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AVANCES EN LA DETERMINACIÓN DE RESIDUOS DE HERBICIDAS Y CEFALOSPORINAS EN MUESTRAS AMBIENTALES Y ALIMENTARIAS MEDIANTE TÉCNICAS MINIATURIZADAS

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Avances en la determinación de residuos de herbicidas y cefalosporinas en muestras ambientales y alimentarias mediante técnicas miniaturizadas

Advances in the determination of residues of herbicides and cephalosporins in environmental and food samples using miniaturized techniques

Por

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Sección Químicas

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CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de *"*AVANCES EN LA DETERMINACIÓN DE RESIDUOS DE HERBICIDAS Y CEFALOSPORINAS EN MUESTRAS AMBIENTALES Y ALIMENTARIAS MEDIANTE TÉCNICAS MINIATURIZADAS*"*, ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de la profesora D[a]. M. Monsalud del Olmo Iruela, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y reúne todos los requisitos para poder optar al Grado de Doctor.

Granada a 15 de Abril de 2013

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Objeto de la memoria

Los objetivos abordados en esta Tesis Doctoral se enmarcan dentro de varios Proyectos de Investigación desarrollados durante el transcurso de este trabajo y forman parte de las líneas de investigación del grupo en el que se ha realizado.

Como objetivo general se ha planteado el desarrollo de nuevos métodos de análisis sensibles y selectivos para la determinación de residuos de herbicidas (sulfonilureas y productos de degradación del metribuzin) y antibióticos (cefalosporinas) en muestras ambientales de distinta procedencia y alimentos de origen animal.

En el caso de los alimentos la legislación vigente establece, para antibióticos y herbicidas, unos Límites Máximos de Residuos (LMR) que no deben ser superados con objeto de garantizar la calidad del producto y permitir su distribución y consumo. Por otro lado, la incorporación constante en el medioambiente de estos residuos orgánicos sin el debido control está provocando serios problemas en los ecosistemas receptores que obligan al desarrollo de metodologías para su cuantificación y posterior evaluación de riesgos.

Teniendo en cuenta las recientes e importantes mejoras de las técnicas separativas miniaturizadas como son el aumento de la eficacia, alta sensibilidad, elevada resolución y bajo consumo de disolventes y muestra, en esta Tesis Doctoral se propone la utilización de la electroforesis capilar (CE) y cromatografía líquida capilar (HPLC capilar) con detección UV para la cuantificación de los compuestos señalados.

Así mismo en esta Tesis se proponen métodos alternativos para el tratamiento de muestras de alimentos de origen animal, frutas, suelos y aguas de distinta procedencia, más eficaces y selectivos.

Los objetivos concretos de esta Tesis son los siguientes:

- Demostrar el potencial de la electroforesis capilar zonal (CZE) con preconcentración en línea para la determinación de los productos de degradación del metribuzin en muestras ambientales de suelo y agua, utilizando para ello la extracción líquida presurizada (PLE) en combinación con la extracción en fase sólida (SPE).

- Emplear la CZE con detección UV para la determinación de sulfonilureas en muestras de agua y uvas de mesa de distinta procedencia, combinando la SPE con la preconcentración en el propio capilar.

- Desarrollar un método de análisis altamente sensible basado en el empleo de CZE con preconcentración en línea y SPE que permita la cuantificación de cefalosporinas en aguas de distinta procedencia a las bajas concentraciones esperadas en este tipo de matrices.

- Utilizar la cromatografía líquida capilar (HPLC capilar) para la determinación de cefalosporinas de uso veterinario y humano en muestras de carne y aguas de distinta procedencia. La extracción líquido-líquido asistida por sales (SALLE) se propone en este caso como alternativa para la extracción de estos compuestos altamente polares.

- Por último, la estancia predoctoral llevada a cabo en el grupo dirigido por el Prof. Philippe Morin, del *Institut de Chimie Organique et Analytique (ICOA),* Université d'Orléans (Orléans, Francia) tuvo como objeto la síntesis y evaluación de un nuevo polímero impreso molecularmente utilizado como sorbente para extracción en fase sólida (MISPE) en el tratamiento de muestra previo al análisis de cefalosporinas en leche por HPLC.

Resumen

En esta Tesis se han desarrollado diferentes métodos analíticos para la determinación de residuos de herbicidas y cefalosporinas, en alimentos y muestras ambientales. Para ello, se han evaluado diferentes técnicas separativas miniaturizadas, como la electroforesis capilar (CE) y la cromatografía líquida capilar (HPLC capilar). Además se han propuesto tratamientos de muestra alternativos a los existentes en bibliografía obteniéndose un incremento en la eficacia y una disminución del tiempo invertido en la etapa de tratamiento de muestra.

En el primer capítulo de esta Tesis doctoral, se ha desarrollado un método para la determinación de los principales productos de degradación del metribuzin, utilizando la electroforesis capilar en zona (CZE) con detección UV. Con el fin de aumentar la sensibilidad de la técnica se ha aplicado un método de preconcentración dentro del propio capilar, denominado apilamiento de gran volumen de muestra (LVSS) con polaridad inversa. El método propuesto se ha aplicado a muestras de suelo previa extracción de los compuestos de interés mediante extracción líquida presurizada (PLE), seguida de una etapa de limpieza y preconcentración basada en el empleo de la extracción en fase sólida (SPE). Para su aplicación en muestras de agua subterránea, se necesitó sólo una etapa de SPE antes del análisis mediante LVSS-CZE. Además se han calculado los valores de las constantes de disociación (pK_a) de estos compuestos utilizando CZE y los valores obtenidos se han comparado con los valores recogidos en bibliografía.

Para demostrar el potencial de la metodología LVSS-CZE-UV, en el segundo capítulo se han determinado cinco sulfonilureas en muestras de uva, donde este tipo de herbicidas se aplica frecuentemente, y también en aguas. En este caso, se utilizó la SPE con relleno de HLB en las muestras de agua y para las muestras de uva se usó C18.

El tercer capítulo presenta un nuevo método para la determinación de cinco cefalosporinas en muestras de agua. En este caso la SPE ha sido utilizada como procedimiento de preconcentración en discontinuo, combinado con LVSS como procedimiento de preconcentración en línea acoplado a CZE con detección UV. El aspecto más notable de esta metodología es la obtención de bajos límites de detección, lo que permite su aplicación a las matrices estudiadas donde estos residuos presentan niveles de concentración muy bajos.

La determinación de ocho cefalosporinas en muestras de carne y agua de distinta procedencia se ha desarrollado en el capítulo cuarto. Para ese propósito, se utilizó la HPLC capilar con detección UV como técnica instrumental y la extracción

líquido-líquido asistida por sales (SALLE) para el tratamiento de la muestra. La formación previa de un par iónico entre las cefalosporinas y el bromuro de hexadeciltrimetilamonio (CTAB) ha mejorado la eficacia de la extracción.

En el quinto capítulo, se describe la síntesis y eficacia de un polímero impreso molecularmente (MIP) para la extracción específica de tres cefalosporinas en muestras de leche, utilizando HPLC-UV para las medidas analíticas. Este trabajo ha sido desarrollado en el Institut de Chimie Organique et Analytique (CIAO) Université d'Orléans (Orleans, Francia) durante una estancia predoctoral.

La revisión bibliográfica acerca de la determinación de cefalosporinas llevada a cabo antes del establecimiento de los nuevos métodos analíticos incluidos en esta Tesis ha permitido elaborar un artículo de revisión donde se discuten los métodos analíticos existentes para la determinación de β-lactamas haciendo uso de HPLC con diferentes sistemas de detección, en diferentes áreas y con tratamientos de muestra distintos.

Summary

In this Thesis, different analytical methods for the determination of residues of herbicides and cephalosporins in foodstuff and environmental samples have been developed. For that purpose, different miniaturized separative techniques have been evaluated, such capillary electrophoresis (CE) and capillary liquid chromatography (capillary HPLC). In addition, alternative sample treatments to those previously reported have been assessed allowing an increased efficiency and sample throughput.

In the first chapter of this Thesis, a method for the determination of the major degradation products of metribuzin has been developed using capillary zone electrophoresis (CZE) with UV detection. In order to increase sensitivity, large volume sample stacking (LVSS) with polarity switching has been applied as on-line preconcentration methodology. The method has been applied to soil samples using pressurized liquid extraction (PLE) followed by an off-line preconcentration and sample clean-up procedure by solid phase extraction (SPE). For groundwater sample applications, only SPE was necessary before LVSS-CZE analysis. In addition, the values of the dissociation constants (pKas) of these compounds have been calculated using CZE, and the obtained values have been compared with those reported in bibliography.

In order to explore the potential of the LVSS-CZE-UV methodology, in the second chapter five sulfonylurea herbicides were determined in grape samples, where this kind of pesticides are frequently used, and also in environmental waters. In this case, SPE with HLB sorbent was used for water samples and C18 sorbent for grape samples.

The third chapter presents a new method for the determination of five cephalosporins in water samples. In this case SPE has been used as off-line preconcentration procedure in conjunction with LVSS as on-line procedure coupled to CZE with UV detection. The most remarkable aspect of this methodology is the very low detection limits obtained, in accordance with the low concentrations levels of these residues usually found in the studied matrices.

The determination of eight cephalosporins in meat and environmental waters is presented in chapter four. For that purpose, capillary HPLC with UV detection was used as instrumental technique and for sample treatment salting-out assisted liquid-liquid extraction was applied (SALLE). The previous formation of an ion-pair between cephalosporins and hexadecyltrimethylammonium bromide improved the efficiency of the extraction.

In the fifth chapter, the synthesis and efficiency of a molecularly imprinted polymer (MIP) for the specific extraction of three cephalosporins from milk samples has been described, using HPLC-UV for analytical measurements. This work has been developed in the *Institut de Chimie Organique et Analytique (ICOA)* Université d'Orléans (Orléans, France) during the predoctoral stay.

The revision of the literature about cephalosporin determination previous to the establishment of the proposed new analytical methods included in this Thesis allowed to produce a review paper discussing analytical methods for the determination of the β-lactam antibiotics by using HPLC with different detection systems in different field of applications using several sample treatments.

Introducción general

1. Plaguicidas

1.1. Generalidades y clasificación

Entre todos los contaminantes orgánicos, son los plaguicidas los que han recibido mayor atención. Su demostrada toxicidad y elevado uso en todo el planeta, así como las posibilidades de contaminación directa en productos de consumo humano, como alimentos o aguas, son las causas de su carácter prioritario como contaminantes a controlar [1]. Los residuos de plaguicidas afectan tanto a la economía como a la salud pública, ya que su presencia, además de constituir una barrera que limita la comercialización de productos en los mercados internacionales, constituye un riesgo para la salud de los consumidores. De este modo, el tema se ha convertido en uno de los problemas ambientales y sanitarios que más interés despierta entre los consumidores de todo el mundo y actualmente recibe un gran esfuerzo investigador en todos los campos.

Muchos de los plaguicidas son contaminantes orgánicos persistentes, encontrándose entre los compuestos más peligrosos producidos. Por esta razón, el uso de muchos de sus derivados ha sido prohibido en todo el mundo. Sin embargo, en medio siglo de producción, la industria química mundial ha generado una elevada cantidad de estos compuestos que se han difundido por todo el planeta, contaminando prácticamente a todos los seres vivos hasta en las tierras más remotas. Probablemente, la consecuencia de mayor alcance derivada del uso de los plaguicidas es su contribución a la contaminación y degradación del ambiente debido a su acumulación en los ecosistemas acuático y terrestre pasando, a través de la cadena alimentaria, a las especies animales y, por último, al hombre. En la figura 1 se muestran las principales rutas de exposición a este tipo de compuestos. Aire, agua y suelo pueden estar en contacto con los plaguicidas así como las fuentes de alimentación, que podrían contribuir a la exposición de pesticidas a los animales, plantas y seres humanos. Lo ideal sería que todas las fuentes de exposición a los plaguicidas fuesen evaluadas para determinar cuál es exactamente el grado de exposición en cada momento.

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[1] M. Correia, C. Delerue-Matos, A. Alves, J. Chromatogr. A, 889 (2000) 59

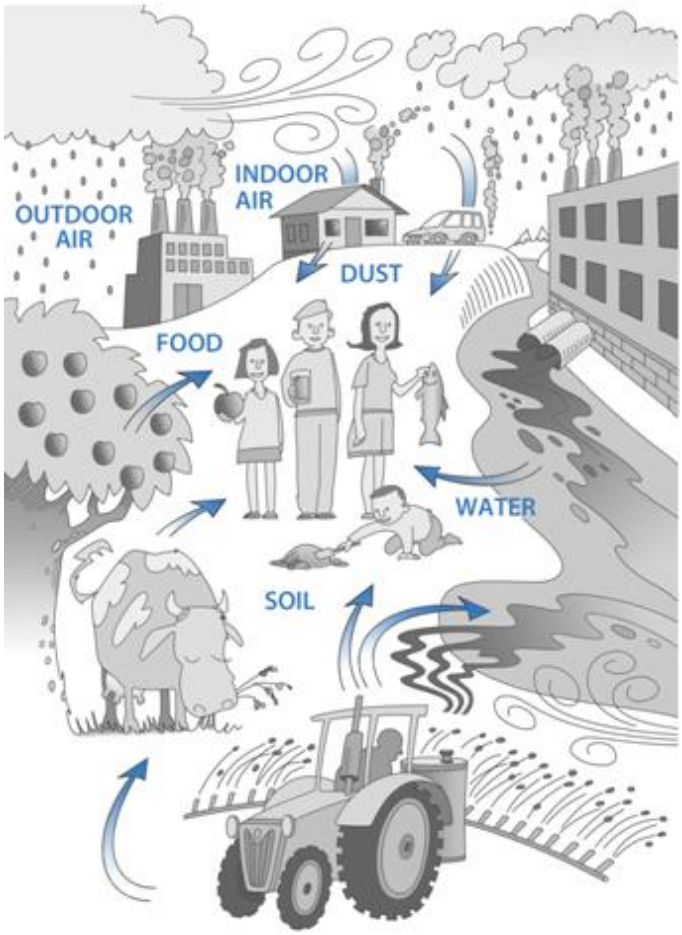

Figura 1. Rutas comunes de exposición a plaguicidas. Adaptado de la página web Carex emissions proyect. Marzo 2013 en: http://carexcanada.uvic.ca/emp/?page_id=827

Según la FAO (Food and Agriculture Organization of United Nations) el término "plaguicida", definido en el *Codex Alimentarius*, incluye cualquier sustancia destinada a prevenir, destruir, atraer, repeler o combatir cualquier plaga, incluidas las especies indeseadas de plantas o animales, durante la producción, almacenamiento, transporte, distribución y elaboración de alimentos, productos agrícolas (entendiendo como tales cereales en bruto, remolacha azucarera y semilla de algodón que no se consideran alimentos) o alimentos para animales, o que pueda administrarse a los animales para combatir ectoparásitos [2,3]. Así, se

[2] Adaptado de la página web de la FAO. Marzo 2013 en: http://www.fao.org/docrep/

incluyen las sustancias destinadas a utilizarse como reguladoras del crecimiento de las plantas, defoliantes, desecantes, agentes para reducir la densidad de fruta o inhibidores de la germinación, y las sustancias aplicadas a los cultivos antes o después de la cosecha para proteger el producto contra el deterioro durante el almacenamiento y transporte. El término no incluye normalmente a los fertilizantes, nutrientes de origen vegetal o animal, aditivos alimentarios ni medicamentos veterinarios.

Según datos de la FAO, dos tercios de la Humanidad están subalimentados, lo que implica que el aumento de la producción agrícola sea una necesidad primordial, para lo cual es necesario incrementar tanto las áreas de cultivo como el rendimiento de las explotaciones. Las pérdidas causadas por las plagas son muy elevadas por lo que se pone de manifiesto la necesidad del empleo de los plaguicidas. Considerando en primer lugar, el elevado número y variedad de parásitos existentes en la naturaleza, y en segundo lugar, la capacidad de algunas especies para desarrollar resistencias a determinados compuestos químicos, se hace necesaria la aplicación de nuevos principios activos para combatirlos.

El uso de los plaguicidas es relativamente reciente, señalándose su introducción a principios del siglo pasado con el descubrimiento de la acción plaguicida de algunos compuestos como el azufre, los arseniatos y el sulfato de cobre, entre otros. A partir de 1922 el desarrollo se hace más rápido y tiene su punto de partida en la utilización de aceites insecticidas en Holanda, descubriéndose también en este periodo la acción insecticida del pelitre y la rotenona. Sin embargo, es a partir del descubrimiento de las propiedades insecticidas del DDT, realizado por Müller en 1940, cuando se suceden con rapidez los descubrimientos de nuevos plaguicidas. Esto ha permitido un aumento en los rendimientos agropecuarios así como en el bienestar humano, pero a la vez, su uso indiscriminado ha provocado en ocasiones un grave problema de contaminación en aguas, suelos, vegetales y aire, entre otros. De aquí el interés que en la actualidad presenta el desarrollo de métodos de análisis de estos compuestos por parte de los investigadores, así como el descubrimiento de nuevas sustancias con acción plaguicida.

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[3] Pesticides, Veterinary and Other Residues in Food, ed. Watson, D., Woodhead Publishing Limited, Cambridge, 2004.

Los plaguicidas se pueden clasificar siguiendo diferentes criterios. Una primera clasificación se puede realizar atendiendo al tipo de parásitos a los que se dirige principalmente su acción [4]:

1. ***Insecticidas***: luchan contra insectos.
2. ***Acaricidas***: combaten la araña roja y los ácaros.
3. ***Antibióticos de uso agrícola***: luchan contra las bacteriosis propias de los cultivos.
4. ***Herbicidas***: luchan contra las malas hierbas, ya sea de un modo general o selectivo, es decir, dejando indemne el cultivo y destruyendo todas o buena parte de las hierbas adventicias (malas hierbas).
5. ***Fungicidas***: actúan contra los hongos causantes de enfermedades en los cultivos.
6. ***Rodenticidas***: se emplean contra las ratas, ratones y topillos.
7. ***Nematicidas***: son usados contra los nematodos.
8. ***Desinfectantes del suelo***: su acción se extiende a nematodos, insectos, hongos y malas hierbas que se encuentran en los suelos destinados a cultivo.

Otra clasificación considera su estructura química de modo que es posible diferenciar las siguientes familias [5]:

1. ***Organoclorados***: Insecticida
2. ***Organofosforados***: Insecticida
3. ***Carbamatos***: Insecticida
4. ***Derivados del ácido carboxílico***: Herbicida
5. ***Triazinas***: Herbicida

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[4] Adapdado de la página web de U.S. Environmental Protection Agency. Marzo 2013 en: http://www.epa.gov/pesticides/about/types.htm

[5] H.S. Rathore, en "Handbook of pesticides, methods of pesticide residues analysis", 1ª Ed. CRC Press 2010, p. 8

6. ***Ureas sustituidas***: Herbicida
7. ***Piretroides***: Insecticida
8. ***Organometálicos***: Funguicida
9. ***Tiocianatos***: Insecticida
10. ***Fenoles***: Insecticida

En la presente Tesis se han propuesto métodos analíticos para la determinación de residuos de herbicidas: productos de degradación del metribuzin, perteneciente a las triacinas y sulfonilureas, incluidas en el grupo de ureas sustituidas.

1.2. Control de residuos de plaguicidas en alimentos

A nivel europeo es posible consultar los aspectos relacionados con la seguridad alimentaria y los contaminantes como los residuos de plaguicidas en la web denominada "Seguridad Alimentaria - De la granja a la mesa" [6], dependiente de la Dirección General Europea para la Salud y los Consumidores. El objetivo fundamental es garantizar un alto nivel de protección de la salud humana y de los intereses de los consumidores en relación con los alimentos.

La FAO y la Organización Mundial de la Salud (OMS) han establecido varios parámetros relacionados con aspectos tóxicos para salvaguardar la salud de los consumidores. Algunos de estos parámetros han sido fijados por las principales agencias de control, y sus definiciones se recogen a continuación [7]:

- **Residuo de plaguicida**

Por "residuo de plaguicida" se entiende cualquier sustancia especificada presente en alimentos, productos agrícolas o alimentos para animales como consecuencia del uso de un plaguicida. El término incluye cualquier derivado de un plaguicida, como productos de conversión, metabolitos y productos de reacción, y las

[6] Adaptado de la página web de la Comisión Europea de Sanidad y Consumo. Marzo 2013 en: http://ec.europa.eu/food/site_map_en.htm

[7] Adaptado de la página web de la FAO. Marzo 2013 en: http://www.fao.org/waicent/faostat/Pest-Residue/pest-s.htm

impurezas consideradas de importancia toxicológica, considerando tanto los residuos de procedencias desconocidas o inevitables (por ejemplo ambientales), como los derivados de usos conocidos de la sustancia química.

- **Buenas prácticas agrícolas en el uso de plaguicidas (BPA)**

Como BPA se entienden los usos inocuos autorizados a nivel nacional, en las condiciones existentes, de los plaguicidas necesarios para un control eficaz y fiable de las plagas. Comprende una gama de niveles de aplicaciones de plaguicidas hasta la concentración de uso autorizado más elevada, de forma que quede la concentración mínima posible del residuo. Los usos inocuos autorizados se determinan a nivel nacional y prevén usos registrados o recomendados en el país que tiene en cuenta las consideraciones de salud pública y profesional, y la seguridad del medio ambiente. Las condiciones existentes comprenden cualquier fase de la producción, almacenamiento, transporte, distribución y elaboración de alimentos para consumo humano y piensos.

- **Ingestión diaria tolerable provisional (IDTP)**

Representa la ingestión humana tolerable de un plaguicida utilizado anteriormente en la agricultura que puede aparecer como contaminante de un alimento, el agua potable y el medio ambiente [8].

- **Ingestión diaria admisible (IDA)**

La IDA de una sustancia química es la dosis diaria que, ingerida durante todo el período vital, parece no entrañar riesgos apreciables para la salud del consumidor, sobre la base de todos los hechos conocidos en el momento de la evaluación de la sustancia química por la Reunión Conjunta FAO/OMS sobre Residuos de Plaguicidas [9]. Se expresa en miligramos de sustancia química por kilogramo de peso corporal.

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[8] Informe de la JMPR de 1995, documento de la serie FAO: Producción y protección vegetal, nº 127, p 5

[9] Informe de 1975 de la Reunión Conjunta FAO/OMS sobre Residuos de Plaguicidas, Estudios FAO: Producción y protección vegetal nº 1, ó OMS: Serie de Informes Técnicos, nº 592

- **Ingestión diaria admisible temporal (IDAT)**

Por IDAT se entiende la dosis de ingestión diaria admisible establecida para un período limitado especificado, con objeto de poder obtener más datos bioquímicos, toxicológicos o de otra índole que se necesiten para estimar una dosis de ingestión diaria admisible. (Nota: Cuando la Reunión Conjunta FAO/OMS sobre Residuos de Plaguicidas estima una IDAT suele aplicar un factor de seguridad más amplio que el utilizado al estimar una IDA).

- **Medida de la toxicidad de un plaguicida**

La toxicidad de los plaguicidas puede expresarse de un modo cuantitativo para animales de experimentación de distintas formas. Así, se denomina dosis letal media, representada como DL_{50}, al número de mg de ingrediente activo, por kg de peso corporal, necesario para producir la muerte del 50% de una gran población de animales de prueba.

Según se realice la administración del producto puede distinguirse entre:

- **DL_{50} oral aguda**: se determina administrando una sola vez una dieta, con una determinada cantidad de tóxico en estudio, a varios grupos iguales de animales.

- **DL_{50} oral crónica**: se determina mediante la observación de los efectos producidos en los distintos grupos de animales de experimentación tras la administración en la dieta diaria de cantidades distintas del producto para cada uno de los lotes durante un tiempo determinado. Se expresa como mg kg^{-1} de plaguicida presente en la dieta alimenticia, durante el tiempo que se especifique, que producen efectos señalados.

- **DL_{50} dérmica**: valora las posibilidades de intoxicación por absorción del plaguicida a través de la piel y representa la cantidad de sustancia necesaria para producir la muerte del 50% de los animales de un lote de investigación, cuando se ha procedido a la fijación del tóxico sobre su piel mediante pincelación del mismo en estado puro o en disolución de la concentración que se indique.

El límite máximo de residuos de plaguicida (LMR) es la concentración máxima de residuos de un plaguicida (expresada en mg kg^{-1}), recomendada por la Comisión del Codex Alimentarius [2], para que se permita legalmente su uso en la superficie

o la parte interna de productos alimenticios para consumo humano y de piensos. Los LMRs se basan en datos de BPA y tienen por objeto lograr que los alimentos derivados de productos básicos que se ajustan a los respectivos LMRs sean toxicológicamente aceptables. Los LMRs del Codex, que se destinan principalmente para ser aplicados a productos que circulan en el comercio internacional, se obtienen basándose en estimaciones hechas por la JMPR (Joint FAO/WHO Meeting on Pesticide Residues), después de:

1. la evaluación toxicológica del plaguicida y su residuo.

2. el examen de datos de residuos obtenidos en ensayos y usos supervisados, en particular usos que se ajustan a las BPA nacionales. En el examen se incluyen datos de ensayos supervisados realizados a la concentración de uso más elevada recomendada, autorizada o registrada en el país. Para tener en cuenta las variaciones introducidas en los requisitos nacionales de control de plagas, en los LMRs del Codex se consideran los niveles más elevados observados en tales ensayos supervisados, que se estima representan las prácticas efectivas de control de plagas.

El examen de las diversas estimaciones y determinaciones, tanto a nivel nacional como internacional, de la ingesta de residuos a través de la alimentación, teniendo en cuenta la ingesta diaria admisible (IDA), debería indicar que los alimentos que se ajustan a los LMRs del Codex son inocuos para el consumo humano. La legislación sobre los LMRs deriva de cuatro Directivas fundamentales, 76/895/CEE, 86/362/CEE, 86/363/CEE y 90/642/CEE. Estas directivas se sustituyeron en 2005 por una legislación única a escala comunitaria [10], con sus correspondientes modificaciones sucesivas [11-13]. El reglamento reúne en un solo texto y armoniza

[10] Reglamento (CE) N. 396/2005 del Parlamento Europeo y del Consejo, de 23 de febrero de 2005 relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal y que modifica la Directiva 91/414/CEE del Consejo. Diario Oficial de la Unión Europea L 70/1-16.

[11] Reglamento (CE) N. 178/2006 de la Comisión de 1 de febrero de 2006 por el que se modifica el Reglamento (CE) N. 396/2005 del Parlamento Europeo y del Consejo con vistas a establecer el anexo I que incluye la lista de alimentos y piensos a los que se aplican contenidos máximos de residuos de plaguicidas. Diario Oficial de la Unión Europea. L 29/3-25.

[12] Reglamento (CE) N. 299/2008 del Parlamento Europeo y del Consejo de 11 de marzo de 2008 por el que se modifica el Reglamento (CE) no 396/2005, relativo a los límites

los límites aplicables a los diferentes productos destinados a la alimentación humana y animal, y fija un límite máximo de 0.01 mg kg^{-1} aplicable por defecto. El texto elimina las diferencias en los LMRs nacionales de los diferentes países de los Estados miembros, facilitando el funcionamiento del mercado interior, así como con terceros países. Existe una base de datos de la UE [14] en la que es posible encontrar una actualización de los LMR de los plaguicidas, toda la legislación al respecto y permite la búsqueda de LMR por sustancias activas y tipo de alimento.

1.3. Control de residuos de plaguicidas en medio ambiente

El problema de la contaminación por plaguicidas es cada vez más grave tanto por la cantidad y diversidad como por la resistencia a ellos que adquieren algunas especies, lo que ocasiona que se requiera cada vez mayor cantidad del plaguicida para obtener el efecto deseado en las plagas. Sin embargo, la flora y fauna oriundas son afectadas cada vez más, destruyendo la diversidad natural de las regiones en que se usan.

La contaminación del ambiente por plaguicidas se da por aplicaciones directas en los cultivos agrícolas, derrames accidentales, lavado inadecuado de tanques contenedores, filtraciones en los depósitos de almacenamiento y residuos descargados y dispuestos en el suelo. Los restos de estos plaguicidas se dispersan en el ambiente y se convierten en contaminantes para los sistemas bióticos (animales y plantas principalmente) y abiótico (suelo, aire y agua) amenazando su estabilidad y representando un peligro de salud pública.

Al introducirlos en el medio ambiente pueden seguir diversos caminos: atmósfera, suelo y agua, pudiendo intercambiarse de un sistema a otro formando un ciclo. Para entender cómo se comportan en el ambiente se necesita conocer cierta información sobre las propiedades físico-químicas de la molécula (solubilidad, presión de vapor, coeficiente de partición octanol-agua, etc.) y su mecanismo de

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máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal, por lo que se refiere a las competencias de ejecución atribuidas a la Comisión.L 97/67-71.

[13] Reglamento (UE) N. 600/2010 de la Comisión de 8 de julio de 2010 por el que se modifica el anexo I del Reglamento (CE) N. 396/2005 del Parlamento Europeo y del Consejo a fin de añadir y modificar ejemplos de variedades u otros productos relacionados a los que se aplica un mismo LMR

[14] Adaptado de la página web de base de datos de pesticidas de la Unión Europea. Marzo 2013 en: http://ec.europa.eu/sanco_pesticides/public/index.cfm

transporte (difusión, lixiviación, evaporación), así como las características medioambientales y la geografía del lugar. Por otra parte, la molécula de plaguicida no permanece intacta por tiempo indefinido en el medio ambiente, ya que con el tiempo sufre una degradación influenciada por microorganismos, actividad química, pH, clima, y contenido de materia orgánica del suelo, entre otros.

La distribución de un plaguicida en la biofase (plantas y microorganismos) depende de la capacidad de absorción de ésta y de la naturaleza del suelo. Un suelo con gran capacidad de absorción puede conducir a la inactividad total del plaguicida, ya que nunca penetrara en la plaga. La contaminación del suelo consiste en la introducción de un elemento extraño al sistema que, por sí mismo o por su efecto sobre los restantes componentes, genera un efecto nocivo para los organismos del suelo, sus consumidores, o es susceptible de transmitirse a otros sistemas. Los aspectos propiamente ecotoxicológicos derivados de la aplicación de los plaguicidas a los suelos comprenden: persistencia de plaguicidas en suelos, producción de metabolitos tóxicos, influencia de los plaguicidas en la microflora del suelo, incidencia sobre las propiedades del suelo y riesgo de contaminación de aguas subterráneas [15].

El agua es un importantísimo componente de los seres vivos y es factor limitante de la productividad de muchos ecosistemas. La contaminación de los cursos de agua por plaguicidas se produce en forma directa por la aplicación de plaguicidas en las aguas, por lavado de envases o equipos y por descarga de remanentes y residuos. Es igualmente importante la contribución indirecta producida por lixiviación (infiltración) de productos, caída por desniveles y por contaminación de suelos. Las aguas contaminadas expanden el tóxico a la flora y fauna produciendo la muerte de especies, el aumento de la intoxicación humana, la pérdida del curso de agua como recurso utilizable y la probable contaminación de las reservas hídricas (acuíferos) [16].

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[15] Evaluación de la contaminación del suelo: Manual de referencia. Depósito de Documentos de la FAO. Documento de campo GCP/INT/650/NET. www.fao.org/docrep/005/x2570s/X2570S00.htm.

[16] Lucha contra la contaminación agrícola de los recursos hídricos. Depósito de Documentos de la FAO. Capítulo 4 - los plaguicidas, en cuanto contaminantes del agua. Marzo 2013 en:
http://www.fao.org/docrep/W2598S/w2598s06.htm

Las aguas superficiales de los continentes fueron las más visiblemente contaminadas durante muchos años, pero precisamente al ser tan visibles los daños que sufren, son las más vigiladas y las que están siendo regeneradas con más eficacia en muchos lugares del mundo, especialmente en los países desarrollados, a través de las denominadas Redes de vigilancia de calidad de las aguas superficiales. En España esta red de control se denomina Red ICA (Red Integrada de Calidad de las Aguas) que desde el año 1992 recoge los datos obtenidos en las distintas redes existentes en ese momento como son la Red COCA (Control de Calidad General de las Aguas), la Red COAS (Control Oficial de Abastecimientos) y la Red ICTIOFAUNA que controla la aptitud del agua para la vida piscícola.

Hoy en día la calidad química del agua en Europa está regulada por la directiva 2000/60/EC (Water Framework Directive, WFD) [17] incluyendo como uno de sus principales objetivos prevenir el deterioro, proteger y mejorar el estado de los ecosistemas acuáticos, terrestres y humedales que directamente dependen de los sistemas acuáticos. Dentro de este marco, la estrategia clave adoptada, en el área de la contaminación química, ha sido la directiva 2455/2001/CE [18], que estableció una lista de 33 sustancias o grupos de sustancias de principal interés debido a su persistencia, toxicidad, bioacumulación y uso generalizado y a su detección en ríos, lagos y aguas de transición y costeras. También, más recientemente, la Directiva 2008/105/CE [19] ha supuesto un nuevo paso en avance de la regulación, ya que establece por vez primera, las normas de calidad de sustancias de interés prioritario y otros contaminantes que también suscitan preocupación, para asegurar una adecuada protección del medio ambiente acuático y la salud humana.

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[17] Directive of the European Parliament and of the council 2000/60/EC establishing a framework for community action in the field of water policy. L 327/1, 22/12/2000.

[18] Decisión n. 2455/2001/CE del parlamento europeo y del consejo de 20 de noviembre de 2001 por la que se aprueba la lista de sustancias prioritarias en el ámbito de la política de aguas, y por la que se modifica la Directiva 2000/60/CE. L 331/1, 15/12/2001.

[19] Directiva 2008/105/CE del parlamento europeo y del consejo de 16 de diciembre de 2008 relativa a las normas de calidad ambiental en el ámbito de la política de aguas, por la que se modifican y derogan ulteriormente las Directivas 82/176/CEE, 83/513/CEE, 84/156/CEE, 84/491/CEE y 86/280/CEE del Consejo, y por la que se modifica la Directiva 2000/60/CE. L 348/84, 24/12/2008.

También en este ámbito se encuentra la Directiva 98/83/EC relativa a la calidad de las aguas destinadas al consumo humano [20], y la Directiva 2006/118/EC relativa a la protección de las aguas subterráneas contra la contaminación y el deterioro [21], donde se establece que la concentración de un plaguicida individual no puede superar los 0.1 μg L^{-1} mientras que la suma de plaguicidas totales no puede exceder de 0.5 μg L^{-1}.

2. Antibióticos

2.1. Generalidades y clasificación

La palabra antibiótico viene del griego αντί – anti y βιοτικός - biotikos, "dado a la vida", es decir que es una sustancia que, producida por un ser vivo o sintetizada en un laboratorio, inhibe el desarrollo y multiplicación de los microorganismos (acción bacteriostática) u origina su destrucción (acción bactericida). Estrictamente hablando, el término antibiótico solo incluiría a las penicilinas, tetraciclinas, macrólidos, aminoglicósidos y anfenicoles ya que inicialmente el término antibiótico sólo se utilizaba para referirse a los compuestos orgánicos producidos por microorganismos (bacterias, hongos y actinomicetos) que resultaban tóxicos para otros microrganismos. Sin embargo, hoy día se utiliza como sinónimo de antibacteria y en la actualidad también se emplea para denominar compuestos sintéticos, como las sulfonamidas y las quinolonas.

Los antibacterianos son la principal categoría de antibióticos, pero se incluye también, en este tipo de fármacos, a los antipalúdicos, antivirales y antiprotozoos. El mecanismo de acción de los antibióticos no se ha conocido de forma científica hasta el siglo XX; sin embargo, la utilización de compuestos orgánicos en el tratamiento de la infección se conoce desde la antigüedad. Los extractos de ciertas plantas medicinales se han usado durante siglos, y también existe evidencia de la aplicación de extractos de hongos que crecen en ciertos quesos para el

[20]Directiva 98/83/CE del Consejo de 3 de noviembre de 1998 relativa a la calidad de las aguas destinadas al consumo humano. Diario Oficial n° L 330 de 05/12/1998 p. 0032 - 0054
[21]Directiva 2006/118/CE del Parlamento Europeo y del Consejo, de 12 de diciembre de 2006, relativa a la protección de las aguas subterráneas contra la contaminación y el deterioro. Diario Oficial n° L 372 de 27/12/2006 p. 0019 - 0031

tratamiento tópico de las infecciones. La primera observación de lo que hoy en día se denominaría efecto antibiótico fue realizada en el siglo XIX por el químico francés Louis Pasteur, al descubrir que algunas bacterias saprofitas podían destruir gérmenes del carbunco (enfermedad también conocida como ántrax). Hacia 1900, el bacteriólogo alemán Rudolf von Emmerich aisló una sustancia capaz de destruir los gérmenes del cólera y la difteria en un tubo de ensayo, no siendo eficaces en el tratamiento de las enfermedades. Alexander Fleming descubrió, de forma accidental, la penicilina en 1928, que es un derivado del hongo Penicillium notatum; esta sustancia demostró su eficacia frente a cultivos de laboratorio de algunas bacterias patógenas como las de la gonorrea, de la meningitis o la septicemia. Este descubrimiento permitió el desarrollo de posteriores compuestos antibacterianos producidos por organismos vivos [22].

Existen diferentes clasificaciones de los antibióticos siendo las más habituales las que se realizan en función de su mecanismo de acción frente a los organismos infecciosos o en función de su estructura química, pero también se pueden clasificar en función de su espectro bacteriano (Gram-positivos, Gram-negativos, de amplio espectro y de espectro selectivo) o según su origen (micóticos, bacterianos, actinomices, sintéticos o semisintéticos).

En función de su estructura química podemos encontrar la siguiente clasificación de los antibióticos en [23]:

- Aminoglucósidos
- Glicopéptidos
- Betalactamas (Penicilinas y Cefalosporinas)
- Carbapenemas
- Macrolidos
- Monobactamas
- Polipectidicos
- Oxazolidinonas

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[22] Adaptado de la página web de la Junta de Andalucía. Marzo 2013 en: http://www.juntadeandalucia.es/averroes/~29701428/salud/anti.htm

[23] R.W. McLawhon, en “Therapeutic Drug Monitoring: Newer Drugs and Biomarkers”, 1ª Ed, Academic Press 2000, p. 199

- Quinolonas
- Sulfonamidas
- Tetraciclinas

Entre todos estos antibióticos, la presente Tesis Doctoral se ha centrado en el desarrollo de métodos analíticos para la determinación de la familia de las llamadas Cefalosporinas, cuyas características se describirán en la introducción al bloque correspondiente a los métodos analíticos propuestos para estos compuestos.

2.2. Control de residuos de antibióticos en alimentos

En los últimos años existe una preocupación pública acerca del empleo de medicamentos en animales productores de alimentos, fundamentalmente de antibióticos debido a la transferencia al humano de bacterias resistentes [24]. Tales sustancias pueden igualmente ser usadas en el animal no sólo para tratar infecciones, en el caso de animales enfermos, sino que hasta el año 2006 también se suministraron como promotores del crecimiento ya que afectan a la flora microbiana y a los procesos de digestión ralentizando éstos y favoreciendo el proceso de absorción de nutrientes, lo que origina el engorde del animal, y por ellos los residuos de estos compuestos han podido estar presentes tanto en animales sanos como enfermos.

Mediante el Reglamento (CE) No 1831/2003 [25], a partir del 1 de Enero de 2006 se prohibió el uso de antibióticos como promotores del crecimiento. Markos Kyprianou, Comisionado para la Salud y la Protección al Consumidor, ha indicado a este respecto: "esta prohibición de los antibióticos como promotores de crecimiento es de gran importancia, no sólo como parte de la estrategia de seguridad alimenticia de la Unión Europea, sino también considerando la salud pública. Necesitamos reducir ampliamente el uso no esencial de antibióticos si efectivamente estamos de acuerdo sobre el problema de los microorganismos que se vuelven resistentes a los tratamientos, en los que hemos confiado por años. El

[24] L.J.V. Piddock, J. Antimicrob. Chemother. 38 (1996) 1

[25] Reglamento (CE) No 1831/2003 del Parlamento Europeo y del Consejo de 22 de septiembre de 2003 sobre los aditivos en la alimentación animal. DOCE L268/29 de 18-10-2003

alimento para animales es el primer paso en la cadena alimenticia, y un buen lugar para actuar tratando de llegar a este objetivo".

Algunas consecuencias de un uso indebido y no controlado de los antibióticos es su incidencia en diferentes aspectos relacionados con la calidad y seguridad alimentaria, tales como: (a) pueden producir reacciones de hipersensibilidad alérgicas en algunas personas; (b) inciden negativamente en procesos industriales de fermentación en alimentos lácteos, tales como la elaboración del yogurt; y (c) la presencia de antibacterianos puede ocultar la existencia de patógenos en alimentos de origen animal cuando se realizan análisis bacteriológicos. Estas son las principales razones para controlar la existencia de tales residuos en alimentos de origen animal [26].

La normativa Europea establece los requisitos necesarios para garantizar la seguridad, eficacia y calidad de los medicamentos veterinarios en todo el proceso de su autorización, elaboración, comercio, almacenamiento, suministro y utilización, en relación con los animales de destino, así como de su seguridad, habida cuenta las repercusiones sobre la salud pública y el medio ambiente. Así, en el R.D. 109/1995 [27] se exponen los requisitos de calidad, eficacia, seguridad y pureza que ha de cumplir un medicamento veterinario para poder ser registrado y autorizado. En este sentido, no podrá autorizarse la puesta en el mercado de un medicamento veterinario, con excepción de los inmunológicos, para ser administrado a animales cuya carne o productos sean destinados al consumo humano si no tiene establecido el correspondiente LMR tal y como está previsto en el Reglamento (CE) Nº 2377/90 [28].

Se define el LMR específicamente en este caso como “la concentración o nivel máximo permitido de un medicamento o sustancia química en un pienso o alimento desde el momento del sacrificio, recolección, procesado, almacenamiento o venta y consumo por animales o el hombre”. Se calcula para cada tejido (matriz) en particular, dividiendo el producto de la IDA y el peso

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[26] J.D.G. McEvoy, Anal. Chim. Acta, 473 (2002) 3

[27] Real Decreto 109/1995, de 27 de enero sobre medicamentos veterinarios. BOE. 53, de 3 de marzo de 1995

[28] Reglamento 2377/90/CEE del Consejo, de 26 de junio de 1990, por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. DOCE L 224 de 18 de agosto de 1990. Modificado por el Reglamento 1338/2000/CE de la Comisión, de 20 de octubre de 2000. DOCE L 269 de 21 de octubre de 2000

corporal promedio humano (60 kg) por la ingesta diaria estimada para ese tejido en particular y se expresa en mg o µg del compuesto por kilo de alimento fresco. Una vez que se establecen los LMRs, es necesario establecer en qué momento a partir de la aplicación de un producto veterinario, tanto el principio activo como sus metabolitos se hayan eliminado hasta encontrarse en un valor inferior al LMR. Este es el fundamento del establecimiento del "periodo de restricción o periodo de retirada". El periodo de retirada de un producto veterinario es el tiempo que debe transcurrir entre la última administración del mismo y el sacrificio o recolección de los productos que se derivan de esos animales tratados destinados al consumo humano.

En el Reglamento (CE) Nº 2377/90, para proteger la salud pública, las sustancias farmacológicamente activas, atendiendo a la evaluación científica de su seguridad, fueron clasificadas en cuatro anexos:

- ***Anexo I****:* sustancias farmacológicamente activas para las que se ha fijado un LMR
- ***Anexo II***: sustancias para las que no es necesario fijar un límite máximo de residuos
- ***Anexo III***: sustancias para las que se ha fijado un límite máximo de residuos provisional
- ***Anexo IV***: sustancias para las que no puede establecerse límite máximo alguno porque sus residuos, sea cual sea su límite, constituyen un riesgo para la salud humana.

La Directiva Comunitaria 96/23/CE [29] establece las medidas de control y los planes de vigilancia que deben aplicarse para la detección de determinadas sustancias y sus residuos, potencialmente tóxicos para el consumidor, en animales vivos o productos de origen animal destinados al consumo humano. Así, obliga a los países terceros a ofrecer garantías en cuanto a la cantidad de residuos que contienen los productos que exportan, en relación con todos los grupos de sustancias enumerados en el anexo I. En el anexo I de esta Directiva se enumeran

[29] Directiva 96/23/EC de Abril 1996 relativa a las medidas de control aplicables respecto de determinadas sustancia y sus residuos en animales vivos y sus productos y por la que se derogan las Directivas 85/358/EEC y 86/469/EEC y las Decisiones 89/18/EEC y 91/664/EEC, Off. J. Eur. Commun. 1996, L125, 10–18

los grupos de sustancias que deben ser controlados en cada especie animal o producto, distinguiendo entre:

Grupo A, que engloba sustancias con efecto anabolizante y la mayoría de las sustancias de uso prohibido por la UE en animales destinados a la producción de alimentos, está compuesto por 6 subgrupos:

A1. Estilbenos, derivados de los estilbenos, sus sales y ésteres.

A2. Agentes antitiroidianos.

A3. Esteroides.

A4. Compuesto de lactona del ácido resorcíclico (incluido zeranol).

A5. β-agonistas.

A6. Sustancias incluidas en el anexo IV del Reglamento (CEE) Nº 2377/90 (o en el cuadro 2 del nuevo Reglamento (UE) Nº 37/2010) (cloranfenicol, cloroformo, clorpromacina, colchicina, dapsona, nitroimidazoles (dimetridazol, metronidazol), nitrofuranos (incluida furazolidona) y ronidazol)

Grupo B, que incluye los residuos de numerosas sustancias farmacológicamente activas, cuyo uso en animales destinados a la producción de alimentos puede estar autorizado en la UE y para los que se fija un LMR (sustancias incluidas en los anexos I, II o III del Reglamento (CEE) Nº 2377/90 o del nuevo Reglamento (UE) Nº 37/2010):

B1. Sustancias antiinfecciosas (antibacterianos y antibióticos): penicilinas, cefalosporinas, quinolonas, macrólidos, tetraciclinas, sulfamidas, lincosamidas, aminoglucósidos, novobiocina, ácido clavulánico, etc.

B2. Otros medicamentos veterinarios:

a) Antihelmínticos: medicamentos utilizados en el tratamiento de las helmintiasis, es decir las infestaciones por vermes, helmintos o lombrices (benzimidazoles como albendazol, mebendazol, tiabendazol, etc.; avermectinas como abamectina, ivermectina, doramectina, moxidectina, emamectina, etc.).

b) Anticoccidiales: medicamento usados para el tratamiento de la coccidiosis, enfermando del tracto intestinal producida por un parásito y que afecta normalmente a aves de corral sometidas a condiciones de hacinamiento (amprolio, halofuginona, diclazurilo, decoquinato, monensina, lasalócido como ionóforos), incluidos los nitroimidazoles.

c) Carbamatos y piretroides.

d) Tranquilizantes.

e) Antiinflamatorios no esteroideos (AINS).

f) Otras sustancias que ejerzan una actividad farmacológica.

B3. Otras sustancias y contaminantes medioambientales:

a) Compuestos organoclorados, incluidos los PCB.

b) Compuestos organofosforados.

c) Elementos químicos.

d) Micotoxinas.

e) Colorantes.

f) Otros.

Como se muestra a continuación, en la tabla 1, en el Anexo II de esta Directiva se indica el grupo de residuos o sustancias que habrán de detectarse según el tipo de animales, sus piensos y agua de beber y en función del tipo de productos animales de origen primario.

Asimismo, el Reglamento (CE) N° 1181/2002 de la Comisión [30] modifica el anexo I del Reglamento (CE) N° 2377/90 del Consejo, sobre los LMR de medicamentos veterinarios en alimentos de origen animal, incorporando nuevas sustancias. Los

[30] Reglamento 1181/2002 de la Comisión, de 1 de julio de 2002, por el que se modifica el anexo I del Reglamento (CEE) nº 2377/90 del Consejo por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. DOCE L 172 de 2 de Julio de 2002

grupos incluidos cuyos LMRs están legislados en diferentes tejidos animales comestibles y productos (grasa, hígado, riñón, leche, músculo, etc.) para diversas especies productoras de alimentos (bovinos, porcinos, caprinos, equinos, aves, conejos, salmónidos, etc.). Dentro de los agentes antiinfecciosos se encuentran los *quimioterapeúticos* (sulfonamidas y derivados de la diaminopirimidina), *antibióticos* (penicilinas, cefalosporinas, quinolonas, macrólidos, flurofenicol y compuestos asociados, tetraciclinas, ansamicina, pleuromutilinas, lincosamidas, aminoglucósidos, etc.) y *agentes antiparasitarios* (salicilanidas, benzimidazoles, derivados fenólicos, ect.). Como otros fármacos de uso veterinario de este grupo se encuentran los *antihelmínticos*, los *anticoccidiales*, incluyendo nitroimidazoles, carbamatos y piretroides, *sedantes*, *antiinflamatorios no esteroideos* y *otras sustancias farmacológicamente activas*. Este reglamento ha ido sufriendo diversas modificaciones, incorporando nuevas sustancias o modificando los LMR. Tales modificaciones quedan recogidas en una versión no oficial consolidada [31].

[31] Reglamento (CEE) Nº 2377/90 del consejo de 26 de junio de 1990 por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. Marzo 2013 en: http://eur-lex.europa.eu/LexUriServ/site/es/consleg/1990/R/01990R2377-20070516-es.pdf

Tabla 1. Anexo II de la Directiva Comunitaria 96/23/CE

Sustancias	Animales RD 147/93*	Aves de corral	Animales de acuicultura	Leche	Huevos	Carne de conejo y caza	Miel
A1	X	X	X			X	
A2	X	X				X	
A3	X	X	X			X	
A4	X	X				X	
A5	X	X	X			X	
A6	X	X	X	X	X	X	
B1	X	X	X	X	X	X	X
B2a	X	X	X	X		X	
Bb	X	X			X		
Bc	X	X				X	X
Bd	X						
Be	X	X		X		X	
Bf							
B3a	X	X	X	X	X	X	X
Bb	X			X			X
Bc	X	X	X	X		X	X
Bd	X	X	X	X			
Be			X				
Bf							

*RD 147/93, de 29 de enero por el que se establecen las condiciones sanitarias de producción y comercialización de carnes frescas. Animales incluidos: bovino, porcino, caprino, y equino

Tal y como se observa, las Cefalosporinas (pertenecientes al grupo B1) deben determinarse en todos los productos recogidos en la tabla 1: carne fresca de ganado bovino, porcino, caprino y equino, aves de corral, animales de acuicultura, leche, huevos, carne de conejo y caza y miel.

Recientemente se dispone de un nuevo documento que sustituye al Reglamento (CE) Nº 2377/90 sobre LMR. Este Reglamento (UE) Nº 37/2010 [32] indica que por razones de simplificación, es necesario incorporar las sustancias farmacológicamente activas y su clasificación por lo que se refiere a los LMRs a un Reglamento de la Comisión. Dado que esta clasificación sigue el sistema previsto

[32] Reglamento (UE) Nº 37/2010 de la Comisión de 22 de diciembre de 2009 relativo a las sustancias farmacológicamente activas y su clasificación por lo que se refiere a los límites máximos de residuos en los productos alimenticios de origen animal. DOUE, 20-1-2010, L15/1.

en el Reglamento (CE) Nº 470/2009 [33], donde se derogaba el Reglamento (CEE) Nº 2377/90, debe ser también tenida en cuenta en relación con la posibilidad de administrar estas sustancias farmacológicamente activas a animales destinados a la producción de alimentos. Así, se incorpora la información existente sobre la clasificación terapéutica de las sustancias farmacológicamente activas contenidas en los anexos del Reglamento (CEE) Nº 2377/90 en una columna específica y para una mayor comodidad de uso, las sustancias farmacológicamente activas se enumeran en un anexo por orden alfabético, estableciéndose dos cuadros distintos: uno para las sustancias autorizadas, que figuran en los Anexos I, II y III del Reglamento (CEE) Nº 2377/90, y otro para las sustancias prohibidas, enumeradas en el Anexo IV del anterior reglamento.

2.3. Control de residuos de antibióticos en medio ambiente

Además de lo expuesto hasta ahora en cuanto al riesgo que supone la presencia de residuos de fármacos en los alimentos y su control, existe otro importante problema adicional y es el de su incorporación constante en el medio ambiente sin el debido control [34,35]. Así, los antibióticos y las sustancias empleadas como aditivos alimentarios para incrementar el crecimiento de animales (como por ejemplo en piscifactorías), junto con los residuos que generan los humanos (ya sea a través de la excreción de los mismos o por su indebido uso y desecho), los vertidos de residuos hospitalarios en la redes urbanas de alcantarillado o los vertidos controlados de las industrias farmacéuticas al medio ambiente, están entre las fuentes de contaminación más significativas sin que se tenga gran conocimiento de las consecuencias de esta exposición, pudiéndose acumular y afectar a los microrganismos acuáticos. Muchas de las sustancias empleadas en medicina, tanto en humanos como en veterinaria, son excretados tal y como son ingeridas en un elevado porcentaje y junto con las otras fuentes señaladas pueden dar lugar a

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[33] Reglamento (CE) Nº 470/2009 del Parlamento Europeo y del Consejo de 6 de mayo de 2009 por el que se establecen procedimientos comunitarios para la fijación de los límites de residuos de las sustancias farmacológicamente activas en los alimentos de origen animal, se deroga el Reglamento (CEE) n o 2377/90 del Consejo y se modifican la Directiva 2001/82/CE del Parlamento Europeo y del Consejo y el Reglamento (CE) n o 726/2004 del Parlamento Europeo y del Consejo. DOUE, 16-6-2009, L152/11.

[34] F.M. Christensen, Regul. Toxicol. Pharmacol. 28 (1998) 212

[35] R.T. Williams, “Human Pharmaceuticals: Assessing the Impacts of Aquatic Ecosystems, Society of Environmental Toxicology and Chemistry (SETAC)”, (Ed) Florida, 2005

concentraciones locales altas.

Una vez en el medio ambiente, los fármacos pueden seguir tres rutas principales [36,37]:

1. El producto se mineraliza a dióxido de carbono y agua.

2. El producto es lipofílico y parte de él no se degrada, quedando en los sedimentos.

3. El producto es hidrófilo o se metaboliza a una forma más hidrofílica, pero aún persistente, terminando en las aguas residuales o en un río, pudiendo afectar a organismos acuáticos, si los metabolitos son biológicamente activos. Puede permanecer en sedimentos o campos de cultivo, terminar formando parte del estiércol y afectar a microorganismos o cultivos.

Desafortunadamente, aún hay falta de información sobre el impacto que pueden producir la presencia, destino y efectos tóxicos en el medio ambiente de disruptores endocrinos, fármacos y productos de cuidado personal ya que muy pocos han sido clasificados a nivel mundial como contaminantes. Los primeros estudios sobre la presencia de fármacos en aguas residuales tratadas y de sus efectos adversos sobre la fauna y la flora fueron realizados en los años 70 en Estados Unidos, y llevaron a la FDA (Agencia de Alimentos y Medicamentos, U.S. Food and Drug Administration) y a la UE a realizar estudios que contemplaban la evaluación del impacto ambiental y el desarrollo de estrategias de tratamiento, estudio de metabolitos, toxicidad, eliminación, biorremediación y técnicas instrumentales para su identificación y cuantificación [38-40].

En los últimos años se han publicado diversos artículos de revisión [41-45] y diversos trabajos de investigación [46-48] concernientes al efecto y determinación

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[36] S.E. Jørgensen, B. Halling-Sørensen, Chemosphere, 40 (2000) 691
[37] B. Halling-Sørensen, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lützhøfl, S.E. Jørgensen, Chemosphere, 36 (1998) 357
[38] K. Fent, A.A. Weston, D. Caminada, Aquat. Toxicol 76 (2006) 122
[39] K. Kümmerer, J. Environmen. Manag. 90 (2009) 2354
[40] M.D. Celiz, J. Tso, D.S. Aga, Environ. Tox. Chem. 28 (2009) 2473
[41] C.J. Cartagena, Revista Lasallista de Investigación, 8 (2011) 143-153
[42] M.S. Díaz–Cruz, M.J. López de Alba, D. Barceló, Trends Anal. Chem. 22 (2003) 340
[43] K. Wille, H.F. De Brabander, L. Vanhaecke, E. De Wulf, P. Van Caeter, C.R. Janssen, Trends Anal. Chem. 35 (2012) 87

de estos “contaminantes emergentes” (como fármacos y productos de cuidado personal) en el medio ambiente, lo que demuestra el creciente interés que este problema está suscitando en la comunidad científica. La mayoría de estos estudios se centran en el estudio de aguas, especialmente aguas de consumo, pero pocos se centran en el estudio de suelos, lodos o sedimentos [45]. A partir de estos estudios se han obtenido nuevos conocimientos sobre los efectos de xenobióticos de uso extendido, que actúan como disruptores endocrinos, lo que ha provocado inquietud en ámbitos relacionados con la salud pública ya que sustancias consideradas como seguras pueden causar efectos desconocidos incluso a concentraciones traza. Así, se han detectado una gran variedad de productos farmacéuticos y cosméticos (como β-bloqueantes, analgésicos, disruptores endocrinos y antimicrobianos) en muestras de aguas subterráneas, de consumo o en plantas de tratamiento de aguas residuales en diferentes países [45-53]. Estos contaminantes emergentes tienen propiedades bioacumulativas, provocando efectos que aún son desconocidos en los ecosistemas acuáticos o terrestres. Por ello, los medicamentos pueden haberse convertido en un problema medioambiental de considerable envergadura. Si hasta hace unos años lo que preocupaba prioritariamente eran los vertidos de carácter industrial, ahora el interés se centra en el impacto de los vertidos que reciben los ríos desde los núcleos de población.

No obstante, a pesar de todas las disposiciones establecidas para el control de sustancias contaminantes en los medios acuáticos naturales aún no se han incluido los antibióticos en ninguna de estas listas porque aún se desconocen con certeza los

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[44] K. Y. Bell, M.J.M. Wells, K.A. Traexler, M.L. Pellegrin, A. Morse,J. Bandy, Water Environ. Res. 83 (2011) 1906
[45] M. Petrovic, E. Eljarrat, M. J. Lopez de Alda, D. Barceló, Anal. Bioanal. Chem. 378 (2004) 549
[46] Y. Yu, L. Wu, Talanta, 89 (2012) 258
[47] K. Wille, M. Claessens, K. Rappé, E. Monteyne, C.R. Janssen, H.F. De Brabander, L. Vanhaecke, J. Chromatogr. A, 1218 (2011) 9162
[48] C.L. Chitescu, E. Oosterink, J. Jong, A.A.M. Stolker, Anal. Bioanal. Chem. 403 (2012) 2997
[49] M.J. Martínez-Bueno, M.J. Gómez, S. Herrera, M.D. Hernando, A. Agüera, A.R. Fernández-Alba, Environ. Pollut. 164 (2012) 267
[50] R.H. Lindberg, P. Wennberg, M.I. Johansson, Environ. Sci. Technol. 39 (2005) 3421
[51] D. Bendz, N. A. Paxeus, T. R. Ginn, F.J. Loge, J. Hazard. Mater. 22 (2005) 195
[52] M. Kuster, M. J. López de Alda, M.D. Hernando, M. Petrovic, J. Martín Alonso, D. Barceló, J. Hydrol. 358 (2008) 112
[53] M. Pedrouzo, F. Borrull, E. Pocurull, R. M. Marcé, Water Air Soil Pollut. 217 (2011) 217

posibles efectos perjudiciales que el uso del producto puede causar al medio ambiente.

En la UE, la legislación en cuanto a la evaluación del riesgo medioambiental de los fármacos veterinarios tuvo su comienzo a principios de los 80 culminando en la Directiva 2001/82/CE del Parlamento y del consejo de 6 de noviembre de 2001 [54] por la que se establece un código comunitario sobre medicamentos veterinarios, y que ha sufrido diversas modificaciones posteriormente. Esta directiva implica que cualquier regulación en materia de producción y de distribución de medicamentos veterinarios deberá tener en cuenta el posible impacto medioambiental. Así, en su Anexo 1 establece la necesidad de evaluar el riesgo medioambiental de medicamentos veterinarios en busca de los posibles efectos dañinos del uso del medicamento veterinario y para determinar el riesgo de tales efectos.

En la evaluación se identificará cualquier medida preventiva que pueda ser necesaria para reducir tal riesgo y se realizará en dos fases: La primera fase de la evaluación estudiará la posible exposición del medio ambiente al medicamento y el riesgo asociado con tal exposición, teniendo especialmente en cuenta los puntos siguientes:

- las especies animales de destino y la utilización propuesta, el modo de administración y, en particular, el grado probable de incorporación directa del producto al ecosistema,
- la posibilidad de que el medicamento, sus principios activos o sus metabolitos pasen de los animales tratados al medio ambiente y su persistencia en las excreciones,
- la eliminación de medicamentos veterinarios no utilizados u otros residuos.

En la segunda fase se investigará, de manera específica, el destino y los efectos del medicamento en ecosistemas particulares, según las directrices establecidas. Se tendrá en cuenta la amplitud y duración de la exposición del medio ambiente al medicamento y la información disponible sobre las propiedades fisicoquímicas,

[54] Directiva 2001/82/CE del Parlamento Europeo y del Consejo de 6 de noviembre de 2001 por la que se establece un código comunitario sobre medicamentos veterinarios. DO L 311/1, 28.11.2001

farmacológicas o toxicológicas de la sustancia en cuestión que se haya obtenido durante la realización de las demás pruebas exigidas.

Para llevar a cabo la evaluación del riesgo medioambiental de los fármacos de uso veterinario, la UE ha publicado diversas guías como, por ejemplo, la *Environmental Risk Assessment for Veterinary Medicinal Products other than GMO containing and Immunological Products*, EMEA/CVMP/055/96) [55], publicada para proporcionar orientación sobre la evaluación del riesgo de la exposición del medio ambiente a un medicamento veterinario, sus ingredientes y los metabolitos, así como la evaluación de los posibles efectos perjudiciales que el uso del producto puede causar al medio ambiente. No obstante y a pesar de lo expuesto, en muchos casos no se han establecido requisitos legales para limitar el posible impacto producido por estos productos en exposiciones a largo plazo y a bajos niveles de concentración, no existiendo actualmente límites máximos de concentración permitidos de fármacos en muestras medioambientales.

En esta Tesis Doctoral se ha abordado el estudio de la presencia de cefalosporinas en aguas de diversa procedencia. Éstas pertenecen a la familia de las β-lactamas y se encuentran entre las 5 clases de antibióticos (β-lactamas, macrólidos, quinolonas, sulfonamidas y tetraciclinas) más frecuentemente encontradas en el medio ambiente [56].

3. Técnicas separativas miniaturizadas para el análisis de contaminantes

En los últimos años las técnicas de separación miniaturizadas han cobrado gran interés debido a las numerosas ventajas que presentan, tales como la reducción del consumo de disolventes, el incremento en la resolución e incluso una mejorada sensibilidad. Igualmente es de destacar los bajos volúmenes de muestras requeridos, lo que constituye una gran ventaja, fundamentalmente en el ámbito

[55] Guideline: Environmental Risk Assessment for Veterinary Medicinal Products other than GMO containing and Immunological Products. Octubre 2012 en: http://ec.europa.eu/health/files/eudralex/vol-7/a/7ar1a_en.pdf
[56] A. Jia, Y. Wan, Y. Xiao, J. Hu, Water Res. 46 (2012) 387

biomédico [57]. En general, dentro de los sistemas miniaturizados se engloban actualmente los que utilizan columnas de bajo diámetro interno o capilares de sílice fundida, y aquellos en los que la separación se realiza en microchips. En esta Tesis se han empleado dos de las técnicas con columnas de reducido diámetro: *la Electroforesis Capilar* y la *Cromatografía Líquida Capilar.*

3.1. Electroforesis Capilar

La *Electroforesis Capilar (CE)* es una técnica separativa miniaturizada competitiva con la cromatografía que presenta como principales características: altas eficacias de separación, tiempos de separación cortos, volúmenes de muestra muy pequeños al emplear capilares muy estrechos y cantidades de reactivos muy pequeñas, por lo que el coste y la contaminación ambiental disminuyen considerablemente, en comparación con HPLC, consiguiendo además mayores eficacias. El medio de separación consiste en tampones en medio acuoso, por lo que las muestras se pueden inyectar directamente sin necesidad de procesos de extracción y se puede trabajar a temperatura ambiente por lo que se evitan procesos de descomposición o desnaturalización de las muestras. Es altamente versátil, pudiendo analizar muchos tipos de muestras (polares, apolares, iónicas, neutras y de alto peso molecular), debido a sus diferentes modos de separación.

La CE ha demostrado su eficacia en los últimos años para la detección de residuos de contaminantes principalmente usando *detección UV-Vis mediante batería de diodos (DAD)* y en menor extensión aplicando otros sistemas de detección. Algunos artículos de revisión recogen las aportaciones en este ámbito, fundamentalmente en la determinación de residuos de plaguicidas [58-61]. En la última década han aumentado las aplicaciones de la CE en la determinación de *medicamentos de uso veterinario* en alimentos de origen animal, fundamentalmente de *antibióticos*. Algunos artículos de revisión recogen las aportaciones en el ámbito de la determinación de antibióticos en alimentos de origen animal y muestras

[57] M. Szumski, B. Buszewski, Crit. Rev. Anal. Chem. 32 (2002) 1

[58] Y. Picó, R. Rodríguez, J. Mañes, Trends Anal. Chem. 22 (2003) 133

[59] J. Hernández-Borges, S. Frías-García, A. Cifuentes, M.A. Rodríguez-Delgado, J. Sep. Sci. 27 (2004) 947

[60] J. Hernández-Borges, A. Cifuentes, F.J. García-Montelongo, M.A. Rodríguez-Delgado, Electrophoresis, 26 (2005) 980

[61] A. Juan-García, G. Font, Y. Picó, J. Sep. Sci. 28 (2005) 793

medioambientales [61-66], incluyendo todos los métodos de análisis establecidos para el análisis de las principales familias de antibacterianos: *sulfonamidas*, *quinolonas*, *tetraciclinas*, *β-lactamicos*, *aminoglucósidos* y *nitroimidazoles*. Principalmente se han utilizado dos modos, la electroforesis capilar zonal (CZE) y la cromatografía capilar electrocinética micelar (MEKC). También se han llevado a cabo algunas aplicaciones empleando disolventes orgánicos en un alto porcentaje en el medio de separación, modo denominado electroforesis capilar no acuosa (NACE).

El principal inconveniente que presenta su aplicación en este ámbito radica en la necesidad de aplicar una técnica de elevada sensibilidad, considerando las bajas concentraciones de residuos en estas matrices, lo cual contrasta con la limitación de la técnica de CE acoplada a la detección UV, debido a los bajos volúmenes de inyección introducidos y a la limitada capacidad de tal detección. Sin embargo aún son escasas las aplicaciones en CE acoplada con fluorescencia inducida por láser (LIF) en la detección de residuos contaminantes [67]. Recientemente se han propuesto métodos para la determinación de antibióticos de la familia de las quinolonas en alimentos y aguas [68,69] o micotoxinas (aflatoxinas) en arroz [70]. En los últimos años la Espectrometría de Masas (MS) ha cobrado una importancia cada vez mayor como método de detección y cuantificación en CE [71]. El acoplamiento de un detector tan selectivo con una técnica tan versátil y de eficacias tan elevadas, hace que la CE-MS sea una potente herramienta que presenta importantes perspectivas en su aplicación en calidad y seguridad alimentaria y ambiental, siendo posible la determinación de residuos de

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[62] C. García-Ruíz, M.L. Marina, Electrophoresis, 27 (2006) 266

[63] M. Castro-Puyana, A.L. Crego, M.L. Marina, Electrophoresis, 29 (2008) 274

[64] A.M. García-Campaña, L. Gámiz-Gracia, F.J. Lara, M. del Olmo Iruela, C. Cruces-Blanco, Anal. Bioanal. Chem. 395 (2009) 967

[65] M. Castro-Puyana, A.L. Crego, M.L. Marina, Electrophoresis, 31 (2010) 229

[66] V. Pérez-Fernández, E. Domínguez-Vega, A.L. Crego, M.A. García, M.L. Marina, Electrophoresis, 33 (2012) 127

[67] A. Juan-García, G. Font, Y. Picó, Electrophoresis, 27 (2006) 2240

[68] M. Lombardo-Agüí, L. Gámiz-Gracia, A.M. García-Campaña, C. Cruces-Blanco. Anal. Bioanal. Chem. 396 (2010) 1551

[69] M. Lombardo-Agüí, A.M. García-Campaña, L. Gámiz-Gracia, C. Cruces-Blanco. J. Chromatogr. A 1217 (2010) 2237

[70] N. Arroyo Manzanares, L. Gámiz Gracia, A.M. García Campaña, J.J. Soto Chinchilla, L.E. García Ayuso. Electrophoresis 31 (2010) 2180

[71] C. W. Klampfl, Electrophoresis, 27 (2006) 3

plaguicidas y antibióticos [72-78], proporcionando una segunda dimensión en la separación ya que los analitos además de separarse por su relación carga/tamaño se separan por su relación masa/carga. En los últimos años CE-MS ha comenzado a utilizarse debido al desarrollo de la instrumentación que ha solventado el principal inconveniente de este acoplamiento: la compatibilidad entre la CE, que trabaja con muestras líquidas con la MS que trabaja con muestras en estado gaseoso. Así se han desarrollado interfases adecuadas que trabajan de forma satisfactoria, siendo una de las más usadas la ionización por electrospray o electronebulización (ESI) usando interfase coaxial con flujo adicional.

Las técnicas convencionales de CE están caracterizadas por el empleo de capilares con una longitud de 20-100 cm y un diámetro interno de 10-100 µm, lo que permite usar volúmenes pequeños de muestra (1-2 µL). Entre las ventajas destaca la reducción de la posibilidad de pérdida de muestra en la superficie del capilar y de los volúmenes muertos pero el mayor inconveniente es la capacidad de carga limitada, lo que conlleva límites de detección altos. Existen algunos métodos que permiten mejorar la sensibilidad mediante el aumento del camino óptico, que se muestran en la figura 2, consistentes en el uso de iluminación axial en lugar de perpendicular, utilizando una celda de flujo de alta detección o el uso de capilares con camino óptico extendido (capilares de burbuja), en los que el diámetro interno está aumentado de 3 a 5 veces en el punto de detección, sin que esto implique un aumento en la difusión del analito. Esta última opción ha sido empleada en esta Tesis Doctoral.

[72] C. Simó, C. Barbas, A. Cifuentes, Electrophoresis 26 (2005) 1306

[73] F.J. Lara, A.M. García-Campaña, F. Alés-Barrero, J.M. Bosque-Sendra, L.E. García-Ayuso, Anal. Chem. 78 (2006) 7665

[74] L. Ravelo-Pérez, M. Asensio-Ramos, J, Hernández-Borges, M.A. Rodríguez-Delgado, Electrophoresis, 30 (2009) 1624

[75] J.J. Soto Chinchilla, A.M. García Campaña, L. Gámiz Gracia. Electrophoresis 28 (2007) 4164

[76] F.J. Lara, A.M. García Campaña, F. Alés Barrero, J.M. Bosque Sendra. Electrophoresis 29 (2008) 2117

[77] M.I. Bailón Pérez, A.M. García Campaña, M. del Olmo Iruela, C. Cruces Blanco, L. Gámiz Gracia. Electrophoresis, 30 (2009) 1708

[78] D. Moreno-González, L. Gámiz-Gracia, J.M. Bosque-Sendra, A.M. García-Campaña J. Chromatogr. A, 1247 (2012) 26

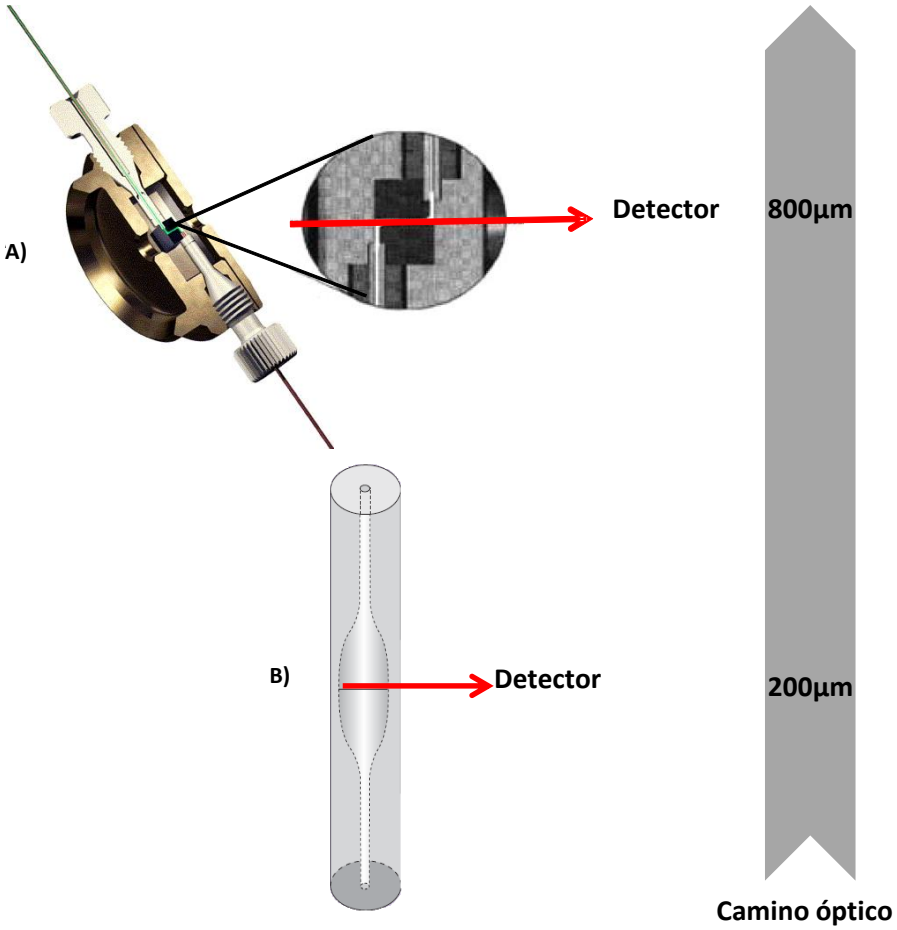

Figura 2. A) Celda de flujo de alta detección y B) capilar de burbuja.

Para superar estos límites de detección también se han desarrollado una serie de técnicas para introducción de muestra que proporcionan un efecto de concentración "on-column" y permiten el uso de mayores volúmenes. La técnica más simple y popular para mejorar la relación señal/ruido es la conocida como apilamiento on-line o "stacking" [79-83] cuya modalidad más usual consiste en

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[79] R.L. Chien, Electrophoresis, 24 (2003) 486

[80] Z. Malá, L. Krivánková, P. Gebauer, P. Bocek, Electrophoresis, 28 (2007) 243

[81] H.Y. Xie, Y.Z. He, W.E. Gan, G.N. Fu, L. Li, F. Han, Y. Gao, J. Chromatogr. A, 1216 (2009) 3353

[82] A. Šlampová, Z. Malá, P. Pantůčková, P. Gebauer, P. Boček, Electrophoresis, 34 (2013) 3

disolver la muestra en agua pura, tampón diluido o disolvente orgánico y colocarla entre dos porciones del tampón empleado en la separación. Esta zona de muestra tendrá una fuerza iónica notablemente inferior al resto del capilar. Cuando se aplica un voltaje elevado a lo largo del capilar, la fuerza de campo eléctrico es considerablemente más alta en la zona de la muestra. Como consecuencia, los iones de la muestra se mueven rápidamente hasta que llegan al tampón donde su velocidad cae debido a la diferencia de fortaleza del campo eléctrico. Por tanto la muestra es focalizada eléctricamente o amontonada ("stacked"), y la concentración dentro del capilar aumenta. Con esta técnica se han llegado a detectar niveles de partes por trillón.

En esta Tesis se ha empleado la modalidad de "*apilamiento de gran volumen de muestra con inversión de polaridad"* (*Large Volumen Sample Stacking, LVSS*). En esta modalidad la muestra se disuelve en un disolvente de baja conductividad y se inyecta la disolución resultante hidrodinámicamente durante un periodo de tiempo prolongado. En esta modalidad el volumen de muestra introducido es alto y la matriz de la muestra debe ser propulsada hacia fuera del capilar con objeto de preservar la eficacia de la separación. Esta expulsión debe llevarse a cabo mediante presión externa o mediante el EOF, el sentido siempre será opuesto al del movimiento de los solutos cargados y la velocidad de expulsión debe de ser menor que la velocidad electroforética de éstos. Una limitación de esta modalidad es que sólo pueden concentrarse a la vez analitos cargados positiva o negativamente. Es posible conseguir factores de concentración mayores de 100, mejorándose los límites de detección en más de 2 órdenes de magnitud. Esta modalidad puede llevarse a cabo invirtiendo o no la polaridad.

La figura 3 muestra un esquema de este proceso para el caso de LVSS con cambio de polaridad para aniones. El cambio de polaridad se realiza con objeto de controlar el EOF y se lleva a cabo en sistemas de CZE que requieren alto EOF para llevar los analitos separados hasta el detector. La polaridad del voltaje durante el apilamiento de la muestra se selecciona en función de la carga de los iones de la muestra, siendo inversa a la polaridad usada para la separación y la detección. Como se muestra en la figura 3, en el caso de separación de aniones en una muestra se usa un electrodo positivo en el vial de salida. En el caso de muestras con cationes, se usa un aditivo en el tampón electroforético (por ejemplo un

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[83] M.C. Breadmore, A.I. Shallan, H.R. Rabanes, D. Gstoettenmayr, A.S. Abdul Keyon, A. Gaspar, M. Dawod, J.P. Quirino, Electrophoresis, 34 (2013) 29

surfactante catiónico) para invertir el EOF y el electrodo negativo se ubica en el vial de salida. En ambos casos, cuando se observa que la corriente alcanza el 90-99 % de la corriente real (corriente obtenida cuando el capilar se llena sólo con el tampón electroforético), la polaridad se invierte de modo que el sentido del EOF es hacia el detector.

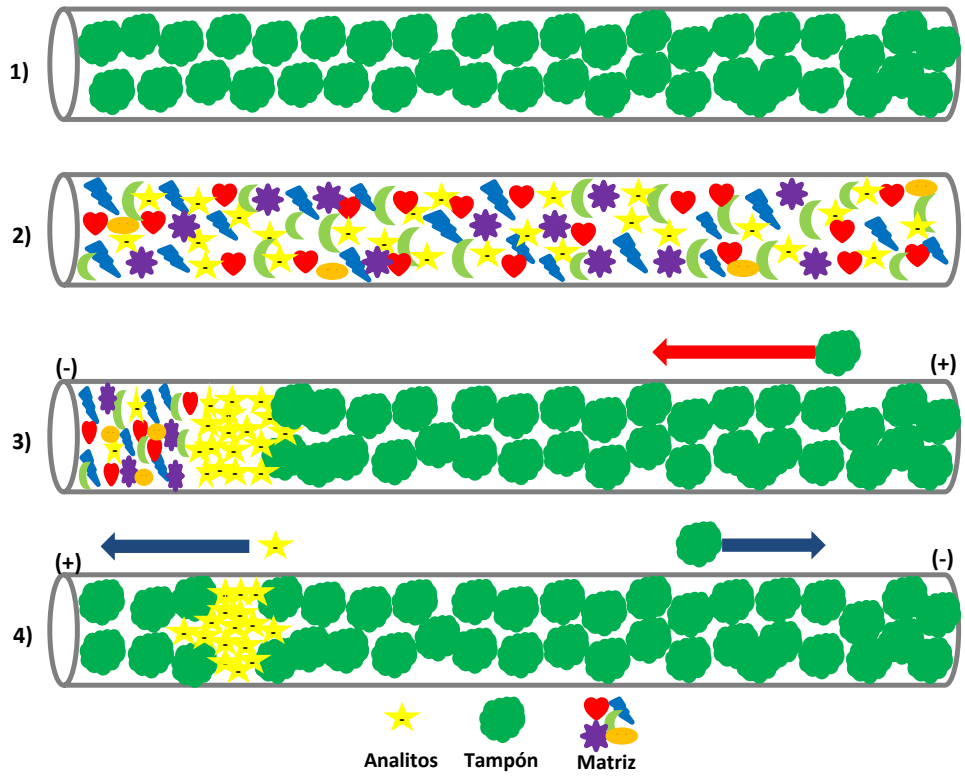

Figura 3. LVSS con cambio de polaridad para aniones. 1) Situación inicial, capilar lleno de tampón de separación; 2) inyección hidrodinámica de la muestra preparada en disolvente de baja conductividad; 3) aplicación de voltaje a polaridad negativa para focalizar zonas y eliminar la matriz de la muestra; los aniones se focalizan completamente y la mayoría de la matriz se elimina, parándose el voltaje; 4) aplicación de voltaje a polaridad positiva para la separación y detección de las zonas focalizadas.

3.2. Cromatografía líquida capilar de alta resolución

En los últimos años, para incrementar la eficacia de la separación, la sensibilidad de la detección y disminuir los tiempos de análisis con columnas convencionales de

HPLC, se ha reducido el diámetro interno de las columnas así como el tamaño de partícula del material de empaquetamiento. Los importantes avances que han tenido lugar en las tecnologías de fabricación de columnas, han permitido el desarrollo de columnas micro y capilares que respecto a las convencionales de diámetro interno 4.6 mm ofrecen considerables ventajas. Así, la combinación de columnas capilares y células de flujo de volumen de nanolitros con gradiente de elución y temperatura, han permitido desarrollar sistemas de *cromatografía líquida capilar de alta resolución (HPLC capilar)*[57,84] que presentan límites de detección comparables a los obtenidos mediante cromatografía de gases capilar.

La introducción de micro-columnas o columnas capilares en HPLC, término que se utiliza en general para referir columnas de pequeño diámetro, se debe a *Horváth y col*., quienes utilizaron en el año 1967 columnas empaquetadas de diámetro interno comprendido entre 0.5 y 1.0 mm para la separación de ribonucleótidos [85]. En la últimas dos décadas, la automatización y la miniaturización han sido muy importantes en el desarrollo de la cromatografía líquida, y en función del diámetro interno de la columna y de los caudales utilizados, las técnicas de HPLC se han clasificado en diversas categorías [57]. La terminología actualmente usada se muestra en la tabla 2.

Tabla 2. Terminología usada en técnicas de cromatografía líquida

Nombre	Diámetro interno de la columna	Velocidad de flujo	Tamaño de partícula (µm)	Longitud (cm)
HPLC convencional	3.2-4.6 mm	0.5-2.0 mL min^{-1}	3-10	3-25
HPLC microbore	1.5-3.2 mm	100-500 µL min^{-1}	3-8	15-25
Micro-LC	0.5-1.5 mm	10-100 µL min^{-1}	3-5	5-15
LC capilar	150-500 µm	1-10 µL min^{-1}	3-5	5-15
Nano-LC	10-150 µm	10-1000 nL min^{-1}	3-5	5-15

Debido al diámetro interno reducido, los caudales de fase móvil utilizados con micro-columnas son muy pequeños, lo que produce una disminución considerable del consumo de reactivos y disolventes, y por ello de los desechos generados y del

[84] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, J. Sep. Sci. 30 (2007) 1589
[85] J. P. C. Vissers, H. A. Claessens, C. A. Cramers, J. Chromatogr. A, 779 (1997) 1

impacto medioambiental [86]. Además, por su posibilidad de trabajar con pequeños volúmenes de muestra y su reducida dilución cromatográfica [87], poseen una excelente capacidad de acoplamiento directo a técnicas de detección como la resonancia magnética nuclear, la espectrofotometría infrarroja con transformada de Fourier y la MS, que requiere la eliminación previa del disolvente [88]. En comparación con los sistemas convencionales de HPLC, este tipo de técnicas mejoran la detección y permiten obtener eficacias de separación elevadas.

Teóricamente, el uso de micro-columnas aumenta la velocidad de la separación por la mayor velocidad linear de la fase móvil. Estas mejoras cinéticas, junto con el pequeño caudal de fase móvil, permiten reducir la sobrepresión, conectar columnas en serie y conseguir altas eficacias, que permiten la resolución de mezclas complejas. Por otra parte, el pequeño volumen en el que se eluyen los analitos proporciona mayor sensibilidad y mejoras de hasta 20 órdenes de magnitud en la respuesta del detector [86]. De acuerdo con el factor teórico de disminución de escala, las sensibilidades se pueden incrementar del orden de 200 veces en detectores másicos, al reducir el diámetro interno de la columna de 4.6 mm a 320 µm [85], de forma que las micro-columnas han sido muy utilizadas para aumentar la sensibilidad de la detección y la resolución cuando el volumen de muestra disponible para la inyección es limitado o los compuestos a determinar se encuentran presentes a bajas concentraciones en las distintas muestras [89]. Recientemente esta técnica se ha aplicado al análisis de residuos de agentes químicos de riesgo en aguas y alimentos; con detección UV al análisis de antibióticos como penicilinas, [90] o plaguicidas como carbamatos [91] y a la determinación de quinolonas [92] y micotoxinas [93,94] con detección LIF.

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[86] A. Braithwaite, F. J. Smith, “Chromatographic Methods”, 5th Edition, Kluwer Academic Publishers, The Netherlands, 1996, p. 1

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[89] P. Molander, A. Tomasen, L. Kristoffersen, T. Greibrokk, E. Lundanes, J.Chromatogr. B 766 (2002) 77

[90] M.I. Bailón-Pérez, A.M. García-Campaña, M. del Olmo-Iruela, L. Gámiz-Gracia, C. Cruces-Blanco. J. Chromatogr. A 1216 (2009) 8355

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[92] M. Lombardo-Agüí, L. Gámiz-Gracia, C. Cruces-Blanco, A.M. García-Campaña. J. Chromatogr. A 1218 (2011) 4966

4. Tratamientos de muestra en análisis de contaminantes

En la actualidad existen una amplia variedad de sistemas de tratamiento de muestra para la determinación de residuos y contaminantes en muestras complejas como los alimentos [95-98] y muestras medioambientales [99-101] basadas fundamentalmente en la digestión de muestra, extracción con disolventes, extracción con adsorbentes y separación por membranas.

Una amplia variedad de métodos han sido aplicados a la extracción de residuos y contaminantes orgánicos de muestras ambientales y alimentarias. A día de hoy técnicas clásicas, como la extracción con Soxhlet y la extracción líquido-líquido (LLE) convencional, se aplican en muchos laboratorios de rutina para el tratamiento de muestras sólidas y líquidas respectivamente. Sin embargo, en los últimos años ha habido importantes avances en el desarrollo de técnicas de extracción tendentes a la automatización, rapidez y bajo consumo de disolventes [102-104]. Todo ello favorece la reducción de la contaminación intralaboratorio y los costes económicos y de tiempo en la etapa de tratamiento de muestra así como el aumento de la seguridad del analista [96,105], consideraciones englobadas en el concepto de Química Analítica Verde [106]. En este sentido, los

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[96] M.D. Marazuela, S. Bogialli, Anal. Chim. Acta 645 (2009) 5
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[100] C. Mahugo-Santana, Z. Sosa-Ferrera, M.E. Torres-Padrón, J.J. Santana-Rodríguez, Trends Anal. Chem. 30 (2011) 731
[101] M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, Trends Anal. Chem. 30 (2011) 749
[102] K. Ridgway, S.P.D. Lalljie, R.M. Smith, J. Chromatogr. A, 1153 (2007) 36
[103] L. Ramos, J. Chromatogr. A, 1221 (2012) 84
[104] L. Ramos, R.M. Smith (Editors), Advances in Sample Preparation, Part I, J. Chromatogr. A, 1152 (2007)
[105] O. Núñez, H. Gallart-Ayala, C.P.B. Martins, P. Lucci, J. Chromatogr. A, 1228 (2012) 298
[106] M. Farré, S. Pérez, C. Gonçalves, M.F. Alpendurada, D. Barcelo, Trends Anal. Chem. 29 (2010) 1347

avances realizados en el análisis de residuos y contaminantes en alimentos [107,108] han propiciado avances en técnicas como la extracción líquida presurizada (PLE), la extracción asistida con microondas (MAE), la dispersión de matriz en fase sólida (MSPD) y la metodología multirresiduo QuEChERS (*Quick, Easy, Efficient, Cheap, Robust, Safe*). Las principales técnicas que están reemplazando a la LLE son la extracción en fase sólida (SPE) y su modalidad basada en el empleo de los polímeros de impresión molecular (MISPE) como sorbentes, microextracción en fase sólida (SPME), extracción por sorción en barrita agitadora (SBSE) o membrana líquida soportada (SLM) y más recientemente la extracción en fase sólida dispersiva (DSPE) o la microextracción líquido-líquido dispersiva (DLLME).

En esta Tesis se han empleado diferentes metodologías de tratamiento de muestra, desde las más convencionales como SPE, los MIP empleados en la metodología MISPE, PLE o la extracción líquido-líquido asistida por sales con formación de par iónico (IP-SALLE). A continuación se describen algunas particularidades de los procedimientos de tratamiento de muestra empleados en esta Memoria.

4.1. Extracción en Fase Sólida (SPE)

La SPE mediante el uso de adsorbentes es ideal para la extracción de analitos presentes en muestras líquidas y para la purificación de los extractos obtenidos a partir de muestras sólidas mediante las distintas técnicas de extracción existentes. Existen diferentes tipos de cartuchos de extracción con distintos rellenos, siendo posible el empleo de adsorbentes polares, no polares o de intercambio iónico, cuya elección depende del tipo de matriz, analito de interés e interferentes. En diversos artículos de revisión se ha detallado el uso de esta metodología y sus aplicaciones para el análisis de alimentos y aguas [109-112]. En concreto, los rellenos de C18 han sido muy utilizados para la extracción de un elevado número de compuestos de diferente polaridad, así como los copolímeros de estirenodivinilbenceno (SVDE,

[107] M. LeDoux, J. Chromatogr. A, 1218 (2011) 1021
[108] B. Gilbert-López, J.F. García-Reyes, A. Molina-Díaz, Talanta, 79 (2009) 109
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[112] O. Zuloaga, P. Navarro, E. Bizkarguenaga, A. Iparraguirre, A. Vallejo, M. Olivares, A. Prieto, Anal. Chim. Acta 736 (2012) 7

también conocidos como Envichrom P o Lichrolut) para compuestos polares, y los de carbón grafitizado, comúnmente conocido como Carbopack, Carbograph o Envicarb. Sin embargo, cada vez se emplea más el sorbente copolimérico hidrofílico-lipofílico (HLB), debido a sus excelentes características para la extracción de compuestos de un amplio rango de polaridad [113,114]. En la figura 4 se muestra un esquema en el que se detallan las etapas del proceso de extracción en fase sólida.

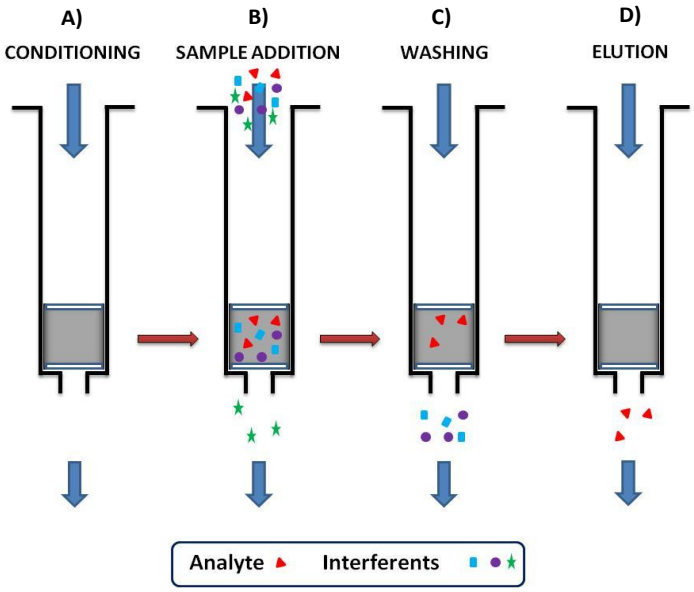

Figura 4. Etapas del proceso de extracción en fase sólida: A) acondicionamiento, B) carga de muestra, C) lavado, D) elución de los analitos con los disolventes adecuados

4.2. Polímeros Impresos Molecularmente (MIPs)

Los polímeros impresos molecularmente (MIPs) son polímeros altamente reticulados, que poseen propiedades de reconocimiento molecular selectivo debido a que los sitios de reconocimiento dentro de la matriz del polímero son complementarios al analito en la forma y posición de los grupos funcionales. Algunos de estos polímeros tienen altas selectividades y constantes de afinidad comparables

[113] P. Pérez-Ortega, B. Gilbert-López, J.F. García-Reyes, N. Ramos-Martos, A. Molina-Díaz, J. Chromatogr. A, 1249 (2012) 32
[114] L.J. Zhou, G.G. Ying, S. Liu, J.L. Zhao, F. Chen, R.Q. Zhang, F.Q. Peng, Q.Q. Zhang, J. Chromatogr. A, 1244 (2012) 123

con los sistemas de reconocimiento que ocurren naturalmente tales como anticuerpos monoclonales o receptores. Esta selectividad es debida a que en el proceso de síntesis del MIP se emplea una molécula "molde" (template), de naturaleza similar a la molécula "diana", que se une al polímero por enlaces covalentes y no covalentes, a través de monómeros que están unidos al polímero a través de "linkers". Una vez formado el polímero la molécula molde es eliminada generando unos espacios moleculares que más tarde pueden ser ocupados por otras moléculas complementarias química y geométricamente y que pueden unirse al polímero mediante diversas interacciones (por ejemplo enlaces por puentes de hidrógeno, interacciones de Van der Waals, interacción hidrofóbica, etc.). Estas interacciones son las responsables de mantener a una determinada molécula o un grupo de moléculas unidas al polímero hasta que se establecen unas condiciones que permiten desligar la molécula del polímero cuando se desee. En la figura 5 se muestra el proceso se síntesis del MIP a partir del monómero, y utilizando una molécula molde; así como la selectividad de la interacción del analito con el polímero sintetizado.

Al poner una muestra que contiene un analito o grupo de analitos en presencia del polímero, éstos pueden unirse a las cavidades formadas durante la obtención del polímero de manera selectiva, presentando una mayor afinidad que otros compuestos no relacionados. Estos analitos pueden ser liberados cuando se establecen las condiciones adecuadas, consiguiendo de esta forma un aislamiento de los mismos del resto de componentes de la matriz.

En la última década, los MIPs se han empleado en Química Analítica en la etapa de tratamiento de muestra como sistema selectivo de extracción, dando lugar a la denominada extracción en fase sólida mediante polímeros impresos molecularmente (MISPE); las etapas del tratamiento de muestra con MISPE son similares a las de la extracción en fase sólida e incluyen: 1º acondicionamiento de la columna o cartucho, 2º carga de la muestra, 3º elución de los interferentes, 4º elución de los analitos.

Existen diversas aplicaciones de MIP sintetizados en laboratorios particulares para la extracción de diversos compuestos en muestras ambientales (aguas y suelos), alimentos o plantas, aunque sus aplicaciones aún son limitadas, como se recoge en

diversos artículos de revisión [115-120]. La casa comercial Supelco ha comercializado algunos sistemas de MISPE [121] (SupelMIP™), para triacinas, cloranfenicol, anfetaminas o fluoroquinolonas. Recientemente se han sintetizado MIPs para determinar penicilinas en muestras de agua [122], pero no existen para cefalosporinas, por lo que su síntesis se ha planteado en esta Tesis.

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[120] A. Beltrán, F. Borrull, R.M. Marcé, P.A.G. Cormack, Trends Anal. Chem. 29 (2010) 1363
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[122] J.L. Urraca, M.C. Moreno-Bondi, A.J. Hall, B. Sellergren, Anal. Chem. 79 (2007) 695

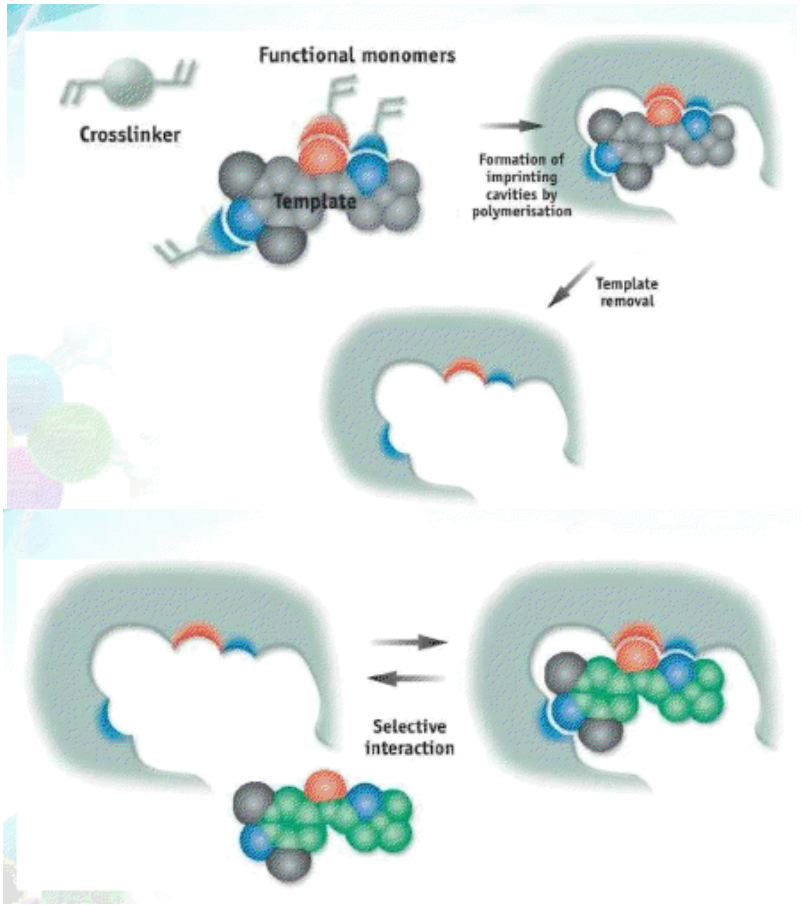

Figura 5. Polimerización y sitios específicos en el MIP para la interacción selectiva, adaptada de la página web de Sigma Aldrich. Marzo 2013 en: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/2/supelmip.pdf

4.3. Extracción líquida presurizada (PLE)

La PLE también denominada comercialmente *extracción acelerada con disolvente* (ASE), fue introducida en 1994 por la compañía Dionex Corporation. Este sistema emplea disolventes orgánicos típicos a alta presión y/o alta temperatura sin alcanzar el punto crítico, para conseguir la extracción de compuestos orgánicos. Las altas temperaturas aceleran la cinética de extracción, mientras que la alta

presión mantiene al disolvente en estado líquido permitiendo así extracciones rápidas y seguras [123].

En la figura 6A se muestra el diagrama de las etapas seguidas en el proceso de extracción líquida presurizada. En la figura 6B se muestra el esquema del equipo utilizado en este tipo de extracción. La muestra se coloca en una celda de extracción y se llena de disolvente (15-40 mL). Posteriormente se aplica una alta temperatura (200°C como máximo) y presión (hasta 20 Mpa). Después de un corto periodo de tiempo (10-15 min), el extracto obtenido se transfiere directamente a un vial para su posterior análisis y la célula se purga con disolvente nuevo para limpiar la muestra o repetir la extracción. Finalmente, el disolvente residual se purga de la muestra al vial usando un gas adecuado que normalmente es nitrógeno. El proceso completo tras colocar la muestra en la celda está totalmente automatizado, pudiendo programarse cada una de las etapas.

Existen en bibliografía un elevado número de aplicaciones en áreas tan diversas como el medio ambiente [124,125], productos farmacéuticos [126], productos alimentarios [127,128], piensos [129] o muestras biológicas [130]. Recientemente se han revisado sus ventajas como técnica verde de extracción, aplicada al análisis de contaminantes y compuestos bioactivos y nutricionales en alimentos y piensos [131].

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[131] H. Sun, X. Ge, Y. Lv, A. Wang, J. Chromatogr. A, 1237 (2012) 1

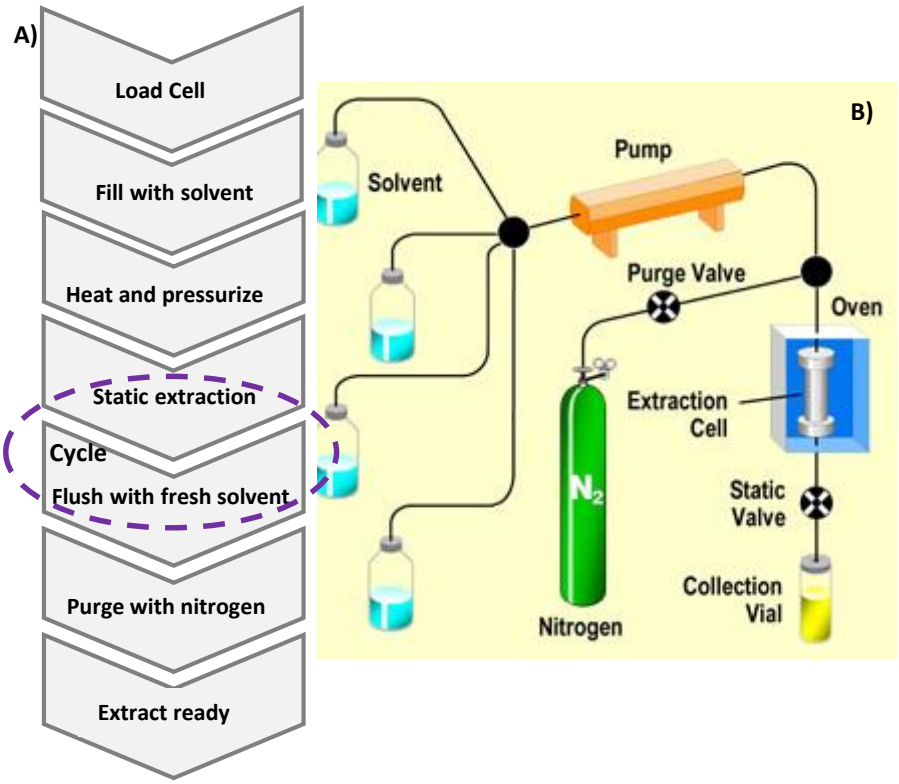

Figura 6. Esquema de un sistema PLE. A) etapas del proceso de extracción líquida presurizada. B) esquema del equipo de extracción líquida presurizada.

Este sistema de extracción ha sido ampliamente utilizado para la determinación de residuos de pesticidas en muestras de suelo, en combinación con diferentes técnicas separativas. Son varios los trabajos en los que se compara la eficacia de la extracción acelerada con disolventes en este tipo de matrices, con la de otros sistemas de extracción sólido-líquido comúnmente empleados como son Soxhlet, Soxtec, ultrasonidos, fluidos supercríticos, microondas o extracción sólido-líquido mediante agitación [132,133]. Los resultados obtenidos ponen de manifiesto que, no sólo se reduce el tiempo de preparación de la muestra y la cantidad de disolvente necesaria, sino que además las condiciones de extracción se mantienen relativamente constantes.

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[132] J. Gan, S.K. Papiernik, W.C. Koskinen, R. Yates, Environ. Sci. Technol. 33 (1999) 3249
[133] S. Sporring, S. Bowadt, B. Svensmark, E. Björklund, J. Chromatogr. A, 1090 (2005) 1

4.4. Extracción líquido-líquido asistida por sales (SALLE)

La extracción líquido-líquido (LLE) convencional se basa en el empleo de disolventes orgánicos inmiscibles con agua. Este tipo de extracción presenta limitaciones en la eficacia de extracción de compuestos polares debido a las bajas constantes dieléctricas de los disolventes comúnmente utilizados. Además el aumento del coste debido al volumen de disolvente consumido en muchas ocasiones y el problema derivado del volumen de residuos generado, han favorecido el desarrollo de metodologías para el tratamiento de muestra, que reducen el consumo de disolventes, en especial los más tóxicos y contaminantes, así como las etapas del tratamiento de muestra, minimizando el tiempo empleado y la manipulación de las muestras.

La LLE convencional se puede modificar con el fin de extraer compuestos orgánicos polares de un medio acuoso, utilizando disolventes orgánicos miscibles con agua como acetonitrilo, acetona, metanol o etanol. Para ello se adiciona un electrolito a la mezcla de disolventes lo que supone la reducción de la miscibilidad entre las dos fases, es lo que se conoce como efecto salino. La separación de fases depende del electrolito utilizado siendo los más frecuentes cloruro sódico, sulfato amónico y sulfato de magnesio.
Las fuerzas intermoleculares débiles, como son los enlaces de hidrógeno entre moléculas y el agua, son interrumpidas fácilmente por la hidratación de los electrolitos. Además la fase orgánica separada contiene agua y sales incrementando así su capacidad aceptora con respecto al disolvente orgánico puro.
Esta metodología, denominada extracción líquido-liquido asistida por efecto salino (SALLE) [134], se ha desarrollado como una alternativa debido a su simplicidad, rápido equilibrio de reparto, y fácil purificación y condensación de extractos, en la que el propio disolvente usado en la extracción puede usarse como fase móvil en HPLC [135]. Además, la fase separada puede inyectarse directamente en la columna sin necesidad de ningún otro tratamiento. La figura 7 muestra un esquema de las etapas que se suceden en un método SALLE.

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[134] M.E. Majors, LCGC North America, 27 (2009) 526
[135] E.A. Podolina, O.B. Rudakov, E.A. Khorokhordina, L.A. Kharitonova, J. Anal. Chem. 63 (2008) 468

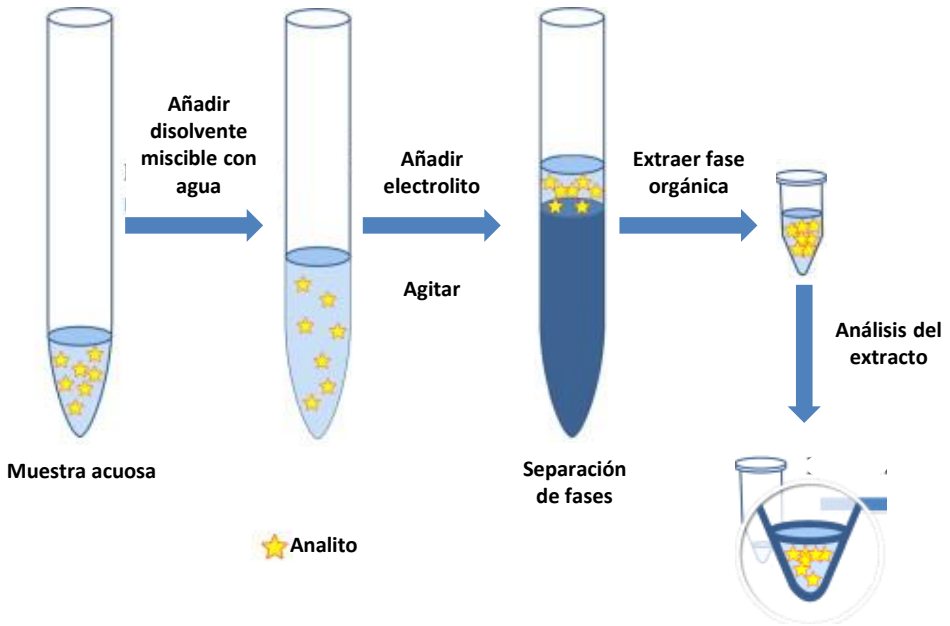

Figura 7. Diagrama de las etapas del proceso SALLE.

El SALLE fue desarrollado para la extracción de quelatos metálicos en acetona mediante el uso de una solución saturada de cloruro de calcio [136,137] más tarde fue aplicado en el análisis de antibióticos [138], análisis clínicos [139] o alimentos [140,141].

En la presente Tesis hemos desarrollado un método para la extracción de cefalosporinas a partir de un medio acuoso utilizando acetona, previa formación de un par iónico. El método de tratamiento de muestra denominado IP-SALLE nos permite una alta eficacia de extracción en un tiempo reducido y minimizando el gasto de disolventes.

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[137]E.C. Matkovich, G.D. Christian, Anal. Chem. 46 (1974) 102
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[140] M. Gupta, A.K.K.V. Pillai, A. Singh, A. Jain, K.K. Verma, Food Chem. 124 (2011) 1741
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Part I:

Determination of residues of herbicides

1. Herbicides

According to the European Environmental Agency (EEA) [1] herbicides are chemicals used to manipulate or control undesirable vegetation. The most frequent application of herbicides occurs in row-crop farming, where they are applied before or during planting to maximize crop productivity by minimizing other vegetation. They also may be applied to crops in the fall, to improve harvesting. Herbicides are used as well in forest management to prepare logged areas for replanting; the total applied volume and area covered is greater but the frequency of application is much less than for farming. In suburban and urban areas, herbicides are applied to lawns, parks, golf courses and other areas. In some cases herbicides are applied to water bodies to control aquatic weeds that impede irrigation withdrawals or interfere with recreational and industrial uses of water.

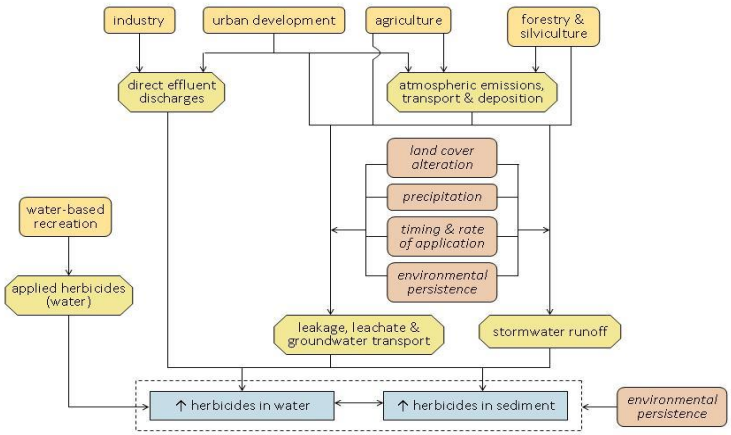

Figure 1. Sources of herbicides in the environment [2]

The anthropogenic activities and sources compiled in figure 1 can supply streams with high concentrations of herbicides and their metabolites, which can lead to lethal and sub-lethal effects on aquatic biota. Typically herbicides are applied to soil or terrestrial vegetation, which can increase herbicides in groundwater

[1] Adapted from European Environmental Agency (EEA) web page, accessed in March 2013: http://glossary.eea.europa.eu

[2] Adapted from Environmental Protection Agency (EPA) web page, accessed in April 2013: http://www.epa.gov/caddis/ssr_herb_int.html

discharge, atmospheric drift, and in runoff (storm or irrigation). The extent to which herbicides reach streams depends on factors such as precipitation patterns, timing and rates of application, and environmental persistence of herbicides and their metabolites. In streams, herbicides may be dissolved in the water column or bound to sediments, and their impact depends on the medium in which they occur. The bioavailability, uptake and toxicity of herbicides vary with environmental conditions.

Until now, extensive research has been conducted regarding the occurrence of herbicides in the environment; however, current understanding of the biological impacts of herbicide use is limited by the fact that most investigations have been focused on the active ingredients (parent compounds) without considering their transformation products (metabolites). For an understanding of the fate of herbicides in soil and water, the inclusion of metabolites is crucial because they can be present at higher levels in the environment than the parent pesticide itself [3-6].

1.1. Consumption

Worldwide consumption structure of pesticides has undergone significant changes since 1960s. The proportion of herbicides in pesticide consumption increased rapidly, from 20% in 1960 to 48% in 2005. The proportion of consumption of insecticides and fungicides declined despite their sales increased as shown in table 1. The rapid increase of herbicide consumption enhanced agricultural intensification and productivity.

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[4] R.K. Juhler, S.R. Sorensen, L. Larsen, Water Res. 35 (2001) 1371
[5] V. Andreu, Y. Picó, Trends Anal. Chem. 23 (2004) 772
[6] E. Herrero-Hernández, E. Pose-Juan, A. Álvarez–Martín, M.S. Andrades, M.S. Rodríguez-Cruz, M.J. Sánchez-Martín, J. Sep. Sci. 35 (2012) 3492

Table 1. Changes of pesticide consumption worldwide in recent years (adapted from [7])

	1960	1970	1980	1990	2000	2005
Pesticide	Sale (%)	Sale (%)	Sale (%)	Sale (%)	Sale (%)	Sale (%)
Insecticide	310 (36.5)	1002 (37.1)	4025 (34.7)	7655 (29.0)	7559 (27.9)	7798 (25.0)
Herbicide	170 (20.0)	939 (34.8)	4756 (14.0)	11625 (44.0)	12885 (47.5)	14971 (48.0)
Fungicide	340 (40.0)	599 (22.2)	2181 (18.8)	5545 (21.0)	5306 (19.6)	7486 (24.0)
Others	30 (3.5)	159 (5.9)	638 (5.5)	1575 (6.0)	1354 (5.0)	936 (3.0)

Sale: million US dollars (Xu, 1997; http://www.docin.com/p-55305172.html)

Over the period 2007 to 2008, herbicides ranked the first in three major categories of pesticides (insecticides, fungicides and herbicides). Fungicides increased rapidly and ranked the second. Europe is now the largest pesticide consumer in the world, seconded by Asia. As for countries, China, the United States, France, Brazil and Japan are the largest pesticide producers, consumers or traders in the world. Most of the pesticides worldwide are used to fruit and vegetable crops. In the developed countries pesticides, mainly herbicides are mostly used to maize. Since the 1980s hundreds of thousands of pesticides have been developed, including various biopesticides [7].

According to the FAO (Food and Agricultural Organization of the United Nations) in Europe, Germany is the largest producer and second largest consumer of pesticides dominated the pesticide markets. France is the largest pesticide consumer in Europe followed by Germany (table 2). Other major pesticide consumers in Europe include Italy, Spain and UK.

According to historical records, herbicides dominated the pesticide markets in Germany. In 2006, Germany exported herbicides worth of 830 million US dollars. Consumption of herbicides is the largest, as it is shown in table 3. It is believed that the domestic market tends to be saturated and the export increases. In 2008 the

[7] W.J. Zhang, F.B. Jiang, J.F. Ou, Proceedings of the International Academy of Ecology and Environmental Sciences, 1 (2011) 125

export to Central America and South America increased by 10%. However the export to Europe declined.

Table 2. Pesticide sales in some European countries (million US dollars)

Country	2006	2007	Increase (%)
France	2424.2	2564.3	5.8
Germany	1566.7	1737.2	10.9
Italy	1003.6	1043.3	4.0
Spain	782.9	810.4	3.5
UK	686.4	770.7	12.3

Data from European Crop Protection Association (ECPA), web page: (http://www.ecpa.eu)

In France fungicides are the most used pesticides, as it is shown table 3. The pesticide sale in 2008 increased by 13.3% and the sale reached 2.959 billion US dollars.

Overall the pesticide consumption in Europe declined by 50% compared to the average in 1980s.

Table 3. Use of pesticides in some countries of EU in recent years (t) [8]

Country	Pesticide	2007	2008	2009	2010
France	Insecticide	2101[1]	1300[1]	1100[2]	N/A[3]
	Herbicide	26808[1]	27200[1]	22600[2]	N/A[3]
	Fungicide	36919[1]	39200[1]	32500[2]	N/A[3]
Germany[1]	Insecticide	1370.4	1175.6	1355.6	1243.8
	Herbicide	17163.7	18626.0	15159.0	16675.0
	Fungicide	10309.4	10752.5	10201.2	9666.7
Italy[1]	Insecticide	13567.0	13523.0	10930.0	10834.0
	Herbicide	9180.0	8435.0	7637.0	9934.0
	Fungicide	49634.0	50629.0	29414.0	42537.0
Spain[3]	Insecticide	N/A	N/A	N/A	N/A
	Herbicide	N/A	N/A	N/A	N/A
	Fungicide	N/A	N/A	N/A	N/A
United Kingdom[1]	Insecticide	1279.5	1200.6	1157.5	589.5
	Herbicide	12159.2	10691.6	10499.0	7488.8
	Fungicide	5746.1	6197.3	6173.6	5521.3

[1]: Official data reported on FAO Questionnaires from countries; [2]: Data reported on country official publications or web sites (Official) or trade country files; [3]: Not available.

1.2. Classification

Herbicides can be classified as soil or foliage-applied compounds, which are normally absorber by roots or leaf tissues respectively. These compounds can be

[8] adapted from FAO web page, accessed in March 2013:
http://faostat.fao.org/site/424/DesktopDefault.aspx?PageID=424#ancor

total or selective herbicides. Total herbicides can kill all vegetation, whereas selective herbicides can control weeds without affecting the crop. These chemical substances may be applied at different crop stages, such a presowing and pre- or post-emergence, and these different treatments can be used depending on the weeds needed in a particularly crop. The selectivity of an herbicide may depend on a differential plant uptake, translocation or metabolism as well as on differences in the site of action. Knowledge of physicochemical properties, that is vapor pressure (V.p.), octanol/water coefficient partition (K_{ow}, expressed in the logarithmic form log P) and solubility in water allows the fate and behaviour of such chemicals in the environment to be predicted.

Herbicides can be grouped by activity, use and application mode, mechanism of action, or chemical family.

1.2.1. Classification by activity

Herbicides can be divided into two categories:

- **Contact herbicides** destroy only the plant tissue in contact with the chemical. Generally, these are the fastest acting herbicides. They are less effective on perennial plants, which are able to regrow from rhizomes, roots or tubers.
- **Systemic herbicides** are translocated through the plant, either from foliar application down to the roots, or from soil application up to the leaves. They are capable of controlling perennial plants and may be slower-acting, but ultimately more effective than contact herbicides.

1.2.2. Classification by use and application mode

In this case, the **soil-applied herbicides** are applied to the soil and are taken up by the roots and/or hypocotyl of the target plant. The three main types are:

- **Preplant incorporated herbicides** are soil applied prior to planting and mechanically incorporated into the soil. The objective for incorporation is to prevent dissipation through photodecomposition and/or volatility.
- **Pre-emergent herbicides** are applied to the soil before the crop emerges and prevent germination or early growth of weed seeds. Pre-emergence

herbicides remain active in the soil for an extended period of time, thereby providing residual control of weeds. Some herbicides can stay active for six moths.

- **Post-emergent herbicides** are applied after the crop has emerged. Sometimes surfactant may be added to this kind of herbicides to enhance the performance of the herbicide. Post-emergence herbicides need a specified drying time, before an irrigation or rainfall, for maximum effectiveness.

Generally, individual crops are treated with two to three herbicides. For example, separate herbicides may be used pre-emergence to control the major broadleaf and grass weeds infesting a crop. Additional herbicides may be used post-emergence to control emerged weeds that are missed by the pre-emergence application.

1.2.3. Classification by mechanism/site of action.

Their classification by mechanism of action (MOA) indicates the first enzyme, protein, or biochemical step affected in the plant following application. Currently there are eight modes of action, they include lipid synthesis inhibition, amino acid synthesis inhibition, seedling growth inhibition, growth regulators, photosynthesis inhibition, cell membrane disruption, pigment inhibition, and for a few herbicides the mode of action is simply listed as 'Unclassified' or 'Unknown'. Notice that most of these modes of action deal with regulation or inhibition of plant functions. The 'Unclassifed' or 'Unknown' herbicides do not fit into any of the other groups.

The site of action is the biochemical pathway which a particular herbicide acts upon in a plant. Currently there are more than fifteen sites of action. Table 4 lists the more common sites of action recognized by the Weed Science Society of America (WSSA).

Table 4. Herbicides classification based on site of action [9]

WSSA Classification	Site of action
ACCase	Acetyl CoA Carboxilase inhibitors
ALS	acetolactate synthase inhibitors
MT	Microtubule asembly
GR	Grown regulator
PSII(A)	Photosystem II, binding site A inhibitors
PSII(B)	Photosystem II, binding site B inhibitors
PSII(C)	Photosystem II, binding site C inhibitors
SHT	Shoot inhibitors
EPSP	enolpyruvyl-shikimate-phosphate synthase inhibitor
GS	glutamine synthetase inhibitor
PDS	phytoene desaturase synthesis inhibitor
DITERP	diterpene inhibitor
PPO	protoporphyrinogen oxidase inhibitor
SHT/RT	shoot and root inhibitor
ED	photosytem 1 electron diverter
HPPD	Hydroxyphenylpyruvate dioxygenase synthesis inhibitor

The site of absorption is the location where the herbicide is taken up by the plant. This should not be confused with site of action which is the biochemical pathway within the plant where the herbicide acts, as described above. The site of absorption may be referred to as the site of uptake. There are three possibilities for the site of absorption: root, shoot and foliar. Root absorption is through the roots of the plants. Shoot absorption refers to uptake by the shoots as it passes through the soil on the way to emergence. Many pre-emergence herbicides will have root absorption, shoot absorption or both as their site of uptake. Foliar absorption is through emerged leaves. Foliar absorption herbicides must be applied so that the herbicide is directed to the plant leaves rather than to the soil. If an herbicide has more than one site of absorption listed then typically the sites will be listed in primary order of absorption. It should also be noted that herbicides within the same chemical families may have different sites of absorption. Do not

[9] Adapted from "Plant and Soil Sciences eLibrary" web page, accessed in March 2013:
http://passel.unl.edu/pages/informationmodule.php?idinformationmodule=1059083105&topicorder=3&maxto=5&minto=1

assume that since a particular herbicide is in a family that it will have the same site of absorption as other members of the family.

1.2.4. Classification by family

Herbicide family can be considered a group of herbicides that is named in relation to its similar chemical properties. Members of the same herbicide family will have the same mode of action and typically the same site of action. Therefore, understanding the mode of action of an herbicide and the chemical family it belongs to will greatly aid in planning effective weed control and preventing herbicide resistance. Table 5 summarizes the herbicide families commonly used in spring wheat and barley and they are related according to their mode of action.

Table 5. Herbicide families commonly used in spring wheat and barley related to their mode of action [10]

Mode of action	Herbicides Family
Amino acids synthesis inhibitor	Sulfonylureas, Imidazolinones, and Amino Acid derivatives
Cell membrane disruptors	Bipyridylium
Growth regulator	Phenoxy-acetic acids, Benzoic acids, and Pyridines
Lipid synthesis inhibitors	Aryloxyphenoxypropionates
Photosynthesis inhibitor	Nitriles
Seedling growth inhibitors	Thiocarbamates and Dinitroanilines
Unclassified	Difenzoquat and Propanil

1.3. Formulation

An herbicide formulation is the total marketed product, and is typically available in forms that can be sprayed on as liquids or applied as dry solids [11]. It includes the active ingredient (or active ingredients), any additives that enhance herbicide effectiveness, stability, or ease of application such as surfactants and other

[10] adapted from University of Minnesota web page, accessed in March 2013: http://www.extension.umn.edu/distribution/cropsystems/components/6967_01f.html
[11] M. Tu, C. Hurd, J.M.Randall, Weed Control Methods Handbook: Tools & Techniques for Use in Natural Areas, The Nature Conservancy Wildland Invasive Species Team, April 2001

adjuvants, and any other ingredients including solvents, carriers or dyes. The application method and species to be treated will determine which formulation is best to use. In most cases, manufacturers produce formulations that make applications and handling simpler and safer. Some herbicides are available in forms that can reduce risk of exposure during mixing, such as pre-measured packets that dissolve in water, or as a liquid form already mixed with surfactant and dye. The most common formulations are:

- **Sprayable/liquid formulation**, only a few herbicidal active ingredients readily dissolve in water. This kind of formulation includes:
    - Water soluble liquids, powders or granules; when these products are mixed with water, the mixture is kept homogeneous.
    - Emulsifiable formulations (emulsifiable concentrations and gels); these products tend to be easy to handle and store, require little agitation, and will not settle out of solution.
    - Liquids suspension that are dispersed in water; these products consist of a particulate or liquid droplet active ingredient suspended in a liquid.
    - Dry solids that are suspended in water; these formulations are some of the most widely used. The active ingredient is mixed with a fine particulate carrier, such as clay, to maintain suspension in water, they require constant agitation to maintain suspension.
- **Dry formulations** include: granulets, pellets or tablets and dusts. They consist of the active ingredient absorbed onto particles of clay or other substance, and are most often used in soil applications. These formulations can persist for some time and may need to be incorporated into the soil.
- **Salts and esters.** Many active compounds are acids that can be formulated as a salt or an ester for application. Once the compound enters the plant, the salt or ester cation is cleaved off allowing the parent acid (active ingredient) to be transported throughout the plant
- **Adjuvants.** An adjuvant is any material added to a pesticide mixture that facilitates mixing, application or pesticide efficacy. It enables an applicator to customize a formulation to be most effective in a particular situation. Adjuvants include surfactants, stickers, extenders, activators, compatibility agents, buffers and acidifiers, deposition aids, de-foaming agents, thickeners and dyes. Surfactants are the most important adjuvants. They

are chemical compounds that facilitate the movement of the active herbicide ingredient into the plant. They may contain varying amounts of fatty acids that are capable of binding to two types of surfaces, such as oil and water

1.4. Behavior in the environment.

Usually the most important factor determining the fate of herbicide in the environment is its solubility in water [12]. Water-soluble herbicides generally have low adsorption capacities, and are consequently more mobile in the environment and more available for microbial metabolism and other degradation processes. Herbicides that are slightly soluble in water, adsorb quickly to soils, penetrate plant tissues readily, and are more volatile than salt and acid formulations [13].

1.4.1. Behavior in soils

An herbicide's persistence in soils is often described by its half-life (also known as the DT_{50}). The half-life is the time it takes for half of the herbicide applied to the soil to dissipate. The half-life gives only a rough estimate of the persistence of an herbicide since the half-life of a particular herbicide can vary significantly depending on soil characteristics, weather and the vegetation at the site. Dissipation rates often change with time [14].

The distribution of an herbicide in the soil is determined firstly by the amount, type and surface area of clays and organic matter in the soil, the amount and quality of soil moisture and soil temperature and soil pH [15]. Most natural soils have pH values between 5 and 8, but rainfall and the amount of leaching could influence these values. In wet areas and/or coarse soils, cations can be leached out, leaving the soil acidic, although in arid and semi-arid regions, soils retain cations and are

[12] O. Hutzinger, in J.D. McKinney (ed.) Environmental Health Chemistry Ann Arbor Science Publishers Inc., Ann Arbor, Michigan (1981)
[13] S.S. Que Hee, R G. Sutherland, The Phenoxyalkanoic Herbicides. Vol 1, CRC series in pesticide chemistry. CRC Press, Boca Raton, Florida (1981)
[14] L.W. Parker, K.G. Doxtader, J. Environ. Qual. 12 (1983) 553
[15] C.S. Helling, P.C. Kearney, M. Alexander, Adv. Agron. 23 (1971) 147

more alkaline. Acidic soils can also be found in bogs where organic acids lower the soil's pH.

1.4.2. Behavior in water

The behavior of an herbicide in water is determined by its solubility in water. Specifically, herbicides with water solubility of 10 mg L^{-1} or higher mainly move to the aquatic environment by dissolving into surface water, while those that dissolve with difficulty or have high soil adsorption move with soil particles suspended in water or sediment to which the chemicals have been adsorbed [16]. Salts and acids tend to remain dissolved in water until degraded through photolysis or hydrolysis. Esters often adsorb to the suspended matter in water, and precipitate to the sediments and once in the sediments, they can remain adsorbed or be degraded through microbial metabolism. Normally the surface waters pH values are between 5 and 9, although highly acidic or alkaline waters can change the chemical properties of an herbicide and change its behavior in water [12].

Environmental water can be contaminated by direct overspray, or when herbicides drift, volatilize, leach through soils to groundwater, or are carried in surface or subsurface runoff. The runoff rate into water systems is about 0.5% of the applied amount, if rain falls the first few days after an application, one estimate puts the total runoff rate two weeks after application at 1 or 2% [17]. High soil adsorption capacity, low rates of application and low rainfall reduce total runoff and contamination environmental waters [18].

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[16] R.F. Turco, E.J. Kladivko, in R.C. Honeycutt, D.J. Schabacker, Studies on Pesticide Mobility: laboratory vs. field, in Mechanisms of Pesticide Movement into Ground Water, CRC Press, Boca Raton, Florida (1994) pp. 63

[17] R.A. Leonard, Movement of Pesticides into Surface Waters, in Pesticides in the soil environment, in Processes, Impacts and Modelling, H.H. Cheng (Ed.), Soil Sci. Soc. Am., Madison, 1990, pp. 303

[18] M. Ueji, Y. Kobar, Environmental Fate of Pesticide in Structure And Function, in Agroecosystems Design and Management, M. Shiyomi, H. Koizumi (Ed), Taylor & Francis Group, Gifu and Mito, 2001, pp. 281

1.5. Toxicology

Herbicides have widely variable toxicity. Some herbicides cause a range of health effects from skin rashes to death. High exposure levels cause an increase of the toxicity, they could become possible carcinogenic substances as well as may cause other problems, such as contributing to breathing or neurological diseases.

1.5.1. Toxicity in the environment

In birds and mammals the herbicide's toxicity is described by its LD_{50}, which is the dose received either by oral ingestion or contact with skin that kills half the population of study animals. The LD_{50} is typically reported in grams of herbicide per kilogram of animal body weight. Dermal LD_{50} values may be more meaningful to herbicide handlers because they are more likely to be exposed to herbicide through their skin rather than by oral ingestion [11].

The LD_{50} does not provide any information about chronic, long-term toxic effects that may result from exposure to lesser doses. Sublethal doses can lead to skin or eye irritation, headache, nausea, and, in more extreme cases, birth defects, genetic disorders, paralysis, cancer and even death.

The most dramatic effects of herbicides on non-target plants and animals often result from the habitat alterations they cause by killing the targeted weeds. In the case of loss of invasive riparian plants can cause changes in water temperature and clarity that can potentially impact the entire aquatic community and the physical structure of the system through bank erosion. Removing a shrubby understory can make a habitat unsuitable for certain bird species and expose small mammals to predation.

In aquatic species, the herbicide's toxicity is quantified with the LC_{50}, which is the concentration of herbicide in water required to kill half of the study animals. The LC_{50} is typically measured in micrograms of pesticide per liter of water.

In general, ester formulations are more dangerous for aquatic species than salt and acid formulations because ester formulations are lipophilic and consequently, can pass through the skin and gills of aquatic species relatively easily. Ester formulations, additionally, are not water soluble and are less likely to be diluted in aquatic systems.

1.5.2. Human toxicology

Agricultural workers are often exposed to herbicides when they unintentionally re-enter a treated area too soon following treatment. People who mix and apply herbicides are at the greatest risk of exposure. The most common routes of exposure are through the skin or by inhalation. Accidental spills or splashing into the eyes is also possible and with some compounds, can result in severe eye damage and even blindness. Agricultural herbicide handlers are typically exposed to herbicide levels ranging from micrograms to milligrams per cubic meter of air through inhalation, but are thought that exposures through the skin will be much greater. Dermal exposure can occur to the hands (directly or through permeable gloves), splashes onto clothing or exposed skin, and anywhere which wipe the hands.

The pathway of attack can arise from intentional or unintentional direct contact, improper application resulting in the herbicide coming into direct contact with people or wildlife, inhalation of aerial sprays, or food consumption prior to the safety period indicated on the herbicide label. Under extreme conditions, herbicides can also be transported via surface runoff to contaminate distant water sources.

The herbicide poisoning depends on the toxicity of the herbicide, the size of the dose, duration of exposure, route of absorption and the efficiency with which the herbicide is metabolized and excreted by the person's body [19,20]. Herbicides can poison the body by blocking biochemical processes or dissolving or disrupting cell membranes. Common symptoms of low-level exposure to many herbicides include skin and eye irritation, headache, and nausea. Higher exposition doses can cause blurred vision, dizziness, heavy sweating, weakness, stomach pain, vomiting, diarrhoea, extreme thirst, and blistered skin, as well as nervous disorders such as apprehension, restlessness and anxiety. Extreme cases may result in convulsions, unconsciousness, paralysis, and death. Some researchers have demonstrated that high and continued exposure to herbicides and pesticides could increase the risk of

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[20] G.W. Ware, Fundamentals of Pesticides: a self-instruction guide, Thomson Publications. Fresno, California (1991) pp. 307

Parkinson's disease [21,22]. The effects are usually reversible, but in extreme cases can be permanently debilitating [19].

All commercially products must be extensively tested prior to approval for sale and labelling. However, because of the large number of herbicides in use, concern regarding health effects is significant. In addition to health effects caused by herbicides themselves or impurities derived from the formulation of the herbicide, commercial herbicide mixtures often contain other chemicals, including additives, which could have negative impacts on human health. Some herbicides were found to contain adjuvants which, even in low concentrations, were capable of killing human embryonic, placental, and umbilical cells in vitro [23,24].

1.6. Degradation processes

Herbicide degradation occurs when it is decomposed to smaller component compounds, and even to CO_2, water, and salts through photochemical, chemical, or by microbial metabolism reactions [25]. When an herbicide degrades, it usually yields several compounds (degradation products or metabolites) each of which has its own chemical properties including toxicity, adsorption capacity and resistance to degradation. Some metabolites are more toxic and/or persistent than the parent compound. In most cases, the behaviour of the metabolites is unknown.

Photodegradation refers to decomposition by sunlight. These studies are often conducted using UV light.

Microbial degradation is decomposition through microbial metabolism. Different microbes can degrade different herbicides, and consequently, the rate of microbial degradation depends on the microbial community present in a given situation

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[26,27]. Soil conditions that maximize microbial degradation include warmth, moisture and high organic content.

Herbicides may be microbially degraded via one of two routes [12]. They may be metabolized directly when they serve as a source of carbon and energy for microorganisms, or they may be co-metabolized in conjunction with a naturally occurring food source that supports the microbes. There is sometimes a lag time before microbial degradation proceeds. This may be because the populations of appropriate microbes or their supplies of necessary enzymes start small, and take time to build-up [28]. If this lag time is long, other degradation processes may play more important roles in dissipation of the herbicide. Degradation rates of co-metabolized herbicides tend to remain constant over time.

Chemical decomposition is degradation driven by chemical reactions, including hydroxylation (reaction with hydrogen), oxidation (reaction with oxygen), and disassociation (loss of an ammonium or other chemical group from the parent molecule).

2. Triazines. Metribuzin and its degradation products

Triazine herbicides belong to the group of the most widely used herbicides worldwide. Chemically, triazine herbicides are comprised of asymmetrical triazines: triazidinones or triazinones (ametridone, amibuzin, ethiozin, hexazinone, isomethiozin, metamitron and metribuzin) and symmetrical triazines (s-triazines or 1,3,5-triazines): chlorotriazines, methoxytriazines, methylthiotriazines, according to the substituent in the 2-position. Metabolites of metribuzin are some of the target analytes of this Thesis.

Triazine herbicides are usually applied as pre and post-emergent weed control to improve the quality of agricultural products. Because of their wide use and relatively high resistance of degradation, both the quality and the quantity of these

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[26] P.J. McCall, S.A. Vrona, S S. Kelley, J. Agric. Food Chem. 29 (1981)100
[27] Voos, G. and P. M. Groffman, Biol. Fertil. Soils 24 (1997) 106
[28] W.J. Farmer, Y. Aoch, in J.W. Biggar, J.N. Seiber (Eds) Fate of Pesticides in the Environment. University of California, Publication 3320 (1987)

compounds present important concerns about the environment and water control. After their introduction in the environment, they are transformed into degradation products, and some of these products are important for the assessment of the overall fate of triazine herbicides and their impact on the environment.

2.1. Toxicity of triazines

Triazine herbicides are generally of low acute toxicity for birds and mammals, although certain species show special vulnerability for some of them, e.g. for sheep the fatal dose of simazine has been reported as 0.5-1.4 g kg^{-1}, while LD_{50} for rats is more than 5 g kg^{-1} [29].

Adverse effects of atrazine and its main transformation products, as endocrine-disrupting properties [30,31], were shown also for rats and on oestrus, and they have been shown to affect immune function in mice [32]. Metribuzin has caused alterations of kidney performance in rainbow trout [33]. Other triazine herbicides are not that extensively studied regarding their chronic toxicity, probably because they are less widely applied. However, USA Environmental Protection Agency (EPA) concludes that triazines and their main transformation products with chlorine attached to the ring have the same common mechanism of toxicity regarding their endocrine-related developmental, reproductive and carcinogenic effects [34].

2.2. Degradation studies. Metribuzin and its metabolites.

The abiotic degradation of triazines in soils is a minor dissipation route, although the biotic degradation by microorganisms and higher plants is the most frequent

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transformation mode of these compounds. Under exposure to solar light and low pH, atrazine and his main transformation products were converted to hydroxy derivates, because of acid hydrolysis [35], however simazine and terbutylazine were found to dissipate faster under solar irradiation of the soil [36]. Numerous studies are available for plant uptake of triazines from the contaminated soils. The C4-metabolism of some plants such as *Polygonum lapathifolium* or *Panicum dichotomiflorum*, show the greatest resistance to triazines and detoxify contaminated soils by hydrolysis [37].

Metribuzin is a selective systemic herbicide used for pre- and post-emergence control of many grasses and broad-leaved weeds in soy beans, potatoes, tomatoes, sugar cane, alfalfa, asparagus, maize and cereals at 0.07-1.05 kg a.i. ha^{-1} [38]. Metribuzin is considered to be of short to moderate persistence in soils, the half-lives measured have been specified between 5 and 50 days. Metribuzin degradation happens through microbiological and chemical processes, generating diketometribuzin (DK), deamino metribuzin (DA) and deaminodiketo metribuzin (DADK) like degradation majority products, and two conjugated glycosides like minority products. The microbiological and photolytic processes of degradation give rise to the deamination and the desulphuration of the original active principle. The total degradation of metribuzin, until transforming into water and carbon dioxide, happens through DADK. Other unidentified metabolites are detected in experiments using ^{14}C-labeled metribuzin. Total degradation of metribuzin to inorganic species (mineralization) is usually below 10% of the metribuzin applied; thus, stable and persistent metabolites may be accumulated in the soil [39]. Figure 1 shows the generally accepted degradation pathway of metribuzin.

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[35] H. Prosen, L. Zupančič-Kralj, Environ. Pollut. 133 (2005) 517
[36] S. Navarro, S. Bermejo, N. Vela, J. Hernández, J. Agricult. Food Chem. 57 (2009) 6375
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[39] T. Henriksen, B. Svensmark, R.K. Juhler, J. Environ. Qual. 33 (2004) 619

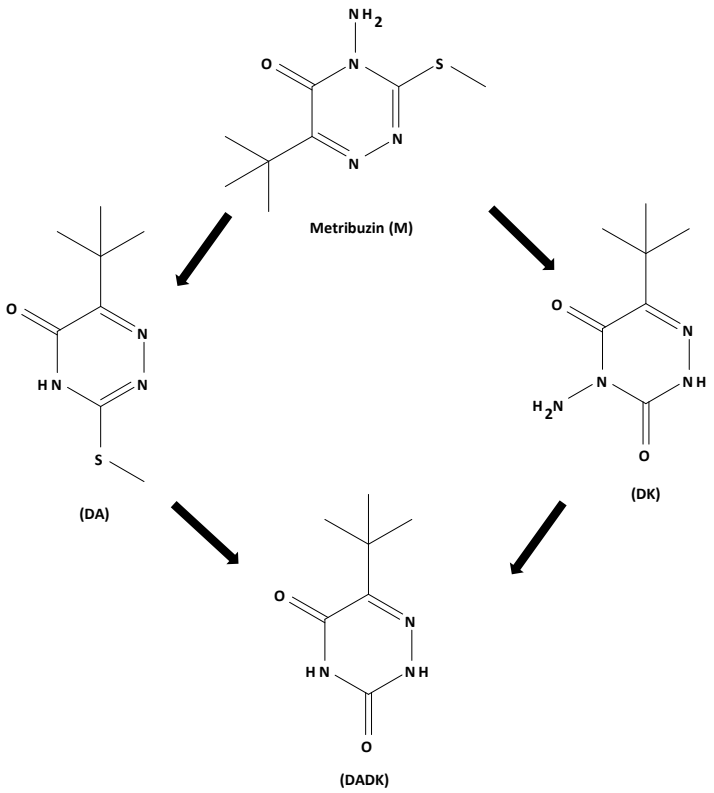
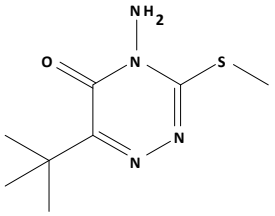


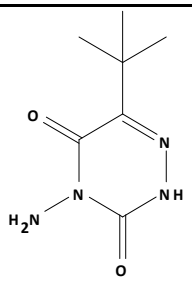
Figure 1. Likely degradation pathways of metribuzin. DA, deaminometribuzin; DK: diketometribuzin; DADK: deaminodiketometribuzin.

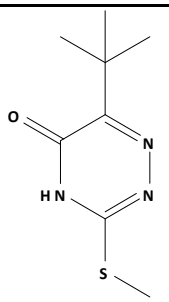
2.3. Physicochemical properties of metribuzin and its degradation products

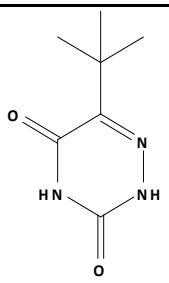
Physico-chemical properties of compounds relevant for their behaviour in the environment are their polarity (expressed as n-octanol-water partitioning coefficient, K_{ow}, log P), linked to water solubility, moreover their acidic-basic properties (expressed as dissociation constant K_a). The structures and some properties of metribuzin and its metabolites, studied in chapter 1, are listed in table 5.

Table 5. Chemical names and properties of the studied compounds

Metribuzin (M)	
IUPAC name	1,2,4-Triazin-5(4*H*)-one, 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)
Water Solubility (g L^{-1}, T: 25°C)	1.0
K_{ow}, log P	1.45
pK_a	-0.16
Molecular weight (Da)	214.3
Half-life in soil (days)	14-60

Diketometribuzin (DK)	
IUPAC name	1,2,4-Triazine-3,5(2*H*,4*H*)-dione, 4-amino-6-(1,1-dimethylethyl)
Water Solubility (g L^{-1}, T: 25°C)	5.70
K_{ow}, log P	0.091
pK_a	10.06; -1.67
Molecular weight (Da)	184.2
Half-life in soil (days)	N/A

Deaminometribuzin (DA)	
IUPAC name	1,2,4-Triazin-5(2*H*)-one, 6-(1,1-dimethylethyl)-3-(methylthio)
Water Solubility (g L^{-1}, T: 25°C)	0.94
K_{ow}, log P	1.74
pK_a	7.47; 1.01
Molecular weight (Da)	199.27
Half-life in soil (days)	N/A

Deaminodiketometribuzin (DADK)	
IUPAC name	1,2,4-Triazine-3,5(2*H*,4*H*)-dione, 6-(1,1-dimethylethyl)
Water Solubility (g L^{-1}, T: 25°C)	4.9
K_{ow}, log P	0.98
pK_a	7.81; -1.43
Molecular weight (Da)	169.18
Half-life in soil (days)	N/A

N/A: Not available

2.4. Methods of analysis

Analytical methodologies for the triazine herbicides have improved significantly since their original development. During the 50-year period in which the triazines have been used as herbicides, analytical equipment and extraction systems have evolved to be able to detect very small amounts of triazines in samples. More

recently, methods have also been developed for extracting and isolating a wide array of metabolites.

The determination of triazines and their main transformation products in solid samples is complicated. The extraction technique frequently used like Soxhlet, microwave assisted extraction (MAE) and pressurized liquid extraction (PLE), extract triazine compounds and many soil components [40-43]. Most of the unwanted organic compounds from the sample would be transferred to the extract and these interferences have to be removed before the analysis by an appropriate clean-up technique. Recently another extraction techniques have been developed, such as dispersive liquid-liquid microextraction (DLLME) or solid-phase microextraction (SPME) [44,45], extraction based on solidification of organic drop [46,47], extraction using ionic liquids (IL) [48-50] or using molecularly imprinted polymer (MIP) [51-53].

Determination of triazines after extraction and clean-up is usually accomplished using either gas (GC) or liquid chromatography (HPLC) [54,55]. Both techniques can be coupled with mass spectrometry (MS) [56,57]. Other detectors used in triazine

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[57] V.W.H. Tsang, N.Y. Lei, M.H.W. Lam, Marine Poll. Bull. 58 (2008) 1462

analysis are diode-array detector for HPLC [58], and nitrogen-phosphorous detector for GC [59].

Besides chromatography, other analytical techniques are applied to triazine determination such as capillary zone electrophoresis (CZE) [60] as micellar electrokinetic chromatography (MEKC) [61] and voltammetry [62]. Biosensors and bioassays are used for preliminary screening of samples or sample extracts, but because of their cross-reactivity the samples with analyte content above the cut-off value should be re-analysed by a more specific analytical technique. The most widely applied is antibody-based ELISA, but some innovative approaches have been developed, such as sensors based on photosystem-II inhibition from plant photosynthetic membranes [63].

Usually the analysis of metribuzin and its degradation products has been accomplished by different chromatographic methods such as reverse phase thin layer chromatography (RP-TLC) with UV detection [64] or HPLC with diode array detection [65,66] or mass spectrometry detection [67,68]. Capillary electrophoresis (CE) presents a very interesting alternative to chromatographic method for the analysis of pesticide residues and by-products in environmental samples, due to its low cost, short separation times, high efficiency and no need of high volumes of organic solvents. Although CE has been used to separate metribuzin from others pesticides in multiresidue determinations using MEKC [69,70] or CE in presence of cyclodextrins

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using laser-induced fluorescence [71], only recently a MEKC procedure, based on the use of sodium dodecyl sulphate (SDS) has been developed for determining simultaneously metribuzin and its degradation products in soil samples [72], considering the neutral nature of metribuzin which prevents its determination by CZE. In this case, detection limits of 23, 22 and 20 μg kg^{-1} were obtained from DA, DK and DADK. However, the anionic properties of the degradation products of metribuzin could allow the determination by CZE, which can be easily combined with several on-column sample pre-concentration methods to increase the amount of the analyte introduced into the capillary in order to obtain an improvement in sensitivity.

3. Urea herbicides. Sulfonylureas

Urea herbicides form, together with phenoxy derivatives and triazines, the most important agricultural herbicide group. The substituted urea herbicides are used for the control of many annual and perennial weeds, for bush and weed control in irrigation and drainage ditches.

Substituted urea pesticides include two kinds of herbicides (phenylureas and sulfonylureas) and a group of insecticides (benzoylureas) [73]. The general structures of urea herbicides are shown in figure 3.

(A)

$H_2NC(=O)NH_2$

(B)

$R_1\text{-}SO_2HNC(=O)NH\text{-}R_2$

(C)

$Ph\text{-}NH\text{-}C(=O)N\text{-}R_1$

(D)

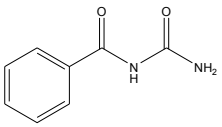

Figure 3. General structure of (A) Urea pesticides, (B) Sulfonylurea herbicides, (C) Phenylurea herbicides and (D) N-benzoylurea, example of benzoylurea insecticide

[71] M. Navarrete-Casas, A. Segura-Carretero, C. Cruces-Blanco, A. Fernández-Gutiérrez, Pest. Manag. Sci. 61 (2005) 197

[72] J.F. Huertas-Pérez, M. del Olmo-Iruela, A.M. García-Campaña, A. González-Casado, A. Sánchez-Navarro, J. Chromatogr. A, 1102 (2006) 280

[73] M. Stoytcheva (Ed.) Pesticides-Strategies for Pesticides Analysis. ISBN 978-953-307-460-3,Publisher: InTech, (2011)

Phenylurea herbicides (PUHs) are largely used in field applications for pre- and post-emergence weed control in a variety of crops. PUHs are used as selective and non-selective herbicides in substantial amounts, including the use as systemic herbicides to control broadleaf and grassy weeds in cereals and other crops, as total herbicides in urban areas, and as algaecides in paints and coatings.
Benzoylurea compounds (BUIs) are promising insecticides used because their ability to act as insect growth regulators that inhibit the synthesis of cuticle chitin in target pests.
The sulfonylurea herbicides (SUHs) have been developed more recently, and they have an herbicidal activity higher than the PUH, with application rates of 10 – 40 g a.i. ha^{-1} instead of kg ha^{-1}. SUHs are low dose herbicides used to control broad leaved weeds in cereals, exhibiting very low acute and chronic mammalian toxicities [74]. They can be absorbed by foliage and roots and they are normally applied in postemergence and in some cases may have observable field persistence [75].

The mechanism of action of all ureas is common, consisting in act inhibiting the photosynthesis producing herbicidal activity, impeding the chlorophyll's function due to the inhibition light reaction at the level of Hill reaction. SUHs act inhibiting acetolactate synthase. This enzyme intervenes in the biosynthesis of branched chain amino acids (valine, leucine, and isoleucine) conducting the photosynthesis inhibition.

3.1. Toxicity of sulfonylurea herbicides

Most of the SUHs are resistant to hydrolysis and oxidation under environmental conditions but they are only moderately persistent because they are degraded by soil microorganisms. SUHs are less persistent in soil, with half-lives ranging from a few days to a few months. Most SUHs have low volatility, are mobile in soil and are slightly to moderately soluble in water, so they can leach into runoff and can affect wild aquatic plant growth [76]. Many SUHs are nontoxic to slightly toxic to birds

[74] Y.S. Wang, W.C. Chen, L.C. Lin, J.H. Yen, J. Environ. Sci. Health Part B, 45 (2010) 449
[75] J.L.Tadeo (Ed.) Analysis of pesticides in food and environmental samples. Taylor and Francis group (2008)
[76] Adapted from Centers for Disease Control and Prevention (CDC) web page, accessed in March 2013:
http://www.cdc.gov/biomonitoring/SulfonylureaHerbicides_BiomonitoringSummary.html

and aquatic insects and vertebrates, although with massive accidental spills into rivers, fish kills have been reported. SUHs have very low mammalian toxicity [77].

General population exposure to SUHs is likely to be uncommon because of the low application rates of these herbicides. Potential routes of human exposure include consumption of foods grown in soil to which the SUHs were applied or drinking contaminated water. Tolerances or acceptable concentrations have been established for various edible food crops to which several of these herbicides may be applied. Human health effects from the SUHs at low environmental doses or at biomonitored levels from low environmental exposures are unknown. Animal studies showed low acute toxicity and little chronic, reproductive or developmental toxicity or teratogenic effects. Developmental or teratogenic effects tended only to occur at high or maternally toxic doses. At high sublethal, chronic oral doses, such nonspecific effects as weight loss and anemia were observed. Some chemical-specific effects were noted at high doses in animals. SUHs were not mutagenic in vitro and most were not carcinogenic in animals [76].

3.2. Degradation studies of sulfonylurea herbicides

The more recent herbicide formulations are designed to offer advantages of the highest selectivity together with the lowest persistence in the environment and SUHs meet these requirements. Although, lower persistence in the environment does not imply lower toxicity, because many herbicides undergo natural degradation reactions in the environment that do not lead to mineralization but to the formation of new species potentially more toxic and stable than the precursors [78].

The most important pathways of degradation of SUH in soil are chemical hydrolysis and microbial degradation, while other dissipation processes such as volatilization and photolysis are relatively insignificant [79,80,74]. SUH typical field dissipation half-lives ($t_{1/2}$) are about 1-8 weeks in some cases, but within a few days in the case of some newer compounds. Chemical hydrolysis is pH and temperature

[77] K. Michael (ed.) Pesticide Profiles-Toxicity, Environmental Impact, and Fact. CRC-Lewis Publishers, Boca Raton (FL), 1997, pp. 377
[78] M. Bottaro, P. Frascarolo, F. Gosetti, E. Mazzucco, V. Giatiotti, S. Polati, E. Pollici, L. Piacentini, G. Pavese, M.C. Gennaro, J Am. Soc. Mass Spectrom. 19 (2008) 1221
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[80] Y.B. Si, L.G. Zhang, K. Takagi, Int. J. Environ. Anal. Chem. 85 (2005) 73

dependent: in most cases the degradation is faster in acidic rather than in neutral or in weakly basic conditions, and at high temperature [74].

Different authors have shown that soil pH is the most important factor in affecting both sorption behaviour and chemical degradation of metsulfuron-methyl in soil because of its ability to influence the ionization state of the herbicide [81]. The mineralization rate was negatively correlated with soil pH, organic carbon contents and clay contents, while it was positively correlated with soil microbial biomass carbon and silt contents.

The dissipation mechanisms of two SUHs, chlorsulfuron and imazosulfuron, were both chemical and biological. The $t_{1/2}$ of chlorsulfuron was 6.8–28.4 days and that of imazosulfuron was 6.4–14.6 days. Persistence is strongly influenced by the temperature and soil pH. Both compounds dissipate faster in a more acidic soil. Both SUHs changed the soil bacterial composition, and the change was larger with imazosulfuron at 50 mg kg^{-1}. The selectivity of survival for bacteria was stronger in more alkaline soils [74]. In soil, the hydroxylation of the aromatic ring of chlorsulfuron has been reported in the presence of the fungus *Aspergillus niger*. Chemical cleavage was the main degradation pathway in aerobic conditions, whereas in anaerobic conditions, microbial degradation was the main degradative pathway to demethylate imazosulfuron [74].

The hydrolysis rate of rimsulfuron was as high as the photolysis rate, and decreased on diminishing the pH values of the solution. The photochemical degradation of the herbicide was strongly affected by retention phenomena, showing that silica and clay minerals can retain and protect rimsulfuron from photodegradation much more than soil. Rimsulfuron is moderately persistent to non-persistent in aqueous solutions/soil suspensions under anaerobic/aerobic conditions, with $t_{1/2}$ of 6–40 days in soil.

The kinetics of hydrolytic degradation of sulfosulfuron was investigated to predict the fate of the herbicide in an aqueous environment. The degradation was dependent on pH and temperature. Hydrolysis rate was faster in acidic condition ($t_{1/2}$=9.24 days at pH 4.0) than alkaline environment ($t_{1/2}$=14.14 days at pH 9.2). Under abiotic conditions, the major degradation mechanism of the compound was

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the breaking of the sulfonylurea bridge yielding corresponding sulfonamide and aminopyrimidine [79].

The UV-induced photodegradation of metsulfuron in water has been studied. The mechanism involved hydrolytic cleavage of the sulfonylurea bridge to form the corresponding phenyl sulfonyl carbamic acid and s-triazine, with the carbamic acid subsequently decarboxylating to form a phenyl sulfonamide and a cyclic derivative [82].

3.3. Physicochemical properties of sulfonylurea herbicides

Knowledge of the chemical structure and the physical characteristics of SUHs is needed to understand the use and behaviour in the environment. SUHs are weak acids, having a pK_a values ranging from 3.3 to 5.2. The log octanol/water partition coefficient (log P, K_{ow}) is dependent of pH value since the neutral form of the molecule is more lipophilic than the anionic form. In the same way, their solubility in water is pH dependent, with greater solubility at more alkaline pH values, were the hydrophilic anionic forms are predominant. The pH effect on Log P and water solubility affects their mobility and soil sorption; in general, greater sorption of SUHs occurs in acidic soils.

They are non-volatile, with vapour pressure ranging from $<10^{-5}$ to 10^{-14} Pa at 20-25°C, which minimizes the loss of SUHs in gas form during storage, mixing and application or from treated soil and plants applications [83].

Table 6 summarizes the physicochemical properties of the SUHs studied in the chapter 2 of this thesis.

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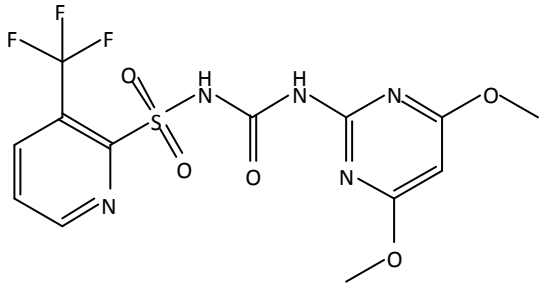
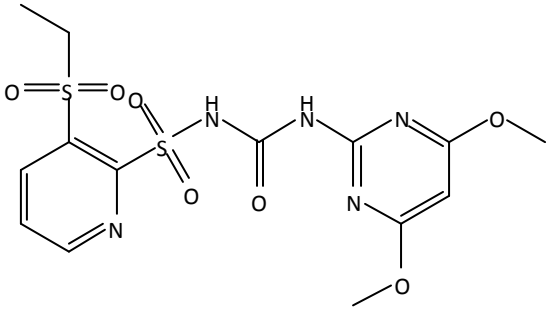
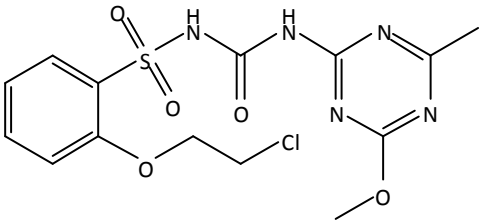
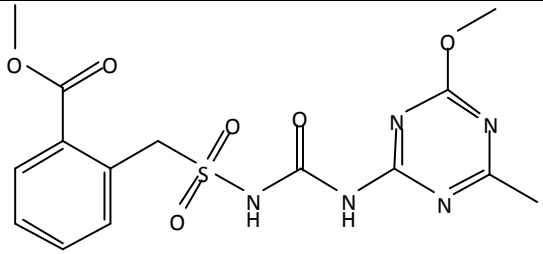
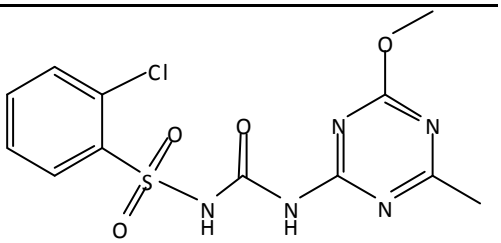
Table 6. Chemical names, structures and properties of the sulfonylurea herbicides.

Flazasulfuron (Flaza-S)	
IUPAC name	1-(4,6-Dimethoxypyrimidin-2-yl)-3-(3-trifluoromethyl-2-pyridylsulfonyl)urea
Water Solubility (g L^{-1}, T: 25°C)	0.77
K_{ow}, log P	2.73
pK_a	3.55; 1.08
Molecular weight (Da)	407.33
Half-life in soil (days)	<7

Rimsulfuron (Rim-S)	
IUPAC name	2-Pyridinesulfonamide, *N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(ethylsulfonyl)-
Water Solubility (g L^{-1}, T: 25°C)	1.2
K_{ow}, log P	1.58
pK_a	3.47; 1.03
Molecular weight (Da)	431.4
Half-life in soil (days)	10-20

Triasulfuron (Tria-S)	
IUPAC name	Benzenesulfonamide, 2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-
Water Solubility (g L^{-1}, T: 25°C)	3.6
K_{ow}, log P	1.91
pK_a	3.34; 2.6
Molecular weight (Da)	401.83
Half-life in soil (days)	19

Metsulfuron-methyl (Met-S)	
IUPAC name	Benzoic acid, 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-, methyl ester
Water Solubility (g L^{-1}, T: 25°C)	5.0
K_{ow}, log P	1.54
pK_a	2.55
Molecular weight (Da)	381.36
Half-life in soil (days)	7-35

Chlorsulfuron (Chlor-S)	
IUPAC name	Benzenesulfonamide, 2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-
Water Solubility (g L^{-1})(T: 25°C; pH:7)	2.6
K_{ow}, log P	2.20
pK_a	4.21; 2.56
Molecular weight (Da)	357.77
Half-life in soil (days)	28-42

3.4. Methods of analysis of urea herbicides

Analytical methodologies for the analysis of urea (phenylurea, sulfonylurea and benzoylurea) pesticides in crops, soil and water samples have been reviewed [84,85,73]. The polar characteristic, low volatility or thermal instability of some SUHs avoid the direct analysis of them by gas chromatography (GC) and usually involved a derivatization procedure with diazomethane or pentafluorobenzyl bromide. The derivative procedure made GC difficult to be a robust tool for monitoring urea pesticides.

HPLC has been the most frequent technique used for analysing SUHs and some of their degradation products in different matrices such as human urine [86], grains, seeds, vegetables, fruits [87,88] or water samples from different origins [89-93] with UV or MS detection.

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Fluorescence detection (FLD) has been closely bound to the important development of LC instrumentation as it is generally more sensitive than classical UV absorption and less expensive that MS detection. It represents a very selective detector, overcoming matrix interferences. However, few compounds are fluorescent, although some of them possess the necessary degree of aromaticity and may be converted to fluorescent species by using derivatization methods. Several authors studied the application of FLD combined with post-column photochemically induced fluorimetry derivatization to determine PUH compounds in groundwater, rice and corn samples [94-96]. Amperometric detector has been also coupled with LC for the analysis of PUH [97].

CE has emerged as an increasingly powerful separation technique, especially for thermally unstable compounds, due to its simplicity, short separation times and lower consumption of reagents and solvents. MEKC has been applied to the analysis of SUHs in water and soil samples [98-100] or in cereals [101] and sulfonylurea together with other urea-derived pesticides in fruits and vegetables [102]. Since sulfonylureas are weak acids, they are particularly amenable to the separation by capillary zone electrophoresis (CZE). An alternative approach to separate sulfonylureas by CZE with MS detection has been reported, which offers a

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very low detection limit [103,104]. The determination of five SUHs in grains [105] and six SUHs in soil [106] have been accomplished using this methodology.

There is still a lack of officially approved methods that would solve the difficulties associated with quantitative isolation of urea pesticides from the various matrices, clean-up of the extract without significant loss of the analyte, separation of all individual pesticides contained in the purified extract, detection of the separated components and unequivocal identification and quantification of the identified compounds. Sample pretreatment processes are very important steps to achieve clean-up and effective enrichment of the target analytes before analysis. For solid samples, traditionally, Soxhlet and manual or mechanical shaking have been used for ureas extraction [95,107-111]. For aqueous samples the classical methodology is liquid-liquid extraction [112], which presents some disadvantages, for example the large volumes of organic solvents required, it is also time-consuming and involves multistep processes that have always the risk of loss of some analytes. Supercritical-fluid extraction (SFE), matrix solid-phase dispersion (MSPD), pressurized liquid extraction (PLE) [113], microwave-assisted extraction (MAE) [114-116] and batch extraction enhanced by sonication (BEES) [117,118] have been developed as alternative techniques to replace classical extraction methods mainly for solid samples. All these methods reduce extraction time and the

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volumes of solvent required, but have the disadvantages of high investment and maintenance costs of the instruments (i.e., SFE, PLE and MAE). Actually, MAE and PLE are applied successfully for urea residue control in soils [114], vegetables [115,116] and in oysters [113]. Low temperatures must be selected due to urea's thermolability. Studies have shown that once optimized, these new extraction techniques are comparably efficient, with similar standard deviations. However, the main disadvantage is the wide range of co-extracted compounds leading usually to more purification steps.

MSPD is relevant for tissue analysis, such as beef fat, catfish muscle or oysters [113]. Matrices are blended with C18 or Florisil phases before analyte elution with an adequate solvent. The major drawback of this procedure is the manual preparation which complicates the routine application. Solid phase extraction (SPE) is a useful preconcentration technique that allows extraction (for liquid samples) and concentration of traces of contaminants. It represents the most often-applied method in environmental and food analysis. The popularity of SPE has increased in the last years because it can be easily automated and a wide range of phases are available. Octadecylsilica is the largely preferred sorbent over other supports for all the urea herbicides [119-121]. Procedures for sample treatment before SUH analysis generally include SPE with many purification steps, rendering the sample treatment laborious. This drawback has been overcome by different authors following alternatives such as SPE with single-walled carbon nanotubes disk [122], coupling on-line SPE [123] or using MIP [124-128]. MIPs are synthetic polymers possessing specific cavities designed for a target molecule. By a

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mechanism of molecular recognition, the MIPs are used as selective tools for the development of various analytical techniques such as SPE. There are applications of MIPs directly to real samples without a preliminary treatment [129,130].

Room temperature ionic liquids (RTILs) containing relatively large asymmetric organic cations and inorganic or organic anions have recently been used as “green solvents” to replace traditional organic solvents for chemical reactions. The application of immobilized ILs in separation and clean-up procedures has recently raised much interest [131,132]. It was showed that cartridges with IL-functionalized silica sorbent allow a better simultaneous quantification of 12 SUHs than that reached with C18 sorbent [133].

The need to reduce the overall sample preparation time and the quantities of organic solvents has led to the emergence of several new extraction approaches, including SPME [96,134,135], liquid phase microextraction (LPME) [136] and DLLME [137,138]. The SPME technique is a solvent-free extraction technique that was successfully coupled to GC and LC [96,135] in order to analyze PUH in fruit juices and groundwater.

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CAPÍTULO 1

Determinación de los productos de degradación del Metribuzin en muestras ambientales mediante electroforesis capilar en zona con preconcentración en línea

CHAPTER 1

Large volume sample stacking in capillary zone electrophoresis for the monitoring of the degradation products of Metribuzin in environmental samples

RESUMEN

Se ha desarrollado un método de separación basado en el empleo de la electroforesis capilar en zona (CZE), con detección UV-Vis para el análisis de los productos de degradación mayoritarios del Metribucin: Dicetometribucin (DK), Desaminometribucin (DA) y Desaminodicetometribucin (DADK). Se han calculado las constantes de disociación de cada compuesto usando CZE, no encontrándose diferencias significativas con los valores obtenidos mediante otras técnicas, descritos en bibliografía. La separación óptima se ha llevado a cabo en menos de 9 minutos, con tampón tetraborato 40 mM a pH 9.5, aplicando un voltaje de 15 KV y a 25°C de temperatura; además el ácido p-aminobenzoico se ha utilizado como patrón interno. Para aumentar la sensibilidad del método analítico se ha utilizado un método de preconcentración en línea con polaridad inversa (*large volumen sample stacking*, LVSS). Los límites de detección alcanzados son de 10, 10, y 20 ng mL^{-1} para DA, DADK y DK respectivamente. El método ha sido aplicado a suelos empleando para ello la extracción con líquidos presurizados (PLE) con metanol como agente extractante y sulfato sódico como desecante, a alta temperatura y presión (103°C y 15000 psi.). La extracción PLE iba seguida por una etapa de limpieza y preconcentración basada en la extracción en fase sólida (SPE) con cartuchos LiChrolut EN. Esta última etapa de SPE es suficiente para el tratamiento de aguas subterráneas, antes del análisis por CZE. La combinación de los dos métodos de preconcentración, en el tratamiento de muestra y durante el desarrollo del método separativo, proporcionó una mejora significativa en la sensibilidad. LVSS proporciona un factor de preconcentración de 4, 28 y 36 para DK, DA, DADK respectivamente, y con la SPE se consigue una preconcentración de 500 veces en aguas subterráneas y 2.5 veces en muestras de suelo. El método es adecuado para el análisis de este tipo de residuos en muestras ambientales, con alta sensibilidad, precisión y recuperaciones satisfactorias.

SUMMARY

A capillary zone electrophoresis (CZE) method with UV-Vis detection has been developed for the simultaneous monitoring of the major degradation products of metribuzin, i.e. deaminometribuzin (DA), deaminodiketometribuzin (DADK) and diketometribuzin (DK). The dissociation acid constants have been also estimated by CZE and no significant differences have been observed with the values obtained by applying other techniques. Optimum separation has been achieved in less than 9 min in 40 mM sodium tetraborate buffer, pH 9.5 by applying a voltage of 15 kV at 25°C and using p-aminobenzoic acid as internal standard. In order to increase sensitivity, large volume sample stacking (LVSS) with polarity switching has been applied as on-line preconcentration methodology. Detection limits of 10, 10 and 20 ng mL^{-1} for DA, DADK and DK, respectively were obtained. The method has been applied to soil samples, for which pressurized liquid extraction (PLE) was applied. Samples were extracted at high temperature (103°C and 1500 psi) using methanol as extraction solvent and sodium sulphate as drying agent. This PLE procedure was followed by an off-line preconcentration and sample clean-up procedure by solid phase extraction (SPE) using a LiChrolut EN sorbent column. These last two procedures were also suitable for the direct treatment of groundwater samples before CZE analysis. The combination of both off-line and on-line preconcentration procedures provided a significant improvement in sensitivity. LVSS provided preconcentration factors of 4, 36 and 28 for DK, DA, and DADK, respectively and with SPE a pre-concentration of 500-fold for the case of water samples and of 2.5-fold in the case of soil samples was obtained. The method is suitable for the monitoring of these residues in environmental samples with high sensitivity, precision and satisfactory recoveries.

1. Materials and methods

1.1. Chemicals

All the reagents used were of analytical grade and the solvents of HPLC grade. The water used to prepare the solutions was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Organic solvents such as acetonitrile, methanol and acetone were purchased from Merck (Darmstadt, Germany).

The separation buffer was prepared from sodium tetraborate supplied by Sigma-Aldrich Química (Madrid, Spain). The pH was adjusted to 9.5 with 1 M sodium hydroxide obtained from Panreac-Química (Madrid, Spain). The reagents used in determination of the dissociation constants were: buthylamine supplied by Merck; phosphoric acid 85%, sodium di-hydrogen phosphate 1-hidrate, di-sodium hydrogen phosphate and sodium chloride, all of these supplied by Panreac-Química; boric acid, formic acid and acetic acid were purchased from Merck; sodium formiate and sodium acetate obtained from Sigma-Aldrich Química. Other reagents were hydrochloric acid supplied by Scharlaub (Barcelona, Spain) and sodium hydroxide supplied by Panreac-Química. Analytical standards of deaminometribuzin (DA), 6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one, CAS RN [35045-02-4], diketometribuzin (DK), 4-amino-6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H)-dione, CAS RN [56507-35-0] and deaminodiketometribuzin (DADK) 6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H)-dione, CAS RN [52236-30-3] were purchased form Dr. Ehrenstorfer (Augsburg, Germany). Purity was 99.0, 99.0 and 98 %, for, DA, DK and DADK, respectively. Stock standard solutions containing 500 mg L^{-1} of each compound were prepared by dissolving accurately weighed amounts in acetonitrile and stored in darkness at 4°C. Working standard solutions were made daily by diluting them with deionised water. p-Aminobenzoic acid (PABA) (Fluka, Sigma-Aldrich Química, Madrid, Spain) was used as internal standard (I.S.). A stock solution of 100 mg L^{-1} of PABA was prepared by dissolving 10 mg of the product in 100 mL of water. The solution was stable for at least 1 month.

The sorbent used for SPE was LiChrolut EN, polymer of styrene-divinylbenzene (200 mg, 3 mL) (Merck).

1.2. Instrumentation and software

CE experiments were carried out with a HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the software provided with the HP ChemStation version A.09.01. Separation was carried out in a bared fused silica capillary 48.5 cm x 75 µm i.d. (effective length 40 cm) with an optical path length of 200 µm (bubble cell capillary from Agilent Technologies, Waldbron, Germany). For pH measurements, a pH meter (Crison model pH 2000, Barcelona, Spain) was employed with a resolution of ± 0.01 pH unit.

An ASE 100 system from Dionex (Sunnyvale, CA, USA) was applied to perform PLE of the analytes from soil samples. The system was operated with pressure resistant steel extraction cells with a volume of 33 mL. Nitrogen at a pressure of 10 bar was supplied to assist the pneumatic system and to purge the extraction cells.

A vacuum manifold system from Supelco (Bellafonte, PA, USA) coupled with a vacuum pump (Büchi model B-169, Switzerland), nylon filters with 0.20 and 0.45 µm pore size (Supelco, Bellafonte, PA, USA) and a rotavapor (Büchi, Flawil, Switzerland) were used for standard solutions and sample clean-up and preconcentration.

Statgraphics Plus for Windows (Rockville MD USA) version 5.0 was used for pK_a calculations and for the application of experimental designs. Excel 2000 was used for the statistical analysis of data.

1.3. Procedure for pK_a determination by CZE

For the characterization of the analytes, the values of dissociation constants, pK_a, were estimated by establishing the relationship between the electrophoretic mobility with the pH value of the applied separation buffer, using a constant ionic strength of 0.05 M. The preparation of the applied buffer solutions for this study was randomized and carried out as described by Tanabe et al. [1].

[1] A. Tanabe, K. Kawata, Anal. Sci. 20 (2004) 227

Table I.1. Buffers recipes for pK_a measurements by CE

Run order	Buffer composition (ionic strength = 50 mM)	pH
1	Butylamine 0.618 mL+ HCl, 1M, 0.672 mL + 658.5 mg NaCl in 0.25 L water	11.42
2	Butylamine 1.235 mL + HCl, 1 M, 7.855 mL + 266.8 mg NaOH in 0.25 L water	10.53
3	H_3BO_3 772.9 mg +NaOH 1M, 9.466 mL + NaCl 907.8 mg, in 0.5 L water	9.63
4	H_3BO_3 772.9 mg + NaOH 1M, 3.802 mL + NaCl, 1238.9 mg, in 0.5 L water	8.71
5	H_3BO_3 618.3 mg + NaOH, 1M, 0.581 mL + NaCl 696.6 mg, in 0.25 L water	7.81
6	$NaH_2PO_4.H_2O$ 317.2 mg + Na_2HPO_4 482.7 mg in 0.25 L water	7.09
7	$NaH_2PO_4.H_2O$ 713.4 mg + Na_2HPO_4 153.3 mg + NaCl 239 mg in 0.25 L water	6.21
8	CH_3COOH 0.0896 mL + $NaCOOCH_3$ 694.3 mg + NaCl 2373.8 mg in 0.25 L water	5.37

To develop the study, the instrumental conditions were: fused silica capillary 48.5 cm x 75 µm i.d.; applied voltage 20 kV; separation temperature 25°C; hydrodynamic injection pressure 50 mbar during 5 s. The samples contained 10 mg L^{-1} of each analyte in water. Acetone 1% was used as a neutral marker to determine the EOF. Before the application of the CE procedure, the capillary was conditioned for each separation buffer before the electrophoretic separation. The pH value of the background electrolyte (BGE) solution and the corresponding migration time can be used to estimate the electrophoretic mobility of the analytes and from these data, the corresponding dissociation constants can be calculated.

1.4. Electrophoretic procedure

Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min at 60°C, then with water for 5 min, and finally with the BGE solution for 20 min. A pressure of 1 bar was applied. At the beginning of each day, the capillary was rinse with an N_2 pressure of 7 bar for 1 min with 0.1 M NaOH, 1 min with

water and 2 min with running buffer. In order to increase migration time reproducibility, after each run, the capillary was post-washed with 7 bar for 0.5 min 0.1 M NaOH, 0.5 min with deionized water and 1 min with buffer. If drastic drifts in electrophoretic current and/or migration times were observed, the capillary was rinsed with an N_2 pressure of 7 bar for 1 min with deionized water followed by 2 min with methanol. At the end of each day, the capillary was rinsed with deionized water for 1 min and dried with air for 0.5 min.

Electrophoretic separation was performed using a 40 mM sodium tetraborate buffer, pH 9.5 adjusted with 1 M sodium hydroxide, by applying a voltage of 15 kV (normal mode). Buffer solution was filtered through a 0.20 μm membrane filter before use. DA was monitored at 220 nm with a bandwidth of 20 nm, and DK and DADK were monitored at 260 nm with a bandwidth of 30. The temperature of the capillary was kept constant at 25°C. Injection of the sample occurred using the following LVSS procedure.

1.5. LVSS procedure

Samples containing the analytes were loaded with a pressure of 50 mbar for 200 s. In this way, 80% of the capillary column was filled with the sample solution. After sample injection, a negative voltage (–25 kV) was applied. Sample matrix removal from the capillary was indicated by monitoring the electric current, which progressively increased to its normal value as the low-conductivity injected zone was eliminated from the capillary. At this stage the stacking process could be considered complete. The high voltage was then switched from negative to positive (15 kV).

1.6. Sample preparation procedures

1.6.1. Extraction in soil samples by PLE

Soil samples were air dried, mixed and sieved through a 2-mm sieve before fortification by adding the appropriate volume of the working standard solutions and processed 24 hours after spiking. A portion of 5 g of spiked soil sample was

transferred into the extraction cell of the ASE system and 10 g of sodium sulfate were added as drying agent. To protect the end cap from particulate substances, a cellulose filter was mounted inside the cell towards the outlet direction. For extraction, methanol was used at 103°C for 5 min static time, in two extraction cycles. Other extraction conditions were as follows: pressure 1500 psi., preheat 3 min, flush volume 100% and purge time 60 s. The total final volume of the extract was approximately 55-60 mL (depending on soil moisture and mass). Then, the extract was filtered through a nylon filter of 0.45 μm, and collected in a 100 mL spherical flask. Filter and flask were washed with 10 mL of methanol. The obtained solution was concentrated to approximately 1 mL in a rotary evaporator at a temperature of 37°C. The extract was diluted with 25 mL of deionized water and pre-concentrated by SPE before being analyzed by CE.

1.6.2. Pre-concentration and clean-up of extracts from soil samples and groundwater samples by SPE.

SPE procedure was similar for groundwater samples and for soil extracts obtained from the PLE procedure above described. SPE was carried out on LiChrolut EN cartridges pre-conditioned with 6 mL of acetone, 6 mL of methanol and 6 mL of deionized water, consecutively. In the case of groundwater samples, a portion of 100 mL was spiked with different concentration levels of standard solutions of the analytes. The water samples or the soil extracts were loaded through the cartridge at the rate of 2 mL min^{-1} by using a vacuum operated pumping system. After sample loading, the cartridges were washed with 5 mL of water and 5 mL of methanol:water (45:55 v/v) and air-dried for 15 min. The elution of solutes was achieved with 4 mL of methanol. The eluates were brought to dryness under a gentle nitrogen current at 20°C. The dry residue was dissolved in 500 μL of methanol:water (10:90 v/v) containing PABA (500 μg L^{-1}) as IS, for groundwater samples and in 2 mL of the same solution containing the same concentration of IS in the case of soil samples. These solutions were injected into the CE system for analysis.

2. Results and discussion

2.1. Determination of acid dissociation constants

Ionization constants of herbicides and their metabolites in general have been shown to be important in studies of soil adsorption and aqueous solubility. Ionic binding to soil constituents, which decreases effective herbicidal activity, has been postulated to be related in a predictable manner to the pK_a values of the herbicides. In addition, ionization constants and their relation to lipophilicity are important parameters to the study of membrane transport and metabolism. Historically, potentiometric titration was the standard method for pK_a measurements. For high-purity, water-soluble compounds available in relatively large quantities, this is still largely the case today [2-4]. Spectrophotometric titrations are generally considered the main alternative to potentiometric titrations for measuring pK_a values of water-soluble compounds of high purity. The main advantage is the higher sensitivity for compounds with favorable molar absorption coefficients. The fundamental limitation of conventional spectrophotometric titrations is the need for an identifiable chromophore shift associated with changes in ionization. Sample impurities and degradation products with similar absorption properties to the target compound may interfere in the measurements [5,6]. CZE was introduced as a tool for characterization of acid-base chemistry in 1991 [7]. Since then, over 60 papers have been published in this field [8-12] and developments in this application have been reviewed [13]. The attractive features of CZE for pK_a measurements are: a) since CE is a separation

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technique, it can handle impure samples, b) instruments are highly automated and require little or no modification for high throughput applications, c) precise information of sample concentration is unnecessary, d) only mobilities are used in calculations, e) sample consumption is minute, f) slightly soluble compounds with suitable chromophore are easily handled, g) no special demands are placed on the purity of electrolyte solutions. Considering these facts, the acid dissociation constants of DA, DK and DADK have been determined by CZE in this chapter.

The determination of pK_a values by CE is based on the observation of the effective mobility of an ionizable compound in a series of electrolyte solutions of constant ionic strength and different pH. The effective electrophoretic mobilities (m_{eff}) of the analytes were calculated from the observed migration times of each analyte and migration times of the EOF marked (acetone) according to this equation [11]:

$$m_{eff} = m_{app} - m_{eff} = \frac{L_{tot} - L_{eff}}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \qquad (1)$$

where m_{app} is the apparent electrophoretic mobility, L_{tot} is the total length of the capillary, L_{eff} the effective length (to the detection window), V is the applied voltage, t_{app} is the observed migration time of each analyte and t_{EOF} the observed migration time of the EOF marker.

The pK_a value can be estimated from the effective electrophoretic mobilities using two different models:

a) Non-linear model [10]:

The electrophoretic mobilities, m_{eff}, calculated using eq. 1 and plotted against the pH, can be fitted with the sigmoidal model represented by eq. 2 using a non-linear regression:

$$m_{eff} = \frac{m_a}{10^{(pK_a - pH)} + 1} \qquad (2)$$

The unknown mobility of the fully ionized species (m_a) and pK_a are the regression parameters. Figure I.1 shows the obtained plots representing the electrophoretic mobility values depending of the pH values, using a non-linear model.

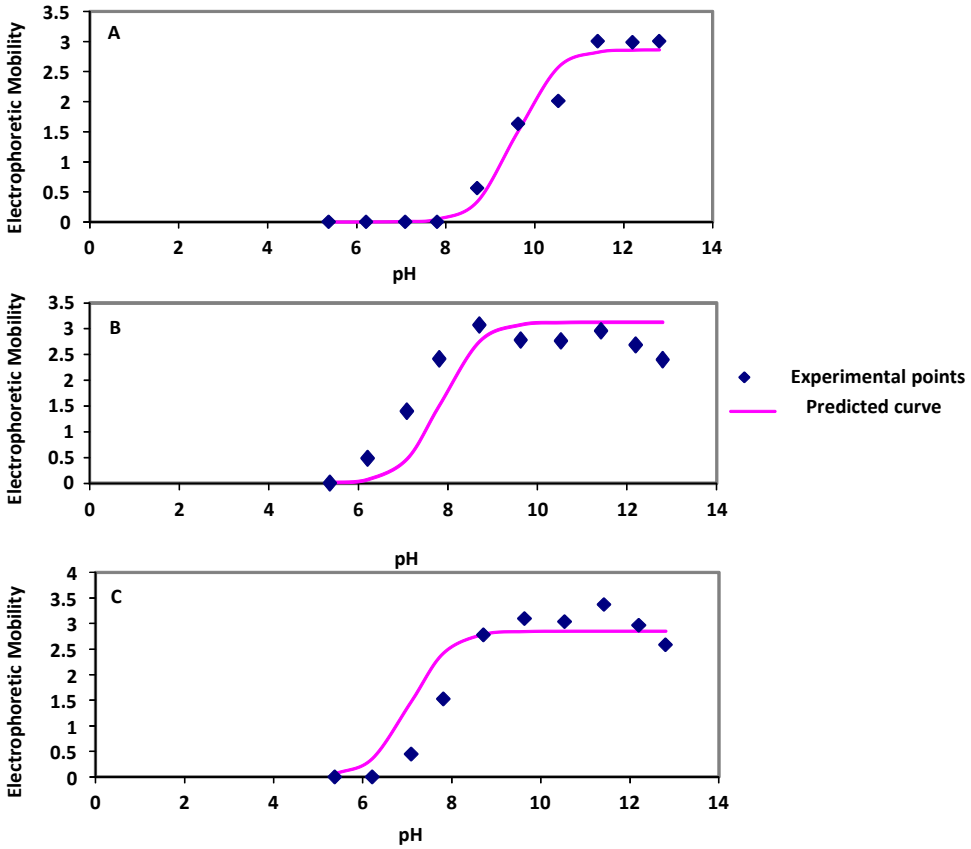

Figure I.1. Electrophoretic mobility values vs pH values for each analyte, using a non-linear model. A) DK; B) DA; C) DADK.

b) Linear model [9]

The inverse of the electrophoretic mobility is plotted in this case against the proton concentration. The value of pK_a is obtained from the slope:

$$\frac{1}{m_{eff}} = \frac{[H^+]}{K_a m_a} + \frac{1}{m_a} \qquad (3)$$

Figure I.2 shows the obtained plots representing the inverse of the electrophoretic mobility values as function of the concentration of proton values, using a linear model.

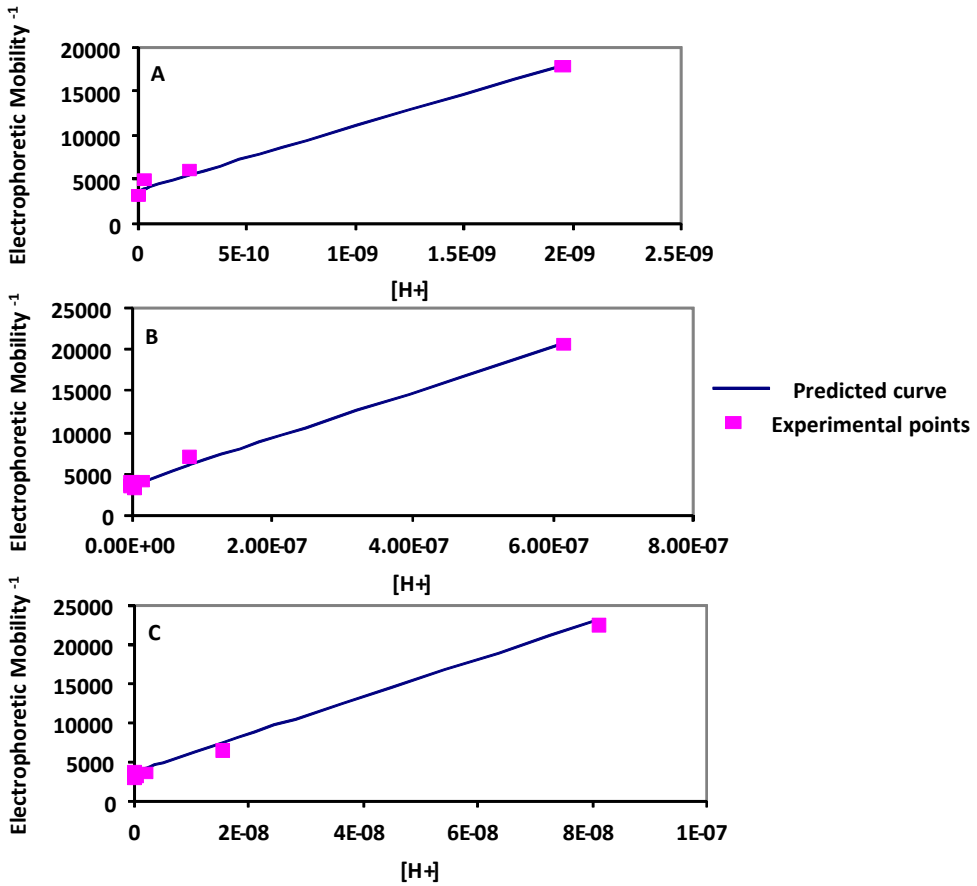

Figure I.2. Reverse of the electrophoretic mobility values vs concentration of protons for each analyte, using a linear model. A) DK; B) DA; C) DADK.

The results from both models obtained using the random run order for buffer solutions indicated in table I.1 are shown in table I.2, along with the data found in bibliography by potentiometric titrimetry and UV spectrophotometry [2]. For the three known metabolites of metribuzin, the pK_a values obtained are in good agreement with the published data.

Table I.2. Results of the pK_a values obtained by CE in this work and those reported in the literature by other techniques [2]

Compound	CE-Non-linear model (RSD, %)	CE-Linear model (RSD, %)	Spectrophotometry	Potentiometry
DK	9.6 (2.1)	9.5 (1.9)	9.9	10.0
DA	7.1 (2.7)	6.9 (2.8)	7.3	7.3
DADK	7.9 (2.5)	7.9 (2.3)	8.4	8.3

2.2. Optimization of the CE separation by applying LVSS with polarity switching procedure

Considering the poor sensitivity of CE using the UV-Vis detection, we have proposed an on-line sample stacking procedure as a way of tracing enrichment in CE for the analysis of major degradation products of metribuzin in environmental samples. LVSS with polarity switching for anions was chosen as the preconcentration technique because the buffer conditions and polarity allowed its use, considering that all the molecules are negatively charged at the working pH. In starting situation, a large volume of sample prepared in a low conductivity matrix is injected, and a voltage at negative polarity is applied for focusing of zones and removal of sample matrix. When the anions are completely focused and most of the sample matrix is removed, voltage is stopped and polarity is reversed. This occurs when the current reached 95-99 % of its value. Finally a voltage at positive polarity is applied in order to separate and detect the focused zones.

In LVSS, apart from the usual variables affecting the CE separation, it is necessary to consider two significant parameters, using water as sample solvent: size of plug sample and current inversion value. In this sense, the following CE-LVSS optimization was carried out:

pH, nature and concentration of the buffer and separation voltage. Phosphate buffer was used to check the full pH range and we observed that with alkaline pH values better resolution of the peaks was obtained. Thus, carbonate (50 mM), phosphate (50 mM) and borate (30 mM) buffer solutions were tested as possible BGE. Unacceptably high Joule heating as a result of a great current generated was observed when phosphate buffer was used. Carbonate buffer provided the worst resolution; therefore borate buffer was selected as the optimum separation BGE. Using this buffer nature, at a concentration of 30 mM, a pH optimization was carried out, selecting a pH interval for the study between 8.5 and 10.5, in steps of 0.5 units, considering the previous screening and the adequate resolution obtained

in this range. Highest peak areas, good resolutions and shorter migration times were obtained at pH 9.5. Figure I.3 shows the obtained electropherograms at the pH values studied. By applying the LVSS procedure, different buffer concentrations were tested (20, 30, 40 and 50 mM), selecting 40 mM as optimum concentration in order to obtain the best resolution between DA and DADK with an adequate current intensity. Figure I.4 shows the variation of the resolution with the increase of the buffer concentration. The values of voltage tested were 20, 15, 12.5, and 10 kV. a value of 15 kV was applied as optimum.

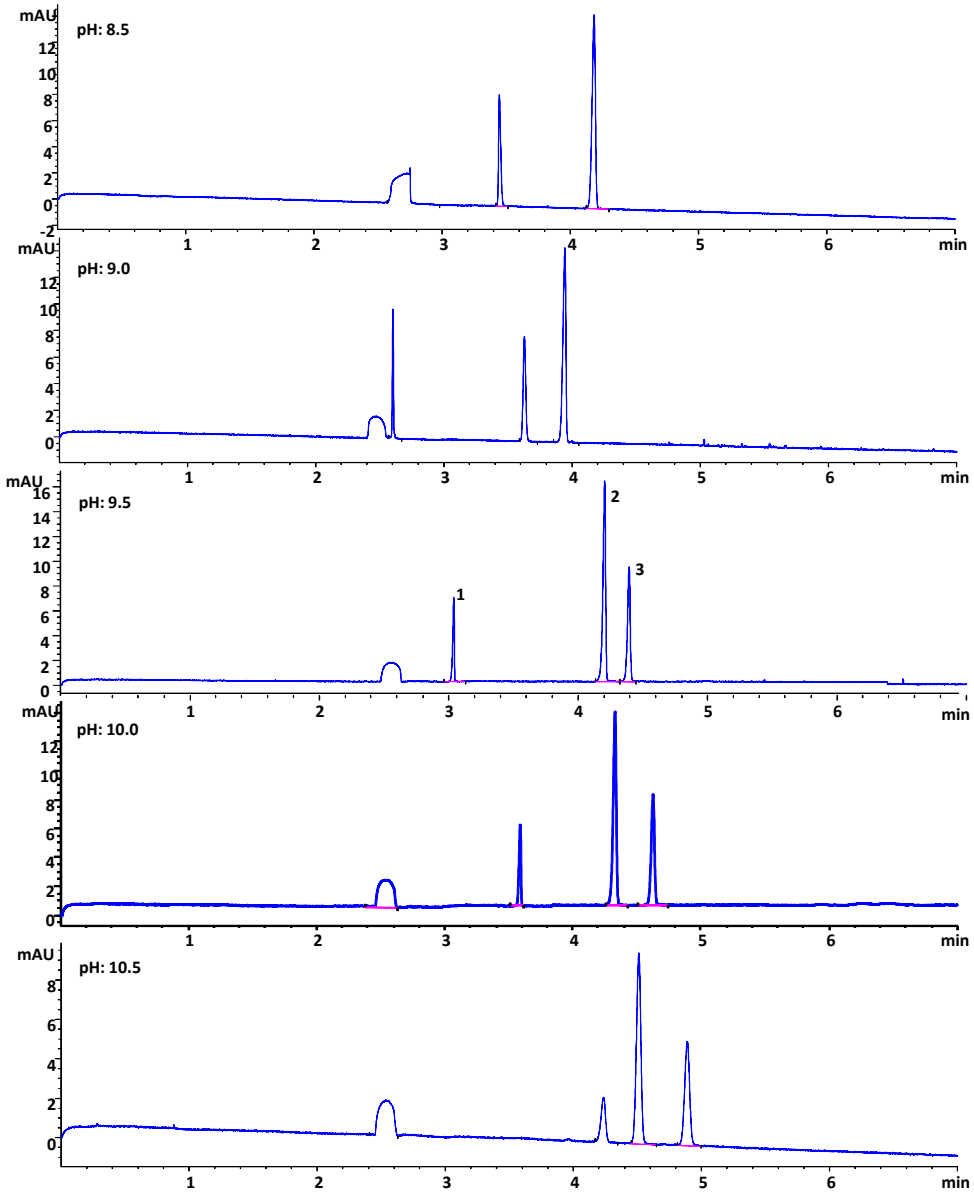

Figure I.3. Electropherograms corresponding to each tested pH value with borate buffer 30 mM. Identification peaks: 1, DK; 2, DA; 3, DADK

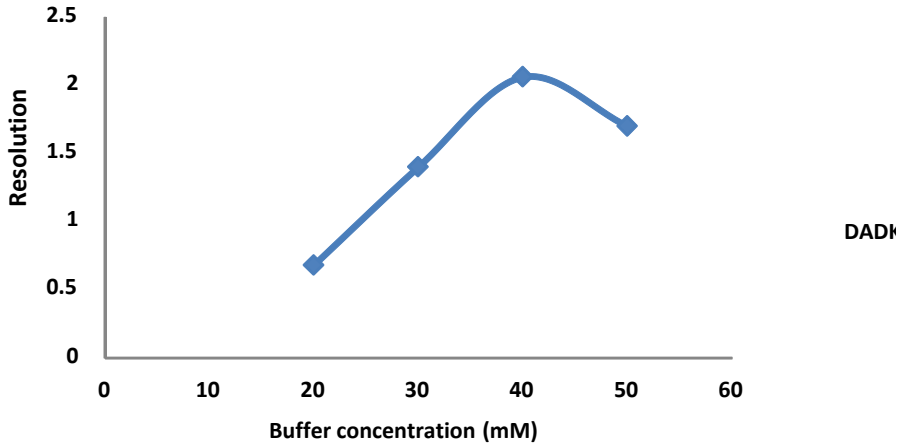

Figure I.4. Variation in the resolution between DA and DADK in relation to the increase of the buffer concentration.

Effect of the temperature. The effect of temperature was investigated in the range of 15-35°C. Higher temperatures were not considered as they increase the noise of the base line. A decrease in the temperature resulted in a decrease in the generated current, in a decrease in EOF, an increase in the migration times, due to the lower electrolyte viscosity and an increase in the resolution. According to these effects, 25°C was selected as suitable.

Optimization of the sample plug (or injection time). To determine the highest sample plug length (or sample volume) to be injected without degrading the separation profile by LVSS, different sample plugs were tested by varying the injection time between 100 s and 250 s, using a 50 mbar hydrodynamic injection. As we can see in figure I.5, the peak area of DA and DADK increase as the injection time increases until 200 s, but poor efficiencies and bad peak shapes were obtained for times longer than 200 s, so it was selected as optimum by applying a pressure of 50 mbar.

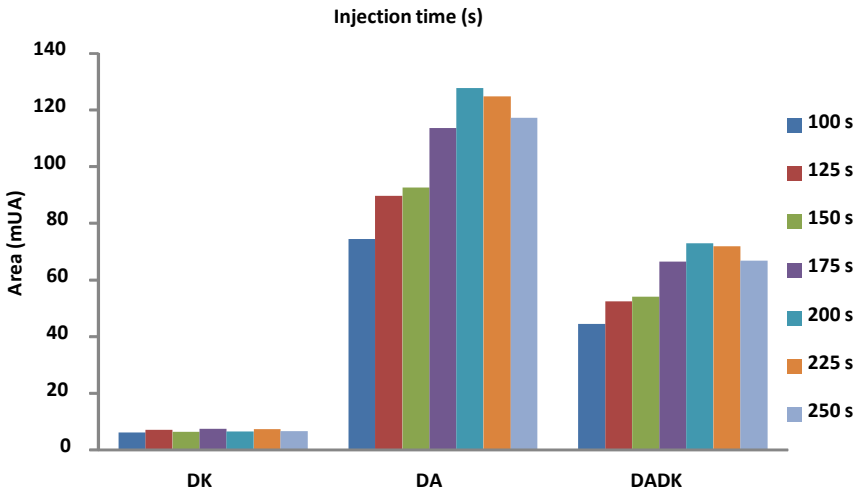

Figure I.5. Effect of the injection time on the analyte signal

Optimization of the current inversion value. In most publications the polarity switching in LVSS is recommended when the electric current reaches 95-99% of the initial current value. In principle, the higher the percentage is, the higher the amount of aqueous matrix is removed, and both, the electric field and the EOF, remain constant during the sample separation step, regardless of the initial sample volume. On the other hand, the current increases very slowly at the beginning of the sample stacking process but the increase rate becomes extremely high when the aqueous sample matrix is practically removed. Thus, a very long inversion time may result in a loss of part of the analytes, especially those with higher mobility [14]. For these reasons, different percentages of the initial current value (at which the polarity is switched to the "normal mode", which is positive at the inlet end) were investigated. Initially, the CE capillary was filled with the BGE and 15 kV were applied; when the current was stabilized, the obtained value was considered the "initial current". As it is shown in figure I.6, higher percentages than 80 % resulted in decreasing sensitivity (peak height) for DK, which shows higher electrophoretic mobility. Therefore, the current inversion value selected was 80% of the initial current value. A very important variable in this kind of methodology is the sample solvent because it requires a very low conductivity to obtain the best focusing of the analytes. In this case, deionized water was the best option for sample solvent,

[14] G. Álvarez-Llamas, A. Rodríguez-Cea, M.R. Fernández de la Campa, A. Sanz-Medel, Anal. Chim. Acta 486 (2003) 183

although 10% of MeOH is added in order to ensure the solubility of the compounds.

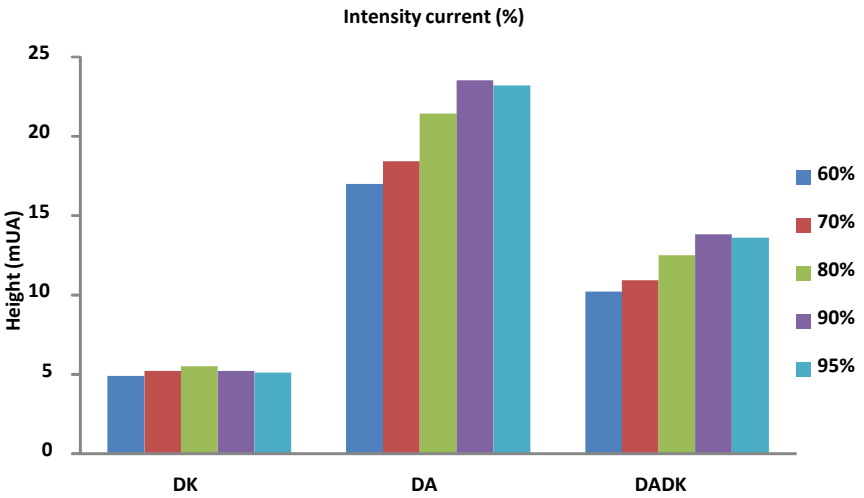

Figure I.6. Optimization of the polarity switching by means of the effect of the current inversion value (in terms of % initial intensity current) on the peak heights

As a summary, the optimized experimental conditions for CZE-LVSS procedure were: fused silica capillary 48.5 cm x 75 µm id, optical path length of 200 µm; separation buffer: sodium tetraborate, 40 mM pH 9.5; separation voltage: 15 kV; T: 25°C; hydrodynamic injection: pressure = 50 mbar, time = 200 s, current inversion limit: 80%; LVSS sample solution: MeOH:water (1:9 v/v); LVSS voltage: - 25 kV.

Figure I.7 shows the electropherogram of the major conversion products of metribuzin under optimized conditions. Comparing with the CE separation without applying any stacking procedure, sensitivity was significantly increased, obtaining preconcentration factors of 4, 36 and 28 for DK, DA and DADK, respectively.

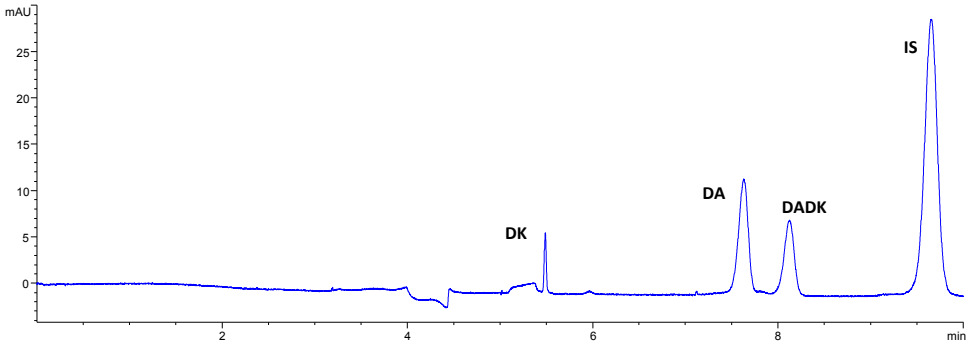

Figure I.7. Electropherogram of a solution containing 400 µg L^{-1} of DK, DA, DADK and 0.5 µg mL^{-1} of PABA as IS at optimum separation conditions

2.3. Optimization of the PLE procedure

For soil analysis, PLE is used as sample preparation technique, which combines elevated temperature and pressure with liquid solvents to achieve fast and efficient extraction of the analytes from a solid matrix [15]. The application of high temperatures implies a reduction in solvent viscosity, increasing the solvent's ability to wet the matrix and to solubilize the analytes. Temperature also assists in weakening and disrupting of strong interactions between analytes and matrix components, improving analyte diffusion to the matrix surface. Since the recent introduction of the first commercial PLE instruments, some applications have been developed based on the extraction of organic contaminants in soils or sediments [16-20] or in food and biological samples [21-24].

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In this study, PLE was selected as extraction technique for soil samples. PLE procedure consists in several steps: filling the cell containing the sample and the dispersing agent with solvent and heating until equilibrium; a static soaking of the sample in the heated solvent; flushing the sample with fresh solvent while collecting the first quantity of solvent and finally purging the sample with nitrogen forcing the remaining solvent out of the cell. When more than one extraction cycle is performed, the cell is flushed with a fraction of the volume and the static step is repeated with fresh solvent. In order to optimize the extraction procedure, the nature of the solvent, temperature, pressure, static time, flush volume, purging time, and number of cycles can all be varied [25].

First, the nature of the extraction solvent was evaluated: methanol, acetonitrile, ethyl acetate, acetone and water were tested; all of them as pure solvent and as 1:1 mixtures of these solvents with water. The extract obtained with pure methanol was the cleanest one and the recoveries were higher for the three compounds, so methanol was selected. Considering that the extraction temperature, static time and flush volume could present interactions among them, we applied the experimental design strategy to optimize simultaneously these variables obtaining the corresponding response surface. Purge time (60-180 s) and number of cycles (1 or 2) were examined in a univariate way. The experimental design applied was a Doehlert design plus three central points (17 runs). This kind of response surface design is scarcely used in analytical chemistry [26] in spite of the advantages such as its high efficiency compared with other designs like Box-Behnken or central composite designs, and because of the possibility to study a higher number of levels for the most significant variables. In our case, the temperature was studied at seven levels in the range between 35-145°C, the static time (5-30 min) at five levels and the flush volume (45-105 % of the total volume of the cell) at three. Using the relative corrected peak areas, the corresponding recoveries were estimated for each analyte, considering these recoveries as analytical responses to be optimized using the surface response methodology.

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Three replicates of the central point were considered to take into account the variability of the system. The total numbers of experiences in a Doehlert design is determined by the following equation: $N = 2^k + 2k + C_0$, where k is the number of variables and C_0 is the number of central points. The real values and the codified values, indicated in brackets, are shown in table I.2.

Table I.2. Matrix of the Doehlert design.

Experiment number	Time (min)	Temperature (°C)	Cell volume (%)
1	18 (0)	90 (0)	75 (0)
2	30 (1)	90 (0)	75 (0)
3	24 (0.5)	42 (0.866)	75 (0)
4	24 (0.5)	74 (0.289)	100 (0.817)
5	5 (-1)	90 (0)	75 (0)
6	11 (-0.5)	138 (-0.866)	75 (0)
7	11 (-0.5)	106 (-0.289)	50 (-0.817)
8	18 (0)	90 (0)	75 (0)
9	24 (0.5)	138(-0.866)	75 (0)
10	24 (0.5)	106 (-0.289)	50 (-0.817)
11	11 (-0.5)	42 (0.866)	75 (0)
12	18 (0)	58 (0.577)	50 (-0.817)
13	11 (-0.5)	74 (0.289)	100 (0.817)
14	18 (0)	122 (-0.577)	100 (0.817)
15	18 (0)	90 (0)	75 (0)

In brackets codified values

In order to reach the optimum values for the studied parameters for which the maximum responses are achieved simultaneously for the three studied degradation products, we applied the desirability function [27], the maximum of which permits the simultaneous optimization of the extraction conditions for each compound from soil samples. The desirability function obtained is shown in figure I.8. The lack of fit P-value for the model was 33% and the determination coefficient (R^2) was 83 %, indicating that the predicted model fitted well the experimental data.

[27] M.C. Ortiz, A. Herrero, S. Sanllorente, C. Reguera, Talanta, 65 (2004) 246

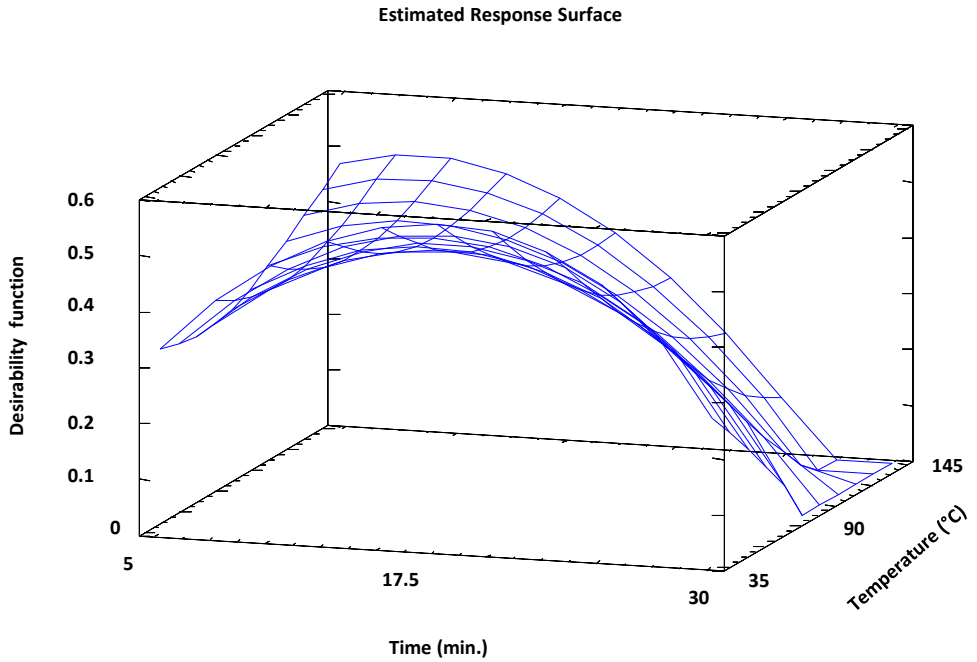

Figure I.8. Plot of Desirability function

Thus, the optimized conditions for the PLE procedure in soils using Doelert design are: T: 103°C; static time: 5 min; flush volume: 100 %.

In addition, a univariate optimization was carried out for selecting the numbers of extraction cycles, purge time and extractant agent volume. Although this last parameter was previously optimized in the Doelert design, the number of the extraction cycles is associated with the extractant volume, for this reason three different possibilities were checked:

- Extraction with: solvent cell volume 50% and 2 cycles.
- Extraction with: solvent cell volume 100% and 1 cycle.
- Extraction with: solvent cell volume 100% and 2 cycles.

Better results in terms of efficiency in the extraction were obtained increasing the number of the extraction cycles to 2 and the solvent cell volume to 100%.
The purge time is the time that a stream of nitrogen passes through the sample for keeping the sample dry thus ensuring that all the solvent is collected. The purge time was increased from 60 s considered initially to 100 and 180 s; finally 60 s was selected as optimum value, because this value improved the extraction yield

without increasing the extraction time. The summary of the optimum conditions of the PLE are shown in the table I.3.

Table I.3. Optimum conditions of the PLE with ASE 100.

Parameter	Optimum
Solvent extraction	Methanol
Temperature	103 °C
Static time	5 min
Solvent volume	100 %
Number of cycles	2
Preheat	3 min
Pressure	15000 psi
Purge time	60 s

Once the extraction was carried out by using PLE, a pre-concentration and clean-up procedure established previously by our research group for these analytes in soil samples was applied to the extracts [28], as it was described in section 2.6.2. For groundwater samples, the treatment applied was the same as for soil extracts, described in the same section.

2.4. Calibration curves and performance characteristics

The linearity of the response was established from six calibration levels corresponding to 25, 50, 100, 250, 500 and 750 μg L^{-1} of each compound. In all cases, 500 μg L^{-1} of PABA was added as IS. Each concentration level was injected by triplicate. Calibration curves were established by considering the relative corrected peak areas (as the ratio analyte peak per migration time to IS peak per migration time) as a function of the analyte standard concentration. The statistical parameters calculated from least-square regression and the performance characteristics are presented in table I.4. The satisfactory determination coefficients (R^2) and the P-value for the lack-of-fit test confirm that analyte responses were linear over the studied range.

[28] J.F. Huertas-Pérez, M. del Olmo-Iruela, A.M. García-Campaña, A. González-Casado, A. Sánchez-Navarro, J. Chromatogr. A, 1102 (2006) 280

In order to test the influence of the matrix of the selected samples (water and soil) we have established matrix-matched calibration curves using treated blank samples following the procedures above mentioned for water or soils but spiked before analysis with the same concentration level of analytes used in the standard solutions. By comparing statistically both curves, no significant differences were obtained from intercept and slopes. This comparison ensures that there are no significant systematic errors due to matrix effect, being possible to use directly the standard calibration curves for quantification purposes.

Table I.4. Statistical parameters and performance characteristics of the LVSS-SPE-CE proposed method.

	Calibration Equation[a]	R^2 (%)	Interday RSD (%)	Intraday RSD (%)	Lack of fit test (P-value)	LOD ($\mu g L^{-1}$)	LOQ ($\mu g L^{-1}$)	LDR ($\mu g L^{-1}$)
DK	A = -0.0031 + 0.0001 · C	97.0	5.1	3.2	10.7 %	20	67	67-750
DA	A = -0.0287 + 0.001 ·C	98.7	4.8	2.5	14.5%	10	32	32-750
DADK	A = -0.008+0.0006 ·C	99.3	4.0	1.0	11.8 %	10	33	33-750

[a] Obtained from four injections of the standard mixture solutions. A: area of the electrophoretic peak / migration time vs Internal Standard Area/migration time C: concentration of the analyte in the standard solution ($\mu g L^{-1}$).R^2: determination coefficient. RSD: Relative Standard Deviation for n = 5 and 250 $\mu g L^{-1}$ of each analyte. LDR: Linear Dynamic Range

Precision was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of repetitive measured to two samples (experimental replicates) at an intermediate concentration level (250 $\mu g L^{-1}$) and each one was injected by triplicate (instrumental replicates). Intermediate precision was assessed for five consecutive days with a similar procedure for repeatability studies. The results, expressed as relative standard deviation (RSD) of relative peak areas, are given in table I.4. As can be observed, acceptable precision was obtained in all cases.

Detection (LOD) and quantification (LOQ) limits have been calculated from the blank standard deviation following the methodology proposed by Muñoz Soto *et al.* [29]. It relies on studying the blank standard deviation in a time interval corresponding to the peak width at its base, extrapolated to zero concentration.

[29] G. Muñoz Soto, A. González Casado, L. Cuadros Rodríguez, E.J. Alonso Hernández, J.L. Vílchez Quero, J. Cap. Electrophoresis 4 (1997) 15

Thus, LOD and LOQ were calculated considering the analytical procedure without taking into account the previous treatment of the sample, and the results obtained are also summarized in table I.4. These obtained limits were improved in relation to those obtained in the previous MEKC method [28]. Also, considering that the applied SPE process for these analytes involves a pre-concentration step of 500-fold for the case of water samples (in which the expected concentrations are lower) and of 2.5-fold in the case of soil samples, the proposed method allows the detection of these compounds in the lower ng L^{-1} range in the case of water samples and at very low μg L^{-1} in the case of soils. In this sense, and taking into account these preconcentration factors, this method is among the most sensitive ones, and comparable with detection limits obtained using LC-diode array detection [30] or LC-MS for the analysis of soils [31] or water [1] samples.

2.5. Trueness of the method: Recovery studies

In order to check the trueness of the proposed method two different kind of samples, free of analytes, were used for recovery studies: A calcareous silt loam soil located in a fertile area, above one of the most important aquifers from Andalucía (Southeastern of Spain) with an organic matter content of 1.29% and groundwater samples from a farm located in the same area.

Samples of soil spiked with each one of the analyte solutions at three different concentration levels (100, 200 and 300 μg Kg^{-1}) were analyzed. Three replicates were prepared at each concentration level, and each one was injected in duplicate. Previously the samples were extracted following the above-described treatment and a sample blank was also analyzed in order to confirm that the analyzed soil samples were free of the conversion products of metribuzin.

Groundwater samples were spiked with a mixture of each one of the major conversion products of the metribuzin at three different levels (0.5, 2.0 and 3.5 ng mL^{-1}, of each one). Each level was prepared by triplicate and it was injected three times. Also, a sample blank was analyzed.

[30] E.N. Papadakis, E. Papadopoulou-Mourkidou, J. Chromatogr. A *962* (2002) 9
[31] T. Henriksen, B. Svensmark, R.K. Juhler, J. Chromatogr. A *957* (2002) 79

The recoveries were calculated by comparing the obtained concentrations for spiked samples at different concentrations levels, and calculated from the calibration curve established with pure standard solution of each analyte, with the true concentration value. The obtained values of the recovery for each compound and the corresponding RSD, are shown in table I.5 and table I.6 for soil and groundwater samples, respectively.

Table I.5. Recovery study using the LVSS-SPE-CE proposed method in soil samples using PLE.

Added (µg kg^{-1})	**DK**		**DA**		**DADK**	
	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
100	81	11	62	12	72	15
200	97	10	62	12	67	11
300	102	14	91	13	70	15

RSD, Relative standard deviation for n = 5

Table I.6. Recovery study using the LVSS-SPE-CE proposed method in groundwater samples.

Added (µg L^{-1})	**DK**		**DA**		**DADK**	
	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
0.5	103	5	111	6	98	6
2.0	85	6	93	7	97	7
3.5	95	5	117	7	99	7

RSD, Relative Standard deviation for n = 3

Figure I.9 Shows the electropherograms of a blank soil sample and a soil sample spiked with 200 µg kg^{-1} of each analyte, using the optimized method.

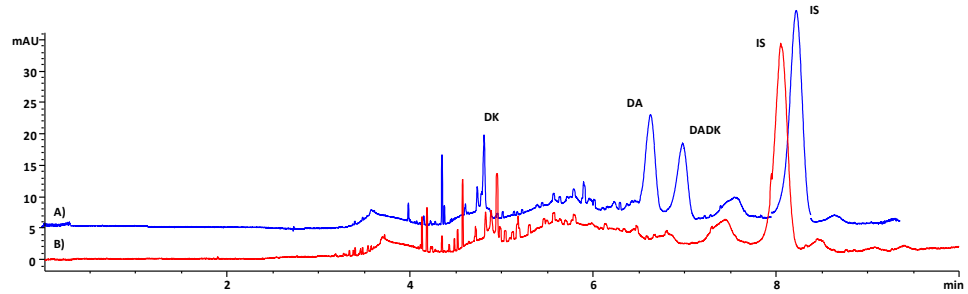

Figure I.9. Electropherograms of A) soil sample spiked with 200 µg kg^{-1} of DK, DA, DADK, and B) blank soil, in both cases, 0.5 µg mL^{-1} of PABA as IS.

Figure I.10 shows the electropherograms of a blank groundwater sample and a groundwater sample spiked with 2 ng mL^{-1} of each analyte, using the optimized LVSS at the selected CE experimental conditions.

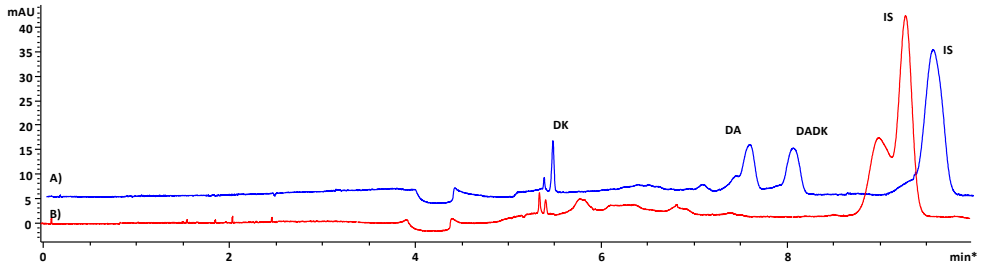

Figure I.10. Electropherograms of A) ground water sample spiked with 2 ng mL^{-1} of DK, DA, DADK and B) blank ground water, 0.5 µg mL^{-1} of PABA as internal standard in both cases.

3. Concluding remarks

A sensitive and rapid electrophoretic method has been developed and validated for the analysis of major degradation products of metribuzin, i.e. deaminometribuzin, deaminodiketometribuzin and diketometribuzin. For the first time, the characterization of the analytes was carried out calculating the dissociation acid constants, K_a, by CE. Previously to the CZE analysis, a PLE procedure was optimized and applied for extraction of the analytes in soil samples, followed by an off-line preconcentration and sample clean-up procedure by SPE. These last procedures are suitable for soil extracts obtained from the PLE and also directly for groundwater samples. For the first time to our knowledge, on-line preconcentration using LVSS has been carried out, improving the sensitivity of the

detection when it is combined with the off-line preconcentration step, and making the method suitable for the monitoring of these residues in environmental samples in the very low ng mL^{-1} and ng g^{-1} range, for waters and soils, respectively. The separation takes place in less than 9 min. This new combined method provides good recoveries, ranging from 62 to 102 % in the selected matrices.

This work was published as:

"Large volume sample stacking in capillary zone electrophoresis for the monitoring of the degradation products of metribuzin in environmental samples". Carolina Quesada-Molina, Ana M. García-Campaña, Monsalud del Olmo. J. Chromatogr. A, 1164 (2007) 320-328.

CAPÍTULO 2

Determinación de sulfonilureas a nivel de trazas en muestras de agua y uva, usando electroforesis capilar en zona con preconcentración en línea

CHAPTER 2

Trace determination of sulfonylurea herbicides in water and grape samples by capillary zone electrophoresis using large volume sample stacking

RESUMEN

Se ha desarrollado y validado un método de separación fiable y sensible usando electroforesis capilar en zona con detección UV con batería de diodos (CZE-DAD), para el análisis de trazas de herbicidas de la familia de las sulfonilureas (SUHs) en muestras de agua y uva de mesa de diverso origen. Los analitos incluidos son Tria-S, Rim-S, Flaza-S, Met-S y Clor-S. La separación óptima se llevó a cabo en un capilar burbuja de 48.5 cm x 50 μm (longitud efectiva 40 cm) usando acetato amónico 90 mM a pH 4.8 como tampón de separación, aplicando un voltaje de 20 KV y una temperatura de 25°C. El ácido p-aminobenzoico (PABA) se empleó como patrón interno. Para aumentar la sensibilidad del método se desarrolló un método de preconcentración de gran volumen de muestra en el propio capilar con polaridad inversa (*large volumen simple stacking,* LVSS). Para las muestras de agua se aplicó previamente un método de preconcentración y limpieza basado en la extracción en fase sólida (SPE) con cartuchos HLB. Para las muestras de uva, se utilizaron los cartuchos C18 después de la extracción asistida por ultrasonidos de los compuestos con MeOH:agua (1:1, v/v). Los límites de detección obtenidos para los compuestos estudiados se encontraron entre 0.04 y 0.12 μg L^{-1} para las muestras de agua y 0.97 y 8.30 μg Kg^{-1} para las muestras de uva, en todos los casos menores que los límites máximos de residuos permitidos por la Unión Europea (EU) en este tipo de alimentos. Se ha demostrado que la metodología desarrollada es adecuada para la determinación de estos residuos en aguas ambientales y uvas de mesa, con gran sensibilidad, precisión y buenas recuperaciones.

SUMMARY

A sensitive and reliable method using capillary zone electrophoresis with UV-diode array detection (CZE-DAD) has been developed and validated for trace determination of residues of sulfonylurea herbicides (SUHs) in environmental water samples and grapes from different origins. The analytes included are Tria-S, Rim-S, Flaza-S, Met-S and Chlor-S. Optimum separation has been achieved on a 48.5 cm x 50 μm (effective length 40 cm) bubble cell capillary using 90 mM ammonium acetate buffer, pH 4.8, by applying a voltage of 20 kV at 25°C and using p-aminobenzoic acid (PABA) as internal standard. In order to increase sensitivity, large volume sample stacking (LVSS) with polarity switching has been applied as on-line preconcentration methodology. For water samples a solid-phase extraction (SPE) procedure based on the use of Oasis HLB cartridges was applied for off-line preconcentration and cleanup. For grape samples, the SPE procedure was achieved with C18 sorbent, after extraction of the compounds with MeOH:water (1:1 v/v) by sonication. The limits of detection for the studied compounds were between 0.04-0.12 μg L^{-1} for water samples and 0.97-8.30 μg Kg^{-1} in the case of grape samples, lower in all cases than the maximum residue limits permitted by the European Union (EU) for this kind of food. The developed methodology has demonstrated its suitability for the monitoring of these residues in environmental water and grape samples with high sensitivity, precision and satisfactory recoveries.

1. Materials and methods

1.1. Chemicals and standard solutions

All the reagents used were of analytical grade and the solvents of HPLC grade. The water used to prepare the solutions was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Organic solvents such as acetonitrile, methanol, ethyl acetate and dichloromethane were purchased from Merck (Darmstadt, Germany).

The separation buffer was prepared from ammonium acetate supplied by Merck (Darmstadt, Germany). The pH was adjusted to 4.8 with acetic acid obtained from Merck.

Analytical standards of rimsulfuron (CAS: 122931-48-0; purity: 99%) and flazasulfuron (CAS: 104040-78-0; purity: 99.5%), were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Triasulfuron (CAS: 82097-50-5; purity: 97.3%, Pestanal), metsulfuron methyl (CAS: 74223-64-6; purity: 99.9%, Pestanal) and chlorsulfuron (CAS: 64902-72-3; purity: 99.8%, Pestanal) were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock standard solutions containing 1000 $mg \cdot L^{-1}$ of each compound were prepared by dissolving accurately weighed amounts in methanol and stored in darkness at 4°C. Working standard solutions were made daily by diluting them with deionised water. p-Aminobenzoic acid (PABA) was used as internal standard (IS) and was obtained from Fluka (St. Louis, MO, USA).

The sorbents used for SPE were Oasis HLB, copolymer of hydrophilic-lipophilic balance (60 mg, 3 mL) from Waters (Milford, MA, USA) and C18 sorbent (DSC-18, 6 mL, 500 mg) from Supelco (Bellefonte, PA, USA). Other phases, such as Lichrolut EN (200 mg, 3 mL) from Merck (Darmstadt, Germany), Strata X (500 mg, 6 mL) from Phenomenex (California, USA), Isolute ENV+ (50 mg, 3 mL) and Evolute ABN (25 mg, 1 mL) from Biotage (Uppsala, Sweden) and Supelclean ENVI-8 (50 mg, 3 mL) from Sigma-Aldrich (St. Louis, MO, USA) were checked.

1.2. Instrumentation and software

CE experiments were carried out with a HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the software provided with the HP ChemStation version A.09.01. Separation

was carried out in a bared fused silica capillary 48.5 cm x 50 μm i.d. (effective length 40 cm) with an optical path length of 200 μm (bubble cell capillary from Agilent Technologies, Waldbron, Germany). For pH measurements, a pH meter (Crison model pH 2000, Barcelona, Spain) was employed with a resolution of ± 0.01 pH unit.

A Bapitaurus food chopper (Taurus, Germany), vacuum manifold system from Supelco (Bellafonte, PA, USA) coupled with a vacuum pump (Büchi model B-169, Switzerland), nylon filters with a 0.20 μm pore size (Supelco, Bellafonte, PA, USA), and a centrifuge (Hettich Zentrifugen, Germany) were used for sample treatment.

Excel 2007 was used for the statistical analysis of data.

1.3. Electrophoretic procedure

Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min at 60°C, then with water for 5 min, and finally with the background electrolyte (BGE) solution for 20 min. A pressure of 1 bar was applied. At the beginning of each day, the capillary was rinse with an N_2 pressure of 7 bar for 4 min with 0.1 M NaOH, 2 min with water and 5 min with running buffer. In order to increase migration time reproducibility, after each run, the capillary was post-washed at 7 bar for 1 min with 0.1 M NaOH, 0.5 min with deionized water and 1 min with buffer. If drastic drifts in electrophoretic current and/or migration times were observed, the capillary was rinsed with an N_2 pressure of 7 bar for 1 min with deionized water followed by 2 min with methanol. At the end of each day, the capillary was rinsed with deionized water for 1 min and dried with air for 0.5 min.

Electrophoretic separation was performed using a 90 mM ammonium acetate buffer, pH 4.8 adjusted with acetic acid, by applying a voltage of 20 kV (normal mode). All the solutions used during the electrophoretic procedure were filtered through a 0.20 μm membrane filter before use. Tria-S was monitored at 226 nm with bandwidth of 20 nm, and the other SUHs were monitored at 240 nm with a bandwidth of 50 nm. The temperature of the capillary was kept constant at 25 °C. Injection of the sample occurred using the following LVSS procedure.

1.4. LVSS procedure

Samples containing the analytes were loaded with a pressure of 7 bar for 1 min. In this way, the whole capillary column was filled with the sample solution. MeOH:water (1:9 v/v) was used as the sample solvent to produce a low-conductivity analyte matrix. After sample injection, a negative voltage of –25 kV was applied and the sample stacking started. Sample matrix removal from the capillary was indicated by monitoring the electric current, which progressively increased to its normal value as the low-conductivity injected zone was eliminated from the capillary. At this stage the stacking process could be considered complete. The applied voltage was then switched from negative to a positive value of 20 kV in order to separate the compounds.

1.5. Sample preparation procedures

1.5.1. Water samples

Two kinds of water samples were analyzed: groundwater and spring water from Sierra Arana, Granada (Southeastern of Spain). The water samples were collected directly in PVC bottles; they were filtered through nylon membranes with 0.2 µm pore size and were stored at 4°C in the dark. SPE was carried out on Oasis HLB cartridges pre-conditioned with 5 mL of methanol and 5 mL of deionized water, consecutively. Portions of 100 mL of water samples were spiked with different concentration levels of standard solutions of the analytes. The water samples were loaded through the cartridge at the rate of 2 mL min^{-1} by using a vacuum operated pumping system. After sample loading, the cartridges were washed with 5 mL of water:MeOH (97:3, v/v) and air-dried for 15 min. The elution of solutes was achieved with 4 mL of methanol. The eluates were brought to dryness under a gentle nitrogen current at 30°C. The dry residue was dissolved in 500 µL of MeOH:water (1:9 v/v) containing PABA (500 µg L^{-1}) as IS. These solutions were filtered through 0.22 µm nylon membrane and injected into the CE system for analysis.

1.5.2. Grape samples

Grape samples were purchased from a local market. Three kinds of grape were analyzed: Victoria variety, Red-globe variety and Sugraone variety. A representative portion of sample (250 g of whole fruit) was chopped and homogenized before fortification by adding the appropriate volume of the working standard solutions. Then, a portion of 5 g of grape sample was extracted with 10 mL of MeOH:water (1:1, v/v) in an ultrasound bath, during 15 min. After that, the extract was centrifuged (7000 rpm, 5 min), the supernatant liquid was transferred to a volumetric flask, and 5 mL of deionized water was added and centrifuge again (7000 rpm, 5 min). Finally, the extracts were brought up to 250 mL with deionized water. SPE was carried out on C18 cartridges pre-conditioned with 6 mL of methanol and 6 mL of deionized water, consecutively. The grape extracts were loaded through the cartridge at the rate of 2 mL min^{-1} by using a vacuum operated pumping system. After sample loading, the cartridges were washed with 5 mL of water and air-dried for 15 min. The elution of solutes was achieved with 10 mL of dichloromethane. The eluates were brought to dryness under a gentle nitrogen current at 30°C. The dry residue was dissolved in 500 µL of MeOH:water (1:9, v/v) containing PABA (500 µg L^{-1}) as IS. These solutions were injected into the CE system for analysis.

2. Results and discussion

2.1. Optimization of the CE separation by applying LVSS with polarity switching procedure

The poor sensitivity of CZE using UV-Vis detection hardly recommends the direct use of this technique because the LODs obtained could not reach the desired levels in pesticides analysis. In order to avoid this aspect we propose the use of an on-line sample stacking procedure as a way of trace enrichment in CE for the analysis of urea-derived pesticides in water and grape samples. LVSS with polarity switching for anions was chosen as the preconcentration technique because the buffer conditions and polarity allowed its use, considering that all the molecules are negatively charged at the working pH. In starting situation, a large volume of sample prepared in a low conductivity matrix is injected, and a voltage at negative polarity is applied for focusing of zones and removal of sample matrix. When the anions are completely focused and most of the sample matrix is removed, voltage

is stopped and polarity is reversed. This occurs when the current reached 95-99 % of its value. Finally a voltage at positive polarity is applied in order to separate and detect the focused zones.

Firstly the effect of pH value was investigated over the range 2.5-10, varying each 0.5 units and using 50 mM phosphate buffer. The best resolution was achieved at a pH of 4.5. Subsequently, we checked the buffer nature at this pH using solutions that present a higher buffering capacity: ammonium acetate, sodium phthalate and sodium citrate, at a concentration of 50 mM. We found better resolution with ammonium acetate for most of the analytes; consequently it was selected as BGE. Then, we checked a reduced pH range between 5.0-4.2 (step 0.2 U), finding an optimum pH value at 4.8 since it provided the best peak resolution in very short analysis time, as it is shown in figure II.1.

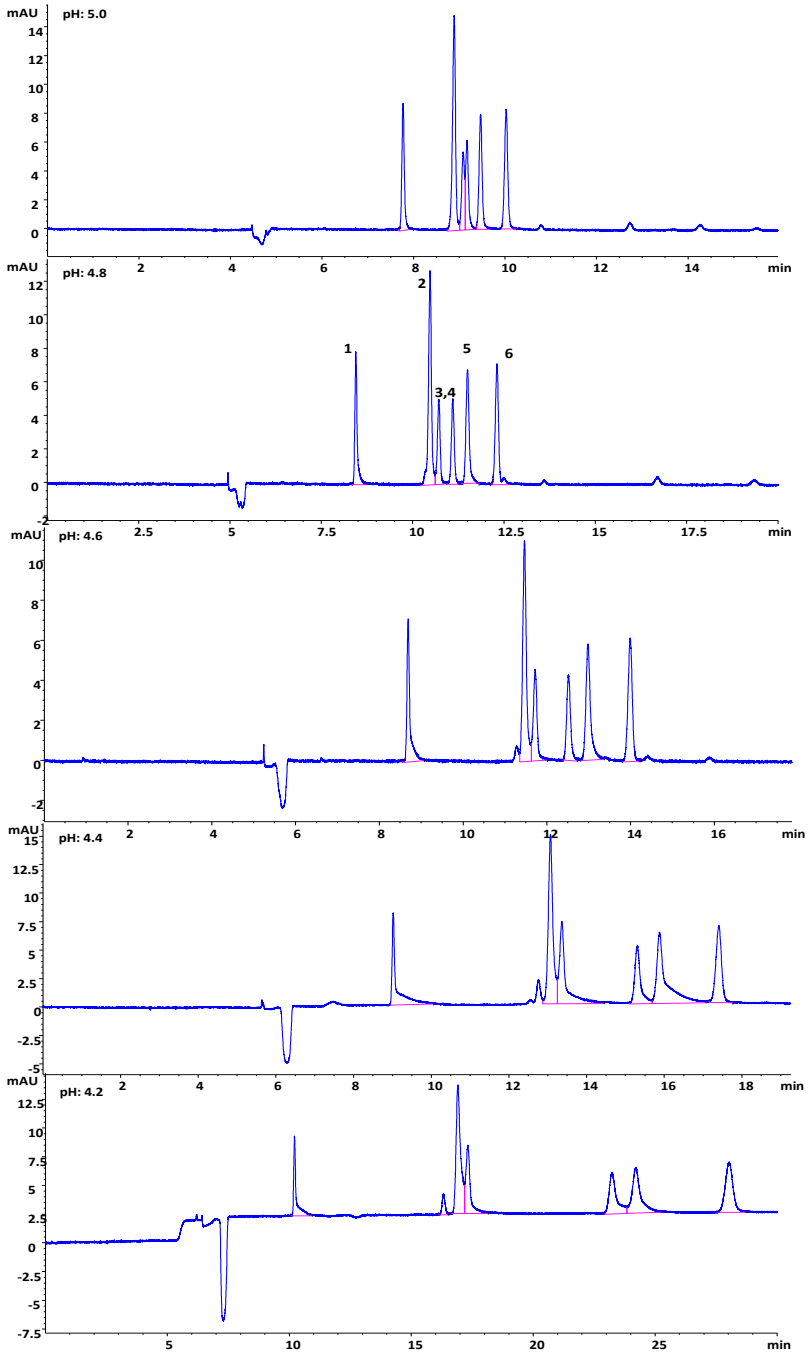

Figure II.1. Electropherograms obtained with ammonium acetate 50 mM as BGE and different pH values. Identification peaks: 1:Tria-S; 2: Rim-S; 3:Flaza-S; 4: Primi-S; 5: Met-S; 6: Chlor-S.

Different buffer concentrations were tested (50, 60, 70, 80, 90, and 100 mM), selecting 90 mM as optimum concentration because, as figure II.2 shows, at this concentration the peak area is very high in general, mainly for Tria-S and Flaza-S, which are less sensitive, besides to obtain the best resolution with an adequate electric current.

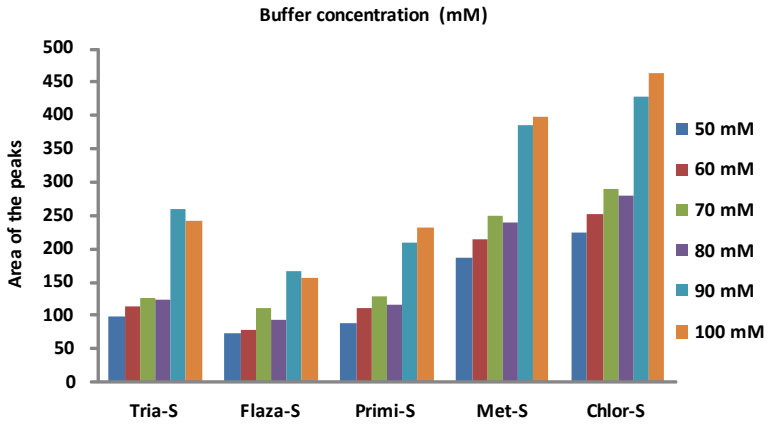

Figure II.2. Influence of the buffer concentration on the peak area for the studied SUHs.

The addition of different percentages of organic solvent (5-10%) was also assessed in order to improve resolution, by using methanol, acetone and acetonitrile, but it didn't provide satisfactory results. The applied voltage for the separation was also studied at different values: 10, 15, 20, and 25 kV; the optimum value found was 20 kV.

The effect of temperature was also investigated in the range of 15-35°C. Higher temperature values were not considered as they increase the noise of the base line. A decrease in the temperature resulted in a decrease in the generated current, a decrease in the EOF, an increase in the migration times due to the lower electrolyte viscosity and an increase in the resolution. According to these effects, 25°C was selected as a compromise.

In LVSS, three significant parameters have to be considered: sample solvent, size of the plug sample, and voltage. The most relevant variable in this type of preconcentration methodology is the sample solvent, because it requires a very low conductivity to obtain the best focusing of the analytes. In this case, deionized water with 10% of MeOH was the best option for sample solvent, in order to ensure the solubility of the compounds. To optimize the size of plug sample,

different percentages (50, 70, 90 and 100%) of the capillary were filled in the experience. These percentages were calculated, based on the diameter and length of the capillary and applying a pressure. The results showed that it was possible to fill the whole volume of the capillary applying a pressure of 7 bar during 1 min. The negative voltage was studied in the interval of -15 to -30 kV. Finally, -25 kV was selected as optimum, as this value permits a rapid filling of the capillary in a stable system. Values higher than -25 kV frequently produce the capillary rupture.

As a summary, the experimental conditions used for CE-LVSS procedure were: fused silica capillary 48.5 cm x 50 µm id, optical path length of 200 µm; separation buffer: ammonium acetate 90 mM at pH 4.8; separation voltage: 20 kV; T: 25°C; hydrodynamic injection: pressure = 7 bar, time = 1 min, current inversion limit: 95%; LVSS solution: MeOH:water (1:9, v/v); LVSS voltage: - 25 kV.

2.2. Optimization of sample treatment

2.2.1. Water samples

The value accepted by the EU for individual herbicide residues with the aim to ensure the quality of water intended for human consumption has not to surpass 0.1 µg L^{-1} [1], consequently a preconcentration step prior to CE determination is mandatory in order to reach sensitivity levels below these limits, and also to quantified these SUHs in waters, where they are suspected to be at trace levels.To examine the feasibility of the SPE procedure for preconcentration and clean up, different sorbent nature were tested, such as the following polymeric sorbents: Lichrolut EN (ethylvinylbenzene-divinylbenzene), Strata X (surface modified styrene divinylbenzene), Oasis HLB, or C18. Once the sample (100 mL) was passed through the different cartridges, we observed that Lichrolut EN did not retain the analytes and HLB provided the best results in terms of extraction efficiency (recoveries) and clean up of the obtained extracts, as it is shown in figure II.3.

[1] Council Directive 98/83/EC on the quality of water intended for human consumption. Official Journal of the European Communities, 3 November 1998, L330/32.

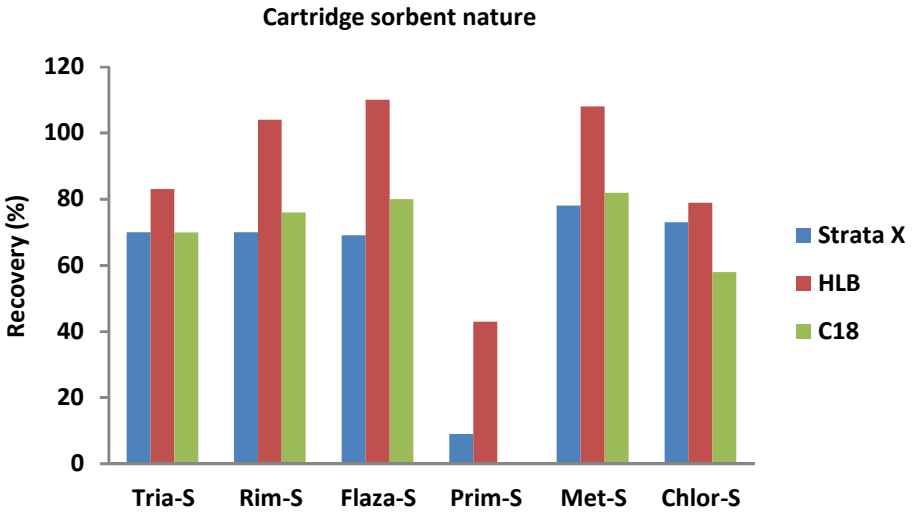

Figure II.3. Obtained recoveries for the analytes in each sorbent used in the SPE procedure in water samples.

For the clean up step, different percentages of methanol in a volume of 5 mL with water were tested (0, 1, 2, 3, 4, and 5 %) as washing solvent. The best recoveries were obtained when using 2% and 3% of methanol in water. Although there are no significant losses of analytes even when using 5% of methanol, we selected as optimum washing step water:MeOH (97:3, v/v) because no peaks of interferents were obtained in the electropherograms comigrating with the analytes. Methanol and acetonitrile were checked as elution solvents and 4 mL of MeOH provided the best recoveries of the compounds without eluting large quantities of observable interferences. Figures II.4.A and II.4.B show the optimization of the clean up and elution steps in relation to the recovery percentages for each compound.

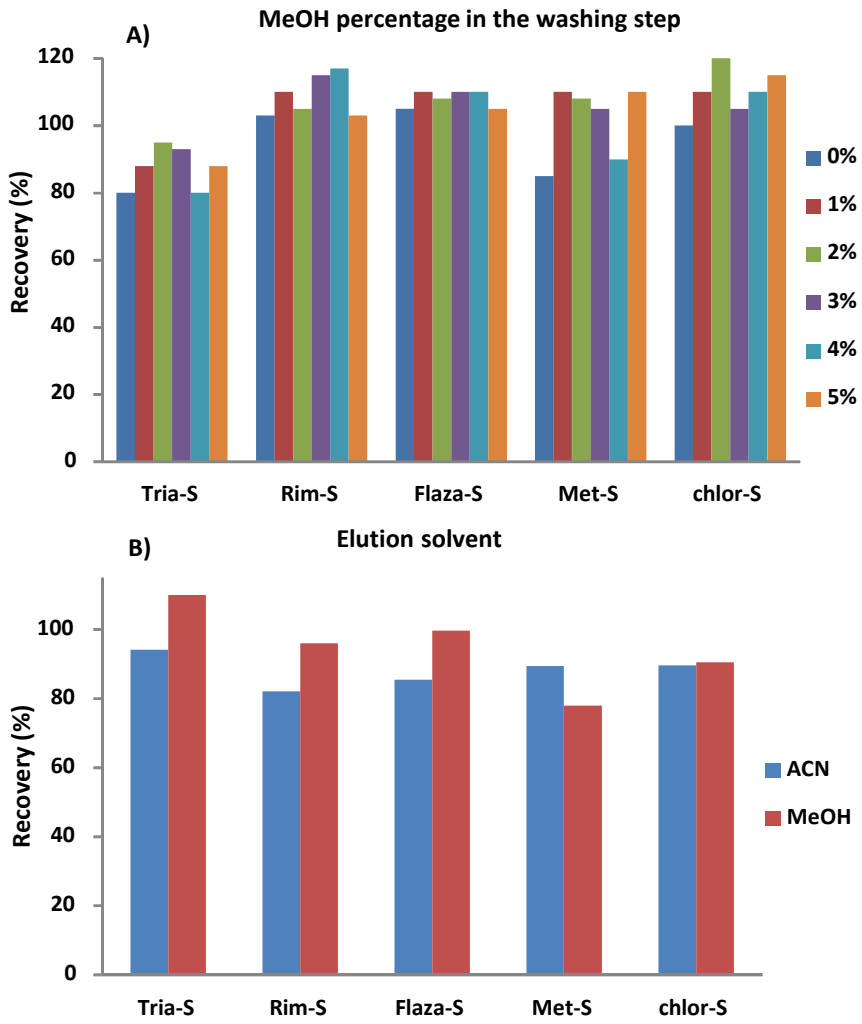

Figure II.4. Study of the SPE procedure. A): Recoveries of the SUHs in relation to the MeOH percentage used in water in the washing step (volume of washing solution 5 mL). B): Recoveries of the SUHs in relation to the kind of elution solvent (volume of the elution solvent 4 mL).

This procedure showed good results in term of recoveries and peak shapes, and a preconcentration factor of 200 was achieved; therefore it was adopted for the validation of the optimized method in water samples. Figure II.5.A shows an electropherogram of a blank of groundwater treated with the developed procedure, and no matrix peaks were found co-migrating with the analytes. Figure II.5.B provides the electropherogram of a groundwater sample spiked with 1 µg L^{-1} of each compound.

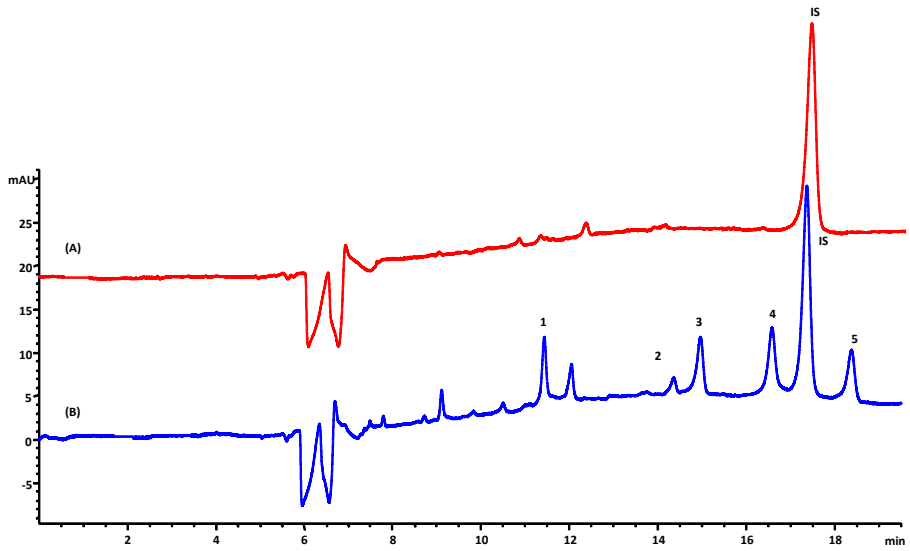

Figure II.5. Electropherogram of A) groundwater blank and B) extract of water fortified with 1 µg L^{-1}. Peak identification: 1: Tria-S; 2: Rim-S; 3: Flaza-S; 4: Met-S; 5: Chlor-S; IS: Internal standard, PABA 500 µg L^{-1}.

2.2.2. Grape samples

On account of the very polar properties of the SUHs and the endogenous compounds of the grape matrix, we optimized the SPE procedure using the Victoria variety as representative matrix. C8, C18, Oasis HLB and other non-polar phases, such as Isolute ENV+ (hydroxylated polystyrene divinylbencene resin), Evolute ABN (surface modified polystyrene divinylbencene) and Supelclean ENVI-8 were tested as solid phases for clean up and preconcentration of the samples. C18 gave the best results in terms of extraction efficiency and the cleaning of the extracts. Methanol, ethyl acetate and dichloromethane were tested as elution solvents. Elution with methanol extracted a high amount of endogenous compounds, which produced several interferent peaks, and the quantification of the compounds was impossible. Ethyl acetate and dichloromethane eluted the SUHs without eluting large quantities of interferences. A volume of 10 mL of dichloromethane was finally chosen because it gave the highest recoveries. Figure II.6 shows the obtained recoveries with different elution solvents.

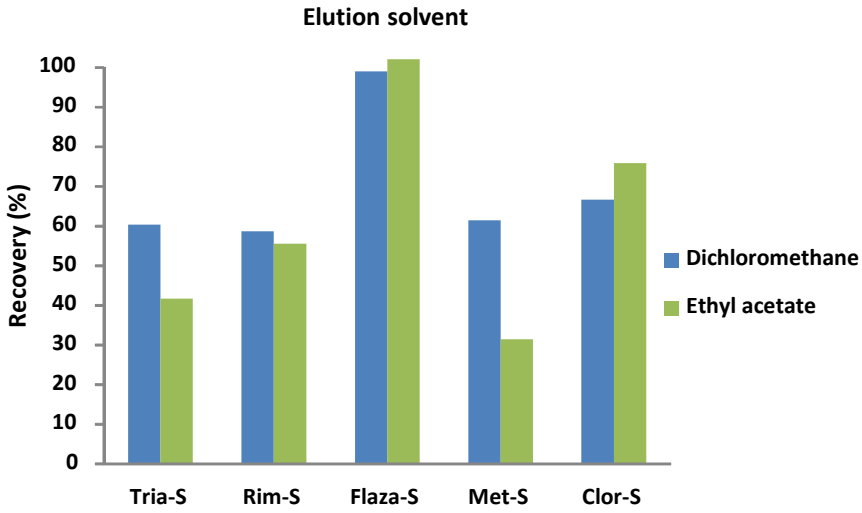

Figure II.6. Recoveries for the studied SUHs in grape sample when using different elution solvents (elution volume: 10 mL) in the SPE procedure.

This procedure was adopted for the validation of the optimized method in grape samples. Figure II.7.A shows an electropherogram of a blank of grape sample. No SUHs were found. Figure II.7.B provides the profile for a grape sample spiked with 100 µg Kg^{-1} of each studied SUH.

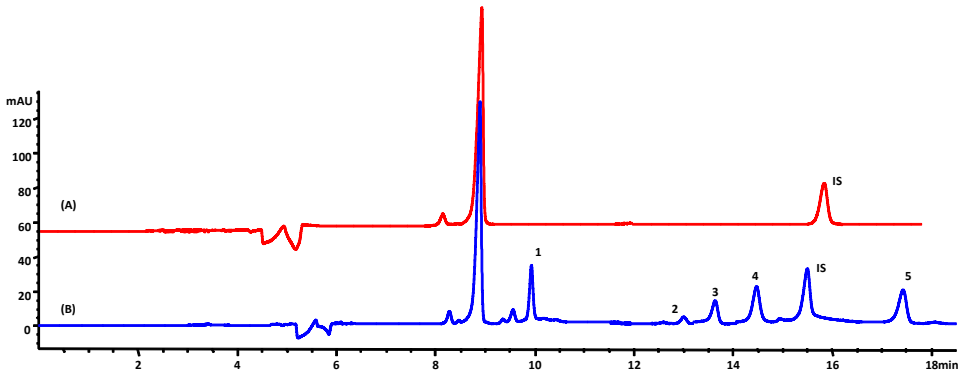

Figure II.7. Electropherogram of (A) blank of grape sample and (B) fortified grape sample with 100 µg Kg^{-1} of each compound. Peak identification: 1: Tria-S; 2: Rim-S; 3: Flaza-S; 4: Met-S; 5: Chlor-S; IS: Internal standard, PABA 500 µg Kg^{-1}.

2.3. Validation of the LVSS-CZE-DAD procedure

2.3.1. Statistical and performance characteristics

The calibration curves were established for the five analytes in each kind of matrix, water and grape samples, treated according to the sample procedure described above. The selected matrices for establishing the matrix-matched calibration curves were groundwater and Victoria grape variety, spiked before the sample treatment.

The calibration curves were established for six different concentration levels corresponding to 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 μg L^{-1} for water matrix and 25, 50, 100, 200, 250 μg Kg^{-1} for grape matrix. In all cases 500 μg L^{-1} of PABA was added as IS. Each concentration level was injected by triplicate. Calibration curves were established taking into account the relative corrected peak areas (as the ratio analyte peak per migration time to IS peak per migration time) as a function of the analyte standard concentration. The statistical parameters, calculated by linear regression and the performance characteristics of the SPE-LVSS-CZE method are shown in table II.1. In all cases, the satisfactory determination coefficients obtained confirmed that SUH responses were linear over the concentration range studied. The LOD was considered as the minimum analyte concentration yielding an S/N ratio equal to three. The LOQ was adopted as the lowest analyte concentration yielding a signal 10 times greater than the noise. As can be seen, for all the studied compounds the LODs obtained by applying both off-line and on-line preconcentration procedures are at the low μg L^{-1} levels, and are below to the MRLs regulated in the EU Directive for this kind of fruits [2] and also allow the monitoring of these herbicides in drinking waters [1]. In this sense, the methodology developed in this work is fitted for the purpose of monitoring these compounds in grapes and waters.

[2] EC council directive 91/414/EEC, Regulation (EC) No 396/2005.

Table II.1. Statistical and performance characteristics for the five herbicides investigated in each kind of sample.

	Groundwater sample				
	Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Chlor-S**
Intercept	0.0036	0.0049	0.0062	0.0262	- 0.0006
Slope	0.2422	0.0652	0.2457	0.3121	0.215
R^2	99.3%	98.4%	99.1%	98.1%	99.2%
LOD (µg L^{-1})	0.046	0.116	0.045	0.045	0.057
LOQ (µg L^{-1})	0.160	380	0.150	0.150	0.190
LDR (µg L^{-1})	0.046-2.5	0.116-2.5	0.045-2.5	0.045-2.5	0.057-2.5
	Victoria grape sample				
	Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Chlor-S**
Intercept	0.0837	0.009	0.015	0.0077	0.0533
Slope	0.0097	0.001	0.0058	0.0153	0.0071
R^2	98.9%	98.2%	97.4%	98.7%	98.9%
LOD (µg Kg^{-1})	0.97	8.3	2.3	1.4	1.9
LOQ (µg Kg^{-1})	3.24	27.8	7.7	4.5	6.4
LDR (µg Kg^{-1})	0.97-200	8.3-200	2.3-200	1.4-200	1.9-200
MRLs (µg Kg^{-1})	50	50	20	50	50

MRL: Maximum residue limit. Regulation (EC) No 396/2005; LDR: Linear dynamic range

2.3.2. Precision study

The precision of the whole method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of repetitive application of the SPE-LVSS-CZE procedure to both kind of samples (experimental replicates) at concentration levels of 50 and 100 µg Kg^{-1} for grape samples, and 0.25, 1.5 and 2.5 µg L^{-1} for water samples, and each one was injected in triplicate (instrumental replicates). Intermediate precision was assessed for three consecutive days with a similar procedure as for repeatability studies. The results related to concentrations and migrations times, expressed as relative standard deviation (RSD) of relative peak areas and migration times, are given in table II.2. As can be observed, acceptable precision was obtained in all cases.

Table II.2. Precision study of the proposed method for different concentration levels and migration times.

Victoria grape sample					
	Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Clor-S**
Interday RSD (%) n=9					
50 μg kg^{-1}	21.4	12.4	17.8	19.7	14.2
100 μg kg^{-1}	19.2	18.0	13.0	17.9	16.8
Migration time (min)	10.4	13.3	14.2	16.1	18.1
RSD (%)	9.2	11.1	11.7	11.9	12.5
Intraday RSD (%) n=6					
50 μg kg^{-1}	5.7	10.0	6.2	11.7	7.6
100 μg kg^{-1}	10.7	14.9	13.5	17.9	10.4
Migration time (min)	10.8	14.2	14.9	15.8	18.8
RSD (%)	5.2	6.1	6.3	7.0	7.0
Groundwater sample					
	Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Clor-S**
Interday RSD % (n=9)					
0.25 μg L^{-1}	3.6	1.8	2.6	4.3	2.3
1.5 μg L^{-1}	6.3	9.1	5.4	4.1	7.6
2.5 μg L^{-1}	6.1	8.4	7.3	5.5	5.4
Migration time (min)	3.1	8.2	9.9	10.8	12.0
RSD (%)	2.8	6.5	8.1	9.2	9.7
Intraday RSD (%) (n=6)					
0.25 μg L^{-1}	10.7	7.6	7.9	12.9	2.4
1.5 μg L^{-1}	5.0	4.0	3.8	8.8	1.9
2.5 μg L^{-1}	1.7	8.8	2.6	1.9	1.5
Migration time (min)	2.8	6.8	8.0	8.5	9.2
RSD (%)	0.7	1.1	1.3	1.4	1.5

2.4. Trueness of the method: Recovery studies

In order to check the trueness and applicability of the proposed methodology for the analysis of SUHs, recovery experiments were carried out in two different analyte-free kinds of water samples and three varieties of grape samples. Grape samples spiked with each one of the analytes at two different concentration levels,

(50 and 100 µg kg^{-1}) were analyzed. Three replicates were prepared at each concentration level, and each one was injected in duplicate. Previously the samples were extracted following the above-described treatment and a sample blank was also analyzed in order to confirm that the analyzed samples were free of the SUHs. The identification of the compounds was based on both their migration times and the absorption spectra. The recoveries for spiked grape samples at different concentration levels were obtained from the calibration curves established with fortified grape samples. The obtained values of the recovery study in grape samples for each compound and the corresponding RSD (%) are shown in table II.3.

Table II.3. Recoveries of SUHs from grape samples spiked at different concentration levels.

		Sugraone grape variety				
		Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Chlor-S**
50 µg kg^{-1}	R (%)	119.8	132.8	116.5	98.0	111.5
	RSD (%)	10.5	10.8	3.3	7.0	20.6
100 µg kg^{-1}	R (%)	145.7	115.3	96.1	114.3	126.9
	RSD (%)	12.1	2.1	6.4	8.6	9.6
		Redglobe grape variety				
		Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Chlor-S**
50 µg kg^{-1}	R (%)	136.7	87.3	82.4	75.4	100.9
	RSD (%)	10.8	16.2	4.9	15.6	13.4
100 µg kg^{-1}	R (%)	161.1	117.6	82.0	87.0	107.4
	RSD (%)	5.6	12.9	4.0	2.9	2.9
		Victoria grape variety				
		Tria-S	**Rim-S**	**Flaza-S**	**Met-S**	**Chlor-S**
50 µg kg^{-1}	R (%)	78.5	130.9	82.5	88.1	81.5
	RSD (%)	6.7	11.9	6.7	10.1	4.0
100 µg kg^{-1}	R (%)	82.0	125.5	72.2	99.3	87.6
	RSD (%)	11.1	5.4	16.9	18.4	13.3

RSD, Relative standard deviation (n=6)

Ground and spring water samples were spiked with a mixture of each one of the analytes at three different levels (0.25, 0.50 and 1.0 µg L^{-1}). Each level was prepared by triplicate and it was injected three times. In order to check possible interferents, blank samples were submitted to the proposed method and no matrix peaks were found co-migrating with the analytes. The obtained values of

the recovery study for each compound and the corresponding RSD (%) are shown in table II.4 for water samples.

Table II.4. Recoveries of SUHs from water samples spiked at different concentration levels.

		Tria-S	Rim-S	Flaza-S	Met-S	Chlor-S
Groundwater samples						
0.5 µg L^{-1}	R (%)	92.7	77.2	84.7	108.9	117.9
	RSD (%)	14.9	10.6	7.5	23	20.5
1.0 µg L^{-1}	R (%)	123.4	117.8	106.5	134.5	133.2
	RSD (%)	10.9	17.8	11.6	9.8	15.8
2.0 µg L^{-1}	R (%)	109.6	106.9	98.6	116.8	115.0
	RSD (%)	6.8	9.4	2.2	2.6	2.3
Spring water samples						
		Tria-S	Rim-S	Flaza-S	Met-S	Chlor-S
0.5 µg L^{-1}	R (%)	60.9	96.2	79.1	104.2	105.8
	RSD (%)	6.4	23.1	12.7	16.8	13.1
1.0 µg L^{-1}	R (%)	76.2	126.6	111.2	101.9	138.7
	RSD (%)	1.3	31.0	3.9	2.3	4.7
2.0 µg L^{-1}	R (%)	59.0	109.1	98.5	119.4	123.2
	RSD (%)	4.9	5.6	3.3	9.1	3.1

RSD, Relative standard deviation (n=9)

The proposed method provides satisfactory results in terms of both, trueness and precision, so method accuracy for the analysis of these kinds of matrices was demonstrated.

3. Concluding remarks

A sensitive and rapid electrophoretic method has been developed and validated for the analysis of five SUHs, i.e. triasulfuron, rimsulfuron, flazasulfuron, metsulfuron methyl, and chlorsulfuron. Prior to the CE analysis, the method involves the extraction of the analytes from grape samples followed by an off-line

preconcentration and sample clean-up procedure of the obtained extracts, which was accomplished by SPE. In the case of water samples, only SPE is necessary for preconcentration and sample clean up. On-line preconcentration using LVSS has been carried out, improving the sensitivity of the detection when it is combined with the off-line preconcentration step, and making the method suitable for the monitoring of these SUHs in water and grape samples in the very low ng L^{-1} and ng g^{-1} ranges, respectively. The separation takes place in less than 19 min. This new combined method provides satisfactory recoveries in the selected matrices: groundwater (77-134%), spring water (59-139%), Sugraone grape variety (96-146%), Redglobe grape variety (75-161%) and Victoria variety (72-131%). The LODs obtained are low enough for detecting these compounds below the legislated MRLs established in grape samples by the EU, being among the fastest and sensitive methods for the simultaneous determination of these SUH herbicides in these kinds of samples.

This work was published as:

"Trace determination of sulfonylurea herbicides in water and grape samples by capillary zone electrophoresis using large volume sample stacking". Carolina Quesada-Molina, Monsalud del Olmo, Ana M. García-Campaña. Anal. Bioanal. Chem., 397 (2010) 2593-2601.

Part II:

Determination of residues of cephalosporins

1. Characteristics of β-lactam antibiotics

The term antibiotic refers to a very diverse range of chemical substances produced from bacteria or fungi in a natural way or in a semisynthetic or synthetic way that possess antibacterial activity, by killing or inhibiting the growth of microorganisms. They are used in human and animal medicine to prevent and treat diseases. Among the different types, β-lactam antibiotics have a long history in the treatment of infectious diseases, though their use was and continues to be confounded by the development of resistance in target organisms. For nearly six decades, penicillins have been widely used to treat bacterial infections. The development of multidrug resistance, however, has reduced the effectiveness of β-lactams and antimicrobial drugs.

β-lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. While traditionally β-lactam antibiotics were mainly active only against Gram-positive bacteria, the development of broad-spectrum β-lactam antibiotics active against various Gram-negative organisms has increased their usefulness [1].

They are bactericidal, and act by inhibiting the growth of sensitive bacteria by inactivating enzymes located in the bacterial cell membrane, being involved in the third stage of cell wall synthesis, the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin binding proteins.

2. Classification of β-lactam antibiotics

This type of antibiotics can be classified into several groups according to their structural characteristics, but their unique structural feature is the presence of the four-membered β-lactam (2-azetidinone) ring. They include: penicillins,

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[1] adapted from Chemeurope web page, accessed in March 2013: http://www.chemeurope.com/en/encyclopedia/Beta-lactam_antibiotic.html#Mode_of_action

cephalosporins, and more recently, carbapenems and monobactams (figure 1). This group also includes β-lactamase inhibitors and they have no general structure, for this reason they are excluded in figure 1.

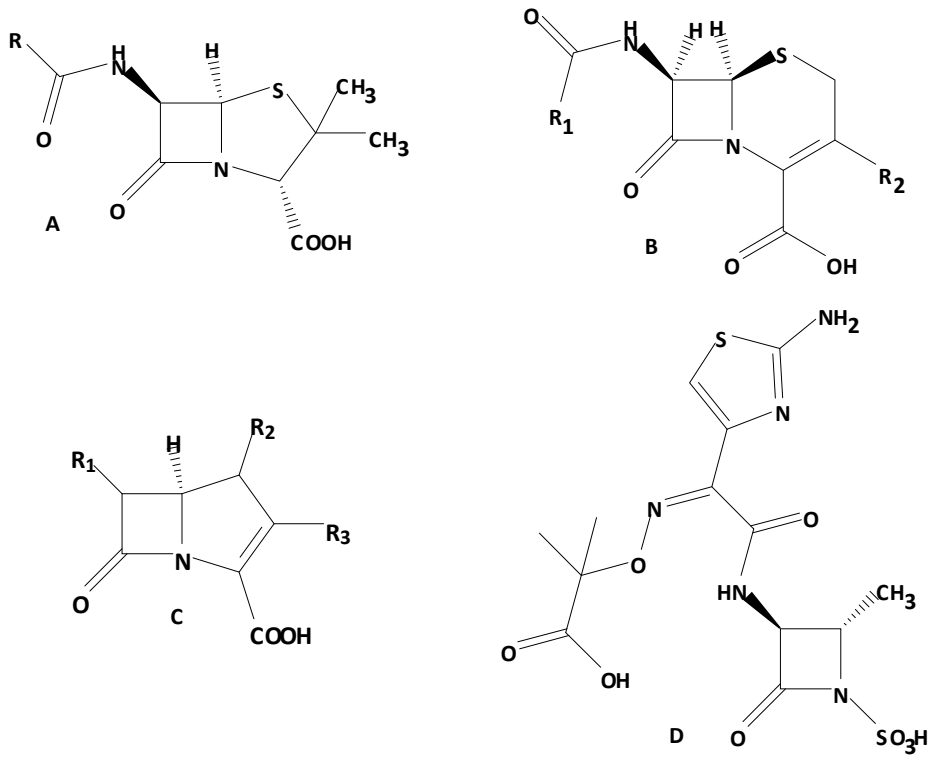

Figure 1. General structure of A) penicillin, B) cephalosporin, C) carbapenem, and D) monobactam antibiotics.

- ***Penicillins***

Penicillins work against bacterial infections, inhibiting the formation of the cell wall in susceptible bacteria. The basic structure of penicillins is a thiazolidizine ring connected to a β-lactam ring, to which a side chain is attached. They are derived from *Penicillium* fungi used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. "Penicillin" is also the informal name of a specific member of the penicillin group *Penam Skeleton*, which consists in a heteroatomic ring structure, containing three carbon atoms and one nitrogen atom, as figure 1 shows. They include penicillin G, procaine penicillin, benzathine, penicillin VK, penicillin V, amoxicillin, ampicillin, dicloxacillin,

cloxacillin, oxacillin, nafcillin, bacampacillin, carbenicillin indanyl, ticarcillin, mezlocillin, and piperacillin.

- ***Cephalosporins***

The cephalosporin ring structure is derived from 7-aminocephalosporinic acid (7-ACA) which is produced from the *cephalosporium acremonium* while the penicillins are derived from 6-aminopenicillanic acid (6-APA). In the case of the cephalosporins the β-lactam ring is fused to a six-membered dihydrothiazin ring. Today, most cephalosporins are produced semi-synthetically deriving from 7-aminocephalosporinic acid (7-ACA). Cephalosporin antibiotics with different pharmacological properties are produced by modification of the side chains at C3 (R2) and C7 (acylamido, R1). The β-lactam ring contains two chiral centres with the absolute configuration 6R and 7R [2]. Figure 2 shows the change that the structure suffers from the β-lactam ring to the cephalosporin skeleton formation.

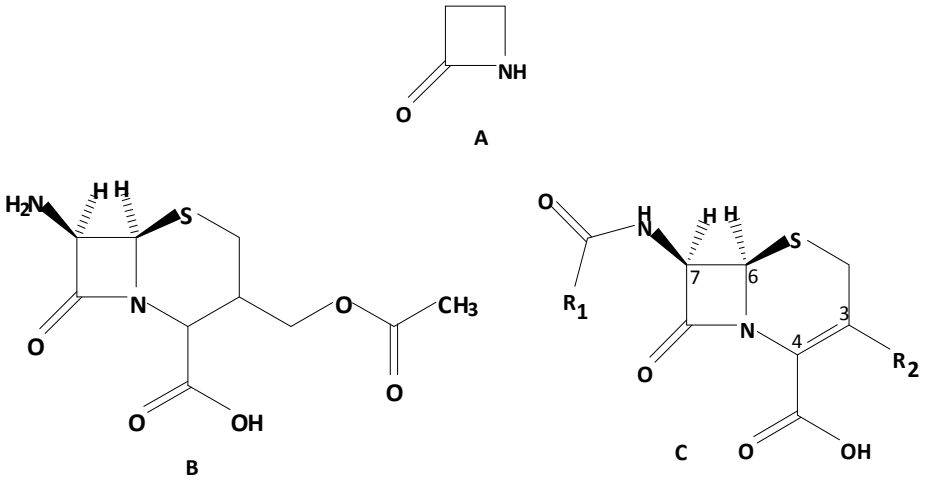

Figure 2. A) The four-membered lactam ring is the common skeleton for penicillins and cephalosporins. B) Microbially produced 7-amino-cephalosporinic acid is used to synthesize cephalosporins with different biological properties (semi synthetic process). C) Common skeleton of cephalosporin antibiotics [2].

[2] P.C. Van Krimpen, W.P. Van Bennekom, A. Bult, Pharm. Weekbl. [Sci], 9(1987) 1

- ***Carbapenems***

Carbapenems are compounds fairly recent added to the class known as β-lactams, and are used against serious infectious diseases or methicillin-resistant *Staphylococcus aureus*. Carbapenems present a broad spectrum of antibacterial activity and have a structure which renders them highly resistant to β-lactamases. Carbapenem antibiotics were originally developed from thienamycin, a naturally-derived product of *Streptomyces cattleya.*

Additionally they are generally resistant to the typical bacterial β-lactamase enzymes which are one of the principal resistance mechanisms of bacteria. They are active against both Gram-positive and Gram-negative bacteria, with the exception of intracellular bacteria, such as the *Chlamydiae*. This group includes imipenem, meropenem, ertapenem, faropenem, doripenem, and paripenem.

- ***Monobactams***

Monobactams are compounds wherein the β-lactam ring is alone and not fused to another ring. They work only against Gram-negative bacteria. Unlike other β-lactams, there is no fused ring attached to β-lactam nucleus. Thus, there is less probability of cross sensitivity reactions. They are named as tigemonam, nocardicin A, and tabtoxin.

- ***β-lactamase inhibitors***

A β-lactamase inhibitor is a molecule used in conjunction with a β-lactam antibiotic to extend its spectrum of activity. Although generally have little antimicrobial properties themselves, their sole purpose is to prevent the inactivation of β-lactam antibiotics by binding the β-lactamases, and as such, they are co-administered with β-lactam antibiotics. They are clavulanic acid, subactam and tazobactam.

3. Characteristics of cephalosporins

The first cephalosporin was discovered by Italian scientist Brotzu in 1945. Cephalosporin antibiotics were found to be active against large number of pathogenic bacteria but the main hindrance in its application is its low stability. Also, occurrence of bacterial strains that are resistant to already existing

antibiotics such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *E. faecalis* (VRE) has led to the search of new semisynthetic cephalosporins with better solubility and new mechanism of action [3].

The cephalosporin antibiotics can be divided into four groups (four generations) based on the time of discovery and pharmacological properties, newer generations are in active research, developed in response to a specific clinical need for a drug with different characteristics than the previous generation. Each generation has a broader spectrum of activity against Gram-negative microbes and shows higher resistance to β-lactamase activity than the one before. Cephalosporin of the first generation (narrow-spectrum) became commercially available in 1960s and showed high activity against Gram-positive bacteria. However, their limitation against Gram-negative bacteria led to the development of second generation cephalosporin in 1970s (expanded-spectrum). The third generation of cephalosporin (broad-spectrum), which were available in early 1980s, showed improved resistance to β-lactamase activity and Gram-negative bacteria, but were generally less effective against Gram-positive microbes. Representatives of the fourth generation of cephalosporin antibiotics (extended-spectrum) have got a quaternary amino group in their side chain (R2), which is responsible for broader activity against Gram-negative bacteria compared to previous generations. The fifth generation cephalosporin is still an unclear picture with many new modified cephalosporins in the research sector. This generation of antibiotic is specifically developed against nosocomial infections of MRSA and Pseudomonas based refractory infection in immuno-compromised patients. Figure 3 shows a diagram of the cephalosporin activity in relation to the generation.

Crucial for the antimicrobial activity of all cephalosporin antibiotics is the presence of an acylated amino group on the C-α-atom [4]. The extensive search for analogous in order to extent the total antibacterial spectrum has lead to multitude

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[3] K. Gaurav, S. Karmakar, K. Kundu, S. Kundu, in Dr. Marina Pana (Ed.), (2012). Design, Development and Synthesis of Novel Cephalosporin Group of Antibiotics, Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium, ISBN: 978-953-51-0472-8, InTech, DOI: 10.5772/29658. Available from: http://www.intechopen.com/books/antibiotic-resistant-bacteria-a-continuous-challenge-in-the-new-millennium/design-development-synthesis-and-in-vitro-antibacterial-activity-of-some-novel-cephem-antibiotics

[4] B. Testa, J.M. Mayer. Hydrolysis in Drug and Prodrug Metabolism - Chemistry, Biochemistry, and Enzymology. Verlag Helvetica Chimica. Acta, Zürich, Wiley-VCH, Weinheim (2003) p.166.

of compounds. Most of these compounds are used in human and veterinary medicine.

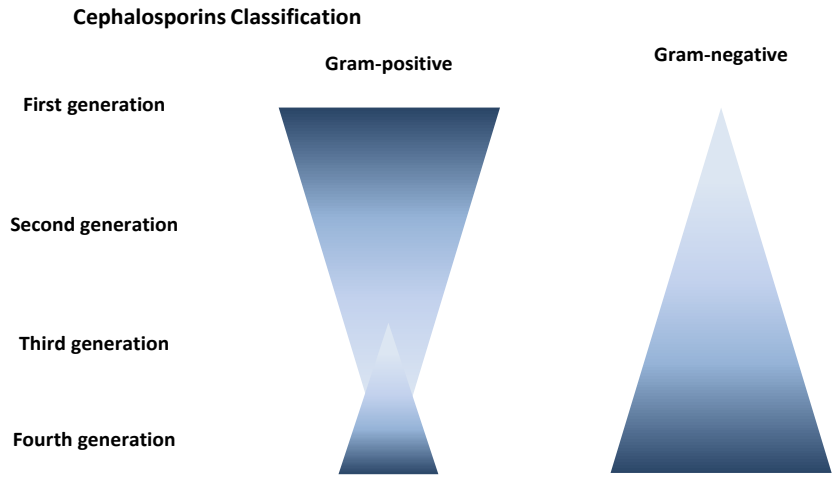


Figure 3. Spectrum activity of the cephalosporins

The physical and chemical properties of the cephalosporins are similar to those of the penicillins, although the cephalosporins are somewhat more stable to pH and temperature changes. Cephalosporins are weak acids derived from 7-ACA. Cephalosporins also contain a β-lactam nucleus that is susceptible to β-lactamase (cephalosporinase) hydrolysis. Modifications of the 7-ACA nucleus and substitutions on the side chains by semisynthetic means have produced differences among cephalosporins in antibacterial spectra, β-lactamase sensitivities and pharmacokinetics [5]. The chemical stability of β-lactams and their resistance to enzymatic and chemical hydrolysis has been subject of numerous studies [6,7], and it is important for the analysis of β-lactam antibiotics. The β-lactam ring is more reactive compared to normal amides and is prone to nucleophilic attack in both acidic and alkaline medium. The reactions and degradation at neutral and basic conditions are rather different from those in

[5] adapted from Merck Veterinary Manual web page, accessed in March 2013: http://www.merckmanuals.com/vet/pharmacology/antibacterial_agents/cephalosporins_and_cephamycins.html?qt=&sc=&alt=

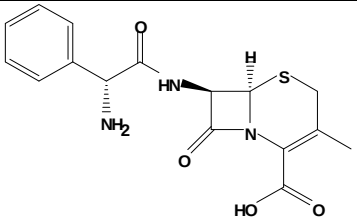
[6] A. Wildfeuer, K. Raeder, International Journal of Antimicrobial Agents (1996), 6(Suppl.), S31-S34

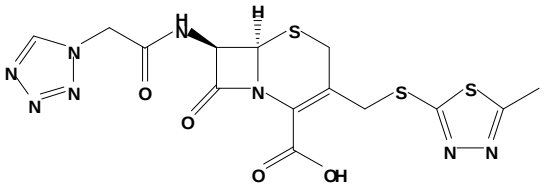
[7] J.C. Rotschafer, B.E. Ostergaard, American Journal of Health-System Pharmacy (1995), 52(6, Suppl. 2), S15-22

acidic medium and are also influenced by the electronegative properties of substituents at R1 and R2 [2].

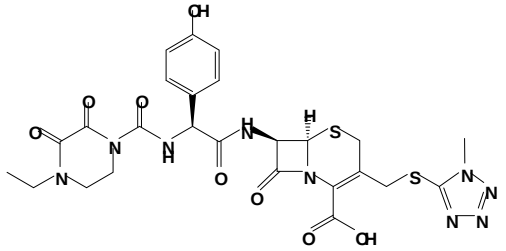
Table 1 summarizes the physicochemical properties [8] of the studies cephalosporins in this part of the Thesis.

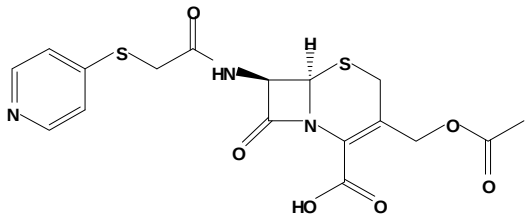
Table 1. Chemical names, structures and properties of the studied cephalosporin antibiotics

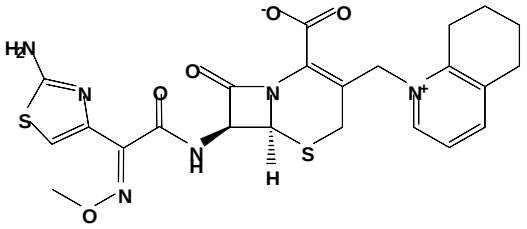
Cephalexin (CL)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-, (6*R*,7*R*)-
Water Solubility (g L^{-1}, T: 25°C)	4.90
K_{ow}, log P	0.35
pK_a	3.12; 6.84
Molecular weight (Da)	347.4
Elimination, half-life (h)	1

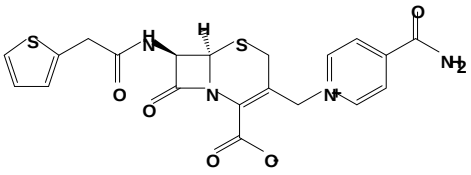
Cephazolin (CZ)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[[(5-methyl-1,3,4-thiadiazol-2-yl)thio]methyl]-8-oxo-7-[[2-(1H-tetrazol-1-yl)acetyl]amino]-, (6R,7R)
Water Solubility (g L-1, T: 25°C)	82
Kow, log P	-0.70
pKa	0.45; 2.60
Molecular weight (Da)	454.5
Elimination, half-life (h)	1.8

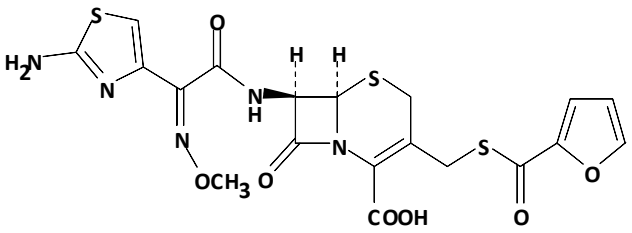
[8] Adapted from SciFinder data base, accessed in March 2013: https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf

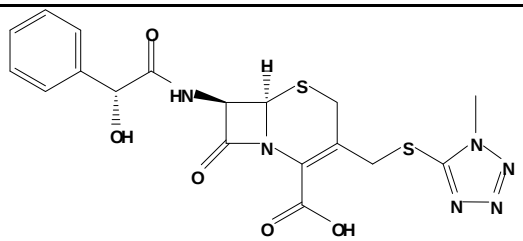
Cephoperazone (CPR)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2*R*)-2-[[(4-ethyl-2,3-dioxo-1-piperazinyl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-, (6*R*,7*R*)
Water Solubility (g L^{-1}, T: 25°C)	170
K_{ow}, log P	-1.11
pK_a	0.67; 2.62
Molecular weight (Da)	645.7
Elimination, half-life (h)	N/A

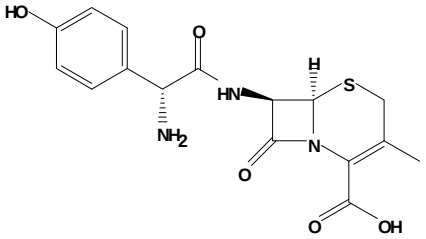
Cephapirin (CP)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[(acetyloxy)methyl]-8-oxo-7-[[(4-pyridinylthio)acetyl]amino]-, (6*R*,7*R*)-
Water Solubility (g L^{-1}, T: 25°C)	180
K_{ow}, log P	-0.41
pK_a	2.67; 4.49
Molecular weight (Da)	423.5
Elimination, half-life (h)	N/A

Cefquinome (CQ)	
IUPAC name	Quinolinium, 1-[[(6*R*,7*R*)-7-[[(2*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-5,6,7,8-tetrahydro-
Water Solubility (g L^{-1}, T: 25°C)	N/A
K_{ow}, log P	N/A
pK_a	2.6
Molecular weight (Da)	528.6
Elimination, half-life (h)	N/A

Cephalonium (CLN)	
IUPAC name	Pyridinium, 4-(aminocarbonyl)-1-[[(6*R*,7*R*)-2-carboxy-8-oxo-7-[[2-(2-thienyl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-
Water Solubility (g L^{-1}, T: 25°C)	N/A
K_{ow}, log P	N/A
pK_a	3.3
Molecular weight (Da)	458.1
Elimination, half-life (h)	N/A

Ceftiofur (CT)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetyl]amino]-3-[[(2-furanylcarbonyl)thio]methyl]-8-oxo-, (6*R*,7*R*)-
Water Solubility (g L^{-1}, T: 25°C)	8.4
K_{ow}, log P	1.66
pK_a	1.7; 2.62
Molecular weight (Da)	523.6
Elimination, half-life (h)	N/A

Cephamandole (CM)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2*R*)-2-hydroxy-2-phenylacetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-, (6*R*,7*R*)-
Water Solubility (g L^{-1}, T: 25°C)	160
K_{ow}, log P	-0.04
pK_a	0.67; 2.62
Molecular weight (Da)	462.5
Elimination, half-life (h)	0.5-1.5

Cephadroxil (CD)	
IUPAC name	5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-, (6*R*,7*R*)-
Water Solubility (g L^{-1}, T: 25°C)	3.1
K_{ow}, log P	-0.25
pK_a	3.12; 7.17
Molecular weight (Da)	363.4
Elimination, half-life (h)	1.5

N/A: Not available

4. Analytical methods for β-lactam antibiotics

Apart from the extensive use of these antibiotics in human medicine for treating infections, its most frequent use in veterinary medicine is to combat mastitis causing-pathogens, a disease which produces significant economic losses in dairy industry. In the last few years, the public concern about the utilization of antibiotics in food-producing animals and their misuse in humans has increased due to the transfer of antibiotic-resistance bacteria to man. In this sense, the control of antibiotic residues in edible animal tissues is mandatory.

On the other hand, personal care products and pharmaceutical substances, including antibiotics, have experienced a fast growing interest as emerging pollutants and recent studies have shown that a multitude of drugs are present in aquatic systems. It is important to consider that the large amount of antibiotics consumed by humans and animals which are continuously introduced to the environment make them potential pollutants that are incorporated from a variety of sources, including discharges from domestic wastewater treatment plants and pharmaceutical companies, run-off from animal feeding operations, infiltration from aquaculture activities or from compost made of animal manure containing

antibiotics [9,10]. However, these antimicrobials have not been included in the list of priority and hazardous substances in the Water Framework Directive of the European Union [11].

The low concentration levels and the complexity of numerous matrices such as groundwater and surface water samples, foods of animal origin, biological fluids like blood, plasma from human or animal origin, etc., makes necessary to use highly sensitive and selective methods for β-lactam antibiotics determination. High performance liquid chromatography (HPLC) has been widely used for the analysis of antibiotics in food and environmental samples, mainly in combination with mass spectrometry (MS), as it has been clearly stated in some general reviews, which included also β-lactams [10-13] and specifically in a review about analytical methodologies for these compounds [14,15].

In this part, an overview of the existing analytical methods using HPLC and capillary electrophoresis (CE) has been developed. Different detection techniques and sample treatments are considered as well as the applications in different fields such as clinical and pharmaceutical, food and environmental analysis.

4.1. Analysis of β-lactam antibiotics by HPLC with UV-detection

4.1.1. Food analysis

UV detection by using diode array (DAD) is a very popular technique coupled with HPLC but it has some limitations to determine β-lactams such as low sensitivity due to the lack of chromophores. Moreover, HPLC-UV is not completely reliable for confirmation, not even using a DAD. That is why HPLC-MS is the preferred technique for identification and quantitation of β-lactams in foodstuffs. In the case

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[9] M.A. Soliman, J.A. Pedersen, I.H. Suffet, J. Chromatogr. A, 1029 (2004) 223
[10] M.S. Díaz-Cruz, M.J. López De Alda, D. Barceló, Trends Anal. Chem. 22 (2003) 340
[11] European Commission, Commission Decision (EEC) 2000/60/EC, Off. J. Eur. Commun. L327 (2000) 1
[12] C. Blasco, Y. Picó, C.M. Torres, Trends Anal. Chem. 26 (2007) 895
[13] A.A.M. Stolker, T. Zuidema, M.W.F. Nielen, M.W.F. Nielen, Trends Anal. Chem. 26 (2007) 967
[14] S.R. El-Shaboury, G.A. Saleh, F.A. Mohamed, A.H. Rageh, J. Pharm. Biomed. Anal. 45 (2007) 1
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of the monitoring of β-lactam antibiotics in food derived from animals, considering the low maximum residue limits (MRLs) established by the international legislations, it is necessary to apply preconcentration steps in sample treatment to reach limits of detection (LODs) close or below the MRLs for monitoring these residues.

Due to the fact that β-lactams antibiotics are usually employed for the treatment of mastitis in cows, milk is the most frequently analyzed sample [16] but, once the chromatographic separation has been developed, sample treatments can be adjusted and optimized for different types of samples. For instance, a capillary HPLC method has been proposed for the simultaneous determination of 10 β-lactams in foods of animal origin, such as milk or chicken muscle [17]. LODs for chicken muscle and milk samples were below the legislated MRLs, established on 25–300 μg kg^{-1} for chicken muscle and 4–200 μg L^{-1} for milk. Capillary HPLC shows several advantages compared to analytical HPLC, such as better resolution, lower LOD and lower solvent consumption, being more environmentally friendly than conventional HPLC. Recently ultrasound-assisted matrix-solid phase dispersion (MSPD) has been applied to isolate β-lactam antibiotics from milk [18,19]. Extraction yield by using this strategy with Nexus polymeric sorbent was higher than other applied solid-phase extraction (SPE) procedures for the same matrix. High recoveries can also be obtained when penicillin G, oxacillin and cloxacillin are extracted from beef and milk using ion-paired extraction (IPE) with tetrabutylammonium bromide as ion pairing agent and binary mixture water:acetonitrile as extractant [20].

Column technology evolution has led to the manufacture of sub-2 μm particle sizes used for ultra-high performance chromatography (UHPLC). Compared to LC, UHPLC improves efficiency, resolution and sensitivity, as well as significantly reducing sample analysis time and mobile phase solvent consumption. This superior performance requires expensive pumps capable of operating at pressures up to 1200 bar. An interesting option not needing new instrumentation is the use of analytical columns with core-shell technology. These columns are packed with 2.6

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[16] V.F. Samanidou, E.D. Tsochatzis, I.N. Papadoyannis, Michrochim. Acta, 160 (2008) 471
[17] M.I. Bailón-Pérez, A.M. García-Campaña, M. del Olmo-Iruela, L. Gámiz-Gracia, C. Cruces-Blanco, J. Chromatogr. A, 1216 (2009) 8355
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[19] E.G. Karageorgou, V.F. Samanidou, L.N. Papadoyannis, J. Sep. Sci. 35 (2012) 2599
[20] C. Kukusamude, R. Burakham, O. Chailapakul, S. Srijaranai, Talanta, 92 (2012) 38

µm core shell particles which consist of a solid core (1.9 µm) and a porous shell. As a result shorter diffusion paths are obtained allowing high efficiency even at high flow rates [21,22]. They are available with different chemistries: phenyl-hexyl, C18, C8, pentafluorophenyl (PFP) and Hilic. This technology has been used to determine penicillins and amphenicols in milk with a sample treatment based on MSPD and QuEChERS as a sorbent [23].

A mixed micelle-cloud point extraction (MM-CPE) has been developed for the analysis of four penicillins (ampicillin, penicillin G, oxacillin, and cloxacillin) in milk samples using Triton X-114 (TX-114) and cethyl trimethylammonium bromide (CTAB) as the mixed micellar extractant [24]. Limits of detection were 2-3 ng mL^{-1}, and 15-40 fold enhancement compared to that without preconcentration.

Recently, the introduction of dispersive liquid-liquid micro-extraction (DLLME) has provided simplicity of operation, rapidity, low sample volume and cost, as well as high preconcentration of a great variety of compounds. In this sense, *Adlnasab et al.* [25] have developed a novel three phase dispersive liquid- liquid microextraction (TP-DLLME) for β-lactam antibiotics using chloroform and acetonitrile as the solvents for extraction and dispersion, respectively, where Aliquat 336 is the carrier. Preconcentration factors were in the range of 80–125.

4.1.2. Environmental analysis

In the case of water analysis, considering the extremely low level of residues that can be found in this kind of matrixes, high volumes of samples are considered in the preconcentration steps, usually by means of SPE, increasing the total analysis time. In this sense, the selection of the sorbents and the volume of sample to be processed are crucial. As example, different types of wastewaters (industrial, influent and effluent sewage treatment plant samples, STP) have been analyzed for the simultaneous determination of different β-lactams antibiotics [26]. Two SPE

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[21] F. Gritti, G. Guiochon, J. Chromatogr. A, 1217 (2010) 1604
[22] V.F. Samanidou, E.G. Karageorgou, Drug Test. Anal. 3 (2011) 234
[23] E.G. Karageorgou, V.F. Samanidou, J. Sep. Sci. 34 (2011) 1893
[24] C. Kukusamude, A. Santalad, S. Boonchiangma, R. Burakham, S. Srijaranai, O. Chailapakul, Talanta, 81 (2010) 486
[25] L. Adlnasab, H. Ebrahimzadeh, Y. Yamini, Microchim. Acta, 179 (2012) 179
[26] E. Benito-Peña, A.I. Partal-Rodera, M.E. León-González, M.C. Moreno-Bondi, Anal. Chim. Acta, 556 (2006) 415

cartridges were compared for sample clean up and preconcentration: a reversed-phase silica-based cartridge (Bond Elut C18, Varian Inc.) and a strong polymeric mixed mode anion exchanger (Oasis MAX, Waters). The matrix components in industrial and urban wastewater samples reduce the preconcentration efficiency in both sorbents, especially for the Bond Elut C18. Capillary HPLC also demonstrated its usefulness in the analysis of different water samples from river and well [17]. In this case, also 250 mL of water were used in the preconcentration step, applying Oasis HLB cartridges.

Molecularly imprinted polymer (MIP) applied to SPE (MISPE) can be also an interesting alternative to achieve selective extraction of the target compound when the commonly applied sorbents lack selectivity. A MISPE procedure has been developed for the selective preconcentration of different β-lactams antibiotics in environmental waters, by using a MIP imprinted with penicillin G [27]. Various parameters affecting the extraction efficiency of the polymer have been evaluated to achieve the selective preconcentration of the antibiotics from aqueous samples and to reduce non-specific interactions. This resulted in an MISPE-HPLC method allowing the direct extraction of the analytes from the sample matrix with a selective wash using just 10% (v/v) organic solvent. On the basis of UV detection only, the method showed good recoveries and precision both for tap and river water, suggesting that this molecular imprinted procedure can be applied to the direct preconcentration of β-lactam antibiotics in environmental waters. Also, a cephalexin MIP was synthetized for the determination of this compound in river waters [28]. In order to increase the sample volume for a higher sensitivity, a tandem SPE system incorporating Oasis HLB sorbent was implemented, being possible the determination of this compound with recoveries higher of 50 %.

4.1.3. Clinical and pharmaceutical analysis

The application of HPLC with UV detection to the analysis of β-lactams in clinical and pharmaceutical matrices has been used at length [29]. It includes the monitoring of the concentrations of β-lactam antibiotics and their precursors for the optimization of their production.

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[27] J.L. Urraca, M.C. Moreno-Bondi, A.J. Hall, B. Sellergren, Anal. Chem. 79 (2007) 695
[28] A. Beltrán, N. Fontanals, R.M. Marcé, P.A.G. Cormack, F. Borrull, J. Sep. Sci. 32 (2009) 3319
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Some progresses in sample treatment include the use of direct injection of samples without any previous sample treatment as in the case of the use of a MISPE procedure to determine amoxicillin and cephalexin in urine, only adjusting the urine sample to acidic pH [28] or the analysis of serum samples without any pre-treatment, integrating the extraction column and the separation column in the same setup [30]. After the matrix passed the extraction column, the retained analyte is quantitatively transferred to the analytical column where separation by isocratic HPLC was performed. In other study, only a single common sample preparation method involving ultracentrifugation and stabilization was used, before HPLC analysis for the determination of β-lactams in human plasma [31]. Other advances are focused in getting simpler designs and very low-cost equipment. In that sense, multisyringe chromatography (MSC) consisting of a multisyringe module, solenoid valves and a chromatographic column has been proved to be a satisfactory and cheap alternative to HPLC for the analysis of three β-lactams antibiotics (amoxicillin, ampillicin and cephalexin) present in a generic formulation of amoxicillin [32]. Selectivity is a key point in these methods because several active principles are usually co-administered. A C18 analytical column with isocratic elution was used to determine cloxacillin together with ciprofloxacin and ibuprofen in human urine without interferences [33]. Sometimes gradient elution must be used to achieve a complete separation, for example to carry out pharmacokinetic studies involving the carboxypenicillin ticarcillin and the β-lactamase inhibitor clavulanate in rabbit serum and tissue cage fluid samples [34]. Columns based on core-shell technology have also been used in pharmacokinetic studies of carbapenem. Retention times were significant decreased and authors refer to this technique as ultra-high performance liquid chromatography even though they do not use sophisticated ultra-high pressure pumps [35].

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[31] S.E. Briscoe, B.C. McWhinney, J. Lipman, J.A. Roberts, P.J. Ungerer, J. Chromatogr. B, 907 (2012) 178
[32] H.M. González-San Miguel, J.M. Alpízar-Lorenzo, V. Cerdá, Anal. Bioanal. Chem. 387 (2007) 663
[33] A. Espinosa-Mansilla, A. Muñoz de la Peña, D. González Gómez, F. Cañada-Cañada, J. Sep. Sci. 29 (2006) 1969
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[35] E. Dailly, R. Bouquié, G. Deslandes, P. Jolliet, R. Le Floch, J. Chromatogr. B, 879 (2011) 1137

Table 2 shows a summary about the proposed methods for the analysis of these compounds in different matrixes above considered using HPLC-UV detection.

Table 2. Determination of β-lactams by HPLC-UV detection

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Milk and veterinary formulation	CTX, CLX	Protein precipitation. Nexus SPE. Elution with ACN. Ev.	**C18** MeOH/acetate buffer (pH 4) λ= 265 nm	84-121	0.1 μg L^{-1} CTX 0.3 μg L^{-1} CLX	[16]
Milk, chicken muscle, waters	AMOX, AMPI, PEN G, PEN V, PIPE, NAFCI, OXA, CLOX, DICLOX, CLAV.ACID	Ex with ACN. SPE with Oasis HLB and Alumina. Elution with H_2O	**Luna C18** (150× 0.5 mm,5μm) Solvent A: water/0.01% TFA Solvent B: ACN/0.01% TFA gradient λ= 220 nm	82.9-98.2	0.04–0.06 μg L^{-1} waters 0.80–1.40 μg L^{-1} foods	[17]
Milk	CDX, CCL, CLX, CTX, CFZ, CXM, CPZ, CTF	UA-MSPD with Nexus sorbent. Elution with MeOH, Ev	**Chromolith RP-18e** (100×4.6 mm,5μm) Solvent A: HCOOH 0.1% Solvent B: MeOH/ACN (75:25) gradient λ= 265 nm except for CFZ and CXM 275 nm	93.8-115.3	103-112.3 μg Kg^{-1}	[18]
Beef, milk	PENG, OXA, CLOX	Ion-paired extraction with TBABr and water-ACN	**Xbridge C18** (250×4.6 mm,5μm) Solvent A: 5 mM phosphate (pH 6.6) Sovent B: ACN Isocratic (75% A, 25% B) λ= 226, 240 nm	72.3-98.9	1-2 ng mL^{-1}	[20]
Milk	AMOX, AMPI, OXA, CLOX, DICLOX	UA-MSDP - QuEChERS	**Core shell Kinetex C18** (150×4.6 mm,2.6μm) Solvent A: 0.05 M CH_3COONH_4 Sovent B: ACN Gradient λ= 226, 240 nm	70-120	6-12 μg Kg^{-}_{1}	[23]
Milk	ampicillin, penicillin G, oxacillin, cloxacillin	MM-CPE: Triton X-114 + CTAB	**C18 column**, Isocratic 5 mmol L^{-1} phosphate buffer (pH 6.6) and methanol (55:45, v/v), flow rate: 1 mL min^{-1}	>80	2-3 ng mL^{-1}	[24]
Wastewater	PENG, AMOX, AMPI, PENV, OXA, CLOX, DICLOX, NAFCI	Anion exchanger SPE	**C18** H_2O/TFA-ACN/TFA, gradient λ= 220 nm	[b]46-91 industrial [b]28-91 influent STP [b]39-114 effluent STP	[c]2.9-25.6 μg L^{-1} industrial [c]2.5-12.4 μg L^{-1} influent STP [c]2.2-12.7 μg L^{-1} effluent STP	[26]

Table 2. Continue

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Environmental water samples	PENG, PENV, AMPI, AMOX, NAFCI, OXA, CLOX, DICLOX	MISPE (template PENG)	**C_{18}** H_2O TFA-ACN TFA λ= 220 nm	93-100 tap water 90-100 river water	[f]0.38-0.98 µg L^{-1} Milli Q [f]0.9-2.9 µg L^{-1} tap water [f]1.3-5.8 µg L^{-1} river water	[27]
River water Human urine	CLX CLX, AMOX	MISPE (template CLX) Urine: pH =3 Water: tandem SPE with HLB	**C_{18}** (250× 4.6 mm,5µm) ACN 1% acetic acid λ= 252 nm	> 50 river water 78 CLX and 60 AMOX	nr	[28]
Pharmaceuticals, human blood serum, urine	CLX, CDX, CCL, CTX	Serum: Add ACN, V, C, Ev, SPE with Diol Bond Elut: Urine: D + F	**C_{18}** Acetate bf (pH 4.0)-MeOH λ= 265 nm	76.3-112.0	0.01 ng μL^{-1} CDX and CLX 0.005 ng μL^{-1} CTX and CCL	[29]
Serum, bronchial secretions	CAZ, MPM	HPLC-ISP: C_8 or C_{18} cartridge and in-line filter. Serum: DI Sputum: Add NaH_2PO_3 bf (pH 5.0), H, C	**C_{18}** ACN-NaH_2PO_3 bf (pH 5.0) λ= 258, 296 nm	Sputum: 56.7-73.4 MPM 49.3-56.9 CAZ Serum: 105.2-89.4 MPM 109.0-95.8 CAZ	<0.5µg mL^{-1}	[30]
Pharmaceutical formulation	AMOX, AMPI, CLX	nr	**FIA-Monolithic C_{18}** MeOH-acetic/acetate (pH 6.2) λ= 250 nm	101.4 AMOX	nr	[32]
Human urine	Ciprofloxacin, CLOX, ibuprofen	D	**C_{18}** Formic/formate (pH 3)-ACN-MeOH λ= 220, 280 nm	92-110	0.41 µg mL^{-1} CLOX	[33]
Rabbit serum, tissue cage fluid (TCF)	TIPC, clavulanate	Add ACN, C Add DCM, C	**C_{18} IP** ACN-phosphate bf pH 4.1 (TBAS), gradient λ= 218, 254 nm	76.8 µg mL^{-1} serum 79.9 µg mL^{-1} TCF	1 µg mL^{-1} (LOQ)	[34]

Table 2. Continue

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Human plasma	DORI, ERTA, IMI, MPM	Add MES bf pH 6 Add ACN, C, Ev Add MES bf pH 6	**Core shell Kinetex PFP** (100×4.6 mm,2.6µm) Solvent A: Methanol Solvent B: 0.1 M phosphate (pH = 7) Gradient λ= 295 nm	87.9-99.7	0.50 mgL^{-1} (LOQ)	[35]

AMOX, Amoxicillin; AMPI, Ampicillin; CAZ, Ceftazidime; CCL, Cefaclor; CDN, Cephradine; CDX, Cefadroxil; CEP, Cephapirin; CFM, Cefepime; CFPM, Cefpirome; CFX, Cefoxitin; CFZ, Cefazolin; CPZ, Cefoperazone CLAV.ACID, clavulanic acid; CLOX, Cloxacillin; CLX, Cephalexim; CMNX, Cefminox; CMZ, Cefmetazole; CTF, Ceftiofur; CTX, Cefotaxime; CXM, Cefuroxime DICLOX, Dicloxacillin; DORI, doripenem; ERTA, ertapenem; IMI, imipenem; MPM, Meropenem; NAFCI, Nafcillin; OXA, Oxacillin; PENG, Penicillin G; PENV, Penicillin V; PIPE, piperacillin; PFP, pentafluorophenyl; TIPC, Ticarcillin

ACN, acetonitrile; bf, buffer; C, centrifugation; Co, concentration; CTAB, cethyl trimethylammonium bromide; D, dilution;DCM, dichloromethane; DI, direct injection; Ev, evaporation; Ex, extraction; F, filtration; H, homogenization; He, heat; HTAB, Hexadecyltrimethylammonium bromide; IP, Ion pair; ISP, integrated sample preparation; ISPR, isopropanol; MIP, molecularly imprinted polymer; MM-CPE, mixed micelle-cloud point extraction; UA-MSPD, ultrasound-assisted matrix solid-pahse dispersion; nr, no reported; S8, octanesulfonate; S12, dodecanesulfonate; STP, sewage treatment plant; TBABr, tetrabutylammonium; TBAS, tetrabutylammonium hydrogen sulphate; TEA, tetraethylammonium; TFA, trifluoroacetic acid; TrEA, triethylamine; ulC, ultracentrifugation; V, vortex;

[a]Abstract; [b]Samples spiked with all the antibiotics at 25 and 75 µg L^{-1}; [c]Processing 250 mL of wastewater samples; [d]At drug concentrations between 0.78 and 100 mg L^{-1}; [e]LOQs; [f]Percolation of 50 mL of water.

4.2. Analysis of β-lactam antibiotics by HPLC with mass spectrometry detection

4.2.1. Food analysis

Food samples such as milk are typically screened for β-lactam antibiotics by nonspecific methods based on enzymatic reactions or microbial growth inhibition. These methods are fast and sensitive but cannot identify individual compounds and also yield false positives. MS is the preferred detection technique to confirm the presence of β-lactam in foodstuffs. In spite of the high selectivity of this detection technique, a previous chromatographic separation is necessary in order to avoid ion suppression of coeluting compounds. Most of the proposed methods are also able to quantify although early designs of mass spectrometers had some

difficulties [36,37]. Both trap and triple quadrupole analyzers can easily achieve the minimum number of identification points required by the EU for unambiguous β-lactam identification in this type of samples. At least two fragments must be detected, being the most intense signal used for quantification and the second one for identification. The ratio between the intensities of both fragments has to be within the permitted tolerance for a successful identification.

β-lactams were first determined by HPLC-MS using thermospray but currently electrospray ionization (ESI) is the preferred interface between both techniques due to the ease of β-lactam ionization. Although β-lactams have a carboxylic group, positive ionization is normally used due to the higher sensitivity achieved in this mode. In some cases, better results have been reported in negative mode avoiding matrix interferences resulting in remarkable signal weakening [38]. Collision-induced fragmentation in the positive ion mode leads to the cleavage of the β-lactam ring. Therefore, two fragments are usually detected: the class-specific fragment m/z 160 and the compound-specific daughter ions $[M+H-159]^+$. The most abundant ion to be fragmented can be either the protonated molecular ion, the sodium adduct [39] or adducts with methanol ($[M+CH_3OH+H]^+$) [40] but it is important to realize that related-metabolites can also be found especially in case of ceftiofur [41] or amoxicillin [42].

On one hand methanol should be avoided in the mobile phase due to the potential for analyte degradation [43] but, on the other hand, it can provide better chromatographic peaks. Usually, if the chromatographic run is fast enough degradation effects are not significant [44]. HPLC-UV methods cannot be directly applied to MS if they use non-volatile compounds in the mobile phase such as

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buffers or ion-pair reagents. Problems can be also anticipated if non-volatile compounds are used as eluting solvent in SPE. Separation capacity of liquid chromatography can be improved tremendously with the new sub-2 micron chromatography columns. This allows the development of multi-residue methods to determine in a short analysis time several analytes from different families likely to be found in a given sample. This is the case of β-lactams, which can be found together with other antibiotics [45-49].

Milk is one of the most often analyzed samples for the monitoring of β-lactams, being usually extracted using acetonitrile. Acidic solutions can also be used but attention must be paid to stability of some of them such as cephapirin [38] and amoxicillin at extreme pHs [50]. β-lactam antibiotics exhibit significant binding to matrix components, especially proteins, but enzymatic degradation of proteins can lead to antibiotic degradation as well. The most detected β-lactams in milk are penicillin G and amoxicillin [38,51]. Once acetonitrile has been evaporated, samples are reconstituted in a suitable solvent and SPE can be applied. On-line SPE has also been proposed for fully automated analysis of β-lactams and satisfactory results were achieved although relative standard deviations were sometimes above 30% [44]. Newer strategies for sample treatment include magnetic MIP extraction which is an interesting alternative when we are interested in class specific analysis rather than multi-residue method [52]. For solid samples such as meat, kidney or liver, sample treatments that have been used include: liquid membrane extraction achieving LODs of 1 ng kg^{-1} for penicillin G and penicillin V [53], pressurized liquid extraction (PLE) to determine cloxacillin, cefalexin and dicloxacillin in meat with LODs below the MRLs [54] and dispersive SPE [43,55,56].

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This last option is an alternative clean up instead of the classic cartridge-based SPE, which simplify and speed up the sample preparation. β-lactam residues were not detected in eggs of laying hens orally dosed with these antibiotics [37].

4.2.2. Environmental analysis

Generally, drugs are absorbed by the organism after intake and are metabolized. However, a significant amount of the original substance will leave the organism unmetabolized via urine or feces. Waste water treatments plants are not able to completely eliminate these pharmaceutical residues and eventually they will contaminate the aquatic environment. Intact β-lactams do not occur frequently in the environment due to the poor stability of the β-lactam ring. Analytical methods, especially sample treatments, must provide very low LODs (ng L^{-1}). Although SPE is still the most used sample treatment for liquid samples [57,58] some variations have been applied to improve sensitivity. These modifications include the use of two different SPE cartridges plugged together [59] or a derivatization step to form penicilloyl methyl esters. This reaction takes place "in situ" during the SPE solvent evaporation and can increase sensitivity of dicloxacillin by a factor of 9 [60]. In case of solid samples such as soil, PLE has been successfully applied using methanol:water mixture (8:2, v/v) as extracting solvent [59]. Analytical methods for environmental samples are usually multi-residue methods and are optimized for a wide variety of antibiotic families including β-lactams and many other pharmaceuticals. That means they have to find a compromise among all the instrumental parameters involved [61,62].

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4.2.3. Clinical and pharmaceutical analysis

Some applications of MS in pharmaceutical analysis include the determination of β-lactam contamination in non-β-lactam pharmaceuticals. Cross-contamination is a critical issue because it may cause unexpected adverse reactions [63]. Another field of application is therapeutic drug monitoring (TDM). The purpose of TDM is to provide a rational dosing of the drug depending on the needs of each individual and based on pharmacokinetic parameters. UHPLC has been applied in TDM to determine six β-lactams in neonate plasma [64] achieving an analysis time of approximately 3.5 min per sample. Also, β-lactams have been determined in plasma [65,66] and serum [67] samples by SPE using Oasis HLB cartridges with good recoveries (>75%) and LODs in the ng mL^{-1} range.

Degradation of β-lactams is quite likely due to the instability of the four-member ring. Therefore, analytical methods are needed to check the purity of these drugs. MS is essential to identify degradation products but, unfortunately, non-volatile buffers and ion-pairing agents not compatible with MS have to be used to achieve a complete chromatographic separation. An interesting alternative to make compatible HPLC conditions with MS is based on multi-stage LC. Briefly, a small volume eluted from the first column is diverted to a second column. The mobile phase of the first column contains all the additives required for the separation, and the mobile phase of the second column is fully MS compatible. Using this approach it was possible to determine the degradation products of a newly synthesised β-lactam antibiotic [68]. In this case, it was necessary to add a third column to avoid the contamination of the ion-pairing reagent with the second column. Coupling two columns in-line has also been used for on-line purification of plasma samples. The first column, used as on-line extraction column, contains big particles (30 μm) and high flow rates are applied (4 mL min^{-1}). The second column is the analytical

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column where separation takes place. Satisfactory precision was obtained and the total analysis time was just 1.6 min [69].

β-lactams often decompose at high temperatures in atmospheric pressure chemical ionization (APCI) interfaces. If softer conditions are applied for the ionization, some β-lactams are not detected unless an ionization accelerating solvent is used. Bromoform has been reported as a satisfactory ionization accelerating solvent being able to form adducts with these labile β-lactams improving sensitivity and making easy to identify $[M+Br]^-$ because of the stable isotope abundance ratio of 1:1 [70]. Nevertheless, ESI is the preferred ionization mode and fragmentation patterns for β-lactams are well known [71].

Table 3 includes the methods for the analysis of these compounds in different fields of application by using liquid chromatography with MS detection.

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Table 3. Determination of β-lactams by HPLC-MS detection

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Natural and wastewater	AMOX, AMPI, OXA, CLOX, CEP	SPE with Oasis HLB, Ev, add mobile phase	**C_{18}-LC/MS/MS** with ESI 0.1% formic acid-ACN-MeOH gradient	>75% except AMOX<40%	8-10 ng L^{-1} surface H_2O 13-18 ng L^{-1} influent 8-15 ng L^{-1} effluent	[57]
Beef kidney tissue	DECEP, AMOX, CEP, DCCD, AMPI, CFZ, PENG, OXA, CLOX, NAFCI, DICLOX	H, Ex H_2O-ACN, V, C, Ev ACN, SPE with C_{18}, Ev	**C_{18}-LC/MS/MS** with ESI 0.1% formic acid in water-0.1% formic acid in MeOH, gradient	nr	(DECEP: 10-50; AMOX: 50-100; CEP: 10; DCCD: 500; AMPI:10; CFZ:10; PENG:10; OXA:10; CLOX: 10; NAFCI:10; DICLOX:100-500) ng g^{-1}	[36]
Eggs	Sulfonamides, tetracyclines, fluoroquinolones and β-lactams (AMOX, CEP, AMPI, PENG, CLOX)	Bl, Add sodium succinate bf, H, C, SPE with Oasis HLB	**LC/MS/MS** with ESI Phenyl-bonded silica column 0.1% formic acid-ACN, gradient	AMOX CEP < 25%; AMPI, PENG, CLOX: 30-50 %	Screening limits approx. 50 ppb	[37]
Bovine milk	AMPI, AMOX, PENG, PENV, CLOX, CEP, CTF	Add ACN, V, C, Ev, add phosphate bf, V, SPE cleanup with Oasis HLB,	**C_{18} LC-IT-MS/MS** H_2O-1% acetic ac/MeOH-1% acetic ac, gradient	115-85[a]	(0.2 AMPI, 0.4 CTF, 0.8 CEP, 1AMOX and PENG, 2 CLOX and PENV)[c] $ngmL^{-1}$	[39]
Raw bovine milk	DCFA	C, add extraction solution (0.4% DTE in borate bf pH 9), V, Dr with iodacetamide, V, pH adjusted to 3.5, C, SPE with Oasis HLB, Ev, C	**LC-ESI MS/MS** Phenylether 0.005% Formic acid-MeOH, gradient	87.0-95.2	96.1 µg Kg^{-1}	[41]
Pig kidney, liver, muscle and fat.	AMOX and related metabolites	Add phosphate bf, V, C, add TCA, SPE with C_{18}, elute with ACN, Ev, R	**RP-LC-PI-ESI-MS/MS** H_2O-9.6 mM PFPA/ACN-H_2O (1:1 v/v) + 9.6 mM PFPA , gradient	39.2-104.0[a]	0.2 – 12.0 ng g^{-1}	[42]
Milk	AMOX, OXA, PENV	Magnetic - MIP	**C_{18}-LC-MS/MS** 0.1% formic acid aqueous solution: methanol (4:6, v/v)	71.6-90.7%	1.6-2.8 ng mL^{-1}	[52]

Table 3. Continue

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Milk	7 β-lactam, 12 macrolide, 2 liconsamide, morantel, orbifloxacin	Add ACN, C, Ev, add ACN (50%), F	**UPLC HSS T3 column** (100 mm×2.1 mm, 1.8 µm) Solvent A: 0.05% FA in water Solvent B: ACN Gradient	140.3-50.5	0.1 – 2.5 ng mL^{-1}	[47]
Bovine milk	PENG, AMPI, OXA, AMOX, DICLOX, CLX, CEP	Add 10% acetic ac, V, C, F	**C_{18} LC-PI-ESI-MS** H_2O-0.1% Formic ac/ACN-0.1% Formic ac, gradient	(PENG: 52, AMPI: 76, OXA: 43.5, AMOX: 52, DICLOX: 28.5, CLX: 82, CEP: 78.5)[b]	PENG,CLX, CEP: 1µgL^{-1}, AMPI:2µgL^{-1}, OXA:5 µgL^{-1}, AMOX:<1 µgL^{-1}, DICLOX:4 µgL^{-1}	[51]
Cow milk, bovine kidney and liver tissues	AMPI, CLOX, PENV, PENG	SLM n-undecane:di-n-hexyl ether	**C_{18} LC-PI-ESI-MS** Acetic ac-MeOH-H_2O	34-66 milk[a] 47-85 kidney, liver[a]	PENG, PENV: 1ng Kg^{-1} in kidney, liver 0.7 µg L^{-1} in milk AMPI: 1.4 µg Kg^{-1} in kidney, liver 1.7 µg L^{-1} in milk	[53]
Bovine kidney tissue	DECEP, AMOX, DCCD, AMPI, CFZ, PENG, OXA, CLOX, NAFCI, DICLOX	H, Ex H_2O-ACN, dispersive SPE cleanup with C_{18}, C, Ev, F	**C_{18}-LC/MS/MS** A: 0.1% formic acid; B: 50% ACN-50% MeOH	70% except DCCD 58%	nr	[55]
Non-β-lactam pharmaceuticals	CMZ, CPDXPR	Di, S, C, F	**RP-LC-PI-ESI-MS** H_2O-ACN-Formic acid	96.7-102.2 CMZ 88.9-94.2 CPDXPR	0.002 mg Kg^{-1}	[63]
Human plasma	CXM	Add 0.2 mM NH_4Ac (pH 5), V, C, SPE with Oasis HLB, Ev, R in mobile phase	**RP-LC-NI-ESI-MS/MS** ACN:0.2 mM NH_4Ac: glacial acetic acid (70:30:0.020, v/v/v)	> 76.4%	LOQ: 81.0 ng mL^{-1}	[65]
Human serum	AMPI, CFZ, CFM, CMZ, CTX, DORI, MPM, PIPE	Add 10 mM ammonium formate, V, SPE with Oasis HLB, Ev, R in 10 mM ammonium formate: methanol (95:5)	**C_{18}-LC/MS/MS with ESI** A: 10mM aqueous ammonium formate containing 0.1% formic acid. B: methanol containing 0.1% formic acid	Relative recoveries: 80.2 - 98.6% Absolute recoveries: 83.7% - 104.9%	CFP: 0.05, DORI: 0.10, MPM: 0.05, CMZ: 0.01, CTX: 0.05, AMPI: 0.01, CFZ: 0.05, PIPE: 0.001 (µg mL^{-1})	[67]

Table 3. Continue

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
Human plasma	A β-lactam candidate (comp I) and its open-ring transformation product (comp II)	Add equal vol. IS in 0.5M NH_4Ac (pH 4), V, C	**C_{18} LC-ESI-MS/MS** High Flow Oasis HLB for online Ex A: 1mM formic acid in water; B: 1mM formic acid in MeOH; C:1mM formic acid in MeOH-H_2O	70% comp I 95% comp II	0.980 ng mL^{-1d}	[69]
No sample	CTX, CPZ, CMX, CPM, CTRX, CPIZ, CMNX, CZX, CFX, CMZ, CTM, CXM, LOMX, AMPI, PENG, CBPC, SBPC, PIPE, ASPC, AMOX, TIPC		**C_{18} LC-APCI-MS** A: ACN-1% acetic acid, B: ACN-Bromoform as ionization acceleration solvent (100:1) Identification by FIA	nr	nr	[70]
Human blood	CCL	Dr Pγ/CIPIC at 80°C C, F	**C_{18}-LC/APCI/MS** 40%ACN-10% 0.1M TrEA	nr	10 nm 50 μgL^{-1}	[78]
Bovine milk	AMOX, AMPI, CLOX, OXA, PENG	C, Ex 100mM phosphate bf pH 9.2, liq-liq Ex with hexane, SPE with C_{18}, vol reduction, F	**C_{18} LC- ESI MS/MS** A: 0.1% formic acid B: 65% ACN, 35% H_2O, 0.1% formic acid, gradient	Stability studies in milk and milk extracts at 4, -20 and -76°	nr	[72]
Fish	AMOX, AMPI, CEP, PENG, OXA, CLOX, DICLOX	Ex ACN-hexane, C, Ev, add H_2O-ACN	**Phenyl-LC-ESI-MS/MS** A: 0.1% formic acid with 10 μM NaOH in water B: ACN	AMOX: 10% AMPI, PENG <50% CEP, OXA, CLOX, DICLOX: >50%	0.1 mg Kg^{-1} AMOX, AMPI, CEP, OXA, CLOX, DICLOX 1 mg Kg^{-1} PENG	[73]
Bovine muscle	AMOX	Ex H_2O, C, Add ACN, C, Add dichloromethane	**C_{18}-LC-MS/MS** A: ACN; B: H_2O + 0.005% FA; C: H_2O.	86%	nr	[74]

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Table 3. Continue

Sample	Analyte	Sample treatment	Chromatographic/detection characteristics	Recovery (%)	LODs	Ref.
ERTA	Dried blood spots	Ex with (3/7, v/v) water:methanol	**LC-MS/MS** C18 (50 mm×1.8 µm, 2.1 mm) Solvent A: 0.1% FA in water Sovent B: 0.1% FA in ACN	94-155%	0.2 mg L^{-1}	[75]

AMOX, Amoxicillin; AMPI, Ampicillin; ASPC, Aspoxicillin; CBPC, Carbenicillin; CCL, Cefaclor; CEP, Cephapirin; CFM, Cefepime; CFPM, Cefpirome; CFX, Cefoxitin; CFZ, Cefazolin; CLOX, Cloxacillin; CLX, Cephalexim; CMNX, Cefminox; CMX, Cefmenoxime; CMZ, Cefmetazole; CPDXPR, cefpodoxime proxetil; CPIZ, Cefpimizole; CPL, Cephalin; CPM, Cefpiramide; CPZ, Cefoperazone; CQM, Cefquinome; CTF, Ceftiofur; CTM, Cefotiam; CTRX, Ceftriazone; CTX, Cefotaxime; CXM, Cefuroxime; CZX, Ceftizoxime; DCCD, Desfuroylceftiofur cysteine disulfide; DCFA, Desfuroylceftiofur free acid;DECEP, Deacetylcephapirin; DICLOX, Dicloxacillin; DORI, Doripenem; ERTA, ertapenem; LOMX, Latamoxef; MPM, Meropenem; NAFCI, Nafcillin; OXA, Oxacillin; PENG, Penicillin G; PENV, Penicillin V; PIPE, piperacillin; SBPC, Sulbenicillin; TIPC, Ticarcillin

APCI, Atmospheric pressure chemical ionization; bf, buffer; Bl, blend; C, centrifugation; CID, collision induced dissociation; CIPIC, 4-(2´-cyanoisoindolyl) phenylisothiocyanate CTAB, cetyltrimethylammonium bromide; Di, disolve; Dr, derivatization; DTE, Dithioerythritol; ESI, Electrospray ionization; Ev, evaporation; Ex, extraction; F, filtered; FA, formic acid; H, homogenize; IT, ion trap; NI, negative ionization ; nr, no reported; PFPA, pentafluoropropionic acid; PI, positive ionization; PPC, perfusive-particle chromatography; Py, Pyridine; R, reconstitution; RP, reverse phase; S, sonicate; SLM, supported liquid membrane; TCA, tricloroacetic acid; TrEA, triethylamine; ulC, ultracentrifugation; V, vortex;

[a] Recovery range covers all the analytes and concentrations. [b] Average recovery. [c] For a nominal sample size of 5 mL. [d] Corresponding to limits of quantification.[e] Abstract

4.3. Analysis of β-lactam antibiotics by HPLC with other detection techniques

4.3.1. Chemiluminescence

β-lactams as a group possess poor chromophores, especially those lacking an aromatic ring, for that reason other detection techniques have been tested such as chemiluminescence detection (CLD), fluorescence and electrochemical detection. CLD has been the most used among them due to its inherent sensitivity and selectivity. β-Lactams can participate in CLD reactions in two ways: 1) either enhance CLD emission or 2) being derivatized with a suitable CLD reagent. In the first group, it was found that dicloxacillin and clavulanic acid exhibit the highest CLD enhancement when luminol is oxidized with H_2O_2 [76]. It appears that a strained β-lactam ring is essential for this particular CLD enhancement. Attention

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must be paid to mobile phase composition as there are buffers (e.g. borate) and organic solvents (e.g. acetonitrile) not supporting the CLD emission. In the second group, it is possible to use derivatization reagents such as 4-(2'-cyanoisoindolyl)phenylisothiocynate (CIPIC). CIPIC reacts with the primary amino group of the drug to form a CIPIC-conjugated which emits CLD when oxidized with H_2O_2. Cefaclor has been determined using this strategy in human serum and better sensitivity was obtained than with fluorescence and MS detection [77,78]. Borate and acetonitrile support this CLD emission.

4.3.2. Fluorescence

Fluorescence detectors have also been tested for the determination of β-lactams. Lower LODs are expected but, because of the lack of a fluorophore in these compounds, derivatization is usually required. This is a tedious process and the efficiency might not be optimum when β-lactam concentration is low. CIPIC derivate used for CL detection was found to be also fluorescence but better sensitivity was achieved with CL [78]. A labelling reaction involving trichloroacetic acid (TCA) and formaldehyde at 100°C for 30 min was used to determine amoxicillin [79]. Previously, the analyte was extracted from catfish and salmon tissues with phosphate buffer (pH 4.5), proteins were precipitated with TCA and β-lactams were recovered with SPE (C18). LODs were 0.5 and 1.2 μg kg^{-1} for catfish and salmon, respectively. Precolumn derivatization with salicylaldehyde avoids some co-eluting HPLC peaks in milk samples and is also a highly sensitive method to determine amoxicillin and ampicillin [80]. Postcolumn derivatization is also possible using fluorescamine 0.1 μg mL^{-1} was the LOD for amoxicillin in human serum with this approach [81]. It is also possible to get good sensitivity for amoxicillin and ampicillin without derivatization, exciting at 235 nm (λ_{em} = 310 nm)

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[80] W. Luo, E.B. Hansen Jr., C.Y.W. Ang, J. Deck, J.P. Freeman, H.C. Thompson Jr., J. Agric. Food Chem. 45 (1997) 1264
[81] H.J. Mascher, C. Kikuta, J. Chromatogr. A, 812 (1998) 221

but a preconcentration step using SPE is required [82].

4.3.3. Electrochemical detection

Sulphur-containing β-lactams can be determined using electrochemical methods such as pulsed electrochemical detection (PED). Sulphur compounds are preadsorbed to the oxide-free noble metal (gold) surface by a non-bonded electron pair from the sulphur group (sulphur cannot be fully oxidized). The adsorbed sulphur moiety is then oxidized concurrently with the gold surface. A detector signal results from analyte oxidation and gold oxide formation. Unfortunately, formation of the gold oxide is associated with unstable baselines and large background signals. While in PED, current is measured after a pulse and a short delay, to allow charging current to decay, in integrated pulsed amperometric detection (IPAD), current is integrated continuously during a cycle where the electrode is oxidized and then reduced back to the original state. The advantage of IPAD is that the contribution of surface oxide formation from the detector signal is removed and the effect on the baseline is greatly minimized. IPAD has been used to determine cephapirin and ampicillin in milk samples and no derivatization was required. Better sensitivity and selectivity was achieved than that obtained using UV detection [83].

The description of the methods for the analysis of β-lactam antibiotics by HPLC using different detection techniques is included in table 4.

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[83] C.O. Dasenbrock, W.R. LaCourse, Anal. Chem. 70 (1998) 2415

Table 4. Determination of β-lactams by HPLC with other detection techniques

Sample	Analyte	Sample treatment	Determination technique	Recovery (%)	LODs	Ref.
No sample	PENG, PENV, DICLOX		**C_{18} LC-CLD** 35% MeOH-0.01M NaH_2PO_4 PDr: 10^{-4} M Luminol in 0.001M NaOH, 0.03% H_2O_2 in 0.001M NaOH	nr	nr	[76]
Human blood	CCL	Dr Py/CIPIC at 80ºC C, F	**C_{18} LC-FL** 34% ACN-10% 0.1M TrEA CL detection H_2O_2, borate bf (pH 9.5), ACN	94-106	10 mol/25 µL 1.0 pmol/25 µL	[78] [77]
Catfish and salmon tissues	AMOX	Ex phosphate bf (pH 4.5) C_{18} SPE Dr TCA-formaldehyde at 100°C	**C_{18} LC-FL** 80% ammonium formate (0.05M, pH 5.8) - 20% ACN.	67.0-82.0	0.5 µg kg^{-1} catfish 0.8 µg kg^{-1} salmon	[79]
Bovine milk	AMOX, AMPI	Add 0.01M KH_2PO_4 + Na_2WO_4 + H_2SO_4 C_{18} SPE Dr salicylaldehyde	**ODS-3 LC-FL** ACN:0.02 M KH_2PO_4 bf, pH 5.5 Gradient	81.6-87.0	1.1 ng mL^{-1} AMOX 1.0 ng mL^{-1} AMPI	[80]
Human serum and plasma	AMOX	Add $HClO_4$ + sodium acetate Dr fluorescamine	**C_{18} LC-FL** 0.02 M methanesulfonic acid:ACN (92:8, v/v)	100.2-106.8	0.1 µg mL^{-1}	[81]
Human urine	AMOX, amoxicilloic acid,	Method 1: Ex MeOH + ACN Method 2: SPE with Bond Elut	**C_{18} LC-FL** 0.1% Formic acid: ACN gradient	Method 1: 55-91.4 Method 2: 46.6-98.0	Method 1: 0.24 µg mL^{-1} AMOX, 0.24 µg mL^{-1} amoxicilloic acid Method 2: 0.02 µg mL^{-1} AMOX, 0.04 µg mL^{-1} amoxicilloic acid	[82]
Raw milk	AMPI, CEP	C, solidification fat layer, add ACN, F, Ev, C_{18} SPE, F	**C_8 LC-IPAD** ACN-acetate bf (pH 3.75)-water	67-80[a]	5 µg L^{-1}	[83]

AMOX, Amoxicillin; AMPI, Ampicillin; CCL, Cefaclor; CDN, Cephradine; CDX, Cefadroxil; CEP, Cephapirin; CLX, Cephalexim; DICLOX, Dicloxacillin; PEN, Penicillin; PENG, Penicillin G; PENV, Penicillin V

ACN, acetonitrile; bf, buffer; C, centrifugation; CLD, chemiluminescence detection; CIPIC, 4-(2´-cyanoisoindolyl) phenylisothiocyanate; Dr, derivatization; Ev, evaporation; Ex, extraction; F, filtration; FL, fluorescence; IPAD, integrated pulsed amperometric detection; MFS, micro-flow system; nr, no reported; PDr, Postcolumn derivatization; Py, pyridine; TCA, tricloroacetic acid; TrEA, triethylamine; V, vortex;

[a]Recovery range covers all the analytes and concentrations.

4.4. Analysis of β-lactam antibiotics by CE with optical detection

4.4.1. Food analysis

In relation to food analysis, residues of some isoxazolylpenicillins (oxacillin, cloxacillin and dicloxacillin) at trace levels have been monitored in milk samples by CZE-UV/Vis [84]. This method comprised the application of an on-line preconcentration technique, large volume sample stacking using the EOF pump (LVSEP) and the use of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as selective complex-forming background electrolyte additive. LVSEP was successfully applied to improve the sensitivity, where the EOF in the buffer zone was suppressed by using an acidic buffer. Based on the relationship between β-CD concentration and migration time, the association constants for isoxazolylpenicillins binding with HP-β-CD were calculated, being 27.3, 34.9, and 48.5 M^{-1} for oxacillin, cloxacillin and dicloxacillin respectively. These values were proportional to their hydrophobic properties and steric hindrances. A simple and easy-manipulative sample preparation method was developed and validated, being necessary only 0.1 mL of milk sample for the analysis of isoxazolylpenicillins to meet EU guideline requirement in relation to MRL. Another method for the simultaneous determination of 7 β-lactam antibiotics (nafcillin, dicloxacillin, cloxacillin, oxacillin, ampicillin, penicillin G, amoxicillin) in milk samples of different origins has been proposed by Bailón-Pérez *et al.*, by using capillary zone electrophoresis (CZE) [85]. Also, the coupling of an off-line solvent extraction SPE method for preconcentration and sample clean-up with the on-line LVSS procedure with polarity switching, similar to that proposed in reference [87], provided LODs for all the studied analytes below their MRLs established in milk by the EU Regulation. Satisfactory recoveries ranging from 86 to 93% were obtained for different kind of milk samples.

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[85] M.I. Bailón-Pérez, A.M. García-Campaña, C. Cruces-Blanco, M. del Olmo-Iruela Electrophoresis, 28 (2007) 4082

4.4.2. Environmental analysis

These compounds are neutral or weakly ionic molecules, so MEKC is often the mode of CE to separate them, although some methods based on CZE have been also published, as it is shown in table 5. In the case of water samples, considering the low level of residues and the inherent lack of sensitivity of UV/Vis detection in CE, two approaches were compared in order to increase the sensitivity in the determination of ceftiofur, one based on the application of an on-line stacking technique, such as large volume sample stacking (LVSS) and other based on an in-line SPE [86]. LVSS allowed on-column electrophoretic pre-concentration, without modifying the separation capillary. In-line SPE-CZE was developed using a home-made micro-cartridge filled with a reversed-phase sorbent (C18) and coupling the micro-cartridge in-line near the separation capillary inlet. LVSS and in-line SPE-CE allowed automated operation and improved sensitivity for ceftiofur determination with respect to conventional CE. When environmental water samples were analyzed, an additional pretreatment step based on off-line SPE was necessary in both cases to further decrease LODs. In terms of ceftiofur determination sensitivity for river water, the combination of off-line SPE with in-line SPE-CE was the most sensitive option. Also off-line SPE for preconcentration and sample cleanup and on-line preconcentration by means of LVSS were applied in the determination of 7 penicillins (nafcillin, dicloxacillin, cloxacillin, oxacillin, ampicillin, penicillin G, amoxicillin) in wastewater, groundwater and river water samples, by using CZE with UV detection [87]. In the case of river and water samples, only an Oasis HLB cartridge, where the analytes were retained, was needed in order to concentrate the samples. However, considering the complexity of wastewater, a second cartridge of alumina N (a polar sorbent) was required for cleanup. With these preconcentration strategies, this method was satisfactorily applied for the monitoring of these residues in water at the low μg L^{-1} levels. Average recovery from water fortified with these penicillin residues at different concentrations was 94-99%, with RSD lower than 10%.

Micellar electrokinetic chromatography (MEKC) was applied in water samples for the separation and quantification of at least 6 penicillins (amoxicillin, ampicillin,

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[86] P. Puig, F. Borrull, M. Calull, F. Benavente, V. Sanz-Nebot, J. Barbosa, C. Aguilar, Anal. Chim. Acta. 587 (2007)208

[87] M.I. Bailón-Pérez, A.M. García-Campaña, C. Cruces-Blanco, M. del Olmo Iruela, J Chromatogr. A, 1185 (2008) 273

penicillin G, oxacillin, penicillin V and cloxacillin), in farmer water samples [88]. Also, in order to know what kind of peniclillin G was administered to the animal (as procaine or benzathine salts) an alternative method was proposed to separate both of them, by another buffer (0.3 M sodium dihydrogen phosphate, pH 5) different to that optimized for the multirresidue method. If a peak corresponding to penicillin G was found when a real sample was analysed by using the multiresidue method, the same sample could be re-analysed by using the alternative one, in order to know if penicillin G-procaine salt or penicillin G-benzathine salt is present in the sample analysed. The determination of penicillins in environmental samples was directly carried out without any preconcentration step, because the main objective of this work was the optimisation of a CE screening method for penicillins. The LOD achieved with this method could be lowered by using a SPE concentration step into the sample preparation process.

To increase sensitivity in the determination of some β-lactam antibiotics (ampicillin, amoxicillin, cephradine and cephalexin) in environmental water, MEKC was coupled with laser induced fluorescence detection (LIF), including a previous derivatization step with sulfoindocyanine succinimidyl ester (Cy5) [89]. Water samples were enriched by SPE by passage through a weak base cation, Amberlite IRA-93, exchange column. Sodium dodecyl sulphate (SDS) micelles play important roles in the experimental steps involved in the whole analytical process by improving yield (sensitivity) and kinetics of the labelling reaction, eluting the retained antibiotics from the SPE pre-concentration system, and electrophoretically resolving their Cy5-derivatives. The optimum procedure included an antibiotic derivatization step at 25°C for 10 min and a direct injection for MEKC analysis, conducted in approximately 15 min. Very low LODs and RSDs (within-day precision) of 3.5-5.9% were obtained for water containing antibiotics, with average recoveries between 96.4-99.4%. This proposed method is a straightforward, sensitive tool to determine antibiotics in environmental waters.

4.4.3. Clinical and pharmaceutical analysis

Some cephalosporins were analysed in human blood plasma by CE with UV detection [90,91] and CZE-SPE on-line was used with a T-split interface [92]. This

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[90] G. Castaneda Penalvo, M. Nelly, H. Maillols, H. Fabre, Anal. Chem. 69 (1997) 1364

separation technique is also used to determine β-lactams in other kinds of biological fluids like serum [93], cerebrospinal fluids using MEKC methodology [94], bronchial secretion [95] or urine using a high sensitivity cell [96]. Deng *et al*. [97] analysed amoxicillin in human urine using on-line coupled with electrochemiluminescence (ECL) detection.

In several pharmaceutical formulations, CE was used for the determination of this kind of compounds [98,99], although MEKC is more frequently used than CZE [100-103].

The description of the methods for the analysis of β-lactam antibiotics by CE using optical detection is included in table 5.

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[94] Y. Chou, Y. Yang, J. Chen, C. Kuo, S. Chen, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 856 (2007) 294
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[102] G. Pajchel, K. Michalska, S. Tyski, J. Chromatogr. A, 1087(2005) 197
[103] K. Michalska, G. Pajchel, S. Tyski, J. Chromatogr. A, 1216 (2009) 2934

Table 5. Determination of β-lactams by CE with optical detection

Sample	Analyte	Sample treatment	CE mode and Characteristics	LOD	Ref.
River water	Ceftiofur	C18 SPE and elution with ACN	**In-line SPE (C_{18})-CZE-DAD or LVSS-CZE-UV**: 25 mM sodium acetate buffer (pH 5); 25 kV; 25°C; Capillary 55 cm (46.5 cm det) × 75 µm i.d. (254 nm)	0.010 μgL^{-1} (in-line SPE) 0.100 μgL^{-1} (LVSS)	[86]
Wastewater, groundwater and river water	7 Penicillins	River and groundwater: Oasis HLB SPE. Wastewater: Oasis HLB and Alumina N SPE cartridges	**LVSS-CZE-UV** (220 nm). 175 mM Tris - 20% ethanol (pH 8); 30 kV; 30°C; Capillary: extended light path (150 µm) 64.5 cm × 75 µm i.d.	0.08-0.80 µg L^{-1}	[87]
Environmental waters	Ampicillin, amoxicillin, cephradine, cephalexim	Amberlite IRA-93 SPE in a continuous-flow system eluted with 30 mM sodium borate at pH 8.35 and 5 mM SDS. Formation of a fluorescence derivative with Cy5	**MEKC-LIF** (635 nm). 35 mM borate - 15 mM SDS (pH 9.3); 15 kV; 25°C; Capillary: 57 cm × 50 µm i.d.	0.03-0.045 µg L^{-1}	[89]
Farmer water samples	Amoxicillin, ampicillin, penicillin G, oxacillin, penicillin V, cloxacillin	Filtration	**MEKC-UV** (210 nm). 40 mM borate -100 mM SDS (pH 8.5); 10 kV; 20°C; Capillary: 57cm × 75 µm i.d.	140-270 µg L^{-1}	[88]
Plasma	Cefoperazone ceftiofur	SPE on-line: C18 micro-precolumn, desorcion with ACN	**CZE-UV:** ammonium acetate, 25 mM pH 7.5	100 µg L^{-1}	[92]

Table 5. Continue

Sample	Analyte	Sample treatment	CE mode and Characteristics	LOD	Ref.
Milk	Oxacillin, cloxacillin, dicloxacillin	Extraction with ethyl acetate, dry with N_2, dissolution with water and rinse with hexane	**LVSEP-CZE-UV:** (206 nm). 50 mM phosphoric acid (pH 3.6) - 5.2 mM HP-β-CD; 30 kV; 25°C; Capillary: 49 cm × 75 µm i.d.	2 µg L^{-1}	[84]
Bovine raw and skimmed milks and goat raw milk	7 Penicillins	Extraction with ACN, evaporation and reconstitution with phosphate buffer (pH 8.5). Oasis HLB and Alumina N SPE.	**LVSS-CZE-UV:** (220 nm). 175 mM Tris - 20% ethanol (pH 8); 25 kV; 30°C; Capillary: extended light path (150 µm) 64.5 cm × 75 µm i.d.	2-10 µg L^{-1}	[85]
Plasma	cefotaxime	ACN for proteins denaturalization	**MEKC-UV:** phosphate buffer, pH 8.00 + 165 mM SDS **CZE**: borate buffer, pH 9.2	MECK: 1 mgL^{-1} CZE: 2mgL^{-1}	[90]
Plasma	Cefpirom, cefotaxim, cefuroxim cefodizim	Dilution in water (1:5, v/v)	**CZE-UV:** Phosphate 20 mM, pH: 7.2	2-6 mgL^{-1}	[91]
Serum	Meropenem	Any pretreatment	**MEKC-UV:** tetraborate 25 mM, pH: 10 + SDS 90 mM	2 mg L^{-1}	[93]
Human plasma and cerebrospinal liquid	Meropenem	Plasma: SPE on C18 cartridge Cerebrospinal liquid: any pretreatment	**MEKC-UV**: Tris buffer 40 mM, pH 8.0 + SDS	Plasma: 0.2 mg L^{-1} Cerebrospinal liquid: 0.3 mg L^{-1}	[94]

Table 5. Continue

Sample	Analyte	Sample treatment	CE mode and Characteristics	LOD	Ref.
Bronchial secretions	cefazolin, cefepim, cefamandol, cefuroxime, ceftazidim, ceftriaxo	Lyophilization and dissolution in methanol:water (1:1, v/v)	**MEKC-UV:** borate, 25 mM, pH9.1 + SDS 50 mM	0.42–0.84 mg mL^{-1}	[95]
Human plasma and urine	meropenem	Urine: dilution with buffer (1:3, v:v) plasma: dilution with ACN (1:3, v:v)	**CZE-UV:** pH 7.2, 10 mM phosphate	Plasma: 0.5 mg L^{-1} Urine: 0.3 mg L^{-1}	[96]
Human urine	amoxicillin	Add: 2 ml of ethylacetate + 50 µl of 0.1 M NaOH, centrifugation, evaporation upper layer and dissolved in water	**CE –ECL:** detection 10 mM, pH 8.5 phosphate buffer	0.31 ngmL^{-1}	[97]
Cefradine for injection	cefradine, cephalexin	Dilution with water	**CZE-UV:** phosphate buffer, 50 mM pH: 6.5	Cefradine: 5.0mgL^{-1} Cephalexin: 1.7 mgL^{-1}	[98]
Pharmaceutical formulations	doripenem	Dilution with water	**CZE-FASS-UV**: phosphate buffer 100 mM, pH 2.9 + 10% (v/v) of methanol	3.0 mg mL^{-1}	[99]
Intravenous preparation	Ticarcillin, clavulanic acid	Dilution with water	**MEKC-UV**: 0.02 M of phosphate solution + 1.44% SDS, pH: 8.66	0.04 mg mL^{-1}	[100]
Pharmaceutical formulations	9 Penicillins	Dilution with water	**MEKC-UV**: Borate buffer 26 mM, pH 8.5 + 100 mM SDS	0.35-1.42 mg L^{-1}	[101]
Pharmaceutical formulations	phenoxymethylpenicillin and its related substances	Dilution with water	**MEKC-UV**: phosphate-borate buffer,0.02 M, pH 6.3, + 69 mM SDS + 12.5 mM PS	1-2 mg L^{-1}	[102]

Table 5. Continue

Sample	Analyte	Sample treatment	CE mode and Characteristics	LOD	Ref.
Pharmaceutical formulations	ertapenem and its impurities	Dilution with water	**MEKC-UV**: 60 mM of NaH_2PO_4 and 20 mM H_3BO_3 buffer, pH 6.0 + 80 mM SDS	0.3 mg L^{-1}	[103]

Cy5, sulfoindocyanine succinimidyl ester; CZE, capillary zone electrophoresis, ECL, electrochemiluminescence; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; LIF, laser induced fluorescence; LVSEP, large volume stacking using the electroosmotic flow pump; LVSS, large volume sample stacking; MEKC, micellar electrokinetic chromatography; SDS, sodium dodecyl sulphate; FASS, field-amplified sample stacking

4.5. Analysis of β-lactam antibiotics by CE with mass spectrometry detection

The coupling of CE with MS detection for monitoring of residues in food and environmental samples [104-107] is an attractive alternative to LC-MS, which combines the advantages of both techniques but this strategy has been scarcely used in the determination of antibiotics [108-111].

The sheath-flow interface is the most commonly used for the coupling of CE with MS, being commercially available and offering flexibility in the selection of CE buffers [112]. The interface uses an additional flow of liquid, known as a sheath or make-up liquid, that mixes with the CE effluent as it exits the separation capillary. The added flow serves for different purposes: 1) to establish electrical contact

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between an electrode and the background electrolyte (BGE) inside the capillary in order to drive the CE separation and the ESI process; 2) to modify the composition of the CE electrolyte to make it more compatible with ESI and MS detection; 3) to increase the liquid flow to levels comparable to those found in liquid chromatography.

The most common interface of this type is the coaxial sheath-flow arrangement, where a continuous flow of sheath liquid is delivered through a tube that surrounds the separation capillary terminus. The advantage in this case is that there is no dead volume, so the capillary separation is undisturbed by additional flows until the analytes have exited the separation capillary. However, significant flow rates for the sheath liquid are required in order to operate stably in this manner, leading to significant dilution of the analytes. Also, the flow of sheath liquid around the capillary terminus can create suction that may lead to a parabolic flow profile and reduced resolution. The currently available commercial CE–MS interfaces employ a three-tube coaxial design. The innermost tube is the separation capillary, which protrudes slightly from the second tube through which the sheath liquid flows. The third, outer tube delivers a flow of sheath gas to help improve spray stability and solvent evaporation from the generated micro droplets.

To ensure a stable ESI flow, the sheath liquid is usually introduced at a greater flow rate (several microliters per minute) than the CE flow rate. The capillary column eluent is thus diluted at the mixing point. The behavior of the MS detector depends on the flow rate and the specific interface design. With the introduction of the dilution factor, the sensitivity of the MS response is lowered, which is a limitation in trace analysis. The distance between the CE capillary and the sheath tubing should be minimized to provide good ESI current stability and a small mixing volume at the tip so that band broadening caused by diffusion is kept to a minimum.

4.5.1. Food and environmental analysis

The coupling of CE-MS has been scarcely used in the determination of β-lactam antibiotics in food and environmental matrices, apart from some multiresidue methods. In our group, a new CE-MS/MS method was developed for the simultaneous identification and quantification of 9 penicillins of human and veterinary use (nafcillin, dicloxacillin, cloxacillin, oxacillin, ampicillin, penicillin G,

amoxicillin, penicillin V and piperacillin) in chicken muscle and water samples [113]. CE separation conditions, sheath liquid composition and ESI parameters were carefully optimized to reach high sensitivity and precision. For confirmatory analysis, MS/MS experiments were carried out using an ion trap as analyzer operating in multiple reaction monitoring (MRM) mode to achieve the minimum number of IPs established by the EU Decision. The use of a SPE procedure in two steps, combining Oasis HLB and Alumina N cartridges provided a satisfactory preconcentration and cleanup treatment for chicken muscle samples after extraction of the compounds with ACN. Only Oasis HLB cartridges were necessary in water samples to obtain adequate recoveries. The method has been characterized for its use in meat and water samples, using matrix matched calibrations. For chicken muscle samples, LODs were below MRLs permitted for this matrix and in the case of water samples, the sensitivity reached ensures its satisfactory application in aquatic samples.

A specific CE-MS method was developed for the simultaneous determination of twelve antibacterial (four sulphonamides, four quinolones and four β-lactams) residues in fish and livestock [114]. Separation conditions, sheath liquid composition and electrospray parameters were optimized to obtain adequate CE separation and high sensitivity. The minimum number of IPs, according to the 2002/657/EC European Decision, was achieved using an IT in MRM mode. For quantification in meat and fish samples, a two-step procedure was developed using ACN to extract the analytes and to precipitate the proteins and dispersive SPE, with C18 to clean up the extract. The LODs were in all cases lower than the maximum residue limits for these compounds in these kinds of matrices. The obtained results indicate the potential of CE-MS for the analysis of multiclass antibacterial residues in food.

4.5.2. Clinical and pharmaceutical analysis

A method for the determination of penicillin V together with its impurities and byproducts formed during biosynthesis, using CE with UV and ESI-MS detection was developed and applied to a real fermentation broth [115]. The obtained

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[114] A. Juan-García, G. Font, Y. Picó, Electrophoresis, 28 (2007) 4180
[115] E.F. Hilder, C.W. Klampfl, W. Buchberger, P.R. Haddad, Electrophoresis, 23 (2002) 414

results indicate the suitability of aqueous and nonaqueous CE with either UV or ESI-MS detection for the determination of penicillin V and its related substances. For the determination of the minor impurities and by products in a typical penicillin V fermentation broth, the aqueous system (using either UV or ESI-MS detection) was found to be superior to non aqueous capillary electrophoresis (NACE) in terms of both selectivity and sensitivity. Additionally, the CE methods developed offer much faster analysis times than any other previous methods for the determination of these analytes in such a mixture, therefore demonstrating a great potential for such samples.

Recently, a CE-MS method has been developed to analyze the profile anionic metabolites of urine from antibiotics treated rats [116]. CE-MS method for the global profiling of anionic metabolites, requiring only dilution (1:1, v/v) with BGE as sample treatment, was applied to urine samples from rats receiving various antibiotic treatments. CE-MS uniquely revealed ten metabolites modulated by antibiotic exposure. This clearly demonstrates the added value of CE-MS for nontargeted profiling of small anionic metabolites in biological samples.

The description of the methods for the analysis of β-lactam antibiotics by CE using MS detection is included in table 6.

[116] M.G.M. Kok, M.M.A. Ruijken, J.R. Swann, I.D. Wilson, G.W. Somsen, G.J. de Jong, Anal. Bioanal. Chem. 405 (2013) 2585

Table 6. Determination of β-lactams by CE with MS detection

Sample	Analyte	Sample treatment	CE mode and characteristics	LOD	Ref.
Chicken muscle River and well waters	9 Penicillins	River and well waters: SPE (Oasis HLB); Chicken muscle: Extraction with ACN and SPE (Oasis HLB and Alumina N for cleanup)	**CZE-MS/MS:** 60 mM ammonium acetate (pH 6.0); 30 kV; 30°C; Capillary: 96 cm × 50 µm i.d. Sheath liquid: 2-propanol/water/formic acid (40:57:3 v/v/v).	8-12 µg Kg^{-1} (chicken muscle) 0.18-0.26 µg L^{-1} (water samples)	[113]
fish and livestock	4 sulfonamides, 4 quinolones, 4 β-Lactams	ACN to ext. and to ppt. the proteins and dispersive SPE, with C18	**CZE-MS/MS:** 60 mM ammonium acetate buffer at pH 8 + 10% of methanol	< 18 $ngkg^{-1}$	[114]
fermentation broth	Penicillin V and its impurities	Filtration and diluted with ACN (1:1)	**CZE-MS/MS:** 20 mM Ammonium acetate injection, pressure, 50 mbar,18 s; detection, negative ion mode; sheath flow, 4 $nLmin^{-1}$ of 0.1% ammonia in (8:2 v/v) 2-propanol/water.	nr	[115]
Rat urine	Penicillin G	Filtration and diluted with BGE (1:1)	**CE-MS (TOF):** 25 mM TEA (pH 11.7). The sheath liquid: 5 mM TEA in water–methanol (1:1, v/v) at 5 µL min^{-1}	nr	[116]

ACN, Acetonitrile; nr, not reported; TEA, triethylamine; TOF, time of flight.

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"*Advances in the determination of β-lactam antibiotics by liquid chromatography*". F.J. Lara, M. del Olmo-Iruela, C. Cruces-Blanco, C. Quesada-Molina, A.M. García-Campaña. Trends in Analytical Chemistry 38 (2012) 52-66.

CAPÍTULO 3

Análisis de residuos de cefalosporinas en muestras de agua mediante electroforesis capilar en zona combinada con técnicas de preconcentración en línea y extracción en fase sólida

CHAPTER 3

Analysis of cephalosporin residues in environmental waters by capillary zone electrophoresis with off-line and on-line preconcentration

RESUMEN

Se ha desarrollado y validado un método sensible y exacto para la determinación de cefalosporinas a nivel de trazas en muestras de agua de manantial, agua de pozo y agua de río utilizando para ello la electroforesis capilar en zona con detección UV por diodos en fila (CZE-DAD). Debido a la falta de sensibilidad de este tipo de detección, se ha aplicado un procedimiento de extracción en fase sólida (SPE) en el que además de limpiar las muestras de agua se preconcentran los analitos y este tratamiento de muestra se ha combinado con el método de preconcentración dentro del propio capilar denominado (large volumen simple stacking, LVSS) ya aplicado en los capítulos anteriores. La determinación simultánea de cinco cefalosporinas de uso veterinario (cefalexina (CL), cefoperazona (CPR), ceftiofur (CT), cefapirin (CP) y cefazolin (CZ)) en muestras acuosas se ha llevado a cabo usando como tampón de separación acetato amónico 70 mM a pH 7, aplicando un voltaje de 22.5 kV y 25°C y utilizando cefadroxil (CD), una cefalosporina de uso humano, como patrón interno. Para el tratamiento de muestra mediante SPE se utilizaron cartuchos HLB. La combinación de los dos procedimientos de preconcentración proporcionó una mejora significativa de la sensibilidad, obteniendo límites de detección de 0.1 μg L^{-1} para CL, 0.2 μg L^{-1} para CPR, CT y CP, y 0.3 μg L^{-1} para CZ. El procedimiento SPE-LVSS-CZE-DAD es adecuado para el control de estos compuestos en muestras de agua consiguiendo alta sensibilidad y precisión y recuperaciones satisfactorias.

SUMMARY

A sensitive and reliable method using capillary zone electrophoresis with UV-diode array detection (CZE-DAD) has been developed and validated for trace determination of cephalosporin antibiotics in spring water, groundwater and river water matrices. Due to the lack of sensitivity of the UV/Vis detection, a solid-phase extraction (SPE) method has been applied for off-line preconcentration and cleanup of water samples, in combination with the on-line preconcentration methodology named large volume sample stacking (LVSS) with polarity switching, also named stacking with matrix removal (SWMR), applied in the previous chapters. The simultaneous determination of five cephalosporins of veterinary use, named cephalexin (CL), cephoperazone (CPR), ceftiofur (CT), cephapirin (CP) and cephazolin (CZ) in environmental water samples has been accomplished using 70 mM acetate buffer, pH 7.0, applying a voltage of 22.5 kV at 25°C and using cephadroxil (CD), a cephalosporin for human use, as internal standard. For SPE procedure a HLB sorbent column was used. The combination of both off-line and on-line preconcentration procedures provided a significant improvement in sensitivity, obtaining detection limits of 0.1 μg L^{-1} for CL; 0.2 μg L^{-1} for CPR, CT and CP, and 0.3 μg L^{-1} for CZ. The SPE-LVSS-CZE-DAD procedure is suitable for the monitoring of these compounds in water samples with high sensitivity, precision and satisfactory recoveries.

1. Materials and methods

1.1. Chemicals and standard solutions

All the reagents used were of analytical grade and the solvents of HPLC grade. The water used to prepare the solutions was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Organic solvents such as acetonitrile, methanol and 2-propanol were purchased from Merck (Darmstadt, Germany).

The separation buffer was prepared from ammonium acetate supplied by Merck (Darmstadt, Germany). The pH was adjusted to 7.0 with hydrochloric acid obtained from Merck.

Analytical standard of cephoperazone (CPR, CAS: 62893-20-3; purity: 94%), was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Cephalexin (CL, CAS: 15686-71-2; purity: 99.8%), ceftiofur (CT, CAS: 80370-57-6; purity: 99.4%), cephapirin (CP, CAS: 24356-60-3; purity: 98%) and cephazolin (CZ, CAS: 27164-46-1; purity: 100%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock standard solutions containing 1000 mg L^{-1} of each compound were prepared by dissolving accurately weighed amounts in ultrapure water and methanol in the case of CT, and stored in darkness at 4°C. Working standard solutions were made daily by diluting them with deionised water. Cephadroxile (CD, CAS: 66592-87-8; purity: 100%), a cephalosporin for human use was used as internal standard and was obtained from Fluka (St. Louis, MO, USA).

The sorbents checked for SPE were Oasis HLB, copolymer of hydrophilic-lipophilic balance (60 mg, 3 mL), Oasis MAX, mixed-mode anion exchange (divinylbenzene-co-N-vinylpyrrolidone, 3 mL, 60 mg) both from Waters Milford (MA, USA), C18 sorbent (DSC-18, 3 mL, 60 mg) from Supelco (Bellefonte, PA, USA) and Lichrolut EN (ethylvinylbenzene-divinylbenzene, 200 mg, 3mL) from Merck (Darmstadt, Germany). Nylon filters with a 0.20 μm pore size (Supelco, Bellafonte, PA, USA) were used.

1.2. Instrumentation and software

CE experiments were carried out with a HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the software provided with the HP ChemStation version A.09.01. Separation

was carried out in a bared fused silica capillary 48.5 cm x 50 µm i.d. (effective length 40 cm) with an optical path length of 200 µm (bubble cell capillary from Agilent Technologies, Waldbron, Germany).

For pH measurements, a pH meter (Crison model pH 2000, Barcelona, Spain) was employed with a resolution of ± 0.01 pH unit.

A vacuum manifold system from Supelco (Bellafonte, PA, USA) coupled with a vacuum pump (Büchi model B-169, Switzerland), an evaporator with nitrogen (System EVA-EC, VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific industries, Bohemia, USA) were used for sample treatment.

Excel 2007 was used for the statistical analysis of data.

1.3. Electrophoretic procedure

Before the first use, the new capillary was rinsed with 1 M NaOH at 60°C for 20 min., then with water for 10 min. and finally with BGE (background electrolyte) solution for 30 min., applying a pressure of 1 bar. After each run, the capillary was flushed with an N_2 pressure of 7 bar for 1 min with 0.1 M NaOH, 1 min with deionized water, and, finally, 2 min with the running buffer to maintain an adequate repeatability of run-to run injections. At the end of the day, the capillary was cleaned with an N_2 pressure of 7 bar for 0.1 M NaOH for 2 min, then with deionized water for 5 min and finally flushed with air for 3 min.

The antibiotics were analyzed using a BGE consisting of an aqueous solution of ammonium acetate 70 mM, pH 6.9. A voltage of 22.5 KV (normal mode) was applied. The cephalosporins were monitored at 250 nm for CL, CPR, and CZ; 270 nm for CP and 292 nm for CT, depending on the maximum absorbance of each analyte. The temperature of the capillary was kept constant at 25°C. Samples were introduced by hydrodynamic injection at a N_2 pressure of 1 bar during 1 min, filling the 100% of the capillary volume. Injection of the samples occurred using the following LVSS procedure.

1.4. LVSS procedure

For the proposed method, firstly standard solutions or samples containing the analytes were loaded at a pressure of 1 bar for 1 min into the electrophoretic system so that the whole capillary was filled with the sample solution. Water: acetonitrile (6:4, v/v) was used as the sample solvent to produce a low-conductivity analyte matrix. A negative voltage (−22 kV) was then applied and the sample stacking started. Sample matrix removal from the capillary was indicated by monitoring the electric current, which progressively increased to its normal value as the low conductivity injected zone was eliminated from the capillary. When the 90% of the normal current was reached, the voltage was stopped and at this stage and the stacking process could be considered complete. A positive voltage (22.5 kV) was then applied to separate the compounds.

1.5. Sample preparation procedure

Different kinds of water samples were analyzed. River water was collected from a local river (Genil River in Granada, Spain); groundwater and spring water were obtained from Sierra Arana (Granada, Spain).

The water samples were collected directly in PVC bottles; they were filtered through nylon membranes with 0.2 µm pore size and were stored at 4°C in the dark. Sample aliquots of 50 mL of water were spiked with different concentration levels of cephalosporins. Then, the pH value was adjusted to 2 with HCl 2.5 M, in order to achieve a pH value lower than pKa values for all the analytes.

A SPE treatment for preconcentration and clean up of the water samples has been developed. An Oasis HLB cartridge was used in order to concentrate the analytes from the samples. It was previously washed sequentially with 5 mL of methanol, 5 mL of ACN and 5 mL of deionized water at pH 2. Without using vacuum, 50 mL of water was applied to the column. A washing step was necessary with 5 mL water: methanol (0.5%). The antibiotics were then eluted with 5 mL of methanol and 5 mL of ACN at 1–2 mL min^{-1}, using a vacuum manifold. This final solution was evaporated to dryness under a gentle N_2 current at 35°C, and then reconstituted with 0.5 mL of a mixture of deionized water: acetonitrile (6:4, v/v), containing 0.2 µg mL^{-1} of CD as IS.

2. Results and discussion

2.1. Optimization of electrophoretic conditions

Firstly the effect of the pH value on the resolution between peaks was investigated over the whole range (2-11) using 50 mM phosphate buffer. The best resolution was achieved at pH 7. Subsequently, we checked the buffer nature which has a high buffering capacity at that pH value: ammonium acetate, ammonium bicarbonate, sodium tetraborate and Tris, at a concentration of 50 mM for all studied buffers. We found better resolution with ammonium acetate buffer for most of the analytes; consequently it was selected as BGE. Then, we checked the pH range of 6-8 (step 0.2 U), finding an optimum pH value of 7.0 since it provided the best peak resolution in a very short analysis time. Different buffer concentrations were tested (50, 60, 70, 80, 90, and 100 mM), selecting 70 mM as optimum concentration in order to obtain the best results, in terms of resolution and analysis time with an adequate electric current. These results are showed in figure III.1.

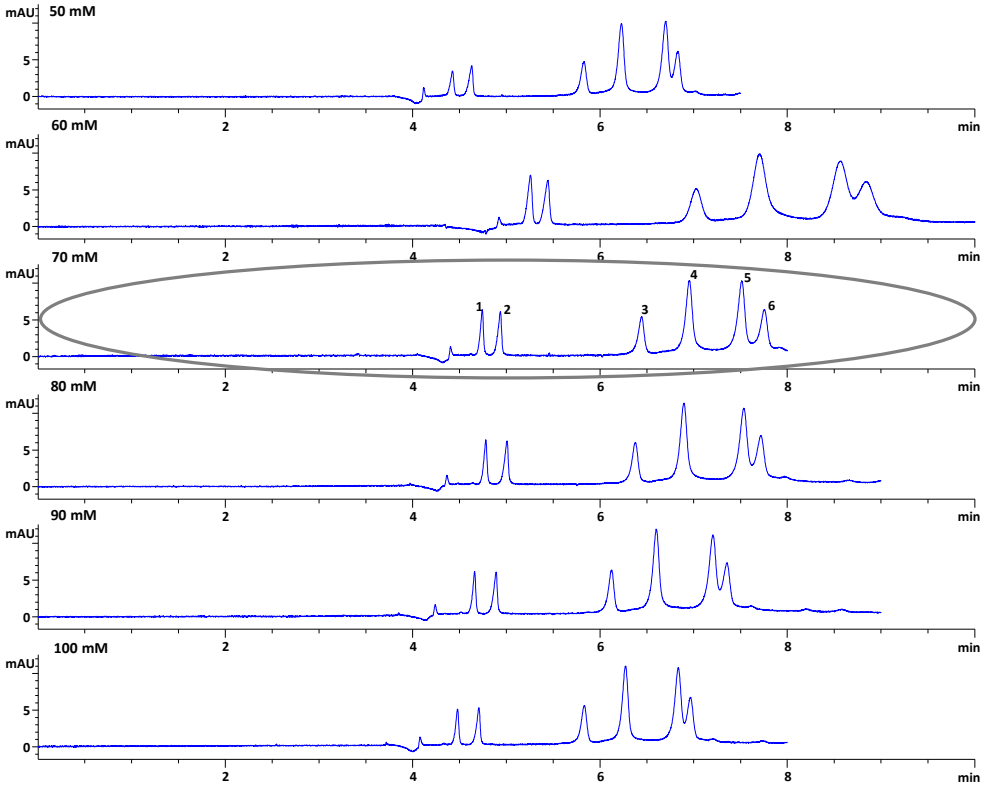

Figure III.1. Electropherograms at different ammonium acetate buffer concentrations. Peaks are numbered for the optimum buffer concentration (70 mM): 1, CD (internal standard), 2: CL, 3: CPR, 4: CT, 5: CP, 6: CZ

The addition of different percentages of organic solvent (5-10%) was also assessed, by using methanol, acetone and acetonitrile, but it did not provide satisfactory results. The applied voltage for the separation was also studied at different values: 15, 17.5, 20, 22.5 and 25 kV, the optimum value found was 22.5 kV.

The effect of temperature was also investigated in the range of 15-35°C. Lower values did not provide an adequate resolution for all the analytes. Higher temperature values were not considered as they increase the noise of the base line. A decrease in the temperature resulted in a decrease in the generated current, a decrease in the EOF, an increase in the migration times and an increase in the resolution. According to these effects, 25°C was selected as a compromise.

2.2. Optimization of variables affecting LVSS with polarity switching

The poor sensitivity of CZE using UV-Vis detection hardly recommends the direct use of this technique because the LODs obtained could not reach the expected levels for the monitoring of cephalosporin residues in environmental samples, such as waters. In order to avoid this inconvenience, we have proposed the use of an on-line sample stacking procedure as a way of trace enrichment in CE for the analysis of these residues in waters. LVSS with polarity switching for anions was chosen as the preconcentration technique because the buffer conditions and polarity allowed its use, considering that all the molecules are negatively charged at the working pH. In starting situation, a large volume of sample prepared in a low conductivity matrix is injected, and a voltage at negative polarity is applied for focusing of zones and removal of sample matrix. When the anions are completely focused and most of the sample matrix is removed, voltage is stopped and polarity is reversed. This occurs when the current reached 90-95 % of its value. Finally a voltage at positive polarity is applied in order to separate and detect the focused zones. With this on-line preconcentration technique we achieved a preconcentration factor of 20 in relation to a normal injection for CZE.

In LVSS, three significant parameters have to be considered: sample solvent, size of the plug sample, and voltage. The most relevant variable in this type of preconcentration methodology is the sample solvent, because it requires a very low conductivity to obtain the best focusing of the analytes. In this case for the CZE method, deionized water was the best option for sample solvent but when applying the stacking procedure, cephalosporins with higher electrophoreric mobility were lost. In order to modify the conductivity, different percentages of some organic solvents were added to the sample solvent: MeOH, ACN and 2-propanol were tested (5, 10, 20, 30, 40 and 50%). Finally ACN 40% was selected because it provides low mobility for CD and CL with high current stability and shorter analysis time. Figure III.2 shows the obtained electropherograms for different percentages of acetonitrile as sample solvent. Even apparently 50 % of ACN provided better resolution, percentages higher of 40% in the sample solvent caused irreproducibility when applying LVSS.

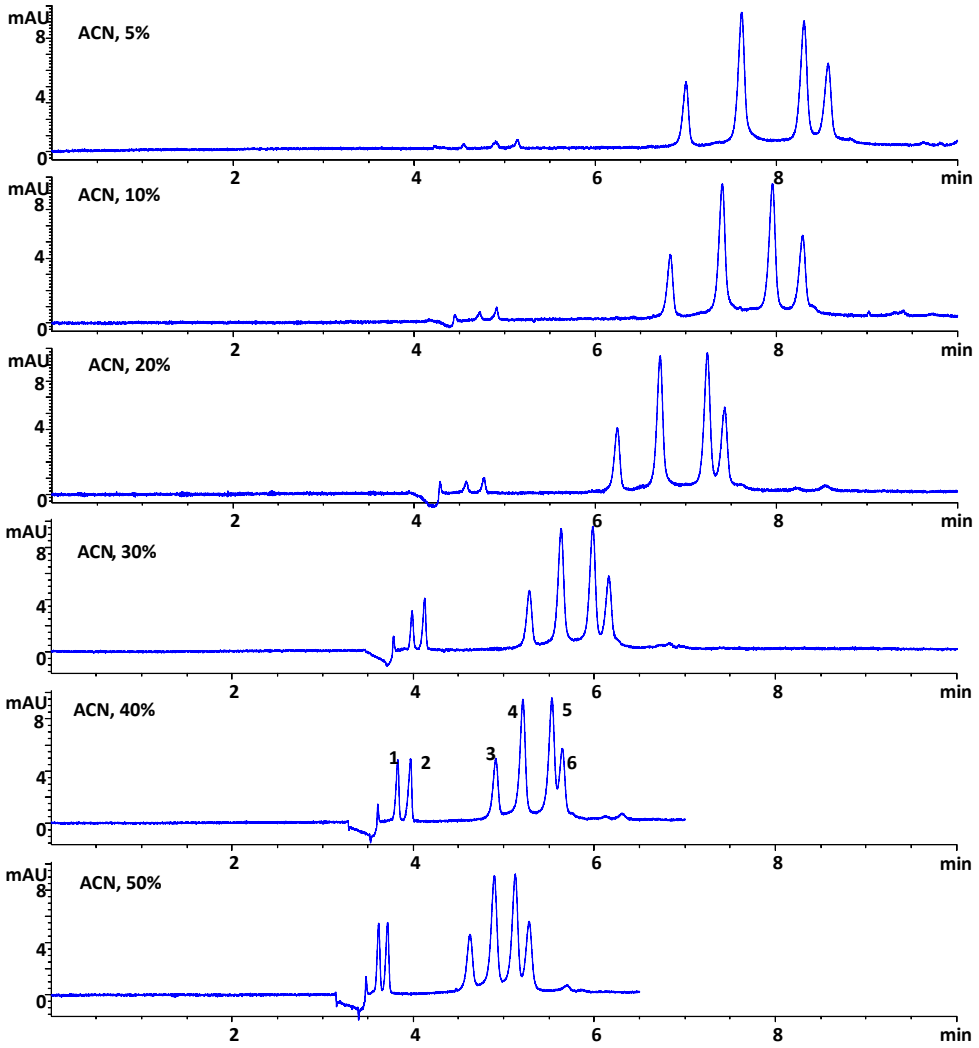

Figure III.2. Electropherograms at different percentages of ACN in the sample solvent. Peaks are numbered for the optimum ACN percentage (40%): 1, CD (internal standard), 2: CL, 3: CPR, 4: CT, 5: CP, 6: CZ

To optimize the size of plug sample, different percentages (70, 80, 90 and 100%) of the capillary were filled in the experience. These percentages were calculated, based on the diameter and length of the capillary and the applied pressure. For this experience, we applied a pressure of 50 mbar and the time was increased from 400 to 600 s. The results showed that it was possible to fill the whole volume of the capillary, so we apply for obtaining the same results with shorter injection time a pressure of 1 bar during 1 min. The negative voltage was studied in the

interval of -15 to -30 kV. Finally, -22 kV was selected as optimum, as this value permits a rapid filling of the capillary in a stable system. Values higher than -25 kV frequently produce the capillary rupture.

As a summary, the optimum experimental conditions used for CZE-LVSS procedure were: fused silica capillary 48.5 cm x 50 µm id, optical path length of 200 µm; separation buffer: ammonium acetate 70 mM at pH 7.0; separation voltage: 22.5 kV; T: 25°C; hydrodynamic injection: pressure = 1 bar, time = 1 min., current inversion limit: 90%; LVSS sample solution: ACN:water (4:6, v/v); LVSS voltage: - 22 kV.

2.3. Optimization of the extraction procedure

To examine the feasibility of the SPE procedure for preconcentration and clean up, sorbents of different natures were tested, such as polymeric sorbents: Lichrolut EN, Oasis HLB, C18 and MAX. Once the water sample (50 mL) had been passed through the different cartridges, we observed that HLB provided the best results in terms of compound retention, recoveries and cleanup of the obtained extracts as figure III.3 shows. Different sample volumes were passed through the HLB cartridge (5, 25, 50, 100 and 250 mL) and we found that for volumes higher than 50 mL the retention capacity of the cartridge was decreased, so 50 mL was selected in order to achieve the maximum sample volume with minimum analyte losses. For the cleanup, different percentages of methanol in water were tested (0, 0.5, 1 and 2%) and the best recoveries were obtained when using 5 mL of water with 0.5% MeOH. Higher percentages of methanol caused losses of analytes in the wash step. Methanol and acetonitrile were checked as elution solvents and we conclude that 5 mL of methanol and 5 mL of acetonitrile provided the best recoveries of the compounds without eluting large quantities of present interferences as it is shown in figure III.4. This procedure showed good results in term of recoveries and peak shapes, achieving a preconcentration factor of 100 at the end of the sample treatment.

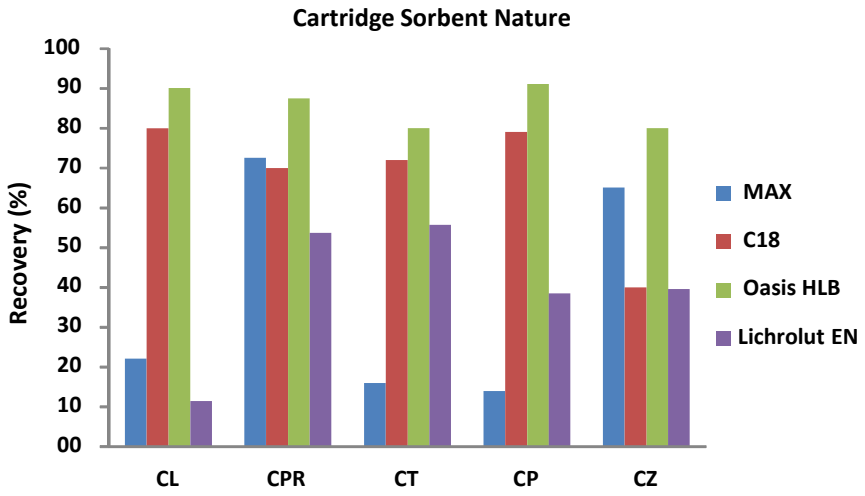

Figure III.3. Obtained recoveries in the extraction of the studied cephalosporins with different sorbents used in the SPE procedure for environmental water samples.

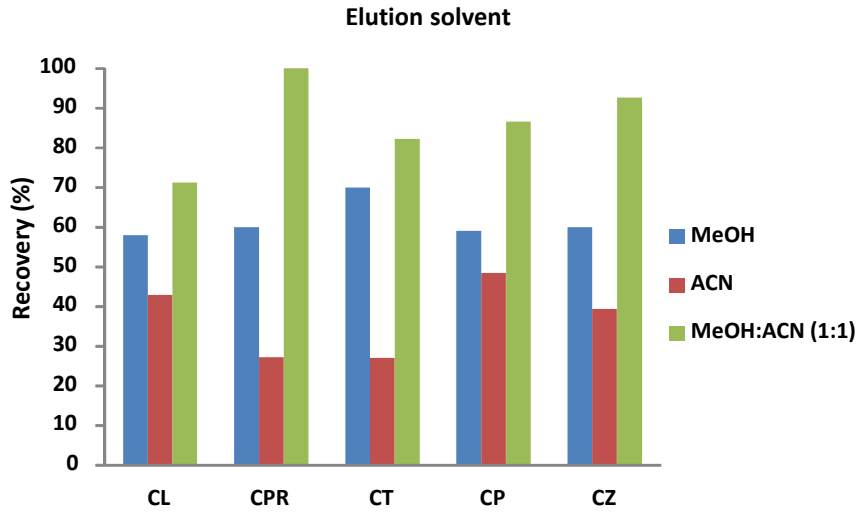

Figure III.4. Obtained recoveries in the extraction of the studied cephalosporins with different elution solvents checked in the SPE procedure for environmental water samples

Figure III.5.A provides the electropherogram of a spring water sample spiked with 3 μg L^{-1} of each compound. Figure III.5.B shows an electropherogram of a blank of spring water treated with the developed procedure, and no matrix peaks were found co-migrating with the analytes.

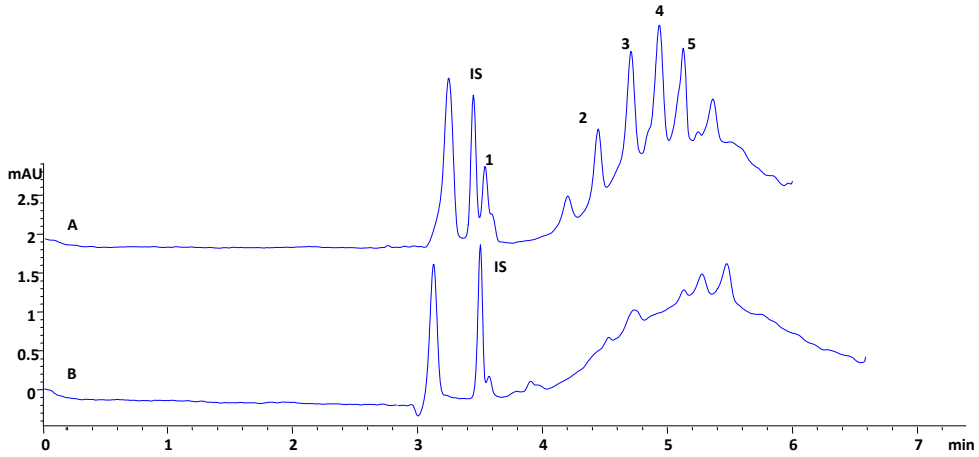

Figure III.5. Electropherogram of A) extract of spring water fortified with 3 µg L^{-1} and B) spring water blank, both spiked with 200 µg L^{-1} of CD as internal standard. Peak identification: 1: CL, 2: CPR, 3: CT, 4: CP, 5: CZ, IS: CD

2.4. Validation of the LVSS-CZE-DAD method

2.4.1. Statistical and performance characteristics

The matrix-matched calibration curves were established for the five analytes in analyte-free spring water samples spiked before the sample treatment and treated according to the sample procedure described above.

The calibration curves were established for six different concentration levels corresponding to 0.5, 1.0, 2.0, 4.0, 6.0 and 10.0 µg L^{-1} for CT and CP; 0.5, 1.0, 2.0, 4.0, 6.0 µg L^{-1} for CL and CPR; 1.0, 2.0, 4.0, 6.0 and 10.0 µg L^{-1} for CZ. In all cases 200 µg L^{-1} of CD was added as IS. Each concentration level was injected by triplicate. Calibration curves were established taking into account the relative corrected peaks areas (as the ratio analyte peak per migration time to IS peak per migration time) as a function of the analyte standard concentration. The statistical parameters, calculated by linear regression and the performance characteristics of the SPE-LVSS-CE method are shown in table III.1. In all cases, the satisfactory determination coefficients obtained confirmed that cephalosporin antibiotics responses were linear over the concentration range studied. The LOD was considered as the minimum analyte concentration yielding an S/N ratio equal to three. The LOQ was adopted as the lowest analyte concentration yielding a signal

10 times greater than the noise. As can be seen, for all the studied compounds the LODs obtained by applying both off-line and on-line preconcentration procedures are at the low μg L^{-1} levels.

Table III.1. Statistical and performance characteristics for the studied cephalosporins in spring water by using the proposed SPE-LVSS-CZE method.

Compound	Intercept	slope	(R^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Linear dynamic range (μg L^{-1})
Cefalexin (CL)	-0.002	0.357	0.992	0.1	0.2	0.1-6
Cefoperazone (CPR)	0.033	0.307	0.992	0.2	0.8	0.2-6
Ceftiofur (CT)	-0.054	0.569	0.995	0.2	0.6	0.2-10
Cefapirin (CP)	0.178	0.653	0.993	0.1	0.5	0.1-10
Cefazolin (CZ)	0.271	0.152	0.992	0.3	1.1	0.3-10

2.4.2. Precision study

The precision of the whole method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of repetitive application of the SPE-LVSS-CZE procedure to spring water samples (three experimental replicates) at concentration levels of 1, 3 and 6 μg L^{-1} for water samples, and each one was injected in triplicate (instrumental replicates). Intermediate precision was assessed for three consecutive days with one water sample injected by triplicate. The results related to concentrations, expressed as relative standard deviation (RSD) of relative peak areas, are given in table III.2. As it can be observed, acceptable precision was obtained in all cases.

Table III.2. Precision study (RSD %) of the proposed method for different concentration levels[a].

Intraday	CL	CPR	CT	CP	CZ
1 μg L^{-1}	2.1	4.5	4.1	4.2	3.4
3 μg L^{-1}	3.3	2.7	2.1	1.1	2.0
6 μg L^{-1}	2.1	2.5	4.8	2.6	0.8
Interday	**CL**	**CPR**	**CT**	**CP**	**CZ**
1 μg L^{-1}	5.8	6.6	5.3	4.9	3.5
3 μg L^{-1}	3.2	3.5	2.2	5.0	4.7
6 μg L^{-1}	4.1	3.3	6.8	4.6	5.6

[a]RSD, Relative standard deviation (n=9)

2.4.3. Trueness of the method: Recovery studies

In order to check the trueness and applicability of the proposed methodology for the analysis of cephalosporins, recovery experiments were carried out in three different analyte-free kinds of water samples. Ground, spring and river water samples were spiked with a mixture of each one of the analytes at three different levels (1, 3 and 6 μg L^{-1}). Each level was prepared by duplicate and it was injected by triplicate. In order to check the presence of possible interferents, blank samples were submitted to the proposed method and no matrix peaks were found co-migrating with the analytes. The obtained values of the recovery study for each compound and the corresponding RSD (%) are shown in table III.3 for all kinds of water samples. As can be seen, the recoveries were satisfactory for most of the compounds in the studied matrixes. Only in the case of CL for groundwater in the two lowest spiked levels the obtained values were low. In this sense, trueness was satisfactory for all the compounds in the whole range of concentration levels studied for river and spring water samples and in the case of groundwater, quantification of CL cannot be carried out with acceptable accuracy.

Table III.3. Recoveries of cephalosporin antibiotics from water samples spiked at different concentration levels.

		River water				
		CL	**CPR**	**CT**	**CP**	**CZ**
1 µg L^{-1}	R (%)	95.2	97.7	111.0	94.6	102.0
	RSD%	9.9	10.0	3.3	3.4	7.9
3 µg L^{-1}	R (%)	99.4	101.9	101.5	100.2	103.5
	RSD%	0.8	3.2	0.5	0.9	7.8
6 µg L^{-1}	R (%)	100.8	84.1	97.9	101.2	100.4
	RSD%	0.9	7.9	2.6	3.5	1.9
		Groundwater				
		CL	**CPR**	**CT**	**CP**	**CZ**
1 µg L^{-1}	R (%)	14.0	112.0	87.4	96.9	95.4
	RSD%	22.9	1.9	18.1	15.8	10.4
3 µg L^{-1}	R (%)	53.9	80.7	98.3	87.3	103.9
	RSD%	30.7	19.2	27.2	7.6	10.7
6 µg L^{-1}	R (%)	104.0	103.6	108.5	98.2	94.5
	RSD%	1.7	20.5	23.5	20.3	14.5
		Spring water				
		CL	**CPR**	**CT**	**CP**	**CZ**
1 µg L^{-1}	R (%)	102.9	93.6	91.7	100.4	97.0
	RSD	1.2	0.8	3.3	3.6	0.6
3 µg L^{-1}	R (%)	100.0	97.4	97.8	90.4	89.8
	RSD	0.1	3.5	1.8	2.9	3.8
6 µg L^{-1}	R (%)	100.6	101.5	109.0	98.9	98.8
	RSD	0.3	2.3	2.1	4.2	1.2

R, Recovery (%) ; RSD, Relative standard deviation (n=6)

3. Concluding remarks

A sensitive and rapid electrophoretic method has been developed and validated for the analysis of five cephalosporins, i.e. cephalexin, cephoperazone, ceftiofur, cephapirin, and cephazolin. Prior to the CE analysis, the method needs only SPE necessary for preconcentration and sample clean-up. On-line preconcentration using LVSS has been carried out, improving the sensitivity of the detection when it is combined with the off-line preconcentration step, making the method suitable for the monitoring of these cephalosporins in water samples in the very low μg L^{-1} range. The separation takes place in less than 7 min. This new combined method provides satisfactory recoveries for most part of the compounds in the selected matrices. The LODs obtained are low enough for detecting these compounds, being among the fastest and sensitive methods for the simultaneous determination of these cephalosporin antibiotics in these kinds of samples.

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CAPÍTULO 4

Extracción de cefalosporinas asistida por sales previa formación de par iónico y análisis mediante cromatografía líquida capilar en muestras de agua y carne

CHAPTER 4

Ion-paired extraction of cephalosporins in acetone prior to their analysis by capillary liquid chromatography in environmental water and meat samples

RESUMEN

En este capítulo se propone un método de extracción líquido-líquido basado en la formación de pares iónicos en medio acuoso con las cefalosporinas seleccionadas, previa a su extracción con acetona, añadiendo sulfato amónico a una mezcla acetona:agua (1:2, v/v), para su posterior análisis usando la cromatografía líquida capilar (HPLC capilar) en fase inversa. La técnica de HPLC capilar muestra algunas ventajas en comparación con HPLC convencional, tales como mejor resolución, límites de detección inferiores y un menor consumo de disolventes, siendo más respetuosa con el medioambiente. Los compuestos analizados son cefoperazona (CPR), cefquinoma (CQ), cefalexin (CL), cefapirin (CP), cefalonium CLN), cefamandol (CM), cefazolin (CZ) y cefadroxil (CD).

Para la formación del par iónico se ha utilizado el bromuro de hexadeciltrimetilamonio (CTAB), de naturaleza catiónica, a una concentración de 0.9 mM y como solución acuosa un tampón fosfato 10 mM a pH 8. La metodología aplicada, denominada extracción líquido-líquido asistida por efecto salino (SALLE) incluye la adición de 1.25 g de sulfato amónico, como agente salino.

La separación de las cefalosporinas se llevó a cabo usando una columna capilar Luna C18 (150 mm×0.3 mm, 5 µm, 100 Å) trabajando en modo gradiente en el que se combinan fracción A (0.1% ácido fórmico en agua, pH 4) y fracción B (acetonitrilo:metanol (1:1, v/v)), con un caudal de 20 µl min^{-1}. La temperatura de la columna fue de 35°C y el volumen de inyección 7 µl, con detección UV a 250 nm. Los límites de detección para los compuestos estudiados estaban comprendidos entre 1.3 y 6.8 µg L^{-1} para las muestras de agua y 1.2 y 10.1 µg kg^{-1} en el caso de muestras de carne, inferiores a los límites máximos de residuos permitidos por la EU para este tipo de alimentos. La metodología desarrollada ha demostrado ser adecuada para el análisis de estos antibióticos, ampliamente utilizados, en muestras de agua y carne, incluyendo músculo de ternera y cerdo, con alta sensibilidad y precisión y recuperaciones satisfactorias.

SUMMARY

In this chapter, ion-pair extraction of cephalosporins from aqueous solution into acetone by the addition of ammonium sulphate to a acetone:water (1:2, v/v) solvent was carried out followed by their determination using reversed-phase capillary liquid chromatography (capillary HPLC). Capillary HPLC shows several advantages compared to analytical HPLC, such as better resolution, lower detection limits and lower solvent consumption, being more environmentally friendly than conventional HPLC. The analytes included are cephoperazone (CPR), cefquinome (CQ), cephalexin (CL), cephapirin (CP), cephaloniun (CLN), cephamandole (CM), cephazolin (CZ) and cephadroxil (CD). In order to form the ion-pair, hexadecyltrimethylammonium bromide (CTAB) was selected as cationic ion-pairing agent, using 10 mM phosphate buffer at pH 8 as the optimum condition for the aqueous solution. The applied methodology, named salting-out assisted liquid-liquid extraction (SALLE) involves the use of 1.25 g of ammonium sulphate as salting-out agent.

The separation of cephalosporins using a Luna C18 (150 mm×0.3 mm, 5 μm, 100 Å) column was achieved under the following conditions: a gradient programme combining solvent A (0.1% formic acid in water, pH 4) and solvent B (acetonitrile:methanol (1:1, v/v)), at a flow rate of 20 μl min^{-1} with a column temperature of 35°C and an injection volume of 7 μl, using UV detection at 250 nm. The limits of detection for the studied compounds were between 1.3 and 6.8 μg L^{-1} for water samples and 1.2 and 10.1 μg kg^{-1} in the case of beef samples, lower than the maximum residue limits permitted by the EU for this kind of food. The developed methodology has demonstrated its suitability for the analysis of these widely applied antibiotics in environmental water and meat samples, including beef and pork muscle, with high sensitivity, precision and satisfactory recoveries.

1. Materials and methods

1.1. Chemicals and standard solutions

All the reagents used were of analytical grade and the solvents of HPLC grade. The water used to prepare the solutions was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Organic solvents such as acetonitrile (ACN), methanol (MeOH), acetone (ACO), 2-propanol, and tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany).

The formic acid (FA) used in the mobile phase was supplied by Sigma Aldrich (St. Louis, MO, USA). The pH was adjusted to 4.0 with sodium hydroxide (10 M), obtained from Merck.

Analytical standards of cephoperazone (CPR, CAS: 62893-20-3; purity: 94%) and cefquinome (CQ, CAS: 118443-89-3; purity: 95%), were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Cephalexin (CL, CAS: 15686-71-2; purity: 99.8%), cephapirin (CP, CAS: 24356-60-3; purity: 98%), cephaloniun (CLN, CAS: 5575-21-3; purity: 99.4%), cephamandole (CM, CAS: 30034-03-8; purity: 99.9%), cephazolin (CZ, CAS: 27164-46-1; purity: 100%) and cephadroxil (CD, CAS: 66592-87-8; purity: 100%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock standard solutions containing 1000 mg L^{-1} of each compound were prepared by dissolving accurately weighed amounts in ultrapure water and acetonitrile:water (1:1, v/v) in the case of CLN, and stored in darkness at 4°C. Working standard solutions were made daily by diluting them with deionised water. Hexadecyltrimethylammonium bromide (CTAB) and tetrabutylammonium bromide (TBABr) were purchased from Sigma Aldrich. The stock solution of CTAB (0.9 mM) and TBABr (6 mM) were prepared in water. Ammonium sulphate, $(NH_4)_2SO_4$ and dipotassium hydrogen phosphate, K_2HPO_4 were purchased from Panreac (Barcelona, Spain).

1.2. Instrumentation and software

The HPLC system consisted of an Agilent HP-1200 series capillary high performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump (20 µl min^{-1} maximum flow-rate), on-line degasser, autosampler (8 µl loop), column thermostat, and a diode array detector (DAD). ChemStation software (A.10.20 [1757] version) was used for data acquisition and processing.

The pH measurements were carried out in a pH-meter model Crison pH 2000 (Barcelona, Spain). A centrifuge Model Universal 320R (Hettich, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, USA), an evaporator with nitrogen System EVA-EC from VLM GmbH (Bielefeld, Germany) and a blender (Taurus, Barcelona, Spain) were used for sample treatment.

Excel 2007 was used for the statistical analysis of data.

1.3. HPLC conditions

The chromatographic separation was performed on a Luna C18 (2) (150 mm×0.3 mm, 5 µm, 100 Å) from Phenomenex (supplied by Jasco Analítica, Spain). A gradient programme was used, combining eluent A (Milli-Q water with 0.1% FA, pH: 4) and eluent B (ACN:MeOH (1:1, v/v)) as follows: 5% B (0 min) to 25% B (10 min), 30% B (15 min), 45% B (20 min) at a flow rate of 20 µl min^{-1}, keeping the column temperature at 35°C. The injection volume was 7 µl and all the compounds were eluted within 22 min. The UV detector was set at 250 nm. Quantification was performed using corrected peak area as analytical signal.

1.4. Sample preparation procedures

1.4.1. Water samples

Spring water collected from Sierra Arana (Granada, Spain) and river water from a local river (Paules, Granada, Spain) were analyzed. The water samples were collected directly in PVC bottles; they were filtered through nylon membranes with 0.2 µm pore size and were stored at 4°C in the dark.

A volume of 200 mL of the natural water samples was spiked with different concentration levels of standard solutions of the analytes (CL, CP, CLN, CQ, CZ, CPR and CM) and an adequate amount of K_2HPO_4 was dissolved in order to obtain a 10 mM phosphate buffer at pH 8. An aliquot of 2.0 mL was taken and 200 µL of CTAB (0.9 mM) were added and stirred in vortex during 1 min. in order to form the ion-pair. Afterward, 1 mL of acetone and 1.25 g of ammonium sulphate were added. The mixture solution was well mixed in vortex during 1 min. and centrifuge at 4500 rpm during 5 min to obtain the separation of the organic phase. The upper phase was evaporated at 30°C under gentle nitrogen current to eliminate the organic

solvent and reconstituted in 200 µL of water with CD (1 mg L^{-1}) as internal standard. These solutions were filtered through 0.2 µm nylon membrane and injected into the capillary HPLC system for analysis.

1.4.2. Meat samples

Beef and pork samples were purchased from a local market in Granada (Spain). A representative portion of sample (250 g of meat muscle) was chopped and homogenized before fortification by adding the appropriate volume of the working standard solutions of the cephalosporins of veterinary use (CL, CP, CLN, CQ, CZ and CPR). After fortification, the samples were kept in dark during 15 min.

Portions of 2.0 g of crushed meat samples were extracted with acetonitrile, by adding 4 mL and mixed in vortex during 1 min. After that, the samples were centrifuged at 9000 rpm for 5 min., the supernatants were transferred to vials and were brought to dryness under a gentle nitrogen current at 30°C. The dry residues were redissolved in 2.0 mL of phosphate buffer at pH 8 (10 mM) and subjected to the treatment described above for water samples. In this case the final dry residues were dissolved in 250 µL of water containing CM (1 mg L^{-1}), a cephalosporin for human use, as IS. in this case. These solutions were filtered through 0.20 µm nylon membrane and injected into the capillary HPLC system for analysis.

2. Results and discussion

2.1. Optimization of the chromatographic separation

Considering a previous work concerning the separation of cephalosporins by analytical HPLC [1] we checked acetonitrile, methanol and mixtures of both of them as the organic phase and formic, citric and acetic acid in different ratios, both in the aqueous and in the organic fraction of the mobile phase. For the best separation, a mixture of acetonitrile:methanol (1:1, v/v) was selected as organic fraction (eluent B) and water with 0.1% of formic acid was fixed as aqueous fraction (eluent A).

[1] E.G. Karageorgou, V.F. Samanidou, J. Sep. Sci. 33 (2010) 2862

The pH of the aqueous fraction of the mobile phase was tested at 3.0, 3.5, 4.0, 4.5 and 5.0 and finally the selected pH value in eluent A was 4.0 adjusted with sodium hydroxide (10 M), because satisfactory resolutions between peaks were obtained. Gradient mode was selected in order to get a better resolution in a shorter analysis time. The flow rate was also optimized testing values from 11 to 20 µL min^{-1}, selecting 20 µL min^{-1} as the optimum value because the retention times were lower and sensitivity was not affected. Temperature was varied from 15 to 40°C selecting a final value of 35°C. Injection volume was increased from 1 to 7 µL and 7 µL was selected as optimum value in order to increase sensitivity without any loss of resolution. The separation of the compounds was carried out within 22 minutes.

2.2. Optimization of the extraction procedure

The extraction of cephalosporins from aqueous media into non-polar organic solvent is a challenging task because of their high polarity and the instability of the β-lactam ring with respect to acid and alkali. Liquid-liquid extraction (LLE) can be modified to extract polar organic compounds like cephalosporins using ion-paired extraction with a water-miscible organic solvent [2]. A phase separation occurs from a mixture of solvents containing water and water-miscible organic solvent like acetonitrile or acetone upon addition of an electrolyte to the mixture of solvents, i.e., salting-out, due to the decrease of the solubility of the organic solvent in the aqueous solution. The separated organic solvent contains water and salts, resulting in large donor and acceptor abilities compared to those of the corresponding pure organic solvent [3]. Thus, the solvent can easily extract ion-paired compounds of cephalosporins. The so called salting-out assisted liquid-liquid extraction (SALLE) is a cost-effective, time-efficient and easy-to-use sample preparation method [4]

2.2.1. Water samples

Based on the method proposed by Kukusamude et al. [2] for the extraction of three penicillins, we accomplished the extraction and preconcentration of the

[2] C. Kukusamude, R. Burakham, O. Chailapakul, S. Srijaranai, Talanta 92 (2012) 38
[3] M. Tabata, M. Kumamoto, J. Nishimoto, Anal Chem. 68 (1996) 758
[4] S. Song, E. Njumbe Ediage, A. Wu, S. de Saeger, J. Chromatogr. A, (2012) http://dx.doi.org/10.1016/j.chroma.2012.10.071

selected cephalosporins from aqueous media using an ion-pairing agent and a water-miscible organic solvent.

For the optimization of the sample treatment in water, a volume of 200 mL of the natural water samples was spiked with different concentration levels of standard solutions of the analytes and an adequate amount of K_2HPO_4 was dissolved in order to obtain a 10 mM phosphate buffer at pH 8. An aliquot of 2.0 mL was taken and subjected to the sample treatment. Firstly, TBABr and CTAB were checked as cationic ion-pairing agents, finding that CTAB provides higher extraction efficiency in terms of recoveries, better efficiency and peak shapes and short analysis time, as is shown in figure IV.1.

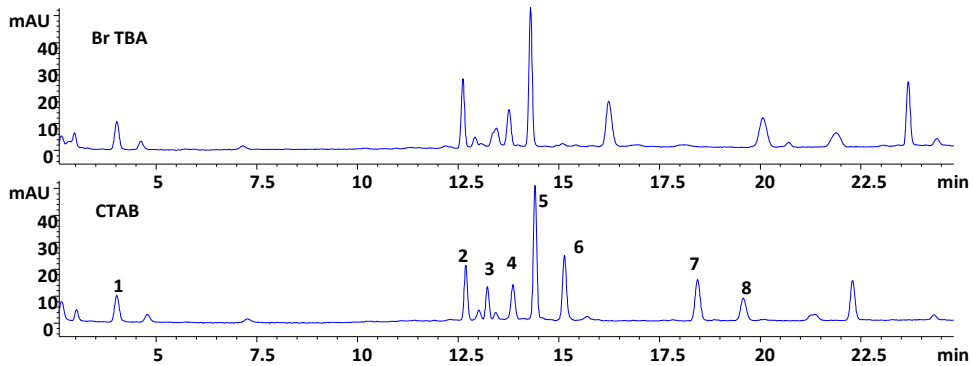

Figure IV.1. Chromatograms obtained with the different ionic-pair agents checked at the optimum separation conditions. Identification peaks: 1: CD as IS; 2: CL; 3: CP; 4: CLN; 5: CQ; 6: CZ; 7: CPR; 8: CM

To select the optimum amount of CTAB the concentration was kept constant at 0.9 mM, below the critical micelle concentration (CMC) which is 1 mM, and 100, 200, 400, 600 and 800 µL were added. It was found that 200 µL was enough to form the ion-pair. In order to select the most appropriate organic solvent for the extraction of the ion-pair formed, we tested ethanol, acetone, 2-propanol, and tetrahydrofuran (THF) finding that the best recoveries were obtained with acetone (see figure IV.2).

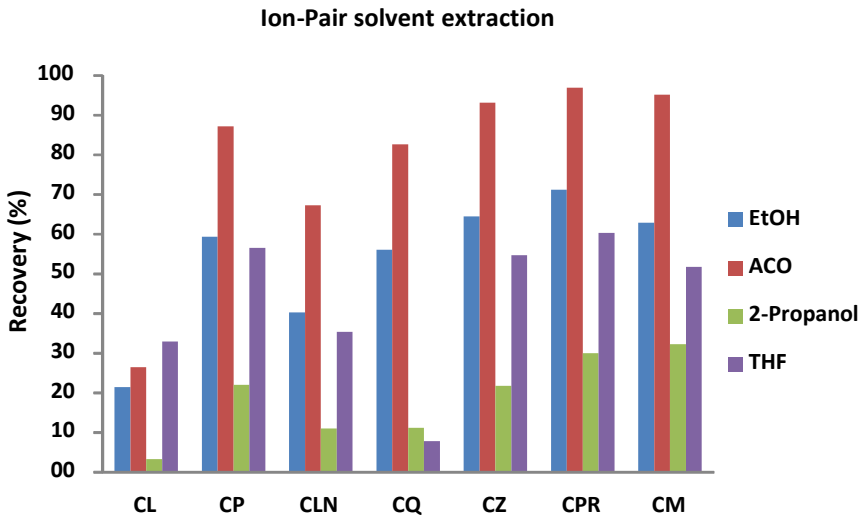

Figure IV.2. Study of the influence of the organic solvent nature on the extraction efficiency of the ion-pair formed with the studied cephalosporins.

The acetone volume was studied by adding 0.5, 1.0, 1.5, 2.0 and 2.5 mL. Finally 1.0 mL of acetone was found to be enough to extract the ion-pair from 2.0 mL of aqueous solution, as is shown in figure IV.3. As salting–out agent, ammonium sulphate was used because in preliminary experiments it was found that using this salt, the volume of organic phase obtained was higher than that obtained when using sodium chloride (a common salting-out agent). The amount of ammonium sulphate was tested by adding 0.5, 0.75, 1.0, 1.25 and 1.5 g of salt. The optimum value was 1.25 g because it provided the best extraction efficiency with a saturated solution of salt.

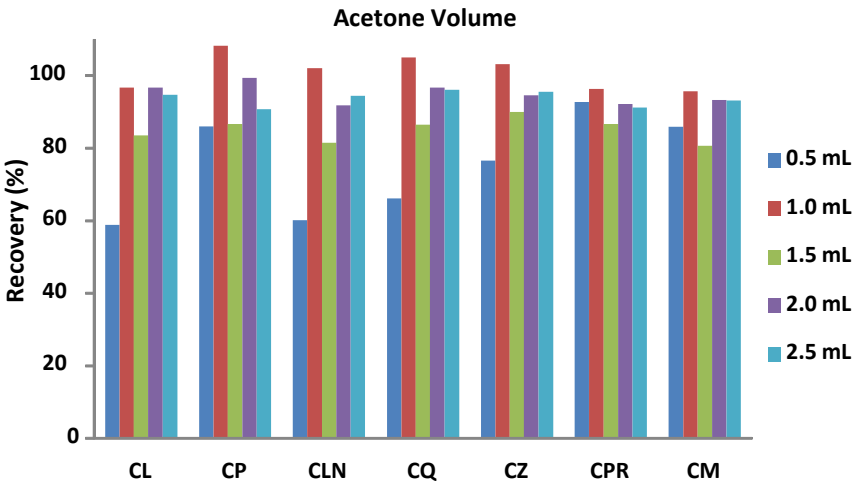

Figure IV.3. Study of the influence of the acetone volume on the extraction efficiency of the ion-pair formed with the studied cephalosporins.

The cephalosporin-CTAB ion-pairs can be more efficiently extracted into the acetone phase in comparison with the original polar forms of these compounds, increasing the recovery values. The optimized extraction procedure named ion-pair salting-out assisted liquid-liquid extraction (IP-SALLE) was used for the application of the method in water samples. Figure IV.4 shows a chromatogram of a blank of water sample, using spring water as representative matrix. No cephalosporins or other interferents were found comigrating with the analytes. Also it is shown the chromatogram for a spring water sample spiked with 100 µg L^{-1} of each studied cephalosporin.

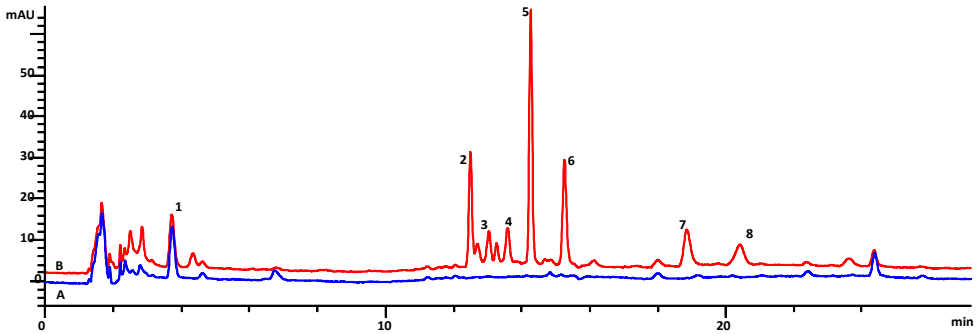

Figure IV.4. Chromatogram of A) a blank of water sample and B) a water sample spiked with 100 µg L^{-1} of each studied cephalosporins. Peak identification: 1: CD as IS; 2: CL; 3 CP; 4: CLN; 5: CQ; 6: CZ; 7: CPR; 8: CM

2.2.2. Meat samples

In the case of meat samples, a portion of 2.0 g of beef muscle spiked with 200 µg kg^{-1} of each cephalosporin was used to evaluate the extraction efficiency. In this case it was necessary to extract the cephalosporins from the matrix before the formation of the ion-pair. Acetone, acetonitrile and mixtures of them (2:8, 4:6, 5:5, 6:4, 8:2, v/v) were checked as extraction solvent; finally 100% acetonitrile provided the best extraction efficiency. The volume of acetonitrile was studied by adding 0.5, 1.0, 2.0, 4.0 and 6.0 mL, finding that 4.0 mL of acetonitrile was the optimum value. After that the organic extract goes to dryness under a gentle nitrogen current at 30°C and the procedure follows as it is described above, by redissolving the dry residues in 2.0 mL of phosphate buffer (10 mM) at pH 8. Figure IV.5 shows a chromatogram of a blank of beef sample. No cephalosporins were found and some endogenous interferents were separated from the selected compounds. Also the chromatogram for a beef sample spiked with 200 µg kg^{-1} of each studied cephalosporin is shown.

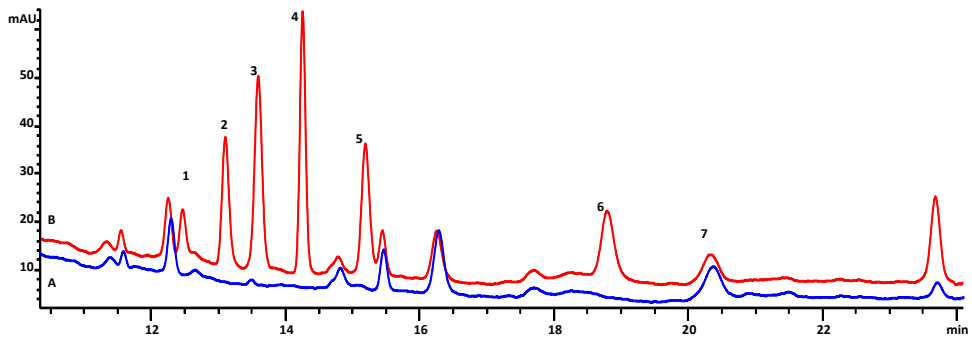

Figure IV.5. Chromatogram of A) blank of beef sample and B) a beef sample spiked with 200 µg kg^{-1} of each studied cephalosporins. Peak identification: 1: CL; 2 CP; 3: CLN; 4: CQ; 5: CZ; 6: CPR; 7: CM as IS

2.3. Validation of the IP-SALLE-capillary HPLC method

2.3.1. Calibration curves and performance characteristics

The method was characterized for different types of matrices of environmental water and meat. With this purpose, calibration curves in the presence of matrix were obtained using spring water and muscle beef samples spiked with the subsequent calibration levels before sample treatment. In all cases, a blank sample

was analysed in order to check if any of the analytes were already present at a concentration above the limit of detection or if some interferences could comigrate with the analytes.

Spring water samples were used for the establishment of matrix calibration curves for the analysis of environmental waters. The calibration levels for the analytes (CL, CP, CLN, CQ, CZ, CPR and CM) were 5, 25, 50, 100, 150 and 200 μg L^{-1} of each analyte in the sample. In all cases 1 mg L^{-1} of CD was added as IS. For muscle beef samples, the matrix-matched calibration curves of the cephalosporins for veterinary use (CL, CP, CLN, CQ and CPR) were established from five levels corresponding to concentrations in sample of 25, 100, 200, 300 and 400 μg kg^{-1} of each cephalosporin; for CZ the calibration curve was established from six concentration levels (25, 100, 200, 300, 400 and 500 μg kg^{-1}). In both cases 1 mg L^{-1} of CM was added as IS.

In both matrix-matched calibration curves the relative corrected peak area (as the ratio of analyte peak area to IS. peak area) as a function of the analyte concentration was considered. Each concentration level was prepared by duplicate and injected by triplicate. Satisfactory determination coefficients confirmed that responses were linear over the studied range for the samples. All the statistical parameters and performance characteristics are shown in table IV.1. Limits of detection (LODs) and quantification (LOQs) have been calculated using S/N of 3 and 10, respectively. As can be seen, for all the studied compounds, very low LODs were obtained. It must be highlighted that LODs for beef muscle samples were below the legislated MRLs [5], established from 20 to 200 μg kg^{-1}. The LODs obtained in water for all the studied compounds by applying this procedure are at the low μg L^{-1} levels. No regulation exists for this kind of emerging pollutants in waters.

[5] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin., Off. J. Eur. Commun. L15 (2010) 1

Table IV.1. Statistical and performance characteristics for the studied cephalosporins in each kind of sample

Spring water						
Compounds	**Intercept**	**Slope**	**R^2**	**LOD ($\mu g\ L^{-1}$)**	**LOQ ($\mu g\ L^{-1}$)**	**Linear dynamic range ($\mu g\ L^{-1}$)**
CL	-0.0093	0.0119	0.996	3.2	10.6	3.2-200
CP	0.0407	0.0038	0.992	5.8	19.4	5.8-200
CLN	0.033	0.0044	0.991	6.8	22.7	6.8-200
CQ	0.0596	0.0209	0.997	1.3	4.3	1.3-200
CZ	0.0053	0.0115	0.995	3.5	11.6	3.5-200
CPR	0.0637	0.0067	0.993	2.0	6.8	2.0-200
CM	0.0034	0.0043	0.992	4.5	14.9	4.5-200
Beef muscle[a]						
Compounds	**Intercept**	**Slope**	**R^2**	**LOD ($\mu g\ kg^{-1}$)**	**LOQ ($\mu g\ kg^{-1}$)**	**Linear dynamic range ($\mu g\ kg^{-1}$)**
CL	-2.28	0.3284	0.991	22.0	73.3	22-400
CP	25.7	0.981	0.993	8.5	28.2	8.5-400
CLN	3.2	1.72	0.993	7.5	25.0	7.5-400
CQ	-7.67	1.68	0.997	4.6	15.5	4.6-400
CZ	73.3	1.19	0.991	1.2	4.1	1.2-500
CPR	3.14	1.04	0.994	10.1	33.8	10.1-400

[a] MRLs established by the Commission Regulation (EU) N. 37/2010 in beef muscle sample: CL: 200 $\mu g\ Kg^{-1}$; CP: 50 $\mu g\ Kg^{-1}$; CQ: 50 $\mu g\ Kg^{-1}$; CLN, CZ, CPR: Not established.

2.3.2. Precision study

The precision of the whole method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of repetitive application of the sample treatment and separation procedure to spring water samples (three experimental replicates) at concentration levels of 25, 100 and 150 μg L^{-1}, and 50, 200, 400 μg kg^{-1} for beef muscle samples, and each one was injected in triplicate (instrumental replicates). Intermediate precision was assessed for five consecutive days with one sample (spring water or beef muscle) for each concentration level and injected by triplicate. The results, expressed as relative standard deviation (RSD) of relative peak areas, are given in table IV.2. As it can be observed, acceptable precision was obtained in all cases.

Table IV.2. Precision study (RSD %) of the proposed method for different concentration levels.

	Spring water samples					
	Intraday			**Interday**		
	Level 1	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
CL	5.0	6.8	2.1	7.8	6.4	5.9
CP	5.4	3.7	3.7	6.5	3.9	7.4
CLN	3.4	3.3	3.2	8.0	5.8	6.0
CQ	3.7	4.2	3.0	7.2	3.8	6.4
CZ	4.1	3.7	4.1	9.5	3.6	9.9
CPR	3.5	3.0	2.3	8.9	6.1	6.6
CM	2.6	3.4	2.1	5.2	7.1	6.6
	Beef muscle samples					
	Intraday			**Interday**		
	Level 1	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
CL	6.8	3.7	2.3	7.5	7.3	8.4
CP	2.4	5.1	4.4	8.4	8.9	6.4
CLN	1.3	7.4	2.7	7.1	6.9	9.2
CQ	0.8	5.8	3.3	9.2	7.9	9.6
CZ	2.1	5.0	2.0	5.8	6.5	5.5
CPR	6.6	8.3	2.7	7.4	8.4	8.7

For water samples: Level 1: 25 μg L^{-1}. Level 2: 100 μg L^{-1}. Level 3: 150 μg L^{-1}.
For beef samples: Level 1: 50 μg kg^{-1}. Level 2: 200 μg kg^{-1}. Level 3: 400 μg kg^{-1}.
RSD, Relative standard deviation (n=9 for intraday study; n=15 for interday study)

2.3.3. Trueness of the method: Recovery studies

In order to check the trueness and the applicability of the proposed methodology for the analysis of cephalosporins, recovery experiments were carried out in different analyte-free kinds of water samples and meat samples. Spring and river water samples spiked with each one of the analytes at three different concentration levels, (25, 100 and 150 μg L^{-1}) were analyzed. Three replicates were prepared at each concentration level, and each one was injected in triplicate. Previously the samples were extracted following the above-described treatment and a sample blank was also analyzed in order to confirm that the analyzed samples were free of the cephalosporins. The identification of the compounds was based on both their migration times and the absorption spectra. The obtained values of the recovery study in river water samples for each compound and the corresponding RSD (%) are shown in table IV.3.

Beff muscle samples were spiked with a mixture of analytes at three differents levels (50, 200 and 400 μg kg^{-1}). In the case of pork muscle samples were spiked at three different levels (50, 200 and 400 μg kg^{-1}) in the case of CL and CPR and 25, 100, and 200 μg kg^{-1} for the rest. Each level was prepared by triplicate and it was injected three times. In order to check the presence of interferences, blank samples were subjected to the proposed method and no matrix peaks were found comigrating with the analytes. The obtained values of the recovery study for each compound and the corresponding RSD (%) are shown in table IV.3 for pork muscle samples.

The proposed method provides satisfactory results in terms of both, trueness and precision for different kind of water and meat of different origins, demonstrating the method accuracy for the analysis of these compounds in these matrices.

Table IV.3. Recoveries of cephalosporin antibiotics from each kind of samples spiked at different concentration levels.

	Spring water					
	Level 1		**Level 2**		**Level 3**	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
CL	76.5	6.3	97.8	5.5	99.9	2.4
CP	90.1	6.2	91.8	6.8	100.8	4.7
CLN	83.9	4.2	93.7	6.8	102.9	5.0
CQ	87.2	7.3	93.1	6.9	103.9	4.6
CZ	81.6	3.0	95.0	7.2	106.6	3.8
CPR	92.7	5.0	95.9	7.0	109.1	5.2
CM	97.7	5.4	96.8	3.9	105.4	4.9
	River water					
	Level 1		**Level 2**		**Level 3**	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
CL	79.7	4.8	100.2	5.6	105.9	2.9
CP	80.6	6.9	105.5	2.4	104.2	2.8
CLN	73.5	4.5	104.1	3.3	97.0	5.3
CQ	88.2	6.6	104.3	2.7	104.0	3.2
CZ	87.8	4.8	104.3	3.4	107.5	3.4
CPR	84.2	5.8	106.8	2.9	105.7	2.7
CM	102.5	5.4	104.5	3.8	105.1	3.2

	Beef muscle					
	Level 1		Level 2		Level 3	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
CL	81.6	6.8	67.9	3.7	70.1	2.3
CP	67.5	2.4	69.5	5.1	68.7	4.4
CLN	74.7	1.3	76.0	7.4	77.2	2.7
CQ	77.1	0.8	78.6	5.8	78.1	3.3
CZ	75.4	2.1	76.7	5.0	77.2	2.0
CPR	87.2	6.6	82.3	8.3	71.3	2.7
	Pork muscle					
	Level 1		Level 2		Level 3	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
CL	70.8	9.4	60.9	3.1	62.7	8.3
CP	99.7	8.2	78.1	3.7	93.2	6.7
CLN	85.1	9.6	72.7	7.9	87.2	6.8
CQ	83.2	9.2	75.0	4.6	92.6	4.8
CZ	91.5	6.4	84.9	7.7	91.3	5.3
CPR	107.6	4.9	87.5	10.1	99.8	2.2

For water samples: Level 1: 25 μg L^{-1}. Level 2: 100 μg L^{-1}. Level 3: 150 μg L^{-1}.
For beef samples: Level 1: 50 μg kg^{-1}. Level 2: 200 μg kg. Level 3: 400 μg kg^{-1}.
For pork samples: Level 1: 50 μg kg for CL and CPR and 25 μg kg for CP, CLN, CQ and CZ. Level 2: 200 μg kg^{-1} for CL and CPR and 100 μg kg^{-1} for CP, CLN, CQ and CZ. Level 3: 400 μg kg for CL and CPR and 200 μg kg^{-1} for CP, CLN, CQ and CZ.

3. Concluding remarks

A sensitive and simple procedure combining IP-SALLE and capillary HPLC with DAD detection has been developed and validated for the analysis of cephalosporins of human and veterinary use (cephalexin, cephapirin, cephalonium, cefquinome,

cephazolin, cephoperazone, and cephamandol) in the case of water samples and only of veterinary use (cephalexin, cephapirin, cephalonium, cefquinome, cephazolin, and cephoperazone) in the case of meat samples. Prior to the HPLC analysis, the method applied an ion-pair formation step necessary for the efficient extraction and preconcentration of the cephalosporins. The preconcentration factor achieved in the sample treatment and the use of a miniaturized technique allow us to obtain very low detection limits and good resolution, showing the proposed method as an efficient and easy alternative for the monitoring of these antibiotics. According to the results obtained in the precision and trueness studies, the method is accurate for the analysis of waters of different origins and animal products and could be satisfactorily applied as a routine procedure to quantify cephalosporins in laboratories of environmental contamination or food quality and safety control.

This work is in revision as: *"Ion-paired extraction of cephalosporins in acetone prior to their analysis by capillary liquid chromatography in environmental water and meat samples*". Carolina Quesada-Molina, Ana M. García-Campaña, Monsalud del Olmo-Iruela, (Talanta, under review).

CAPÍTULO 5

Síntesis y evaluación de un polímero impreso molecularmente para la extracción en fase sólida de cefalosporinas en leche

CHAPTER 5

Synthesis and evaluation of a molecularly imprinted polymer for the solid phase extraction of cephalosporins from milk

RESUMEN

En este capítulo se propone el desarrollado un polímero impreso molecularmente (MIP) mediante un procedimiento de impresión molecular no-covalente y su aplicación a la extracción en fase sólida (MISPE) de cefalosporinas de uso veterinario (cefalexin (CL) y cefapirin (CP)). Para la síntesis del MIP se utilizó como analito molde la sal tributilamónica de cefadroxil (TBA-CD). Las condiciones óptimas de preparación del MIP se determinaron considerando la alta polaridad de CD y además su baja solubilidad en disolventes orgánicos comunes usados para aumentar las interacciones no covalentes entre la molécula molde y el monómero. El ácido metacrílico y el dimetilacrilato de etilenglicol fueron seleccionados como monómero y agente reticular respectivamente. La reacción de polimerización se llevó a cabo mediante activación térmica a 60°C y como disolvente porógeno se utilizó una mezcla de acetona:metanol (92:8, v/v). El protocolo MISPE se optimizó inicialmente con patrones antes de mostrar su potencial en leche.

La selectividad del MIP para los analitos CL, CD, y CP se comparó con la selectividad del polímero no impreso (NIP), tanto en disoluciones patrón como en muestras de leche. La aplicación de MISPE proporcionó recuperaciones mayores del 60% para los tres analitos estudiados en muestras de leche dopada. Según la bibliografía consultada, ésta es la primera aplicación de MIP a la extracción de cefalosporinas de muestras de leche.

SUMMARY

In this chapter, a molecularly imprinted polymer (MIP) for cephalosporin molecules (cephalexin (CL) and cephapirin (CP)) of veterinary concern using cefadroxil (CD) as template for the imprinted polymer synthesis, was prepared by non-covalent molecular imprinting approach and applied to solid phase extraction (MISPE). The best conditions of MIP preparation were determined considering the high polarity of CD and, therefore, its lack of solubility in usual organic solvents which are used to enhance non-covalent interactions between the template and the monomer. For MIP synthesis, a tributylammonium cefadroxil salt (TBA-CD) was used as template, methacrylic acid and ethylene glycol dimethacrylate were selected as monomer and cross-linker, respectively. The polymerisation was performed by thermal activation at 60°C in a porogenic solvent constituted by an acetone:methanol (92:8, v/v) mixture. Then, the MISPE protocol was optimised with standard solutions before to show the potential application to milk samples.

The selectivity of MIP versus non imprinted polymer (NIP) was confirmed for CL, CD and CP in standard solutions as well as in milk samples. The MISPE recoveries were higher than 60 % for the three target analytes in spiked milk. To our knowledge, it is the first time that a MIP is applied to cephalosporin extractions from milk samples.

1. Materials and methods

1.1. Chemical and standards

Methacrylic acid (MAA), 2-(trifluoromethyl)acrilic acid (TFMAA), ethylene glycol dimethacrylate (EDMA), azobisisobutyronitrile (AIBN), tributylamine (TBA) (99.5+%), methanol (MeOH) and acetone (HPLC grade) were provided by Aldrich (St-Quentin-Fallavier, France). Cefadroxil (CD), cephalexin (CL) hydrate and cephapirin (CP) sodium salt were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Before use, MAA was distilled under vacuum and AIBN was re-crystallised from methanol.

Acetonitrile (ACN) of HPLC grade and glacial acetic acid (AcOH) 100 % were from SDS (Carlo-Erba, Val de Reuil, France).

Ortho-Phosphoric acid 85 % and sodium hydroxide pellets, puriss. were provided by Riedel-de Haën (Hanover, Germany). Deionized water (18 MΩ) was obtained from an Elgastat UHQ II system (Elga, Antony, France).

The phosphoric acid – sodium hydroxide (pH 3) buffer solution (10 mM ionic strength) was prepared with the help of Phoebus software (Analis, Namur, Belgium).

1.2. Instrumentation and softare

The HPLC analyses were performed on a (150 mm x 3 mm i.d., 5 µm) reversed-phase column Waters Symmetry® C18 (Waters Corporation, Milford, USA). The mobile phase was delivered by a Merck-Hitachi LaChrom HPLC system L-7100 pump (Merck KGaA, Darmstatd, Germany). A Kratos (Applied Biosystems, Les Ulis, France) Spectroflow 783 UV spectrophotometric detector. Data were collected and analyzed using EZ-Chrom Elite software (Version 2.5). A manual injection with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 20 µL loop was used for injection.

The extraction was carried out by using a 12-Port Visiprep vacuum manifold (Supelco, Sigma-Aldrich, St Quentin Fallavier, France).

1.3. Preparation of (TBA-CD) salt

Tributylammonium cefadroxil (TBA-CD) salt was prepared by addition of 2.12 mL of TBA (1 M) in MeOH to a methanolic suspension of CD 2.12 mmol (768 mg of CD in 2 mL of MeOH). Anhydrous $MgSO_4$ was added to the reactive mixture that was stirred at room temperature for two hours. Then, $MgSO_4$ was discarded and the solvent was evaporated by rotative evaporator giving a light yellow homogeneous solid. This powder was washed in acetone until a white powder appeared. The amount of obtained TBA-CD was 712.8 mg (1.3 mmol).

1.4. Preparation of MIP

TBA-CD imprinted polymer was synthesized by the non-covalent approach. In a glass test tube (8 mL), were added amounts of template (TBA-CD, 0.315 mmol / 173 mg), functional monomer MAA (1.59 mmol / 135 µL), cross-linker (EDMA, 7.90 mmol / 1520 µL) and acetone (2.20 mL). The homogeneity of the reactive mixture was achieved by addition of 200 µL of MeOH. After sonication, 0.17 mmol (28.5 mg) of AIBN was added to the solution. The glass tube was cooled in ice and the mixture was purged with nitrogen for ten minutes and sealed. The polymerisation was carried out in a water bath (60°C) for twenty hours, until the polymerisation was complete. The monolith polymer was crushed into particles with a pestle and mortar and wet-sieved through a 45 µm metal sieve. Then, the particles were washed with a water:acetic acid (4:1, v/v) mixture and with MeOH. Fine particles were removed by repeated sedimentations in acetone. Finally, the recovered polymer particles (25 – 45 µm size range) were dried under vacuum and stored at ambient temperature. The non-imprinted polymer (NIP) was prepared by the same procedure without adding the template to the initial polymerisation mixture.

Thus, 833.9 mg of MIP and 879.3 g of NIP were obtained.

Moreover, another MIP was prepared with TFMAA as monomer. The TFMAA-MIP and NIP were prepared following the same protocol as MAA-MIP synthesis. The amounts of MIP and NIP obtained were equal to 898 mg and 851 mg, respectively.

1.5. Solid phase extraction procedure on MIP

A 100 mg amount of polymer was poured into 1 mL-volume SPE cartridges (Isolute IST, Hengoed, UK). The sorbent was conditioned with 7.5 mL of H_3PO_4 (1.2 mmol L^{-1}). A 1 mL-volume of H_3PO_4 (1.2 mmol L^{-1}) standard solution spiked with CD, CL and CP (20 µg mL^{-1}) was loaded on the MISPE cartridge. After a washing step with ACN (1 mL), the analytes were eluted with 4 mL of the mixture MeOH:AcOH (9:1, v/v). All fractions were evaporated until dryness at 20°C under a stream of nitrogen and dissolved in 1 mL mobile phase prior HPLC analysis.

1.6. Milk sample treatment

A step of protein precipitation was carried out before MISPE. Thus, equal volumes (5 mL) of milk and ACN were vortexed for 1 min. Then, 10 mL more of ACN were added to the previous mixture. Supernatant extract was isolated after centrifugation (1800 rpm for 10 min) and filtrated through 0.2 µm PTFE filter. A portion of 2 mL of the clear filtrate was evaporated until dryness under a gentle steam of nitrogen at ambient temperature.

1.7. HPLC procedure

The mobile phase was a mixture of MeOH and H_3PO_4-NaOH buffer (pH 3, 10 mmol L^{-1} ionic strength) (25:75, v/v) [1]. Methanol was preferred to acetonitrile for economic reasons and chromatograms displayed satisfactory peak resolutions. The flow rate was 0.4 mL min^{-1}. UV absorbance signal was recorded at 252 nm which was a compromise between the wavelengths of maximum absorbance of each analyte.

[1] H. Niu, Y. Cai, Y. Shi, F. Wei, J. Liu, S. Mou, G. Jiang, Anal. Chim. Acta 594 (2007) 81

2. Results and discussion

2.1. Synthesis of MIP

The MIP of CD has been synthesized following a non-covalent approach. In order to achieve the formation of specific imprints inside the polymer matrix, two functional monomers have been tested: methacrylic acid (MAA) and 2-(trifluoromethyl)acrylic acid (TFMAA). Indeed, electrostatic interactions and hydrogen bonds can exist between the acid and basic functions of MAA, or TFMAA, and CD.

Whatever is the type of interaction between the template and the monomer, either electrostatic or hydrogen bond, the solvent of the polymerisation has to display a limited competitor effect. For instance, a non protic solvent with low dielectric constant has been favoured. However, the polarity and hydrophilic character of the template was a limiting factor for the choice of the solvent. Only MeOH, protic solvent, could solubilise CD at the concentration level required for the MIP synthesis, i.e. about 50 mg of template per milliliter of solvent. For this reason, the polarity of CD was modified by the formation of its less polar tributylammonium salt; the structure of the formed salt (TBA-CD) is shown in, figure V.1 [2]. According to pK_A values of TBA (pK_A= 9.9) and CD (pK_A of carboxylic acid = 2.9; pK_A of phenol = 7.1), we assumed that the stoichiometry of the TBA-CD salt was 1:1, since the phenol group of CD was not acidic enough to react with TBA.

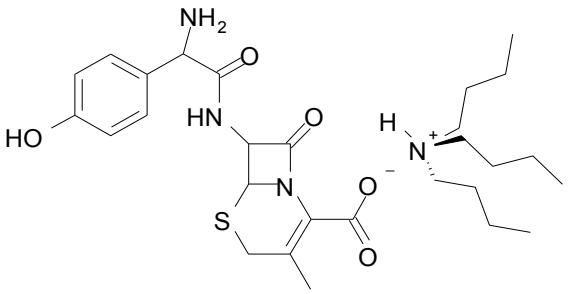

Figure V.1. Structure of the TBA-CD used as a template in MIP synthesis

[2] A. Beltran, R.M. Marcé, P.A.G. Cormack, D.C.S Herrington, F. Borrull, J. Sep. Sci. 31 (2008) 2868

Solubility tests were performed with ACN and acetone. Finally acetone was selected as the porogenic solvent of MIP synthesis because TBA-CD salt was not soluble in ACN.

After polymer grinding and sieving, the extraction of the template was performed at room temperature by several washes in water:AcOH (8:2, v/v), followed by a last washing step in MeOH. Then, the MIP particles were dried in vacuum and stocked at 4°C. The MAA-MIP and NIP yields were 49 % and 51.5 % respectively.

TFMAA (pK_A = 2.3) is more acidic than MAA (pK_A = 5) due to the electron attraction effect of the trifluoromethyl group. For this reason, TFMAA was selected as a monomer for the preparation of a MIP with high affinity for cephalosporins [3].

2.2. Evaluation of MIP selectivity on standard solution

The MIP selectivity is based on the existence of CD specific cavities inside the polymer matrix. To evaluate this, a SPE protocol was applied simultaneously to MIP and NIP cartridges filled with 100 mg of polymer and the extracts were submitted to the chromatographic method above-mentioned. After conditioning of MIP and NIP by H_3PO_4 (1.2 mM, pH 3), a solution of CD, CP and CL (20 mg L^{-1}) in H_3PO_4 (1.2 mmol L^{-1}, pH 3) was loaded. At this step, the analytes were totally retained on MIP and NIP as proved in figure V.2. A washing-step was carried out by ACN percolation and showed the MIP selectivity since the imprinted polymer retained higher quantities of analytes than NIP. Indeed, the hydrophobic and electrostatic non-specific interactions were broken by the organic solution constituted by ACN and residual quantities of H_3PO_4 (1.2 mmol L^{-1}, pH 3). However, the optimal spatial repartition of the acidic functions inside the imprinted polymer specific cavities succeeded in keeping quantitatively the analytes on MIP until the following step. MIP elution enabled to recover more than 58 % of analytes by percolation 4 mL of MeOH:AcOH (9:1, v/v).

[3] J .Matsui, O. Doblhoff-Dier, T. Takeushi, Anal. Chim. Acta 343 (1997) 1

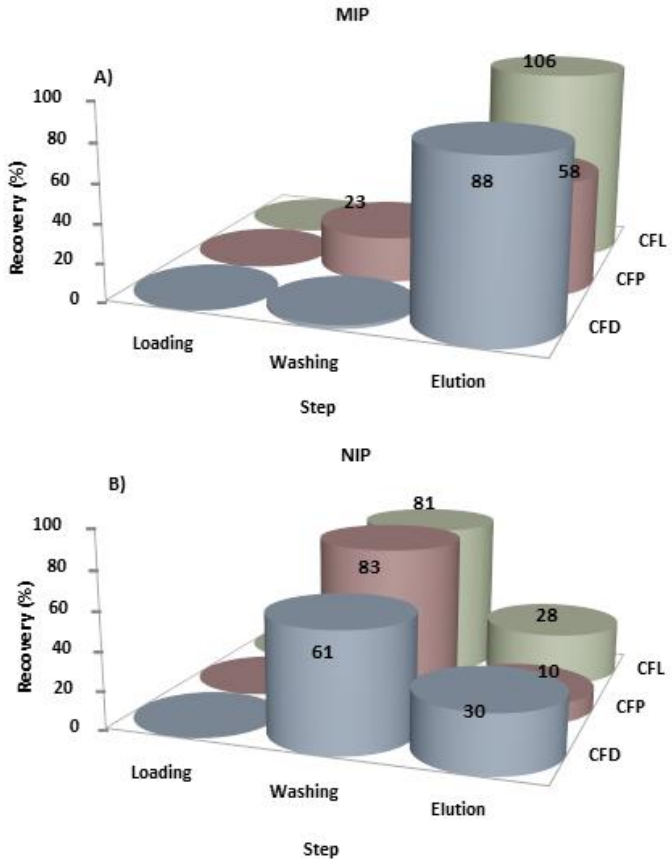

Figure V.2. Recoveries of CD, CP and CL obtained by SPE for A) MIP and B) NIP. Standard solution: CD, CP and CL (20 µg mL^{-1}) in H_3PO_4 (1.2 mmol L^{-1}, pH 3), (n = 2)

Imprinting parameter (I.P.) obtained from the ratio of elution recoveries of MIP versus NIP was equal to 3 for CD, 5.6 for CP and 3.8 for CL. These values, which are superior to the unity, are a proof of the specific interactions between the analytes and MIP.

Using the MISPE protocol described before, the synthesized polymer from TBA-CD as template and TFMAA as functional monomer was checked but selectivity between MIP and NIP was not proved because of high retention of the cephalosporins by NIP. The addition of AcOH (1 and 5%, v/v) to ACN as washing step solvent did not achieve to decrease the non-specific interactions between TFMAA polymer and two target analytes, CD and CL. CP was partially eliminated (55 %) from MIP and NIP during the ACN-AcOH (95:5, v/v) washing-step; thus, CP was less retained by the polymer than CD and CL. However, whatever was the

analyte and its retention during the washing-steps, the MIP and NIP recoveries at the elution step were equal. In conclusion, no TFMAA-MIP/NIP selectivity was proved either with or without AcOH addition to ACN.

2.3. MISPE application to milk

Milk is a complex aqueous matrix in which proteins are among the main constituents. These macromolecules are assumed to prevent optimal interaction between target molecules and MIP specific sites. Therefore, a preliminary step of protein precipitation was carried out before MISPE. Protein precipitation from the milk samples was tested using different volumes of ACN. Thus, equal volumes (5 mL) of milk and ACN were mixed by vortex for 1 min. Then, 10 mL more of ACN were added to the previous mixture in order to favour the protein precipitation. During optimization of the sample preparation procedure, different vortex and centrifuging times were evaluated in order to maximize the recovery and minimize preparation time. Finally, supernatant extract was isolated after centrifugation (1800 rpm for 10 min) and filtrated through 0.2 µm PTFE filter. A portion of 2 mL of the clear filtrate was evaporated until dryness under a gentle steam of nitrogen at ambient temperature. The solid residue was then dissolved in 1 mL of H_3PO_4 (1.2 mmol L^{-1}, pH 3). This sample was subjected to the same MISPE protocol as the previous standard solutions and submitted to the chromatographic method above-mentioned. The results are reported in figure V.3, and confirmed MIP selectivity and the quantitative extraction of the selected cephalosporins from a reconstituted complex matrix (recovery percentages between 60-66 %).

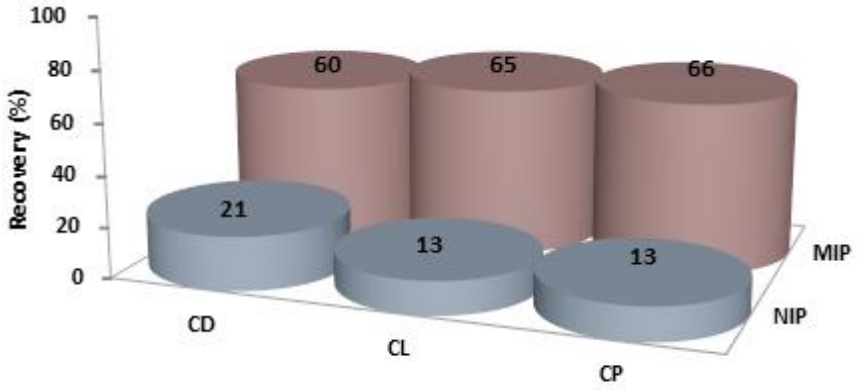

Figure V.3. Recoveries of CD, CP and CL obtained at the elution step (MeOH:AcOH (9:1, v/v)) of MIP and NIP–SPE, (n = 2). Extract from milk sample: CD, CP and CL (20 µg mL^{-1}).

A chromatogram from a spiked milk sample (final concentrations in the extract for CD, CP and CL, 20 µg mL^{-1}) treated using the proposed MIP procedure is shown in figure V.4. It can be seen that no interferences are coeluting with the analytes.

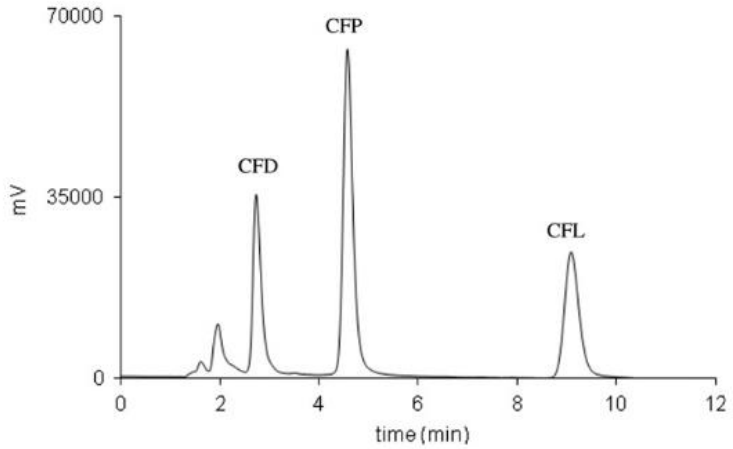

Figure V. 4. HPLC–UV analysis of milk sample using MISPE (final extract CD, CP and CL, 20 µg mL^{-1}). Column: WATERS Symmetry® C18 (150x 3 mm, 5 µm). Mobil phase: MeOH:H_3PO_4–NaOH (pH 3, 10 mmol L^{-1} ionic strength) 25:75 (v/ v). Flow: 0.4 mL min^{-1}. Loop volume: 20 µL. Detection wavelength: 252 nm

From these preliminary results, we consider that the synthesized MIP could be an effective and simple approach for the selective extraction of these antibiotics in milk samples of different origins, being also possible to increase the nature of matrixes to be applied. Further work will be done in this address.

3. Concluding remarks

A MIP for the cephalosporin antibiotics CD, CL and CP has been successfully synthesized, in spite of the polar character of the analytes. The MIP demonstrates useful cross-selectivity, being able to extract three structurally related compounds from complex samples, such as milk, with satisfactory recoveries in these preliminary experiments. The results were evaluated by using HPLC with DAD detection. This strategy could be a powerful alternative to SPE sorbent used in commercial cartridges, considering the high polarity of CD, CL and CP. Further work could be carried out to optimize the extraction step of analytes from other animal origin food samples.

This work was developed in ICOA, Université d'Oléans, Orléans (France) and was published as:

"Convenient solid phase extraction of cephalosporins in milk by using a molecularly imprinted polymer". Carolina Quesada-Molina, Bérengère Claude, Ana M. García-Campaña, Monsalud del Olmo-Iruela, Philippe Morin. Food Chem. 135 (2012) 775-779.

Conclusions

In this Thesis different methods for determination of herbicides (sulfonylureas and degradation products of metribuzin) and antibiotics (cephalosporins), in food and environmental samples, have been developed. Liquid chromatography and different miniaturized analytical techniques have been evaluated, such as CE and capillary HPLC coupled with UV detection. In addition, different sample treatments have been assessed as an alternative to existing treatments in order to improve efficiency and sample throughput. It has contributed to increase the analytical methods available for the control of residues of herbicides and cephalosporins in food, water and soil.

The advantages and drawbacks of each methodology have been emphasized below:

- CE methods implied lower consumption of solvents and reagents, especially organic solvents, than chromatographic methods and that means lower cost than LC methods as well as a lower impact on the environment. In addition, CE methods have a shorter analysis time than capillary HPLC method.

- The values of the dissociation constants (pK_as) of the major degradation products of metribuzin have been calculated using the CE technique, and no significant differences have been observed with the values obtained by applying other techniques.

- The CE technique has allowed the development of an on-line preconcentration methodology (LVSS) which has been suitable for increasing the sensitivity in the proposed methods for the determination of sulfonylureas, degradation products of metribuzin and cephalosporins in the selected matrices.

Another aim of this Thesis has been the study of alternative sample treatments in order to increase the selectivity and the sensitivity. Specific sample treatments based on MIPs and generic sample treatments, such as SPE or PLE have been assessed.

- The PLE procedure was optimized and applied for extraction of the major degradations products of metribuzin in soil samples, followed by an off-line preconcentration and sample clean-up procedure by SPE. This last procedure is suitable for soil extracts obtained from the PLE and also directly for water samples.

- When SPE was used to analyse samples, a high preconcentration factor allowed very low LODs, even combined with CZE-UV, for the analysis of sulfonylureas in grapes and waters and cephalosporins in water and meat samples.

- In terms of selectivity, MISPE is the most selective sample treatment, since the MIP used was specifically synthetized for the CL, CP and CD extraction. However, it did not allow the use of high sample volumes and high preconcentration factors could not be achieved. When MISPE was used for milk samples together with HPLC-UV, very clean chromatograms were obtained, and no peaks were found co-eluting with the cephalosporins.

- A simple and rapid procedure combining IP-SALLE has been developed for the efficient extraction of the cephalosporins from aqueous solution using polar organic solvents. The ion-pair formation step is necessary for the efficient extraction of the cephalosporins.

- The detection limits obtained for the studied cephalosporins in water samples by applying CZE in combination with SPE as off-line preconcentration and LVSS as on-line preconcentration were one order of magnitude lower than that corresponding to capillary HPLC with IP-SALLE as sample treatment.

- IP-SALLE with capillary HPLC showed higher selectivity and enough sensitivity to detect concentrations of cephalosporins lower than the maximum residue limits permitted by the EU in meat samples.

As a summary, the most significant analytical characteristics of the developed methods are shown in table C.1.

Table C.1. Summary of the developed methods in this Thesis.

Analytes	Matrix	Sample treatment	Instrumental technique and experimental conditions	Analysis time (min)	LOD
DA, DK, DADK	Soil waters	Soil: PLE (methanol) + SPE (LiChrolut EN) Water: SPE (LiChrolut EN)	**CE-LVSS** sodium tetraborate buffer (40 mM, pH 9.5)	< 9	Water: 10-20 ng mL^{-1}
Tria-S, Rim-S, Flaza-S, Met-S, Chlor-S	Grapes Waters	SPE: Grape: C18 Water: HLB	**CE-LVSS**: ammonium acetate buffer (90 mM, pH 4)	< 20	Grape: 0.97-8.30 µg kg^{-1} Water: 0.04-0.12 µg L^{-1}
CL, CPR, CT, CP, CZ	Environmental waters	SPE: HLB	**CE-LVSS:** ammonium acetate buffer (70 mM, pH 7.0)	< 6	0.1-0.3 µg L^{-1}
CL, CP, CLN, CQ, CZ, CPR, CM	Beef and pork muscle Environmental waters	(IP-SALLE)	**Capillary HPLC**: gradient programme: solvent A (0.1% formic acid in water, pH 4) solvent B (ACN:MeOH (1:1, v/v))	<23	Beef: 1.2-10.1 µg kg^{-1} Water: 1.3-6.8 µg L^{-1}
CD, CL, CP	Milk	MISPE	**HPLC:** isocratic mode: MeOH:fosfate buffer (pH 3, 10 $mmolL^{-1}$ ionic strength) (25:75, v/v)	< 10	nr

nr: not reported

Conclusiones

En esta Tesis se han desarrollado diferentes métodos para la determinación de herbicidas (los productos de degradación mayoritarios del metribuzin y sulfonilureas) y antibióticos (cefalosporinas), en muestras de alimentos y medioambientales. Se han evaluado diversas técnicas como la cromatografía líquida y técnicas analíticas miniaturizadas, tales como la electroforesis capilar y HPLC capilar ambas acopladas a detección UV. Además, se han evaluado diferentes tratamientos de muestra alternativos a los tratamientos existentes con el fin de mejorar la eficacia y rendimiento. De este modo se ha contribuido a aumentar los métodos analíticos disponibles para el control de residuos de herbicidas y cefalosporinas en alimentos, agua y suelo.

A continuación se muestran las ventajas y desventajas de cada método que se ha desarrollado en esta Tesis.

- Los métodos de CE implican menor consumo de disolventes orgánicos y reactivos que los métodos cromatográficos, lo que significa un menor coste que los métodos de LC, así como un menor impacto sobre el medio ambiente. Además, los métodos de CE tienen un tiempo de análisis más corto que el método de HPLC capilar.
- Se han calculado los valores de las constantes de disociación (pK_a) de los principales productos de degradación de metribuzin, utilizando CE, y no se han observado diferencias significativas con los valores obtenidos mediante la aplicación de otras técnicas.
- La técnica CE ha permitido el desarrollo de una metodología de preconcentración en línea (LVSS) que ha sido adecuada para aumentar la sensibilidad en los métodos propuestos para la determinación de sulfonilureas, productos de degradación de metribuzin y cefalosporinas en las matrices seleccionadas.

Otro objetivo de esta Tesis ha sido el estudio de tratamientos de muestra alternativos con el fin de aumentar la selectividad y la sensibilidad. Así se han evaluado tratamientos de muestra específicos, como los MIPs y tratamientos genéricos de muestra.

- Se ha optimizado un procedimiento de PLE y se ha aplicado en la extracción de los productos de degradación del metribuzin, en muestras de suelo, seguido por una etapa de preconcentración y limpieza previo de

las muestras por SPE. Este último procedimiento es adecuado para los extractos de suelo obtenidos mediante la PLE y también directamente para las muestras de agua.

- Cuando la SPE se utiliza para analizar las muestras se consigue un elevado factor de preconcentración, permitiendo alcanzar LOD muy bajos, incluso usando CZE-UV, para el análisis de sulfonilureas en uvas y aguas y cefalosporinas en muestras de aguas.
- En términos de selectividad, MISPE es el tratamiento de la muestra más selectivo, puesto que los MIPs utilizados son sintetizados específicamente para la extracción de CL, CP y CD. Sin embargo, no permite el uso de grandes volúmenes de muestra y no consigue factores de preconcentración elevados. Los cromatogramas obtenidos cuando se utiliza MISPE junto con HPLC-UV son muy limpios, y no se encontraron interferentes que co-eluyan con las cefalosporinas estudiadas.
- Se ha desarrollado y validado un procedimiento rápido y sencillo que combina IP-SALLE para la extracción de cefalosporinas en solución acuosa utilizando disolventes orgánicos polares. La formación previa del par iónico (IP) es necesario para conseguir la extracción y preconcentración de las cefalosporinas.

A modo de resumen, en la tabla C.1 se muestran las características más notables de los métodos de análisis desarrollados.

Tabla C.1. Resumen de los métodos desarrollados en esta Tesis.

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental y condiciones experimentales	Tiempo de análisis (min)	LOD
DA, DK, DADK	suelo aguas	Suelo: PLE (metanol) + SPE (LiChrolut EN) Agua: SPE (LiChrolut EN)	**CE-LVSS** Tampón tetraborato sódico (40 mM, pH 9.5)	< 9	agua: 10-20 ng mL^{-1}
Tria-S, Rim-S, Flaza-S, Met-S, Chlor-S	uvas aguas	SPE: Uva: C18 Agua: HLB	**CE-LVSS**: Tampón acetato amónico (90 mM, pH 4)	< 20	uva: 0.97-8.30 μg kg^{-1} agua: 0.04-0.12 μg L^{-1}
CL, CPR, CT, CP, CZ	aguas	SPE: HLB	**CE-LVSS:** Tampon acetate amónico (70 mM, pH 7.0)	< 6	0.1-0.3 μg L^{-1}
CL, CP, CLN, CQ, CZ, CPR, CM	ternera cerdo agua	(IP-SALLE)	**Capillary HPLC**: gradiente: disolvente A (0.1% ácido fórmico en agua, pH 4) disolvente B (ACN:MeOH (1:1, v/v))	<23	ternera: 1.2-10.1 μg kg^{-1} agua: 1.3-6.8 μg L^{-1}
CD, CL, CP	leche	MISPE	**HPLC:** isocratico: MeOH:fosfato (pH 3, 10 $mmolL^{-1}$ ionic strength) (25:75, v/v)	< 10	nc

nc: no calculado

Abreviaturas y acrónimos

Abbreviations and acronyms

En la mayoría de los casos se ha empleado la abreviatura o acrónimo anglosajón debido a su empleo más generalizado.

In most cases, the anglo-saxon abbreviation or acronym have been employed because their use is more widespread.

6-APA: 6-Aminopenicillanic acid

7-ACA: 7-Aminocephalosporinic acid

AIBN: azobisisobutyronitrile

APCI: Atmospheric pressure chemical ionization

BGE: Backgruond electrolyte

BPA: Buenas prácticas agrícolas en el uso de plaguicidas

BUI: Benzoylurea insecticide

CD: Cephadroxil

CE: Electroforesis capillar

Chlor-S: Chlorsulfuron

CIPIC: 4-(2´-cyanoisoindolyl) phenylisothiocyanate

CL: Cephalexin

CLD: Chemiluminiscence detection

CLN: Cephalonium

CM: Cephamandole

CMC: Critical micelle concetration

CP: Cephapirin

CPR: Cephoperazone

CQ: Cefquinome

CT: Ceftiofur

CTAB: Cethyl trimethyl ammonium bromide

CZ: Cephazolin

CZE: Electroforesis capilar en zonal

DA: Deaminometribuzin

DAD: Diode array detection

DCM: Dichloromethane

DADK: Deaminodiketometribuzin

DDT: dichloro diphenyl trichloroethane (1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane)

DK: Diketometribuzin

DL_{50}: Dosis letal media

DLLE: Extracción líquido líquido dispersiva

DSPE: Extracción en fase sólida dispersiva

ECL: Electrochemiluminiscence

EDMA: Ethylene glycol dimethacrylate

EEA: European environmental agency

EOF: Flujo electroosmótico

EPA: Environmental Protection Agency (USA)

ESI: Electrospray

EU: European union

FAO: Food and Agriculture Organization of United Nations

Flaza-S: Flazasulfuron

FLD: Fluorescence detection

HLB: Balance hidrofílico-lipofílico

HPLC: Cromatografía líquida de alta resolución

HP-β-CD: 2-hydroxypropyl-β-cyclodextrin

IDA: Ingestión diaria admisible

IDAT: Ingestión diaria admisible temporal

IDTP: Ingestión diaria tolerable provisional

IL: Ionic liquid

IPAD: Integrated pulsed amperometric detection

IPE: Ion paired extraction

IP-SALLE: Extracción líquido-líquido asistida por sales con formación de par iónico

IS: Internal standard

IT: Ion trap

JMPR: Joint meeting on pesticide residues

LIF: Fluorescencia inducida por laser

LLE: Extracción líquido líquido

LMR: Límite máximo de residuo

LOD: Limit of detection

LOQ: Limit of quantification

LVSEP: Large volume sample stacking usig EOF pump

LVSS: Large volumen sample stacking

MAA: Methacrylic acid

MAE: Extracción asistida por microondas

MEKC: Micellar electrokinetic chromatography

Met-S: Metsulfuron methyl

MIP: Polímero impreso molecularmente

MISPE: Molecularly imprinted solid phase extraction

MM-CPE: Mixed micelle cloup point extraction

MOA: Mechanism of action

MS: Mass spectrometry

MSPD: Matrix solid phase dispersion

NACE: Non aqueous capillary electrophoresis

NIP: Non imprinted polymer

OMS: Organización Mundial de la Salud

PABA: p-Aminobenzoic acid

PED: Pulsed electrochemical detection

PFP: pentafluorophenyl

PLE: Extracción líquida presurizada

PUH: Phenylurea herbicide

Rim-S: Rimsulfuron

RP-TLC: Reversed phase thin layer chromatography

RSD: Relative standard deviation

RTIL: Room temperature ionic liquids

SALLE: extracción líquido líquido assitida por sales

SBSE: extraction por sorción sobre barra agitadora

SDS: Sodium dodecylsulphate

SFE: Supercritical fluid extraction

SLM: membrane líquida soportada

SPE: extracción en fase sólida

SPME: microextracción en fase sólida

SUH: Sulfonylurea herbicide

SWMR: Stacking with matrix removal

TBA: tributylamine

TBABr: Tetrabutylammonium bromide

TCA: Trichloroacetic acid

TDM: Therapeutic drug monitoring

TFMAA: 2-(trifluoromethyl)acrilic acid

THF: Tetrahydrofuran

TOF: Time of flight

Tria-S: Triasulfuron

Tx-100: Triton X-100

UE: Unión europea

UHPLC: Ultra high performance liquid chromatography

UV: Ultraviolet

Vis: Visible

WSSA: Weed science society of America