106	1-2	[65]
NPX				34-81	7-9	
IBP				63-111	8-12	
DCF				60-102	2-10	
CLF	Wastewater and surface water	SPE	LC-QqLIT	101-112	0.1-0.3	[66][66].
NPX				71-113	0.3-21	
IBP				82-107	0.4-9	
DCF				84-105	1-4	
NPX	Wastewater	SPE	GC-MS	117	2.7	[67]
IBP				92	9.6	
DCF				98	2.4	
CLF	Wastewater and surface water	SPE	LC-UV/FLD	79-107	UV: 366-3333	[29]
NPX				92-97	FL: 6.67-1517	
IBP				79-100		
DCF				87-101		
CLF	Wastewater, tap water and surface water	SPE	HPLC-MS/MS	15-44	0.96	[24]
IBP				53-70	0.52	
DCF				55-86	0.12	
NPX	Wastewater, surface water and drinking water	SPE	LC-MS/MS	76-103	49 [a]	[28]
IBP				90-124	642 [a]	
DCF				83-98	137 [a]	
NPX	Wastewater and surface water	SPE	GC-MS/MS	112	0.5	[25]
IBP				64	0.8	
DCF				99	1	
CLF	Wastewater	SPE	GC-MS	98	10	[32]
NPX				101		
IBP				99		
DCF				87		
NPX	Wastewater	SPE	HPLC-DAD/FLD	98	20	[26]
IBP				89	250	
DCF				78	380	
IBP	Wastewater	SPE	GC-MS	78 - 98	23	[21]
DCF					100	
NPX	Wastewater	SPE	GC-MS	97		[31]
IBP				100		
DCF				99		
CLF	Effluent and influent wastewater	XAD-bag samplers	GC-MS	32	25, 55	[68]
NPX				69	12, 20	
IBP				61	12, 16	
DCF				60	22, 50	

[a]MLOQ

Table 4.4. Comparison of methods for CLF, NPX, IBP and DCF reported in aqueous samples (continued).

Analyte	*Matrix*	*Extraction technique*	*Analytical determination*	*Recovery (%)*	*MDL (ng L^{-1})*	*Ref.*
CLF **NPX** **IBP** **DCF**	Wastewater, surface water and drinking water	SBSE	GC-MS	83-109 77-111 97-105 94-112	14 19 20 88	[23]
NPX **IBP** **DCF**	Wastewater effluent	Continuous flow HF-LPME	HPLC-DAD/FLD	74 91 83	10 30 50	[37]
IBP **DCF**	Wastewater	HF-LPME	HPLC-MS/MS	50-53 71-73	300 100	[35]
CLF **NPX** **IBP** **DCF**	Wastewater	HF-LPME	LC-MS/MS	93-123	0.3[a] 10[a] 14[a] 25[a]	[36]
CLF **NPX** **IBP**	Wastewater and tap water	Dynamic HF-LPME	GC-MS	98-106 98-103 97-102	20 10 10	[41]
IBP **NPX**	Wastewater, drinking water and surface water	Dynamic HF-LPME	GC-FID	90-108 89-95	1 2	[42]
IBP	Wastewater	Liquid-liquid-liquid	HPLC-UV	73.1	100	[64]
IBP **DCF**	Surface water	SBME	HPLC-UV	44-50 47-52	900 900	[34]
CLF **NPX** **IBP** **DCF**	Wastewater, drinking water and surface water	HF-LPME	HPLC-UV	61-98 77-94 63-95 60-98	10 0.5 5 5	Proposed method

[a]MLOQ

4.3.5. EFFECT OF DISINFECTION TREATMENTS IN NSAIDS REMOVAL

Disinfection is carried out in WWTPs for the elimination of microorganisms in treated water; furthermore it is also interesting to evaluate disinfection in WWTPs for the elimination of pharmaceuticals. As has been explained in the experimental section, three disinfectant treatments were evaluated: 1) PAA, 2) UV and 3) PAA combined with UV in the laboratory and in a WWTP.

4.3.5.1. Laboratory experiments

The degradation of the four pharmaceuticals was studied in spiked water. First, different doses (1, 5 and 10 mg L^{-1}) of both PAA and NaClO were tested. No

degradation was observed after 24 h independently of the dose. Photodegradation processes were then studied applying only UV radiation and combining the addition of oxidants (PAA or NaClO) with UV radiation. No differences were found when applying UV radiation and combined PAA/UV or NaClO/UV treatments as can be seen in Figure 4.14 for CLF, NPX and IBP. In all cases, elimination of the pharmaceuticals increased over time. 94% of DCF was eliminated in just 10 minutes whereas an identical amount of CLF was eliminated in 60 minutes. Degradations of higher than 95% were observed in the case of NPX but 4 hours were required. IBP had the lowest photodegradation rate, requiring 8 hours to remove just 70%. These results broadly agree with other published studies which have found that CLF and DCF photodegrade faster than NPX [10,61] and, for example, that DCF can be totally degraded in 15 min [69]. Kim et al. [61] studied the efficiency of UV treatment with removal values of 90% and 40% in ten minutes for DCF and NPX, respectively.

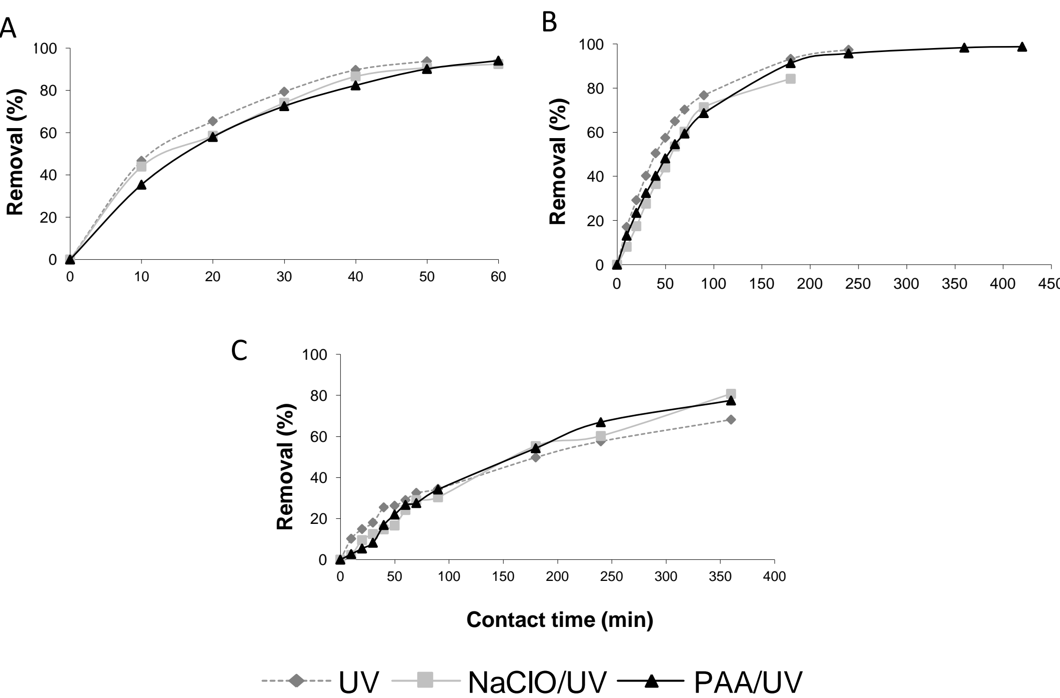

Figure 4.14. Removal (%) of the compounds in reagent water spiked at 0.2 mg L^{-1} by type of treatment. A) CLF, B) NPX and C) IBP.

Spiked secondary wastewater samples were treated with 5 mg L^{-1} of PAA for 24 hours. As in spiked reagent water, no degradation was observed. Finally, the effects of UV

radiation and combined treatments (PAA or NaClO followed by UV radiation) were studied in spiked secondary wastewater without significant differences being found (Figure 4.15). DCF and CLF were photodegraded in 20 and 180 minutes, respectively. NPX was completely eliminated after 5 hours. 70% removal of IBP was attained after 8 hours.

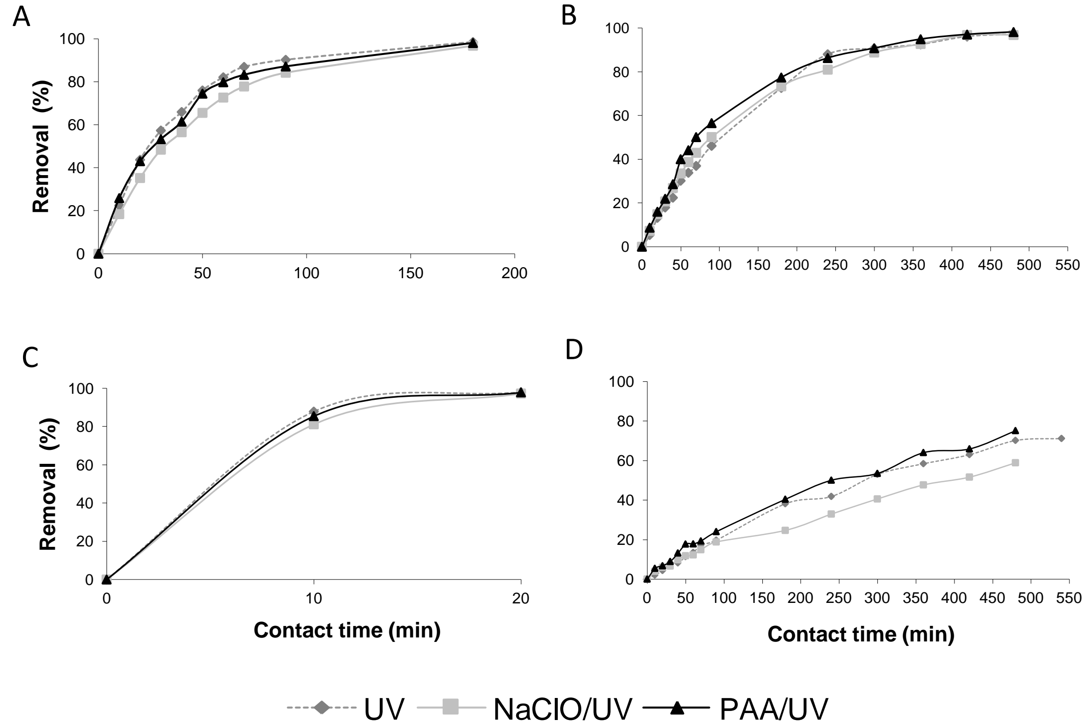

Figure 4.15. Removal (%) of the compounds in secondary clarifier wastewater spiked at 2 mg L^{-1} by type of treatment. A) CLF, B) NPX, C) DCF and D) IBP.

For UV experiments without PAA or NaClO, the data obtained for reagent water and secondary wastewater fitted to pseudo-first-order kinetic law according to Eq. (3.1) [61]:

$$ln\frac{C_t}{Co} = -kt \qquad (3.1)$$

Where C_o is the initial concentration, C_t is the concentration at time t, k is the pseudo-first-order rate constant or degradation rate constants and t is the irradiation time in minutes.

A higher degradation rate constant was observed for all the compounds in reagent water, especially in the case of DCF and CLF (Table 4.5). In wastewater these rate constants were always lower as a result of the competition of organic matter for the UV radiation [62,70,71]. For example, Yuan et al. [72] determined constant values for IBP with an 11 W low-pressure mercury vapor lamp (21.2 W m^{-2}) at a concentration of 5 µmol L^{-1}, pseudo-first order rate constants were about 0.03 and 0.018 min^{-1} in deionized water, and in the presence of humic acids, HCO_3^- and NO_3^- , respectively. Therefore, a decrease was observed in the presence of other substances. For IBP, similar values to those obtained in the present study were reported by Matamoros et al. [73] under sunlight (0.003 min^{-1}). Finally, Kim et al. [10] observed values of 0.5 and 0.03 min^{-1} for DCF and NPX under an 8 W low-pressure mercury lamp (0.384 mW cm^{-2}) and degradation rate constants were 0.576 and 0.072 min^{-1} under an 10 W low-pressure mercury lamp (0.388 mW cm^{-2}). Finally, Giri et al. [74], applying a low pressure tubular mercury lamp (10 W), observed degradation of all the compounds studied with a higher degradation rate constant for DCF (0.74 min^{-1}), followed by CLF (0.11 min^{-1}), NPX (0.04 min^{-1}) and IBP (0.01 min^{-1}). Therefore, different degradation rate constants were observed depending on the type of UV lamp used and the energy applied. However, various different studies have always found higher rates for DCF and the lowest rates for IBP, and our results are consistent with those findings.

Table 4.5. Pseudo-first order rate constants (k) and coefficient of determination (r^2) by UV radiation of pharmaceuticals in reagent water and secondary wastewater.

Analyte	*Reagent water*		*Secondary wastewater*	
	k (min^{-1})	***r^2***	***k (min^{-1})***	***r^2***
CLF	0.049	0.985	0.028	0.997
NPX	0.008	0.994	0.007	0.996
DCF	0.46	0.999	0.180	0.998
IBP	0.005	0.948	0.003	0.986

4.3.5.2.Study in WWTP

4.3.5.2.1.Occurrence of drugs in WWTP

Field experiments were carried out between November 2008 and July 2009 in El Port de la Selva WWTP. The average concentrations of pharmaceuticals in influent and

secondary wastewater during the sampling period are summarized in Table 4.6. The highest concentrations in the influent wastewater correspond to IBP followed by NPX with average values of 3,547 and 2,088 ng L^{-1}, respectively. CLF and DCF were detected with average concentrations of 275 and 123 ng L^{-1}, respectively. These results agree with previous reports: Jelić et al. [13] detected NPX and DCF at similar concentrations between 0.4 – 7.2 µg L^{-1}; Carballa et al. [9] reported values for NPX and IBP in Galicia (Spain) with maximum concentrations of 5.7 µg L^{-1}, and Zorita et al. [16] determined average concentrations of 53.5, 4,900, 230 and 6,900 ng L^{-1} for CLF, NPX, DCF and IBP, thus showing the same tendency as observed in our study.

Table 4.6. Average concentrations (ng L^{-1}) and relative deviation of the pharmaceuticals detected in influent and secondary clarifier.

Analyte	*Influent (N=12)*		*Secondary clarifier (N=28)*			
	Concentration (ng g^{-1})		*Concentration (ng g^{-1})*		*Removal (%)*	
	Average	*Range of values*	*Average*	*Range of values*	*Average*	*Range of values*
CLF	275	(<LOD–885)	<LOD	(<LOD–290)		
NPX	2,088	(100–3,728)	150	(39–642)	80	(54–93)
DCF	123	(n.d–337)	360	(99–640)	37	(-6–85)
IBP	3,547	(101–6,361)	510	(51–943)	91	(82–99)

n.d.: not detected for the presence of some interference.

In secondary wastewater, NPX, DCF and IBP were detected at levels of 150, 360 and 510 ng L^{-1}, respectively. However, in the WWTP studied, CLF was below the method limit of detection in most of the samples. This agrees with Kim et al. [61], who found CLF at concentrations of 2 - 6 ng L^{-1} in secondary wastewater. Although IBP and NPX are susceptible to biodegradation in biological treatment, concentrations between 510 and 150 ng L^{-1} were found. Maximum and minimum concentration levels of NPX were 70 and 500 ng L^{-1} (November and February), for IBP they were 100 and 730 ng L^{-1} (January and June), while for DCF the minimum was below the method limit of detection (<10 ng L^{-1}) and the maximum was 431 ng L^{-1} (December and May) (Figure 4.16). High concentrations in spring can be explained by the increase of the population in the town. Concentration variations during the day were studied on different occasions (Figure 4.17): the lowest concentrations of NPX, DCF and IBP were at 9 am

whereas peaks were reached at around 12 noon. Samples were always taken at the same time.

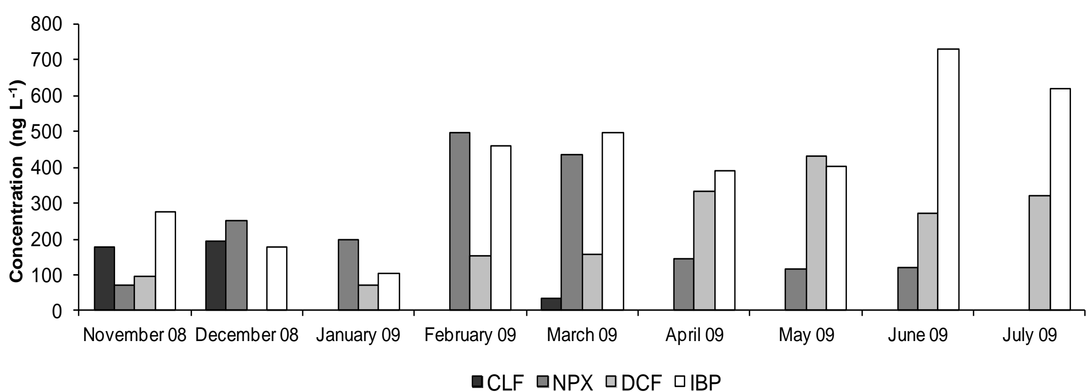

Figure 4.16. Temporal evolution of the average concentrations of the pharmaceuticals detected in secondary clarifier wastewater.

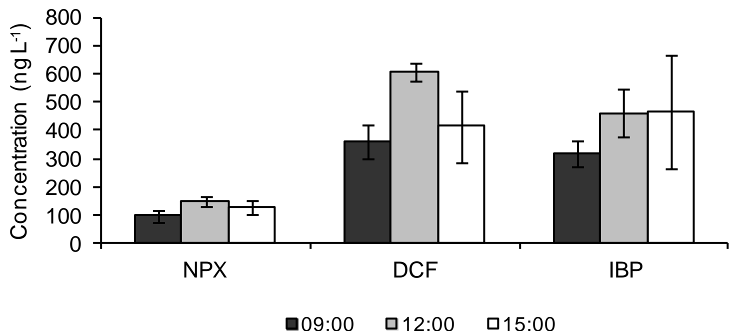

Figure 4.17. Temporal evolution during the same day of the average concentrations of the pharmaceuticals detected in secondary clarifier wastewater in May 2009 (N=3).

Biological treatment with activated sludge where pollutants can be removed by sorption onto sludge or biodegradation shows high average removal efficiencies for IBP and NPX with values of about 91 and 80%, respectively (Table 4.7). These values agree with previous studies that have reported removal efficiencies of biological treatments of about 90 and 70% for IBP and NPX [4,61,76]. Biological treatment seems to be the main removal treatment for IBP and NPX. On the other hand, only 37% of DCF was removed; this can be explained by the fact that DCF is a recalcitrant compound with a low degradation constant [16,77]. These results are consistent with other published findings of DCF; such as Gómez et al. [21] who determined a removal rate of 59% or Sui et al. [5] who reported removal efficiencies of 28 - 53% in activated sludge [6,8,14]. Joss et al. [4] reported removal values of higher than 90% for IBP and

between 50 – 80% and 20 – 40% for NPX and DCF, respectively. In their study, no influence of the contact time between wastewater and sludge, and no influence of temperature in sludge age and reactor configuration (conventional activated-sludge, membrane bioreactor and suspended-biofilm reactors) were observed.

Table 4.7. Removal efficiencies (%) of some pharmaceuticals applying different treatments in WWTP.

Analyte	*UV removal (%) (N=2)*		*PAA/UV removal (%) (N=5)*	
	Average	*Range of values*	*Average*	*Range of values*
NPX	44	22–66	35	10–79
DCF	53	21–84	36	5–75
IBP	35	19–50	53	31–85

4.3.5.2.2.Removal of pharmaceuticals in the disinfection treatment

Different PAA doses (1, 3 and 5 mg L^{-1}) were applied after secondary wastewater treatment with a contact time of 5 minutes. Although removal efficiencies could not be calculated for all the compounds as CLF was not detected, no differences were found between the different doses, as was also the case in the laboratory experiments.

The possible photo-chemical degradation by UV and combined PAA/UV for the removal of pharmaceuticals in real wastewater was investigated in WWTP. Table 4.7 shows the efficiency with which NPX, DCF and IBP are removed from effluent wastewater after UV radiation, whether or not combined with 5 mg L^{-1} of PAA. When applying only UV radiation, the average removal ranged from 35 - 53%, thus showing the same degradation as in the PAA/UV process. Unlike the case of the laboratory studies, the lower degradation of DCF can be explained by the shorter contact time in the tertiary system of the WWTP and the presence of other substances such as DOM (dissolved organic matter) that can adsorb UV radiation and so decrease the degradation of pharmaceuticals. The irradiation used in the tertiary system (1.32 W m^{-2}) for bacterial treatment appeared to be too low to completely eliminate the pharmaceutical substances remaining in secondary treated water along with the low hydraulic residence time (5 minutes) in the tertiary system. In contrast to PAA, other advanced oxidation processes such as ozonation have been successfully applied with high removal values of near to 90% and it seems a good alternative to chlorine [5,6].

4.3.5.2.3.Overall removal of pharmaceuticals in WWTP

The overall removal efficiency of pharmaceuticals in WWTP, under the previously explained conditions, was calculated. The removal rates were calculated from the average concentrations determined for each compound both on entry to the plant and at the effluent. Average removal values of 79 ± 29% for NPX and 90 ± 24% for IBP were achieved. For DCF, maximum removal (100%) was found in January whereas no removal was found in April. These values are consistent with those reported by Lindqvist et al. [8] with values of 82% for IBP and 81% for NPX, Zorita et al. [16] with values of 99% and 94% for IPB and NPX or Gros et al. [12] with values of 91% for IBP and NPX. For DCF, controversial values ranging from 0 to 80% are reported, which can be related to wastewater characteristics and treatment techniques applied in the WWTP. In the case of DCF, higher removal rates are observed when tertiary treatment is applied [12,14,71,76]. Jelić et al. [13] observed higher DCF removal when coagulation/flocculation and chlorination were applied (from less than 24 to 60%) [8]. Furthermore, high removal efficiencies (< 61%) were observed for DCF in four treatment plants with different tertiary treatments: lagoons, chlorination and UV radiation [78].

4.4. CONCLUSIONS

A method based on three-phase HF-LPME followed by HPLC-DAD has been developed for the determination of clofibric acid, naproxen, ibuprofen and diclofenac in wastewater and environmental waters. Recoveries were between 84 – 98% in surface and drinking water, 68 – 84% in effluent wastewater and 60 – 77% in influent wastewater due to the presence of organic matter. The method was also compared to SPE and showed similar results and cleaner chromatograms. Due to the high selectivity and high preconcentration achieved (about 11,000) of HF-LPME, procedure method limits of detection obtained are of low ng L^{-1} and comparable to the LODs of the methods developed by LC-MS. The method was applied to the analysis of environmental waters and wastewater during the different steps of the wastewater treatment processes in WWTP study.

A tertiary system combining peracetic acid and UV radiation was evaluated for the degradation of CLF, NPX, IBP and DCF in the laboratory and in a WWTP. In laboratory experiments, PAA was compared to NaClO but degradation was not observed in either case. The effect of UV radiation was also evaluated and the degradation of all the compounds was observed; taking into account the degradation rate constant calculated for pseudo-first-order kinetics, DCF photodegraded faster followed by CLF, NPX and IBP. When comparing the effect of UV radiation on reagent water and secondary wastewater in the elimination of DCF and CLF acid, a lower degradation rate constant was calculated in secondary wastewater. This can be explained by the presence of organic matter which acts as a filter. Peracetic acid and UV radiation were also tested in WWTP without differences between them. The results obtained were compared to biological and secondary treatment. In biological and secondary treatment, NPX and IBP were the most removed (80 - 90%) whereas a removal rate of 37% was observed for DCF. In tertiary treatment, similar removals were observed for the pharmaceuticals (35 – 53%). In order to achieve removal rates of about 99% under UV radiation in WWTP, an exposure of 26, 165, 658 and 1,535 minutes is required for CLF, NPX and IBP. Finally, in WWTP overall removals of about 54% for DCF, 79% for NPX and 90% for IBP were observed.

4.5. REFERENCES

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CHAPTER 5:

Determination of anti-inflammatory drugs in sewage sludge by direct hollow fiber liquid-phase microextraction and liquid chromatography-mass spectrometry

5.1. INTRODUCTION

One of the most consumed groups of pharmaceuticals is non-steroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen (KTP), naproxen (NPX), diclofenac (DCF) and ibuprofen (IBP), which are used in humans and animals all over the world [1,2]. As has been explained in the introduction, it is well known that one of the most important routes of pharmaceuticals getting into the environment is through wastewater treatments plants (WWTPs), either through effluent wastewater or sewage sludge. In WWTPs, some authors have reported a removal efficiency of these compounds at levels ranging from 20 to 90% in biological treatment [2,3]. In conventional activated sludge treatment, high levels of IBP and NPX have been removed from the water phase (up to 72%) whereas KTP and DCF were only removed at 55% and 22%, respectively [4]. This is in agreement with our results which showed that under aerobic conditions high removal efficiencies of IBP (97%) and NPX (70%) were achieved, while for DCF a low elimination was observed. Two processes can be responsible for this reduction, either sorption onto sludge resulting in distribution between solid and aqueous phases [2,5,6,7] or biodegradation [8,9,10].

Some studies suggest that biodegradation is the main process in the removal of NSAIDs in biological treatment [11,12], therefore little or no sorption onto sewage sludge is to be expected as a consequence of the low solid-water distribution coefficients (K_d) [13,14]. Hörsing et al. [15] also observed little sorption of IBP and very low sorption of DCF. Different K_d values are reported in the literature: for DCF, Ternes et al. [16] reported K_d values of 458 L Kg^{-1}, Carballa et al. [14] between 19 and 151 L Kg^{-1} and Radjenović et al. [4] obtained values of 194 and 118 L Kg^{-1} in primary and secondary sludge. Radjenović et al. [4] also calculated values of 9.5 L Kg^{-1} for IBP, while for KTP they obtained K_d values of 226 and 16 L Kg^{-1} in primary and secondary sludge, respectively. Lin et al. [17] showed that for DCF and IBP there was no sorption, while for NPX strong sorption at pH 9 was observed. At pH 9 the analytes are dissociated and negatively charged and the difference between the sorption values can be explained

by the π-π interactions of the diaromatic ring of NPX with aromatic moieties in humic substances.

Although it seems that biological degradation is the main removal process, NSAIDs have been detected in sewage sludge. Sewage sludge is the main solid produced in WWTPs and the European Union (EU) promotes its use as a fertilizer on agricultural land. Therefore, it is important to know the occurrence of contaminants in biosolids [7,18–23]. Several methodologies such as ultrasonic solvent extraction (USE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) have been applied for the extraction of NSAIDs from sludge samples, while for the clean-up and enrichment of the analytes, solid-phase extraction (SPE) is the most used technique [24]. Table 5.1 shows some of the methods described in the literature for the determination of KTP, NPX, IBP and DCF in sediment and sewage sludge.

Table 5.1. Comparison of methods reported for the determination of NSAIDs in sediments and sewage sludge.

Analyte	*Extraction technique (clean-up)*	*Analytical determination*	*Sample size (g)*	*MLOD ($ng\ g^{-1}$)*	*Ref.*
KET **NPX** **IBP** **DCF**	USE (SPE)	LC-UV/FLD	1-1.5	7.7-18.7 0.4-7.53 166-355 3.7-100	[24]
NPX, IBP, DCF	USE (SPE)	LC-MS/MS	5	-	[17]
KET, NPX, IBP, DCF	USE (SPE)	GC-MS	5	-	[26]
KET **NPX** **IBP** **DCF**	USE (SPE)	GC-MS	1	5 2.2 22 11	[27]
KET **NPX** **IBP** **DCF**	USE (SPE)	GC-MS	0.04	7 83 61 62	[28]
KET, **NPX** **IBP** **DCF**	MAE (DME + SPE)	GC-MS	0.5	19 15 20 22	[30]
KET, NPX, IBP	MAE (SPE)	GC-MS	3	-	[31]
KET **NPX** **IBP** **DCF**	MAE (SPE)	GC-MS	5	80 50 60 30	[32]
KET **NPX** **IBP** **DCF**	PLE (SPE)	LC-MS/MS	1	7 9 30 7	[33]
KET **NPX** **IBP** **DCF**	PLE (SPE)	LC-MS/MS	1	0.56 -0.93 0.07-0.84 0.1-0.12 0.03-0.94	[23]
KET **NPX** **IBP** **DCF**	PLE (SPE)	LC-MS/MS	1	25.7-51.8[a] 65.2-70.4[a] 63.6-89.2[a] 68.7-96.3[a]	[35]
NPX **IBP** **DCF**	PLE (-)	LC-MS	5	32[a] 29[a] 22[a]	[36]
KET **NPX** **IBP** **DCF**	PHWE (HF-LPME)	LC-MS	0.5	3.7 1.7 1.4 0.4	[37]

[a]Method limit of quantitation

USE combined with SPE has been widely applied for the determination of NSAIDs in sludge [25]. Lin et al. [17] applied a modified EPA Method 1694 for studying the sorption and degradation of pharmaceuticals in soil samples. The method consists of USE with a phosphate buffer at pH 2/methanol (3/4, v/v) followed by centrifugation, filtration and rotary evaporation of the extract. Then SPE (Oasis HLB) and HPLC-MS/MS were applied. The recoveries obtained were 85 – 88% for DCF, 94 - 107% for IBP and 95 - 105% for NPX with an instrumental limit of detection (ILOD) of 5 µg L^{-1}. Xu et al. [26] applied USE and SPE followed by GC-MS for the determination of the four NSAIDs in soil. The best recovery values were about 85 – 103%, 75 – 111%, 85 – 105% and 50 - 100% for IBP, NPX, KTP and DCF and were achieved with a mixture of acetone-ethyl acetate for clay and sandy soil. Using the same techniques, Yu et al. [27] developed a multi-residue method for the determination of the target NSAIDs, other pharmaceuticals, triclosan and endocrine disruptors in sludge. Under the best extraction conditions, they tested three sample sizes (0.5, 1 and 3 g) and observed that with 3 g low recoveries and worse peak shapes were obtained while for 0.5 and 1 g no differences were observed. Moreover, it was necessary to apply derivatization because the determination was carried out by GC-MS and they observed that clean-up of the sample was necessary in order to achieve complete derivatization of the extracts and improve the profile of the chromatograms. Recoveries were between 84 and 99% with method limits of detection (MLODs) of 2, 2.2, 5 and 11 ng g^{-1} for IBP, KTP, NPX and DCF. The compounds were determined in four sewage sludge samples with concentration levels of 11.1 - 35.1 ng g^{-1} for NPX and <MLOQ - 23.2 ng g^{-1} for KTP, and a wide range concentration for IBP (27.1 – 208 ng g^{-1}) and DCF (86.6 – 421 ng g^{-1}). Samaras et al. [28] also applied USE combined with SPE for NSAIDs with MLODs between 18 - 25 ng g^{-1} for the four NSAIDs in sewage sludge using a compromise determination sample amount of 0.04 g. They studied 0.04, 0.07 and 0.1 g with similar results for NSAIDs.

Microwave assisted extraction has been used as an extraction technique in several studies for the determination of organic pollutants such as flame retardants, alkylphenol ethoxylates, biocides or antibiotics [29]. Dobor et al. [30] developed a new

method for the determination of the selected NSAIDs in activated sludge and mixed sludge (from primary and secondary treatment) by MAE using water as extractant and thus avoiding the use of organic solvents. However, fats, oils and detergents were present in the extracts. For this reason two clean-up steps were applied, colloidal fraction was removed by dispersive solid-phase extraction with alumina, and then SPE was carried out. Recoveries were of about 85% and MLODs of 15 - 22 ng g^{-1}. In the study by Rice et al. [31] MAE using methylene chloride:methanol as solvent mixture and clean-up with a silica microcolumn was applied for the determination of NPX, IBP and KTP in soil with recoveries of lower than 35%. Antonić et al. [32] compared soxhlet, PLE combined with supercritical fluid extraction and MAE for the extraction of NPX, IBP, KTP and DCF in sediments; and although they found similar results for PLE and MAE this last technique was preferred because of the low solvent consumption.

PLE combined with SPE is often applied for the determination of pharmaceuticals in solid samples according to several reports [33,34]. Jelić et al. [23] optimized a multi-residue method with PLE, SPE and LC-MS/MS equipped with a hybrid quadrupole-linear ion trap mass spectrometer. Standard addition and internal standard were used in order to correct ion suppression and low limits of detection were achieved with values of 0.03 - 0.93 ng g^{-1} for KTP, NPX, IBP and DCF. Concentration levels in sludge were determined in the following ranges: <MLOD -21.1, <MLOD – 5.9, 43 - 117 and 27 - 69 ng g^{-1} for KTP, NPX, IBP and DCF, respectively. PLE combined with SPE and LC-MS/MS with standard addition were also applied by Radjenović et al. [35] with MLOQs for the determination of IBP, KTP, NPX and DCF of 63.6, 51.8, 65.2 and 96.3 ng g^{-1} in treated sludge, and 89.2, 25.7, 79.4, 68.7 ng g^{-1} in primary and secondary sludge. Mean concentrations found in treated sludge were 299.3 ng g^{-1} for IBP, 30.3 ng g^{-1} for KTP and 192.8 ng g^{-1} for DCF.

Another approach based on PLE without a clean-up step has been developed by Nieto et al. [36]. NPX, DCF and IBP and other pharmaceuticals were extracted by PLE with a mixture of MeOH/water (H_3PO_4 50 mM). Recovery values were about 89% with MLOQs

of 32, 29 and 22 ng g^{-1} and concentrations below 242, 183 and 99 ng g^{-1} for NPX, DCF and IBP, respectively.

Moreover, pressurized hot water extraction (PHWE) followed by HF-LPME and LC-ESI-MS has been applied recently for the extraction of the four target NSAIDs in sewage sludge. Saleh et al. [37] developed a method based on PHWE with 0.01 M NaOH with native recovery yields of 39, 48, 60 and 90% for KTP, IBP, NPX and DCF. For HF-LPME, enrichment factors (Ee) were in the range 947 – 1,213. Ion suppression for KTP and NPX (-3.8 and -8.9%) and enhancement for DCF (14.6%) were observed and for this reason standard addition was applied. MLODs were 0.4, 1.4, 1.7 and 3.7 ng g^{-1} dry weight and the concentrations found were 51.3 - 89.6 ng g^{-1} (KTP), 7.7 - 14.1 ng g^{-1} (NPX), 13.7 - 22.9 ng g^{-1} (DCF) and 304 - 588 ng g^{-1} (IBP).

The objective of the present study was to develop a simple method based on three-phase HF-LPME combined with LC-MS for the determination of some NSAIDs in sewage sludge in order to reduce the number of steps required for the determination of pharmaceuticals in solid samples. In this way, pre-treatment, extraction and clean-up steps can be combined into one step as shown in Figure 5.1.

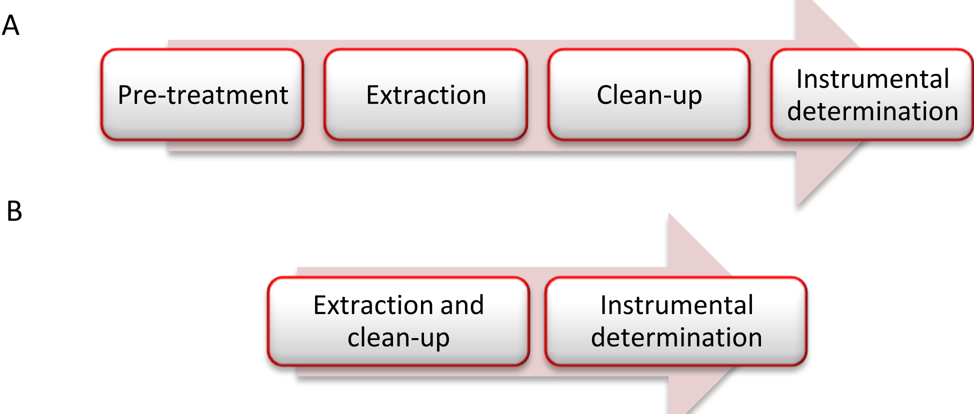

Figure 5.1. Diagram of the typical analytical process for solid samples (A) and the one applied in this chapter (B).

5.2. PRINCIPLE

As has been explained in Chapter 4, three-phase HF-LPME can be used as a clean-up and enrichment technique for acidic pharmaceuticals in water samples. In this system a combination of two processes was carried out: extraction of the analytes from an aqueous sample to the organic phase and back-extraction from the organic phase to the acceptor phase. For the extraction of analytes from water samples, two equilibriums are achieved: donor phase-organic phase and organic phase-acceptor phase.

In this Chapter, three-phase HF-LPME has been used as the extraction, clean-up and preconcentration technique by adding a new phase. A solid phase (sludge) is added in the aqueous solution (donor solution) without modifying the hollow fiber system, as can be seen in Figure 5.2.

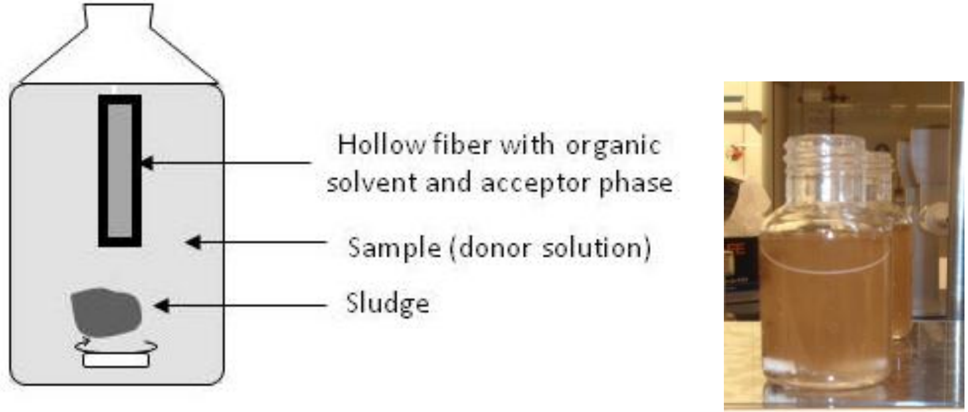

Figure 5.2. Diagram of the HF-LPME system for the determination of analytes in solid samples.

Therefore, four phases are present in the system: a solid phase (sludge), an aqueous phase (donor solution), a membrane phase (organic solvent) and an aqueous phase (acceptor solution). In this system, the analytes are distributed from the solid phase to the aqueous phase, then extracted from the aqueous phase to the organic solvent and, finally, transferred from the organic phase to the acceptor phase which is analyzed by LC. Hence, in the HF-PLME procedure there are three equilibriums which are related as follows:

$$\mathrm{A_{Se}} \leftrightarrow \mathrm{A_{De}} \leftrightarrow \mathrm{A_{Me}} \leftrightarrow \mathrm{A_{Ae}} \qquad (5.1)$$

Where A_{Se} is the target analyte in the sewage sludge, A_{De} is the target analyte in the donor solution, A_{Me} is the target analyte in the membrane phase and A_{Ae} is the analyte in the acceptor solution.

In order to achieve the equilibrium between the solid phase and the donor phase, sludge has to be mixed in water before the extraction process. For this reason the solid sample and the water are left in contact overnight which would be enough to attain equilibrium.

5.3. THEORETICAL BASIS

In three-phase HF-LPME the analytes are extracted from the aqueous sample or donor solution through the organic solvent to the acceptor phase present inside the lumen of the hollow fiber. After some time, equilibrium of the compound between the acceptor solution, organic solvent and donor solution is achieved and can be written as:

$$K_{AD} = \frac{C_{\mathrm{Ae}}}{C_{\mathrm{De}}} = \frac{m_{\mathrm{Ae}} . v_{\mathrm{D}}}{m_{\mathrm{De}} . v_{\mathrm{A}}} \qquad (5.2.)$$

Where K_{AD} is the acceptor-donor partition coefficient for the compound, which is determined by the conditions in the donor and acceptor phases, C_{Ae} is the concentration in the acceptor solution at equilibrium, C_{De} is the concentration in the donor solution at equilibrium, m_{Ae} is the amount in the acceptor solution at equilibrium, m_{De} is the amount in the donor solution at equilibrium, v_{D} is the volume of the donor solution and v_{A} is the volume of the acceptor phase.

Moreover, in slurry samples under equilibrium conditions the concentration in the solution is assumed to be proportional to the concentration in the sludge (Eq. (5.3)):

$$K_{\mathrm{SD}} = \frac{C_{\mathrm{Se}}}{C_{\mathrm{De}}} = \frac{m_{\mathrm{Se}} . v_{\mathrm{D}}}{m_{\mathrm{De}} . w_{\mathrm{S}}} \qquad (5.3)$$

Where K_{SD} is the sludge-donor partition coefficient, C_{Se} is the concentration in the sludge at equilibrium, m_{Se} is the amount in the sludge at equilibrium and w_{S} is the total

amount of sludge. It has to be taken into account that K_{SD} is not the sludge-water distribution coefficient which is relevant in the environment, since the charge of the NSAIDs, as well as ionizable groups in the sludge, are pH dependant. Moreover, at a pH of below 2 the properties of sludge, such as color, smell and physical appearance, change [30].

In this study, a mass balance between the initial and final amount of each compound in the whole system is used for the determination of the initial concentration of the compound in sewage sludge:

$$m_{\mathrm{Si}} + m_{\mathrm{Di}} = m_{\mathrm{Se}} + m_{\mathrm{De}} + m_{\mathrm{Ae}} + m_{\mathrm{Me}} \quad (5.4)$$

where m_{Si} is the initial amount of the compound in the sludge, m_{Di} is the spiked amount of the compound in the slurry sludge and m_{Me} signifies the amount of the compound in the membrane liquid.

According to Eq. (5.2) and (5.3), m_{Ae}, m_{De}, m_{Se} and m_{Me} can be defined as:

$$m_{\mathrm{Ae}} = C_{\mathrm{Ae}} \cdot v_{\mathrm{A}} \quad (5.5)$$

$$m_{\mathrm{De}} = \frac{m_{\mathrm{Ae}} \cdot v_{\mathrm{D}}}{K_{\mathrm{AD}} \cdot v_{\mathrm{A}}} \quad (5.6)$$

$$m_{\mathrm{Se}} = \frac{m_{\mathrm{De}} \cdot w_{\mathrm{S}} \cdot K_{\mathrm{SD}}}{v_{\mathrm{D}}} \quad (5.7)$$

$$m_{\mathrm{Me}} = \frac{m_{\mathrm{De}} \cdot v_{\mathrm{M}} \cdot K_{\mathrm{MD}}}{v_{\mathrm{D}}} \quad (5.8)$$

where K_{MD} is the partition coefficient between the membrane liquid and the water sample (donor) in analogy with Eq. (5.2) and v_M is the volume of the membrane liquid.

Eq. (5.5), (5.6), (5.7) and (5.8) combined with Eq. (5.4) we get:

$$m_{\mathrm{Si}} + m_{\mathrm{Di}} = C_{\mathrm{Ae}} \cdot v_{\mathrm{A}} \cdot (1 + \frac{K_{\mathrm{SD}} \cdot w_{\mathrm{S}}}{K_{\mathrm{AD}} \cdot v_{\mathrm{A}}} + \frac{v_{\mathrm{D}}}{K_{\mathrm{AD}} \cdot v_{\mathrm{A}}} + \frac{K_{\mathrm{MD}} \cdot v_{\mathrm{M}}}{K_{\mathrm{AD}} \cdot v_{\mathrm{A}}}) \quad (5.9)$$

which can be written as:

$$C_{\mathrm{Ae}} = \frac{m_{\mathrm{Di}}}{A} + \frac{m_{\mathrm{Si}}}{A} \quad (5.10)$$

$$A = (v_A + \frac{K_{\mathrm{SD}} \cdot w_{\mathrm{S}} + v_{\mathrm{D}} + K_{\mathrm{MD}} \cdot v_{\mathrm{M}}}{K_{\mathrm{AD}}}) \quad (5.11)$$

Thus, the initial amount of the compound in the sludge can be predicted if Eq. (5.10) is used to plot a calibration curve with the spiked m_{Di} as *x* and the measured C_{Ae} as *y*. Then the amount of the compound in the sludge m_{Si} can be obtained as the intercept divided by the slope of the line.

5.4. EXPERIMENTAL

5.4.1. CHEMICALS AND STANDARDS

Methanol (MeOH) analytical reagent grade was obtained from Fisher Scientific (Pittsburgh, PA, USA). Ammonium carbonate ($(NH_4)_2CO_3$, containing 30 - 33% NH_3), di-*n*-hexyl ether (DHE) and sulfuric acid (95 - 97% pure) were supplied by Fluka (Buchs, Switzerland). KTP, NPX, IBP and diclofenac sodium salt (DCF) were obtained from Sigma Aldrich Inc (St. Louis, MO, USA). Glacial acetic acid, ammonium acetate and formic acid (98 - 100% pure) were purchased from Merck (Darmstadt, Germany). Reagent water was obtained from a MilliQ water purification system (Millipore, Billerica, MA, USA).

Individual stock standard solutions containing 100 mg L^{-1} of KTP, NPX, IBP and DCF were prepared in MeOH. Working solutions of IBP alone and a mixture of all four NSAIDs studied were prepared by the appropriate dilution of individual stock solutions in reagent water. The standards for calibration curves were prepared by diluting individual stocks in 0.1 mol L^{-1} ($(NH_4)_2CO_3$). The solutions were stored under

refrigeration at 4 ºC in the dark. The acceptor buffer solution (0.1 mol L^{-1} $(NH_4)_2CO_3$) was prepared by dissolving the appropriate amount of salt in reagent water.

The dry weight of the sludge was determined by weighing and drying at room temperature for several days.

5.4.2. SAMPLING SITE AND SAMPLING PROCEDURE

Källby WWTP is situated in the city of Lund in southern Sweden and treats the sewage of a population of 84,000. The sewage water undergoes primary sedimentation, biological treatment with activated sludge and finally chemical precipitation of phosphate. The sludge from the biological and chemical treatment steps are returned to incoming water and sludge is only removed from the system during primary sedimentation. After dewatering the sludge is anaerobically digested under mesophilic conditions (37 ºC) for 20 - 30 days, after which it undergoes further dewatering; resulting in a final product with a dry substance content of approximately 25%. Källby WWTP produces approximately 5,000 tons of sludge each year and since 2009 this sludge has been used as a fertilizer in agriculture. In a previous study [38], it was shown that all four NSAIDs occur in the incoming sewage water at Källby in concentrations ranging from 0.2 µg L^{-1} for DCF to 4 µg L^{-1} for IBP. The removal efficiency was calculated to be 22% for DCF, 65% for KTP, 93% for NPX and 96% for IBP. However, since no sludge analysis was performed, it was not determined to what extent these substances are adsorbed onto the sludge.

Sampling of the final sludge was performed in October 2009. The samples were collected in plastic bottles and transported to the laboratory. The samples were stored refrigerated in sealed bottles at 4 ºC until analysis.

5.4.3. HF-LPME

HF-LPME was performed by 50/280 Accurel polypropylene hollow fiber membranes with a wall thickness of 50 µm (0.1 µm pore size) and an internal diameter of 280 µm (Membrana, Wuppertal, Germany).

Before the membrane extraction, an amount of homogeneous sewage sludge (0.5, 1 or 1.5 g) was put into 50 mL of reagent water and stirred for 17 hours at 660 rpm to reach equilibrium. Afterwards, some of the slurry samples were spiked at three levels (0.5, 0.8 and 1 ng mL^{-1}) and the spiked and non-spiked samples were subjected to the extraction procedure. All experiments were performed with the same sludge with a dry weight of 29.2%.

For HF-LPME, a fiber length of 18 cm was selected to provide a volume of 10 µL of acceptor phase consisting of 0.1 mol L^{-1} of $(NH_4)_2CO_3$ at pH 9. After cutting the fiber, one of the ends was connected to a syringe (BDM Micro-Fine, Sweden) with a needle diameter of 0.3 mm, holding 0.5 mL of acceptor phase and the lumen of the hollow fiber was filled with the acceptor phase. Next, the fiber was dipped into DHE for 1 minute to impregnate the fiber pores and the excess of organic solvent in the lumen was rinsed with 0.3 mL of the remaining acceptor phase in the syringe. Then, the membrane was held by a metal wire and the two ends were folded with a piece of aluminum foil to seal the ends. Excess organic solvent was removed by the immersion of the fiber in reagent water for 30 seconds and the fiber was immersed into 50 mL of the donor solution adjusted to pH 1.5 with sulfuric acid in glass bottles covered with aluminum foil to prevent photodegradation.

Extraction experiments were carried out using magnetic stirring (RO10 power, IKA-Werke, Staufen, Germany) at 660 rpm for several hours and after the extraction, the acceptor solution was collected in vials by pushing air through the fiber with a syringe. The final volume of the acceptor phase was about 10 µL and it was analyzed directly by liquid chromatography. Prior to analysis some of the acceptor solutions obtained were diluted with 0.1 mol L^{-1} $(NH_4)_2CO_3$.

To compare experiments, both enrichment factor *(Ee)* and extraction efficiency *(E)* were used. These are defined as in Eq. (1.1) and Eq. (1.2) from Chapter 1.

5.4.4. LC-ESI-MS

For the determination of NSAIDs in sludge, an LC system composed of two Waters 515 pumps (Waters, Milford, MA, USA), a vacuum degasser, a Triathlon autosampler (Spark-Holland, Emmen, Netherlands), an ODS-2 Hypersil column (5µm, 100 × 2.1 mm, (Thermo Scientific, Waltham, MA, USA), a C_8 precolumn (Phenomenex, Torrance, CA, USA) and a single quadrupole mass spectrometer (Waters Micromass ZMD) with electrospray ionization interface (ESI) was used.

Chromatographic separation of the four analytes was achieved by an isocratic elution using a mobile phase of 65% of MeOH and 35% of 10 mmol L^{-1} ammonium acetate adjusted to pH 4 with acetic acid in reagent water at a flow rate of 0.4 mL min^{-1}. The injection was made in ”pick-up” mode with 5 µL of sample followed by 10 µL of mobile phase.

Data acquisition was performed in negative ion mode and MS parameters for the analysis were the following: capillary voltage 3.08 kV, cone voltage 9 V, ESI source block temperature 150 ºC, desolvation temperature 325 ºC, desolvation gas flow 535 L h^{-1}. Single ion monitoring was used to detect ions with m/z ratios of 253 (KTP), 229 (NPX), 294 (DCF) and 205 (IBP).

5.5. Results and discussion

5.5.1. LC-MS method development

For chromatographic analysis by LC-MS, linearity was assessed by using standard solutions ranging from 0.15 to 0.8 mg L^{-1} with an upper limit of 1 mg L^{-1}. Calibration curves were constructed for each compound with r^2 >0.99, see Table 5.2. ILODs and ILOQs were calculated as 3 and 10 times the background noise with values of about 10 µg L^{-1} and 33 µg L^{-1}, respectively for KTP, NPX, DCF and IBP. The intra-day precision and inter-day precision for each compound are shown in Table 5.2. A chromatogram of reagent water spiked at 0.4 mg L^{-1} is shown in Figure 5.3.

Table 5.2. Coefficient of determination (r^2), intra-day precision and inter-day precision of NSAIDs in LC-MS method (N=3).

Analyte	r^2	*Intra-day precision (%)*	*Inter-day precision (%)*
KTP	0.991	1.9	17
NPX	0.994	2.5	14
DCF	0.992	0.5	19
IBP	0.993	7	13

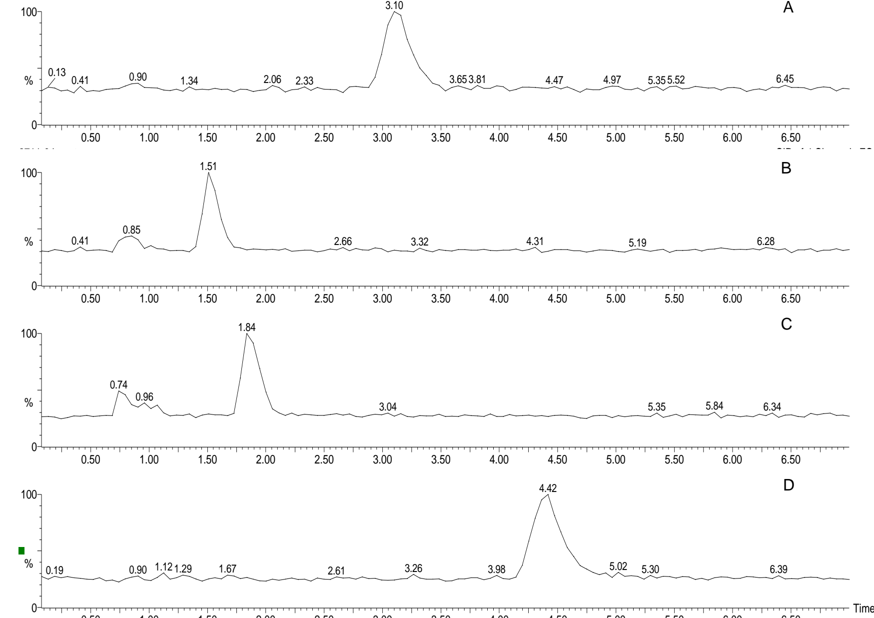

Figure 5.3. Single ion monitoring (SIM) chromatograms obtained by LC-MS from reagent water spiked at 0.4 mg L^{-1}. (A) m/z=294 (DCF), (B) m/z=253 (KTP), (C) m/z=229 (NPX) and (D) m/z= 205 (IBP).

5.5.2. EXTRACTION OF IBUPROFEN BY HF-LPME

5.5.2.1. Extraction time

In order to test the influence of the extraction time, reagent water spiked at 1 µg L^{-1} of IBP and sludge slurry samples with 1% of sludge spiked at 1 µg L^{-1} of IBP were extracted at different times of between 1 and 8 hours. As can be seen in Figure 5.4, the enrichment factor increased from 1 to 2 hours while no increase was observed afterwards indicating that equilibrium was attained. After 5 h the performance of the system shows a decrease of stability due to possible organic losses. The extraction time chosen for further experiments was 3 hours with average enrichment factors of 3,052 and 1,363 times for reagent water and sludge slurry, respectively. The decrease

of the enrichment factor in sludge slurry is assumed to be due to sorption of the analyte by the sludge particles.

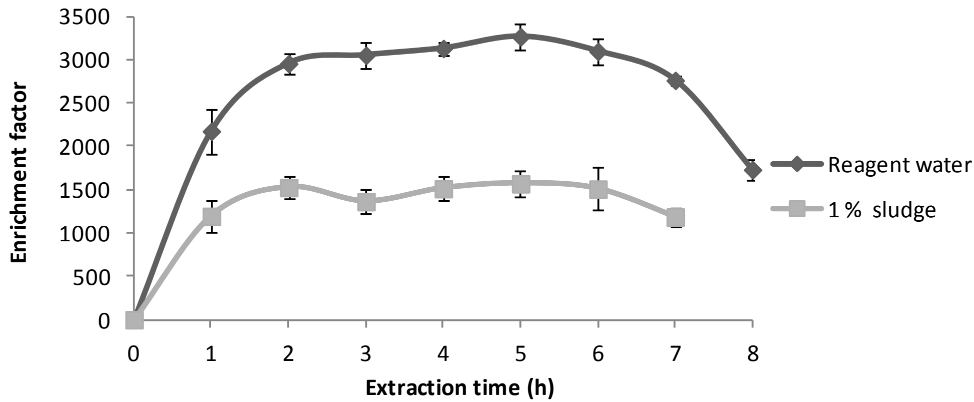

Figure 5.4. Extraction time profiles for ibuprofen in spiked reagent water and sludge slurry samples with 1% of sludge at a concentration of 1 μg L^{-1} (N=2).

In reagent water the average extraction efficiency was 67% and for the acceptor-donor partition coefficient (K_{AD}) a value of 7,821 was obtained. This value was calculated from Eq. (5.2), assuming that the influence of the membrane liquid can be neglected.

5.5.2.2. Analytical performance

Under the aforementioned conditions, the performance of HF-LPME extraction was evaluated with reagent water spiked at 3 different levels (0.5, 0.8 and 1 μg L^{-1}) with an extraction time of 3 hours. Good linearity (r^2=0.9939) was obtained with an intra-day precision (N=2) and inter-day precision (N=3) of 3% and 5%, respectively, while for 1% slurry samples, values of 3% and 10%, respectively, were obtained.

5.5.2.3. Concentration in sludge

The method developed was applied for the determination of the concentration of IBP in sewage sludge. For this purpose, different amounts of the analyte were added to slurry samples with three different quantities of sludge (1, 2 and 3%, corresponding to 0.5, 1.0 and 1.5 g wet weight, respectively). The results obtained can be observed in Figure 5.5, which shows that when the amount of sludge increased, a decrease of concentration in the acceptor phase was found. It can be observed that for reagent

water (0% sludge) the extraction efficiency was higher than for sludge. For each amount of sludge a linear regression between the amount spiked and the concentration obtained in the acceptor can be found as expected from Eq. (5.10).

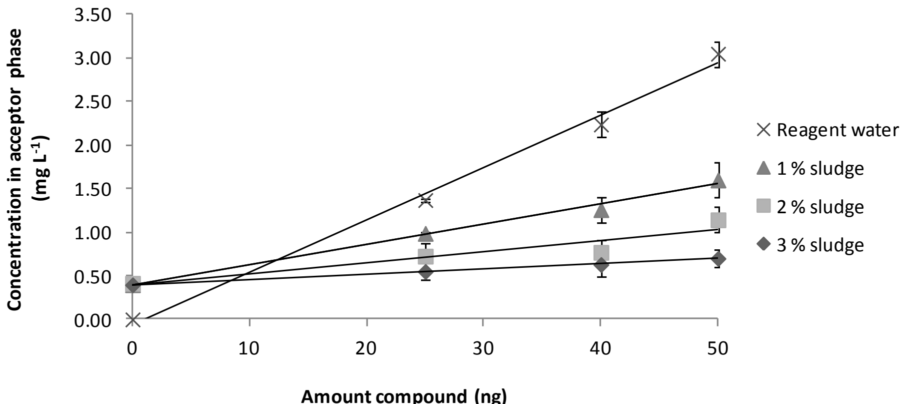

Figure 5.5. Concentration of IBP obtained in the acceptor phase as a function of the amount of analyte added in the slurry sample for different quantities of sludge (N=3).

In Table 5.3, coefficients of determination are presented with values of higher than 0.88. Also, the values of the slope and intercept are shown with an intercept close to 0 for reagent water and practically equal intercepts for the sludge samples. By applying Eq. (5.10), the initial amount of IBP was calculated for each amount of sludge added. The average concentration found in wet sludge was 36 ng g^{-1} with a relative standard deviation of 20%.

Table 5.3. Coefficient of determination (r^2), slope and intercept for the regression lines in Figure 5.5, m_{Si}, C_{Si} and average C_{Si} with relative standard deviation (RSD) in brackets.

Sludge (%)	r^2	*Slope*	*Intercept*	m_{Si} *(ng)*	C_{Si} *(ng g^{-1})*	**Average C_{Si} (ng g^{-1})**
0	0.994	0.0598	-0.055			
1	0.992	0.0233	0.3922	17	34	
2	0.884	0.0129	0.3923	30	30	36 ± 7 (20%)
3	0.999	0.0060	0.3971	66	44	

5.5.3. EXTRACTION OF NSAIDS BY HF-LPME

5.5.3.1. Extraction time

Figure 5.6 shows the influence of the extraction time on the enrichment factor for all the NSAIDs chosen. The extraction was carried out with reagent water spiked at 1 μg L^{-1}. The analysis was made with LC-MS as described above. Optimum enrichment factors were obtained after 3 h for naproxen, diclofenac and ibuprofen while for ketoprofen the maximum value was achieved after 5 hours. However, during prolonged extraction time a decrease in the stability of the system was observed, so, consequently, an extraction time of 4 h was selected.

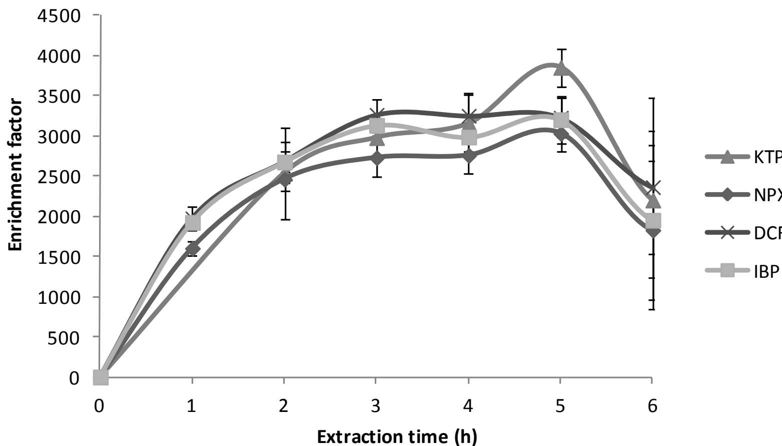

Figure 5.6. Extraction time profiles for spiked reagent water at concentrations of 1 μg L^{-1} (N=2).

In spiked reagent water the average enrichment factor values for all the studied compounds ranged from 2,761 to 3,158 (Table 5.4). In Table 5.4 the extraction efficiency is shown with values of between 53% and 61%. Acceptor-donor partition coefficients are also shown. These are calculated from Eq. (5.2), assuming that the influence of the membrane liquid can be neglected.

Table 5.4. Average enrichment factor (Ee), extraction efficiency (E) and acceptor-donor partition coefficient (K_{AD}) for NSAIDs extracted from reagent water (N=2).

Analyte	*Ee*	*E (%) (RSD in brackets)*	K_{AD}
KTP	3,158	61 (8.5%)	8,569
NPX	2,761	53 (10.1%)	6,164
DCF	3,254	62 (9.7%)	9,318
IBP	2,989	57 (8.7%)	7,433

5.5.3.2. Analytical performance

To evaluate the hollow fiber technique for KTP, NPX, DCF and IBP after 4 h of extraction, intra-day precision and inter-day precision in reagent water and slurry samples at 1% of sludge were tested (Table 5.5). Values for intra-day precision and inter-day precision were 2.5 - 12% and 6 - 12% for reagent water and 10 - 18% and 7 - 16% for slurry sludge at 1%, respectively.

Table 5.5. Method intra-day precision and inter-day precision (N=2) as standard deviations for reagent water and slurry spiked at 1 μg L^{-1}.

Analyte	*Intra-day precision (%)*		*Inter-day precision (%)*	
	Reagent water	*1% sludge*	*Reagent water*	*1% sludge*
KTP	2.5	14.0	11.7	6.8
NPX	7.1	17.7	7.9	9.2
DCF	12	9.7	8.7	15.5
IBP	5.3	10.3	5.8	10.7

5.5.3.3. Concentration in sludge

Figure 5.7 shows the correlation obtained for KTP and DCF with different amounts of sludge and two different spiked levels. It can be seen that in non-spiked samples KTP was not detected, therefore linear correlation was obtained using only 2 points. For the other compounds r^2 was better than 0.965. Also, for NPX in slurry samples with 2% sludge an instrumental problem occurred and for this reason the corresponding values are not shown in Table 5.6. The relative standard deviations of the concentrations in the acceptor phase range from 2 to 15%, 12 to 18%, 2 to 15% and 6 to 14% for KTP, NPX, DCF and IBP, respectively. Method limits of detections in dried sludge were calculated as taking instrumental limits of detection as a concentration in the y-axis to the x-axis, where it becomes an amount in the sludge, and then divided it by the sludge volume; the values were between 1.1 - 1.9 ng g^{-1} for KTP, 1.2 - 1.9 ng g^{-1} for NPX, 3.4 - 5.6 ng g^{-1} for IBP and 2.8 – 3.1 ng g^{-1} for DCF; which are in the same range as the methods reported in the literature (Table 5.1).

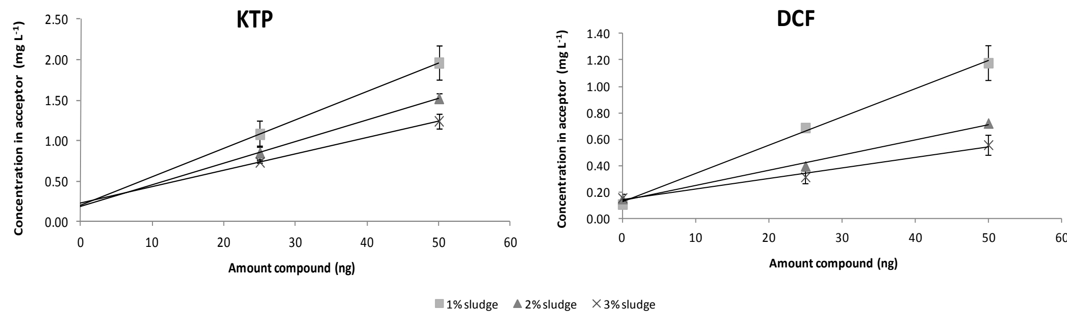

Figure 5.7. Concentration obtained in the acceptor phase as a function of the amount of analyte added in the slurry sample for different quantities of sludge for KTP and DCF (N=2).

Table 5.6. m_{Si}, C_{Si} and average C_{Si} with standard deviation of NSAIDs found in wet sewage sludge and concentration in dry sludge (RSD in brackets).

Analyte	*Sludge (%)*	*m_{Si} (ng)*	*C_{Si} (ng g^{-1})*	*Average C_{Si} (ng g^{-1})*	*Concentration in dry sludge (ng g^{-1} d.w.)*
	1	5.7	11.5		
KTP	2	6.7	6.7	9 ± 3 (30%)	29 ± 9 (30%)
	3	11.3	7.5		
NPX	1	20	40	40.4 ± 0.6 (1.4%)	138 ± 2 (1.4%)
	3	61	40.8		
	1	5.1	10.2		
DCF	2	12.9	12.9	12 ± 1 (11.5%)	39 ± 5 (11.5%)
	3	16.9	11.3		
	1	16.9	33.7		
IBP	2	37.7	37.7	36 ± 2 (5.6%)	122 ± 7 (5.6%)
	3	53.3	35.5		

When applying Eq. (5.10), the average concentrations detected in sludge were obtained and are given in Table 5.6. It should be noted that the result for IBP is similar to the one obtained when IBP was determined without the other NSAIDs (see 5.5.2.3). Finally, concentrations of these compounds in dried sewage (d.w.) sludge were calculated.

Earlier reported data regarding occurrence of NSAIDs in digested sludge from other Swedish WWTP [38] shows concentrations in the range: 5 - 580 ng g^{-1} for KTP, 3 - 350 ng g^{-1} for NPX, 4 - 77 ng g^{-1} for DCF and 4 - 560 ng g^{-1} for IBP. Another study in Källby WWTP from April to August 2010 by Saleh et al. [37] shows similar concentration levels for the same analytes as the ones obtained in this study with values in the range 57.7 - 89.6 ng g^{-1} for KTP, 7.7 – 14.1 ng g^{-1} for NPX, 18.8 - 22.9 ng g^{-1} for DCF and 304.3 - 587

ng g^{-1} for IBP. The values obtained in this study lie quite well within these ranges, however it has to be noted that the ranges of these literature values are very wide. For ketoprofen, similar concentrations to our study have been determined with values of below 21.1 ng g^{-1} [23], below 23.2 ng g^{-1} [27], 67 - 76 ng g^{-1} [30] and 30.3 ng g^{-1} [35]. For naproxen, McClellan et al. [39] reported mean concentrations of 119 ± 79 ng g^{-1} in biosolids from USA whereas other studies have detected levels of a few ng g^{-1} [23,27,30,35,36]. For diclofenac, Jelić et al. [23] and Dobor et al. [30] determined it in ranges from 64 - 73 ng g^{-1} and 27 - 69 ng g^{-1}, whereas Radjenović et al. [35] found it with a mean concentration of 192.8 ng g^{-1} and Yu et al. [27] a wide range from 86.6 and 142 ng g^{-1}. Finally, the same tendency has been observed for IBP; high and low concentrations have been reported with values of 299.3 ng g^{-1}, 246 ± 121 ng g^{-1}, 27.1 - 208 ng g^{-1}, 43 - 117 ng g^{-1} and 21 - 28 ng g^{-1} [23,27,30,35,39]. This could, however, be attributed to differences in the treatment processes at the different WWTPs and in the sludge composition investigated or perhaps in the analytical methods applied. It definitely underlines the need for further investigation of the pharmaceutical content of sludge, a process in which the method developed in this study, which is simple, environmentally friendly and does not require too much manipulation, could aid. Several tons of sewage sludge are today spread on farmland each year and precise and accurate measurements of its pharmaceutical content is a crucial parameter in conducting high quality risk assessments of this use.

5.6. CONCLUSIONS

A new method for the determination of some NSAIDS in sewage sludge was developed. Hollow fiber liquid-phase microextraction was applied successfully as an extraction and clean-up technique for acidic compounds. High enrichment factors, about 3,000, were obtained for all analytes in reagent water.

The method developed allows the application of water for the extraction of the analytes from sewage sludge and the quantitation of ketoprofen, naproxen, diclofenac and ibuprofen using an equilibrium system. Different amounts of sludge were used and the same concentrations were found in all cases. Concentrations of about 29, 39, 122

and 138 ng g^{-1} d.w. were measured for ketoprofen, diclofenac, ibuprofen and naproxen, respectively. This is in agreement with other reports which have found similar concentrations of these analytes in sewage sludge.

5.7. REFERENCES

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CHAPTER 6:

Comparison of two extraction approaches for the determination of antidepressants in sewage sludge using hollow fiber liquid-phase microextraction

6.1. INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs), which include citalopram (CTP), paroxetine (PRX), fluoxetine (FLX) and sertraline (SRT), are among the most prescribed psychiatric drugs worldwide [1–6]. This class of pharmaceuticals is not completely metabolized in the human body and they are excreted as the parent compounds or as metabolites reaching wastewater where they are detected, although the percentage of non-metabolized compounds in urine is low [4,7–10]. Norfluoxetine (NFX) is one N-desmethyl product of FLX which is also biologically active, but it is less potent than the parent compound itself.

As has been explained previously, sludge is a potential route for pharmaceuticals to reach the environment. The adsorption coefficients of SSRIs on sediments are high and, as a consequence, the distribution of these compounds between solid and aqueous phases can be done rapidly [11]. The most studied SSRI is FLX. Zorita et al. [12] evaluated the fate of FLX in a wastewater treatment plant (WWTP) and observed a high removal of FLX in primary sludge. Furthermore, different wastewater treatment processes have been evaluated for the removal of FLX with reported efficiencies of 92% in adsorption processes [4]. FLX is recalcitrant to biodegradation processes, photolysis and hydrolysis [13–14], and in the first 24 h it is rapidly partitioned to sediment (37 - 65%); therefore sorption seems to be the main removal path. Kwon et al. [15] studied the sorption of other SSRIs in sediment and sludge and found that pH is an important parameter influencing sorption.

There is little information about the presence of SSRIs in sewage sludge from WWTPs. FLX has been detected at high concentrations in biosolids in the 100 - 4700 ng g^{-1} range [16], with mean and maximum concentrations of 171 and 258 ng g^{-1} [17]. Radjenović et al. [18] determined the concentration of FLX at levels of 72, 92 and 123 ng g^{-1}, and for PRX the concentrations found were 52, 60 and 41 ng g^{-1} in primary, secondary and treated sludge, respectively. In another study, CTP was measured at 168 and 317 ng g^{-1} in sewage sludge [19].

Furthermore, while SSRI concentrations found in environmental matrices might not be high enough to cause negative effects, the organisms are also exposed to other compounds that might exert additive effects when combined with SSRIs [4,17,18,20]. Therefore, it is necessary to determine the presence and levels of pharmaceuticals in sewage sludge. To this end, different analytical methodologies have been applied.

6.1.1. SAMPLE PREPARATION

The most commonly used techniques for the extraction and clean-up of pharmaceuticals and personal care products in solid samples are pressurized liquid extraction (PLE), microwave assisted extraction (MAE) or ultrasound solvent extraction (USE) used alone or in combination with solid-phase extraction (SPE) [21–24].

For SSRIs, PLE has been applied in several studies. Schultz et al. [2] determined the target compounds in sediments by PLE followed by LC-MS/MS. The extraction process was carried out with 20 g of wet sediment using acetonitrile/water (70:30, v/v) as solvent at 130 ºC and performing 3 extraction cycles. The obtained recoveries ranged from 32 to 66% and method limits of quantitation (MLOQs) were between 0.25 and 2.5 ng g^{-1} for sediments. The same technique was applied by Kinney et al. [16] for the extraction of FLX in biosolids with method limits of detection (MLODs) of 2.2 ng g^{-1} and recoveries of about 20 - 90% depending on the matrix. Kown et al. [13] also applied PLE to the study of the sorption of FLX and NFX in creek and lake sediments. Different extraction solvents (MeOH or a mixture of dichloromethane:acetone (70:30, v/v)) were necessary depending on the sample properties (organic matter content or ion-exchange capacity). Under these conditions recoveries were in the ranges of 87 - 94% and 80 – 81% for FLX and NFX, respectively, while MLOQ was 12 ng g^{-1} in wet sediment.

Langford et al. [19] determined CTP and FLX in sludge (1 g) and sediment (10 g) by PLE under the following conditions: MeOH, 70 ºC, static time 10 min and 2 extraction cycles. Next, extracts were evaporated and diluted with water prior to LC-MS/MS. Recoveries varied for the different matrices: for CTP they were in the ranges of 8 – 88% and 11 – 126% in sediments and sludge, respectively; while for FLX, recovery was 111%

in sediments. MLODs were 4 - 26 ng g^{-1} and 10 ng g^{-1} for CTP and FLX, respectively. Furthermore, in this study SPE was applied for the clean-up but no important improvement in terms of ion suppression in mass spectrometry detection was observed. PLE was also combined with SPE for the determination of pharmaceuticals, including PRX and FLX, in different types of sludge [18]. In this case, 1 g of sludge was extracted by PLE with MeOH/water (2:1, v/v) at 100 ºC. Then the extracts were submitted to clean-up using Oasis HLB cartridges. The SPE clean-up step applied did not prevent ion suppression in the LC-MS/MS analysis, therefore standard addition was used. In this study, it was also observed that for FLX and PRX (the less polar compounds) more than one cycle was needed in PLE. Recovery yields were in the 16 - 35% and 54 – 67% ranges, while MLOQs were 1.20 or 3.9 ng g^{-1}, and 0.66 or 1.74 ng g^{-1} for FLX and PRX, respectively. Chu et al. [25] determined PRX, FLX and NFX in fish by PLE using MeOH as the extraction solvent at 100ºC and 1500 psi followed by clean-up with a mixed-mode cation exchange sorbent (Oasis MCX). For water samples, Schultz et al. [9] compared Oasis HLB and Oasis MCX to better recoveries with Oasis HLB, although it was expected that antidepressants were positively charged. In all the cases, a wide range of recoveries was obtained depending on the sample matrix characteristics, with MLODs close to a few ng g^{-1}.

Finally, USE followed by SPE has also been used for the determination of FLX in biosolids. McClellan et al. [17] applied EPA method 1694 for FLX in biosolids; which consists of USE with acetonitrile, centrifugation, rotary evaporation and SPE with Oasis HLB followed by LC-MS/MS. In this method, 1 g of sludge was treated, obtaining a FLX recovery of 89% and a MLOD of 8.2 ng g^{-1}. Redshaw et al. [14] studied the biodegradation of FLX in sewage sludge-amended soil. The analysis started with the extraction with acetonitrile containing 1% of formic acid, centrifugation, and followed by a tandem SPE with Strata-SAX (anion exchange) and STRATA-X (polymeric reversed phase). In both methods high manipulation was required.

As has been explained in Chapter 1, an alternative technique for the extraction of organic pollutants in solid samples is pressurized hot water extraction (PHWE), which is

an organic solvent free extraction technique. PHWE is based on the use of water as the extraction solvent at elevated temperatures and under pressure to keep it in a liquid state. Under these conditions, the polarity of water can be reduced to close to the polarity of alcohols [26–29] and the aqueous extract obtained can be directly submitted to the clean-up step and subsequently to analysis by HPLC [26].

On the other hand, HF-LPME has been successfully applied in the extraction of five SSRIs from sewage sludge [8]. In this case, the analytes were first allowed to distribute between the sludge and acidified reagent water. Next, the sludge was removed by centrifugation and the remaining aqueous phase was subjected to HF-LPME.

The first goal of the present study was to evaluate direct HF-LPME as a combined extraction, clean-up and enrichment technique, followed by LC-ESI-MS for the simultaneous determination of CTP, PRX, SRT, FLX and NFX in sewage sludge. The second objective was to develop and validate an alternative procedure for the determination of the selected analytes by applying PHWE as the extraction technique followed by HF-LPME as a clean-up and concentration technique and LC-ESI-MS for the final determination. Finally, PHWE-HF-LPME and direct HF-LPME procedures were compared.

6.1.2. PRINCIPLES OF THE HF-LPME SYSTEM

The analytes of interest are basic compounds and can thus be extracted by three-phase HF-LPME by applying a pH gradient. The extraction principle is based on using a basic sample donor phase and an acidic acceptor phase. In this way, the analytes are uncharged in the donor sample and amines can pass through the membrane to the acceptor phase where the compounds are protonated and trapped as ions [30–32]. As the analytes end up in an aqueous solution, liquid chromatography can be applied directly for the analysis of SSRIs (Figure 6.1).

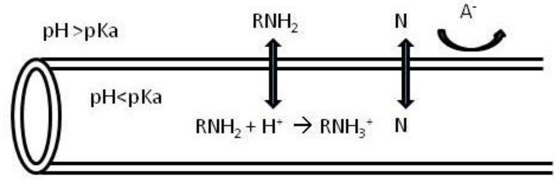

Figure 6.1. Diagram of the HF-LPME procedure for basic compounds.

Several studies have applied HF-LPME for the determination of SSRIs in environmental samples. In the method developed by Vasskog et al. [8] for the determination of SSRIs and NFX in sewage sludge, di-*n*-hexyl ether (DHE) was used as an organic solvent and the acceptor phase was formic acid at pH 2. Zorita et al. [31] optimized a method based on HF-LPME for the determination of FLX and NFX in 1 L of wastewater basified at pH 12.5. Several acceptor phases were tested and the best results were obtained from 10 mM sulfuric acid at pH 2.1, but a 0.5 M phosphate buffer at pH 2.1 was selected in order to keep a constant pH during the extraction process. Several organic solvents were also evaluated and DHE was chosen as the organic solvent. Esrafili et al. [33] used HF-LPME for the extraction and preconcentration of SRT and other antidepressants from biological fluids. The analytes were extracted from 11 mL of aqueous solution at pH 12 into an organic solvent (*n*-dodecane) and back extracted into 24 µL of 0.1 M H_3PO_4 at pH 2.1; 100 mL of tap water spiked with the target compounds were also subjected to HF-LPME with enrichment factors of 606 for SRT without improvement when sodium chloride was added to the donor phase.

6.2. EXPERIMENTAL

6.2.1. CHEMICALS AND STANDARDS

CTP, PRX, FLX, SRT, NFX, di-*n*-hexyl ether (DHE), ammonium acetate and triethylamine were purchased from Sigma-Aldrich Chemie GmbH (Steinhem, Germany). Acetonitrile (ACN) and methanol (MeOH), both of analytical reagent grade, were obtained from Honeywell B&J brand (Sleeze, Germany). Reagent grade sodium hydroxide was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Glacial acetic acid (HAc), formic acid (98 – 100% pure), phosphoric acid (85% pure), potassium phosphate, ammonia and ammonium phosphate, reagent grade, were purchased from Merck

(Darmstadt, Germany). Reagent water was obtained from a MilliQ water purification system (Millipore, Billerica, MA, USA).

Individual stock standard solutions containing 200 mg L^{-1} of CTP, 100 mg L^{-1} each of PRX, FLX and SRT and 30 mg L^{-1} of NFX were prepared in methanol. For the optimization and application of the method, working solutions of a mixture of all five SSRIs studied were prepared by the appropriate dilution of individual stock solutions in either methanol or 0.1 M ammonium phosphate at pH 2.1. Calibration curves for LC-ESI-MS were obtained by measuring standard solutions prepared by diluting individual stocks in the acceptor phase used for HF-LPME. The solutions were stored under refrigeration at 4 ºC in the dark.

6.2.2. SAMPLING SITE AND SAMPLING PROCEDURE

Treated sludge samples were collected from Källby WWTP (Lund, Sweden) in October 2010. This plant treats the sewage from a population of 84,000 people. The sewage water undergoes primary sedimentation, biological treatment with activated sludge and, finally, chemical precipitation of phosphate. The sludge from the biological and chemical treatment steps are returned to the incoming water and sludge is only removed from the system during primary sedimentation. After dewatering, the sludge is anaerobically digested under mesophilic conditions (37 ºC) for 20 - 30 days where it then undergoes further dewatering, resulting in a final product with a dry substance content of about 30%. Källby WWTP produces approximately 5,000 tons of sludge each year.

The samples were collected in plastic bottles, transported to the laboratory and kept at 4 ºC in the dark until analysis. All experiments were performed with the same sludge. For the direct HF-LPME method, the sludge was analyzed directly without any previous treatment. For the PHWE-HF-LPME method, the sludge was dried at 40 ºC, grounded with a mortar and sieved through a 0.5 mm sieve.

6.2.3. HF-LPME

Different experiments were performed with sludge slurry samples. Aliquots of homogenized sewage sludge (0.25, 0.5 or 1 g wet weight) were mixed with 50 mL of reagent water and stirred overnight at 660 rpm to reach partitioning equilibrium of the analytes between the solid and the aqueous phase. Afterwards, some of the slurry samples were spiked with the SSRI analytes at four levels (0.2, 0.5, 0.8 and 1 µg L^{-1}) and spiked and non-spiked samples were subjected to the HF-LPME extraction procedure.

HF-LPME as an extraction, clean-up and enrichment technique (direct HF-LPME) was performed with 50/280 Accurel polypropylene hollow fiber membranes with a wall thickness of 50 µm (0.1 µm pore size) and an internal diameter of 280 µm (Membrana, Wuppertal, Germany) (Membrane A). A fiber length of 20 cm was selected. After cutting the fiber, one of the ends was connected to a syringe (BDM Micro-Fine syringe, BD Consumer Healthcare, Sweden) with a needle diameter of 0.3 mm. The lumen of the hollow fiber was filled with the acceptor phase (0.1 M ammonium phosphate at pH 2.1). Next, the fiber was dipped into DHE for 1 minute to impregnate the fiber pores and the excess of organic solvent in the lumen was rinsed with 0.2 mL of the remaining acceptor phase in the syringe. Then, the two ends of the fiber were sealed with a piece of aluminum foil and inserted into a small glass tube. The end of the membrane fiber and the glass tube were fixed with Teflon tape. The excess of organic solvent from the surface of the fiber was removed by the immersion of the fiber into reagent water for 30 seconds. Finally, the fiber was immersed in the sample solution adjusted to pH 12.4 with 5 M sodium hydroxide. The extraction experiments were carried out using a multi-station magnetic stirrer (IKA-Werke, Staufen, Germany). After the extraction, the fiber was removed from the sample, the closed end was opened and the acceptor solution was collected in a vial by pushing air through the fiber with a syringe. 5 µL were used for LC-ESI-MS analysis.

6.2.4. PHWE

PHWE was carried out on a Dionex ASE-300 instrument (Sunnyvale, CA, USA). The method was based on a previous study with some modifications [26]. 0.5 g of dried

sludge was transferred into a 33 cm^3 stainless steel extraction cell containing 20 g of sea sand (previously washed with acetone and water and dried at 200 ºC). For spiked samples, sludge was spiked at 200 ng g^{-1} with the standard solution at the appropriate concentration in methanol inside the cell with sea sand and the cell was then left stirring and drying overnight.

The extraction conditions were as follows: the extraction solution consisted of 0.05 M of phosphoric acid in water at pH 2, an extraction temperature of 120 ºC, an extraction pressure of 1500 psi, a preheating time of 6 min, 5 min static extraction time, 5 extraction cycles, a flush volume of 90% of the cell volume, and 60 s for the nitrogen purge time. The final extract volume was about 90 mL, which was transferred into a 100 mL volumetric flask and adjusted to pH 12.4 with sodium hydroxide prior to the HF-LPME extraction.

The extraction solvent, extraction temperature, number of cycles and flush volume were optimized using two PHWE extractions for each level of each parameter: one spiked sludge sample and one non-spiked sludge sample. For non-spiked samples, after diluting the extract to 100 mL, two portions of 40 mL of the extract were submitted to HF-LPME and one of them was spiked at 1 µg L^{-1} with a mixed standard solution of the pharmaceuticals in methanol. For spiked samples, after diluting the extract to 100 mL, a portion of 40 mL of the spiked extract was processed by HF-LPME. Therefore, three samples in duplicate (non-spiked, spiked after PHWE and spiked before PHWE) were extracted using HF-LPME and used to calculate the recovery of PHWE for each level of the target parameter. Using this method, the different matrix effects due to the different PHWE conditions used were compensated in the HF-LPME system and in the mass spectrometer because the co-extracted interference could be different.

For the hollow fiber liquid-phase microextraction clean-up, Q3/2 Accurel polypropylene hollow fiber membranes with a thickness of 200 µm (0.2 µm pore size) and an internal diameter of 600 µm (Membrana, Wuppertal, Germany) (Membrane B) were used. Hollow fibers were cut into 10 cm pieces and one of the ends was

connected to a 100 µL syringe (Hamilton, Bonaduz, Switzerland) holding the acceptor phase (0.1 M ammonium phosphate at pH 2.1). Next, the lumen of the hollow fiber was filled with acceptor phase and after, the fiber was dipped into DHE for 15 seconds to impregnate the fiber pores. Then, the lumen was rinsed with the remaining acceptor phase in the syringe and the end of the hollow fiber was sealed with a piece of aluminum foil. The excess of organic solvent from the surface of the fiber was removed by the immersion of the fiber into reagent water for 30 seconds. Finally, the fiber was immersed in the sample solution, which had been adjusted to pH 12.4 as described above, and the syringe holding the fiber was fixed above the sample with a screw clamp. After the extraction, the closed end was opened and the acceptor solution was collected by withdrawing it into the syringe and transferring it to an autosampler vial. 10 µL were injected on the LC-ESI-MS system.

6.2.5. LC-ESI-MS

An LC system composed of two Waters 515 pumps (Waters, Milford, MA, USA), a vacuum degasser, a Triathlon autosampler (Spark-Holland, Emmen, Netherlands), an ODS-2 Hypersil (5 µm, 100 x 2.1 mm) column (Thermo Scientific, Waltham, MA, USA), a C_8 precolumn (Phenomenex, Torrance, CA, USA) and a single quadrupole mass spectrometer (Waters Micromass ZMD) with electrospray ionization interface (ESI) were used. Chromatographic separation was carried out using a gradient of mobile phases A (10 mM ammonium acetate adjusted to pH 4 with acetic acid) and B (acetonitrile). The gradient started with a linear increase of mobile phase B from 35% to 40% in 13 min and a decrease to 35% in 2 min with an equilibration time of 5 min. The flow rate was 0.3 mL min^{-1}. The injection was made in µL pick-up mode with an injection volume of 5 µL for samples after direct HF-LPME and 10 µL for sludge samples extracted by PHWE followed by HF-LPME. As the membrane used had different internal diameters, different acceptor phase volumes were obtained and different volumes were injected.

Data acquisition was performed in positive ion mode and optimized MS parameters were the following: capillary voltage 3.6 kV, cone voltage 15 V (for FLX, NFX and SRT)

and 30 V (for CTP and PRX), ESI source block temperature 150 ºC, desolvation temperature 350 ºC, desolvation gas (N_2) flow 540 L h^{-1}. Selective ion monitoring was used to detect ions with m/z ratios of 325 (CTP), 330 (PRX), 296 (NFX), 310 (FLX) and 306 (SRT). Instrumental intra-day and inter-day precision were determined by five repeated injections of 0.5 mg L^{-1} standard solutions in acceptor phase during the same day and on five successive days, respectively.

6.3. RESULTS AND DISCUSSION

6.3.1. LC-MS METHOD DEVELOPMENT

To optimize chromatographic separation, different columns were used; the best separation was achieved with an ODS-2 Hypersil (5 μm, 100 x 2.1 mm) column. Also, different mobile phases were evaluated; MeOH and ACN as the organic mobile phase and the effect of the addition of triethylamine in the aqueous phase were tested. 5 mM triethylamine was added to avoid silanol interaction with the amines in order to reduce peak tailing but its use caused ion suppression on the MS. After injecting a standard solution, the best obtained mobile phase composition for the detection and separation of SSRIs in positive mode was ACN and 10 mmol L^{-1} NH_4Ac/HAc at pH 4.

Calibration parameters of LC-ESI-MS were evaluated by injecting 5 and 10 µL. Calibration curves for each compound were obtained with determination coefficients (r^2) higher than 0.996 in both cases for all the compounds. The precision of the method was calculated by intra-day and inter-day analysis with values in the range of 1 - 4% and 2 - 10% for the injection of 5 µL and 3 - 6% and 4 - 10% for 10 µL. Instrumental limits of detection and quantitation were calculated as 3 and 10 times the signal-to-noise ratio, respectively. Instrumental limits of detections and quantitation for 5 µL injection were 1, 5, 10, 30 and 50 µg L^{-1} and 3, 17, 33, 100 and 167 µg L^{-1} , while for 10 µL injection they were 0.7, 2, 5, 10 and 25 µg L^{-1} and 2, 7, 17, 33 and 83 µg L^{-1} for CTP, PRX, FLX, SRT and NFX, respectively.

6.3.2. HF-LPME OPTIMIZATION

In order to achieve maximum sensitivity, some HF-LPME parameters such as the pH of the donor solution, acceptor solution and extraction time were optimized with reagent water. There have been several studies examining the effect of organic solvent on the extraction efficiency of the analytes. Di-*n*-hexyl ether was selected as the organic solvent according to some studies which reported the best extraction efficiencies for some SSRIs by HF-LPME [31].

As has been explained above, the pH of the donor phase is an important parameter because the analytes must be in their neutral form in order to be soluble in the organic solvent and be extracted from the donor phase to the acceptor phase. Due to the high pKa value of the compounds, the donor phase was adjusted to either pH 10 or 12.4 with NaOH. At pH 12.4 the enrichment factors were between 1.4 and 5 times higher than the values obtained at pH 10; therefore the pH of the acceptor solution was kept at 12.4 for subsequent experiments. These results are consistent because the pKa values of the compounds are between 9 and 10, therefore at pH 10 analytes would not be completely uncharged and the affinity to the organic solvent decreases.

In order to avoid the re-extraction of the target compounds to the organic phase, the acceptor phase has to trap the analytes as ions. For this reason an acidic solution was selected to ensure the protonation of amines. Several acidic solutions have been tested previously such as formic acid, sulfuric acid or phosphoric acid for the extraction of some SSRIs, but a solution with a buffer capacity is preferred in order to keep the acceptor pH constant [8,31,33]. Two volatile buffers were tested (ammonium formate and ammonium acetate) but, as can be seen in Figure 6.2, they were not able to extract the analytes. Ammonium phosphate at pH 2.1 gave the best enrichment factors for all the compounds; therefore it was used in the subsequent extractions.

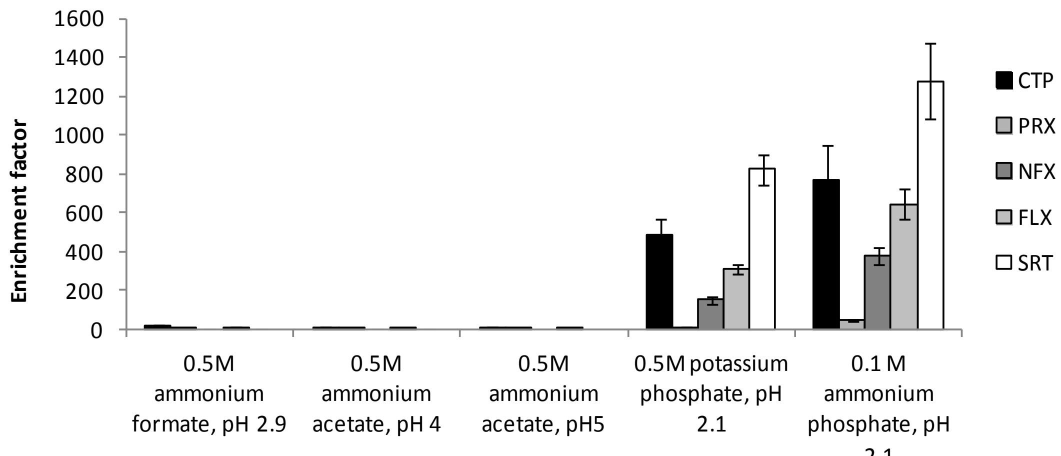

Figure 6.2. Effect of acceptor solution for spiked reagent water at a concentration of 5 μg L^{-1}, extraction time of 4 h and NaOH 0.1 mol L^{-1} as sample solution (N=3).

Finally, the extraction time was studied for both membranes, A and B, employed in this study and the results are shown in Figure 6.3. For membrane A, using 50 mL of donor solution, the extraction increases rapidly within the first 4 hours, while from 4 to 6 hours extraction, a slight increase was observed for almost all of the compounds, except for SRT. In order to obtain the highest enrichment factor for PRX, 6 hours was chosen as the optimal extraction time. For membrane B, with 100 mL of donor solution, the enrichment factor increases during the first 7 hours and then increases slowly until 8 hours when equilibrium conditions are achieved. A decrease of the extraction efficiency was observed after 8 hours, which can be explained by a decrease in the stability of the membrane. Therefore, 8 hours was selected as the optimum extraction time. The differences in the extraction kinetics observed for the two membranes can be explained by the different diffusivity through the membrane wall due to their different thicknesses. The membrane A setup, with a thinner membrane wall, leads to a shorter equilibrium time.

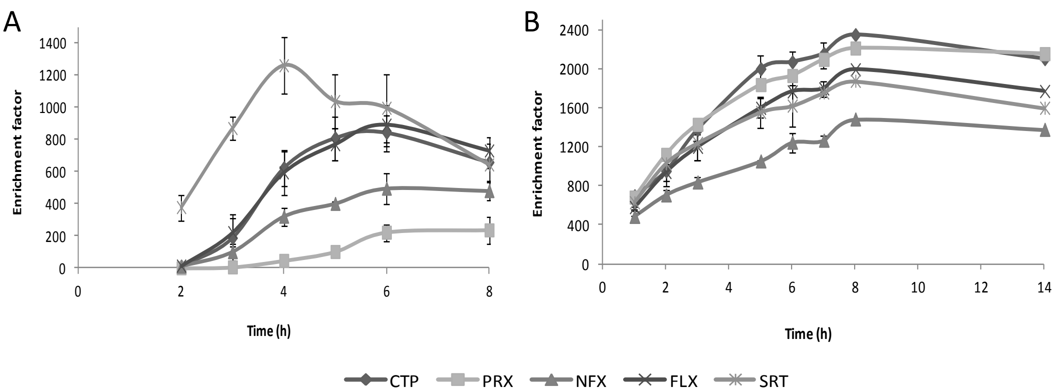

Figure 6.3. Extraction time profiles for spiked reagent water at a concentration of 5 µg L^{-1}. (A) 50/280 Accurel polypropylene hollow fiber membrane with a thickness of 50 µm (0.1 µm pore size) and an internal diameter of 280 µm. (B) Q3/2 Accurel polypropylene hollow fiber membrane with a thickness of 200 µm (0.2 µm pore size) and an internal diameter of 600 µm (N=2).

In Table 6.1 the enrichment factors and extraction efficiency for both membranes in reagent water are presented. As can be seen, for both systems high preconcentration of the analytes can be achieved. Enrichment factors between 200 and 1,000 times were obtained with membrane A, while for membrane B values of around 2,000 were obtained. For membrane A, which was used for the direct HF-LPME procedure after the equilibration of the slurry samples, intra-day precision and inter-day precision relative standard deviation values in reagent water were in the range of 3 - 12% and 10 - 18%, respectively. These values are similar to the ones obtained by Vasskog et al. [8].

Table 6.1. Average enrichment factor (Ee) and extraction efficiency (E) obtained for SSRIs and NFX for membrane A and B. Method intra-day precision and inter-day precision (N=3) for reagent water spiked at 5 µg L^{-1} for HF-LPME method.

Analyte	*Membrane A*				*Membrane B*	
	Ee	*E (%)*	*Intra-day precision (%)*	*Inter-day precision (%)*	*Ee*	*E (%)*
CTP	842	17	2.7	13.3	2,068	47
PRX	221	5	11.5	12.4	1,928	44
NFX	495	10	8.7	18.4	1,244	29
FLX	890	17	10.4	11.6	1,766	40
SRT	995	19	4.5	9.7	1,620	38

6.3.3. PHWE OPTIMIZATION

To obtain the best extraction conditions for SSRIs from sewage sludge, the pH of water as the extraction solvent and instrumental parameters such as temperature, number

of cycles and flush volume were investigated. In order to maintain water in its liquid state, extraction pressure was fixed at 1500 psi for all PHWE experiments. Moreover, a short static time (5 min) was fixed because it is preferable to have several cycles than have a long static time; in this way fresh solvent is introduced into the cell and the extraction is more efficient.

The effect of water pH on the extraction of basic analytes was evaluated by testing three different aqueous solutions: 0.05 M phosphoric acid at pH 2, reagent water only and 0.01 M NaOH. As can be seen in Figure 6.4, better recoveries for all the analytes studied were achieved with phosphoric acid at pH 2. At this pH the analytes are charged and are more soluble in aqueous solutions. This is in agreement with a previous study by Llop et al. [29] in which pH 4 was chosen for the extraction of amines by PHWE.

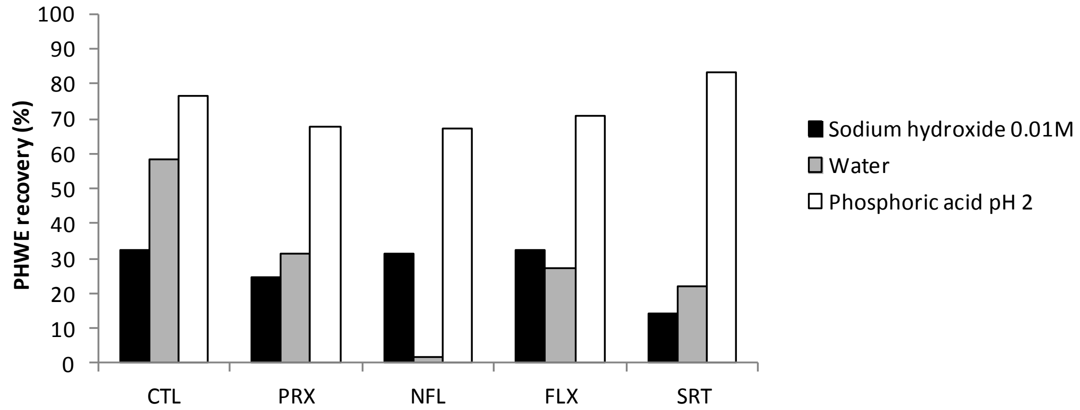

Figure 6.4. Effect of extraction pH on the PHWE recoveries for spiked sludge.

Temperature is also an important parameter because at higher temperatures the polarity of the water decreases [27], thus facilitating the desorption of the analytes from the matrix and improving mass transfer. The effect of the temperature was investigated at 80, 100 and 120 ºC. Figure 6.5 shows that the highest recoveries were obtained at the highest temperature evaluated. Therefore, 120 ºC was selected as the best temperature.

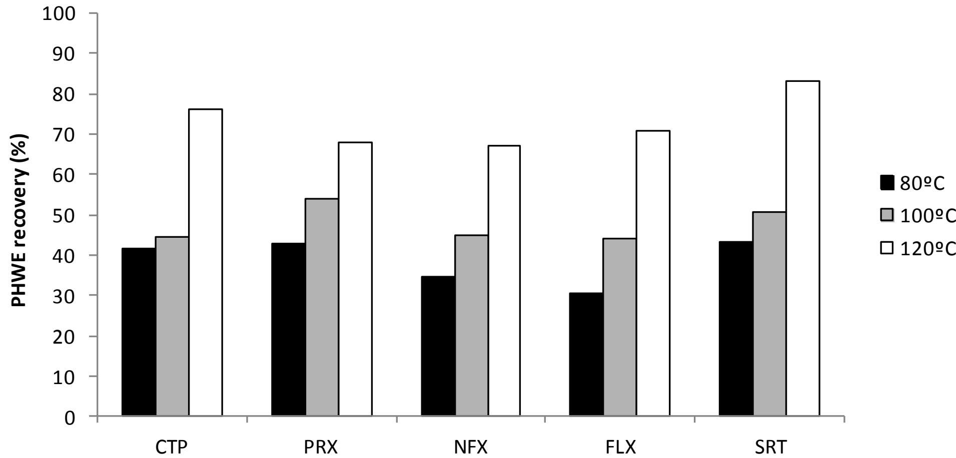

Figure 6.5. Effect of temperature on the PHWE recoveries for spike sludge.

For the evaluation of the number of extraction cycles, 1, 3 and 5 cycles were tested. By increasing the number of cycles, higher recoveries were obtained. Therefore, 5 extraction cycles were carried out in the subsequent experiments. Finally, the effect of the flush volume was studied with 30, 60 and 90% of the extraction cell volume. No differences were observed for the studied compounds, except for NFX which has a higher recovery when a flush volume of 90% of the extraction cell was used. Therefore, subsequent experiments were performed with this flush volume. The recoveries achieved for spiked samples under optimum conditions were between 67 and 83%.

It is to be expected that spiked analytes would be less retained than native ones in solid samples, for this reason, it is interesting to determine recoveries in non-spiked samples (native recoveries). These native recoveries were determined by six consecutive extractions of non-spiked sludge and calculated using Eq. (6.1).

$$PHWE\,recovery = \frac{PA_1}{\sum_{i=1}^{6} PA_i} \times 100 \qquad (6.1)$$

where *PHWE recovery* is the native recovery in percent for the first extraction and, PA_i is the peak area of the analyte after the extraction *i.* The sum of the peak areas for all six extractions are assumed to represent the total extractable analyte amount and, as can be seen in Figure 6.6, only low amounts of analytes were extracted after the first

extraction. The total native recoveries for the compounds were higher than 72% (Table 6.2).

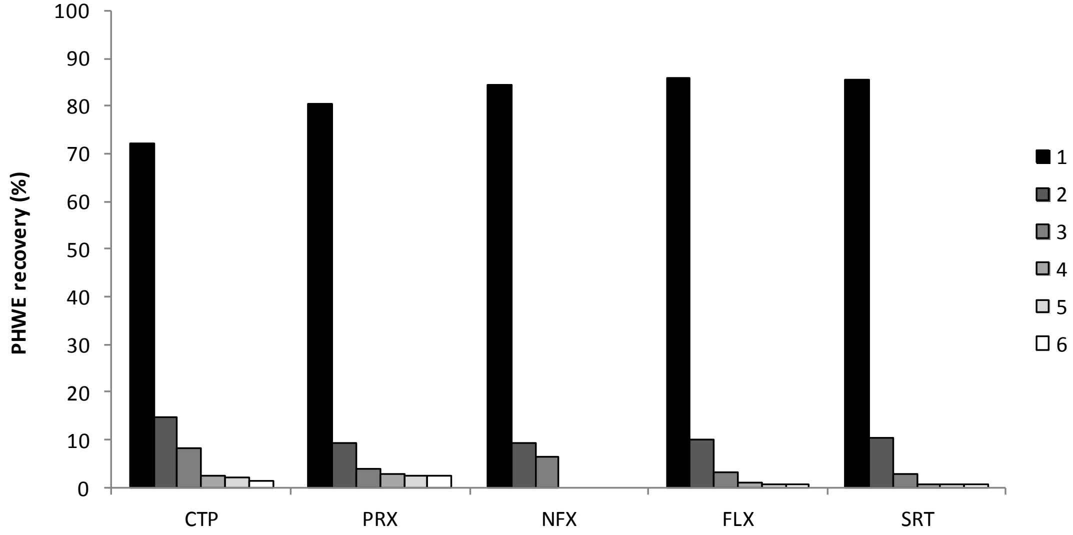

Figure 6.6. PHWE recoveries for the six consecutive extractions in duplicate.

Method limits of detection (MLOD) and method limits of quantitation (MLOQ) were determined as the minimum detectable amount of analyte with a signal-to-noise ratio (S/N) of 3 and 10, respectively. MLODs and MLOQs were about 6 and 20 ng g^{-1} for all the compounds, respectively. The obtained MLODs are similar to the ones reported previously [13,16,18,19]. Table 6.2 shows intra-day precision and inter-day precision, with values in the range of 5.4 to 12.4% and 11.3 to 20.8%, respectively.

Table 6.2. PHWE-HF-LPME method intra-day precision and inter-day precision (N=5).

Analyte	*Intra-day precision (%)*	*Inter-day precision (%)*	*Native PHWE recovery (%)*
CTP	8.1	17.2	72.2
PRX	9.1	20.8	80.4
NFX	5.4	19.5	84.4
FLX	12.1	11.3	85.8
SRT	12.4	18.8	85.5

6.3.4. Application of HF-LPME and PHWE-HF-LPME to sludge

For the application of the direct HF-LPME method to sewage sludge, a modified standard addition procedure developed in Chapter 5 was used for the determination of the concentration of CTP, PRX, NFX, FLX and SRT in sewage sludge by applying the following equations:

$$C_{Ae} = \frac{m_{Di}}{A} + \frac{m_{Si}}{A} \quad (6.2)$$

$$A = (v_A + \frac{K_{SD} \times w_S + v_D + K_{MD} \times v_M}{K_{AD}}) \quad (6.3)$$

Where C_{Ae} is the concentration in the acceptor solution at equilibrium, m_{Di} is the spiked amount of the compound in the slurry sludge, m_{Si} is the initial amount of the compound in the sludge, v_A is the volume of the acceptor phase, v_D is the volume of the donor solution, v_M is the volume of the membrane liquid, K_{SD} is the sludge-donor partition coefficient, K_{MD} is the partition coefficient between the membrane liquid and the water sample (donor), K_{AD} is the acceptor-donor partition coefficient, and w_S is the total amount of sludge.

Figure 6.7 shows the correlation obtained after applying Eq. (6.2) for CTP and FLX with different amounts of sludge and four different spiking levels. For all the compounds, determination coefficients were higher than 0.94, therefore Eq. (6.2) was used in order to calculate the amount of analyte in sludge (m_{Si}), taking into account that A^{-1} is the slope and m_{Si} A^{-1} is the intercept of the calibration curve obtained for each amount of sludge used. Table 6.3 shows the values of concentrations obtained for each compound when different amounts of sludge (C_{si}) were analyzed. The average concentrations determined in wet sludge and the corresponding relative standard deviations are also given. Relative standard deviations were lower than 10% in all cases. Method limits of detections were calculated as the concentration in the y-axis to the x-axis, where it becomes an amount in the sludge, divided it by the sludge volume; the values were between 1 and 12 ng g^{-1}.

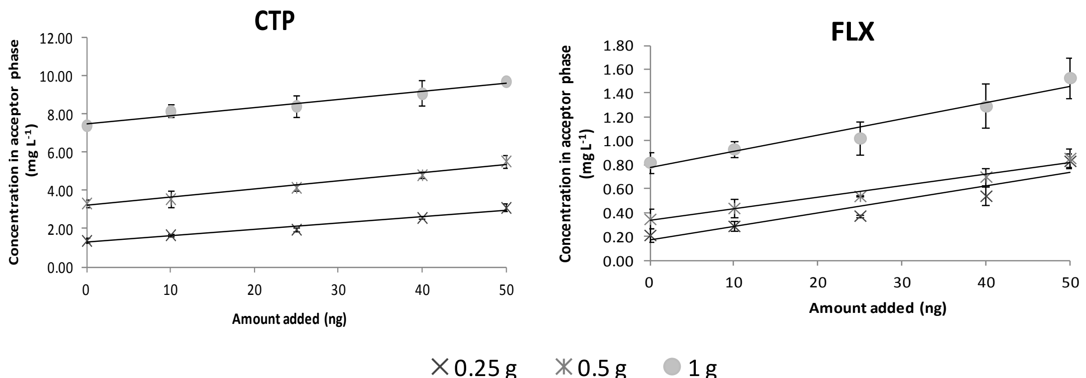

Figure 6.7. Concentration obtained in the acceptor phase as a function of the amount of analyte added in the slurry sample for different quantities of sludge for CTP and FLX (N=3).

Table 6.3. m_{Si}, C_{Si} and average C_{Si} with standard deviation of NSAIDs found in wet sewage sludge (N=3).

Analyte	*Sludge (g)*	*m_{Si} (ng)*	*C_{Si} (ng g^{-1})*	*Average C_{Si} (ng g^{-1})*
CTP	0.25	39.5	157.9	161 ± 16 (9.7%)
	0.5	73.8	147.6	
	1	178.4	178.3	
PRX	0.25	2.6	10.4	10 ± 1 (6.6%)
	0.5	4.8	9.7	
	1	9.1	9.1	
NFX	0.25	38.0	152.0	139 ± 13 (9.1%)
	0.5	68.3	136.5	
	1	127.1	127.1	
FLX	0.25	14.5	57.9	61 ± 5 (8.8%)
	0.5	33.4	66.9	
	1	57.4	57.4	
SRT	0.25	15.0	60.1	63 ± 2 (3.7%)
	0.5	32.3	64.5	
	1	63.5	63.5	

For the PHWE-HF-LPME method, and due to matrix effects observed in LC-ESI-MS measurements for sludge samples, the standard addition calibration method was performed by adding 10, 25, 50, 100 and 200 ng of the analytes to the extract [18,26]. The PHWE recoveries obtained in the analysis of the native target compounds were used to correct the analyte concentration determined in dried sludge. Figure 6.8 shows the LC-ESI-MS chromatogram of a non-spiked sludge sample; as can be seen, all the analytes can be detected and quantified. Several other peaks are seen, due to unknown compounds present in the exceedingly complex sludge samples.

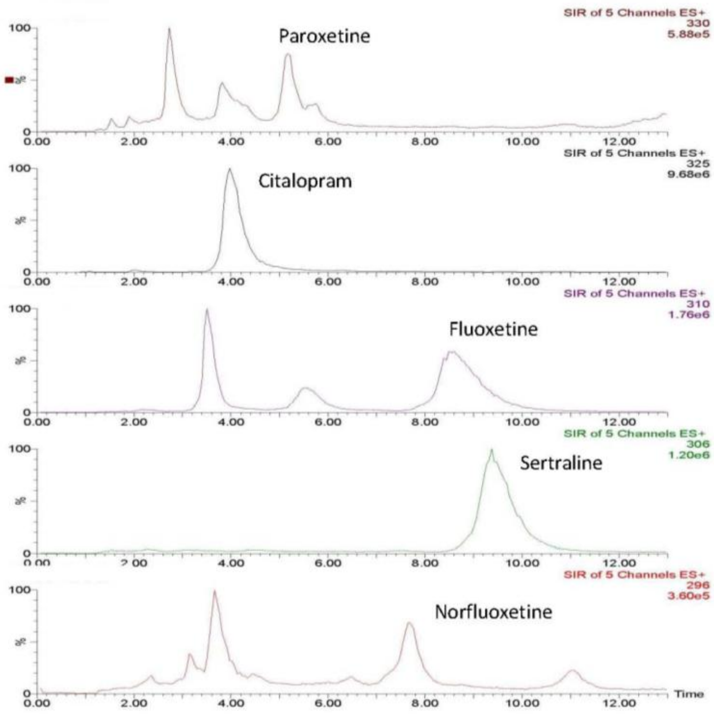

Figure 6.8. Single ion monitoring (SIM) chromatograms obtained by LC-ESI-MS from non-spiked sludge under optimized PHWE conditions.

The concentrations of SSRIs and NFX determined in dried sludge by using both methods, the direct HF-LPME method and the PHWE-HF-LPME method, are shown in Table 6.4. All the SSRIs and NFX were found in the sludge samples at levels of ng g^{-1}. With direct HF-LPME, similar concentrations were found when compared to PHWE-HF-LPME for CTP, PRX and FLX. These results confirm the good performance of the measurement, as different extraction principles were applied. In both cases, CTP was the compound present at highest concentrations with a value of approximately 530 ng g^{-1}. For FLX and PRX, concentrations of approximately 200 and 40 ng g^{-1} were determined, which is similar to the concentrations reported by Radjenović [18] and McClellan [17]. For SRT and NFX, the results differ between the two methods, with factors of 2 - 3 times, and present different tendencies. In the case of SRT, the concentration found in sludge using HF-LPME was lower than the one found for PHWE-HF-LPME. This can probably be explained by the low aqueous solubility of SRT resulting in an incomplete transport to the water phase in direct HF-LPME. For NFX, the

concentration determined by PHWE-HF-LPME is lower than that obtained by the direct method, suggesting a possible degradation of NFX.

Table 6.4. Comparison of the concentration obtained by HF-LPME and PHWE-HF-LPME for analytes in dried sewage sludge (N=3).

Analyte	***Concentration in dry sludge(ng g^{-1})***	
	PHWE-HF-LPME	***HF-LPME***
CTP	527 ± 146	539 ± 52
PRX	50 ± 11	32 ± 2
NFX	144 ± 45	463 ± 42
FLX	200 ± 45	203 ± 18
SRT	488 ± 95	209 ± 8

6.4. CONCLUSIONS

Two different methods were developed for the determination of citalopram, paroxetine, fluoxetine, sertraline and norfluoxetine in sewage sludge: a direct, simple hollow fiber liquid-phase microextraction method applied after distribution between sludge-water and combined with LC-ESI-MS; and pressurized hot water extraction combined with HF-LPME and LC-ESI-MS. Hollow fiber liquid-phase microextraction was applied successfully as an extraction and clean-up technique for basic compounds and both methods have a very low organic solvent consumption. Good linear correlations were achieved for the HF-LPME method and high recoveries were obtained for PHWE. The results obtained by both methods are similar for the three analytes and concentrations of approximately 530, 40 and 200 ng g^{-1} were found for citalopram, paroxetine and fluoxetine respectively.

Even though detected concentrations differ a little between the two methods for two of the analytes (SRT and NFX), all five compounds were detected in digested sewage sludge by using both methods, meaning that, if spread onto farmland, the sludge can contribute to the release of SSRIs into the environment. However, to determine if the measured concentrations pose a threat to the soil ecosystems, a comparison with effect data is required. To date, no or very few studies have been published regarding potential effects of SSRIs on terrestrial organisms which makes it difficult to perform a

relevant risk assessment. However, the presence of these compounds in sludge, shown by this study, underlines the need for such studies to generate data for accurate risk assessments, especially since toxic effects have been shown in aquatic organisms. In such assessments, the further treatment of the sludge and the application onto farmland also has to be taken into account. Long-time storage of the sludge might lead to degradation of the analytes thus giving lower concentrations in the sludge actually applied onto farmland, but potentially higher concentrations of known as well as unknown degradation products. Also, the mixing of the sludge with soil during application leads to a dilution and exposure of the soil ecosystem to concentrations lower than those in pure sludge. All such parameters need to be considered for accurate risk assessments to be performed.

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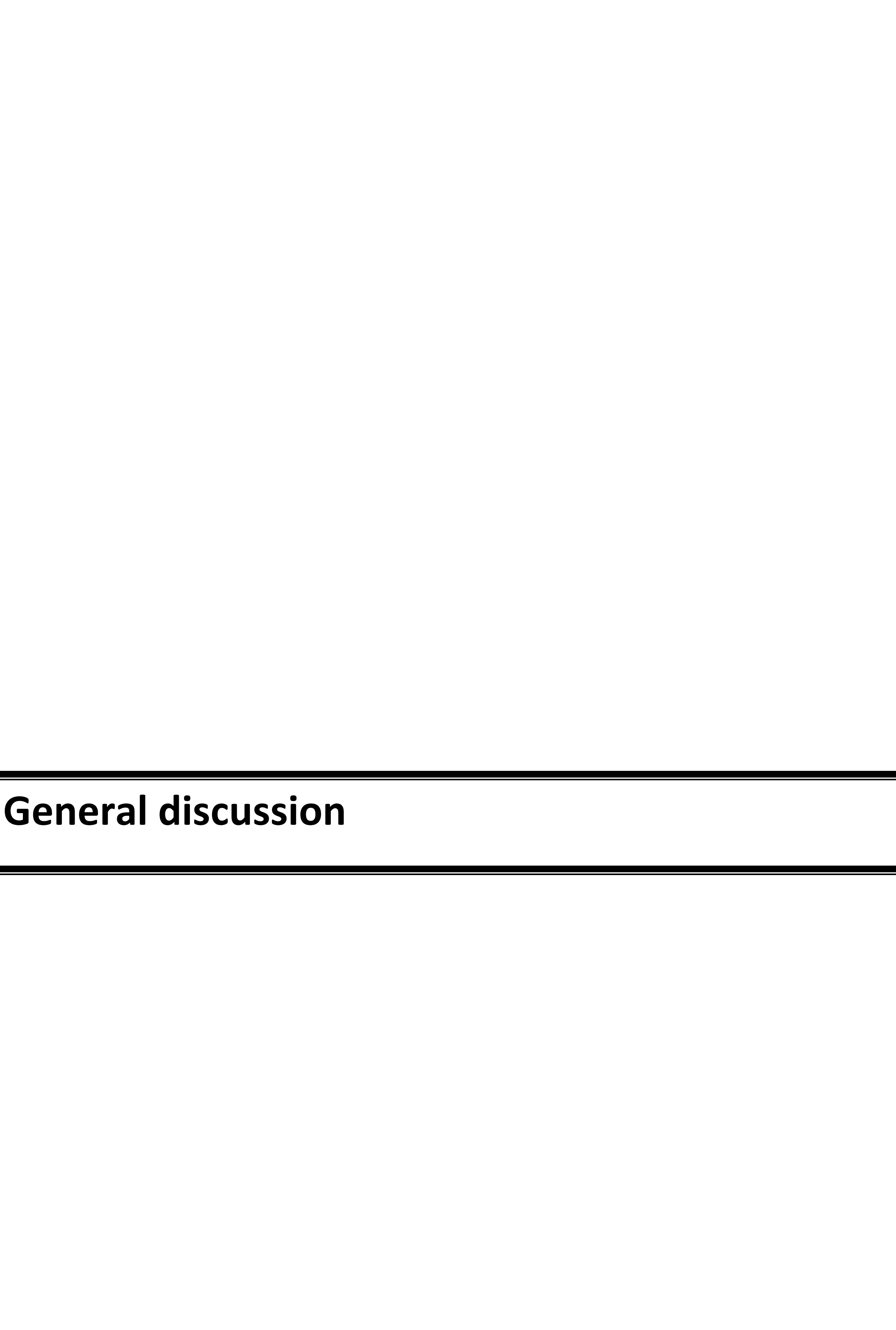


General discussion

As conventional wastewater treatment plants (WWTPs) are not designed to remove pharmaceuticals and personal care products these compounds can reach environmental aqueous systems and solid lands. In order to provide measured concentrations and evaluate their effects on wildlife and human health, it is necessary to develop analytical methods for their determination in environmental samples. The complexity of the matrices and the trace level presence of pharmaceuticals and personal care products make necessary to employ extraction techniques for the enrichment and clean-up of both liquid and solid samples. In this thesis, novel procedures based on hollow fiber liquid-phase microextraction (HF-LPME), as extraction, clean-up and enrichment techniques, combined with liquid chromatography have been developed and applied for the determination of several types of anthropogenic chemicals in aqueous and solid complex matrices.

For neutral organic compounds, a two-phase HF-LPME system has been developed for the determination of triclosan, triclocarban and methyl-triclosan in aqueous samples (Chapter 2) and solid samples (Chapter 3).

In order to obtain a more selective and sensitive method a three-phase HF-LPME method based on pH gradient was applied for the determination of acidic pharmaceuticals (clofibric acid, naproxen, ibuprofen, diclofenac and ketoprofen) in aqueous samples (Chapter 4) and biosolids (Chapter 5). In this case, donor and aqueous phases were adjusted to pH 1.5 and 13, respectively. A pH gradient based on three-phase HF-LPME has also been used for the determination of basic pharmaceuticals (citalopram, paroxetine, fluoxetine, norfluoxetine and sertraline) in biosolids (Chapter 6) with a donor phase at pH 12 and an aqueous acceptor phase at pH 2.

- **TWO-PHASE HF-LPME**

Despite the poor selectivity of the technique, solid-phase extraction (SPE) is the most commonly used technique for the extraction and enrichment of pharmaceuticals and personal care products in aqueous samples. In Chapter 2, we developed a two-phase

HF-LPME method for the extraction of triclosan, triclocarban and methyl-triclosan from aqueous samples combined with HPLC-DAD, and a wide range of parameters were evaluated. The most important parameters affecting the extraction procedure were organic solvent, extraction time and salting-out effect. With regards to the organic solvent, triclocarban was only extracted by di-*n*-hexyl ether:decane (1:1) and to a lesser extent by amylbenzene. Extraction time was 24 hours and the addition of sodium chloride increased the extraction efficiency of hydrophobic analytes. Although, extraction efficiency was lower than 25% in 500 mL of reagent water, high enrichment factors (430 – 707) and limits of detection of 5 - 10 ng L^{-1} were obtained. The effect of organic matter content and particulate matter was also evaluated resulting in a decrease in the extraction efficiency. Recoveries of spiked samples were higher than 77% in effluent wastewaters, surface water and drinking water whereas in influent wastewater recoveries were 18, 47 and 85% for triclocarban, methyl-triclosan and triclosan, respectively. This recovery decrease is explained by the interaction of the analytes with organic and particulate matter. Therefore, only the free content of analytes in water can be determined.

Pressurized liquid extraction (PLE), microwave accelerated solvent extraction and ultrasound solvent extraction combined with clean-up and enrichment techniques, such as solid-phase extraction followed by GC-MS or LC-MS are usually applied for the determination of pharmaceuticals and personal care products in solid samples. Liquid chromatography coupled to mass spectrometry with an electrospray ionization interface is a sensitive and selective technique widely employed for the detection and quantification of pharmaceuticals and personal care products. To overcome the drawback of ion suppression, some studies apply a standard addition method or internal standards for correction. Furthermore, the use of a clean-up technique is preferable for removing co-extracted organic interferences.

In Chapter 3, a method for the determination of personal care products in solid samples was developed, PLE was combined with two-phase HF-LPME as the clean-up and preconcentration technique followed by LC-MS for triclosan and triclocarban, and

HPLC-DAD for methyl-triclosan. As methyl-triclosan is a hydrophobic compound that cannot be ionized for mass spectrometry analysis, it is necessary to use DAD detection.

The effect of several parameters of pressurized liquid extraction on the analytical process was evaluated with 0.5 g of biosolid. MeOH, 60 ºC, 1 cycle and 60% of flush volume were found to be the best conditions. Organic solvent is usually evaporated prior to clean-up. However, in this case higher recoveries were obtained without evaporation, possibly as a result of the low solubility of the analytes with reagent water. For HF-LPME, conditions were the same as in the case of the method developed for aqueous samples except that 100 mL of reagent water was used. After setting of all the parameters, two different methods with the same PLE conditions were used: one for soil samples with high organic content (biosolids) and another with lower organic content (sludge amended soil). In the first one, after PLE, the extracts were diluted to 50 mL in MeOH and an aliquot of 5 mL was diluted to 100 mL with reagent water and spiked for standard addition prior to HF-LPME. Different sample sizes were evaluated by this procedure. For 0.5 g of biosolids, when the PLE extract was diluted with reagent water, the presence of a colloidal fraction that was immiscible in water was observed. This fact may explain the low recoveries obtained as no colloidal fraction was formed with 0.2 g. With 0.2 g, recoveries were 95, 84 and 43% for triclosan, triclocarban and methyl-triclosan, respectively. For sludge amended soil with 1% organic content, an external calibration curve and 0.5 g of soil were used with recoveries higher than 80% for all the compounds. For triclosan and triclocarban, recoveries were confirmed by isotopic labeled compounds with similar recovery values of 91% in biosolids and 84 - 102% in sludge amended soil. Moreover, detection limits of soil samples are of the same order as those reported (1.2 - 2.1 ng g^{-1}) whereas detection limits for biosolids are higher (98 - 172 ng g^{-1}). Nevertheless, to the best of our knowledge, this is the first time that triclosan, triclocarban and methyl-triclosan have been extracted together from solid samples.

- **THREE-PHASE HF-LPME**

HPLC-DAD has also been used for the detection and quantitation of clofibric acid, naproxen, ibuprofen and diclofenac in aqueous samples (Chapter 4). In the method developed, the analytes were extracted from samples by three-phase HF-LPME in 14 hours (overnight) using di-*n*-hexyl ether as the organic solvent, 0.1 M NaOH as the acceptor phase and without sodium chloride as no improvement had previously been observed. After the optimization of the HF-LPME method high enrichment factors (10,397 – 11,740) were obtained. As in the two-phase mode, the effect of the addition of organic matter like humic acids was evaluated. A decrease in the extraction efficiency of the analytes was observed using HF-LPME procedure. When HF-LPME method was compared to solid-phase extraction, cleaner chromatograms for HF-LPME procedure were obtained and similar recoveries were achieved for both methods. Recoveries were higher than 60% in influent and effluent wastewater, drinking water and surface water. Although, HPLC-DAD was used, detection limits of few ng L^{-1} (0.5 – 10 ng L^{-1}) were achieved.

The method developed for acidic pharmaceuticals was used for the evaluation of three disinfectant agents (chlorine, peracetic acid and UV radiation) in the elimination of clofibric acid, naproxen, ibuprofen, and diclofenac. Chlorine is widely used for deactivating bacteria, viruses and protozoan cysts but toxic disinfection by-products (DBPs) such as trihalomethanes and haloacetics are formed because of its interaction with wastewater components. Peracetic acid has been proposed as an alternative and in this thesis its effect on the elimination of pharmaceuticals has been evaluated in laboratory and in a wastewater treatment plant. In laboratory studies, no degradation of clofibric acid, naproxen, diclofenac and ibuprofen was observed after treatment by chlorine and peracetic acid. UV radiation, on the other hand, degraded all the analytes following a pseudo-first order kinetics. When wastewater was used instead of reagent water, the degradation rate constants decrease due to the presence of other substances that compete with the analytes to absorb UV radiation. A possible synergic effect between peracetic acid and chlorine with UV radiation was evaluated but no

effect was observed. The application of UV radiation and peracetic acid in a wastewater treatment plant was evaluated and the lack of utility of peracetic acid was confirmed. Removal efficiencies were just 35 and 53% for naproxen, diclofenac and ibuprofen under UV radiation.

For sewage sludge, another different approach has been developed in this thesis. A novel, simple and low cost method based on three phase hollow fiber liquid-phase microextraction as the extraction, clean-up and enrichment technique for the determination of pharmaceuticals in biosolids, without the extraction of these compounds from biosolids, was developed (Chapter 5 and 6). Solid samples were placed in contact with reagent water overnight in order to achieve a distribution equilibrium of the analytes between sludge and water. Slurry sludge (spiked or not) was then adjusted to pH 2 (acidic pharmaceuticals) or to pH 12 (basic pharmaceuticals) and analytes were extracted into an organic solvent and finally back-extracted into a basic (acidic analytes) or acidic (basic analytes) aqueous solution located inside the lumen of the fiber. By applying the described system (direct HF-LPME), and a modified standard addition method, the following linear regression equation was obtained from the calculation of the mass balance:

$$C_{\mathrm{Ae}} = \frac{m_{\mathrm{Di}}}{A} + \frac{m_{\mathrm{Si}}}{A} \qquad \text{(G.1)}$$

Where C_{Ae} is the concentration in the acceptor solution at the end of the extraction procedure, m_{Si} is the initial amount of the compound in the sludge, m_{Di} is the spiked amount of the compound in the slurry sludge, and $1/A$ is the slope which is constant. Hence, if the data fits to a regression line where C_{Ae} is the y-axis and m_{Di} is the x-axis, the intercept allows the calculation of the initial amount of analyte.

HF-LPME was used to extract different contents of the analytes from spiked samples and the acceptor phase was then analyzed by LC-MS. Different amounts of sludge were used to verify the applicability of the system. Pressurized liquid extraction, microwave assisted extraction, or evaporation steps were not required and so the possibility of analyte loss during the analytical process was avoided.

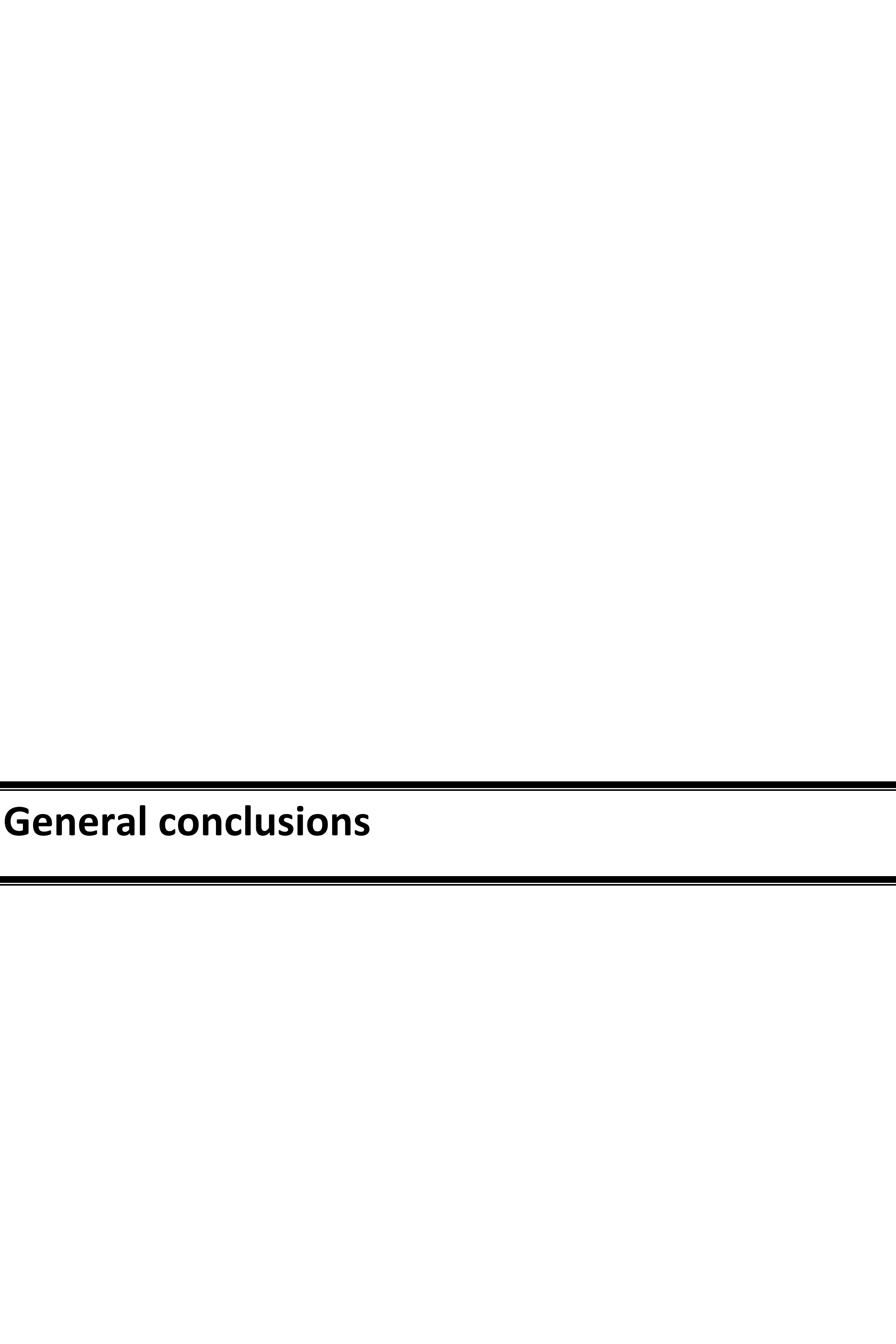
A membrane system was developed for the determination of acidic pharmaceuticals (naproxen, diclofenac, ibuprofen and ketoprofen) in sewage sludge (Chapter 5). The optimum extraction time was found to be 4 hours in reagent water and enrichment factors were of 2,761 to 3,254. After HF-LPME optimization, direct HF-LPME was carried out for the determination of acidic pharmaceuticals. As was expected, the same concentration for each analyte was obtained for the different sludge sizes with determination coefficients higher than 0.88. Moreover, low detection limits were achieved: 1.1 -1.9 ng g^{-1} for ketoprofen, 1.2 - 1.9 ng g^{-1} for naproxen, 3.4 - 5.6 ng g^{-1} for ibuprofen and 2.8 – 3.1 ng g^{-1} for diclofenac. The concentrations obtained were in the same ranges as those reported in the literature: 5 - 580 ng g^{-1} for ketoprofen, 3 - 350 ng g^{-1} for naproxen, 4 - 77 ng g^{-1} for diclofenac and 4 - 560 ng g^{-1} for ibuprofen.

Finally, the same direct HF-LPME method was applied for the determination of citalopram, paroxetine, fluoxetine, norfluoxetine and sertraline in sewage sludge and compared to a method using pressurized hot water extraction (PHWE) as the extraction technique and HF-LPME as the clean-up and enrichment technique (Chapter 6). Evaluation of two donor phases with pHs of 10 and 12 revealed that pH 12 was the most efficient in increasing the extraction capacity of the HF-LPME system. Several acceptor phases were tested and the most efficient was found to be 0.1 M ammonium phosphate at pH 2. Another important parameter is the extraction time. For direct HF-LPME the extraction time was set at 6 hours with enrichment factors of 221 – 995 in reagent water. For HF-LPME as the clean-up technique, 8 hours was chosen as the extraction time and preconcentration factors were 1,244 - 2,068 in reagent water. The difference between preconcentration values can be explained by the fact that different membranes were used.

In the case of PHWE-HF-LPME method, some PHWE parameters affecting the extraction were also evaluated. The best extraction conditions were: phosphoric acid at pH 2 as the extraction solvent, 120 ºC, 5 cycles, and 90% flush volume. Recoveries were then evaluated by applying consecutive extractions. It was observed that in the first extraction a high percentage (72 - 86%) of the compound was extracted from the

final sewage sludge. One extraction was therefore selected. After PHWE optimization and the determination of native recovery, the method was applied to the determination of the analytes to real samples. The standard addition method was applied to estimate the concentration of the analytes in sewage sludge, taking into account the recoveries obtained.

In both methods, similar concentrations were found for citalopram, paroxetine and fluoxetine while concentrations were very different for norfluoxetine and sertraline. We hypothesized that this difference may be due to norfluoxetine degradation as experiments were carried out some months after sampling and in the case of sertraline its low solubility limited the extraction by water. Despite these limitations, the detection limits of the developed methods were between 1 and 12 ng g^{-1}, and 6 and 20 ng g^{-1} for direct HF-LPME and PHWE-HF-LPME, respectively.



General conclusions

In this thesis, several analytical methods based on the use of hollow fiber liquid-phase microextraction combined with liquid chromatography have been developed for the determination of pharmaceuticals and personal care products in complex environmental samples such as wastewaters and solid samples.

Even though conclusions obtained from this research have been included in each chapter, the main conclusions are summarized below:

1. An HPLC-DAD method has been developed for the simultaneously detection and quantitation of triclosan, triclocarban and methyl-triclosan.
2. Two-phase HF-LPME is a suitable technique for the simultaneously extraction of biocides and methyl-triclosan from environmental waters. This technique was combined with HPLC-DAD and applied to environmental aqueous samples and effluent wastewater with acceptable recoveries allowing only the free part of the analyte to be extracted by HF-LPME. Method limits of detection were 5 – 10 ng L^{-1} and recoveries were in 75 - 99% range in effluent wastewater, surface water and drinking for all the analytes.
3. The method was applied to influent and effluent wastewater with triclocarban concentrations below its method limits of quantitation (MLOQs) in all the samples whereas methyl-triclosan was quantified only in two effluent samples. For triclosan minimum and maximum concentrations were 120 and 883 ng L^{-1} in influent wastewater and in effluent were below method limit of detection (MLOD) - 308 ng L^{-1}. In surface water only triclosan was quantified and in drinking water any of the target compounds was observed.
4. A method by pressurized liquid extraction followed by two-phase HF-LPME and LC-MS/MS for triclosan and triclocarban, and HPLC-DAD for methyl-triclosan was developed and employed successfully for the determination of the previously analytes in solid samples:
   a. For sludge with high organic content, low sample sizes (0.2 g) were required in order to obtain good recoveries. Standard addition on HF-LPME was carried out in order to compensate ion suppression on LC-

ESI-MS/MS. Method limits of detection were 29 - 51 ng g^{-1} and recoveries were 42, 84 and 95% for methyl-triclosan, triclocarban and triclosan, respectively.

   b. A simplest method without standard addition was employed for sludge amended soil with 0.5 g and lower limits of detection (1.2 - 2.1 ng g^{-1}). Recoveries were between 80 - 92%. Any analyte was detected in sludge-amended soil.

5. The determination of acidic pharmaceuticals as naproxen, diclofenac, ibuprofen and clofibric acid in complex aqueous matrices has been achieved by three-phase HF-LPME combined with HPLC-DAD. A sensitive and very selective method has been developed with limits of detection in 0.5 - 10 ng L^{-1} range and very high enrichment factors up to 11,740 were obtained. Recoveries were 84 – 98% in surface and drinking water, 68 – 84% in effluent wastewater and 60 – 77% in influent wastewater due to organic matter. Average concentrations in influent wastewater were 3,547, 2,088, 275 and 123 ng L^{-1} for ibuprofen, naproxen, clofibric acid and diclofenac, respectively.
6. Evaluation of disinfectant agents by peracetic acid, chlorine and UV radiation on the removal of naproxen, ibuprofen, diclofenac and clofibric acid, in laboratory and wastewater treatment plant studies, has been carried out. Experiments have shown that peracetic acid and chlorine has no utility in both type of studies. For UV radiation, under laboratory conditions, high degradation rate constant were observed for all the compounds in reagent water, especially in the case of diclofenac and clofibric acid, observing a reduction of the degradation rate constant when secondary wastewater was used. In wastewater treatment plant removals between 35 and 53% were achieved under UV radiation disinfection.
7. A method for the determination of ketoprofen, naproxen, diclofenac and ibuprofen in sewage sludge was developed. Hollow fiber liquid-phase microextraction was applied successfully as an extraction, clean-up and preconcentration technique followed by LC-MS. Using a modified standard

addition method for different amounts of sludge, the same concentration was found for each analyte. Limits of detection were between 1.1 - 5.6 ng g^{-1}. Concentrations about 29, 39, 122 and 138 ng g^{-1} in dried weight were measured for ketoprofen, diclofenac, ibuprofen and naproxen, respectively.

8. The determination of some selective serotonin reuptake inhibitors and norfluoxetine in sewage sludge has been carried out by three-phase HF-LPME by two different approaches:
    a. A simple direct HF-LPME as an extraction, clean-up and preconcentration technique followed by LC-MS was applied using a modified standard addition method, limits of detection were between 1 and 12 ng g^{-1}
    b. A method based on pressurized hot water extraction combined with HF-LPME applying standard addition method and LC-MS has been developed. Recoveries were in 72 – 86% range and limits of detection were between 6 and 20 ng g^{-1}.
    c. The results obtained by both methods are similar for three analytes. Concentrations about 530, 40 and 200 ng g^{-1} were found for citalopram, paroxetine and fluoxetine. Further studies on the parameters affecting direct HF-LPME need to be studied.