UNIVERSIDAD DE GRANADA
FACULTAD DE CIENCIAS
DEPARTAMENTO DE QUÍMICA ANALÍTICA

NUEVAS METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE QUINOLONAS Y OTROS RESIDUOS EN MUESTRAS ALIMENTARIAS Y AMBIENTALES

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Por

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MEMORIA presentada para aspirar al Grado de Doctor en Ciencias, Sección Químicas

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CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de “*NUEVAS METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE QUINOLONAS Y OTROS RESIDUOS EN MUESTRAS ALIMENTARIAS Y AMBIENTALES*”, ha sido realizado en los laboratorios del citado grupo bajo la dirección de las Profesoras Dª. Laura Gámiz Gracia, Dª Carmen Cruces Blanco y yo misma, 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 en Ciencias, con mención de “Doctorado Internacional”, dentro del programa de doctorado “Química”.

Granada de de

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- *"Nuevas estrategias basadas en técnicas miniaturizadas acopladas con detección por fluorescencia y espectrometría de masas para el control de pesticidas y fármacos en muestras ambientales y biológicas"* (Ref.: CTM2006-06363, Ministerio de Educación y Ciencia).

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Objeto de la memoria

OBJETO DE LA MEMORIA

Los objetivos de esta Tesis Doctoral se enmarcan dentro de diversos Proyectos de Investigación que se han desarrollado a lo largo del transcurso de este trabajo y que forman parte de las líneas de investigación del grupo en el que se ha realizado, así como del Proyecto de la Beca Predoctoral disfrutada durante la realización de la misma. Así, se ha llevado a cabo la puesta a punto de nuevos métodos de análisis sensibles y selectivos para la determinación de quinolonas y otros residuos en muestras de alimentos y aguas de diversa procedencia.

En el caso de los alimentos, la legislación vigente establece para los antibióticos y otros contaminantes unos Límites Máximos de Residuos que no deben ser superados con objeto de garantizar la calidad del producto y permitir su distribución y consumo. En el caso de las aguas, el interés de la determinación de antibióticos radica en la importancia que están tomando los fármacos como contaminantes emergentes en el medioambiente, ya que su presencia puede generar cierta resistencia bacteriana o reacciones alérgicas en la población.

Considerando las recientes e importantes mejoras de las técnicas separativas en cuanto a miniaturización y aumento de la eficacia, y con objeto de explorar sus indudables ventajas (bajo consumo de disolventes, alta sensibilidad, elevada resolución, tiempos de análisis reducidos y baja cantidad de muestra), en esta Tesis Doctoral se proponen nuevos métodos analíticos para la determinación de residuos de quinolonas y otros contaminantes empleando técnicas de separación miniaturizadas – Electroforesis Capilar (CE) y Cromatografía líquida capilar (HPLC capilar)– y de alta eficacia –Cromatografía Líquida de Ultra Resolución (UHPLC)– con objeto de explorar las ventajas mencionadas. Estas técnicas, acopladas a sistemas de detección altamente sensibles y selectivos, como la fluorescencia inducida por laser (LIF), la espectrometría de masas en tándem (MS/MS) o de alta eficacia (HRMS), permiten la cuantificación e identificación inequívoca de estos compuestos a las bajas concentraciones esperadas en estas matrices.

Asimismo, en esta Tesis doctoral se proponen nuevos métodos alternativos a los ya existentes para el tratamiento de muestras de diversos alimentos y aguas, más eficaces y respetuosos con el medioambiente, de acuerdo con las nuevas tendencias de la Química Verde.

Como objetivos concretos de esta Tesis Doctoral destacan los siguientes:

- Explorar el potencial del acoplamiento CE-LIF para la determinación de quinolonas de uso humano y veterinario en aguas de diversa procedencia, y de quinolonas de uso veterinario en alimentos.
- Emplear HPLC capilar-LIF para la determinación de quinolonas en leche, como alternativa a la HPLC convencional.
- Demostrar el potencial de la UHPLC-MS/MS como potente herramienta para la identificación inequívoca y cuantificación de quinolonas de uso humano y veterinario en aguas de diversa procedencia, y de quinolonas de uso veterinario en productos apícolas.
- Proponer nuevos tratamientos de muestra para la determinación de quinolonas basados en extracción en fase sólida, extracción en fase sólida dispersiva y polímeros de impresión molecular, como alternativa a los ya existentes, para conseguir la selectividad y sensibilidad requeridas en este tipo de análisis.
- Finalmente, la parte de esta Memoria desarrollada en el *RIKILT Institute of Food Chemistry* (Wageningen, Holanda) tuvo como objetivo el desarrollo de un método de *screening* para la detección multirresiduo de diversas familias de fármacos y plaguicidas en pollo mediante LC-HRMS.

Resumen

En esta Tesis se han desarrollado diferentes métodos para la determinación de contaminantes, Qns y otros residuos, en alimentos y muestras medioambientales. Para ello se han evaluado diferentes técnicas instrumentales separativas, la CE, la HPLC capilar y la UHPLC, acopladas a diferentes métodos de detección de gran sensibilidad y selectividad como son la fluorescencia inducida por laser (LIF), la espectrometría de masas en tándem (MS/MS) y la espectrometría de masas de alta resolución (HRMS). Además se han estudiado y propuesto metodologías de tratamientos de muestra alternativas a las ya existentes que permitan aumentar la eficacia y el rendimiento de los tratamientos.

En el primer capitulo se ha desarrollado un método para la determinación de 6 quinolonas de uso veterinario y humano en aguas utilizando la CE-LIF como técnica instrumental y la extracción en fase solida como método de tratamiento de muestra comparando diferentes sorbentes.

Para demostrar el potencial de la CE-LIF con muestras de mayor complejidad, en el segundo capitulo se determinaron 4 quinolonas de uso veterinario en muestras de leche de vaca y riñón de cerdo, empleando la extracción en fase solida con polímeros impresos molecularmente (MIPSPE) para la extracción y limpieza de la muestra.

En el tercer capitulo se ha llevado a cabo una comparación de dos tratamientos de muestra, MIPSPE y QuEChERS, para la determinación de 7 quinolonas en leche. En este caso, la técnica instrumental empleada ha sido la HPLC capilar-LIF.

En el capitulo cuarto se ha llevado a cabo la determinación de 8 Qns en productos apícolas Se ha utilizado la metodología QuEChERS para la extracción y la UHPLC-MS/MS para el análisis instrumental.

En el quinto se ha desarrollado un método de análisis para la determinación de 19 Qns en muestras de agua. Para el tratamiento de muestra se ha utilizado un método basado en la metodología QuEChERS y para el analisis se ha usado la UHPLC-MS/MS.

En el ultimo capitulo se recoge el trabajo desarrollado en el RIKILT Institute of Food Chemistry (Wageningen, Holanda) consistente en un método de cribado para la detección multirresiduo de diversas familias de fármacos y plaguicidas en pollo mediante LC-HRMS.

Summary

In this thesis different analytical methods for the determinationof contaminants, Quinolones and other residues, in foodstuff and environmental samples have been developed. For that purpose different separative techniques have been assessed, capillary electrophoresis (CE), capillary liquid chromatography (capillary HPLC) and ultra-high performance liquid chromatography (UHPLC), coupled to very sensitive and selective detection techniques as laser induced fluorescence (LIF), tandem mass spectrometry (MS/MS) and high resolution mass spectrometry (HRMS). In addition, alternative sample treatment to those previously reported have been assessed, making possible an increased efficiency and sample throughput.

A method for the determination of 6 Qns of veterinary and human use has been developed in the first chapter. CE-LIF has been used as instrumental technique and different sorbents for solid phase extraction have been compared for sample treatment.

In order to show the potential of CE-LIF when more complex sample are analyzed, in the second chapter 4 Qns were determined in cow milk and pig kidney. In this case, SPE using molecularly imprinted polymer (MISPE) as sorbent has been employed for sample treatment.

In the third chapter two sample treatments, MISPE and QuEChERS, have been compared for the analysis of milk. Capillary HPLC-LIF has been used as an alternative to conventional HPLC.

The determination of 8 veterinary Qns in bee products has been achieved in the fourth chapter. For that purpose, QuEChERS methodology has been used for sample treatment and UHPLC-MS/MS for instrumental analysis.

The same methodology has been used in the fifth chapter; in this case determine 19 Qns of human and veterinary use in environmental water samples.

In the last chapter the work carried out in RIKILT Institute of Food Chemistry (Wageningen, The Netherland) is described. It consisted on a multiresidue screening

method for the determination of veterinary drugs and pesticides in chicken meat using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) and a generic sample treatment.

Introducción

1. Antibióticos: 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 (Qns) [1].

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 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 saprofíticas 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 [1].

---

[1] "Antibióticos" en Microsoft, Encarta 2012, Microsoft Corporation.

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 [1].

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 (Grampositivos, Gramnegativos, 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 [2]:

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

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

Entre todos estos antibióticos, la presente Tesis Doctoral se ha centrado, fundamentalmente, en el desarrollo de métodos analíticos para la determinación de la familia de las llamadas Qns, cuyas características indicaremos a continuación.

2. Quinolonas

Las Qns químicamente son estructuras bicíclicas heteroaromáticas, constituidas por un núcleo piridona-β-ácido carboxílico y un anillo aromático. La denominación de este grupo como Qns se debe al hecho de que la mayor parte de los nuevos antimicrobianos del grupo (flumequina, ácido oxolínico, ofloxacino, fleroxacino, lomefloxacino, ciprofloxacino, etc.) eran derivados de la quinolina sustituida (4-quinolona, figura 1).

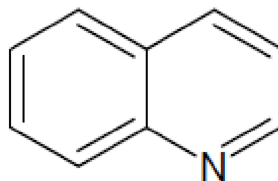

Figura 1. Estructura de la 4-quinolona.

2.1 *Actividad Antibacteriana de las Quinolonas*

La actividad antibacteriana de las Qns se basa en la inhibición de la topoisomerasa II bacteriana (ADN girasa) y de la topoisomerasa IV en especies Grampositivas, inhibiendo el superenrollamiento negativo terciario del ADN bacteriano. Este efecto, probablemente asociado con la unión de las Qns a un complejo de la ADN girasa, es el que ejerce el efecto bactericida. Hay que señalar que aunque existe el mismo tipo de topoisomerasa en las células eucariotas, en los humanos éstas no se ven afectadas por la acción de estos antibióticos, ya que están formadas por solo dos subunidades en lugar de cuatro como es el caso de las células bacterianas. Las Qns rara vez muestran efectos sinérgicos o antagónicos con otros agentes. Tienen un

papel importante en el tratamiento de infecciones moderadas y severas, causadas por un amplio rango de patógenos. En algunas áreas (infecciones urinarias, gonorrea, fiebre tifoidea o infecciones producidas por P. aeruginosa), se han convertido en el antibiótico más comúnmente empleado [3].

En general, las afecciones en las que las Qns son los antibióticos de uso más frecuente son las siguientes:

- Enfermedades del tracto urinario
- Enfermedades de transmisión sexual
- Infecciones respiratorias
- Infecciones gastrointestinales
- Infecciones cutáneas y los tejidos blandos
- Infecciones óseas
- Neutropenia en pacientes con cáncer
- Profilaxis

Cabe destacar que en los últimos años se ha desarrollado resistencia bacteriana a algunos de estos agentes, habiéndose estudiado casos como el de la disminución de la susceptibilidad de cepas de neumococo a varios de estos agentes [4].

2.2 *Desarrollo histórico y clasificación de las quinolonas*

En cuanto a su desarrollo histórico, la primera Qn se descubrió fruto de la casualidad durante la síntesis de la cloroquina. Así, en el año 1962 Lescher sintetizó el ácido nalidíxico (figura 2), una molécula con cierta actividad contra organismos Gramnegativos [5], dando lugar al desarrollo de este importante grupo de antibióticos. Sin embargo, este compuesto presentaba una baja penetración intracelular y su

[3] P. Ball, “The Quinolones: History and overview” en “The Quinolones”, 3ª Ed., Academic Press 2000 p. 1.

[4] D.K. Chen, A. Mc Geer, J.C. de Azavedo, D.E. Low, New Engl. J. Med. 341 (1999) 233.

[5] G.Y. Lesher, E.D. Froelich, M.D. Gruet, H, Bailey, R.P. Brundage, J. Med. Pharmacol. Chem. 5 (1962) 1063.

farmacocinética sólo producía una adecuada concentración en las vías urinarias, por lo que su uso se limitó al tratamiento de infecciones urinarias sencillas.

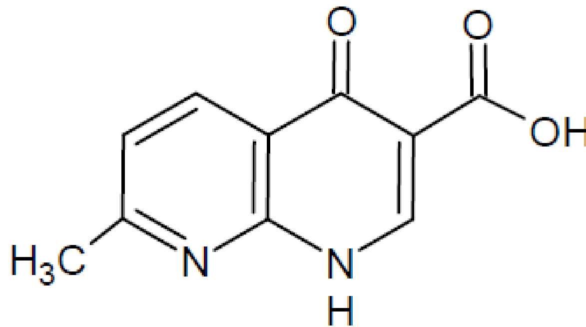

Figura 2. Estructura del ácido nalidíxico

Sobre la estructura de esta molécula se intentaron nuevas modificaciones buscando antimicrobianos con mayor espectro y propiedades farmacocinéticas superiores. El mayor avance en este aspecto correspondió a la síntesis de compuestos que presentaban los sustituyentes 6-fluoro, 7-piperazinil (norfloxacino, ciprofloxacino y pefloxacino), que dieron lugar al grupo que genéricamente se ha denominado fluoroquinolonas, 4-quinolonas o simplemente Qns. Este grupo de antimicrobianos es uno de los que han presentado un mayor avance en las últimas décadas [6].

Así, la relación entre la estructura química y la actividad biológica de las Qns dio lugar a la síntesis de compuestos con distintos radicales en la estructura básica (figura 3), con objeto de mejorar su actividad.

[6] S. Mella, G. Acuña, M. Muñoz, C. Pérez, J. Labarca, G. González, H. Bello, M. Domínguez, R. Zemelman, Rev. Chil. Infect. 17 (2000) 53.

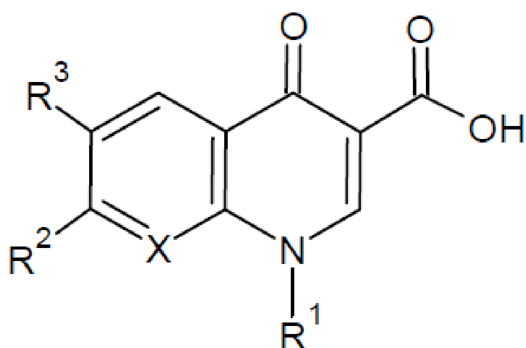

Figura 3. Estructura general de las Qns: X = C o N, R^1= ciclopropil, etil, fluoroetil, metilamino, fluorofenilo, anillo tiazínico u oxacínico. R^2= piperacino-4-ilo, 3-metilpiperacino-1-ilo; R^3 = flúor.

A partir de estas modificaciones experimentadas en el tiempo, uno de los criterios más ampliamente aceptados hoy en día es el de clasificar a las Qns en generaciones, dependiendo del momento de su síntesis y de los radicales utilizados [6]:

- Así, las Qns de Primera Generación son las moléculas más antiguas y que definen los núcleos químicos básicos de estos compuestos. Comprenden al ácido nalidíxico, el ácido oxolínico, cinoxacino, ácido piromídico, ácido pipemídico y flumequina, y se caracterizan por la ausencia de un radical 6-fluoro (con la excepción de la flumequina) (figura 4). Estos agentes son útiles sólo en infecciones del tracto urinario. Así, el espectro de actividad de las Qns de primera generación se limita a organismos entéricos, sin ser eficaces contra las *Pseudomonas aeruginosa* [7].

[7] M.L. Herrera, A. Vargas, C. Pérez, T. Moya, Rev. Méd. Hosp. Nac. Niños, 39 (2004) 45.

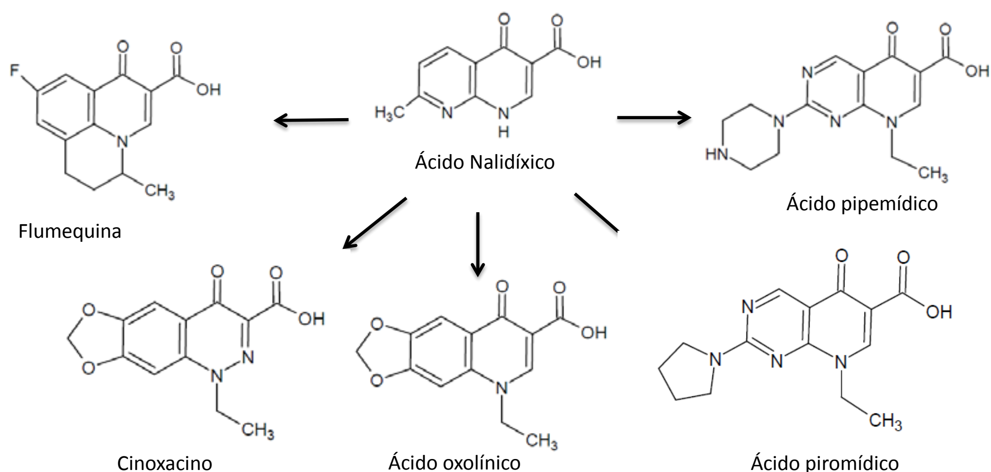

Figura 4. Estructura de algunas Qns de la primera generación.

- Las Qns de Segunda Generación (figura 5) se caracterizan por la presencia constante del flúor en la posición 6 y de piperazina o metil piperazina en la posición 7 de la molécula. Aparecieron en los años 80 cuando se sintetizó la molécula de norfloxacino, la primera que incluía el átomo de flúor en posición 6, lo cual aumenta su acción contra la ADN girasa y facilita la penetración en la célula bacteriana [8]. Este átomo de flúor se ha mostrado de gran importancia ya que ha permanecido en el desarrollo de posteriores Qns tanto de la tercera como de la cuarta generación. En esta segunda generación se incluyen el ciprofloxacino, norfloxacino, enoxacino, pefloxacino, fleroxacino, lomefloxacino, ofloxacino y rufloxacino. La segunda generación, se caracteriza por una excelente actividad contra bacterias Gramnegativas y algunos patógenos menos típicos, por una escasa actividad contra gérmenes Grampositivos (excepto *Staphylococcus aureus*), por una excelente biodisponibilidad oral, una muy buena tolerancia gástrica y con pocos efectos adversos en

[8] J.M. Domagala, L.D. Hanna, C.L. Heifetz, M.P. Hutt, T.F. Mich, J.P. Sanchez, M. Solomon, J. Med. Chem. 29 (1986) 394.

el paciente [7]. Este grupo es útil en el tratamiento de infecciones graves, respiratorias, digestivas, de tejidos blandos y de transmisión sexual.

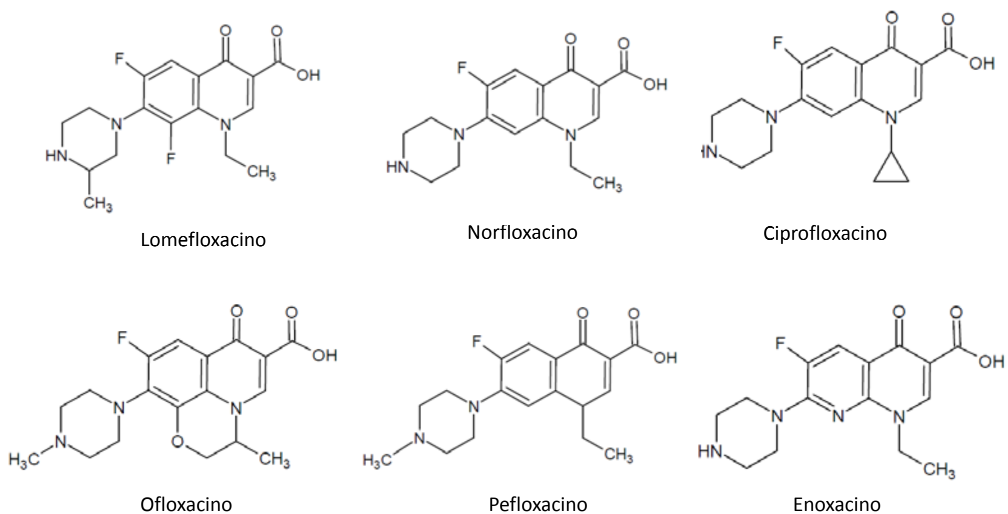

Figura 5. Estructura de algunas Qns de segunda generación.

- La Tercera Generación de Qns (figura 6), desarrollada en la década de los 90, comprende compuestos caracterizados por la presencia de grupos cíclicos aminados en la posición 7, son bi o trifluoradas. Comprende a sparfloxacino, levofloxacino, tosufloxacino, gatifloxacino, pazufloxacino y grepafloxacino. En ellas se mejoró la biodisponibilidad oral, la vida media y su acción contra bacterias Grampositivas (en especial *Streptococcus pneumoniae*), presentando en general una actividad superior a las de la segunda generación sobre bacterias anaerobias [7].

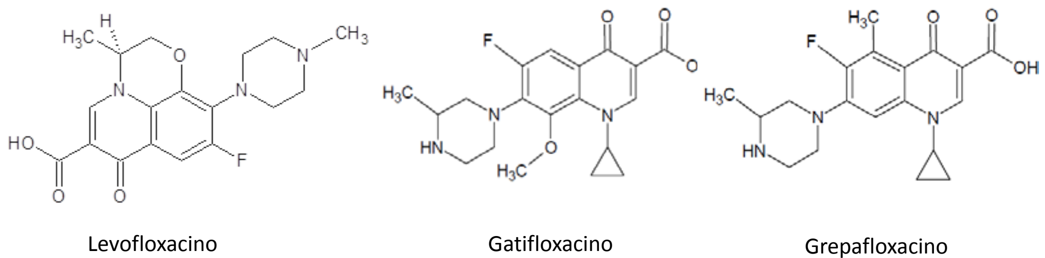

Figura 6. Estructura de algunas Qns de tercera generación.

- Finalmente, las Qns de Cuarta Generación (ver figura 7) muestran mayor actividad frente a organismos Grampositivos y buena actividad frente a los anaerobios (como *Clostridium sp.* y *Bacteroides fragilis*). Entre ellas están trovafloxacino, clinafloxacino, sitafloxacino, gemifloxacino y moxifloxacino. Por su definitiva actividad antianaerobia, se consideran como Qns de Cuarta Generación [7,9].

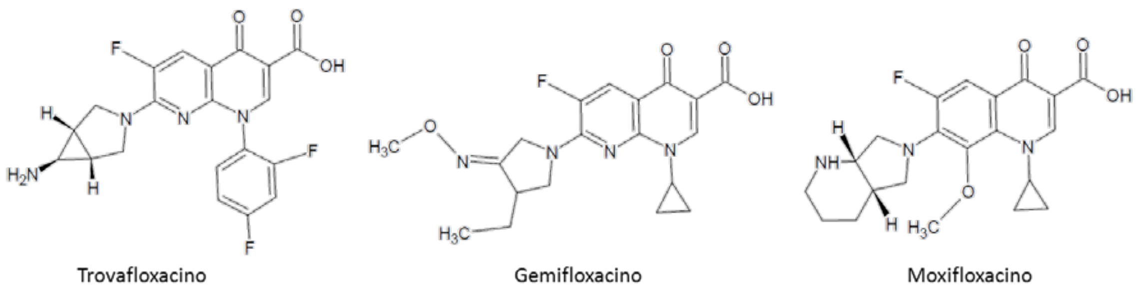

Figura 7. Estructura de algunas Qns de cuarta generación.

Tal y como se ha mencionado, las Qns han mostrado tener una clara relación entre su estructura básica, sus cadenas laterales y su actividad, así como sobre sus posibles efectos adversos; por ello, el diseño de nuevas Qns suele evitar los problemas que caracterizaban a Qns de generaciones anteriores [10].

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[9] D.E. King, R. Malone, S.H. Lilley, Am. Fam. Physician 61 (2000) 2741.

[10] K.E. Brighty, T.D. Gootz, “Chemistry and mechanism of action of the quinolone antibacterials” en “The Quinolones”, 3ª Ed., Academic Press 2000,p. 33.

2.3 *Difusión y excreción de las quinolonas en organismos*

Las Qns, al igual que otros antibióticos, se administran a los seres humanos con el fin de alcanzar y atacar a las bacterias que residen en el cuerpo. El máximo efecto bactericida se consigue cuando los antibióticos, incluyendo las Qns, llegan a los tejidos ocupados por el patógeno y permanecen allí. El éxito de éstos depende de la concentración y del tiempo que los antibióticos residen en el tejido afectado sin interferir en el normal funcionamiento de otros tejidos. La farmacocinética se encarga del estudio del tránsito del antibiótico desde su administración hasta el lugar infectado y su eliminación del cuerpo [11].

Una vez que los antibióticos alcanzan las bacterias dentro del cuerpo humano, el objetivo del tratamiento es matar a la bacteria e inhibir el desarrollo de bacterias resistentes. Para conseguirlo, los antibióticos deben alcanzar su elemento diana en la bacteria. Así, deben llegar al lugar infectado y atacar el sitio adecuado en la bacteria; en el caso de las Qns sería la ADN girasa o la topoisomerasa IV, y dar lugar a la inhibición [11,12,13]. Si los antibióticos no pueden destruir las bacterias completamente, las supervivientes pueden desarrollar factores de resistencia y transmitirlos a otras bacterias. Para maximizar la eficacia de la acción de los antibióticos y evitar la aparición de la resistencia, los antibióticos deben atacar un número de sitios activos suficiente y permanecer allí el tiempo necesario para realizar su función. En general, las Qns tienen una excelente absorción oral y distribución tanto en los tejidos como en fluidos orgánicos, uniéndose a las proteínas en un porcentaje inferior a un 50%. La mayoría de ellas se metabolizan por vía hepática y excreción renal, por lo que gran parte de ellas se elimina sin alteración. A través de

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[11] K. Myo-Kyoung, C.H. Nightingale, "Pharmacokinetics and pharmacodynamics of the fluoroquinolones" en "The Quinolones", 3ª Ed., Academic Press 2000, p. 169.

[12] C. Janoir, V. Zeller, M.D. Kitzis, N.J. Moreau, L. Gutmann, Antimicrob. Agents Chemother. 40 (1996) 2760.

[13] T.D. Gootz, R. Zaniewski, S. Haskell, B. Schmieder, J. Tankovic, D. Girard, P. Courvalin, R.J. Polzer, Antimicrob. Agents Chemother. 40 (1996) 2691.

esta ruta se pueden eliminar diferentes porcentajes de la dosis administrada para muchas de ellas como por ejemplo ciprofloxacino (57-62%) o moxifloxacino (15-21%) [11].

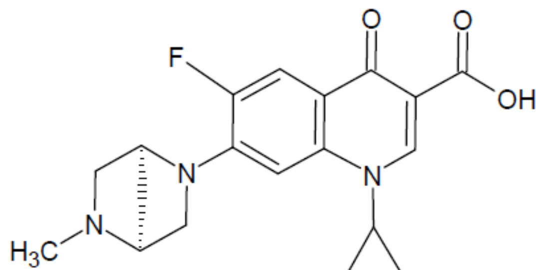
2.4 *Características físico-químicas generales de las quinolonas*

En general, las Qns son escasamente solubles en agua y la mayoría presentan carácter zwitteriónico, debido a la presencia de un ácido carboxílico y una amina en su estructura. Los valores de pKa para estos grupos funcionales están comprendidos entre 5.5–6.3 para los ácidos carboxílicos y 7.6–9.3 para el grupo amino. A pH ácido, tanto la amina como el ácido carboxílico están protonados, dando a la molécula una carga global positiva. Por otro lado, a pH básico, la amina se encuentra en forma de base libre, mientras que el grupo carboxílico se encuentra en forma de anión carboxilato, dando a la molécula una carga neta negativa. Por este motivo, las Qns tienden a ser más solubles en agua a pH ácidos o básicos, con una solubilidad escasa a pH neutro (fisiológico); por ejemplo, el tosufloxacino presenta una solubilidad en agua a pH fisiológico de sólo 8 mg/l. Este punto es particularmente importante en el caso de administración intravenosa de estos agentes [3].

Las Qns se caracterizan por sus elevados puntos de fusión (generalmente >200°C), indicando que su estructura cristalina es altamente estable.

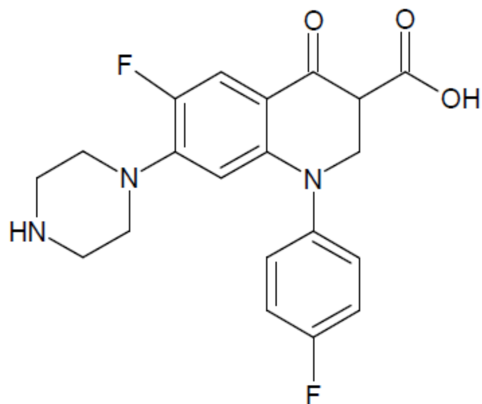
A continuación se muestran las propiedades físico-quimicas de las Qns que han sido objeto de estudio en la presente tesis: danofloxacino, sarafloxacino, difloxacino, ofloxacino y su isómero levofloxacino, flumequine, norfloxacino, cinoxacino, pefloxacino, ácido nalidíxico, ácido pipemídico, enoxacino, lomefloxacino, marbofloxacino, ciprofloxacino, enrofloxacino, fleroxacino, orbifloxacino, moxifloxacino, y ácido oxolínico. Los datos han sido obtenidos de la base de datos Scifinder a excepción de los LMR que han sido obtenidos de Reglamento (EU) Nº 37/2010 [24].

Danofloxacino (DANO)



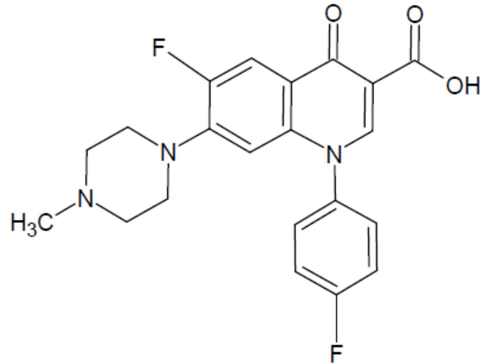
Nombre químico:	Ácido 1-ciclopropil-6-fluoro-1,4-dihidro-7-[(1S,4S)-5-metil-2,5-diazabiciclo[2.2.1]hept-2-il]-4-oxo- 3-quinolincarboxílico
Solubilidad:	12 g/l a pH 1.
Log P:	1’9
pKa:	6’0 y 9’0
Peso molecular:	357
LMR:	30 µg/kg en leche, 200 µg/kg en riñon de cerdo y carne de ave

Sarafloxacino (SARA)



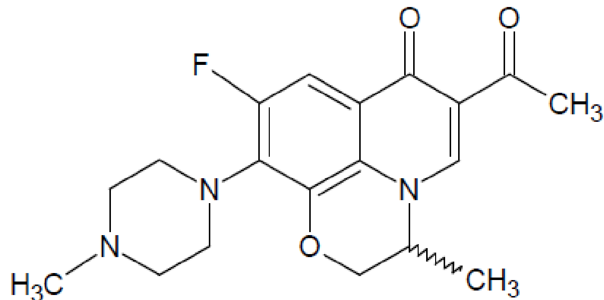
Nombre químico:	Ácido 6-fluoro-1-(4-fluorofenil)-1,4-dihidro-4-oxo-7-(1-piperazinil)- 3-quinolincarboxílico.
Solubilidad:	7.1 g/l a pH 10.
Log P:	3.0
pKa:	5.6 y 8.4
Peso molecular:	385
LMR:	No existe MRL en las matrices estudiadas

Difloxacino (DIFLO)



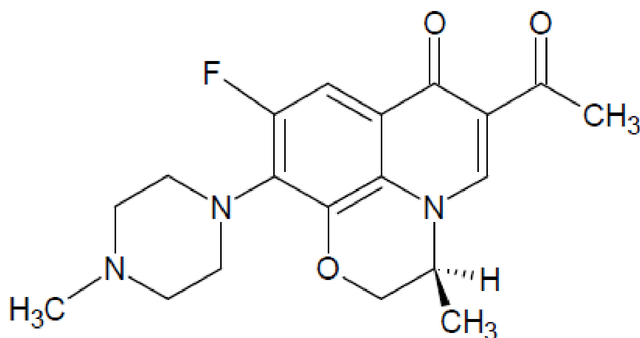
Nombre químico:	Ácido 6-fluoro-1-(4-fluorofenil)-1,4-dihidro-7-(4-metil-1-piperazinil)-4-oxo- 3-quinolincarboxílico
Solubilidad:	380 g/l a pH 10
Log P:	3.7
pKa:	5.9 y 7.4
Peso molecular:	399
LMR:	800 µg/kg en riñon de cerdo y 300 µg/kg carne de ave

Ofloxacino (OFLO)



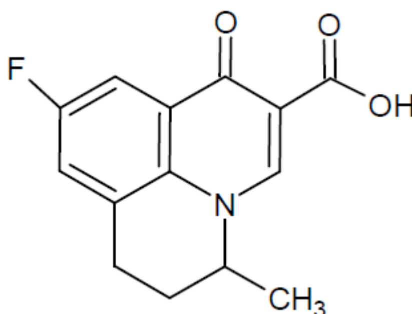
Nombre químico:	Ácido 9-fluoro-2,3-dihidro-3-metil-10-(4-metil-1-piperazinil)-7-oxo-7H-pirido[1,2,3-de]-1,4-benzoxazina-6-carboxilico
Solubilidad:	87 g/l a pH 1
Log P:	1.9
pKa:	5.2 y 7.4
Peso molecular:	361
LMR:	No existe MRL en las matrices estudiadas

Levofloxacino (LEVO)



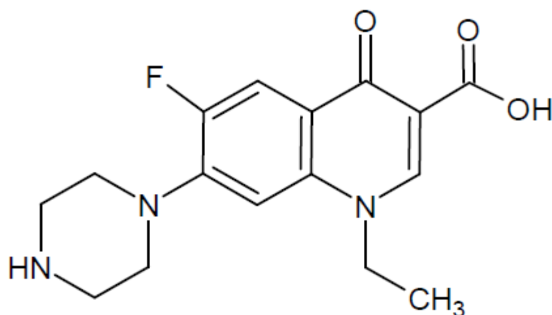
Nombre químico:	Ácido 9-fluoro-2,3-dihidro-3-metil-10-(4-metil-1-piperazinil)-7-oxo-(3S)- 7H-pirido[1,2,3-de]-1,4-benzoxazina-6-carboxilico
Solubilidad:	87 g/l a pH 1
Log P:	1.9
pKa:	5,2 y 7,4
Peso molecular:	361
LMR:	No existe MRL en las matrices estudiadas

Flumequina (FLUME)



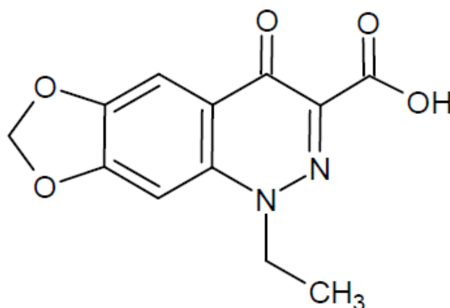
Nombre químico:	Ácido 9-fluoro-6,7-dihidro-5-metil-1-oxo- 1H,5H-benzo[ij]quinolizina-2-carboxílico
Solubilidad:	1000 g/l a pH 10
Log P:	2.4
pKa:	-1.9 y 5.7
Peso molecular:	261
LMR:	No existe MRL en las matrices estudiadas

Norfloxacino (NOR)



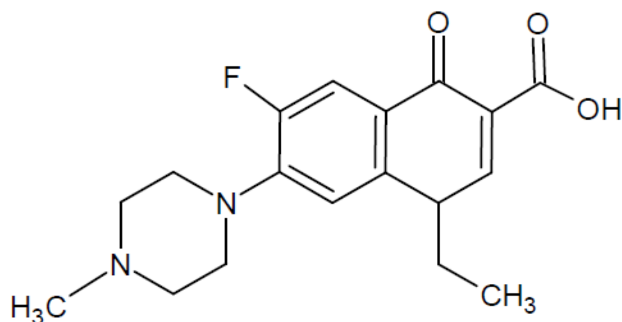
Nombre químico:	Ácido 1-etil-6-fluoro-1,4-diidro-4-oxo-7-(1-piperazinil)3-quinolincarboxilico,
Solubilidad:	220 g/l a pH 10
Log P:	1.7
pKa:	0.2 y 8.7
Peso molecular:	319
LMR:	No existe MRL en las matrices estudiadas

Cinoxacino (CINO)



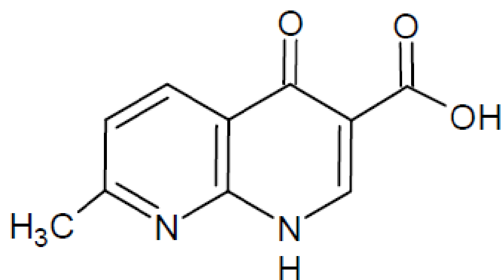
Nombre químico:	[1,3]Dioxol[4,5-g]cinnolina-3-carboxi,1-etil-1,4-dihidro-4-oxo-1-etil-3-carboxi-6,7-metilendioxi-4-cinnolona
Solubilidad:	999 g/l a pH 10
Log P:	0.5
pKa:	4.33
Peso molecular:	262
LMR:	No existe MRL en las matrices estudiadas

Pefloxacino (PEFLO)



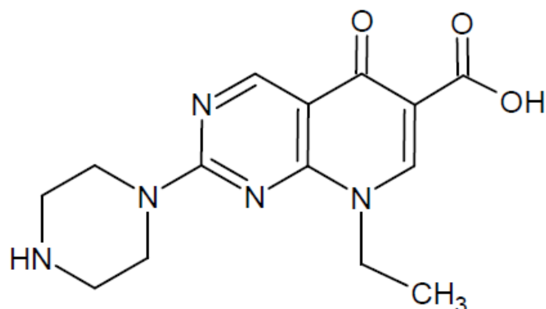
Nombre químico:	Ácido 1-etil-6-fluoro-1,4-dihidro-7-(4-metil-1-piperazinil)-4-oxo-3-quinolincarboxilico
Solubilidad:	190 g/l a pH 1
Log P:	1.9
pKa:	0.2 y 7.4
Peso molecular:	333
LMR:	No existe MRL en las matrices estudiadas

Ácido nalidíxico (NALI)



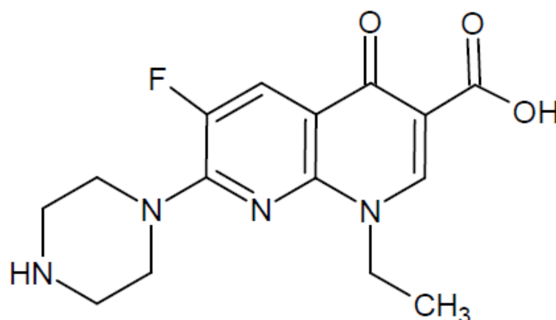
Nombre químico:	Ácido 1-etil-1,4-dihidro-7-metil-4-oxo-1,8-naftiridin-3-carboxilico
Solubilidad:	1000 g/l a pH 10
Log P:	0.025
pKa:	3.5 y 6.12
Peso molecular:	232
LMR:	No existe MRL en las matrices estudiadas

Ácido pipemídico (PIPE)



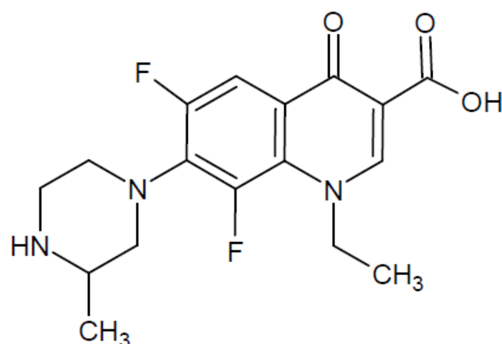
Nombre químico:	Ácido 8-Etil-2-(piperazin-1-il)-5-oxo-5,8-dihidropirido[2,3-d]pirimidine-6-carboxilico
Solubilidad:	1000 g/l a pH 1
Log P:	-0.194
pKa:	4.14 y 8.5
Peso molecular:	303
LMR:	No existe MRL en las matrices estudiadas

Enoxacino (ENO)



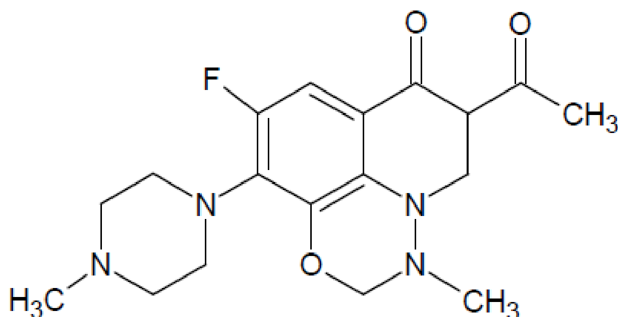
Nombre químico:	Ácido 1-etil-6-fluoro-1,4-dihidro-4-oxo-7-(1-piperazinil)-1,8-naftiridin-3-carboxilico
Solubilidad:	999 g/l a pH 1
Log P:	1.6
pKa:	6.0 y 8.2
Peso molecular:	320
LMR:	No existe MRL en las matrices estudiadas

Lomefloxacino (LOME)



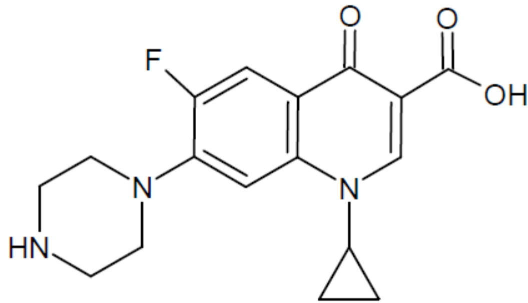
Nombre químico:	Ácido 1-etil-6,8-difluoro-1,4-dihidro-7-(3-metil-1-piperazinil)-4-oxo-3-quinolincarboxilico
Solubilidad:	33 g/l a pH 1
Log P:	2.5
pKa:	-0.3 y 8.6
Peso molecular:	351
LMR:	No existe MRL en las matrices estudiadas

Marbofloxacino (MARBO)



Nombre químico:	Ácido 9-fluoro-2,3-dihidro-3-metil-10-(4-metil-1-piperazinil)-7-oxo-7H-pirido[3,2,1-ij][4,1,2]benzoxadiazin-6-carboxilico
Solubilidad:	1000 g/l a pH 1 y pH 10
Log P:	0.2
pKa:	6.1 y 7.4
Peso molecular:	362
LMR:	75 µg/kg en leche, 150 µg/kg en riñon de cerdo

Ciprofloxacino (CIPRO)



Nombre químico: Ácido 1-ciclopropil-6-fluoro-1,4-dihidro-4-oxo-7-(1-piperazinil)-3-quinolíncarboxílico.

Solubilidad: 56 g/l a pH 1.

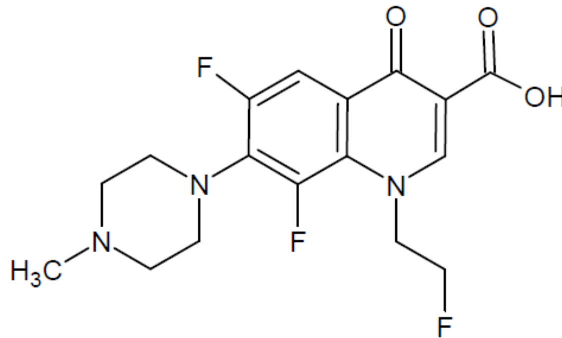
Log P: 1.3

pKa: 6.0 y 8.4

Peso molecular: 331

LMR: Expresado como suma de ENRO y CIPRO. 100 µg/kg en leche, 300 µg/kg en riñon de cerdo y 100 µg/kg en carne de ave

Fleroxacino (FLERO)



Nombre químico: Ácido-6,8-difluoro-1-(2-fluoroetil)-1,4-dihidro-7-(4-metil-1-piperazinil)-4-oxo-3-quinolincarboxilico.

Solubilidad: 170 g/l a pH 10

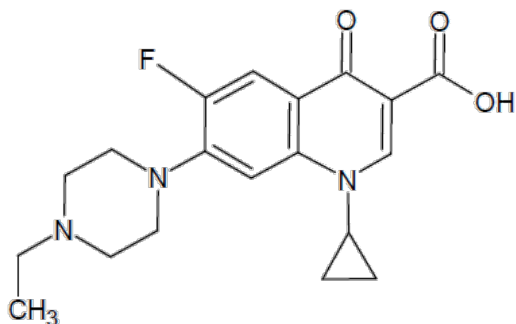
Log P: 1.8

pKa: -0.54 y 7.15

Peso molecular: 369

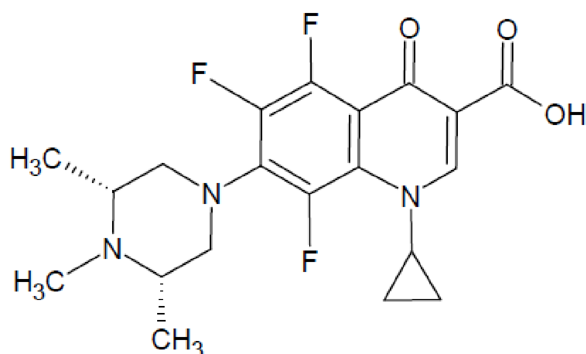
LMR: No existe MRL en las matrices estudiadas

Enrofloxacino (ENRO)



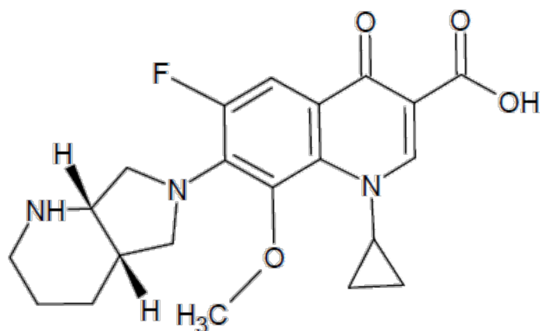
Nombre químico:	Ácido-1-ciclopropil-6-fluoro-7-(4-etil-1-piperazinil)-1,4-dihidro-4-oxo-3-quinolincarboxilico
Solubilidad:	25 g/l a pH 1 y pH 10
Log P:	2.5
pKa:	6.0 y 6.9
Peso molecular:	359
LMR:	Expresado como suma de ENRO y CIPRO. 100 µg/kg en leche, 300 µg/kg en riñon de cerdo y 100 µg/kg en carne de ave

Orbifloxacino (ORBI)



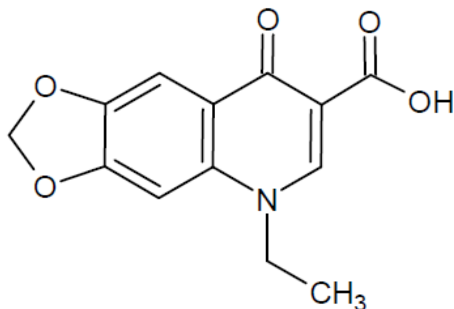
Nombre químico:	Ácido-1-ciclopropil-7-[(3R,5S)-3,5-dimetil-1-piperazinil]-5,6,8-trifluoro-1,4-dihidro-4-oxo-3-quinolincarboxilico
Solubilidad:	1.6 g/l a pH 10
Log P:	3.0
pKa:	6.4
Peso molecular:	8.5
LMR:	No existe MRL en las matrices estudiadas

Moxifloxacino (MOXI)



Nombre químico:	Ácido-1-ciclopropil-6-fluoro-1,4-dihidro-8-metoxi-7-[(4aS,7aS)-octahidro-6H-pirrol[3,4-b]piridin-6-il]-4-oxo-3-quinolincarboxilico
Solubilidad:	20 g/l pH 1
Log P:	2.8
pKa:	6.4 y 10.6
Peso molecular:	401
LMR:	No existe MRL en las matrices estudiadas

Ácido oxolínico (OXO)



Nombre químico:	Ácido 1-Etil-1,4-dihidro-6,7-metilendioxi-4-oxo-3-quinolincarboxlico
Solubilidad:	3.83 mol/l a pH 10
Log P:	0.9
pKa:	-2.0 y 5.9
Peso molecular:	261
LMR:	150 µg/kg en riñon de cerdo y 100 µg/kg en carne de ave

3. *Interés y legislación sobre el control de residuos de medicamentos de uso veterinario en alimentos*

El sector agroalimentario tiene gran importancia en el conjunto de la economía europea. La industria productora de alimentos y bebidas es uno de los sectores industriales más destacados de la Unión Europea (EU) con cerca del 15 % de la producción industrial total, siendo además la EU el mayor productor mundial de productos alimenticios y bebidas. Esta industria es el tercer mayor empleador en el sector industrial europeo y ocupa a más de 2.6 millones de empleados, un 30 % de los cuales trabajan en pequeñas y medianas empresas. Por otro lado, el sector agrícola tiene una producción anual de alrededor de 220000 millones de € y proporciona el equivalente a 7.5 millones de puestos de trabajo a tiempo completo. Las exportaciones de productos alimenticios y bebidas ascienden a 50000 millones de € al año.

La importancia económica, la ubicuidad de los alimentos en nuestra vida y la cada vez más compleja cadena de producción de alimentos sugieren que la seguridad alimentaria ha de ser uno de los principales intereses del conjunto de la sociedad y, especialmente, de los poderes públicos y de los productores del sector [14]. Entre estas preocupaciones, en los últimos años ha aumentado la relativa acerca del empleo de medicamentos (fundamentalmente antibióticos) en animales destinados al consumo o productores de alimentos, debido a la posibilidad de transferencia al humano de bacterias resistentes [15]. Si bien para garantizar la salud y el bienestar de los animales es completamente necesario disponer de medicamentos veterinarios, su utilización en animales productores de alimentos puede dar lugar a la permanencia de residuos en los productos alimenticios obtenidos a partir de animales tratados. Tales sustancias pueden igualmente ser usadas en el animal no sólo para tratar infecciones,

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[14] Libro blanco sobre seguridad alimentaria, Comisión de las Comunidades Europeas, Bruselas, 12.1.2000 COM (1999) 719 final.

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

en el caso de animales enfermos, sino que también se suministraban como promotores del crecimiento ya que afectan a la flora microbiana y a los procesos de digestión, ralentizándolos y favoreciendo el proceso de absorción de nutrientes, lo que originaba el engorde del animal. Por ello, los residuos de estos compuestos han podido estar presentes tanto en animales sanos como enfermos. No obstante, mediante el Reglamento (CE) No 1831/2003 [16], a partir del 1 de Enero de 2006 se prohibió el uso de antibióticos con este fin.

Algunas consecuencias del uso indebido y no controlado de los antibióticos es su incidencia en diferentes aspectos relacionados con la Calidad y Seguridad Alimentaria, tales como [17]:

a) pueden producir reacciones de hipersensibilidad en personas alérgicas;

b) inciden negativamente en procesos industriales de fermentación en alimentos lácteos, tales como la elaboración del yogurt;

c) la presencia de antibacterianos puede ocultar la existencia de patógenos en alimentos de origen animal cuando se realizan análisis bacteriológicos.

Partiendo de esta situación, y con el fin de proteger la salud de los consumidores, se hace indispensable realizar una evaluación de la seguridad de estas sustancias teniendo en cuenta los riesgos toxicológicos, la contaminación medioambiental, y los efectos farmacológicos y/o microbiológicos no deseados de sus posibles residuos. Así, 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

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[16] 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 L268/29,18-10-2003.

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

repercusiones sobre la salud pública y el medio ambiente. En el R.D. 109/1995 [18], y sus posteriores modificaciones, se exponen los requisitos de calidad, eficacia, seguridad y pureza que ha de cumplir un medicamento veterinario para poder ser registrado y autorizado.

Además, una sustancia farmacológicamente activa sólo podrá utilizarse en animales productores de alimentos si ha sido objeto de una evaluación de riesgo, con sólida base científica, y resultado favorable. Con objeto de proteger la salud humana, cuando se considera necesario se establece un MRL (limite máximo de residuo), definido como "la máxima concentración de una sustancia química determinada que puede admitirse en un alimento sin que signifique riesgo para la salud" y más específicamente es “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”. 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 MRL [19]. El MRL se calcula para cada tejido (matriz) en particular, dividiendo el producto de la Ingesta Diaria Admisible (IDA) y el peso corporal promedio humano (60 kg) por la ingesta diaria estimada para ese tejido en particular y se expresa en mg/Kg o µg/Kg sobre la base del peso del alimento fresco. Una vez que se fija el MRL, es necesario establecer en qué momento, a partir de la aplicación de un producto veterinario, tanto el principio activo como sus metabolitos se han eliminado hasta encontrarse en un valor inferior al MRL. Este es el fundamento del establecimiento del "periodo de restricción o periodo de retirada", que es el tiempo

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[18] Real Decreto 109/1995, de 27 de enero sobre medicamentos veterinarios. BOE. 53, 3-03-1995 y posteriores modificaciones.

[19] Agencia Española de Seguridad Alimentaria y Nutrición. http://www.aesan.msc.es/AESAN/web/cadena_alimentaria/subseccion/residuos_medicamentos_veterinarios.shtml

que debe transcurrir entre la última administración del producto veterinario y el sacrificio o recolección de los productos que se derivan de esos animales tratados destinados al consumo humano.

Actualmente la norma básica que recoge y regula estos MRLs es el Reglamento (CE) Nº 470/2009 del Parlamento Europeo y del Consejo [20] por el que se establecen procedimientos comunitarios para la fijación de MRL de las sustancias farmacológicamente activas en los alimentos de origen animal, se deroga el Reglamento (CEE) Nº 2377/90 del Consejo y posteriores modificaciones [21] y se modifican la Directiva 2001/82/CE del Parlamento Europeo y del Consejo y el Reglamento (CE) Nº 726/2004 del Parlamento Europeo y del Consejo.

Este Reglamento (CE) Nº 470/2009 tiene dos objetivos fundamentales [19]:

1. La protección de la salud humana y animal, para lo cual se establece que:
    a. Una sustancia farmacológicamente activa sólo podrá utilizarse en animales productores de alimentos (carne, pescado, leche, huevos y miel) si ha sido objeto de una evaluación favorable por la EMEA (Agencia Europea de Medicamentos). La evaluación científica de los riesgos tendrá en cuenta el metabolismo y la eliminación de las sustancias activas, el tipo de residuos, así como la IDA. En este sentido, cabe destacar que como novedad el reglamento obliga a que el dictamen de la EMEA deberá tener en cuenta todo hallazgo

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[20] 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) no 726/2004 del Parlamento Europeo y del Consejo. DOUE L152/11,16-6-2009.

[21] 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 224,18-08-1990.

científico pertinente de la EFSA (Autoridad Europea de Seguridad Alimentaria).

b. Como consecuencia de lo anterior, se establece una clasificación de las sustancias activas y las categorías terapéuticas a las cuales pertenecen, indicándose: un MRL, un MRL provisional, la ausencia de establecer un MRL, o bien la prohibición de uso de esa sustancia.

2. Garantizar la disponibilidad de medicamentos veterinarios adecuados para enfermedades que afecten a animales productores de alimentos, proponiéndose para ello:
   a. Establecer un procedimiento de extrapolaciones de MRL ya fijados para una sustancia activa en un alimento/especie a otros alimentos derivados o especies animales diferentes.
   b. La incorporación, sin necesidad de dictamen previo de la EMEA de aquellos MRLs adoptados mediante Decisión del Codex Alimentarius (CLXs) para los cuales la delegación de la EU no haya expresado una objeción.

Así, en el Reglamento (CE) Nº 2377/90 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 MRL
- Anexo II: sustancias para las que no es necesario fijar un MRL
- Anexo III: sustancias para las que se ha fijado un MRL 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.

Este reglamento sufrió diversas modificaciones, entre ellas la introducida por el Reglamento (CE) N° 1181/2002 de la Comisión [22], que modificó el Anexo I incorporando nuevas sustancias cuyos MRLs 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 este grupo se encuentran los agentes antiinfecciosos: quimioterapéuticos (sulfonamidas y derivados de la diaminopirimidina), antibióticos (penicilinas, cefalosporinas, Qns, macrólidos, flurofenicol y compuestos asociados, tetraciclinas, ansamicina, pleuromutilinas, lincosamidas, aminoglucósidos, etc.) y agentes antiparasitarios (salicilanidas, benzimidazoles, derivados fenólicos, etc.). Además de ésta, el Reglamento (CE) Nº 2377/90 sufrió otras modificaciones, incorporando nuevas sustancias o modificando los MRLs. Tales modificaciones han quedado recogidas en una versión no oficial consolidada [23].

De forma complementaria al Reglamento (CE) Nº 470/2009, se ha publicado el Reglamento (EU) Nº 37/2010 [24] que recoge la información contenida en los anexos del ya derogado Reglamento 2377/1990, esto es, los MRLs de sustancias farmacológicamente activas en los alimentos de origen animal en la que se introducen, además, varias novedades:

- Se crean únicamente dos listas en lugar de los cuatro anexos anteriores: MRLs para sustancias permitidas (las que figuraban en los Anexos I, II y III

[22] 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, 2-07-2002.

[23] http://eur-lex.europa.eu/LexUriServ/site/es/consleg/1990/R/01990R2377-20070516-es.pdfhttp:/eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1990R2377:20080120:ES:PDF.

[24] 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 L15/1, 20-01-2010.

del Reglamento (CEE) Nº 2377/90), y lista de sustancias prohibidas (que recogen las del Anexo IV).

- Se introduce información acerca de la clasificación terapéutica de las sustancias, así como posibles condiciones o restricciones de su utilización.
- Las sustancias farmacológicamente activas se enumeran en un anexo por orden alfabético.

En cuanto al control de estas sustancias en animales vivos o productos de origen animal destinados al consumo humano, la Directiva Comunitaria 96/23/CE [25] 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 estas matrices. Así, obliga a los países terceros a ofrecer garantías en cuanto a la cantidad de residuos que contienen los productos que exportan. En el anexo I de esta Directiva se enumeran los grupos de sustancias que deben ser controlados en cada especie animal o producto, distinguiendo entre:

- *Grupo A*: engloba sustancias con efecto anabolizante y la mayoría de las sustancias de uso prohibido por la EU en animales destinados a la producción de alimentos; comprende 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.

[25] Directiva 96/23/EC, de 29 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. L125/10, 23-05-1996.

A6. Sustancias incluidas en el anexo IV del Reglamento (CEE) Nº 2377/90 (o en el cuadro 2 del nuevo Reglamento (EU) Nº 37/2010) (cloranfenicol, cloroformo, clorpromacina, colchicina, dapsona, nitroimidazoles, nitrofuranos 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 EU y para los que se fija un MRL (sustancias incluidas en los anexos I, II o III del Reglamento (CEE) Nº 2377/90 o del nuevo Reglamento (EU) Nº 37/2010). Engloba a:

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

B2. Otros medicamentos veterinarios:

a) Antihelmínticos.

b) Anticoccidiales.

c) Carbamatos y piretroides.

d) Tranquilizantes.

e) Antiinflamatorios no esteroideos (NSAIDS).

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.

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, como se recoge en la tabla 1.

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	
B2b	X	X			X		
B2c	X	X				X	X
B2d	X						
B2e	X	X		X		X	
B2f							
B3a	X	X	X	X	X	X	X
B3b	X			X			X
B3c	X	X	X	X		X	X
B3d	X	X	X	X			
B3e			X				
B3f							

*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 Qns (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.

En cuanto a los métodos de análisis para la determinación de residuos de fármacos, la Decisión 2002/657/EC [26] relativa a la validación de los métodos analíticos de control de tales residuos en animales vivos y sus productos y la interpretación de los resultados, en el marco de la Directiva 96/23/EC no establece obligación de usar métodos normalizados en dicho control. Sin embargo, se establecen distintos criterios de funcionamiento y requisitos que deben cumplir los métodos analíticos, en cuanto a manipulación de muestras, veracidad (siempre que se pueda se utilizarán materiales de referencia certificados y cuando no se disponga de ellos se valorará mediante recuperación de adiciones de cantidades conocidas de analitos a una matriz en blanco, estableciéndose valores mínimos de veracidad), especificidad (se analizarán muestras en blanco para detectar posibles interferencias y valorar sus efectos) o criterios y requisitos de confirmación de los residuos y contaminantes de los métodos de cuantificación y cribado (puntos de identificación mediante espectrometría de masas (MS) u otros requisitos en los casos de otros sistemas de detección como UV-Vis, infrarrojos, etc.). Así, en el caso de requerir confirmación inequívoca de los residuos encontrados se indica que la detección por MS es la adecuada tras la separación cromatográfica, empleándose un sistema de puntos de identificación para interpretar los datos, basado en el uso de la MS mediante análisis de las masas moleculares (en baja y alta resolución) de cada compuesto y de los fragmentos obtenidos en la fragmentación molecular producida en el detector cuando se trabaja con MS en tándem. La confirmación de las sustancias clasificadas en el grupo A (anabolizantes y sustancias no autorizadas) de la Directiva 96/23/EC requiere un mínimo de 4 puntos de identificación y las clasificadas en el grupo B (medicamentos de uso veterinario con MRL establecido) un mínimo de 3 puntos. En este sistema de

[26] Decisión 2002/657/CE de la Comisión, de 12 de agosto de 2002, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados. DOCE L 221/8,17-08-2002.

puntos la detección del ion precursor es contabilizado con 1 punto y la de los iones producto, producidos tras la fragmentación del ion precursor, con 1.5, de modo que para las sustancias del grupo B, donde se incluyen las Qns objeto de estudio de esta Tesis, es posible realizar la confirmación monitorizando sólo dos iones fragmento.

4. Interés sobre el control de residuos de medicamentos en muestras medioambientales

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 [27,28]. 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 mas 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 concentraciones locales altas. Así, por ejemplo, se han encontrado concentraciones del orden de varios mg/kg de Qns en muestras de excrementos de cerdo, pollo y pavo [29], que pueden alcanzar suelos dedicados a la agricultura y acuíferos subterráneos y superficiales.

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[27] F.M. Christensen, Regulatory Toxicology and Pharmacology 28 (1998) 212.

[28] R.T. Williams, “Human Pharmaceuticals: Assessing the Impacts of Aquatic Ecosystems”, 2ª Ed, Society of Environmental Toxicology and Chemistry (SETAC), 2005.

[29] E. M. Carballo, C. González-Barreiro, S. Scharf, O. Gans, Environmental Pollution, 148 (2007) 570.

Una vez en el medio ambiente, los fármacos pueden seguir tres rutas principales [30,31]:

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.

Por desgracia, 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 EU 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 [32,33,34].

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[30] S.E. Jorgensen, B. Halling-Sørensen, Chemosphere 40 (2000) 691.

[31] B. Halling-Sorensen, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lützhofl, S.E. Jorgensen, Chemosphere 36 (1998) 357.

[32] K. Fent, A.A. Weston, D. Caminada, Aquat. Toxicol. 76 (2006) 122.

[33] K. Kümmerer, J. Environmen. Manag. 90 (2009) 2354.

[34] M.D. Celiz, J. Tso, D.S. Aga, Environmen. Tox. Chem. 28 (2009) 2473.

En los últimos años se han publicado diversos artículos de revisión [35,36,37,38,39] y diversos trabajos de investigación [40,41,42] concernientes al efecto y determinación 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 [39]. 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 [39,43,44,45,46,47]. Estos contaminantes emergentes tienen propiedades bioacumulativas, provocando efectos que aún son desconocidos en los

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[35] C.J. Cartagena, Revista Lasallista de Investigación 8 (2011) 143-153.

[36] M.S. Díaz–Cruz, M.J. López de Alba, D. Barceló, Trends Anal. Chem. 22 (2003) 340.

[37] K. Wille, H.F. De Brabander, L. Vanhaecke, E. De Wulf, P. Van Caeter, C.R. Janssen, Trends Anal. Chem. 35 (2012) 87.

[38] K. Y. Bell, M.J.M. Wells, K.A. Traexler, M.L. Pellegrin, A. Morse, J. Bandy, Water Environment Research 83 (2011) 1906.

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[42] C.L. Chitescu, E. Oosterink, J. Jong, A.A.M. Stolker, Anal. Bioanal. Chem. 403 (2012) 2997.

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

[47] M. Pedrouzo, F. Borrull, E. Pocurull, R.M. Marcé, Water Air Soil Poll. 217 (2011) 217.

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.

Hoy en día la calidad química del agua en Europa esta regulada por la directiva 2000/60/EC (Water Framework Directive, WFD) [48] 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 [49], 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 [50] 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. Además de estos, otros productos químicos están siendo objeto de estudio para clasificarlos como sustancias

[48] Directiva 2000/60/CE del Parlamento Europeo y del Consejo, de 23 de octubre de 2000, por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas. L 327/1, 22/12/2000.
[49] Decisión 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.
[50] 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 semodifican 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.

peligrosas [51]. 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 posibles efectos perjudiciales que el uso del producto puede causar al medio ambiente.

En EU, 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 [52] 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 medio ambiental. 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,

[51] Propuesta del Parlamento Europeo y de la comisión de modificacion de las directivas 2000/60/EC y 2008/105/EC respecto a las sustâncias de interes prioritario em el marco de la politica de aguas, Bruselas, 31.1.2012 COM (20011) 876 final.
[52] 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. L 311/1, 28.11.2001.

- 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, 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 EU 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 [53], 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.

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[53] Guia: Environmental Risk Assessment for Veterinary Medicinal Products other then GMO containing and Immunological Products. Octubre 2012 en: http://ec.europa.eu/health/files/eudralex/vol-7/a/7ar1a_en.pdf.

En esta Tesis Doctoral se ha abordado el estudio de la presencia de Qns en aguas de diversa procedencia. Éstas se encuentran entre las 5 clases de antibióticos (β-lactamas, macrólidos, Qns, sulfonamidas y tetraciclinas) más frecuentemente encontradas en el medioambiente [54]. Su presencia en aguas superficiales y residuales ha sido constatada en diversos artículos [43,45,54,55,56,57] e incluso en aguas de consumo [58] a concentraciones en torno a µg/l y ng/l. La presencia de las Qns en el medio ambiente está unida al aumento de la resistencia bacteriana, lo cual puede conllevar al desarrollo de una mayor resistencia por parte tanto de humanos como de animales [59]. Además, algunos estudios basados en ensayos in vitro has demostrado que las Qns promueven el desarrollo de genotoxicidad [60,61] por lo que parece claro que la presencia de estos compuestos en el medio ambiente debería controlarse para minimizar o evitar la innecesaria exposición tanto de humanos como de animales a este grupo de compuestos.

5. *Métodos analíticos parala determinación de antibióticos en alimentos y muestras medioambientales*

Tras la problemática previamente descrita, se hace patente la necesidad de desarrollar métodos analíticos sensibles y selectivos para la determinación de residuos de fármacos en muestras tanto de alimentos como medioambientales. Como queda reflejado en diversos artículos de revisión, las técnicas tradicionalmente empleadas para este fin son la (HPLC [62,63,64,65] y en menor medida la CE

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[54] A. Jia, Y. Wan, Y. Xiao, J. Hu, Water Res. 46 (2012) 387.

[55] R. López-Roldán, M.J. López de Alda, M. Gros, M. Petrovic, J. Martín-Alonso, D. Barceló, Chemosfere 11 (2010) 1337.

[56] M.S. Díaz-Cruz, D. Barceló, Anal. Bioanal. Chem. 386 (2006) 973.

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[58] Z.Q. Ye, H.S. Weinberg, M.T. Meyer, Anal. Chem. 79 (2007) 1135.

[59] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, J. Chromatogr. A 815(1998) 213.

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[61] J. Hu, W.Wang, Z. Zhu, H. Chang, F. Pan, B. Lin, Environ. Sci. Technol. 41 (2007) 4806.

[62] F. Belal, A.A. Al-Majed, A.M. Al-Obaid, Talanta 50 (1999) 765.

[63] V. Andreu, C. Blasco, Y. Picó, Trends Anal. Chem. 26 (2007) 534.

[63,66,67,68]. En cuanto a las técnicas de detección, las más utilizadas han sido la espectrofotometría UV-Vis [63,69,70], la fluorescencia [62,63,69,70] y la MS [71,72,73]. Cabe destacar el gran aumento de métodos publicados con MS desde el año 2000, debido a la mejora de los sistemas de acoplamiento con las técnicas separativas, especialmente en el caso de la CE. Así, en los últimos años los métodos basados en el empleo de LC-MS y CE-MS se han impuesto sobre los demás como se refleja en recientes artículos de revisión [37,66,72,73,74,75].

En la presente Tesis Doctoral se han empleado técnicas separativas de reciente desarrollo, como son las técnicas miniaturizadas y de ultra resolución, acopladas a sistemas de detección sensibles y selectivos, que pasan a comentarse brevemente en los siguientes apartados.

5.1 *Aplicación de técnicas miniaturizadas en la determinación de antibióticos en alimentos y muestras medioambientales*

Entre las técnicas separativas mencionadas anteriormente, 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 o una mejora en la sensibilidad. Igualmente es de destacar los pequeños

[64] M.D. Marazuela, S. Bogialli, Anal. Chim. Acta 645 (2009) 5.

[65] F.J. Lara, M. del Olmo-Iruela, C. Cruces-Blanco, C. Quesada-Molina, A.M. García-Campaña, Trends Anal. Chem. 38 (2012) 52.

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

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

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

[69] G. Carlucci, J. Chromatogr. A 812 (1998) 343.

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[72] A.A.M. Stolker, T. Zuidema, M.W.F. Nielen, Trends Anal. Chem. Vol. 26 (2007) 967.

[73] V. Rodríguez Robledo, W.F. Smyth, Electrophoresis 30 (2009) 1.

[74] C. Blasco, C.M. Torres, Y. Picó, Trends Anal. Chem. 26 (2007) 895.

[75] A.K. Malik, C. Blasco, Y. Picó, J. Chromatogr. A, 1217 (2010) 4018.

volúmenes de muestras requeridos, lo que constituye una gran ventaja, fundamentalmente en el ámbito biomédico [76]. En general, dentro de los sistemas miniaturizados se engloban actualmente los que utilizan columnas de pequeño diámetro interno o capilares de sílice fundida, y aquellos en los que la separación se realiza en microchips. En la presente Tesis Doctoral se han utilizado dos técnicas pertenecientes al primer grupo: la CE y HPLC capilar, que pasan a comentarse brevemente a continuación.

5.2 *Electroforesis capilar*

La CE es una técnica separativa miniaturizada que presenta como principales características unas altas eficacias de separación, tiempos de separación cortos, volúmenes de muestra y disolventes muy pequeños al emplear capilares muy estrechos, por lo que el coste y la contaminación ambiental disminuyen considerablemente, en comparación con la HPLC, consiguiendo además mayores eficacias en muchas ocasiones. El medio de separación consiste generalmente en disoluciones tampón acuosas, aunque cada vez es posible encontrar más aplicaciones llevadas a cabo en medios no acuosos (non-aqueous CE, NACE) [77,78], por lo que en muchos casos las muestras se pueden inyectar directamente sin necesidad de procesos de extracción, pudiendo trabajar a temperatura ambiente y evitando así procesos de descomposición o desnaturalización de las muestras. Además, es una técnica altamente versátil, pudiendo analizar muchos tipos de muestras (polares, apolares, iónicas, neutras y de alto peso molecular), debido a sus diferentes modos de separación.

En la última década han aumentado las aplicaciones de la CE en la determinación de medicamentos de uso veterinario (fundamentalmente antibióticos) recogidas en

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

[77] M.L. Riekkola, Electrophoresis 23 (2002) 3865.

[78] L. Geiser, J.L. Veuthey, Electrophoresis 30 (2009) 36.

artículos de revisión [63,66,67,68,79,80,81]. Principalmente se han utilizado dos de los modos de separación que presenta la CE: la electroforesis capilar zonal (CZE) y la cromatografía capilar electrocinética micelar (MEKC).

En lo que respecta a las Qns, éstas se han determinado en carne de cerdo [82], en pollo [83,84,85,86,87], en pescado [88,89] en leche [90,91,92,93] o en riñón de cerdo [94], esta última aplicación mediante el empleo de NACE. Sin embargo, el número de trabajos publicados en los que se determinan Qns en muestras medioambientales mediante CE es mucho menor aunque es posible encontrar algunos artículos en los que se lleva a cabo su determinación en muestras de agua como es el caso M. Ferdig y col. que determinan 9 Qns en aguas superficiales mediante CE con detección fluorescente [95] o de A.V. Herrera-Herrera y col. que determina 8 Qns en agua mineral y agua de escorrentía mediante NACE-UV [96].

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[79] M. Hernández, F. Borull, M. Calull, Trends Anal. Chem. 22 (2003) 416.

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

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

[82] H. Sun, P. He, Y.K. Lv, S.X. Liang, J Chromatogr B 852 (2007) 145.

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

[87] H. Hai-Bo, L. Xiao-Xia, Y. Qiong-Wei, F. Yu-Qi, Talanta 82 (2010) 1562.

[88] A. Juan-García, G. Font, Y. Picó, Electrophoresis 28 (2007) 4180.

[89] H. Sun, Y. Zuo, H. Qi, Y. Lv, Analytical Methods 4 (2012) 670.

[90] H. Sun, W. Zhao, P. He, Chromatographia 68 (2008) 425.

[91] I.S. Ibarra, J.A. Rodriguez, M.E. Páez-Hernández, E.M. Santos, J.M. Miranda, Electrophoresis 33 (2012) 2041.

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

[93] M.Y. Piñero, R. Garrido-Delgado, R. Bauza, L. Arce, M. Valcárcel, Electrophoresis 33 (2012) 2978.

[94] M. Hernández, F. Borrull, M. Calull, Electrophoresis 23 (2002) 506.

[95] M. Ferdig, A. Kaleta, T.D. Thanh Vo, W. Buchberger, J. Chromatogr. A 1047 (2004) 305.

[96] A.V. Herrera-Herrera, J. Hernández-Borges, T.M. Borges-Miquel, M.A. Rodríguez-Delgado, Electrophoresis 31 (2010) 3457.

El principal inconveniente que presenta la aplicación de la CE para la determinación de residuos de contaminantes radica en la necesidad de usar estrategias para aumentar la sensibilidad, considerando las bajas concentraciones de analitos en estas matrices y debido a los pequeños volúmenes de inyección introducidos y a la limitada capacidad de la detección UV-Vis, normalmente empleada en CE. Estas estrategias implican el uso de técnicas de preconcentración de la muestra off-line en la etapa previa de tratamiento de muestra, el uso de sorbentes de extracción en fase sólida (SPE) acoplados on-line a modo de preconcentradores en el interior del capilar [85,97] o el acoplamiento con técnicas de detección más sensibles, como la fluorescencia inducida por láser (LIF), de utilidad en el análisis de Qns debido a la fluorescencia nativa que éstas presentan.

De forma general dos son los mayores beneficios de la detección fluorescente acoplada con la CE o con HPLC, estas son la sensibilidad y la slectividad.

- Sensibilidad. La detección fluorescente puede dar lugar a una mayor sensibilidad que la medida de la absorbancia porque la detección fluorescente tienen una señal de fondo muy baja. Normalmente un detector fluorescente puede proporcionar límites de detección 2 o 3 órdenes de magnitud mayores que los detectores de absorbancia. Cuando se utiliza la detección fluorescente, la señal de una muestra se compara con una muestra no fluorescente (el electrolito de fondo), por ello la señal de fondo es cero o muy próxima a cero. En cambio, cuando se utiliza un detector de absorbancia la trasmitancia de la muestra se compara con la de un blanco. Por ello, a bajas concentraciones la diferencia la diferencia entre ambas es pequeña y error en la medida puede ser significativo.

  Además, la sensibilidad del detector está íntimamente ligada a la intensidad de la fuente de radiación utilizada para excitar al compuesto, y el laser es una

[97] P. Puig, F. Borrull, M. Calull, C. Aguilar, Anal. Chim. Acta 616 (2008) 1.

fuente de excitación extremadamente intensa, con un haz colimado con una potencia del orden de los milliwatios.

- Selectividad. La selectividad va a depender en este caso de lo estrecha que sea la banda de longitud de onda utilizada para la excitación. Este hecho hace posible la excitación a 325 nm, esta es la longitud de onda del laser empleado estea tesis, y la reducción de las posibles interferencias en la matriz. Así, cuando se emplea un detector de absorbancia, las medidas se realizan a una sola longitud de onda al mismo tiempo, mientras que en el caso de la fluorescencia se usan dos. Así, en el caso de dos analitos que eluyan al mismo tiempo y absorban radiación a la misma longitud de onda pero diferentes longitudes de onda de emisión (o uno de ellos no emite fluorescencia), un detector fluorescente puede diferenciar las señales analíticas mientras que uno de absorbancia no.

En la presente Tesis Doctoral se ha usado el acoplamiento CE-LIF. Esta fuente de excitación tiene como particularidad el poder incidir en una zona muy reducida, siendo posible su enfoque en el diámetro interno del capilar. En este sentido, la técnica puede alcanzar límites de detección del orden de los 10^{-18}-10^{-21} moles utilizando detección "on-column", es decir, en el mismo capilar. Existen distintos tipos de láseres, siendo los más utilizados los de He-Cd (emisión a 325 y 442 nm), ión Ar (de varios tipos, emitiendo entre 350-360 nm, 476, 488 y 514 nm) o He-Ne (emisión 633 nm). Para su selección es necesario considerar que la longitud de onda que emiten sea lo más próxima a la longitud de onda de excitación del analito, que sean de elevada potencia y que puedan enfocar un punto determinado que se corresponda con el diámetro interno del capilar que se vaya a utilizar.

A continuación se incluye un esquema del sistema óptico del detector LIF (ZETALIF Evolution, Picometrics) acoplado con la CE o con la HPLC.

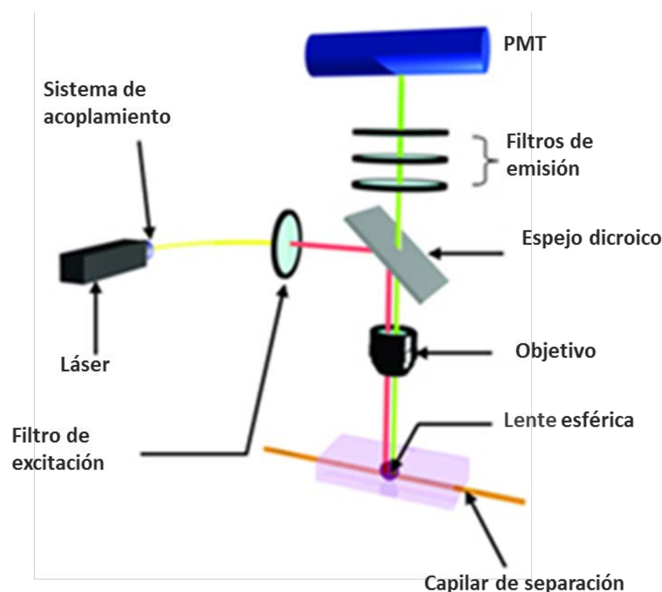

Figure 1.1. Optical layout of the LIF system.

El acoplamiento CE-LIF se ha utilizado para la determinación de compuestos muy diferentes como son los aminoácidos, péptidos, proteínas, compuestos farmacéuticos, etc. Gracias as u fluorescencia nativa, o en menor proporción gracias a la derivatización en el caso de compuestos sin fluorescencia nativa [98,99,100].

Existen aún escasas aplicaciones de este sistema de detección en el control de residuos de antibióticos [66,67,68,101,102], siendo casi inexistentes las aplicaciones al análisis de alimentos y muestras medioambientales. Un ejemplo de la aplicación de esta técnica es la determinación de Qns (ENRO y CIPRO) en carne de pollo [103] o la

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[98] H.M. Tseng, Y. Li, D.A. Barrett, Bioanalysis 2 (2010) 1641.
[99] E. Szöko, T. Tábi, J. Pharm. Biomed. Anal. 53 (2010) 180.
[100] A.M. García-Campaña, M. Taverna, H. Fabre, Electrophoresis 28 (2007) 208.
[101] C.L. Flurer, Electrophoresis 22 (2001) 4249.
[102] C.L. Flurer, Electrophoresis 24 (2003) 4116.
[103] C. Horstkötter, E. Jiménez-Lozano, D. Barrón, J. Barbosa, G. Blaschke, Electrophoresis 23 (2002) 3078.

aplicación en fluidos biológicos, como la determinación de OFLO y sus metabolitos [104], MOXI [105] y CIPRO y sus metabolitos [106].

5.3 *Cromatografía líquida capilar*

La HPLC sigue siendo una técnica imprescindible en la mayoría de los laboratorios de ensayo. Su continuo desarrollo ha conseguido una mejora en los métodos de análisis establecidos con anterioridad. Así, en los últimos años, para incrementar la eficacia de la separación, la sensibilidad de la detección y disminuir los tiempos de análisis de las 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 (tabla 3) que respecto a las convencionales de diámetro interno 4.6 mm ofrecen diversas ventajas [76,107]. Así, 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 impacto medioambiental. 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 [108]. En comparación con los sistemas convencionales de HPLC, este tipo de técnicas mejoran la detección y permiten obtener eficacias de separación elevadas. Por ello, 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

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[104] C. Horstkötter, G. Blaschke, J. Chromatogr. B 754 (2001) 169.

[105] J.G. Moller, H. Stass, R. Heinig, G. Blaschke, J. Chromatogr. B 716 (1998) 325.

[106] K.H. Bannenfeld, H. Stass, G. Blaschke, J. Chromatogr. B 692 (1997) 453.

[107] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, J. Sep. Sci. 30 (2007) 1589.

[108] A. Braithwaite, F.J. Smith, “Chromatographic Methods”, 5ª Edición, Kluwer Academic Publishers, 1996, p. 1.

distintas muestras. Estas ventajas, junto con la facilidad de acoplamiento con MS, se han explotado fundamentalmente en el campo de la proteómica [107,109].

Tabla 3. Terminología usada en técnicas de cromatografía líquida [76].

Nombre	Diámetro interno de columna	Velocidad de flujo	Tamaño de partícula (µm)	Longitud (cm)
HPLC convencional	3.2-4.6 mm	0.5-2.0 ml/min	3-10	3-25
HPLC microbore	1.5-3.2 mm	100-500 µl/min	3-8	15-25
Micro-LC	0.5-1.5 mm	10-100 µl/min	3-5	5-15
HPLC capilar	150-500 µm	1-10 µl/min	3-5	5-15
Nano-HPLC	10-150 µm	10-1000 nl/min	3-5	5-15

Respecto a la determinación de Qns, a pesar de que las aplicaciones en HPLC convencional con diversos sistemas de detección, principalmente MS y MS/MS [74,110,111], o UV-Vis y fluorescencia [63,72,112] son muy numerosas, aún no se han explorado las ventajas de la HPLC capilar para la determinación de este tipo de compuestos. Además es de considerar la potencialidad que ofrece el acoplamiento de esta técnica con la detección LIF combinado las ventajas de la separación con la elevada capacidad de detección que proporciona la fuente láser, como ya se ha comentado en el apartado anterior. En esta Memoria también se ha propuesto esta estrategia para la determinación de Qns en muestras de alimentos de origen animal.

[109] Y. Ishihama, J. Chromatogr. A 1067 (2005) 73.

[110] S. Bogialli, A. Di Corcia, Anal. Bioanal. Chem. 395 (2009) 947.

[111] A.A.M. Stolker, P. Rutgers, E. Oosterink, J.J.P. Lasaroms, R.J.B. Peters, J.A. van Rhijn, M.W.F. Nielen, Anal. Bioanal. Chem. 391 (2008) 2309.

[112] J. Sousa, G. Alves, A. Fortuna, A. Falcão, Anal. Bioanal. Chem. 403 (2012) 93.

5.4 *Aplicación de la cromatografía líquida de ultra resolución para la determinación de antibióticos en alimentos y muestras medioambientales*

Además de la miniaturización, otro paso importante dentro del avance de la HPLC ha sido el desarrollo de fases estacionarias de partícula híbrida, con un componente inorgánico (sílice) y un componente orgánico (organosiloxanos) [115,113] y con un tamaño de partícula inferior a los 2 μm [114], originando lo que se ha denominado "Cromatografía Líquida de Ultra Resolución (UHPLC)" [115]. Esta reducción del tamaño de partícula, disminuye significativamente la altura equivalente de plato teórico (HETP), lo que hace posible mejorar la eficacia del proceso cromatográfico, permitiendo trabajar a elevados caudales de fase móvil sin pérdida de la calidad en la separación cromatográfica [116].

En la figura 8 se representa una gráfica de Van Deemter en función de los tamaños de partícula desarrollados en las últimas décadas. Como se puede observar, la disminución del tamaño de partícula por debajo de 2.5 μm ha dado lugar a un gran aumento de la eficiencia de la separación ya que se produce una disminución notable de la HETP, manteniéndose ésta prácticamente constante al aumentar el caudal o la velocidad lineal [115]. Esta posibilidad de aumentar el caudal sin disminuir la resolución conlleva una reducción del tiempo de análisis, llegándose a determinar más de 100 compuestos en menos de diez minutos [115,117].

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[113] E.S. Grumbach, T.E. Wheat, M. Kele, J.R. Mazzeo, LC-GC N. Am., 23 (2005) 40.

[114] D.T.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, J. Sep. Sci. 29 (2006) 1836.

[115] M.E. Swartz, J. Liq. Chromatogr. Rel. Tech. 28 (2005) 1253.

[116] A.D. Jerkovich, J.S. Mellors, J.W. Thompson, J.W. Jorgenson, Anal. Chem. 77 (2005) 6292.

[117] I.D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, E.M. Lenz, J. Chromatogr. B 817 (2005) 67.

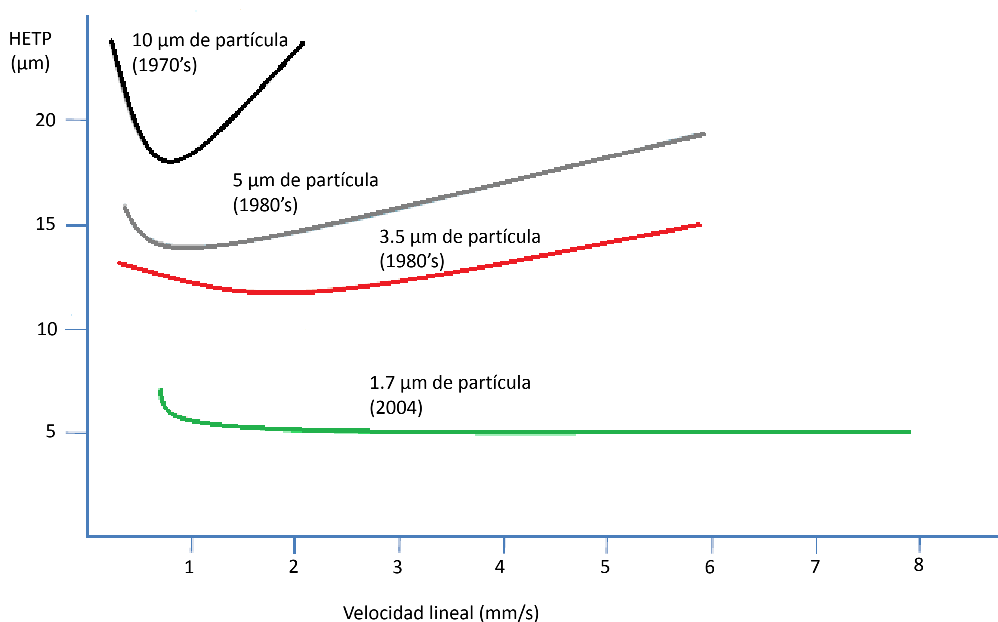

Figura 8. Grafica de van Deemter ilustrando la evolución del tamaño de partícula en las últimas décadas [115].

En resumen, los beneficios de la UHPLC son la mejora de la resolución entre picos cromatográficos, la disminución de los tiempos de análisis, además de la mejora de la sensibilidad. No obstante, el uso de un tamaño de partícula menor (< 2 µm) ha obligado a desarrollar tanto bombas que puedan ejercer la presión suficiente para trabajar a caudales adecuados, soportando presiones superiores a 10000 psi [118], como inyectores más rápidos capaces de inyectar cantidades cada vez menores y capaces de soportar presiones mucho mayores, y detectores mucho más rápidos que sean capaces de proporcionar suficientes puntos por pico y con celdas de detección mucho más pequeñas para evitar el ensanchamiento de pico.

En cuanto a los sistemas de detección, la mayoría de las aplicaciones de UHPLC desarrolladas hasta ahora han implicado la utilización de MS como detector [119]. El

[118] M.I. Churchwell, N.C. Twaddle, L.R. Meeker, D.R. Doerge, J. Chromatogr. B 825 (2005) 134.

[119] D. Guillarme, J. Schappler, S. Rudaz, J.L. Veuthey, Trends Anal. Chem. 29 (2010) 15.

acoplamiento de ambas técnicas mejora considerablemente la detección, puesto que la reducción de la anchura de pico aumenta la altura del mismo, lo que conlleva una mejora en la sensibilidad del método analítico. Sin embargo, debido a los picos considerablemente más estrechos obtenidos en UHPLC (normalmente entre 1–3 s), el acoplamiento con sistemas de detección de MS es crítico. Por esta razón se han comercializado instrumentos específicos con tiempos de adquisición suficientemente rápidos que pueden garantizar un número de puntos por pico adecuado para una correcta detección [119]. El acoplamiento UHPLC-MS se ha convertido así en una poderosa alternativa a la HPLC convencional en el ámbito del análisis multirresiduo en alimentos, reduciendo los tiempos de análisis y suministrando los puntos de identificación necesarios para una confirmación inequívoca de los analitos acorde con la legislación de la EU [26]. Los analizadores de triple cuadrupolo (QqQ), que permiten el modo de trabajo de MS en tándem (MS/MS), son los que claramente se han impuesto para llevar a cabo la determinación de un número elevado de analitos con una buena sensibilidad y selectividad gracias a la monitorización de transiciones MS/MS operando en el modo de detección múltiple de fragmentos (multiple reaction monitoring, MRM).

En los últimos años se han publicado diversas aplicaciones de UHPLC-MS para la detección de residuos de fármacos y productos de cuidado personal en aguas [120,121,122,123]; así como métodos aplicados al análisis de alimentos de origen animal, como la determinación de tetraciclinas y Qns en carne de cerdo [124] y de

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[120] M. Petrovic, M. Gros, D. Barceló, J. Chromatogr. A 1124 (2006) 68.

[121] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Talanta 74 (2008) 1299.

[122] M. Farré, M. Gros, B. Hernández, M. Petrovic, P. Hancock, D. Barceló, Rapid Comm. Mass Sp. 22 (2008) 41.

[123] W.L. Shelver, H. Hakka, G.L. Larsen, T.M. DeSutter, F.X.M. Casey, J. Chromatogr. A 1217 (2010) 1273.

[124] B.Shao, X. Jia, Y. Wu, J. Hu, X. Tu, J. Zhang, Rapid Comm Mass 21 (2007) 3487.

macrólidos en huevos, leche y miel [125,126], así como métodos multirresiduo para leche [127], miel [128], huevo [129] o pollo [130], respectivamente.

5.5 *Aplicación del acoplamiento lc-ms para la determinación multirresiduo de contaminantes en alimentos y muestras medioambientales*

En los últimos tiempos, los requerimientos de los laboratorios de análisis alimentario y medioambiental están aumentando considerablemente, ya que el número de compuestos susceptibles de ser analizados en este tipo de muestras es mayor cada día. No obstante, cuando se realiza el análisis de una muestra, no se determinan todos los posibles contaminantes que ésta podría contener, sino que se realiza la búsqueda de unos pocos compuestos, por lo general aquellos que están recogidos en la legislación. Este análisis de compuestos conocidos, que se buscan en la muestra en cuestión, se conoce como "target analysis". Sin embargo, puede haber muchos otros contaminantes en la muestra no incluidos en la búsqueda inicial. Desafortunadamente no existen métodos universales de análisis y se puede pasar por alto la existencia de diversos contaminantes que simplemente no se han buscado. Es por ello que el análisis multirresiduo, que permita determinar un número elevado de compuestos de la misma o diversas familias simultáneamente, se muestra como una herramienta cada vez más potente y necesaria en los laboratorios de análisis. Así, en los últimos años se ha observado un importante aumento del número de publicaciones

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[125] J. Wang, D. Leung, Rapid Comm. Mass 21 (2007) 3213.

[126] J. Wang, D. Leung, Drug Testing and Analysis 4 (2012) 103.

[127] M.M. Aguilera-Luiz, J.L. Martínez Vidal, R. Romero-González, A. Garrido Frenich, J. Chromatogr. A 1205 (2008) 10.

[128] J.L. Martínez-Vidal, M. Aguilera-Luiz, R. Romero-González, A. Garrido-Frenich, J. Agric. Food Chem. 57 (2009) 1760.

[129] A. Garrido Frenich, M.M. Aguilera-Luiz, J.L. Martínez-Vidal, R. Romero-González, Anal. Chim. Acta 661 (2010) 150.

[130] R.P. Lopes, R.C Reyes, R. Romero-González, A. Garrido Frenich, J.L. Martinez Vidal, Talanta 89 (2012) 201.

con metodologías multirresiduo en el análisis de alimentos [111,127,128,129,131,132,133] y aguas [36,37,40,41,42]. Este aumento ha sido posible en gran parte gracias a los avances de la instrumentación analítica, entre los que cabe destacar la mejora de la sensibilidad y aumento de la velocidad de medida de los instrumentos de MS, la cual se ha utilizado tanto para el análisis de uno o unos pocos compuestos como para el análisis multirresiduo, donde se incluyen gran número de compuestos de diferentes familias y diferentes naturalezas [134,135]. Hoy día no es posible realizar target analysis demás de entre 150 y 200 compuestos en un mismo análisis, dependiendo del tipo de instrumentación de que se disponga, mediante LC-MS/MS [136], aunque en muchos casos las muestras son susceptibles de estar contaminadas o de poseer residuos en un número mucho mayor.

El acoplamiento de la cromatografía con la MS es probablemente la técnica analítica instrumental más extendida hoy en día para el análisis en los campos de la salud, alimentación y medioambiente. El principio de la MS es la producción de iones en estado gaseoso a partir de compuestos neutros o cargados, y la detección de los iones o los fragmentos de esos iones producidos en el proceso de ionización o en el espectrómetro antes de llegar a la zona de alto vacío. Cuando se trata de un acoplamiento LC-MS, como es el caso de los métodos descritos en esta Tesis, los analitos han de pasar a estado gaseoso antes de producirse la ionización. Estos iones descompuestos (fragmentos que también poseen carga) o no descompuestos se dirigen rápidamente a través del espectrómetro para ser "clasificados" de acuerdo a

[131] V. Samanidou, S. Nisyriou, J. Sep. Sci. 31 (2008) 2068.
[132] M.J. Schneider, K. Mastovska, S.J. Lehotay, A.R. Lightfield, B. Kinsella, C.E. Shultz, Anal. Chim. Acta 637 (2008) 290
[133] J. García-Reyes, M. Hernando, A. Molina-Díaz, A. Fernández-Alba, Trends Anal. Chem. 26 (2007) 828.
[134] V. Di Stefano, G. Avellonea, D. Bongiorno, V. Cunsolo, V. Muccillib, S. Sforzac, A. Dossenac, L. Drahosd, K. Vekeyd, J. Chromatogr. A 1259 (2012) 74
[135] M Farré, L. Kantiani, M. Petrovic, S. Pérez, D. Barceló, J. Chromatogr. A 1259 (2012) 86.
[136] M. Mezcua, O. Malato, J.F. García-Reyes, A. Molina Díaz, A.R. Fernández-Alba, Anal. Chem. 81 (2009) 913.

su relación m/z. El espectrómetro de masas no solo clasifica los fragmentos, sino que además mide la cantidad de ellos que se forman. Finalmente se produce una amplificación de la señal producida por cada molécula medida y se genera el espectro de masas. Los espectrómetros de masas constan, básicamente, de cuatro partes [137]:

1. Un sistema de introducción de muestra, que puede ser entre otros un cromatógrafo de líquidos (empleado en esta Tesis Doctoral) o un cromatógrafo de gases. No es más que la herramienta que conduce las moléculas previamente separadas hasta la entrada del espectrómetro de masas.
2. Una fuente de ionización, que es donde se produce el paso al estado gaseoso de la fase móvil y los analitos transportados por ésta y donde las moléculas neutras pasan al estado ionizado. Las fuentes de ionización desarrolladas para el acoplamiento LC-MS han tenido la doble función de eliminar el disolvente (normalmente presente en órdenes de 0.1-2 ml/min) y vaporizar la muestra e ionizarla. En este acoplamiento, la ionización se produce a presión atmosférica. Aunque existen otros modos de ionización como la ionización por termospray (TSP) o la fotoionización a presión atmosferica (*Atmospheric Pressure Photoionization*, APPI), las técnicas de ionización más frecuentemente empleadas hoy en día en los acoplamientos LC-MS son la ionización por electrospray (ESI) y la ionización química a presión atmosférica (APCI). En la tabla 4 se recogen las características más importantes de estas fuentes y en la figura 9 la aplicabilidad de estos modos de ionización.
3. Un analizador o filtro de masas que separa y/o selecciona las moléculas ionizadas así como los fragmentos iónicos generados en función de su relación m/z.

[137] Agencia Estatal Antidopaje. Disponible en noviembre 2012 en: http://www.aea.gob.es/media/126618/espectrometr%C3%ADa%20de%20masas-nuevo.pdf.

Tabla 4. Técnicas de ionización más comunes en el acoplamiento LC-MS [137].

Tipo	Principales características	Limitaciones
IONIZACIÓN POR ELECTROSPRAY (ESI)	• Permite obtener iones cargados de moléculas de muy diferente peso molecular • Funciona bien con ácidos y bases en disolución y con compuestos a los que se les pueda inducir carga. • Puede utilizar temperaturas elevadas para evaporar gran cantidad de fase móvil (permite análisis de moléculas lábiles). • Se pueden obtener espectros de masas de iones positivos y	• Suelen formarse aductos con los tampones utilizados en la fase móvil de LC. • La sensibilidad es muy dependiente del flujo de fase móvil de HPLC y del pH de esta.
IONIZACIÓN QUÍMICA A PRESIÓN ATMOSFÉRICA (APCI)	• Es una ionización química mediante reacciones ion-molécula a presión atmosférica. • Permite análisis de moléculas con bajo peso molecular no volátiles. • Su sensibilidad no depende del pH ni del flujo de fase móvil. • Más sensible que ESI. • Permite obtener espectros de masas de iones positivos y negativos.	• Es una ionización suave. Genera pocos fragmentos. • Mayor facilidad de que se descompongan moléculas lábiles.

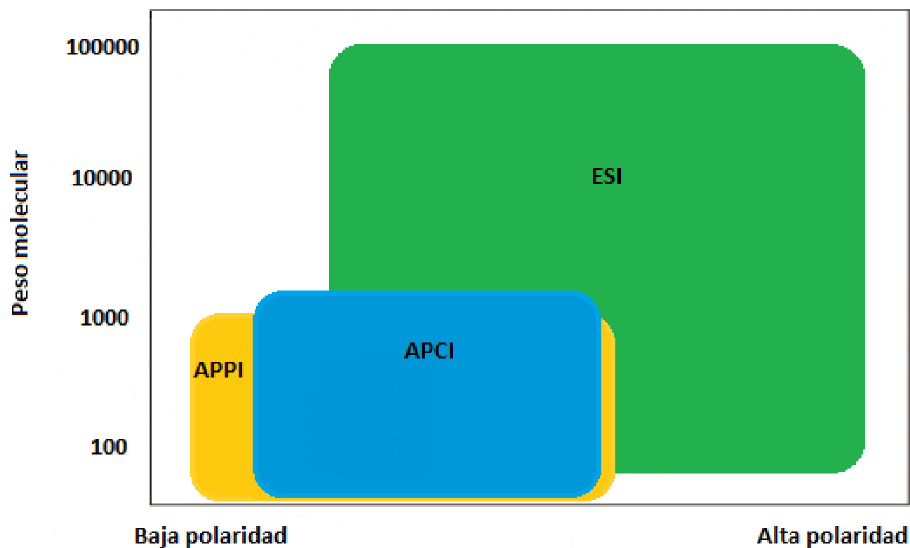

Figura 9. Relación entre polaridad, peso molecular y técnicas de ionización mediante ESI, APCI y APPI.

4. Un detector que recoge y caracteriza los fragmentos iónicos que salen del analizador. Los iones procedentes del analizador llegan al detector el cual generalmente esta constituido por un cátodo emisor que al recibir el impacto producido por las partículas cargadas emite electrones. Estos electrones son acelerados hacia un dínodo el cual emite varios electrones más al recibir el impacto de cada electrón. Este proceso se repite hasta obtenerse una cascada de electrones que llega al colector lográndose una corriente amplificada, por un procedimiento similar al que se utiliza en los tubos fotomultiplicadores, que se lleva a un sistema registrador.

En general, los distintos tipos de espectrómetros de masas se clasifican en función del analizador que posean: cuadrupolo simple (Q), triple cuadrupolo (QqQ), trampa de iones (Ion Trap, IT), Orbitrap, resonancia de ion ciclotrón (Ion Cyclotron Resonance, ICR), sector magnético y tiempo de vuelo (Time of flight, TOF). Cada analizador utiliza una tecnología diferente, lo cual le confiere unas características que lo hacen diferir en

cuanto a sus aplicaciones. Se caracterizan por el rango de masas que pueden medir, resolución de masa (que puede ser de una unidad, como es el caso de los cuadrupolos, en torno a 15000 para los TOF o de hasta 100000 en el caso del Orbitrap), exactitud de masa (la cual varía enormemente de un instrumento a otro siendo los equipos TOF y Orbitrap los que mayor exactitud pueden proporcionar llegando hasta valores inferiores a las 5 ppm), rango dinámico lineal, velocidad de barrido y sensibilidad, en la tabla 5 se puede ver un resumen de estas características [138]. Además, en los últimos años se han comercializado espectrómetros de masas que combinan algunos de los analizadores nombrados, como es el caso de los espectrómetros de tipo Q-TOF, donde se combina el analizador de cuadrupolo con el de TOF, o el Q-Trap, el cual combina el cuadrupolo con la IT.

Tabla 5. Especificaciones de espectrómetros de masas usados en LC–MS [138].

Tipo de MS[a]	Resolución [×10³]	Exactitud de masa (ppm)	*Rango de m/z* (hasta) [×10³]	Velocidad de adquisición (Hz)	Rango dinámico lineal	Precio
Quadrupolo	3–5	Low[b]	2–3	2–10	10^5–10^6	Bajo
IT	4–20	Low	4–6	2–10	10^4–10^5	Moderado
TOF	10–60	1–5	10–20	10–50	10^4–10^5	Moderado
Orbitrap	100–240	1–3	4	1–5	5×10^3	Muy alto
ICR	750–2500	0.3–1	4–10	0.5–2	10^4	Alto

a) TOF, Orbitrap e ICR tambien incluyen configuraciones hibridas con cuadrupolos o IT lineales.
b) Los Cuadrupolos hiperbolicos proporcionan exactitude de masas inferior a las 5 ppm.

En esta Tesis Doctoral se han propuesto métodos de análisis basados en LC-MS utilizando dos modalidades de MS: se ha empleado un analizador de triple cuadrupolo para la determinación de Qns en muestras de productos apícolas y de aguas de diversa procedencia, y un analizador Orbitrap para la determinación multirresiduo de fármacos y pesticidas en muestras de carne de pollo. A continuación se comentan brevemente las características principales de estos analizadores.

[138] M. Holcapek, R. Jirásko, M. Lísa, J. Chromatogr. A 1259 (2012) 3.

Analizador de triple cuadrupolo:

En el analizador tipo cuadrupolo, se emplean campos eléctricos para separar los iones de acuerdo con su relación m/z cuando pasan a través del espacio central entre 4 barras longitudinales (o polos) dispuestas en torno a un eje central y equidistantes sobre las que se aplica una corriente continua y un voltaje de radiofrecuencia que permite que solo las moléculas con una relación m/z determinada atraviesen el espacio cuadrupolar hasta llegar al detector. Todo el sistema se encuentra sometido a alto vacío para evitar colisiones entre los fragmentos iónicos generados o con otras moléculas, que podrían dar lugar a la fragmentación de la molécula o los fragmentos ya generados, algo que es totalmente indeseable.

En MS/MS con analizadores QqQ se acoplan dos cuadrupolos y entre ellos se coloca una celda de colisión (figura 10). En esta celda se puede producir, si se desea, la fragmentación de los iones conducidos a través del primer cuadrupolo por colisión del ion seleccionado con moléculas de un gas inerte, y tanto las no fragmentadas como los fragmentos producidos se pueden conducir a través del segundo cuadrupolo hasta el detector.

Así, trabajando en modo MRM, se pueden medir los fragmentos generados en la celda de colisión. La adquisición en esta modalidad de MS/MS ofrece así una gran selectividad, ya que ofrece la posibilidad de aislar un ion en la celda de colisión, eliminando otros iones o fragmentos que puedan interferir. Asimismo, gracias a la selectividad se mejora mucho la sensibilidad ya que se puede tener una mejor relación señal/ruido. Por todo ello, la información proporcionada por los sistemas de MS/MS aumenta la selectividad y la información generada para la elucidación estructural.

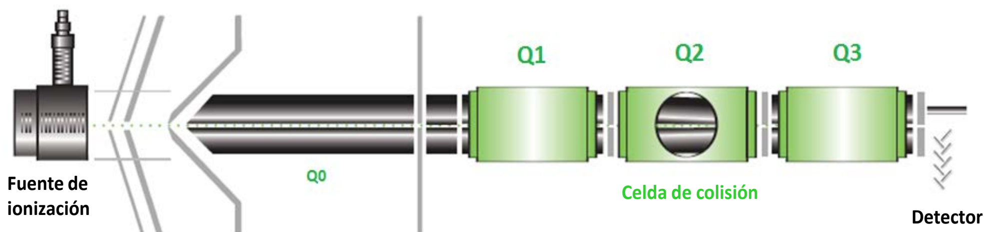

Figura 10. Esquema de un espectrómetro de masas de triple cuadrupolo. (Figura adaptada de http://www.absciex.com/, ABSciex).

La HPLC acoplada con el triple cuadrupolo ha sido ampliamente empleada en la determinación de Qns tanto en muestras de alimentos como medioambientales [63,139,140], gracias a que es un instrumento con una excelente sensibilidad y permite llevar a cabo la detección y cuantificación con muy buena exactitud y precisión. Por todo ello es muy utilizado como herramienta de confirmación.

No obstante, cuando esta técnica se emplea en la determinación multirresiduo de un gran número de analitos, presenta algunas limitaciones:

- el tiempo que requieren es elevado puesto que hay que caracterizar cada analito (tanto iones precursores como iones fragmento) individualmente y hay que construir ventanas de tiempo para la monitorización de cada analito, ya que las medidas en *full scan* (modo de barrido continuo) no permiten medir tan elevado numero de compuestos al mismo tiempo;
- cuando el número de analitos estudiados es muy elevado el *"dweell time"* (periodo de tiempo que el instrumento dedica a la medida de cada ión) ha de ser menor y esto dificulta el análisis ya que se reduce el tiempo dedicado por el detector a la medida de cada analito;
- las ventanas de tiempo para la adquisición de cada analito tienen que ser

[139] V. Carretero, C. Blasco, Y. Picó, J. Chromatogr. A, 1209 (2008) 162.
[140] G. Stubbings, T. Bigwood, Anal. Chim. Acta 637 (2009) 68.

actualizadas cada cierto tiempo ya que los tiempos de retención pueden variar incluso dentro de la misma serie de análisis;

- la resolución alcanzada con equipos de cuadrupolo es de tan solo la unidad, lo cual no es suficiente en muchos casos en los que la matriz es compleja. Además cuanto más compleja es la matriz estudiada mayor es la posibilidad de encontrar compuestos isobaros que dificultan la identificación.

Orbitrap®:

Estas limitaciones que se acaban de comentar y que surgen cuando se quiere estudiar un gran número de compuestos simultáneamente, se pueden solventar, en parte, a través el empleo de técnicas de medida de alta resolución (HRMS) trabajando en modo full scan [141,142]. La HRMS aporta algunas ventajas importantes como son la precisión de masas, posibilidad de hacer medidas de la relación isotópica de las moléculas, no es necesaria la optimización de los parámetros instrumentales para cada analito, se puede obtener información de cualquier compuesto ya que se realiza análisis de untargeted (desconocidos), y es posible estudiar compuestos de los que no se dispone de patrones. Además, maximizar el número de compuestos que pueden determinarse en cada análisis puede suponer un ahorro económico y de tiempo muy importante.

Uno de estos analizadores de alta resolución es el Orbitrap. Este es un tipo de IT ideado por Alexander Makarov, que logra mejorar ampliamente algunas de las características de este tipo de analizadores. Así, aunque los espectrómetros de masas de tipo IT son importantes y ampliamente utilizados, suelen tener algunas desventajas como su baja exactitud de masas, un rango dinámico lineal no muy grande o capacidad de carga reducida.

[141] J. Wang, D. Leung, J. Agric. Food Chem. 57 (2009) 2162.

[142] M. Taylor, G. Keenan, K. Reid, D. Fernández, Rapid Commun. Mass Spectrom. 22 (2008) 2731.

El Orbitrap (figura 11) consiste fundamentalmente en una serie de multipolos, similares a los cuadrupolos descritos para el sistema QqQ, que son utilizados para conducir los iones producidos en una fuente de ionización previa, hasta un último cuadrupolo que actúa como trampa de iones (conocido como C-Trap por su forma curvada y no lineal como es habitual), desde donde pueden ser transferidos directamente al analizador o a una celda de colisión para producir su fraccionamiento. Esta celda de colisión es conocida como HCD (higher energy colision induced dissociation) y consiste en un multipolo conectado con la C-Trap. Este analizador consiste en un electrodo exterior con forma de barril y un electrodo interior coaxial con forma de huso que forma un campo electrostático. Ambos electrodos están conectados a fuentes de voltaje independientes. El electrodo exterior se divide en dos partes, una de ellas se utiliza para la excitación de los iones y otra para su detección. El espacio libre entre el electrodo interno y el externo forma la cámara de medida. Esta cámara se encuentra conectada a una bomba de vacío que permite alcanzar presiones de vacío de aproximadamente 10^{-8} torr e incluso menores. En el analizador, los iones son inyectados tangencialmente entre los electrodos y quedan atrapados girando alrededor del electrodo central. Al mismo tiempo los iones también oscilan a lo largo del eje del electrodo central. Así, los iones con una relación m/z específica se mueven en anillos que oscilan alrededor del huso central; la frecuencia de estas oscilaciones armónicas es independiente de la velocidad del ion y es inversamente proporcional a la raíz cuadrada de la relación m/z. La frecuencia de las oscilaciones axiales de cada molécula girando alrededor del electrodo central induce una corriente en los electrodos externos. En realidad pueden ser millones de iones los que originen una corriente en los electrodos y sus frecuencias son determinadas usando la Transformada de Fourier.

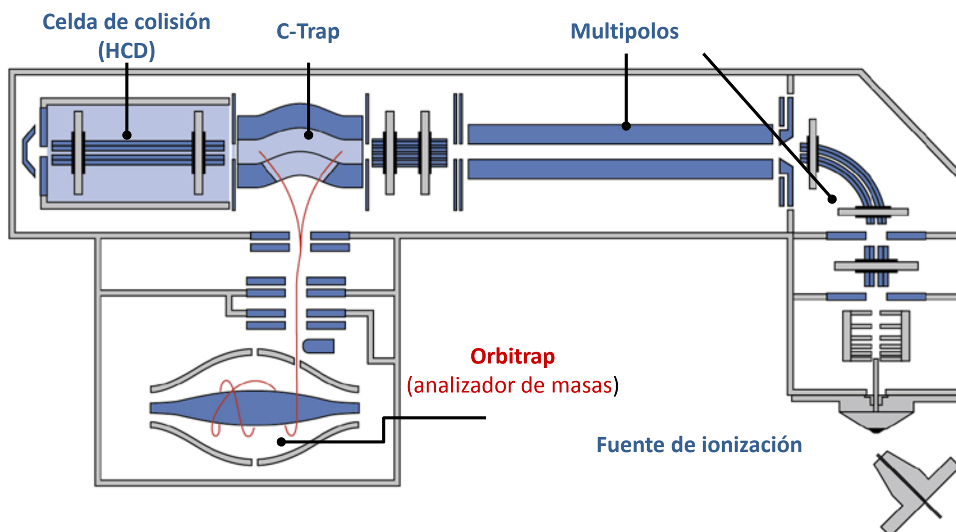

Figura 11. Esquema del analizador de masas Orbitrap (Figura adaptada de http://planetorbitrap.com/q-exactive#tab:schematic, Thermo Fisher)

Aunque el uso del Orbitrap esta menos extendido que el resto de espectrómetros de masas debido a su más reciente comercialización y elevado coste, el número de trabajos publicados en los que se utiliza este tipo de analizador ha crecido rápidamente en los últimos años. Así, en análisis alimentario se ha empleado para la determinación multirresiduo tanto de pesticidas como de compuestos farmacéuticos [143,144,145,146] y otros compuestos de interés [146,147].

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[144] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Anal. Chim. Acta, 700 (2011) 86.

[145] H.G.J. Mol, P. Zomer, M. de Koninng, Anal. Bioanal. Chem. 403 (2012) 2891.

[146] H.G.J. Mol, P. Plaza-Bolaños, P. Zomer, T.C. de Rijk, A.A.M. Stolker, P.P.J. Mulder, Anal. Chem. 80 (2008) 9450.

[147] R.J.B. Peters, A.A.M. Stolker, J.G.J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulos, M.W.F. Nielen, Trends Anal. Chem. 29 (2010) 1250.

6. *Tratamientos de muestra en análisis de contaminantes en alimentos y medioambiente*

El principal objetivo del tratamiento de muestra es eliminar posibles interferentes, y llevar a cabo una preconcentración de los analitos en estudio, haciendo de los mismos una especie detectable mediante las técnica deseada. Por ello una adecuada selección del tratamiento de muestra es un aspecto clave que puede afectar enormemente los resultados finales del análisis. A pesar de los avances realizados en los últimos 20 años para mejorar los tratamientos de muestra, éstos frecuentemente necesitan de muchas etapas y de una gran parte de trabajo manual que los hacen tediosos. Este es el caso de las técnicas clásicas, como la extracción con Soxhlet y la extracción líquido-líquido (LLE) convencional, las cuales se aplican en muchos laboratorios de rutina para el tratamiento de muestras sólidas y líquidas, respectivamente. No obstante, en los últimos años se han producido importantes avances en el desarrollo de técnicas de extracción dirigidos, principalmente, al desarrollo de métodos más rápidos y con los que se consiga una mayor preconcentración [64,148,149].

Las ultimas tendencias en tratamientos de muestra incluyen nuevos objetivos como son la simplificación, reducción del tiempo necesario para los tratamientos, el uso de porciones de muestra lo mas pequeñas posibles, aumento de la selectividad, mejora de la automatización, un menor consumo de reactivos y residuos generados, así como el aumento de la seguridad del analista [148,150], consideraciones englobadas en el concepto de Química Analítica Verde [151]. Por ello, en los últimos años, muchos laboratorios están desarrollando nuevas metodologías de trabajo, encaminadas hacia

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[148] L. Ramos, J. Chromatogr. A 1221 (2012) 84.

[149] L. Ramos, R.M. Smith (Editors), Advances in Sample Preparation, Part I, J. Chromatogr. A 1152 (2007)

[150] O. Núñez, H. Gallart-Ayala, C.P.B. Martins, P. Lucci, J. Chromatogr. A 1228 (2012) 298.

[151] M. Farré, S. Pérez, C. Gonçalves, M.F. Alpendurada, D. Barceló, Trends Anal. Chem. 29 (2010) 1347.

el uso de técnicas de extracción alternativas como la SPE, microextracción en fase sólida (SPME), extracción por sorción en barrita agitadora (SRSE), extracción líquida presurizada (PLE), extracción asistida con microondas (MAE), membrana líquida soportada (SLM) y más recientemente la extracción en fase sólida dispersiva (DSPE) o la microextracción líquido-líquido dispersiva (DLLME). En este sentido se han publicado algunos artículos de revisión donde se subraya la importancia de la etapa de preparación de muestra, y se describen el empleo de los tratamientos nombrados anteriormente para el análisis de alimentos y muestras medioambientales como aguas y suelos [63,64,66,148,152].

Centrándonos en el análisis de alimentos, en la actualidad existe una amplia variedad de sistemas de tratamiento de muestra para la determinación de residuos de contaminantes en estas muestras tan complejas como reflejan diversos artículos de revisión [63,64,66,152]. En cuanto a muestras medioambientales se han descrito tratamientos de muestra similares y, generalmente, se suelen emplear métodos adaptados de otras aplicaciones desarrolladas previamente para alimentos [153,154,155].

Centrándonos en la determinación de Qns, probablemente el método más extendido es la SPE con diferentes sorbentes [63,66,156]. Aunque en los últimos años se pueden encontrar publicaciones en las que se han empleado otras metodologías más novedosas como los polímeros impresos molecularmente [157,158], la DLLME [96,159,160] o la metodología QuEChERS [140].

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[152] K. Ridgway, S.P.D. Lalljie, R.M. Smith, J. Chromatogr. A 1153 (2007) 36.

[153] M. Petrovic, M. Farre, M.J. López de Alda, S. Perez, C. Postigo, M. Kock, J. Radjenovic, M. Gros, D. Barceló, J. Chromatogr. A, 1217 (2010) 4004.

[154] C. Mahugo-Santana, Z. Sosa-Ferrera, M.E. Torres-Padrón, J.J. Santana-Rodríguez, Trends Anal. Chem. 30 (2011) 731.

[155] M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, Trends Anal. Chem. 30 (2011) 749.

[156] J. Blesa, L.J.G. Silva, C. M. Lino, G. Font, A. Pena, J. Sep. Sci. 35 (2012) 832.

[157] E. Turiel, A. Martín-Esteban, J.L. Tadeo, J. Chromatogr. A 1172 (2007) 97.

En la presente Tesis se proponen algunas alternativas para mejorar la eficacia de los procedimientos de extracción y limpieza de para la determinación de Qns, considerando las tendencias actuales que persiguen una reducción del tamaño de muestra, reducción o eliminación del consumo de disolventes orgánicos, procedimientos de extracción genérica multirresiduo y posible automatización y rapidez de procesado sin olvidar el coste económico. A continuación se describen algunas particularidades de los procedimientos de tratamiento de muestra empleados en esta Memoria.

6.1 *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 [161,162,163,164]. 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 (también conocidos como Envichrom P o Lichrolut) para compuestos polares, y los de carbón grafitizado, comúnmente también conocido como Carbopack, Carbograph o Envicarb.

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[158] M. Díaz-Alvárez, E. Turiel, A. Martín-Esteban, Anal. Bioanal. Chem. 393 (2009) 899.

[159] M.M. Parrilla Vázquez, P. Parrilla Vázquez, M. Martínez Galera, M.D. Gil García, Anal. Chim. Acta748 (2012) 20.

[160] H. Yana, H. Wanga, X. Qinb, B. Liua, J. Dua, J. Pharmaceut. Biomed. 54 (2011) 53.

[161] H. Kataoka, H.L. Lord, J. Pawliszyn, J. Chromatogr. A 880 (2000) 35.

[162] A. Sides, K. Robards, S. Helliwell, Trends Anal. Chem. 19 (2000) 322.

[163] D. Pragney, U.V.R. Vijaya Saradhi, Trends Anal. Chem. 37 (2012) 73.

[164] O. Zuloaga, P. Navarro, E. Bizkarguenaga, A. Iparraguirre, A. Vallejo, M. Olivares, A. Prieto, Anal. Chim. Acta 736 (2012) 7.

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 [165,166].

La SPE ha sido ampliamente utilizada para la determinación de Qns tanto en alimentos como en muestras medioambientales empleando cartuchos HLB [63, 93,167], aunque también se han utilizado otros sorbentes de intercambio iónico [63,156] o incluso ambos combinados [92] para extracción y limpieza.

6.2 *Polímeros Impresos Molecularmente (MIPs)*

Los polímeros impresos molecularmente (MIPs) son una clase de elementos de reconocimiento molecular basados en 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 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,

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[165] P. Pérez-Ortega, B. Gilbert-López, J.F. García-Reyes, N. Ramos-Martos, A. Molina-Díaz, J. Chromatogr. A 1249 (2012) 32.

[166] 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.

[167] S. Zorita, L. Larsson, L. Mathiasson, J. Sep. Sci. 31 (2008) 3117.

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 (figura 12).

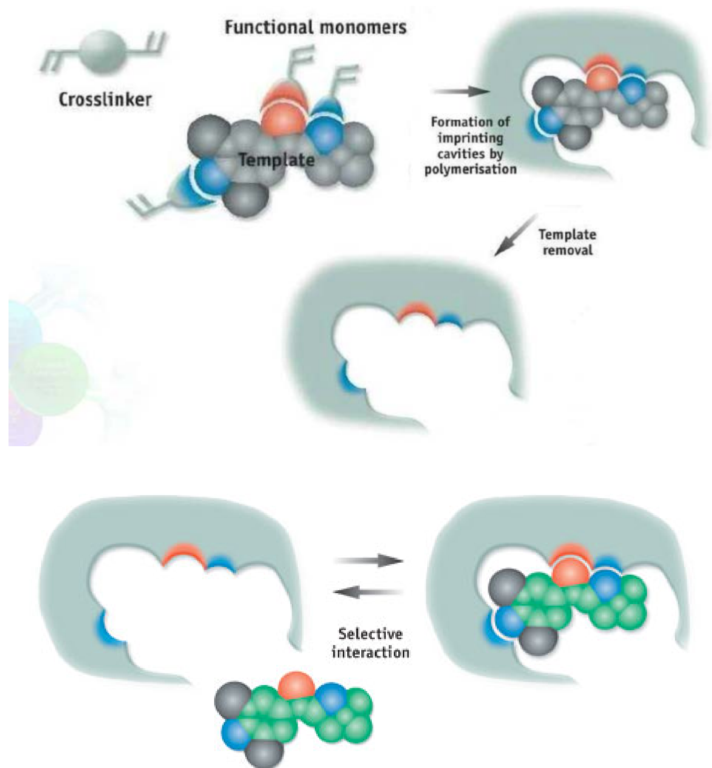

Figura 12. Polimerización y sitios específicos en el MIP para la interacción selectiva (Figura adaptada de http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/2/supelmip.pdf, Sigma Aldrich).

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 de 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 impresa 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 MIPs 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 [168,169,170,171,172,173]. Para determinación de antibióticos, los MIPs se han empleado en la extracción de β-lactámicos en muestras de aguas [174] o leche [175], sulfametacina en leche [176] o Qns en suelos [157] y alimentos infantiles [158].

La casa comercial Supelco ha comercializado algunos sistemas de MISPE [177] (SupelMIP™), para triacinas, cloranfenicol, anfetaminas o fluoroquinolonas (los cuales han sido evaluados para la determinación de Qns en la presente Tesis).

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[168] E. Turiel, A. Martin-Esteban, Anal. Bioanal. Chem. 378 (2004) 1876.

[169] J.O. Mahony, K. Nolan, M.R. Smyth, B. Mizaikoff, Anal. Chim. Acta 534 (2005) 31

[170] E. Caro, R.M. Marcé, F. Borrull, P.A.G. Cormack, D.C. Sherrington, Trends Anal. Chem. 25 (2006) 143.

[171] V. Pichon, F. Chapuis-Hugon , Anal. Chim. Acta, 622 (2008) 48.

[172] E. Turiel, A. Martín-Esteban, Anal. Chim. Acta 668 (2010) 87.

[173] A. Beltrán, F. Borrull, R.M. Marcé, P.A.G. Cormack, Trends Anal. Chem. 29 (2010) 1363.

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[175] C. Quesada-Molina, B. Claude, A.M. García-Campaña, M. del Olmo Iruela, P. Morin. Food Chem. 135 (2012) 775.

[176] A. Guzmán-Vázquez de Prada, P. Martínez-Ruiz, A.J. Anal. Chim. Acta. 539 (2005) 125.

[177] Pagina web de Sigma Aldrich. Accedido en Octubre 2012 en:

6.3 *Extracción en Fase Sólida Dispersiva (DSPE) - QuEChERS*

La DSPE es un método rápido y sencillo de limpieza de muestras, actualmente muy extendido para el análisis multirresiduo de plaguicidas en matrices de frutas y vegetales, pero que muestra interesantes perspectivas en otras matrices y con otras familias de compuestos.

Basándose en este tipo de extracción, Anastassiades y col. [178] desarrollaron el procedimiento comúnmente conocido como QuEChERS – Quick (rápido), Easy (fácil), Cheap (barato), Effective (eficaz), Rugged (robusto), Safe (seguro)– (figura 13) que implica un primer paso en el que se lleva cabo una extracción simple con acetonitrilo, seguida de un fenómeno de partición líquido-líquido por adición de $MgSO_4$ anhidro y NaCl. El sulfato magnésico en concentraciones superiores a las de saturación, ayuda a mejorar las recuperaciones en el proceso de extracción, al favorecer el reparto de los analitos en la capa orgánica. El NaCl ayuda a controlar la polaridad, aunque demasiada sal podría impedir el reparto. Además, el acetato sódico y otras sales actúan controlando el pH.

El segundo paso de esta metodología es el basado en la DSPE propiamente dicha e implica la eliminación de agua residual y limpieza (clean-up) mediante la adición de una mezcla basada en $MgSO_4$ (con objeto de eliminar el agua) y un sorbente, generalmente C18, una amina primaria y secundaria (PSA) o carbono grafitizado, que proporciona una elevada capacidad de limpieza de los componentes de la matriz (azúcares, ácidos grasos, ácidos orgánicos, pigmentos, etc.). Después de este proceso de limpieza, se lleva a cabo una centrifugación y el extracto está listo para ser directamente analizado o sometido a evaporación y recomposición en el disolvente apropiado para su análisis.

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http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/supelmip.html.

[178] M. Anastassiades, S.J. Lehotay, D. Stajbaher, F.J. Schenck, J. AOAC Int., 86 (2003) 412.

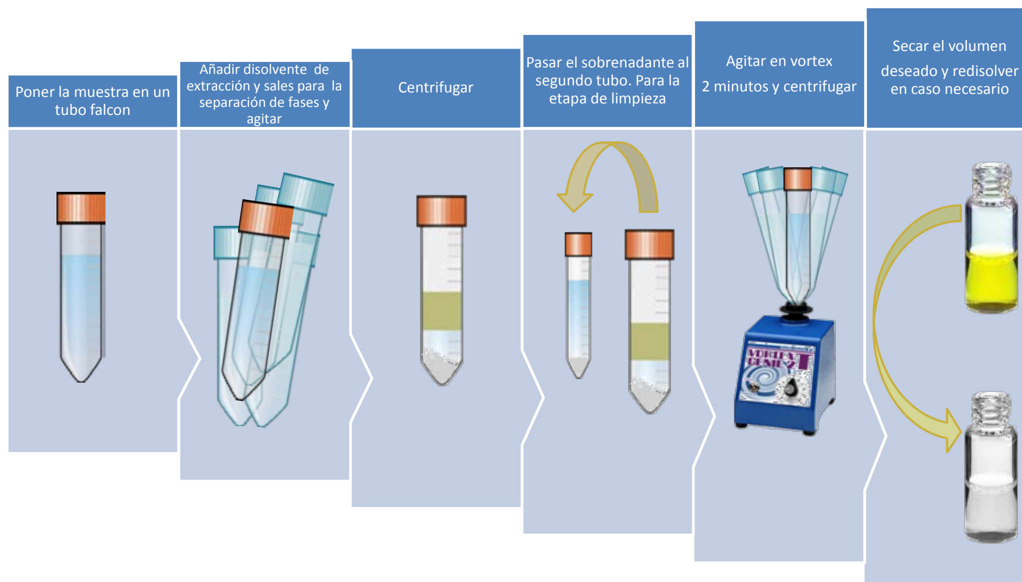

Figura 13. Método QuEChERS

Existen diversas aplicaciones de la metodología QuEChERS para la determinación de residuos de medicamentos veterinarios, pudiéndose encontrar algunas aplicaciones multirresiduo en huevo [129], leche [127], alimentos infantiles [179], pescado [180] o carne de pollo [130]. Actualmente existen kits comercializados por diversas empresas para desarrollar este tratamiento de muestra [181,182] que ha sido establecido como método normalizado europeo para plaguicidas [183].

En esta introducción se ha pretendido abordar algunos principios y antecedentes de las

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[180] R.P. Lopes, R.C. Reyes, R. Romero-González, J.L. Martinez-Vidal, A. Garrido-Frenich, J. Chromatogr. B 895 (2012) 39.

[181] Pagina web de Agilent Technologies, accedido en agosto 2012 en: http://www.chem.agilent.com/en-US/Products/consumables/samplepreparation/

[182] http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/dispersive-spe-tubes.html

[183] Norm EN 15662:2008. “Foods of plant origin — Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE — QuEChERS-method”, 01-02-2009.

técnicas y procedimientos de tratamientos de muestra que se proponen en los siguientes capítulos, así como la problemática del análisis de residuos de fármacos, especialmente de Qns en muestras de alimentos y medioambientales. En cada capítulo experimental igualmente se incluye una introducción específica en función de la problemática abordada, tipo de métodos de análisis y detección, matriz y procedimiento de tratamiento de muestra, incluyendo algunos antecedentes bibliográficos con objeto de facilitar la comprensión independiente de los mismos.

Capítulo 1

Determinación de residuos de quinolonas en aguas mediante CE-LIF.

Chapter 1

Sensitive determination of quinolone residues in waters by CE-LIF.

Resumen

En el primer capítulo de esta Tesis Doctoral se ha desarrollado un método muy sensible para la determinación de seis quinolonas de uso humano (ofloxacino, lomefloxacino y norfloxacino) y veterinario (danofloxacino, enrofloxacino y sarafloxacino) mediante electroforesis capilar acoplada con un detector de fluorescencia inducida por laser en diferentes tipos de aguas (agua de pozo y de grifo). Para ello, se ha utilizado un láser de He-Cd con una longitud de onda de excitación de 325 nm. La separación se ha llevado a cabo empleando un capilar de sílice fundida y se han optimizado las condiciones experimentales con el fin de conseguir una adecuada separación, con la mejor sensibilidad posible. Se ha empleado un capilar de 70 cm de longitud y 75 μm de diámetro interno, con una longitud efectiva de 55 cm, y un medio de separación compuesto por una disolución tampón de fosfato sódico 125 mM a pH 2.8 con un 36% de metanol (MeOH). El tratamiento de las muestras de agua consistió en la extracción y preconcentración de los analitos mediante SPE. Se han evaluado dos tipos de cartuchos de extracción: Oasis HLB (de equilibrio hidrofílico-lipofílico) y Strata-X con fase inversa polimérica. Éste último dio lugar a mejores valores de recuperación para los analitos estudiados. El método desarrollado presenta unos límites de detección muy bajos (0.3–1.9 ng/l) y adecuados valores para las recuperaciones y la precisión.

Abstract

A sensitive capillary electrophoresis–laser induced fluorescence method has been developed for the determination of six quinolones of human (ofloxacin, lomefloxacin and norfloxacin) and veterinary use (danofloxacin, enrofloxacin and sarafloxacin) in different kinds of water (well and tap water samples). Fluorescence detection was achieved using a He-Cd laser, with an excitation wavelength of 325 nm. Separation was performed in a fused-silica capillary, and conditions were optimized to obtain the most adequate separation and the best sensitivity. The separation was carried out in a 70-cm-long capillary (75 μm internal diameter, effective length 55 cm) by using a 125 mM phosphoric acid separation buffer at pH 2.8, with 36% of methanol. The water sample pretreatment involved the separation and preconcentration of the analytes by solid phase extraction. Two reverse-phase cartridges have been evaluated, namely Oasis hydrophilic-liphophilic balance and Strata-X polymeric reversed phase; the latter provided the best recoveries for the selected analytes. The method shows very low detection limits (0.3–1.9 ng/l) with acceptable recoveries and precision.

1.1 Introduction

The extensive use of pharmaceuticals (including antibiotics) in livestock production and human medicine and the frequent detection of them in the aquatic environment has supposed their consideration as “emerging pollutants”, because of the negative impacts they may have on ecosystems [1,2]. These products reach the environment via domestic and hospital sewages or industrial discharges. Moreover, a high percentage of antibiotics consumed are excreted unchanged via urine and faeces and are discharged to wastewater treatment plant effluent into the aquatic environment [3,4,5,6]. Sewage treatments are not good enough to completely remove small amounts of these compounds from treated waters, which lead to the introduction of them into surface waters, making them potential pollutants.

Once in the environment, pharmaceuticals may have unpredicted and unknown side effects on different organisms, particularly after long-term exposure to low concentrations, as well as they may provoke bacterial resistance from the release of antibiotics to the environment [7]. Thus, in order to fulfil quality standards set by the European Union (EU) for water [8], it is necessary to increase our knowledge about the occurrence of all these emerging contaminants in water.

These problems have attracted the attention of the scientific community; thus, in recent years, major attention has been paid to the environmental fate of pharmaceuticals and substances used in personal care products in the aquatic environment [9,10,11]. Several

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[1] N.J. Morley, Environ Toxicol Pharmacol 27 (2009) 161.

[2] V. Christen, S. Hickmann, B. Rechenberg, K. Fent, Aquat Toxicol, 96 (2010) 167.

[3] J.B. Ellis, Environmental Pollution 144 (2006) 184.

[4] M. Cha, S. Yang, K.H. Carlson, J. Chromatogr. A 1115 (2006) 46.

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[7] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, J. Chromatogr. A 815 (1998) 213.

[8] Council Directive 91/271/EEC concerning urban waste-water treatment and amending. Official Journal of European Communities, L135/40. 30-05-1991.

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reviews have been published in the last years relating to determination of emerging contaminants in environment, including antibiotics [12,13,14,15,16]. Moreover, the occurrence of numerous pharmaceuticals and personal care products in the natural environment has been reported in different countries [17,18,19,20,21].

Qns are among the five classes of antibiotics (β-lactam, macrolides, Qns, sulfonamides and tetracyclines) frequently detected in the environment [22]. Qns residues can enter the environment mainly because of their excretion in the urine of humans and animals (as a high percentage is excreted unaltered) as well as of aquaculture treatments and the direct discharge of aquaculture products. Their presence in surface, ground and wastewater samples has been reported in different publications [17,20,21,23,54,24] and even in drinking water [25] at typical concentrations in the range of low µg/l or ng/l. Once Qns reach the aquatic environment, they are affected by many abiotic and biotic factors and may undergo various degradation processes. As commented before, the presence of

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Qn antibiotics in the environment is potentially linked to resistant bacteria, which can make humans and animals more susceptible to antibiotic resistance [7]. In addition, several studies based on an in vitro bioassay have reported that Qns promote the development of genotoxicity [26,27].

Qns have been mainly analyzed by HPLC and CE combined with UV–visible detection, fluorescence, or mass spectrometry (MS). Different sample treatments, including solvent extraction, pressurized liquid extraction, and SPE, have been applied depending on the characteristics of the sample and on the different Qn residues found. Some reviews summarize some of these analytical strategies [28,29,30] and some specific applications has been developed for environmental waters mainly using HPLC [31,32,33,34].

Concerning the CE analysis of these compounds, the chemical structure of Qns, with a carboxylic group (acidic Qns) and in most cases with an amino group as well (zwitterionic Qns), allows their separation in simple buffers used in CZE. However, depending on the Qns mixture to be resolved, it could be necessary to use non-aqueous CE in order to achieve a complete resolution of signals [35]. MEKC has also been used to improve sensitivity rather than to improve resolution by means of on-line sweeping [36]. UV–Vis is the preferred detection mode for the monitoring of the residues of these compounds by

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CE [35,36,37,38,39,40,41,42,43,44] although fluorescence [45,46], MS [47,48,49], amperometry [50], and electrochemiluminescence [51] have also been applied.

Taking into account the low concentrations expected in environmental samples, very sensitive methods for their quantification are required. Apart from off-line SPE procedures, several strategies have been used to improve sensitivity in CE for the determination of Qns, as the on-line coupling of SPE sorbents [49] or the use of an extremely sensitive detection technique, such as LIF detection, considering the native fluorescence of some Qns. CE-LIF have been applied for the determination of some Qns like ofloxacin and its metabolites [52], moxifloxacin [53] and ciprofloxacin and its metabolite [54] in body fluids, and enrofloxacin and ciprofloxacin in chicken muscle [45].

In this chapter we propose a specific and sensitive method based on the use of CE coupled to LIF detection for the simultaneous quantification of residues of six commonly used Qns of human (OFLO, LOME, and NOR) and veterinary use (DANO, ENRO, and

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SARA) that could be present in water samples. A previous SPE step has been optimized in order to improve selectivity and to achieve very low detection limits. The obtained results demonstrate the possibilities of CE-LIF for the quantification of residues of these compounds at very low concentrations in waters.

1.2 Experimental

1.2.1 *Chemicals*

All reagents were of analytical reagent grade. Solvents were HPLC grade and Qns were of analytical standard grade. Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Acetonitrile (AcN), methanol (MeOH), citric acid monohydrate, ammonium acetate, and tris(hydroxymethyl)aminomethan (Tris) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide, sodium dodecyl sulphate (SDS), sodium dihydrogen phosphate monohydrate, and phosphoric acid (85%) were obtained from Panreac-Química (Barcelona, Spain). Qns (OFLO, LOME, NOR, DANO, ENRO, and SARA) and hexadecyltrimethylammonium bromide (CTAB) were obtained from Sigma (St. Louis, MO, USA).

Extraction cartridges containing an Oasis hydrophilic–lipophilic balance (HLB; 200 mg, 6 ml; Waters, Milford, MA, USA) and Strata-X Polymeric Reversed Phase (200 mg, 6 ml; Phenomenex, supplied by Micrón, Madrid, Spain) were used for sample treatment.

A solvent filtration system from Millipore and nylon membranes (0.2 μm, 47 mm; Supelco, Bellefonte, PA, USA) were used for sample filtration, and Acrodisc Nylon membrane syringe filters (0.2 μm, 13 mm, Pall Corp., MI, USA) were used to filter the final extract and the background electrolyte (BGE) before CE analysis.

1.2.2 *Preparation of solutions*

Stock standard solutions (100 mg/l) of each Qn were prepared by dissolving the appropriate amount of each substance in an AcN/H_2O 50/50 (v/v) solution, and they were

stored in the dark at 4°C. Under such conditions, they were stable for at least 1 month. Working standard solutions were freshly prepared by dilution of the stock solutions with AcN/H_2O 50/50 (v/v).

BGE consisted on 125 mM phosphoric acid solution adjusted to pH 2.8 with 4 M sodium hydroxide, containing 36% MeOH.

1.2.3 *Instrumentation*

CE experiments were carried out on an HP3D CE instrument (Agilent technologies, Waldbronn, Germany) coupled to a LIF detector (Zetalif Evolution model LIF UV-01, Picometrics S.A., Ramonville, France), equipped with an He-Cd Laser (excitation wavelength 325 nm), shown in figure 1.1. Data were collected using the software provided with the instrument (ChemStation version A.09.01). Separation was performed in an uncoated fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA).

Figure 1.1. Coupling of Agilent CE instruments and Picometrics laser detector.

For pH measurements, a pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit was used. SPE was carried out on a Visiprep™ DL vacuum manifold for 12 cartridges (Supelco). A vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) was used for recomposition of the extracts after SPE treatment.

1.2.4 *Electrophoretic procedure*

Half an hour before starting a series, the laser was switched on. Before the first use, the new capillary (70 cm length × 75 µm ID, effective length 55 cm) was conditioned by rinsing with 1 M NaOH at 60 °C for 15 min, then with water at room temperature for 10 min, and finally with the BGE for 25 min, all at 1 bar N_2 pressure. Every day, the capillary was prewashed with 0.1 M NaOH for 8 min, water for 1.5 min, and BGE for 15 min, at a N_2 pressure of 1 bar. After each run, the capillary was washed with 0.1 M NaOH for 2 min, water for 1 min, and BGE for 2 min, to maintain an adequate repeatability of run-to-run injections. At the end of the day, the capillary was rinsed with water for 4 min and with air for 4 min. BGE consisted on an aqueous solution of 125 mM phosphoric acid adjusted to pH 2.8 with 4 M sodium hydroxide and 36% of MeOH. The electrophoretic separation was achieved by applying a voltage of 26 kV (normal mode). Sample was injected by hydrodynamic injection using a pressure of 50 mbar for 10 s. The temperature of the capillary (35 cm thermostatized plus 35 cm at room temperature, corresponding to the distance from the CE to the detector) was kept constant at 15 °C. It is recommended to maintain the room temperature at a value around 20 °C, as changes in the room temperature caused irreproducibility in migration times and changes in peak shapes.

1.2.5 *Sample treatment*

Two different well waters (a mountain well and an irrigated-land well, both from Alomartes, Granada, Spain) and one tap water (Alomartes, Granada, Spain) were analyzed. Samples of 250 ml were spiked with appropriates volumes of the Qn standard solutions, and their pH values were adjusted to 6. Then, samples were shacked for 1 min, left in the dark for at least 30 min, and then filtered to remove suspended matter. The samples were then passed at a flow rate of 5 ml/min through a Strata-X cartridge (33 µm polymeric reversed phase, 200 mg, 6 ml) previously conditioned with 2 ml of MeOH plus 2 ml of water at a flow rate of 0.5 ml/min; the cartridge was then dried, and the analytes were eluted with 2 ml of AcN/H_2O 75/25 (5% acetic acid) plus 2 ml MeOH/AcN 50/50.

This extract was dried under a gentle N_2 stream at 35 °C. The extract was recomposed in 0.5 ml of AcN/BGE 25/75, vortexed for approximately 1 min, and filtered.

1.3 Results and discussion

1.3.1 *Optimization of CE-LIF experimental conditions*

Electrophoretic conditions have been optimized considering the compatibility and requirements of the LIF detection and CE separation, in order to obtain the best sensitivity and resolution, with a low generated current. Thus, the following parameters were optimized: pH, BGE nature and concentration, organic modifier, voltage, and temperature. Qns are zwitterions with two different pKa so pH affects their separation in CE. The pH was studied between 2 and 9. Although it has been stated that better resolutions are achieved at alkaline pH values [55], it was observed that fluorescence intensity of the selected Qns decreased at alkaline conditions; thus, the pH was further studied in the range of 2.0–7.5, using a concentration of 100 mM of different buffers solution, namely: phosphoric acid, citric acid, phosphate buffer and Tris. Acidic pH was selected as optimum because of the higher sensitivity of Qns. At this pH, citric and phosphoric acid solutions were tested, obtaining the best result for the later. Finally, an optimum pH of 2.8 was selected. Nevertheless, an exact adjustment of the pH was not so critical, because the electrophoretic mobility of the Qns hardly changes with pH values between 2.0 and 4.0 [38,55,56]. Subsequently, in order to improve the resolution, the phosphoric acid concentration for the BGE was modified between 100 and 150 mM (adjusting the pH with NaOH), and the best results in terms of resolution, migration time, and current intensity were found at a concentration of 125 mM. Higher concentrations increased the current intensity considerably without an improvement on the separation.

The effect of different modifiers in the BGE, such as micellar media (SDS and CTAB),

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AcN, MeOH, and ethanol was studied (all of them included in 5%). MeOH provided the best results, and therefore its influence was studied in the range of 5–40%. The migration times decreased when increasing the MeOH percentage, obtaining the best resolution and a shorter analysis time with a percentage of 35% of MeOH in the BGE, in agreement with the results found in the literature [57]. Later on, percentage of MeOH was studied in detail and finally 36% was selected, as it provided slightly narrower peaks (see figure 1.2).

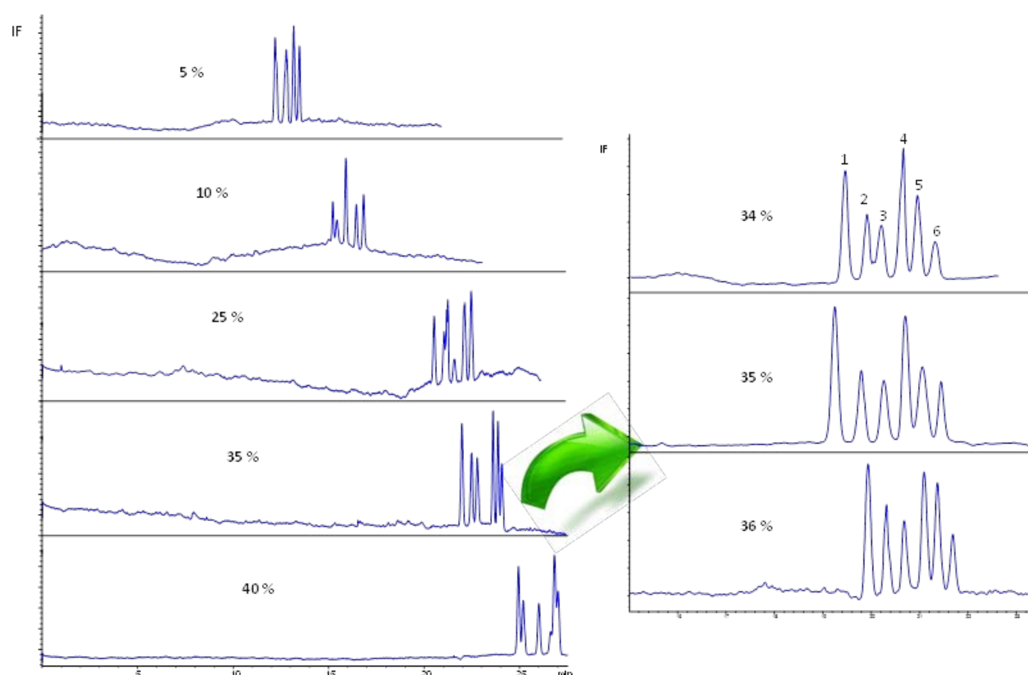

Figure 1.2. Influence of MeOH percentage added to BGE in peak resolution. (1) OFLO, (2) LOME, (3) NOR, (4) DANO, (5) ENRO and (6) SARA.

Then, the separation voltage and the effect of temperature on the separation were studied. Voltage was varied from 20 to 28 kV. A voltage of 26 kV was selected as optimum, as it provided the best compromise between resolution, migration time, and

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electric current. The temperature was tested in the range of 15–30 °C, selecting 15 °C as optimum, as this temperature provided the best resolution.

1.3.2 *Optimization of sample treatment*

In order to obtain suitable limits for the determination of Qns in environmental waters (expected to be at very low concentration levels), a SPE procedure was proposed for preconcentration of the analytes in real samples, using aqueous solutions of the analytes. This alternative has proved to be suitable for the determination of Qns in different samples such as meat [41,58], milk [48,59], biological fluids [60,61], or waters [46,62,63,64]. Based on these applications, we decided to carry out a comparative study for SPE using two polymeric sorbents commonly used (Oasis HLB and Strata-X), which could provide procedures for clean-up and preconcentration adequate to obtain low enough limits of detection for water samples. For preconditioning of the cartridges, commercial specifications (similar for both sorbents) were used: 2 ml of MeOH plus 2 ml of water at a flow rate of 0.5 ml/min.

Since the solvent for extract recomposition is an important variable, different solvents were studied, namely, AcN, H_2O, BGE, can/H_2O 50/50, and AcN/BGE 25/75. In all cases, a volume of 0.5 ml was used, and the mixture was vortexed for 1 min. Due to the high percentage of MeOH included in the BGE, the conductivity of the sample was critical. Pure organic solvent could not be used because conductivity in the capillary decrease until almost zero and separation was not possible. Thus, aqueous solutions and mixtures of aqueous/organic solvents were studied. The use of BGE did not produce good signals,

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while the mixtures AcN/BGE 25/75 and AcN/ H_2O 50/50 gave better results; however, the lower conductivity of the mixture AcN/ H_2O 50/50 caused eventual current fall. So, AcN/BGE 25/75 was selected as optimum recomposition solvent. Recoveries obtained with this study are shown in figure 1.3.

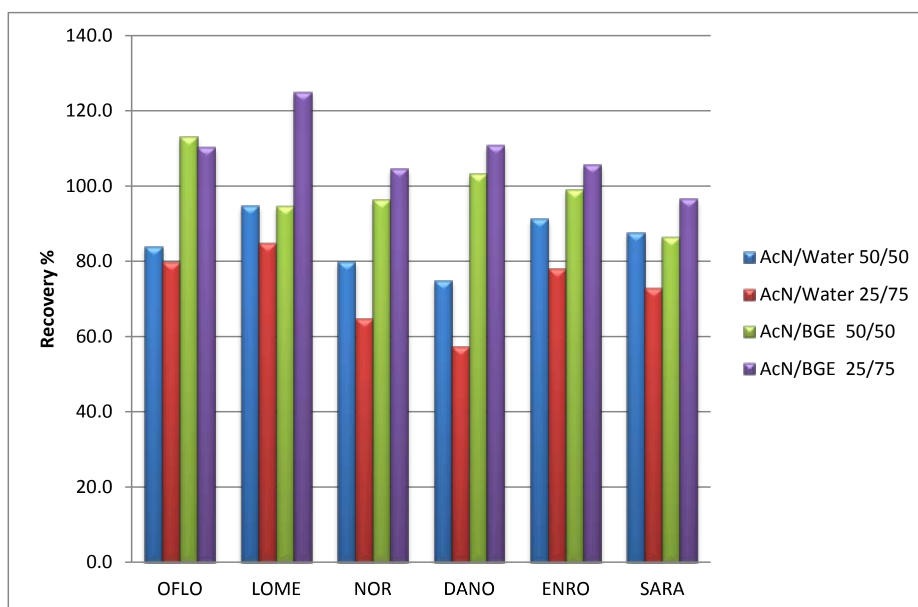

Figure 1.3. Comparison of recoveries obtained for each Qn using different solvents in the recomposition step.

To achieve a complete analyte elution from the sorbents, four different compositions for the elution solution were tested in aqueous standard solutions, namely, (a) 4 ml of MeOH, (b) 2 ml MeOH/ H_2O 75/25 plus 2 ml MeOH, (c) 2 ml AcN/H_2O 75/25 (5% acetic acid) plus 2 ml AcN/MeOH 50/50, and (d) 2 ml H H_2O/MeOH 75/25 (5% acetic acid) + 2 ml MeOH.

The selected elution solvent was (c) because the highest recoveries were obtained, with no significant differences for both cartridges. Once selected the elution solvent, the percentage of acetic acid was studied in the range from 0% to 5%, and the best recoveries (>90%) were obtained for 5%.

As it has been previously commented, Qns are amphoteric compounds that can be in a

cationic, anionic, or zwitterionic form; thus, considering the reversed phase nature of the selected sorbents, the pH of the sample was studied between 3 and 8 (analytes as zwitterions), achieving the highest retention for both cartridges when the pH was comprised between 6 and 8.

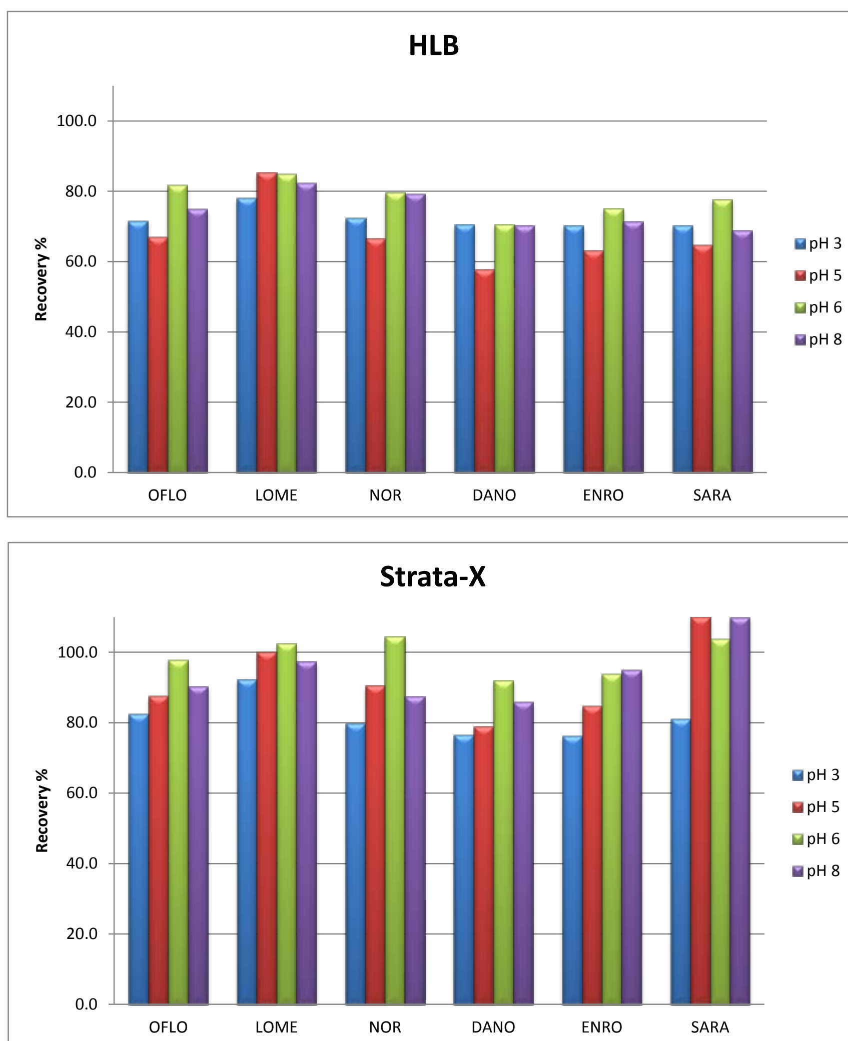

Figure 1.4. Recovery study of the SPE procedure at different pHs of the sample using HLB and Strata-X cartridges.

A pH value of 6 was selected, as it was closer to the original pH of the samples. At this pH, slightly better recoveries were obtained with the Strata-X (92.0–104.4%), in comparison with HLB (70.6–84.9%) cartridge as is shown in figure 1.4, so the former was selected as the sorbent for the SPE procedure. Once the optimum SPE procedure was applied, the final eluate was dried under a gentle N_2 stream and recomposed in 0.5 ml of AcN/BGE 25/75.

Finally, different water sample volumes (50, 100, 250 and 500 ml) were used in the application of the SPE procedure for preconcentration purposes. No significant differences in recovery percentages were obtained when 50, 100 or 250 ml were employed and slightly lower when 500 was employed (figure. 1.5), so 250 ml was selected as optimum volume in order to obtain a high preconcentration factor (500) with satisfactory recovery in an adequate analysis time.

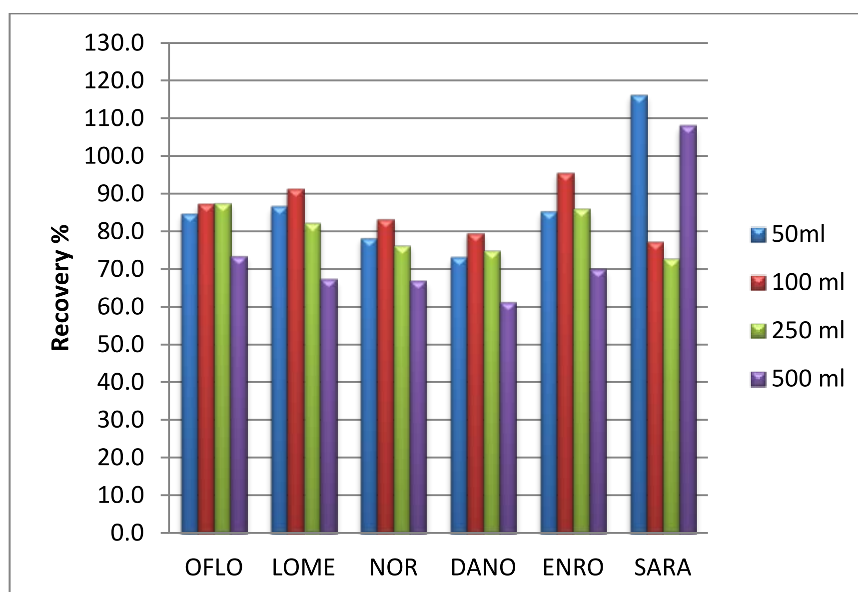

Figure. 1.5. Recovery obtained with different volume of sample.

1.3.3 *Analytical and performance characteristics*

1.3.3.1 Calibration curves, detection and quantification limits

The whole analytical method was validated using matrix-matched calibration curves with

irrigated-land well water samples as a representative matrix spiked with different concentration levels of analytes (2, 10, 20, 40, and 60 ng/l for DANO and 10, 30, 50, 100, and 150 ng/l for the others Qns). Each concentration level was injected by triplicate. Considering the preconcentration factor (500), those concentration levels correspond to final concentrations in the test samples of 1, 5, 10, 20, and 30 µg/l for DANO and 5, 15, 25, 50, and 75 µg/l for the others Qns. A blank sample was also analyzed, and no matrix peaks were found co-migrating with the analytes. Calibration curves were established considering peak areas as a function of the analyte concentration. The statistic parameters calculated by least-square regression, limits of detection (LODs) and limits of quantification (LOQs) of the method are shown in table 1. LODs and LOQs were considered as the minimum analyte concentration yielding a S/N ratio equal to 3 and 10, respectively. As can be seen, very low limits were obtained in all cases, making possible the quantification of the analytes at the lower concentration expected in these kinds of samples.

Table 1.1. Calibration curves and Performance Characteristics of the proposed method.

	Linear range (ng/l)	**Intercept**	**Slope**	**R^2**	**LOD (ng/l)**	**LOQ (ng/l)**
OFLO	0.9-150	226.4	19.4	0.983	0.9	3.2
LOME	1.7-150	57.5	12.6	0.986	1.7	5.3
NOR	1.9-150	176.9	10.9	0.991	1.9	6.6
DANO	0.3-60	384.4	60.0	0.986	0.3	1.0
ENRO	1.2-150	180.8	19.6	0.982	1.2	4.0
SARA	1.8-150	116.3	11.9	0.990	1.8	6.3

1.3.3.2 Precision and trueness studies

The precision of the whole method was evaluated in terms of repeatability (intraday

precision) and intermediate precision (interday precision), using irrigated-land well water. Repeatability was assessed by means of repetitive application of the whole procedure to five samples (experimental replicates) spiked at two concentration levels (10 and 40 ng/l for DANO and 30 and 100 ng/l for the rest), each one injected by triplicate (instrumental replicates) on the same day. Intermediate precision was assessed with a similar procedure, but the five samples were analyzed in five different days. The results, expressed as relative standard deviation (RSD) of peak areas and migration times, are given in Table 2. As can be observed, acceptable precision was obtained in all cases.

Table 1.2. Precision study

	Repeatability RSD (%)				**Intermediate precision, RSD (%)**			
	Level 1[a] (n=15)	**Level 2[b] (n=15)**	**Mig. Time (min) (n=30)**	**RSD (%)**	**Level 1[a] (n=15)**	**Level 2[b] (n=15)**	**Mig. Time (min) (n=30)**	**RSD (%)**
OFLO	7	6.7	21.7	4.4	8.5	9.2	21.8	3.6
LOME	12.6	14.1	22.2	4.3	10.9	10.1	22.2	3.5
NOR	9.7	9.4	22.5	4.4	9.3	8.7	22.5	3.6
DANO	7.4	8.2	22.9	4.3	8.5	6.6	22.9	3.6
ENRO	12.7	8.6	23.5	4.2	9.7	9.9	23.2	3.5
SARA	9.3	9.9	23.2	4.2	8.6	8.9	23.6	3.5

a) Level 1: 10 ng/l for DANO and 30 ng/l for the others Qns.
b) Level 2: 40 ng/l for DANO and 100 ng/l for the others Qns.

In order to check the trueness of the proposed methodology, recovery experiments were carried out in three different types of water: two different well waters (mountain well and irrigated-land well) and one tap water. Water samples were spiked with a mixture of the six studied Qns at different levels (10 and 40 ng/l for DANO and 30 and 100 ng/l for all the rest). Each level was prepared in triplicate, submitted to the proposed SPE method, and analyzed by duplicate. Blank samples were also analyzed, and in none of the Qn

residues were detected. The results are shown in Table 3. Typical electropherograms of a blank sample and spiked water samples are shown in figure 1.6. The others blank samples did not differ from this one presented. As can be seen, the proposed method provides satisfactory results in terms of trueness and precision, so method accuracy for the analysis of these samples was demonstrated.

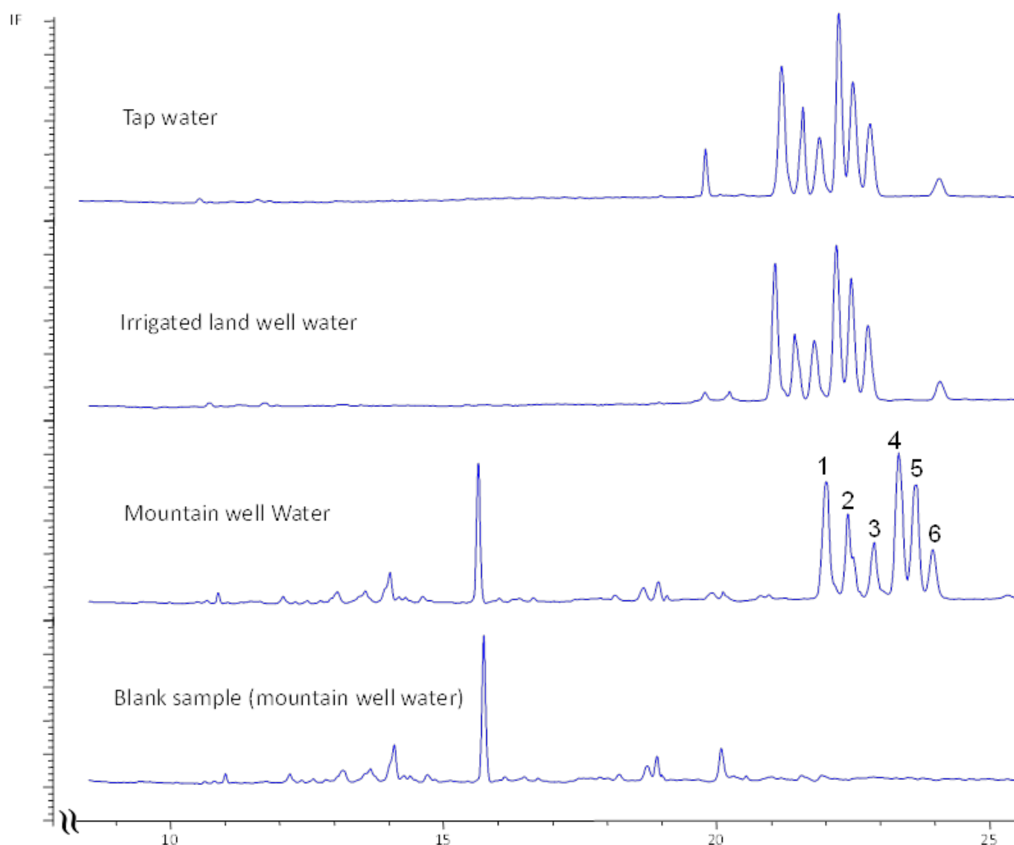

Figure 1.6. Electropherograms at optimum conditions of a blank sample of mountain well water, spiked mountain well water, spiked irrigated-land well water and spiked tap water. Spiked analyte concentrations are 100 ng/L for (1) OFLO, (2) LOME, (3) NOR, (5) ENRO, and (6) SARA and 40 ng/L for (4) DANO.

Table 1.3 Recovery study for the three studied water, n=6

	Mountain well water		Irrigated-land well water		Tap water	
	Level 1[a]	Level 2[b]	Level 1[a]	Level 2[b]	Level 1[a]	Level 2[b]
	R % (RSD %)	R % (RSD %)	R % (RSD %)	R % (RSD %)	R % (RSD %)	R % (RSD %)
OFLO	104.3 (2.6)	102.2 (9.8)	102.1 (6.9)	106.6 (6.2)	103.9 (7.9)	100.6 (7.4)
LOME	93.1 (6.6)	94.7 (5.5)	100.9 (9.4)	93.9 (8.7)	93.5 (4.2)	84.8 (7.5)
NOR	117.5 (5.6)	90.7 (10.3)	102.2 (7.3)	91.3 (6.4)	100.8 (4.1)	83.3 (5.7)
DANO	94.2 (4.8)	100.3 (7.9)	93.8 (8.7)	107.1 (8.8)	96.0 (6.6)	97.3 (6.8)
ENRO	97.0 (4.0)	93.0 (5.4)	89.7 (7.4)	98.8 (8.4)	88.4 (8.8)	91.4 (8.5)
SARA	93.6 (3.1)	99.1 (4.5)	95.6 (5.9)	101.5 (6.7)	92.5 (7.4)	94.2 (7.6)

a) Level 1: 10 ng/l for DANO and 30 ng/l for the others Qns.

b) Level 2: 40 ng/l for DANO and 100 ng/l for the others Qns.

1.4 Conclusions

A simple, sensitive, and selective method for the determination of six Qns in different water samples has been developed, showing LIF as a very attractive detection technique in CE, since it is very selective and sensitive reducing the interference possibilities from the matrix. SPE methodology has shown to be very useful for the extraction and preconcentration of these Qns, with the potential of being applied for the determination of other Qns. Moreover, a comparison between two different sorbent for sample treatment has been carried out, showing a slight improvement using Strata-X cartridges. LOQs were in the very low ng/l and could be easily improved to pg/l range by just increasing the sample volume or diminishing the volume of recomposition solvent after SPE process. This is one of the most sensitive proposed methods for the analysis of these compounds. The recoveries and precision obtained are good enough and show the suitability of this procedure for the monitoring of Qn residues in water samples and for providing data of the occurrence of these compounds in the environment.

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Capítulo 2

Determinación de quinolonas en alimentos de origen animal empleando MIPs y CE-LIF.

Chapter 2

CE-LIF for the determination of quinolones in foods of animal origin using MIPs.

Resumen

En este capítulo se ha utilizado la electroforesis capilar y la detección por fluorescencia inducida por láser para desarrollar y validar un método analítico para la determinación simultánea de cuatro quinolonas de uso veterinario (ciprofloxacino, danofloxacino, enrofloxacino y sarafloxacino), en dos matrices diferentes como son la leche de vaca cruda y el riñón de cerdo. El hecho de que los analitos objeto de estudio presenten fluorescencia nativa ha permitido el acoplamiento de la CE con un sistema de detección tan sensible como es LIF. Para conseguir una elevada selectividad en el proceso del tratamiento de muestra se ha empleado un polímero molecularmente impreso, que se encuentra disponible comercialmente, para realizar la extracción en fase sólida. Una vez optimizados los procesos de retención y elución de los analitos en el polímero, el extracto final se analizó mediante CE-LIF usando un láser de He-Cd de longitud de onda de 325 nm, próxima a la longitud de onda de excitación de los analitos. La separación se llevó a cabo en un capilar de 70 cm × 75 µm, empleando una disolución tampón de fosfato sódico 25 mM a pH 2.8 con un 36% de MeOH como electrolito de fondo. El método presenta unos límites de detección muy bajos que van desde 0.17 a 0.98 µg/Kg en el caso de la leche y desde 1.10 a 10.5 µg/Kg en el caso de la muestra de riñón, con precisiones y recuperaciones satisfactorias.

Abstract

In this chapter we have employed capillary electrophoresis and laser induced fluorescence detection to develop and validate a method for the simultaneous determination of four quinolones of veterinary use (ciprofloxacin, danofloxacin, enrofloxacin y sarafloxacin) in two complex matrixes, such as raw bovine milk and pig kidney. The method is based on the use of capillary electophoresis coupled with a very sensitive detection mode, such as LIF detection, due to the fact that all the selected compounds show native fluorescence. In order to achieve high selectivity in the sample treatment procedure, a commercially available molecularly imprinted polymer has been used for the solid phase extraction of the analytes. Once the retention and elution processes were optimized, the final extract was analyzed by CE-LIF using a 325 nm He-Cd laser, a wavelength close to the excitation wavelength of the analytes. Optimum separation was obtained in a 70 cm × 75 μm capillary using a 125 mM phosphoric acid solution at pH 2.8 with 36% methanol as background electrolyte. The method provided very low detection limits, ranging from 0.17-0.98 μg/Kg for milk and 1.1-10.5 μg/Kg for kidney, with acceptable precision and satisfactory recoveries.

2.1 Introduction

Quinolones are antibacterial compounds effective against a wide range of gram positive and negative bacteria. Their activity is based on the inhibition of bacterial DNA, which prevents bacterial multiplication. In the last years the extensive use of Qns in veterinary has promoted the persistence of these residues in foods derived from animals, which can represent an increment in adverse reactions for human health. Consequently the European Union (EU) has established MRLs for several Qns in foodstuffs of animal origin through the Council Regulation 2377/90//EEC [1] recently changed by the Commission regulation No. 37/2010 [2]. As an example, these limits have been established in 30 µg/Kg for DANO, and 100 µg/Kg for the sum of ENRO and CIPRO in bovine milk, while 200 µg/Kg for DANO, ENRO and CIPRO in pig kidney.Diverse methods have been described for the analysis of Qns in different matrices of animal origin, mainly animal tissues [3,4,5,6,7,8,9], milk [10,11,12,13] or infant foods [14]. Specifically laser induced fluorescence (LIF) detection has been coupled with CE to propose a very sensitive

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method for the determination of ENRO and CIPRO in chicken muscle [15].

Taking into account the low MRLs established for food samples by EU legislation, a sample pretreatment is mandatory, being SPE in the off-line mode the preferred sample preparation technique. These methods involve the use of different SPE cartridges, such as Oasis HLB [3,93], Strata X [14,16], ENV+ Isolute or Oasis MAX [3]. Also, a tandem of MAX and HLB cartridges was used to ensure a convenient preconcentration step and a very clean extract [11]. These alternatives have proved to be suitable for the determination of Qn residues by CE or HPLC in food of animal origin such as meat [3,17], milk [11,93,18,19,20] or eggs [21]. Also, the in-line coupling of a SPE sorbent for on-line preconcentration has also been used in CE [7].

Others materials with highest selectivity, such as molecularly imprinted polymers (MIPs) can provide cleaner sample extract and easier process than usual SPE sorbents. MIPs are synthetic materials with artificially generated recognition sites able to specifically capture target molecules. The use of MIPs as selective sorbent materials allows performing a customized sample treatment step prior to the final determination. Thus, the strong retention between the MIP and its target molecules makes it ideal for the selective extraction of compounds at trace levels, being of special interest when the sample is complex. Several reviews show the characteristics of these materials and their applications in analytical chemistry [22,23,24,25,26,27]. In the last years MIPs have

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been employed successfully in the extraction of different antibiotics, such as β-lactam antibiotics in environmental water [28] or milk [29] and sulfamethazine in milk [30]. Some MIPs have been synthesized in several laboratories using different templates for the determination of Qns. CIPRO has been used as template for the selective analysis of Qns in soils [31] or baby food [32]. Also, an ENRO-imprinted polymer was applied to the selective extraction of this Qn and CIPRO from urine and pig liver [33]. A novel highly selective sample clean-up procedure combining molecular imprinting and matrix solid-phase dispersion (MI-MSPD) was developed for the simultaneous isolation of OFLO, PEFLO, NOR, CIPRO, and ENRO in chicken eggs and swine tissues [34] and ofloxacin-imprinted polymers were used as SPE sorbents for the selective extraction of nine Qns from urine samples [35] and six Qns in serum samples [36]. Recently, water-compatible pefloxacin-imprinted monoliths synthesized in a water-containing system were used for the selective extraction of Qns (ciprofloxacin, difloxacin, danofloxacin and enrofloxacin) in milk samples [37]. Also, automated on-line MISPE has been applied in LC using for the first time tailor-made molecularly imprinted microspherical polymer particles prepared via precipitation polymerization and the method has been satisfactorily applied to the simultaneous detection of six Qns (enrofloxacin, ciprofloxacin, norfloxacin, levofloxacin,

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danofloxacin, and sarafloxacin) in water samples [38].

In this chapter we propose a useful alternative to quantify very low concentrations of four Qns of veterinary use (CIPRO, DANO, ENRO and SARA) using the commercially available MIPs as sorbent for SPE (MISPE) in the treatment of complex matrices, such as bovine raw milk and pig kidney. The use of CE coupled to LIF detection is also proposed as a way of increasing sensitivity. Extraction using MIPs has been optimized in order to improve analyte recoveries. The obtained results demonstrate the possibilities of CE-LIF for the quantification of residues of these compounds in foodstuffs of animal origin at the concentrations required by their MRLs.

2.2 Experimental

2.2.1 *Chemicals*

All reagents were of analytical reagent grade. Solvents were HPLC grade and Qns were analytical standard grade. Ultrapure water (Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (AcN), sodium hydroxide, ammonia (30%), sodium dihydrogen phosphate monohydrate and phosphoric acid (85%) were obtained from Panreac-Química (Barcelona, Spain). Acetic acid (96%) was purchased from Merck (Darmstadt, Germany). CIPRO, DANO, ENRO, and SARA were obtained from Sigma (St. Louis, MO, USA). Chemical structures of Qns included in this study are shown in a previous part of this thesis.

A vacuum manifold system from Supelco (Bellefonte, USA) was used for SPE procedure. Extraction cartridges containing molecularly imprinted polymer (SupelMIP floroquinolones SPE Column, 25 mg, 3 ml) supplied by Supelco (Bellefonte, PA, USA) were used for extraction and clean-up process. Sterile syringe filters with 0.45 µm polyethersulfone membrane (VWR, Barcelona, Spain) were used for kidney extract filtration after solid-liquid extraction and 13 mm filters with 0.2 µm Nylon membrane (Bulk Acrodisc®, Pall

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corp., MI, USA) were used for filtration of the final extracts before CE analysis.

2.2.2 *Preparation of solutions*

Background electrolyte (BGE) consisted on 125 mM phosphoric acid solution adjusted to pH 2.8 with 4 M sodium hydroxide, containing 36 % methanol. For milk sample treatment, 10 mM ammonium acetate buffer pH 5 was prepared from acetic acid by using 3% ammonium hydroxide solution to adjust the pH. The 50 mM phosphate buffer pH (7.4) was prepared from NaH_2PO_4, adjusting the pH using 4 M sodium hydroxide solution.

Stock standard solutions (100 mg/l) of each Qn were prepared by dissolving the appropriate amount of each analyte in AcN, and were stored in the dark at 4 °C. Under such conditions, they were stable for at least one month. A 1000 µg/l working mixed-standard solution of CIPRO, ENRO and SARA was prepared daily in AcN by diluting the stock standard solutions and an independent solution was prepared for DANO under the same conditions.

2.2.3 *Instrumentation*

CE experiments were carried out on an HP3D CE instrument (Agilent Technologies, Waldbronn, Germany) coupled to a LIF detector (Zetalif Evolution model LIF UV-01, Picometrics S.A., Ramonville, France). Data were collected using the software provided with the HP ChemStation version A.09.01. Fused-silica capillaries (75 µm I.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

SPE was carried out on a VisiprepTM DL vacuum manifold (Supelco) for 12 cartridges. A pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, a Universal 320R centrifuge (Hettich, Zentrifugen) and a vortex-2 Genie (Scientific Industries, Bohemia (NY, USA) were also used.

2.2.4 *Electrophoretic procedure*

Before the first use, the new capillary (70 cm × 75 µm I.D., effective length 55 cm) was

conditioned by rinsing with 1 M NaOH at 60 ºC for 15 min, then with water at room temperature for 10 min, and finally with the BGE for 25 min. Every day the capillary was prewashed with 0.1 M NaOH for 8 min, with water for 1.5 min and with BGE for 15 min. After each run, the capillary was washed with 0.1 M NaOH for 2 min, water for 1 min and BGE for 2 min, to maintain an adequate repeatability of run-to-run injections. At the end of the day, the capillary was cleaned with water for 4 min and finally flushed with air for 4 min. In all cases, a N_2 pressure of 1 bar was applied.

Half an hour before starting a serie, the laser was shut on. The electrophoretic separation was achieved using the described BGE, and applying a voltage of 26 kV (normal mode). Samples were injected by hydrodynamic injection using a pressure of 50 mbar for 10 seconds. The temperature of the capillary (35 cm thermostatized plus 35 cm at room temperature, corresponding to the distance from the CE to the detector) was kept constant at 15 ºC, and the room temperature at a value of around 20 ºC.

2.2.5 *Sample treatment*

The method has been applied to two types of foodstuffs of different origin: bovine raw milk and pig kidney.

2.2.6 *Preparation of milk samples*

Samples of 10 gr of bovine raw milk (obtained from a local farm) were spiked at different concentration levels using the working standard solutions. After spiking and homogenizing in vortex, samples were added 50 µl of acetic acid, diluted with 10 ml of 10 mM ammonium acetate buffer pH 5.0, shaken for 5 min and then centrifuged at 5000 rpm. The pH of the supernatant was adjusted to 7.0 with 3% ammonium hydroxide solution, and ammonium acetate buffer was added to obtain a final volume of 25 ml; the pH of the final solution was checked to be 7.0. SupelMIP fluorquinolones SPE Column was used for the selective SPE procedure. For the cartridge conditioning, 1 ml of MeOH, 2 ml of water and 0.5 ml of 10 mM ammonium acetate buffer were successively applied.

An aliquot of 1 ml of the final solution was charged into the preconditioned cartridge at a flow rate of approximately 0.2 ml/min. After that, the cartridge was washed with 3 ml of water and 1 ml of AcN, both of them at a flow rate lower than 0.5 ml/min. After each washing step, vacuum (400 mbar) was applied for 2 min. The elution was achieved using 3 ml MeOH/ H_2O (50/50, v/v) with 3% ammonium hydroxide. This extract was evaporated to dryness at 35 ºC under a stream of nitrogen and the residue was redissolved in 400 µL of AcN/BGE (25/75, v/v), filtered and analysed by the proposed CE-LIF method.

2.2.7 *Preparation of kidney samples*

Pig kidney was purchased from a local market. Kidney was crushed and homogenized and portions of 2 g were spiked at different concentration levels of Qns using the working standard solutions. Solvent was evaporated under N_2 stream, and the kidney sample was homogenized again. For sample pretreatment, this portion was mixed with 30 ml of 50 mM NaH_2PO_4 pH 7.4, shacked for 5 min and centrifuged for 5 min at 5000 rpm. An aliquot of 2 ml of the resulting supernatant was filtered and 1 ml of this final solution was charged into the SupelMIP fluoroquinolone cartridge, previously preconditioned in the same way indicated before for milk sample, at a flow rate of approximately 0.2 ml/min, and submitted to the washing and elution process above described for milk samples.

2.3 Results and discussion

Considering that the difference in this method with respect to the proposed in Chapter 1 is that we focused the analysis only on the Qns of veterinary use (DANO, SARA and ENRO, including now CIPRO as metabolite of ENRO), the optimization of the CE separation is similar to that described in the previous chapter and identical conditions have been applied for the proposed CE-LIF determination of these compounds.

2.3.1 *Optimization of MISPE procedure for milk and kidney samples*

The use of MIPs can simplify the extraction method in these complex matrices, providing

higher selectivity and lower sample manipulation. Initially, the protocols proposed by Supelco for the SupelMIP fluoroquinolones SPE Columns for the analysis of SARA, ENRO, CIPRO, NOR, LOME, and OFLO in milk and kidney samples by LC-MS [39], were tested for our analytes, but we tried to simplify them in order to reduce the extraction steps, considering the intrinsic selectivity of the LIF detection. In addition, when we tested the protocols, low recoveries were observed for all the Qns under study. It involves that an optimization was necessary in order to increase absolute recoveries.

Based on the commercially proposed protocol [39], a re-optimization of the MISPE was carried out in order to adapt it to the CE-LIF method for the analysis of milk and kidney samples.

Considering the low recoveries percentages after apply commercial protocol to the milk samples (above 10% for SARA, ENRO and CIPRO and above 35% for DANO) the inclusion of an additional step in the cartridge conditioning consisting of 0.5 ml of 10 mM ammonium acetate buffer (pH 5) (see Table 2.1) improved significantly the efficiency of the process.

Moreover, the sample pretreatment proposed was changed in order to get a milk protein precipitation previously to the MISPE loading. With this purpose 50 µl of acetic acid was added to 10 g of milk and then was vortexed before centrifugation. The addition of this step provided a much cleaner extract to be loaded into the MIP cartridge than the one obtained following the proposed protocol. Later on, milk sample supernatant was mixed with 10 ml of 10 mM ammonium acetate buffer (pH 5), shaken for 5 min and then centrifuged at 5000 rpm. Supernatant pH was adjusted to 7 and ammonium acetate buffer was added to obtain a final volume of 25 ml. The supernatant was prepared to load into the MIP cartridge. As can be seen in Table 2.1, the commercially proposed protocol suggested five washing steps with different solvents after loading of 1 ml of the supernatant from the sample pretreatment. However, we observed that satisfactory

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[39] SupelMIP SPE Fluoroquinolones Instruction sheet. In http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/supelmip/literature-and-references.html

recoveries (even better than those reported by the fabricant) were obtained with only the first and second washing steps. However, the elution step proposed in the commercial protocol did not produce a complete elution of the analytes, so different aqueous mixtures of MeOH (25–90%) were studied in presence of a 2% ammonium hydroxide solution, selecting a MeOH/H_2O mixture (50/50; v/v) as optimum. Different percentages (1–5%) of ammonium hydroxide solution in the mixture were studied and the best results were obtained using 3%. Finally, different elution volumes (1–4 ml) were tested in order to increase the recovery percentages. The highest recoveries were obtained with a volume of 3 ml. This final extract was evaporated under gentle nitrogen stream and the residue was recomposed in 400 µL of AcN/BGE (25/75) solution [25]. This procedure does not involve any preconcentration step, but it was not necessary due to the high sensitivity of the proposed method.

As in the case of milk samples, the commercially proposed protocol for kidney samples (see table 2.1) was tested but low recoveries were obtained for Qns under study. Therefore the same optimized MISPE protocol for milk samples was applied to kidney samples. Only the sample pretreatment was different in this case (the sample was mixed with 30 ml of 50 mM NaH_2PO_4 pH 7.4, shaken for 5 min and centrifuged for 5 min at 5000 rpm) and a final step in the cartridge conditioning, consisting of 0.5 ml of 50 mM phosphate buffer (pH 7.4) was included. Better results were obtained compared to those obtained from the commercially proposed protocol. Using this procedure, no interferences comigrating with the analytes were observed in the electropherogram. As a summary, figure 2.1 shows a diagram of the optimized extraction methods using MIPs previous to the CE-LIF analysis for Qns in milk and kidney samples.

Table 2.1. MISPE protocols for the extraction of Qns after the sample pretreatment. Comparison between the commercially proposed and the optimized protocol in this work.

	Commercially proposed protocol	**Protocol proposed in this work**
1. Conditioning / Equilibrate:	1 ml MeOH 2 ml ultra-pure water.	1 ml MeOH 2 ml ultra-pure water 0.5 ml extraction buffer [a]
2. Load:	1 ml of sample supernatant.	1 ml of sample supernatant.
3. Wash in the described order*:	- 3 ml ultra-pure water - 1 ml AcN - 1 ml 15% AcN in ultra pure water [b] - 1 ml 0.5% acetic acid in AcN - 1 ml 0.1% ammonia in ultra pure water *A strong vacuum was applied through the cartridge for 2 min between each wash step.	- 3 ml ultra-pure water - 1 ml AcN * Vacuum was applied through the cartridge for 2 min between each wash step.
4. Elute:	Elute Qns with 1 ml 2% ammonium hydroxide in methanol	Elute Qns with 3 ml 3% ammonium hydroxide in methanol/water (50/50).
5. Evaporate / Reconstitute:	The SPE eluent was evaporated gently under nitrogen at 35 °C and reconstituted in 150 µL 50% AcN in 0.1% formic acid prior to analysis.	The SPE eluent was evaporated gently under nitrogen at 35 °C and reconstituted in 400 µL AcN/BGE (25/75) prior to analysis.

a) 10 mM ammonium acetate buffer pH 5.0 for milk samples and 50 mM phosphate buffer pH 7.4 for kidney samples.
b) Only for milk samples.

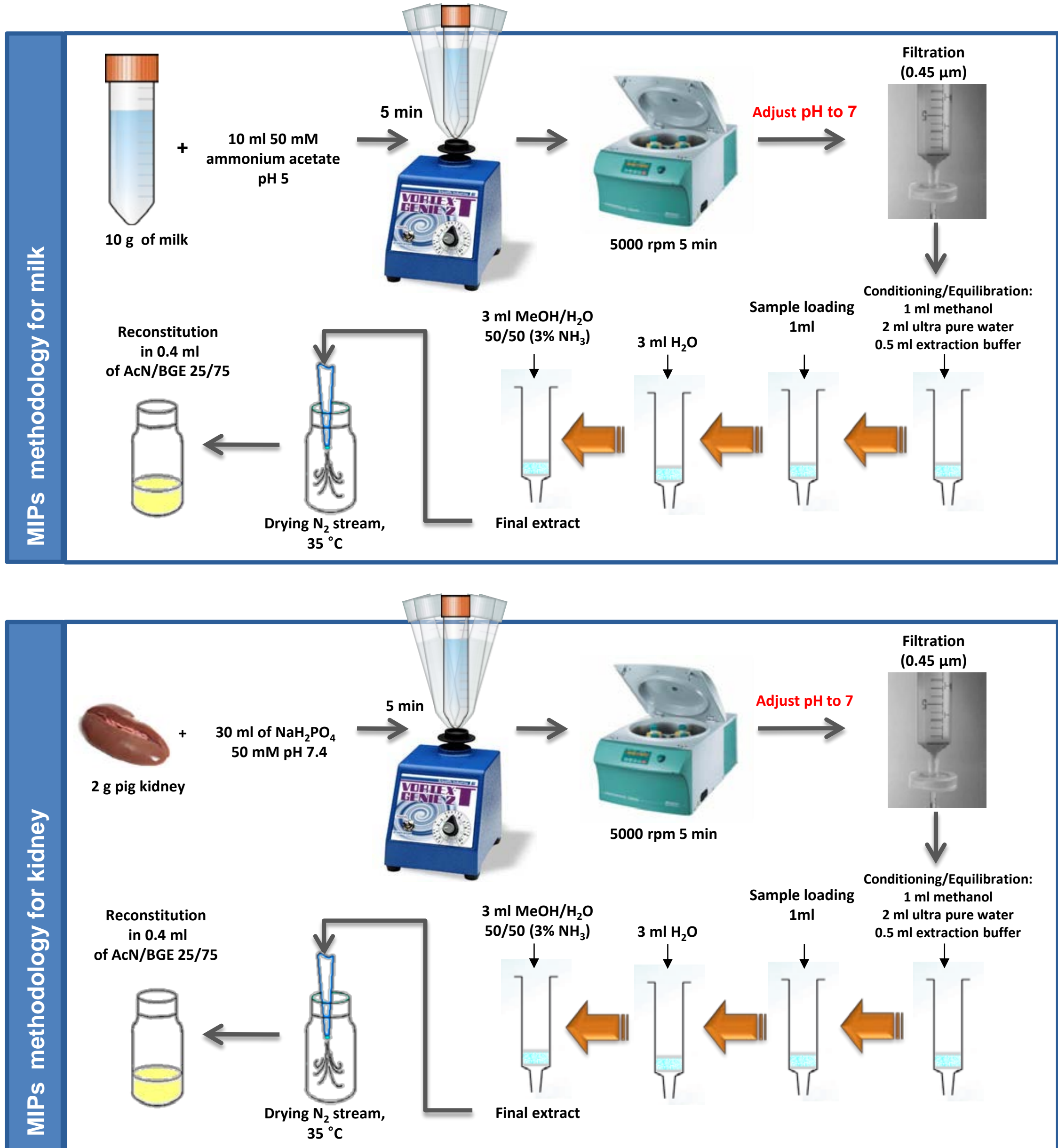

Figure 2.1. Diagram of optimized MIPSPE methods for milk and kidney samples.

2.3.2 *Analytical and Performance Characteristics*

2.3.2.1 Calibration curves, detection and quantification limits

Matrix-matched calibration curves for raw cow milk and pig kidney samples free of analytes and spiked with different concentration levels were established, considering peak areas as analytical signal.

Raw cow milk samples were spiked at 1, 5, 10, 20 and 30 µg/kg for DANO and 5, 15, 25, 50 and 75 µg/kg for the others Qns, and pig kidney samples were spiked at 10, 50, 100, 150 and 200 µg/kg for DANO and 50, 100, 200, 300 and 400 µg/kg for the others Qns. Each concentration level was prepared by triplicate, and all spiked samples were submitted to the MISPE procedure. A blank sample was also analysed, and no peaks were found comigrating with the analytes. Limits of detection (LODs) and limits of quantification (LOQs) were considered as the minimum analyte concentration yielding an S/N ratio equal to 3 and 10, respectively. The statistical parameters calculated by least-square regression, LODs and LOQs of the method for milk and kidney samples are shown in table 2.2. As can be seen, very low LOQs were obtained in all cases, allowing the quantification of the analytes at concentrations much lower than the established MRLs for these samples, without any preconcentration step.

2.3.2.2 Precision and trueness studies

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision), for peak areas and migration times.

Table 2.2. Calibration curves, statistical parameters, LODs and LOQs of the proposed method.

		Linear range (µg/Kg)	Slope	Intercept	R^2	LOD (µg/Kg)	LOQ (µg/Kg)	MRLs (µg/Kg)[a]
Bovine raw milk	**CIPRO**	1.78-75	13.2	93.3	0.991	0.5	1.8	100[b]
	DANO	0.55-30	75.4	75.6	0.994	0.2	0.6	30
	ENRO	1.14-75	30.0	11.1	0.992	0.3	1.1	100[b]
	SARA	3.26-75	9.3	17.0	0.994	1.0	3.3	Not established
Pig kidney	**CIPRO**	19.5-400	2.1	67.2	0.990	5.9	19.5	200[b]
	DANO	3.5-200	14.8	-93.5	0.990	1.1	3.5	200
	ENRO	10.4-400	4.9	-116	0.983	3.1	10.4	200[b]
	SARA	35-400	1.5	21.2	0.990	10.5	35.0	Not established

a) MRLs established by the commission regulation (EU) No 37/2010.
b) Quantified as the sum of CIPRO and ENRO.

Repeatability was assessed by means of repetitive application of the whole procedure to three samples (experimental replicates) spiked at two concentration levels: 5 and 20 µg/kg for DANO, and 15 and 50 µg/kg for the others Qns for milk samples, and 50 and 150 µg/kg for DANO, and 100 and 300 µg/kg for the rest of Qns for kidney samples. Each one was injected by triplicate (instrumental replicates) on the same day. Intermediate precision was assessed with a similar procedure, with five samples analysed in 5 different days. The results, expressed as RSD of peak areas and migration times, are given in table 2.3. As can be observed, satisfactory precisions were obtained in all cases. In order to check the trueness of the proposed methodology, recovery experiments were carried out in raw cow milk and pig kidney samples.

Table 2.3. Precision study.

	Raw cow milk				Pig kidney			
	CIPRO	**DANO**	**ENRO**	**SARA**	**CIPRO**	**DANO**	**ENRO**	**SARA**
	Intraday RSD (%) (n = 9)							
Level 1[a]	4.9	4.7	7.5	5.9	10.0	7.8	5.9	7.0
Level 2[b]	3.2	3.1	5.5	4.7	7.3	8.5	10.5	8.5
Mig. time (min)	22.5	22.9	23.2	23.6	22.6	23.1	23.4	23.7
RSD (%) (n=18)	1.7	1.7	2.0	1.7	4.8	4.7	4.6	4.6
	Interday RSD (%) (n = 15)							
Level 1[a]	5.3	8.3	11.6	8.7	13.9	11.6	12.8	12.2
Level 2[b]	7.7	10.9	8.8	6.9	12.5	12.9	10.1	12.5
Mig. time (min)	24.1	24.5	24.9	25.2	23.1	23.6	23.9	24.2
RSD (%) (n=30)	4.4	4.3	4.3	4.2	3.1	3.0	3.0	3.2

a) Level 1: 5 µg/Kg for DANO and 15 µg/Kg for the other Qns in milk samples; 50 µg/Kg for DANO and 100 µg/Kg for the others Qns in kidney samples.
b) Level 2: 20 µg/Kg for DANO and 50 µg/Kg for the others Qns in milk samples; 150 µg/Kg for DANO and 300 µg/Kg for the others Qns in kidney samples.

Samples were spiked with a mixture of the four Qns at two levels, similar to those used in the precision study. Each level was prepared by quintuplicate, submitted to the proposed method and injected by triplicate. Blank samples were also analysed, and none of them gave a positive result for Qn residues. The results are shown in table 2.4.

Table 2.4. Recovery study (n = 15).

	Raw cow milk				Pig kidney			
	CIPRO	**DANO**	**ENRO**	**SARA**	**CIPRO**	**DANO**	**ENRO**	**SARA**
Level 1[a]	91.1	93.8	86.5	95.2	98.6	91.4	85.5	96.4
RSD (%)	3.3	1.7	4.9	3.2	9.1	5.6	10.1	8.2
Level 2[b]	88.1	94.0	85.2	86.2	97.3	89.7	87.2	97.5
RSD (%)	5.8	4.5	3.8	3.5	7.0	6.3	7.8	9.5

a) Level 1: 5 µg/Kg for DANO and 15 µg/Kg for the others Qns in milk samples; 50 µg/Kg for DANO and 100 µg/Kg for the others Qns in kidney samples.
b) Level 2: 20 µg/Kg for DANO and 50 µg/Kg for the others in milk samples; 150 µg/Kg for DANO and 300 µg/Kg for the others Qns in kidney samples.

Typical electropherograms of blank and spiked samples are shown in figure 2.2 for milk and in figure 2.3 for kidney samples, respectively. As can be seen, an electropherogram free of interference signals was obtained using this MISPE procedure in such a complex matrixes, being a very selective and efficient analyte extraction method. This advantage, combined with the highly sensitivity of the LIF detection provided a very useful method for the analysis of this four Qns in these kind of samples of animal origin. The proposed method provided also satisfactory results in terms of trueness and precision, so the accuracy for the analysis of these samples was demonstrated.

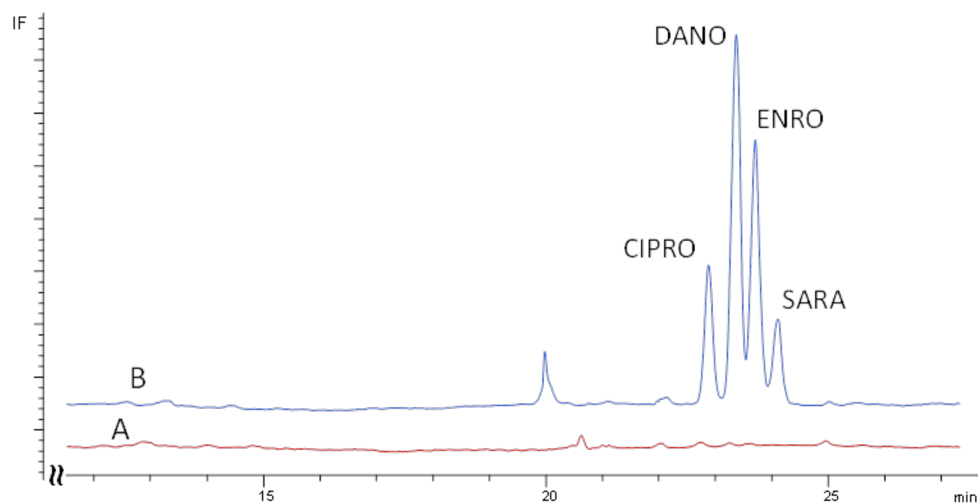

Figure 2.2. Electropherograms at optimum conditions of (A) blank sample of milk; (B) spiked milk sample (50 µg/kg for CIPRO, ENRO and SARA and 20 µg/kg for DANO).

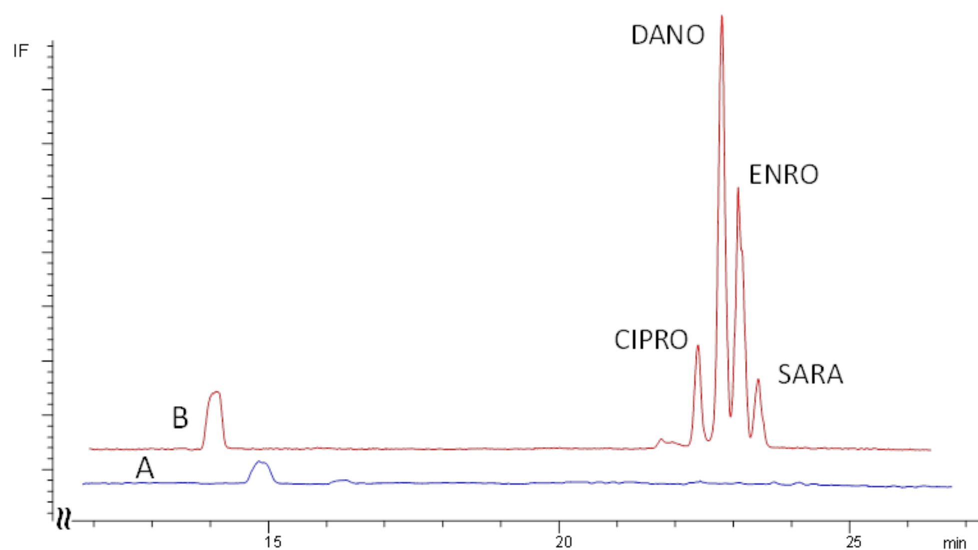

Figure 2.3. Electropherograms at optimum conditions of (A) blank sample of pig kidney; (B) spiked pig kidney sample (300 µg/kg for CIPRO, ENRO and SARA and 150 µg/kg for DANO).

2.4 Conclusions

A simple, selective and sensitive strategy for the determination of four Qns in two complex matrices has been developed, showing the usefulness of MIPs as a powerful tool for extraction and sample clean-up. Some steps of the MISPE procedure have been eliminated from the initial technical note, without any decreases in the recovery percentages. Additionally, LIF is proposed as a very attractive detection technique in CE, showing very high sensitivity and selectivity. LOQs were much lower than the MRLs established by EU, without any preconcentration step, and could be easily improved by just increasing the sample volume loaded into the MIP cartridge or by decreasing the final recomposition volume after the elution process since the electropherogram after sample treatment look very clean. The recoveries and precisions obtained are good enough, and show the suitability of this procedure for the monitoring of Qn residues in foodstuffs of animal origin (milk and kidney). This study opens interesting perspectives of the application of MIPs for the monitoring of these compounds in other samples and with other analytical techniques.

This work was published as:

"Laser induced fluorescence coupled to capillary electrophoresis for the determination of fluoroquinolones in foods of animal origin using molecularly imprinted polymers". M. Lombardo-Agüí, A.M. García-Campaña, L. Gámiz-Gracia, C. Cruces-Blanco. Journal of Chromatography A 1217 (2010) 2237.

Capítulo 3

Comparación de tratamientos de muestra para el análisis de quinolonas en leche mediante HPLC capilar-LIF.

Chapter 3

Comparison of different sample treatments for the analysis of quinolones in milk by capillary HPLC-LIF.

Resumen

Se ha desarrollado un método muy sensible para la determinación simultánea de siete Qns de uso veterinario en leche, empleando cromatografía liquida capilar con detección mediante fluorescencia inducida por láser. Además, se ha realizado una comparación entre dos tratamientos de muestra propuestos diferentes basados en el empleo de QuEChERS (quick, easy, cheap, effective, rugged and safe) y polímeros molecularmente impresos en términos de eficacia del método de extracción (número de analitos extraídos y ausencia de interferencias), rapidez del análisis, rango dinámico lineal en matriz, límites de detección y cuantificación, y veracidad a través de estudios de recuperación. Los resultados han mostrado que el procedimiento QuEChERS es más eficiente y rápido, proporciona mejores recuperaciones, mejor sensibilidad y exactitud para los analitos estudiados. Empleando este método se han obtenido unos límites de detección muy bajos, comprendidos entre 0.4 µg/kg para DANO y 6 µg/kg para SARA.

Abstract

A simple and very sensitive capillary liquid chromatography method coupled with laser induced fluorescence detection has been developed for the simultaneous determination of seven Qns of veterinary use in milk. Moreover, a comparison between two different sample treatments based on the use of QuEChERS (quick, easy, cheap, effective, rugged and safe) and molecularly imprinted polymers (MIPs) has been carried out in terms of efficiency of the extraction (number of analytes to be analyzed and absence of interferences), throughput, linear dynamic range in matrix-matched calibrations, detection and quantification limits and trueness, by means of recovery assays. The results showed that the QuEChERS procedure was more efficient and faster, showing good recoveries, sensitivity and precision for all the studied compounds. Employing this proposed method, very low detection limits, between 0.4 µg/kg for DANO, and 6 µg/kg for SARA, have been obtained.

3.1 Introduction

The determination of trace residues and contaminants in complex matrices, such as food, often requires extensive sample extraction and preparation prior to instrumental analysis. Sample preparation is often the bottleneck in analysis and there is a need to minimise the number of steps to reduce both time and sources of error. There is also a trend towards more environmentally friendly techniques, which use less solvents and smaller sample sizes [1]. In the field of antibiotic residues, different procedures have been proposed to improve the clean-up process and pre-concentration from different matrices [2]. Specifically in the determination of Qns, solid-liquid extraction [3], microwave extraction [4], pressurized liquid extraction [5,6,7], SPE [8,9,10,11,12,13], molecular imprinted-SPE (MISPE) [14,15,16], dispersive SPE [17], stir rod sorptive extraction (SRSE) [18], supramolecular solvents in microextraction [19] or dispersive

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liquid-liquid microextraction [20] have been used depending on the characteristics of the sample and the extracted Qns.

New methodologies have been proposed for the treatment of samples containing Qns. Together with MISPE, previously introduced and used in chapter 2, a fast and inexpensive method, so called Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) methodology are promising alternatives. The QuEChERS methodology presents some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning-up complex samples [21]. It involves two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE step that involves further clean-up using combinations of $MgSO_4$ and different sorbents (such as C18, graphitized carbon or primary-secondary amines), to remove interfering substances. This methodology has been extensively use for extraction of pesticides residues in vegetables [22,23], and recently, different QuEChERS procedures have been applied in the multidetermination of veterinary drugs residues, including Qns in animal tissue [24], milk [25,26] or eggs [27].

The purpose of this work is to develop a sensitive and selective method for the determination of seven Qns of veterinary use, namely CIPRO, DANO, ENRO, SARA,

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DIFLO, OXO and FLUME, using capillary HPLC-LIF detection as an alternative to quantify traces of these analytes in milk. Capillary HPLC is a miniaturized technique in which columns of internal diameter of typically 500 μm and flow rates up to 20 μl/min are used. This technique 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. It is recommended when sample volume is limited, and especially to gain sensitivity, which can be greatly increased when LIF detection is coupled as detection technique. These veterinary compounds have been selected considering that some of them such as DIFLO, SARA and OXO, are not allowed in animals producers of milk for human consumption, and for the others, MRLs have been established by EU legislation [28]. Furthermore, a comparison between both different sample treatments (QuEChERS and MISPE) has been carried out, in terms of efficiency, recovery, sensitivity and precision.

3.2 Experimental

3.2.1 *Reagents and materials*

Solvents were HPLC grade, reagents were analytical grade and Qns were analytical standard grade. Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (AcN), ammonium hydroxide (30%), sodium dihydrogen phosphate monohydrate and phosphoric acid (85%) were obtained from Panreac-Química (Barcelona, Spain). Acetic acid, formic acid and citric acid were supplied by Merck (Darmstadt, Germany). DANO, SARA and DIFLO were supplied by Riedel-de Haën (Seelze, Germany), FLUME by Sigma (St. Louis, MO, USA) and CIPRO, ENRO and OXO by Fluka (Steinheim, Germany).

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

Kits SampliQ QuEChERS (kindly supplied by Agilent Technologies Inc., Wilmington, DE, USA) consisted on 50 ml buffered QuEChERS extraction tubes (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive tubes (15 ml, 150 mg C18 and 900 mg $MgSO_4$). Extraction cartridges containing MIPs (SupelMIP Qns SPE Column, 25 mg, 3 ml, Supelco, Bellefonte, PA, USA) were used in the MISPE treatment.

Filters of 13 mm with 0.2 µm nylon membrane (Bulk Acrodisc®, Pall corp., MI, USA), were used for filtration of the final extracts before analysis. The mobile phase was filtered before use in 47 mm filters with 0.2 µm nylon membranes (Supelco, Bellefonte, PA, USA).

3.2.2 *Preparation of solutions*

Stock standard solutions (100 mg/l) of each Qns were prepared by dissolving the appropriate amount of each analyte in AcN, and were stored in the dark at 4 ºC. Under such conditions, they were stable for at least 1 month. Working solutions (containing all Qns) were prepared diary from the individual stock solutions and diluted with Milli-Q water.

3.2.3 *Instrumentation*

Separation was performed on a 1200 Series Capillary LC System from Agilent Technologies, coupled to a LIF detector (Zetalif Evolution model LIF UV-01, Picometrics S.A., Ramonville, France), with a He-Cd laser with an excitation wavelength of 325 nm (figure 3.1). Data were collected using the software provided with the HP ChemStation version A.09.01. A fused-silica capillary (75 µm ID) from Polymicro Technologies (Phoenix, AZ, USA) was used to couple the LC and the LIF detector. Different chromatographic columns were tested for separation of Qns, namely: Luna C18,

150×0.3 mm, 5 μm; Luna C18, 150×0.5 mm, 5 μm; and Luna C8, 150×0.3 mm, 3 μm (all of them from Phenomenex, Torrance, CA, USA).

A pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320R from Hettich Zentrifugen, Tuttlingen, Germany), a VisiprepTM DL vacuum manifold for SPE (Supelco) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used.

Figure 3.1. Coupling of the capillary HPLC instrument (Agilent technologies) with the LIF detector (Picometrics).

3.2.4 *Chromatographic conditions*

The mobile phase consisted on 10 mM aqueous citric acid solution pH 4.75 (pH adjusted with ammonium hydroxide) (eluent A) and 10 mM citric acid in AcN (eluent B). The gradient elution started with 11% B followed by a linear gradient to 20% B (6 min)

and to 90% B (11 min). The initial conditions were reestablished by a 5 min linear gradient, followed by an equilibration time of 10 min. Analysis were performed at a flow rate of 15 µl/min and a column temperature of 35 °C, using an injection volume of 8 µl. Half an hour before starting the measurements, the laser was switched on and the room temperature was kept constant at approximately 19 ºC.

3.3 Sample treatment

In this study we have tested two different methodologies for extraction and clean-up of whole cow milk samples: QuEChERS and MISPE.

3.3.1 *Use of QuEChERS for the treatment of milk samples*

The QuEChERS procedure was adapted from that described by Agilent Technologies for the determination of some Qns in bovine liver by LC-MS [29]. Samples of 2 g of milk were spiked at different concentration levels of Qns using the working standard solutions. They were placed into 50 ml centrifuge tubes, spiked and homogenized in vortex; then the sample was left stand for 15 min. After that 8 ml of 30 mM NaH_2PO_4 buffer pH 7.0 was added, shacking by hand for 10 s. Subsequently, 10 ml of 5% formic acid in AcN was added to the tube, shaking by hand for 30 s. Agilent SampliQ EN QuEChERS extraction kit was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 9000 rpm for 5 min and 4 ml of the upper AcN layer was transferred to another tube containing the dispersive SPE (C18 and $MgSO_4$) and stirred in vortex for 1 min. The tube was centrifuged at 9000 rpm for 5 min. Then, 1 ml of supernatant was transferred to a vial, dried at 35 ºC under a stream of nitrogen and reconstituted with 1 ml of 10 mM aqueous citric acid solution pH 4.75 (pH adjusted with ammonium hydroxide). Finally, the samples were filtered with a 0.2 µm filter before injection (see figure 3.2 for the whole treatment sketch).

[29] L. Zhao, J. Stevens, Application Note 5990-5085EN, Agilent Technologies INC. http://www.chem.agilent.com/Library/applications/5990-5085EN.pdf

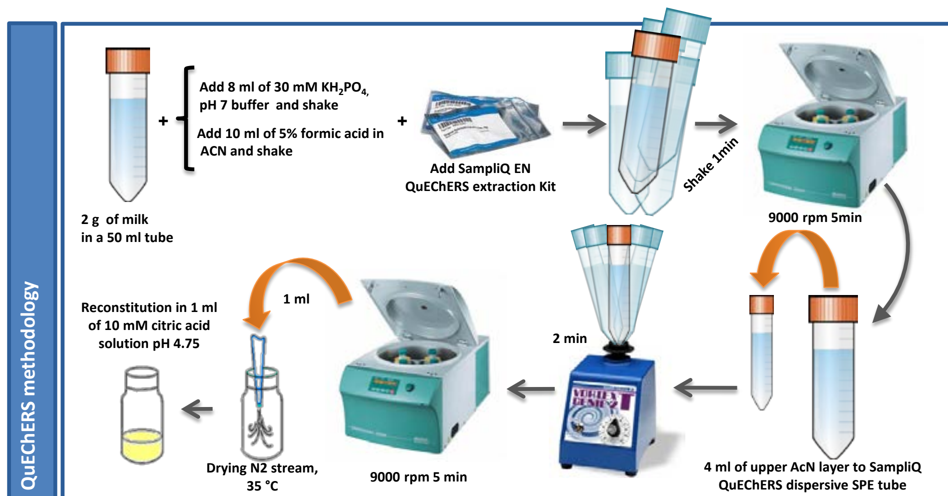

Figure 3.2. Optimized QuEChERS procedure for milk sample.

3.3.2 *Use of MISPE for the treatment of milk samples*

Samples of 10 g of milk were spiked at different concentration levels using the working standard solutions. After homogenizing in vortex, samples were diluted with 10 ml of 10 mM ammonium acetate buffer pH 5.0, shaken for 5 min and then centrifuged for 5 min at 5000 rpm. The pH of the supernatant was adjusted to 7.0 with 3% ammonium hydroxide solution, and 10 mM ammonium acetate buffer pH 7.0 was added to obtain a final volume of 25 ml. For MIP cartridge conditioning, 1 ml of MeOH, 2 ml of water and 0.5 ml of 10 mM ammonium acetate buffer pH 7.0, were successively applied. An aliquot of 1 ml of the final sample solution was charged into the preconditioned cartridge at a flow rate of approximately 0.2 ml/min. After that, the cartridge was washed with 3 ml of water at a flow rate lower than 0.5 ml/min and vacuum (400 mbar) was applied for 2 min. The elution was achieved with 3 ml MeOH/H_2O (50/50, v/v) with 3% ammonium hydroxide. This extract was evaporated to dryness at 35 ºC under a stream of nitrogen and the residue was reconstituted in 1 ml of 10 mM citric acid pH 4.75, filtered and analyzed by the proposed method (see figure 3.3 for the whole treatment sketch).

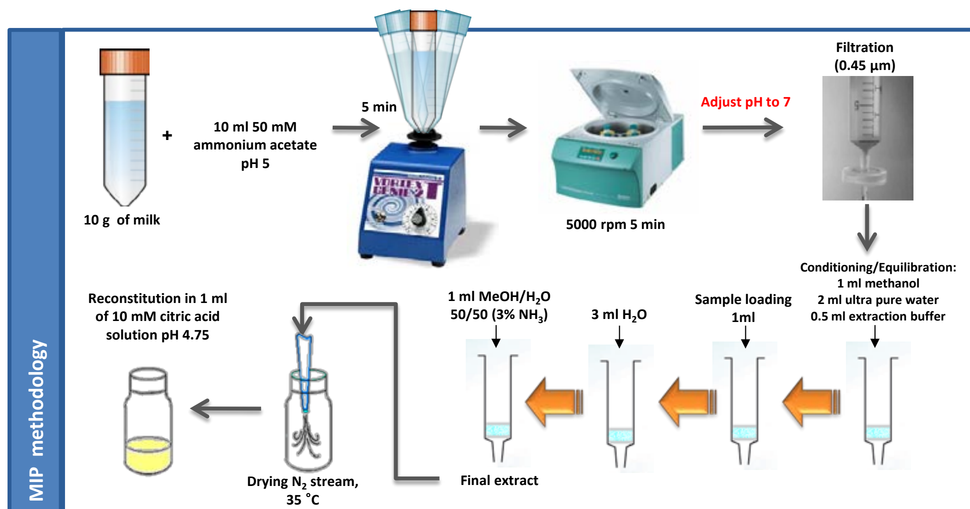

Figure 3.3. Optimized MISPE procedure for milk samples.

3.4 Results and discussion

3.4.1 *Optimization of the chromatographic separation*

Qns have been commonly analyzed by HPLC using citric acid solution and AcN as mobile phase [4,8]. So, using these solvents the separation method was optimized. First of all, the pH of the mobile phase was studied between 2.5 and 5.5, using a concentration of 10 mM citric acid in both, the aqueous and organic phases (A and B, respectively), and a Luna C18 150×0.3 mm, 3 µm column. Best compromise between signal intensity and resolution was found when pH 4.75 was used (see figure 3.4).

Concerning the chromatographic columns, usually C8 and C18 have been used to get a good separation for Qns [8]. Thus, three columns were tested, namely: (1) Luna C8 150×0.3 mm, 3 µm; (2) Luna C18 150×0.3 mm, 5 µm; and (3) Luna C18 150×0.5 mm, 5 µm. Narrow peaks and better resolutions, especially between SARA and DIFLO, were obtained when C18 columns (2 and 3) were used. Slightly higher peaks were obtained for most of the analytes when the third column was used. However, the separation took place shortly when the smaller diameter column (2) was used, as can be seen in figure

3.5. Thus, column 2 was selected as a compromise between resolution and analysis time.

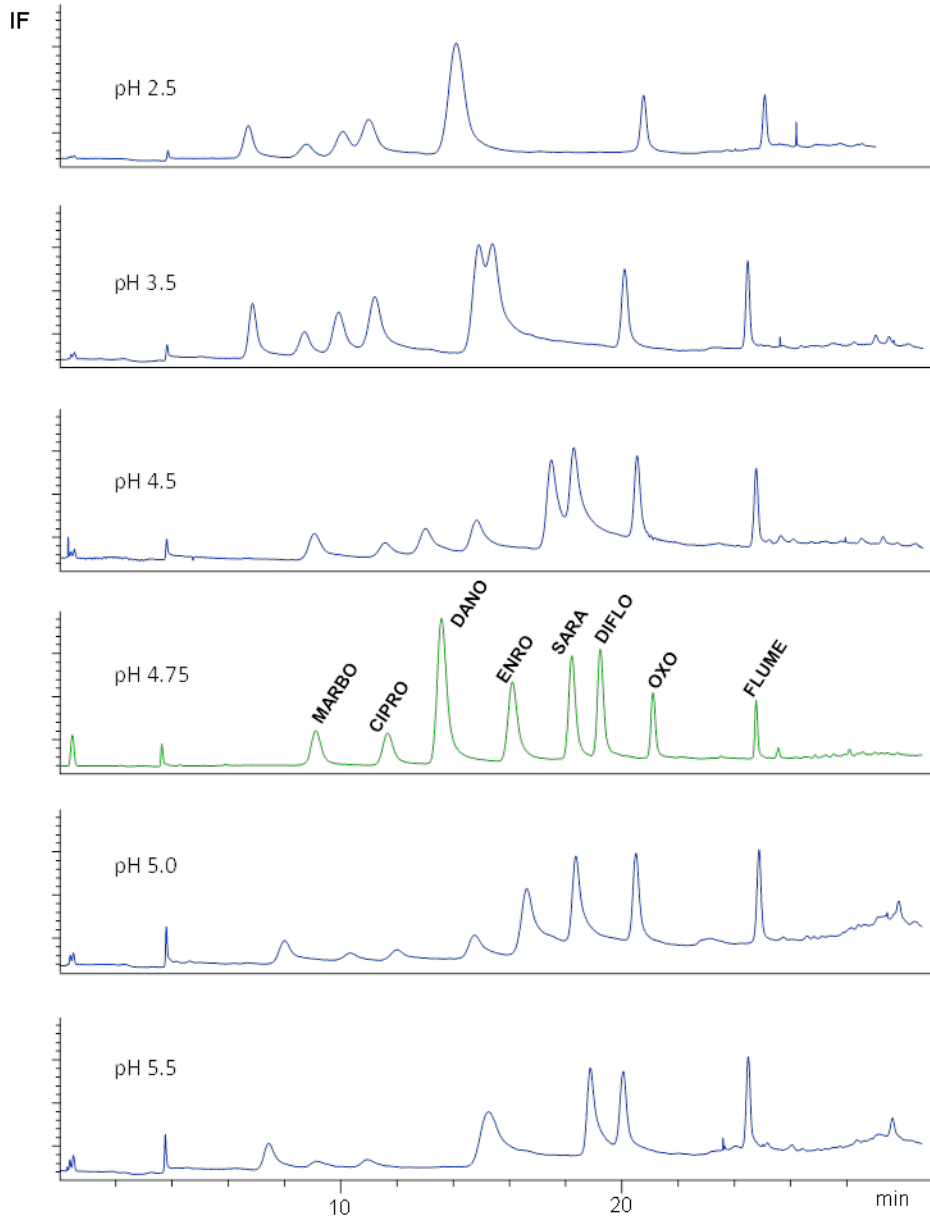

Figure 3.4. Study of the pH of the mobile phase.

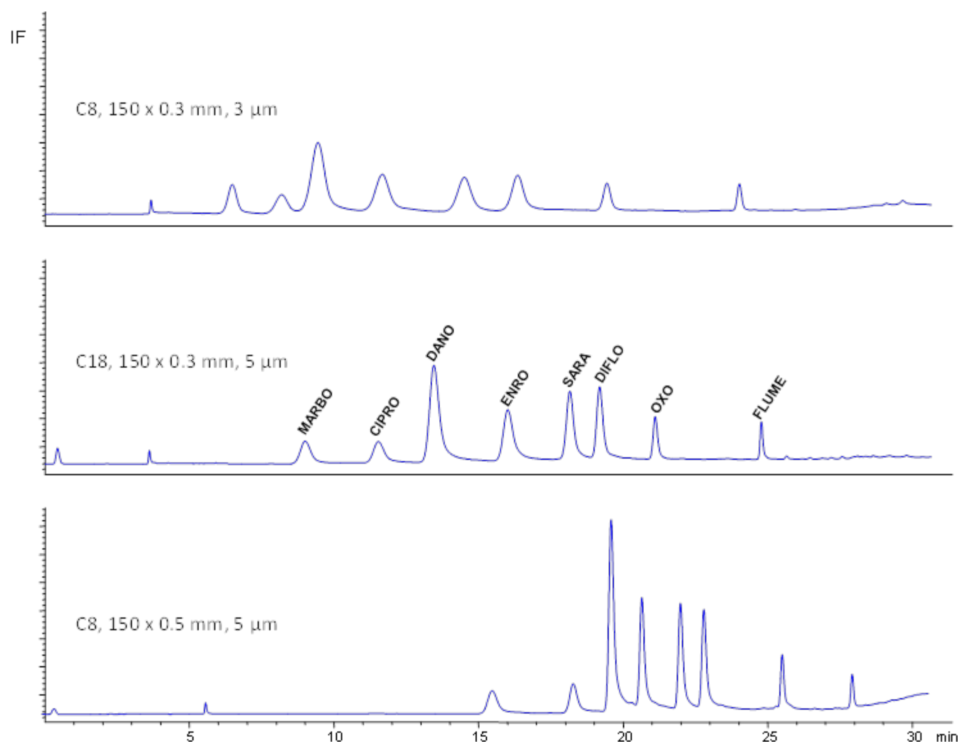

Figure 3.5. Results obtained with different tested column (mobile phase: 10 mM citric acid pH 4.75 aqueous phase and 10 mM citric acid in ACN).

Subsequently, different buffers were tested as aqueous phase (A), namely: citric acid, formic acid and acetic acid (adjusting the pH to 4.75 with ammonium hydroxide solution in all cases) and citric acid/Na_2HPO_4, all of them at the same concentration (10 mM). Using acetic acid, the complete separation between SARA and DIFLO was not achieved, while with the other buffers good and very similar results were obtained. 10 mM citric acid was finally selected for the rest of the work. Then, the gradient was optimized to get a satisfactory separation of the Qns. It was set at 11% B (0 min) followed by a linear gradient to 20% B (6 min) and to 90% B (11 min). The flow rate was increased up to 15 µl/min. This reduced the analysis time and produced narrower peaks which supposed an increase in sensitivity.

The temperature of the column was also studied in the range of 30-45 ºC. In all cases resolution was good enough and no significant differences were observed. Finally 35 ºC was selected. Injection volumes between 1 and 8 µl were tested and 8 µl was selected in order to get the maximum sensitivity. Under these optimal conditions, the separation of the seven compounds was achieved in less than 14 minutes, as can be seen in figure 3.6.

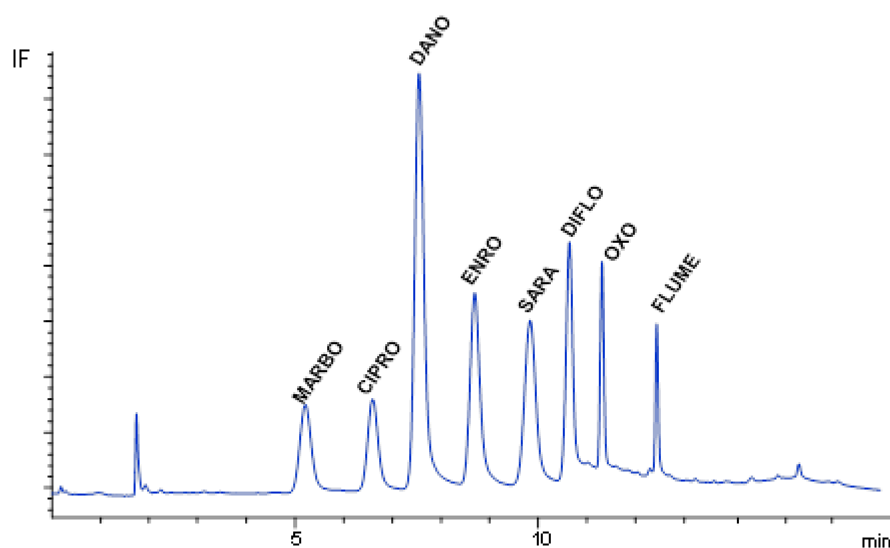

Figure 3.6. Chromatographic separation under optimum conditions.

3.4.2 *Optimization of sample treatments*

The aim of this work was the development of a sensitive method for the simultaneous determination of the eight Qns of veterinary use in whole cow milk combined with an optimum extraction procedure based on more recently used strategies for sample treatment such as, QuEChERS and MISPE methodologies, so an adequate optimization study was carried out with each sample treatment.

3.4.3 *Optimization of the QuEChERS procedure*

The QuEChERS procedure described by Agilent Technologies for the determination of Qns in bovine liver [30] has been adapted in this work for milk samples but in our case the final reconstitution step consisted on 1 ml of 10 mM aqueous citric acid solution (pH 4.75 adjusted with ammonium hydroxide). The procedure is clearly described in section 3.3.1. Figure 3.7 shows both chromatograms of a blank and a spiked milk sample treated following the proposed QuEChERS procedure. As can be seen, only in the case of MARBO an important interference eluting in the same retention time (approx. 5 min) was observed, prevented its detection and determination.

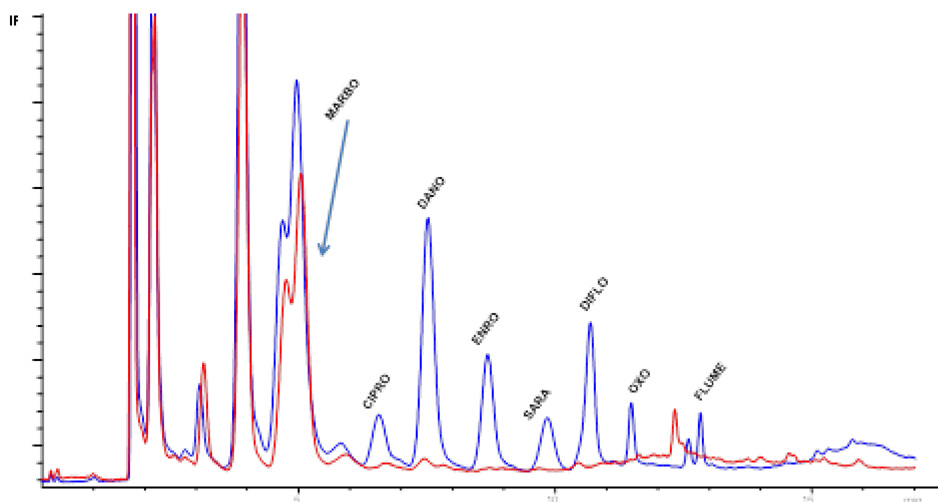

Figure 3.7. Chromatograms of blank (red) and spiked (blue) milk samples (25 µg/Kg for DANO and 50 µg/Kg for the other Qns), extracted with QuEChERS and obtained under optimum conditions.

3.4.4 *Optimization of the MISPE procedure*

A procedure previously developed in our laboratory for the MISPE of CIPRO, DANO, ENRO and SARA (described in Chapter 2, section 2.2.6), was used as starting point for

[30] L. Zhao, J. Stevens, Application Note 5990-5085EN, Agilent Technologies Inc. http://www.chem.agilent.com/Library/applications/5990-5085EN.pdfReferences

MISPE treatment. This procedure involved two washing steps of the cartridge containing the MIP, the first one using 3 ml of water and the second one using 1 ml of AcN. However, when this procedure was tested on spiked milk samples using this proposed capillary HPLC-LIF method for the selected analytes, low recoveries for OXO and FLUME were obtained, as well as some interferents were co-migrating with MARBO, DIFLO and OXO. In order to avoid these drawbacks, alternative washing steps were tested, namely: (a) 3 ml of H_2O; (b) 3 ml of H_2O and 1 ml of AcN; and (c) 3 ml of water, 1 ml of ammonium hydroxide 0.1% in water and 1 ml of AcN. Unfortunately, none of these alternatives was useful to completely avoid these problems; finally 3 ml of water was used to wash the cartridge containing MIP so as to get a compromise between a clean extract with an adequate recovery of compounds. From this fact, we conclude that MISPE procedure could be useful for the determination of CIPRO, DANO, ENRO and SARA, due to the high losses of OXO and FLUME during the treatment and the impossibility of removing interferences that overlaps with MARBO, DIFLO and OXO. Figure 3.8 shows the chromatograms of a blank and a spiked milk sample extracted with MISPE. As can be observed in this figure compared with results showed in figure 3.7, a cleaner chromatogram than that obtained with QuEChERS was achieved, but on the other side co-migrating interference with our analytes were not removed from the extract. The low recoveries of OXO and FLUME could be due to a weaker specific retention of both compounds to the MIP, as their structures differs from the basic structure of the others Qns more effectively retained; this fact could produce a lower degree of interaction with the specific cavities created into the MIP in the synthesis procedure.

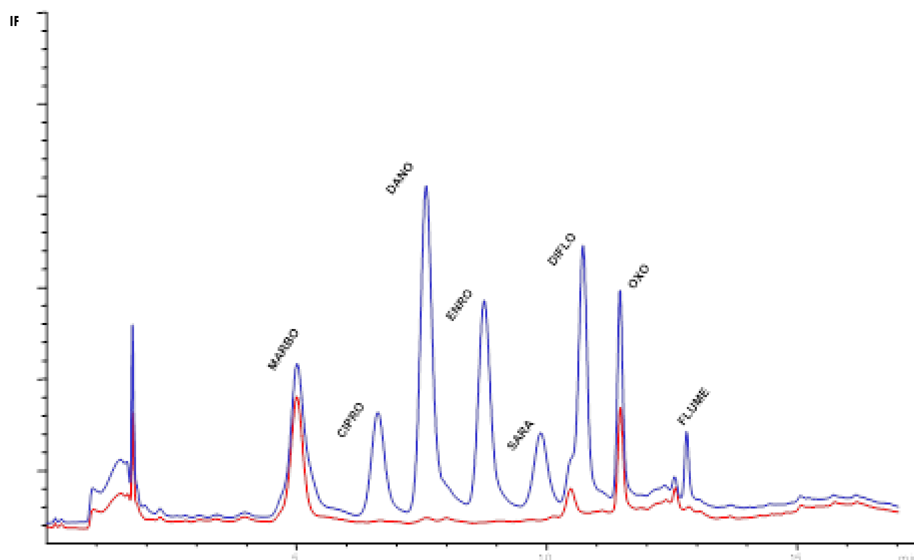

Figure 3.8. Chromatogram of blank (red) and spiked (blue) milk samples (25 μg/Kg for DANO and 50 μg/Kg for the other Qns), extracted with MISPE and obtained under optimum conditions.

3.4.5 *Comparison of the proposed extraction procedures*

The QuEChERS and MISPE procedures were compared in terms of efficiency of the extraction (number of analytes to be analyzed and absence of interferences), throughput, linear dynamic range in matrix-matched calibrations, detection and quantification limits and trueness.

- Extraction efficiency: As can be observed in figures 3.7 and 3.8, an overlapping peak with MARBO was found in both cases, QuEChERS and MISPE; so it was not possible to determine this analyte. Much cleaner extracts were obtained when the MISPE procedure was used, but overlapping peaks with DIFLO and OXO were found, precluding their quantification. Moreover, low recoveries were observed for OXO and FLUME. Thus, it can be concluded that QuEChERS is more efficient for the quantification of seven of the selected Qns in milk samples.

- Throughput: Due to the adjustment of pH, conditioning of the MIP cartridge, elution and evaporation steps, MISPE procedure required at least 3 hours for the same number of samples (about 12) than the QuEChERS procedure, which required less than one hour. Thus, QuEChERS is more than three times faster than MISPE procedure.

- Linear ranges, detection and quantification limits: matrix-matched calibration curves for whole cow milk samples free of analytes and spiked with different concentration levels of Qns (25, 50, 100, 150 and 250 µg/Kg for CIPRO, ENRO, SARA and DIFLO and 10, 25, 50, 150 and 250 µg/Kg for DANO, OXO and FLUME) were established, considering peak areas as analytical signal. Each concentration level was prepared by triplicate, submitted to the QuEChERS or MISPE procedure and analyzed by the proposed capillary HPLC-LIF method. In the case of DIFLO, OXO and FLUME, the calibration curve was not established with the MISPE treatment, due to the existence of overlapping peaks or poor recoveries. LODs and (LOQs) were considered as 3×S/N ratio and 10×S/N ratio, respectively. Lower LOD and LOQ were obtained for CIPRO, ENRO and SARA when MISPE procedure was used and nearly the same results were obtained for DANO with both procedures. In all cases, the LOQs were lower enough for the quantification of the analytes below their MRLs. table 3.1 summarizes the results obtained for the analytes that could be properly quantified with both sample treatments.

- Trueness: Samples were spiked at two concentration levels: 25 µg/Kg for DANO, OXO y FLUME and 50 µg/Kg for the others Qns (Level 1) and 150 µg/Kg of each Qns (Level 2). Three samples were prepared at each concentration level, submitted to the QuEChERS or MISPE procedure and injected by triplicate in the capillary HPLC-LIF system, following the proposed method. As can be observed in table 3.2, higher recoveries were obtained with the QuEChERS extraction procedure, although slightly better relative standard deviations (%RSD) were obtained with the MISPE procedure.

Table 3.1. Matrix-matched calibration curves and performance characteristics using QuEChERS and MISPE treatments in milk samples.

	QuEChERS procedure				**MISPE procedure**				
	Calibration Curve	**R^2**	**LOD (µg/Kg)**	**LOQ (µg/Kg)**	**Calibration Curve**	**R^2**	**LOD (µg/Kg)**	**LOQ (µg/Kg)**	**MRL (µg/Kg)**
CIPRO	y =1026 x + 7526	0.994	5.0	16.8	y= 2160 x + 1926	0.994	2.6	8.7	100
DANO	y = 6880 x + 25681	0.992	0.4	1.4	y = 9074 x + 7567	0.996	1.5	1.9	30
ENRO	y = 2372 x - 27100	0.991	3.0	10.0	y = 4521 x + 20912	0.992	1.4	4.6	100
SARA	y = 961 x - 2190	0.992	6.0	20.0	y = 1721 x + 4304	0.990	3.4	11.7	(b)
DIFLO	y = 1953 x + 532	0.991	1.9	6.2	(a)	(a)	(a)	(a)	(c)
OXO	y = 1090 x + 831	0.990	2.7	9.0	(a)	(a)	(a)	(a)	(c)
FLUME	y = 554 x + 4022	0.992	1.9	6.5	(a)	(a)	(a)	(a)	50

a) Not calculated due to existence of overlapping peaks or poor recoveries.
b) MRL not established for milk samples.
c) Not for use in animals from which milk is produced for human consumption.

Table 3.2. Recovery obtained with QuEChERS and MISPE procedures in milk samples (n=9).

	QuEChERS % Recovery (RSD)		**MIPs % Recovery (RSD)**	
	Level 1 (a)	**Level 2 (b)**	**Level 1 (a)**	**Level 2 (b)**
CIPRO	83.4 (5.4)	85.5 (1.4)	74.8 (0.8)	77.4 (2.2)
DANO	92.1 (2.7)	98.3 (1.9)	66.5 (0.8)	74.5 (1.0)
ENRO	97.6 (2.2)	99.5 (3.1)	70.4 (2.7)	76.0 (1.4)
SARA	92.6 (3.1)	103.0 (3.2)	72.0 (5.0)	77.3 (2.4)
DIFLO	103.9 (1.5)	99.9 (2.6)	(c)	(c)
OXO	99.8 (8.9)	97.3 (2.7)	(c)	(c)
FLUMI	92.5 (6.4)	98.2 (2.3)	< 37.0 (5.0)	< 34.0 (8.3)

a) Level 1: 25 µg/Kg for DANO, OXO and FLUME and 50 µg/Kg for the other Qns.
b) Level 2: 150 µg/Kg for each Qn.
c) Not calculated due to existence of overlapping peak.

3.4.6 *Precision study of the QuEChERS extraction procedure*

From the previous study, we deduced that the QuEChERS procedure can be considered as the most suitable approach for the simultaneous extraction of the selected compounds. For such reason, in order to complete the validation of the proposed method QuEChERS-capillary HPLC-LIF for the Qns analysis, the precision of the method has only been estimated using the QuEChERS extraction procedure. The precision of the method has been evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by means of repetitive application of the whole procedure to five whole milk samples (experimental replicates) spiked at two concentration levels: 25 µg/Kg for DANO, OXO y FLUME and 50 µg/Kg for the oyhers Qns (Level 1) and 150 µg/Kg of each Qn (Level 2). Each sample was injected by triplicate (instrumental replicates) on the same day. Intermediate precision was assessed with a similar procedure, but the samples were

analyzed in five different days. The results, expressed as RSD of peak areas, are given in table 3.3. As can be observed, very good results were obtained in all cases.

Table 3.3. Precision of the method using the QuEChERS treatment in milk samples (n=15).

	Intraday precision RSD (%)		Interday precision RSD (%)	
	Level 1[a]	**Level 2[b]**	**Level 1[a]**	**Level 2[b]**
CIPRO	5.6	8.3	7.5	9.0
DANO	5.8	6.3	11.2	9.7
ENRO	6.4	7.6	10.2	7.5
SARA	10.5	8.3	11.2	11.7
DIFLO	5.4	6.3	7.0	6.2
OXO	8.3	9.1	7.4	7.9
FLUME	5.8	10.6	10.4	5.9

a) Level 1: 25 µg/Kg for DANO and 50 µg/Kg for the other Qns.
b) Level 2: 150 µg/Kg for each Qn.

3.5 Conclusions

LIF has been proposed as a very attractive detection technique in capillary HPLC for the analysis of Qns, showing high sensitivity and selectivity. However, the simultaneous extraction of different Qns from milk presents several difficulties due to the complexity of the matrix. Relatively recent strategies, such as QuEChERS and MISPE methodologies have been compared. Although MISPE provides very clean extracts, QuEChERS has shown to be a better alternative for the simultaneous extraction of a higher number of Qns from milk (thus, MISPE is adequate for the analysis of only four compounds while use of QuEChERS make possible the analysis of seven compounds), reducing extraction time, increasing sample throughput and providing good recoveries and precisions, showing the suitability of this procedure for the monitoring of Qn residues in foodstuff and being environmentally friendly. The obtained LOQs for the

whole proposed method were in the very low µg/kg level and could be easily improved to ng/kg range by just decreasing the solvent volume in the reconstitution step used in the QuEChERS procedure. This method is one of the most sensitive proposed for the analysis of these compounds in comparison to conventional fluorescence, being able to determine seven analytes simultaneously. In addition, to our knowledge, this is the first time that capillary HPLC is coupled to LIF for the determination of Qns. This study shows interesting perspectives of the application of QuEChERS for the monitoring of antibiotics in other food samples and with different analytical methodologies.

This work was published as:

"Comparison of different sample treatments for the analysis of quinolones in milk by capillary-liquid chromatography with laser induced fluorescence detection". M. Lombardo-Agüí, L. Gámiz Gracia, C. Cruces Blanco, A.M. García Campaña. Journal of Chromatography A 1218 (2011) 4966.

Capítulo 4

Determinación de quinolonas de uso veterinario en productos apícolas mediante UHPLC-MS/MS con extracción con QuEChERS.

Chapter 4

Determination of quinolones of veterinary use in bee products by UHPLC-MS/MS using a QuEChERS extraction procedure.

Resumen

En este capítulo se ha desarrollado un método eficaz y rápido para la determinación de las ocho Qns de uso veterinario reguladas por la Unión Europea (marbofloxacino, ciprofloxacino, danofloxacino, enrofloxacino, sarafloxacino, difloxacino, flumequina y ácido oxolínico) mediante cromatografía de ultra resolución acoplada a la espectrometría de masas en tándem como sistema de detección. Las condiciones cromatográficas se han optimizado con el fin de mejorar la sensibilidad y disminuir el tiempo de análisis. La separación se completó en 3 minutos usando una columna Zorbax Eclipse Plus C18 (50×2.1 mm, 1.8 µm) y una fase móvil compuesta por agua con un 0.02% de ácido fórmico y AcN. La detección de los compuestos se llevó a cabo mediante ionización positiva, utilizando la técnica de electroespray y empleando el modo de monitorización de respuesta múltiple (multiple reaction monitoring, MRM). Se seleccionaron los fragmentos adecuados para la cuantificación y confirmación, y a continuación se optimizaron las condiciones de detección MS/MS para conseguir la máxima sensibilidad. Para la extracción de los compuestos de las diferentes muestras de miel, jalea real y propoleo se ha estudiado la metodología QuEChERS. El método se ha validado para cada matriz, evaluándose la linealidad, veracidad, precisión y limites de detección (LOD) y cuantificación (LOQ). Los LODs obtenidos se encuentran entre 0.2 y 4.1 µg/kg y la precisión se mantuvo por debajo del 12% con buenas recuperaciones en la mayoría de los casos. El método ha sido aplicado a diferentes marcas comerciales de los tres tipos de muestras estudiadas.

Abstract

A reliable and rapid ultra-high performance liquid chromatography-tandem mass spectrometry method has been developed for the determination of the eight quinolones of veterinary use regulated by European Union (marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, flumequine y oxolinic acid). Chromatographic conditions were optimized in order to increase sample throughput and sensitivity. The separation was achieved in 3 min, using a Zorbax Eclipse Plus C18 column (50×2.1 mm, 1.8 μm), with a mobile phase of 0.02% aqueous formic acid solution and AcN. The antibiotics were detected by electrospray ionization in positive ion mode with multiple reaction monitoring (MRM) and MS/MS conditions were optimized in order to increase selectivity, selecting the corresponding product ions for quantification and confirmation. A QuEChERS methodology was evaluated for extraction of the quinolones from bee products such as honey, royal jelly and propolis. The method was validated for each matrix in terms of linearity, trueness, precision, limits of detection (LODs) and quantification (LOQs). LODs ranged between 0.2 and 4.1 μg/kg, with precisions lower than 12 % and satisfactory recoveries in most cases. The method was also applied for studying the occurrence of these antibiotics in several market samples.

4.1 Introduction

The Codex Alimentarius Commission [1] defines honey as “the natural sweet substance produced by honeybees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature”. Although, antimicrobial drugs are authorized for the treatment of honey bees in many third countries, it is not the case in the European Union (EU) [2]. In several countries, the beekeeping industry uses antibiotics to control outbreaks of diseases in honeybees, and as growth promoters to increase production. This becomes honey in not so natural product as we on principle though, as these antibiotics can reach the house-holder’s table. Although EU has regulated the use of antibiotics in animal which products are destined to food consumption [3], bee products are not included among the matrices having an established MRL for antibiotic residue. That means that EU does not allow the use of antibiotics for treatment of honeybees, however EU imports honey from third countries where the use of antibiotics for beekeeping is allowed. So, EU has established Reference Points for Action (RPAs, residue concentrations which are technically feasible to detect by food control laboratories). Thus, if antibiotics are detected by a laboratory, the consignments must be rejected. Until date, RPAs have been established in honey for substances such as chloramphenicol and nitrofuran and has also set a provisional MRL of 25 ppb for oxytetracycline.

The incurrence of Qns in bee products could be produced also by a wrong or illegal use of these antibiotics to treat bees. Despite the fact that their use are not allowed by EU,

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[1] Codex Standard for honey, Codex Stan 12-1981, Rev 1(1987), Agriculture and Consumer Protection. FAO.

[2] European commission, Health and Consumers, Manual on residue requirements for non-EU countries exporting to the EU. Accessed on July 2012. Available at: http://ec.europa.eu/food/food/chemicalsafety/residues/docs/requirements_non_eu.pdf.

[3] European Commision Regulation No. 37/2010 Off. J. Eur. Commun. L15 (2010) 1.

ENRO and CIPRO have been found in honey from third countries [4,5]. Therefore, sensitive methods for their determination in bee products are necessary to prevent contaminated product reaching the consumers.

Different methods have been published for the determination of several families of antibiotics in honey and royal jelly, e.g. sulfonamides [6,7], tetracyclines [8,9,10], nitrofurans [11] or macrolides [12,13,14], mainly using HPLC-MS/MS. In the case of Qns, some methods based on HPLC-MS have been developed for the analysis of 16 Qns in honey by using turbulent flow chromatography automated on-line extraction [15], 4 Qns by combining HPLC-MS with a stir rod sorptive extraction with a monolithic polymer as coating [16] and 19 Qns SPE [17] or 7 Qns in royal jelly by ultrasonic assisted extraction and HPLC with fluorescence detection [18]. However, as far as we know, no applications for the analysis of Qns in propolis have been reported. Also, multiclass/multiresidue LC-MS/MS methods have been proposed for the analysis of

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different veterinary drug residues in honey, such as 42 veterinary drugs, including tetracyclines, macrolides, aminoglycosides, β-lactams, amphenicols and sulfonamides [19]. Other multiclass methods included also some veterinary Qns, such as CIPRO, DANO, DIFLO, ENRO and SARA [20]. Recently, UHPLC-MS/MS methods for the determination of antibiotics in honey have been reported, including six macrolides [13] and a mixture of macrolides, tetracyclines, sulfonamides and Qns (MARBO, ENRO, DANO, DIFLO and SARA) [21]. However, as far as we know, nor UHPLC-MS/MS methods specifically for the determination of the eight regulated Qns for veterinary use, neither the analysis of these compounds in royal jelly and propolis have been proposed.

UHPLC shows several advantages compared to conventional HPLC, associated with the use of columns of less than 2.0 μm porous stationary phase able to withstand very high pressures, which allows an increased efficiency with a shortened analysis. UHPLC provides higher peak capacity, greater resolution, increased sensitivity and a higher speed of analysis and it is recommended especially to reduce analysis time and sample preparation [22,23], mainly in combination with MS/MS.

Concerning sample treatment, several methods have been proposed for the determination of Qns in different sample matrices, being SPE the most common methodology also when using UHPLC [24,25] and reported for the analysis of Qns in honey [17,21]. More recently, new methodologies showing higher selectivity and efficiency, being less time-consuming or environmentally friendly have been proposed for the determination of Qns in different matrixes, such as Such as MIPs or

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microextraction techniques, as indicated in previous chapters. In the case of bee products turbulent flow chromatography automated on-line extraction in honey [15] or ultrasonic-assisted extraction combined with SPE for clean-up in royal jelly [18] has been published. As it was shown in Chapter 3, in the last few years, dispersive SPE based on the use of QuEChERS has shown its usefulness in the analysis of residues in foods, presenting some advantages, such as its simplicity, minimum steps, and effectiveness for extraction from complex samples. QuEChERS have been used for the determination of veterinary residues (including Qns) in animal tissues [26,27], milk [28,29,30] and eggs [31], as indicated in chapter 3. However, as far as we know, it had not been used for the analysis of bee products.

The purpose of this work was the development of a simple, sensitive, selective and efficient UHPLC-MS/MS method for the simultaneous determination of the eight Qns of veterinary use regulated by EU (MARBO, CIPRO, DANO, ENRO, SARA, DIFLO, FLUME and OXO) in bee products using a simple and fast extraction procedure (QuEChERS methodology) that reduces sample handling and increase sample throughput.

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4.2 Experimental

4.2.1 *Chemicals*

Solvents were LC-MS grade and Qns were analytical standard grade. Ultrapurewater (Milli-Q Plus system, Millipore Bedford, MA, USA) was used to prepare buffer and standard solutions. Sodium hydroxide and sodium dihydrogenphosphate monohydrate were obtained from Panreac-Química (Madrid, Spain). Formic acid eluent additive for LC-MS, acetonitrile (AcN) and water were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany). DANO, SARA and DIFLO were supplied by Riedel-de Haën (Seelze, Germany), FLUME by Sigma Aldrich and MARBO, CIPRO, ENRO and OXO by Fluka (Steinheim, Germany).

SampliQ QuEChERS kits (Agilent Technologies Inc., Wilmington, DE, USA) consisted on 50 ml tube and buffered QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive tubes (15 ml, 150 mg C18 and 900 mg $MgSO_4$).

Filters of 13 mm with 0.2 µm nylon membrane (Bulk Acrodisc®, Pall corp., MI, USA), were used for filtration of the final extracts before analysis.

4.2.2 *Preparation of solutions*

Individual stock standard solutions (100 mg/l) of each Qn were prepared by dissolving the appropriate amount of each analyte in AcN/0.02% formic acid aqueous solution (50/50, v/v) and were stored in the dark at 4 ºC. Formic acid (analysis grade) was added to each standard to increase solubility of analytes in this solvent mixture. Under such conditions, they were stable for at least 1 month. Working solutions (containing all Qns) were prepared daily from dilution of the individual stock solutions with Milli-Q water.

A 30 mM phosphate buffer solution pH 7.1 was prepared by dissolving 2.07 g of dihydrogenphosphate monohydrate in 500 ml of water and the pH was adjusted with 4M NaOH solution. A 0.02% formic acid aqueous solution was prepared by adding 20 µl of formic acid (eluent additive for LC-MS) to 100 ml of water (LC-MS grade). The 5% formic acid solution in AcN was obtaining by adding 25 ml of formic acid (analysis grade) to 500 ml of AcN.

4.2.3 *Instrumentation*

Separation was performed on an Extreme Pressure Liquid Chromatography (XLC) system (two pumps, oven, auto sampler, mixer and degasser units) from Jasco (Easton, MD, USA). The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB SCIEX, Darmstadt, Germany) with electrospray ionization (ESI) (figure 4.1). The instrument data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (AB SCIEX). Different chromatographic columns were tested to achieve the separation all of them of C18 nature: Zorbax Eclipse Plus HHRD (50×2.1 mm, 1.8 µm), Kinetex (50×2.1 mm, 1.7 µm) and Varian Pursuit UPS (50×2.1 mm, 1.9 µm).

A pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain), a Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a Visiprep TM DL vacuum manifold for SPE (Supelco), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and a evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used.

Figure 4.1. UHPLC and MS/MS instrumentation.

4.2.4 *UHPLC-MS/MS analysis*

UHPLC separations were performed in a C18 column (Zorbax Eclipse Plus HHRD 50×2.1 mm, 1.8 µm) using a mobile phase consisting of 0.02% aqueous formic acid solution (solvent A), and AcN (solvent B). The gradient profile started at 15% of B until 1.5 min; then, to 55% B in 0.1 min and kept until 3 min; then it went to 90% B in 0.1 min and kept until 4 min. Finally it was back to 15% B in 0.1 min for equilibration. The temperature of the column was 35 °C and the injection volume was 5 µl (full loop). Under optimum conditions, all analytes were eluted in 3 min. The mass-spectrometer was working with an electro spray ion source (ESI) in positive mode under the multiple reaction monitoring (MRM) conditions shown in table 4.1. The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 35 psi, ion spray voltage 5500 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 60 psi.

4.2.5 *Extraction Procedure*

Honey, royal jelly and propolis samples were purchased in local markets from Granada (Spain) and stored at room temperature. The QuEChERS procedure was adapted from that described by Agilent Technologies for the determination of Qns in bovine liver [32], previously reported for the analysis of milk [28] and explained in depth in Chapter 3. In all cases, preliminary analyses were performed to the selected matrixes in order to check that they were free from analytes. These samples were used as blank samples in the preparation of calibration standards and during the validation study.

Samples (1 g of honey, a 10 ml vial of royal jelly with an equivalent quantity of 1 g of fresh royal jelly, or 1.5 ml of commercial propolis extract) were placed into 50 ml centrifuge tubes and spiked by adding the proper volume of a solution containing each Qns at a concentration of 1 mg/l (FLUME and OXO) or 10 mg/l (for the other Qns). In order to achieve a proper homogenization of samples, honey was warmed before spiking and vortexed, while royal jelly and propolis extract samples were directly spiked and vortexed without warming, since their density is much lower than that of honey. Then, 8 ml of 30 mM NaH_2PO_4 buffer pH 7.0 were added and the sample was dissolved in this medium. Subsequently, 10 ml of 5% formic acid in AcN were added and the mixture was homogenized in vortex. Agilent SampliQ EN QuEChERS extraction kit was added and the tube was shaken vigorously for 1 min. The sample was centrifuged at 9000 rpm for 5 min and 4 ml of the upper AcN layer was transferred into the SampliQ QuEChERS dispersive tube, stirred in vortex for 1 min and centrifuged (9000 rpm for 2 min). An aliquot of 1 ml of supernatant was transferred to a vial, dried under a stream of nitrogen and the residue was redissolved with 1 ml of H_2O/AcN/formic acid (88/10/2), filtered and analysed by UHPLC-MS/MS (see figure 4.2).

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[32] L. Zhao, J. Stevens, Application Note 5990-5085EN, Agilent Technologies Inc. http://www.chem.agilent.com/Library/applications/5990-5085EN.pdf

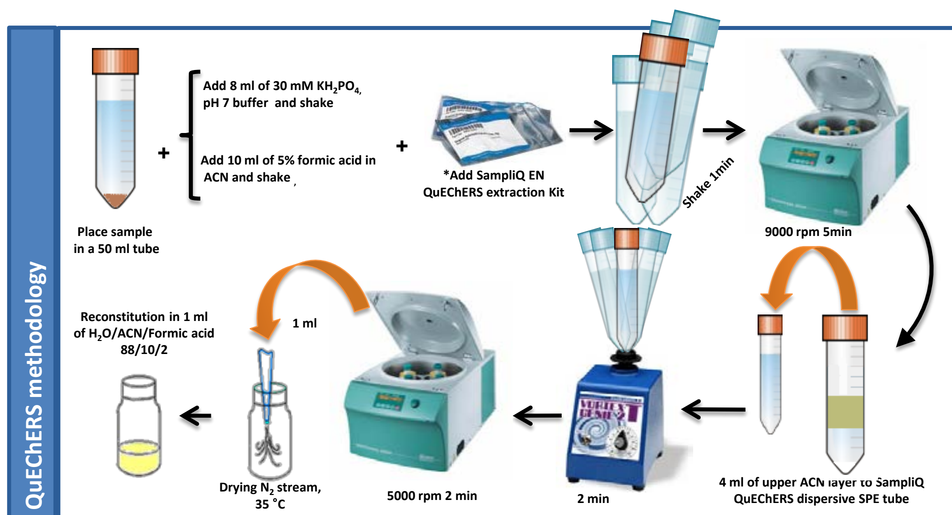

Figure 4.2. QuEChERS methodology for bee products.

4.3 Results and discussion

4.3.1 *Optimization of MS/MS detection and chromatographic separation*

In order to get high sensitivity, MS/MS detection was optimized for every analyte. For that purpose, each analyte was individually infused as a standard solution of 1 mg/l in 0.1% aqueous formic acid solution/AcN (50/50; v/v), directly into the mass spectrometer. All compounds were tested using ESI positive/negative mode. As it was expected from previous data [25, 29,31,33] ESI operating in positive mode showed the best results in term of sensitivity. During the infusion, the parameters Declusterin potential (DP), Entrance potential (EP), Collision Cell Entrance Potential (CEP), Collision Cell Exit Potential (CXP) and Collision Energy (CEN) were optimised for each compound in order to obtain the maximum sensitivity (see table 4.1).

[33] M.P. Hermo, D. Barrón, J. Barbosa, J. Chromatogr. A 1104(2006) 132-139.

Table 4.1. MS optimized parameters.

	Precursor ion (m/z)	DP	EP	CEP	Product ions	CEN	CXP
SARA	386.0	45	5	19.5	299.1 (Q)	35	2.2
					368.1 (I)	29	6.0
DANO	358.0	48	5	18.8	340.0 (Q)	30	5.5
					255.2 (I)	31	4.2
CIPRO	332.0	43	5	18.2	231.2 (Q)	29	6.0
					245.0 (I)	32	2.0
ENRO	360.0	45	5	18.9	316.0 (Q)	26	5.5
					245.0 (I)	36	2.0
DIFLO	400.0	50	5	19.9	356.3 (Q)	24	6.0
					299.3 (I)	43	4.6
FLUME	262.0	37	5.5	16.3	244.2 (Q)	23	3.0
					202.3 (I)	43	3.0
MARBO	363.0	38	4.8	18.9	72.0 (Q)	42	2.5
					320.2 (I)	19	6.0
OXO	262.0	36	4.5	18.0	244.2 (Q)	19	6.0
					216.0 (I)	43	4.0

(Q) transition used for quantification, (I) Transition employed to complete the identification

Each compound was characterized by its retention time and by two precursor-product ion transitions. The most intense product ion was used for quantification (see figure 4.3 and table 4.1), whereas the second one was used to complete the identification. The dwell time established for each transition was 0.1 s. Under the experimental conditions, protonated molecules $[M+H]^+$, were observed for all the compounds.

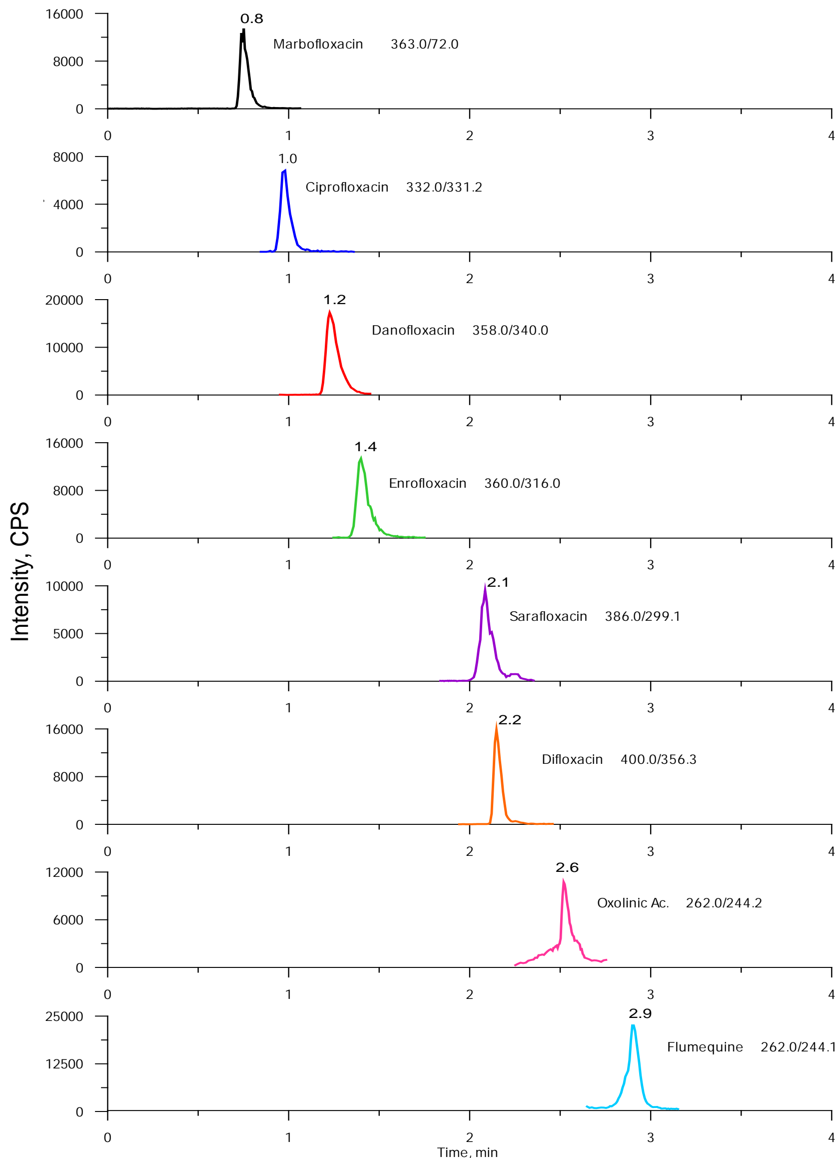

Figure 4.3. UHPLC-MS/MS extracted ion chromatograms of a spiked honey sample at 20 µg/kg for FLUME and OXO and 200 µg/kg for the other quinolones.

In relation to the chromatographic conditions, aqueous standard solutions of Qns were used during the optimization of chromatographic separation. The mobile phase consisted of 0.02% aqueous formic acid solution (solvent A) and AcN (solvent B). The gradient was studied in order to get the best separation, peak shape and sensitivity.

According to previous papers [28,33] aqueous and AcN phases were selected as solvents for the separation of Qns by HPLC. The gradient was studied to get the best separation in the shortest time and finally it was found that a rising gradient until 55% of AcN was necessary to get a good separation and elute the most retained analyte (FLUME). The AcN percentage was increase until 95% after the elution of FLUME to elute others possible components included in the final sample extract. The use of acid in the mobile phase is required to improve the ionisation step in ESI (+). Therefore, different acids (formic and acetic acid) in solvent A were tested. Formic acid provided better signal than acetic acid for some of the compounds so it was selected for the rest of the experimental work, evaluating different percentages (0% to 0.1%). Aqueous mobile phase with 0.02% formic acid gave the higher signals and the better peak shape. The addition of acid in both phases (solvent A and B) was also studied, but worse signals were observed, so it was added only in solvent A.

Once selected the mobile phase and gradient, three different reversed-phase (C18) chromatographic columns were studied: Zorbax Eclipse Plus HHRD (50×2.1 mm, 1.8 μm), Kinetex (50×2.1 mm, 1.7 μm) and Varian (50×2.1 mm, 1.9 μm). The former gave slightly better separation of SARA and DIFLO, and much better peak shapes (see figure 4.4), so it was selected for the rest of the experimental work.

The flow rate was studied from 0.3 ml/min to 0.5 ml/min and finally 0.4 ml/min was selected as a compromise between signal, peak shape and run time. The temperature of the column was studied between 25 °C and 45 °C and 35 °C was selected, as it provided the higher peak height and area with the best resolution and good analysis time. The injection volume was 5 μl (full loop) for all the experiments. Using these conditions, only two peaks (SARA and DIFLO), were not completely resolved but the use of MS/MS enabled their accurate determination.

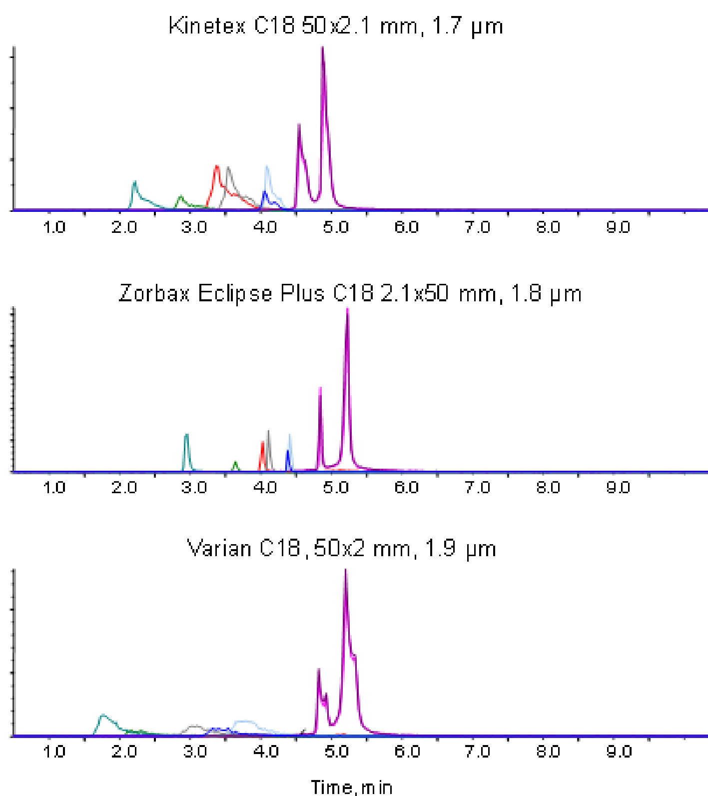

Figure 4.4. Chromatograms obtained using different columns in UHPLC.

After chromatographic optimization the study of ionization source parameters was carried out, considering: Dry Gas Temperature, Curtain Gas™, Ion Spray Voltage and GS1 (Gas 1) y GS2 (Gas 2).

- Dry Gas Temperature (TEM).

The TEM parameter controls the temperature of the turbo gas in the TurboIonSpray™ source or the temperature of the probe in the heated nebulizer (or APCI) source. It helps to evaporate the solvent to produce gas phase sample ions.

Temperatures from 300 to 600 °C were studied in steps of 100 °C. Temperatures from 300 to 400 °C are recommended by AB SCIEX when a 100 to 300 µl/min flow is employed and from 400 °C with a flow between 300 and 1000 µl/min. The result of

study TEM did not show to have a very significant influence on the signal obtained for Qns. Only for FLUME a higher signal was observed when temperature increased, whereas no change or a slightly decrease was observed for the other compounds. Finally, 500 °C was selected for the rest of the work.

- Curtain Gas™ (CUR).

The CUR parameter controls the gas which flows between the curtain plate and the orifice. Curtain Gas™ prevents solvent droplets from entering and contaminating the ion optics. The Curtain Gas™ should be maintained as high as possible without losing sensitivity and compound stability. In this way the entrance of small droplets of mobile phase is avoided so far as possible.

CUR was optimized between 25 and 45 psi in steps of 5. Attending the results, 35 psi was selected, as higher values produced a slightly decrease in the signal.

- Ion spray voltage (IS).

The IS parameter controls the voltage applied to the needle that ionizes the sample in the ion source. It depends on the polarity, and affects the stability of the spray and the sensitivity. This parameter was studied between 5000 and 5500 V, as recommended by AB SCIEX, in step of 250 V. The best results, in terms of signal, were obtained when 5500 V was used.

- Gas 1 (GS1) and Gas 2 (GS2).

The GS1 parameter controls the nebulizer gas, which helps to generate small droplets of sample flow and affects spray stability and sensitivity. The GS2 parameter controls the auxiliary, or turbo, gas. It is used to help evaporate the spray droplets and prevent solvent from entering the instrument. This parameter was not optimized and, regarding

the recommendations from the user manual, a value of 60 psi for GS1 and GS2 was set.

4.3.2 *Optimization of sample preparation*

For the extraction procedure, QuEChERS methodology was selected in order to achieve a quick and effective extraction method. This methodology has been previously used for the extraction of Qns of veterinary use from liver [32] and milk [28] and some of these Qns with others antibiotics in eggs [25], but, to the best of our knowledge, it has not been tested for bee products. Thus, our purpose was to investigate if this methodology is suitable for extracting Qns from honey, royal jelly and propolis.

Honey was selected as sample for the optimization of the QuEChERS procedure. Initially, 2 g of honey were treated using the procedure previously described [28], but the final extract was too dirty and almost all the peaks were split in two, so we try out with only 1 g of honey. In this case the extraction was achieved satisfactorily, as a cleaner extract was obtained and splitting peaks disappeared. As a summary, the first part of the QuEChERS procedure (extraction) consisted of the next steps:

- 1 g of honey was placed into 50 ml centrifuge tubes.
- Then 8 ml of 30 mM NaH_2PO_4 buffer pH 7.0 was added, shaking by hand.
- Subsequently, 10 ml of 5% formic acid in AcN was added to the tube, shaking by hand for 30 s.
- Agilent SampliQ EN QuEChERS extraction kit was added and the tube was shaken vigorously for 1 min and centrifuged.
- Then 4 ml of the upper AcN layer was transferred to another tube containing the dispersive SPE and stirred in vortex for 1 min.

Subsequently, in order to get the cleanest extract, different possibilities in the clean-up step procedure (dispersive tube composition) were tested, all of them supplied by Agilent Technologies:

- C18 (150 mg)+ $MgSO_4$ (900 mg);
- C18 (150 mg) + PSA (150 mg) + $MgSO_4$ (900 mg);
- C18 (400 mg) + PSA (400 mg) + $MgSO_4$ (1200 mg);
- C18 (400 mg) + PSA (400 mg) + GPC (400 mg) + $MgSO_4$ (1200 mg)

(PSA = primary-secondary amine, GPC = graphitized carbon).

When GPC was included in the dispersive tube, no signals for the analytes were obtained; thus, this dispersive phase composition was discarded. When PSA was included the recoveries percentages vary between 41.9 to 88.5%. Dispersive tube with only C18 as sorbent gave the highest recoveries for all the compounds, comprised between 70.1 to 93.7%, so it was selected.

After clean-up, 1 ml aliquot of the obtained extract from the dispersive tube was dried under a N_2 stream and redissolved in 1 ml of solvent. The recomposition medium was studied and different possibilities were tested: H_2O; 1% formic acid aqueous solution; 2% formic acid aqueous solution; H_2O/AcN (90/10; v/v); H_2O/AcN/HCOOH (89/10/1); H_2O/AcN/HCOOH (88/10/2); and H_2O/AcN/$HClO_4$ (88/10/2). The highest recoveries were obtained when 1% and 2% formic acid aqueous solution were used, but slightly higher signal were obtained with the second one, so this was selected for the rest of the work. Finally, the extracts were filtered with a 0.2 µm filter before injection.

4.3.3 *Analytical and performance characteristics*

4.3.3.1 Calibration curves, detection and quantification limits

Each compound was analysed in MRM mode, selecting the two highest precursor ion/product ion transitions, which, with retention times, were used to ensure adequate

analyte identification, fulfilling the requirements of current EU legislation [34]. Performance characteristics of the method (linearity, limits of detection (LOD) and quantification (LOQ), intra and interday precision, and trueness) were established with fortified samples, previously analysed to ensure the absence of Qns. Matrix-matched calibration curves were established for the three selected apiculture products, fortifying samples at five concentration levels (from 5 to 30 μg/kg for OXO and FLUME and from 50 to 300 μg/kg for the others Qns). Thus, 1 g of multifloral honey, 10 ml of royal jelly (containing 1000 mg of royal jelly, water, fructose, and preservatives) and 1.5 ml of commercial propolis extract (containing 68.5% of propolis, water and alcohol) were used as matrices to establish the calibration curves. LODs and LOQs were provided by the software Analyst, as 3×S/N and 10×S/N, respectively. Determination coefficients, LODs and LOQs are shown in table 4.2, showing that the method is sensitive enough for the determination of very low levels of these compounds in the selected matrices.

4.3.3.2 Precision and trueness studies

Intraday precision was performed fortifying honey, royal jelly and propolis samples at three concentration levels (10, 15 and 20 μg/kg for OXO and FLUME and 100, 150, and 200 μg/kg for the other Qns), using three replicates for each concentration level, analysed by triplicate the same day. Interday precision was evaluated in a similar way, but the samples were analysed by triplicate during five consecutive days. The results are summarised in table 4.3.

Trueness was evaluated by analyzing fortified blank samples by triplicate with concentrations similar to those used in the precision study. As can be observed in table 4.4, the results obtained for propolis are worse (in terms of recoveries and sensitivity) than those obtained for honey and royal jelly. This could be due to the high complexity

[34] Commission Decision (2002/657/EC) of 12 August 2002. Implementing Council Directive (96/23/EC) concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Communities 2002, L221: 8.

of propolis samples. Thus, the extracts were dirtier than those from the other samples, because most of the components of propolis showed a high solubility in the organic solvent (AcN) used for extraction. Moreover, the clean-up step was not as effective as for the other samples. This could mean a higher number of interfering substances make more difficult the extraction and detection of Qns in propolis. In the case of honey and royal jelly, the results demonstrated that the method can be applied to these samples.

Table 4.2. Linearity, detection and quantification limits of the QuEChERS-UHPLC-MS/MS proposed method for the different samples

	Honey			**Royal jelly**			**Propolis**		
	R^2	**LOD (µg/kg)**	**LOQ (µg/kg)**	**R^2**	**LOD (µg/kg)**	**LOQ (µg/kg)**	**R^2**	**LOD (µg/kg)**	**LOQ (µg/kg)**
SARA	0.991	1.6	5.5	0.992	0.9	2.9	0.991	2.1	7
DANO	0.994	1.4	4.6	0.995	0.9	2.9	0.992	4.1	13
CIPRO	0.990	1.7	5.5	0.994	2.5	8.3	0.990	4	13.4
ENRO	0.995	1.1	3.7	0.995	1.6	5.5	0.992	3.5	11.6
DIFLO	0.994	1.2	3.9	0.993	0.8	2.7	0.991	1.6	5.4
FLUME	0.984	0.2	0.8	0.990	0.2	0.6	0.993	0.7	2.4
MARBO	0.994	0.7	2.2	0.996	0.5	1.5	0.990	1	3.4
OXO	0.990	0.5	1.6	0.993	0.3	0.8	0.992	1.2	3.9

Table 4.3. Intraday (n=9) and interday precision (n=15) expressed as RSD (%)

	Intraday/Interday (low spiking level)[a]			Intraday/Interday (medium spiking level)[b]			Intraday/Interday (high spiking level)[c]		
	Honey	**Royal Jelly**	**Propolis**	**Honey**	**Royal Jelly**	**Propolis**	**Honey**	**Royal Jelly**	**Propolis**
SARA	7.3/3.2	5.6/6.0	4.0/13.8	6.5/9.2	4.4/2.6	5.6/12.9	5.6/7.3	2.9/3.2	6.6/8.2
DANO	5.0/8.6	3.3/7.2	7.9/9.7	4.9/10.7	3.7/8.8	7.3/12.6	4.1/10.7	3.5/9.6	8.4/7.1
CIPRO	7.7/9.0	5.2/7.3	5.1/13.2	5.4/6.3	5.5/6.0	9.2/12.3	6.2/6.7	3.7/2.9	7.7/10.0
ENRO	4.9/11.9	8.2/8.3	4.6/11.1	3.7/10.5	3.0/7.9	6.1/7.4	5.4/11.3	2.2/7.6	8.2/11.3
DIFLO	3.7/6.0	3.6/11.8	5.1/11.2	1.7/5.1	2.8/4.4	4.7/11.5	4.4/5.9	1.8/5.6	5.4/8.6
FLUME	17.1/6.3	4.9/6.3	5.2/12.9	6.5/13.9	2.0/5.9	4.9/5.0	1.5/12.9	1.7/7.1	4.2/5.6
MARBO	5.4/7.4	5.7/2.8	4.4/8.1	4.7/6.4	3.9/4.3	8.0/9.1	6.2/7.6	2.9/4.2	8.0/14.7
OXO	3.4/6.0	3.2/3.7	7.7/8.9	8.5/8.5	1.7/6.5	5.2/6.3	2.9/8.1	7.2/9.2	2.6/7.7

a) Low spiking level 10 µg/kg for FLUME and OXO and 100 µg/kg for the other Qns.
b) Medium spiking level 15 µg/kg for FLUME and OXO and 150 µg/kg for the other Qns.
c) High spiking level 20 µg/kg for FLUME and OXO and 200 µg/kg for the other Qns.

Table 4.4. Recovery study and RSD (n=9) for the different studied samples

	% R (RSD %) at low spiking level			**% R (RSD %) at medium spiking level**			**% R (RSD %) at high spiking level**		
	Honey	**Royal Jelly**	**Propolis**	**Honey**	**Royal Jelly**	**Propolis**	**Honey**	**Royal Jelly**	**Propolis**
SARA	74.0 (7.3)	74.5 (5.5)	59.1 (4.0)	85.5 (6.5)	80.8 (4.4)	48.2 (5.6)	79.3 (5.6)	90.7 (2.9)	56.0 (6.6)
DANO	72.2 (5.0)	74.8 (3.3)	55.8 (7.9)	79.1 (4.9)	73.7 (3.7)	40.2 (7.3)	72.9 (4.1)	80.4 (3.5)	51.2 (8.4)
CIPRO	61.3 (7.7)	56.7 (5.2)	61.7 (5.1)	77.1 (5.4)	61.7 (5.5)	45.0 (9.2)	61.2 (6.2)	63.2 (3.7)	63.2 (5.9)
ENRO	72.7 (4.9)	77.9 (8.2)	57.7 (4.6)	86.8 (3.7)	86.2 (3.0)	42.9 (6.1)	73.2 (5.4)	88.4 (2.2)	55.0 (8.2)
DIFLO	76.8 (3.7)	81.0 (3.6)	52.7 (5.1)	88.8 (1.7)	84.7 (2.8)	48.1 (4.7)	78.1 (4.4)	94.6 (1.8)	51.9 (5.4)
FLUME	99.8 (3.1)	90.2 (4.9)	39.7 (5.2)	92.3 (6.5)	92.9 (2.0)	55.2 (4.9)	93.9 (1.5)	95.2 (1.7)	55.5 (4.2)
MARBO	70.0 (5.3)	68.9 (5.7)	64.0 (4.4)	78.3 (4.7)	63.7 (3.9)	60.3 (8.0)	70.4 (6.2)	68.3 (2.9)	69.2 (8.0)
OXO	76.0 (3.4)	82.0 (3.2)	65.2 (7.7)	82.5 (8.5)	85.5 (1.7)	49.5 (5.2)	75.6 (2.9)	96.5 (7.2)	62.8 (2.6)

a) Low spiking level 10 μg/kg for FLUME and OXO and 100 μg/kg for the other Qns.
b) Medium spiking level 15 μg/kg for FLUME and OXO and 150 μg/kg for the other Qns.
c) High spiking level 20 μg/kg for FLUME and OXO and 200 μg/kg for the other Qns.

4.3.4 *Analysis of real samples*

Twelve different samples of commercial honey, royal jelly and propolis were analyzed in order to demonstrate the applicability of the method.

Six different samples of honey were checked; among them, three were multifloral honeys from different regions of Spain -Valencia, Badajoz and Las Alpujarras (Granada)-, and three were monofloral honeys -rosemary and orange blossom honey from Valencia, and heather honey from Burgos (all of them from Spain). None of the samples gave a positive result for Qns by using the proposed method.

Four different samples of royal jelly (one of them containing also propolis) were analyzed. The first and second one containing 1 g of fresh royal jelly royal jelly, water, fructose, and preservatives; the third one contained 0.6 g of royal jelly, water, fructose, citric acid and preservatives, while the fourth one was composed by royal jelly, alcoholic extract of propolis (0.6 g), water, fructose, vitamin C, citric acid and preservatives. No Qn residues were found in the samples.

Two different propolis samples were analysed (in addition to the sample containing also royal jelly): the first one containing propolis (68.5%), water and alcohol; and the second one dewaxed propolis (20%), water, fructose, sodium lactate, aroma, lactic acid, ascorbic acid, soybean lecitine, xanthan gum, neoesperidine, and potassium sorbate. Once again, no Qn residues were found in the samples.

4.4 Conclusions

A new method, based on a QuEChERS extraction procedure and UHPLC–MS/MS, has been developed for the simultaneous determination of the eight Qns of veterinary use regulated by EU. The extraction procedure is quick, effective and cheap showing high throughput. The separation of the compounds is achieved in only three minutes and the full analysis (including sample treatment) takes no more than 40 minutes for a batch of

six samples. The results show the suitability of this procedure for the monitoring of the eight Qn residues in bee products in a single run and for providing data of the occurrence of these compounds in a wide range of bee products.

This work was published as:

"Determination of quinolones of veterinary use in bee products by ultra-high performance liquid chromatography-tandem mass spectrometry using a QuEChERS extraction procedure". M. Lombardo-Agüí, A.M. García-Campaña, L. Gámiz-Gracia, C. Cruces-Blanco. Talanta 93 (2012) 193.

Capítulo 5

Análisis multirresiduo de quinolonas en aguas mediante UHPLC-MS/MS con tratamiento de muestra basado en la metodología QuEChERS.

Chapter 5

Multiresidue analysis of quinolones in water samples by UHPLC-MS/MS using a QuEChERS-based method for sample treatment.

Resumen

En este capítulo se ha desarrollado un método rápido, sencillo y robusto para el análisis de 19 quinolonas en muestras medioambientales de agua mediante cromatografía de ultra resolución acoplada a la espectrometría de masas en tándem como sistema de detección. Se ha realizado una optimización cromatográfica y de los parámetros implicados en la detección con el fin de conseguir la mejor separación, sensibilidad e identificación. El método analítico desarrollado logra la separación cromatográfica y detección en menos de 4 minutos utilizando una columna de nueva generación, rellena de partículas superficialmente porosas, que da lugar a menores presiones de trabajo consiguiendo igual o superior resolución que las rellenas de partículas totalmente porosas. La detección de los compuestos se llevó a cabo mediante ionización positiva mediante electroespray y empleando el modo de monitorización de respuesta múltiple (*multiple reaction monitoring*, MRM). Para ello se seleccionaron los fragmentos mas intensos para la cuantificación y confirmación, y a continuación se optimizaron las condiciones de detección MS/MS para conseguir la máxima sensibilidad. En la etapa de tratamiento de muestra se ha empleado un procedimiento basado en la metodología QuEChERS para realizar una extracción rápida y sencilla que permite el análisis multirresiduo o multianalito. El método se ha validado para muestras de agua de pozo procedentes de una zona de montaña, consiguiéndose muy buenos límites de detección (entre 10 ng/l y 90 ng/l) con una precisión menor de 15.6% y con unas recuperaciones superiores al 73% en todos los casos. Además se han estudiado diferentes aguas de pozo de zonas de regadío y de costa obteniéndose resultados similares a los mencionados.

Abstract

In this chapter a rapid, easy and robust method has been developed for the determination of 19 quinolones in environmental water samples using ultrahigh performance liquid chromatography coupled to MS/MS detection. Chromatographic and detection conditions have been optimized in order to get the best separation, detection and identification, which have been achieved in less than 4 minutes. The separation was carried out using a new generation column, filled with superficially porous particles, resulting in lower backpressure and similar or even higher resolution than totally porous particle. The quinolones were detected by electrospray ionization in positive mode using multiple reaction monitoring mode (MRM) for acquisition. The most intense fragments were monitored for quantification and confirmation, and MS/MS conditions were optimized in order to get maximum sensitivity. A sample treatment based on QuEChERS methodology was employed to achieve a fast and simple extraction that enables the multiresidue/multianalite analysis. The method has been validated for an environmental well water sample from a mountain area, achieving very low detection limits (between 10 and 90 ng/l) with precisions lower than 15.6 % and recoveries higher than 73% in all cases. Moreover, well waters from different origins, irrigated-land and coast areas have been evaluated and similar results were obtained.

5.1 Introduction

As it was discussed in the Introduction and Chapter 1, the wide application range and the extensive use of Qns in veterinary and human medicine represent a potential hazard for the environment.

For the determination of Qns in different kinds of water, different chromatographic methods have been reported. For example, liquid chromatography (LC) coupled to fluorescence detection [1,2,3,4], or diode array detection [1,5,6]; however, LC coupled to mass spectrometry (LC-MS) maybe is the most extensively used due to its high sensitivity and specificity [1,7,8,9,10,11]. Concerning the sample treatment, SPE has been used extensively for isolation, purification, and preconcentration of Qns in environmental waters involving commercial sorbents such as Strata X, Oasis HLB, etc. [1,12,13,14]. Other sample treatments that have been explored with good results in different matrices are solid-phase microextraction (SPME) [15,16], liquid-liquid

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microextraction (LLME) [17,18,19], molecularly imprinted polymers (MIPs), commented and used in chapters 2 and 3 for water and milk samples, and QuEChERS (quick, easy, cheap, effective, rugged and safe), introduced and used in chapter 3 for the analysis of Qns in honey. However, as far as we know, QuEChERS based methodology has not been used to determinate Qns in environmental samples. Maybe, the reason is that applying this methodology, usually samples are diluted and very low or no preconcentration is achieved. In addition, cleaning is not exhaustive, while with the extensively used SPE it is possible to achieve cleaner extracts and a high preconcentration factor.

The aim of this chapter was to develop a simple and fast method for the determination of 19 Qns of different generations (DANO, SARA, DIFLO, FLUME, NOR, CINO, PEFLO, NALI, PIPE, ENO, LOME, MARBO, CIPRO, ENRO, FLERO, ORBI, MOXI and OXO) by UHPLC-MS/MS using electrospray ionization (ESI) in positive mode. To achieve the chromatographic separation, a partially porous column has been employed. Although superficially porous particles have been introduced long time ago in LC, now they have re-emerged to rival with totally porous particles, especially in UHPLC applications. This has been reflected in an important increase of publications signing the advantages of superficially porous columns for achieving high efficiency, high resolution and fast analysis at a very wide range of pressure [20,21,22]. The proposed extraction method has involved a very fast and effective liquid-liquid extraction based on partitioning via salting-out extraction, similar to that used in QuEChERS, involving the equilibrium between an aqueous and an organic layer. Further clean-up was not

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necessary since MS selectivity make possible a sensitive determination, allowing an increase in the sample throughput compared with classical sample treatments. To show the applicability of the present method, water samples from different origins have been analyzed.

5.2 Experimental

5.2.1 *Chemicals*

Solvents were LC-MS grade and Qns were analytical standard grade. Ultrapurewater (Milli-Q Plus system, Millipore Bedford, MA, USA) was used to prepare buffer and standard solutions. Sodium hydroxide, sodium dihydrogenphosphate monohydrate, magnesium sulphate, and sodium chloride were obtained from Panreac-Química (Madrid, Spain). SampliQ QuEChERS consisted on QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl) and were purchased form Agilent Technologies Inc. (Wilmington, DE, USA). Formic acid eluent additive for LC-MS and acetonitrile (AcN) were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany). DANO, SARA, and DIFLO were supplied by Riedel-de Haën (Seelze, Germany); FLUME, NOR, CINO, PEFLO, NALI, PIPE, ENO and LOME by Sigma Aldrich; and MARBO, CIPRO, ENRO, FLERO, ORBI, MOXI, and OXO by Fluka (Steinheim, Germany). Filters of 13 mm with 0.2 µm nylon membrane (Bulk Acrodisc®, Pall corp., MI, USA), were used for filtration of the final extracts before analysis.

5.2.2 *Preparation of solutions and samples*

Individual stock standard solutions (100 mg/l) of each Qn were prepared by dissolving the appropriate amount of each analyte in AcN/0.02% formic acid aqueous solution (50/50, v/v) and were stored in the dark at -20 ºC. Formic acid (analysis grade) was added to each standard to increase solubility of analytes in this solvent mixture. Under

such conditions, they were stable for at least 3 months. Working solutions (containing all Qns) were prepared daily by dilution of the individual stock solutions with water.

A 0.1 M phosphate buffer solution pH 7.1 was prepared by dissolving adequate quantity of dihydrogenphosphate monohydrate in water and the pH was adjusted with 4M NaOH solution. A 0.02% formic acid aqueous solution was prepared by adding 50 µl of formic acid (eluent additive for LC-MS) to 250 ml of water. The 5% formic acid solution in AcN (extraction solvent) was obtained by adding 25 ml of formic acid (analysis grade) to 500 ml of AcN.

Samples were collected in different locations. Two of the samples came from Granada (Spain) –sample 1: a mountain well water and sample 2: irrigated-land well water– and the third one (sample 3) from a well near to the coast of Málaga (Spain). After sample collection, they were stored at 4 ºC and left to room temperature before analysis.

5.2.3 *Instrumentation*

Separation was performed on an Agilent 1290 Infinity LC from Agilent Technologies, and the mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (Applied Biosystems, Darmstadt, Germany) with electrospray ionization (ESI). The instrument data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (ABSCIEX). Different C18 chromatographic columns were tested to achieve the separation: a totally porous column, Zorbax Eclipse Plus HHRD (50×2.1 mm, 1.8 µm) and a partially porous, Poroshell 120 EC-C18 (50×2.1 mm, 2.7 µm), both of them from Agilent Technologies.

A pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain), a Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a rotary evaporator (Buchi R200, Flawil, Switzerland) coupled to a heating bath (Buchi B490, Flawil Switzerland)

and an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used.

5.2.4 *UHPLC-MS/MS analysis*

The chromatographic separation was carried out using an UHPLC system consisting of a quaternary pump, autosampler and thermostated column oven. Separation was performed in a C18 column (Poroshell 120 EC-C18, 50×2.1 mm, 2.7 μm), using a mobile phase consisting of 0.02% aqueous formic acid solution (solvent A), and AcN (solvent B). The gradient profile started at 5% of B; 15% B at 1 min; 95% B at 3 min, with a final hold for 1 min. Finally, it was back to 5% B in 0.5 min for equilibration. The flow rate was 600 μl/min, the temperature of the column was 35 °C and the injection volume was 5 μl. Under these conditions all the analytes were distributed along 3.6 min of chromatogram. The UHPLC system was coupled to a mass-spectrometer operating with an electrospray ion source (ESI) in the positive ion mode, under the multiple reaction monitoring (MRM) conditions shown in table 5.1. The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 45 psi, ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 80 psi.

5.2.5 *Extraction Procedure*

The extraction of antibiotics from water was achieved using liquid-liquid extraction based on partitioning via salting-out. This corresponds to the first step of a QuEChERS-based sample preparation, which has been previously reported in chapter 3 for the analysis of milk [23] and in chapter 4 for honey [24]. In this case, the second step of QuEChERS (based on the dispersive SPE clean-up) was unnecessary since extracts were clean enough to be injected directly after extraction.

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Samples of 100 ml of water were spiked with proper volumes of a standard solution of 2 mg/l of each Qn and vortexed for 10 second. The pH of the samples was adjusted to pH 7 by adding 5 ml of 0.1 M phosphate buffer solution. Then an aliquot of 5 ml of water was placed into a 50 ml centrifuge tube, 10 ml of 5% formic acid in AcN was added and the mixture was homogenized in vortex. Agilent SampliQ EN QuEChERS extraction kit, containing 1 g of NaCl and 4 g of $MgSO_4$, was added and the tube was shaken vigorously for 1 min. The sample was centrifuged at 9000 rpm at 4 ºC for 5 min and 9 ml of the upper AcN layer was transferred into a round bottom flask. This extract was evaporated in the rotary evaporator at 40 ºC for about 5 min, and completely dried under N_2 stream; finally, the residue was redissolved with 1 ml of H_2O/AcN/formic acid (88/10/2), filtered, and analysed by UHPLC-MS/MS. The sample treatment is summarized in figure 5.1.

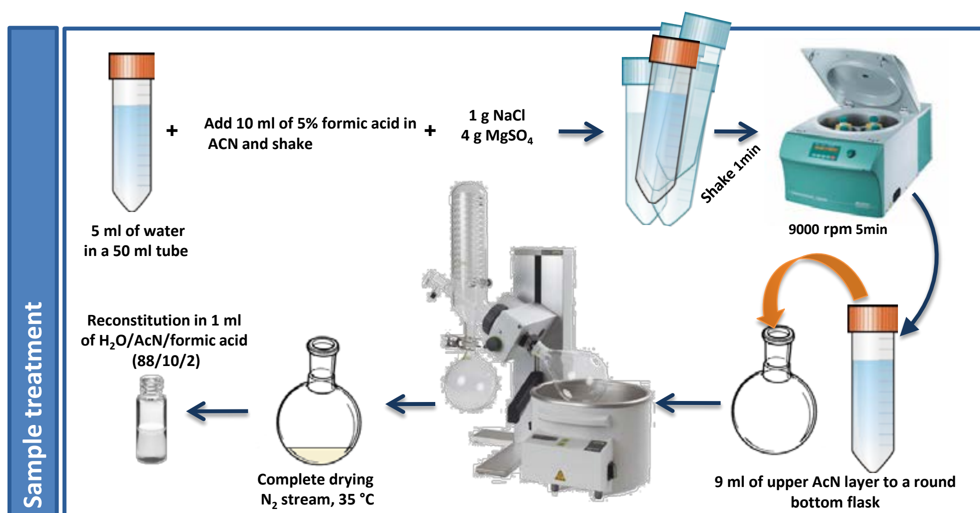

Figure 5.1. Sample treatment scheme.

Table 5.1. MS optimized parameters.

Compound	$[M+H]^+$ (m/z)	Rt	DP	EP	CEP	Product ions	CEN	CXP
PIPE	304.0	1.5	41	7.0	16	217.0 (Q) 189.2 (I)	25 47	6.0 6.0
MARBO	363.2	1.8	38	4.8	16	72.1 (Q) 320.2 (I)	42 19	2.5 5.5
ENO	321.0	1.9	45	8.5	16	234.0 (Q) 206.0 (I)	27 41	4.0 4.0
FLERO	370.2	1.9	60	5.0	26	326.2 (Q) 369.2 (I)	27 31	6.0 4.0
NOR	320.0	2.0	41	6.5	15	276.0 (Q) 233.0 (I)	21 29	8.0 4.0
PEFLO	334.2	2.0	50	6.5	14	290.1 (Q) 233.1 (I)	29 31	10.0 4.0
LEVO	362.4	2.0	50	6	14	318.2 (Q) 261.1 (I)	29 33	4.0 6.0
CIPRO	332.3	2.1	43	5	18	288.1 (Q) 245.2 (I)	23 32	5.0 2.0
LOME	352.0	2.2	55	6.0	24	265.0 (Q) 308.2 (I)	33 23	4.0 6.0
DANO	358.2	2.4	48	5.0	15	340.0 (Q) 314.1 (I)	30 24	5.5 5.5
ENRO	360.2	2.5	45	5.0	15	316.2 (Q) 245.0 (I)	26 26	5.5 2.0
ORBI	396.2	2.5	50	7.5	16	352.3 (Q) 295.1 (I)	23 33	6.0 4.0
SARA	386.1	2.6	45	5	16	342.1 (Q) 299.1 (I)	35 35	5.0 5.0
DIFLO	400.2	2.7	50	5.0	22	356.2 (Q) 299.2 (I)	24 38	6.0 5.5
MOXI	402.2	2.8	50	11.5	22	384.2 (Q) 261.3 (I)	27 37	6.0 4.0
CINO	263.0	2.9	26	5.0	22	245.0 (Q) 189.1 (I)	19 41	4.0 4.0
OXO	262.2	3.1	32	4.5	18	244.1 (Q) 216.1 (I)	24 36	4.0 3.0
FLUME	262.2	3.5	37	5.5	24	244.2 (Q) 202.3 (I)	23 43	3.0 3.0
NALI	233.1	3.5	26	11.0	14	215.2 (Q) 187.1 (I)	19 35	4.0 6.0

(Q) transition used for quantification, (I) Transition employed to complete the identification

5.3 Results and discussion

5.3.1 *Optimization of MS/MS detection and chromatographic separation*

First of all, MS/MS instrumental settings were optimized in order to detect correctly every analyte and their fragments, and to maximize the signal for precursors and most intense product ions. With that purpose, each analyte was individually infused directly into the mass spectrometer, using standard solutions of 1 or 0.5 mg/l in AcN/0.02% aqueous formic acid solution (50/50; v/v). From previous experiments in our laboratory, ESI operating in positive mode was used for MS detection [24]. Each compound was characterized by its retention time and by two precursor-product ion transitions. The most intense product ion was used for quantification whereas the second one was used to complete the identification. All the optimized instrumental settings are detailed in table 5.1.

Although MS/MS allows the determinations of different analytes even though they are not fully resolved, a good separation is recommended, as it provides less overlapped detection windows, which involves a better detection and higher number of points per peak. Therefore, the optimization of the chromatographic separation was developed in order to get the best separation, peak shape and sensitivity, taking the advantages of the UHPLC features. According to bibliography [1,24] aqueous and AcN phases were selected as solvents for the separation of Qns by HPLC. Thus, the mobile phase consisted of 0.02% aqueous formic acid solution (solvent A) and AcN (solvent B). The gradient was studied to get the best separation in the shortest time and it was found that a rising gradient until 95% of AcN in 3 min affords a good compromise between resolution and analysis time.

In this work, a key point of the separation was the selected column. Most of the HPLC methods reported for the determination of Qns are performed with totally porous columns. Nevertheless, superficially porous columns are being adopted by more

chromatographers every day as a way to save time and increase laboratory throughput. Thus, two different columns were tested in order to check possible differences: (i) a totally porous particle column, employed in Chapter 4 for separation of 8 Qns (Zorbax Eclipse Plus HHRD, 50×2.1 mm, 1.8 µm, 95 Å); (ii) a superficially porous particle column (Poroshell 120 EC-C18, 50×2.1 mm, 2.7 µm, 120 Å). A standard solution of Qns was analysed using both columns and, although resolution was similar, peaks were slightly narrower and the backpressure was much lower with the superficially porous column. Therefore, with this option it is possible to work at a higher flow rate, decreasing analysis time and increasing sample throughput. In this sense, the flow rate was studied between 300 and 600 µl/min, and 600 µl/min was selected as optimum; higher flow rates were not studied to avoid a decrease in the number of points per peak.

Finally, injection volume was studied between 5 and 20 µl in order to increase the sensitivity of the method. A volume of 5 µl was finally selected, as higher volumes lead to peak broadening.

Figures 5.2 and 5.3 shows the extracted ion chromatograms of a spiked water sample. As can be observed, the analysis was carried out in 3.6 minutes and good separation for the 19 compounds under study was obtained.

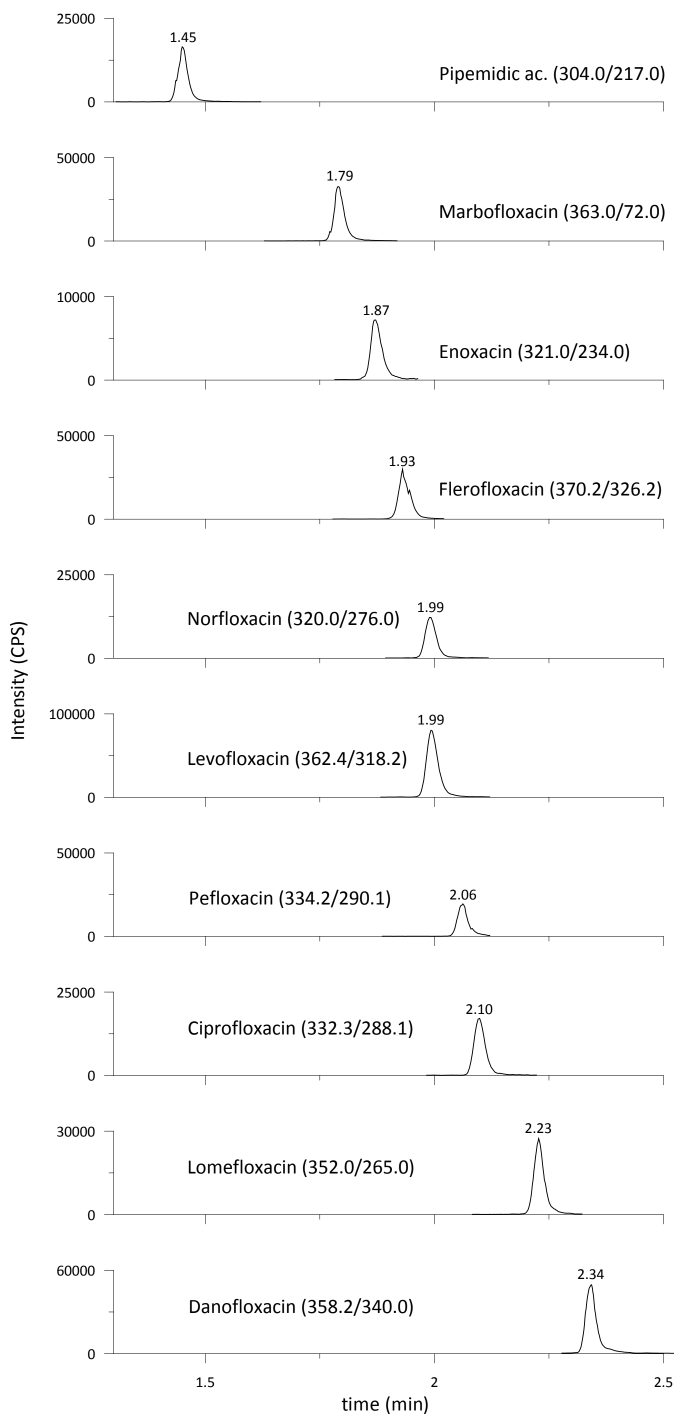

Figure 5.2. UHPLC–MS/MS extracted ion chromatograms of a spiked water sample at 20 µg/l (1 to 2.5 min).

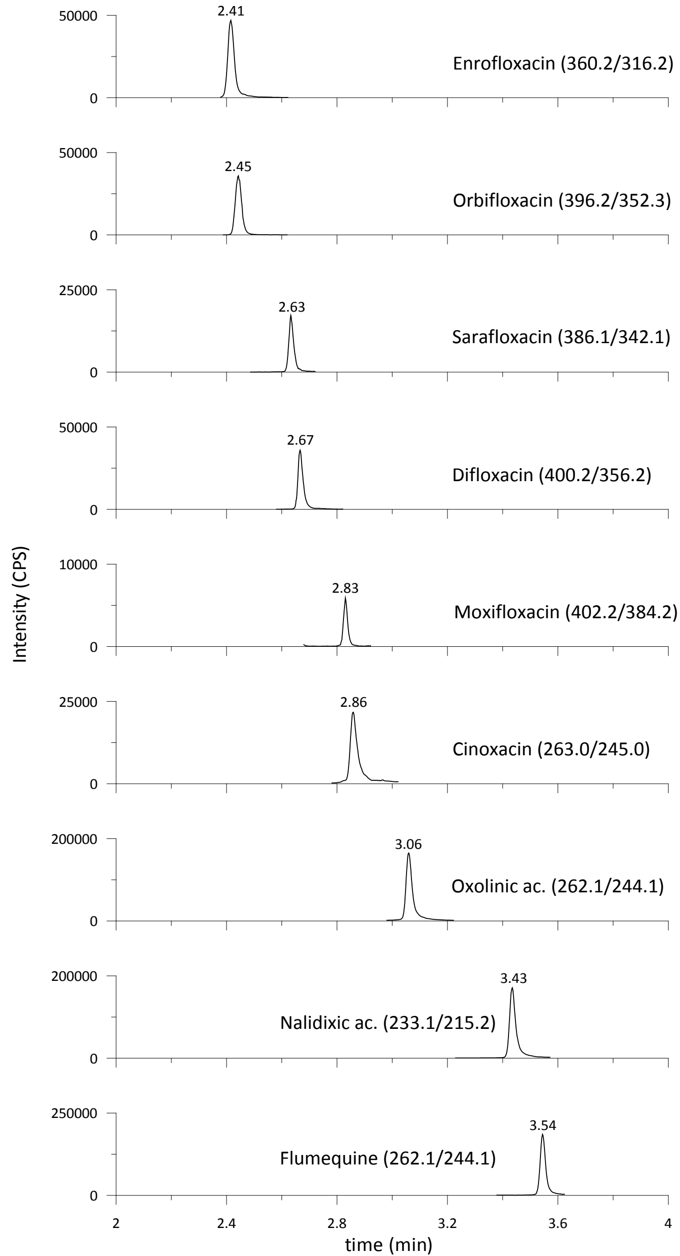

Figure 5.3. UHPLC–MS/MS extracted ion chromatograms of a spiked water sample at 20 µg/l (2 to 4 min).

5.3.2 *Optimization of sample treatment*

Different sample treatments or extraction approaches for the determination of residues of drugs in water have been described in the literature [25,26]. However, when a high number of analytes are determined, polar and non-polar compounds can be present in the same sample a generic treatment is required. Taking into account the good results obtained in previous works for the determination of Qns in other matrices (described in previous chapters), QuEChERS methodology was chosen for the analysis of environmental waters. However, only the first step of this methodology (partitioned liquid-liquid extraction) was necessary in the case of water samples, without further clean-up.

During the optimization, different extraction kits were tested: buffered (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium citrate sesquihydrate), and non-buffered (1 g $MgSO_4$, 1 g NaCl). Similar recoveries were obtained with both options, observing that some compounds were slightly beneficed by buffered and some by non-buffered extraction media. So, a non-buffered kit was selected since a lower residue was produced.

Furthermore, in order to reach low detection limits, different water sample volumes (5, 10, 20 and 30 ml) were assessed in the application of the extraction procedure. When 30 ml of water was used a proper phase separation was not achieved, so this volume was directly discarded. Despite the fact that higher detection limits were reached with higher volumes, finally 5 ml was selected as optimum volume, since recoveries for most of the compounds were higher (near 100%) than those obtained with the other volumes.

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After these optimizations, the final extraction process was as previously described in section 5.2.5. and figure 5.2.

5.3.3 *Analytical and performance characteristics*

5.3.3.1 Calibration curves, detection and quantification limits

Each compound was analysed in MRM mode, selecting the two highest precursor ion/product ion transitions, which, with retention times, were used to ensure adequate analyte identification. Performance characteristics of the method (linearity, limits of detection (LOD) and quantification (LOQ), intra and interday precision, and trueness) were established with spiked samples. Matrix-matched calibration curves were established using the dirtiest water sample (sample 1), spiked at five concentration levels (from 0.2 to 20 µg/l for all the Qns). This sample presented suspended solid material, but filtration before sample treatment was not required. LODs and LOQs were provided by the software Analyst, as 3×S/N and 10×S/N, respectively. Determination coefficients, LODs and LOQs are shown in table 5.2, indicating that the method is sensitive enough for the determination of very low levels of these compounds in water samples.

Table 5.2. Linearity, detection and quantification limits of the UHPLC-MS/MS proposed method for water samples.

Compound	R^2	LOD (µg/l)	LOQ (µg/l)
SARA	0.999	0.02	0.07
DANO	0.994	0.02	0.06
CIPRO	0.996	0.04	0.15
ENRO	0.999	0.02	0.08
DIFLO	0.998	0.03	0.09
FLUME	0.998	0.01	0.02
MARBO	0.996	0.03	0.09
OXO	0.994	0.01	0.03
CINO	0.996	0.06	0.20
ENO	0.998	0.06	0.19
LOME	0.993	0.05	0.16
NOR	0.994	0.04	0.12
PIPE	0.998	0.02	0.05
ORBI	0.998	0.02	0.05
FLERO	0.996	0.02	0.06
PEFLO	0.995	0.09	0.31
MOXI	0.996	0.06	0.20
LEVO	0.997	0.04	0.12
NALI	0.997	0.03	0.10

5.3.3.2 Precision and trueness studies

Intraday precision was performed by using spiking water samples (sample 1) at three concentration levels (level 1= 2 µg/l, level 2= 5 µg/l and level 3= 10 µg/l), using five experimental replicates for each concentration level, analysed by triplicate the same day. Interday precision was evaluated in a similar way, but the samples were analysed by triplicate during five different days. The results are summarised in table 5.3.

Table 5.3. Intraday and interday precision expressed as RSD (%) for sample 1 (n=15).

	Intraday precision[a]			Interday precision[a]		
	Level 1	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
SARA	7.8	3.8	4.4	2.1	3.8	2.4
DANO	12.8	2.3	9.0	13.0	5.4	9.8
CIPRO	8.0	5.6	5.8	10.6	4.9	10.8
ENRO	7.4	2.1	6.3	4.0	7.4	2.6
DIFLO	7.0	3.6	5.3	2.7	2.3	3.2
FLUME	4.5	2.6	4.4	1.3	9.2	5.3
MARBO	11.2	4.4	7.0	2.8	2.7	6.6
OXO	5.7	3.6	7.5	2.3	5.3	4.0
CINO	4.4	3.2	6.4	2.4	6.4	1.0
ENO	7.3	3.6	6.1	2.8	1.8	2.0
LOME	6.5	4.1	4.9	6.8	10.2	13.4
NOR	4.8	2.5	7.5	4.8	3.8	1.1
PIPE	5.1	3.9	4.1	1.2	2.6	2.5
ORBI	3.9	4.6	3.6	2.7	0.9	4.0
FLERO	5.2	1.7	4.8	2.9	1.4	5.0
PEFLO	15.6	14.7	6.7	14.3	12.0	16.5
MOXI	9.7	3.0	7.5	8.1	5.4	8.1
LEVO	7.5	2.7	5.7	6.6	4.8	2.9
NALI	5.7	1.6	3.8	1.8	2.4	7.3

a) level 1= 2 µg/l, level 2= 5 µg/l and level 3= 10 µg/l

Trueness was evaluated for the three selected waters by analyzing five fortified blank samples with concentrations similar to those used in the precision study. As can be observed in tables 5.4, 5.5 and 5.6, satisfactory recoveries (from 73.6 to 107.8 %) were obtained for the three different selected waters. Therefore, the method has shown its suitability for the analysis of water samples of different origin.

Table 5.4. Recovery study and RSD for sample 1 (n = 15)

	Level 1 [a]		Level 2 [a]		Level 3 [a]	
	Recovery	**RSD (%)**	**Recovery**	**RSD (%)**	**Recovery**	**RSD (%)**
SARA	86.4	7.8	99.0	3.8	94.7	4.4
DANO	83.8	12.8	92.1	2.3	80.7	9.0
CIPRO	73.8	8.0	93.5	5.6	93.8	5.8
ENRO	90.3	7.4	95.7	2.1	92.4	6.3
DIFLO	91.0	7.0	97.3	3.6	93.4	5.3
FLUME	105.1	4.5	96.7	2.6	97.7	4.4
MARBO	74.8	11.2	91.1	4.4	81.0	7.0
OXO	99.5	5.7	99.8	3.6	91.4	7.5
CINO	101.5	4.4	96.1	3.2	93.8	6.4
ENO	81.4	7.3	91.0	3.6	83.5	6.1
LOME	98.9	6.5	100.0	4.1	90.7	4.9
NOR	106.4	4.8	85.2	2.5	89.3	7.5
PIPE	73.6	5.1	79.3	3.9	78.0	4.1
ORBI	90.4	3.9	99.3	4.6	95.5	3.6
FLERO	85.9	5.2	91.1	1.7	91.3	4.8
PEFLO	86.6	15.6	77.8	14.7	86.4	6.7
MOXI	100.0	9.7	95.5	3.0	90.6	7.5
LEVO	88.1	7.5	91.7	2.7	91.0	5.7
NALI	102.9	5.7	97.5	1.6	99.1	3.8

a) level 1= 2 μg/l, level 2= 5 μg/l and level 3= 10 μg/l

Table 5.5. Recovery study and RSD for sample 2 (n = 15)

	Level 1 [a]		Level 2 [a]		Level 3 [a]	
	Recovery	**RSD (%)**	**Recovery**	**RSD (%)**	**Recovery**	**RSD (%)**
SARA	104.1	9.8	107.8	0.9	96.5	0.7
DANO	91.8	6.8	91.0	0.9	94.7	5.2
CIPRO	98.0	4.4	92.4	4.2	83.2	12.0
ENRO	97.5	6.2	92.7	1.2	97.0	1.8
DIFLO	99.4	9.4	105.0	2.8	97.2	0.8
FLUME	103.5	6.3	100.6	2.3	104.7	2.8
MARBO	85.4	13.9	80.9	3.0	85.7	0.6
OXO	94.6	9.6	100.2	1.0	96.6	4.7
CINO	105.5	6.9	103.7	0.4	99.8	3.6
ENO	86.3	8.2	94.2	3.5	83.8	1.7
LOME	88.8	5.6	93.0	1.2	89.3	0.8
NOR	92.9	11.1	97.4	1.7	98.2	6.8
PIPE	78.9	8.4	81.4	0.2	78.4	2.5
ORBI	94.0	7.0	103.8	4.2	96.7	2.8
FLERO	86.8	5.1	103.0	5.7	94.8	1.6
PEFLO	88.1	9.5	89.5	5.3	88.5	4.2
MOXI	97.6	1.2	102.9	2.9	100.6	6.5
LEVO	96.4	1.6	107.5	9.2	92.3	5.6
NALI	104.8	0.9	100.4	1.2	98.5	7.1

a) level 1= 2 µg/l, level 2= 5 µg/l and level 3= 10 µg/l

Table 5.6. Recovery study and RSD for sample 3 (n = 15)

	Level 1 [a]		Level 2 [a]		Level 3 [a]	
	Recovery	**RSD (%)**	**Recovery**	**RSD (%)**	**Recovery**	**RSD (%)**
SARA	100.1	5.9	90.1	1.3	98.1	2.2
DANO	92.1	6.4	83.1	4.7	90.8	2.7
CIPRO	85.4	2.4	77.4	6.6	97.4	5.6
ENRO	86.9	4.1	86.3	0.2	96.3	0.4
DIFLO	99.6	0.9	89.5	2.4	94.7	1.0
FLUME	98.1	3.4	95.0	0.1	101.8	0.7
MARBO	85.1	9.9	79.7	2.1	89.8	1.5
OXO	94.9	5.8	83.6	2.0	94.4	1.0
CINO	85.7	14.3	84.8	0.4	99.4	0.5
ENO	83.4	1.7	80.1	1.9	85.9	5.7
LOME	91.2	1.7	86.3	5.2	86.6	4.8
NOR	90.9	0.4	88.4	2.7	86.4	7.7
PIPE	80.0	1.8	89.7	1.8	76.0	2.2
ORBI	95.1	2.4	92.6	2.0	93.7	0.2
FLERO	91.0	2.4	84.0	7.2	94.6	2.1
PEFLO	92.6	9.1	83.6	0.4	96.2	5.1
MOXI	99.5	2.9	91.7	2.2	93.6	1.9
LEVO	97.8	0.5	88.9	2.4	91.6	0.4
NALI	94.4	5.3	98.1	1.6	104.8	0.4

a) level 1= 2 μg/l, level 2= 5 μg/l and level 3= 10 μg/l

5.4 Conclusions

A very fast and simple multiresidue method to determinate 19 compounds belonging to the Qns family has been developed. This work responds to the growing interest in the development of multianalyte methods in the environmental field. UHPLC is especially powerful for a high number of analytes and the identification capability of the detection by MS/MS compensates the lack of selectivity of a generic sample treatment like QuEChERS. The superficially porous column provided much lower backpressures than the sub-2-μm columns working at approximately a half of the pressure and using MS/MS unambiguous confirmation of found residues is achieved, reaching very low detection limits, as those obtained in this work.

On the other side, the use of a generic extraction method like the QuEChERs methodology makes possible the simultaneous extraction of compounds with different properties. Thus, an important number of Qns with different chemical properties, such as polarity, has been extracted by a quick, effective, and cheap method, showing a high throughput when combined with UHPLC-MS/MS. The obtained LOQs were in the ng/l level, meaning that sensitivity is good enough for the monitoring of these pharmaceutical compounds in environmental samples.

This work has been sent for publication to Analytica Chimia Acta

Capítulo 6

Análisis multirresiduo de fármacos y pesticidas en pollo mediante cromatografía líquida acoplada con espectrometría de masas de alta resolución.

Chapter 6

Multiresidue analysis of pharmaceutical and pesticides in chicken by liquid chromatography coupled to full scan high resolution mass spectrometry.

Resumen

En este capítulo se ha desarrollado un método de cribado para la determinación multirresiduo de fármacos veterinarios y plaguicidas en carne de pollo usando cromatografía líquida acoplada a espectrometría de masas de alta resolución (LC-HRMS). Además, se ha confeccionado una base de datos en la que se ha incluido la masa exacta de cada analito y de sus posibles aductos, la composición elemental, el tiempo de retención y el patrón isotópico de cada compuesto. Asimismo, se ha realizado un estudio de las condiciones para la extracción conjunta de los analitos de muestras de carne previamente fortificadas, empleándose finalmente un método genérico como es la extracción con AcN, adicionando EDTA. Empleado LC-HRMS, tras el tratamiento de muestra se han podido detectar un total de 147 compuestos (77 fármacos y 70 plaguicidas) de los 166 (79 fármacos y 87 plaguicidas) estudiados inicialmente. Como sistema de detección de masas de alta resolución se empleó el Orbitrap, proponiéndose distintos umbrales de decisión basados en diferentes intensidades de señal para la determinación del número de falsos positivos/negativos, comparándose los resultados obtenidos en cada caso. Los límites del método de cribado obtenidos para los 147 compuestos fueron de 10, 50 y 100 µg/kg para 127, 17 y 3 respectivamente. Asimismo, se ha propuesto el empleo del ión isotópico más abundante como criterio adicional de confirmación.

Abstract

In this chapter a multiresidue screening method for the determination of veterinary drugs and pesticides in chicken meat using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) has been developed. A database including all the parameters related to the molecular masses of each compound, elemental composition, exact mass of the compound and the different adducts that can be measured, retention time and the isotopic pattern, has been set up. Sample treatment of meat has been studied, selecting a generic treatment using AcN with the addition of EDTA. Using LC-HRMS 147 compounds (77 veterinary drugs and 70 pesticides) were determined from a total of 166 compounds (79 veterinary drugs and 87 pesticides) included in the study. Using LC-RHMS, after sample treatment 147 compounds were detected (77 veterinary drugs and 70 pesticides) from the 166 set (79 veterinary drugs and 87 pesticides) included in the study initially. Orbitrap was used as HRMS system and for the assessment of false positives and negatives different decision thresholds based on signal intensity were established, comparing the results. Screening detection limits for the 147 detected compounds were 10, 50 y 100 µg/kg for 127, 17 y 3, respectively. Also, as additional criteria for confirmation the most intense isotope ion has been proposed.

6.1 *Introduction*

Veterinary drugs (VDs) and pesticides are widely used in farming and agriculture to treat infections in livestock production and to increase production, respectively. As commented in previous chapters, the extensive use of VDs can involve a risk for both human and animal health, as their residues in foodstuffs can cause allergic reactions or antibiotic resistance [1,2,3]. However, disease prevention and control are necessary, as animal diseases can cause big economic problems to animal producers, which make the different treatments necessary for intense livestock production. On the other hand, pesticides are commonly used to combat plagues and to treat crops that are consumed later by animals; thus, pesticide residues can be present in food products of animal origin as a consequence of indirect exposure through intake of feeding stuffs. So, it is necessary to ensure that such residues are not found in food or feed at levels presenting an unacceptable risk to humans [4]. Thus, as food products of animal origin may contain residues of VDs and pesticides, EU has established MRLs for most VDs [5] and pesticides [6,7] in different matrices to protect consumers from exposure to

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[5] Council Regulation (EEC) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L 15/1, 20-1-2010.

[6] Regulation (EC) No. 396/2005 of the European Parliament and of the council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal of the European Union L 70/1, 16.3.2005, and subsequent amending.

[7] Commission Regulation (EU) No. 270/2012 of March 2012 amending Annexes II and III to Regulation (EC) No 396/2005 of European Parliament and of the Council as regards maximum residue levels for amidosulfuron, azoxystrobin, bentazone, bixafen, cyrpoconazole, fluopyram, imazapic, malathion propiconazole ans spinosad in or on certain products. Official Journal of the European Union L 89/5, 27.3.2012.

unacceptable levels of contaminants, and ensure compliance with good agricultural practices (GAP).

For enforcement of MRLs and to gain more insight of the occurrence of both types of residues (VDs and pesticides) efficient and effective analytical methods are required. Although different methods have been published to control VDs or pesticides in different matrices [8,9,10,11,12,13], development of screening methods including a very high number of those analytes (which can vary widely in physical chemical properties and biological activities) in just one method is of especial interest. Screening methods aredefined as "methods used to detect the presence of an analyte or class of analytes at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential noncompliant results. They are specially designed to avoid false compliant results" [14]. Screening methods can be classified either according to the principle of detection (biological methods, biochemical methods and physicochemical methods) or according to their degree of quantification, whether they are qualitative, semi-quantitative or quantitative. While semi-quantitative methods give an approximate indication of the concentration of the putative analyte, quantitative methods give precise concentration of the analyte [15].

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Once the screening method has been carried out, the analytical strategy would involve the subsequent analysis of positive samples to confirm and quantify the possible contaminant. Historically, in the field of multianalyte analysis, MS/MS methods have been employed for the determination of a single class of analytes or a relatively small multiresidue set of analytes. Although some publications have described the simultaneous determination of compounds used for the same purpose but belonging to different families [16,17,18,19,20], the analysis of a very high number compounds is more difficult for current LC-MS/MS instruments. LC-HRMS is a very good alternative to LC-MS or LC-MS/MS when screening methods are performed because, by using HRMS in full-scan mode, targeted and untargeted analysis can be achieved. In this way a higher number of analytes can be studied than with targeted LC-MS/MS [21,22,23]. Thus, up to 100-200 compounds (depending on the scan speed/dwell-time) can be analyzed in a single run by LC-MS/MS in multiple reaction monitoring (MRM) mode with a specific chromatographic method [24]. Besides that only analytes included in the MRM methods are susceptible to be measured, no information about non targeted analysis can be obtained, while with HRMS untargeted analysis can be carried out. These are some of the reasons that makes HRMS, using time of flight (TOF) mass

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spectrometers and the single stage Orbitrap mass spectrometer, really useful for targeted and untargeted analysis, increasing number of analytes and sample throughput [23,25,26]. In this context, the full scan approach using HRMS is emerging as a promising alternative that allows the development of sensitive wide-range screening of contaminants residues overcoming the limitations of MS/MS analysis [23,26,27,28].

Another aim in multianalyte methods is to find a generic sample preparation, generally involving only an extraction step or dilution of sample, which entails obtaining a complex sample extract [29]. Moreover, after instrumental analysis, an efficient data processing must be achieved. Methods based on HRMS methods involve an additional effort to generate databases containing at least detected adducts, exact mass and retention time. These parameters are necessary in order to study each compound (or mass) from their extracted ion chromatograms obtained from the original raw data file. In addition, other parameters must be established for unequivocal identification, such as accepted deviations for accurate mass, retention time or isotope ratio.

In this chapter the development of a multianalyte screening method including VDs and pesticides in chicken meat using LC-HRMS for detection is described in order to assess the potential of this technique for qualitative purposes. The validation of the method was achieved keeping in mind the guidelines established by EU in SANCO 12495/2011 (Method validation and quality control procedures for pesticide residues analysis in food

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and feed) [30] and the Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) [15].

Chicken meat was selected because it is one of the most consumed kinds of meat and could contain residues of both groups of compounds. In addition, chicken meat is classified as a representative commodity of white meat in SANCO 12495/2011 [30]. That means that once a method has been validated for this commodity, it could be applied for the analysis of different kinds of white meat from poultry. Sample treatment has been studied in order to optimize method sensitivity and assess ionic suppression in the ionization step before MS detection. The analysis of tetracyclines has been emphasized since they are one of the most used antibiotics in livestock and poultry breeding. A database containing the most relevant data about the analytes, basically exact mass of the potential detectable adducts, retention time and possible adducts has also been generated. Finally, the number of false positives and negatives has been evaluated using different approaches and thresholds, since they represent an important issue in the screening of contaminant in food sample with LC and full scan HRMS.

6.2 *Experimental*

6.2.1. *Reagents and materials*

Methanol (MeOH) and water (both of them LC-MS grade) and acetonitrile (AcN, HPLC-grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid for LC-MS and ammonium formiate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Primary secondary amine (PSA) for sample treatment was purchased from Agilent Technologies, Inc. (Delaware, US). The sodium salt of ethylenediaminetetraacetic acid

[30] SANCO/12495/2011. Method validation and quality control procedures for pesticide residues analysis in food and feed. http://ec.europa.eu/food/plant/protection/pesticides/docs/qualcontrol_en.pdf. Accessed on 8 October 2012.

(Na_2EDTA), citric acid and sodium phosphate to prepare EDTA-McIlvain buffer were purchased from Merck (Darmstadt, Germany).

6.2.2. *Preparation of solutions*

Solution of 2000 µg/ml of the pesticides aminopyralid, asulam, bifenthrin, bixafen, boscalid, bromoxynil, carbaryl, carbosulfan, chlorpropham, chlorpyrifos, chlorpyrifos-methyl, cypermethrin, cyproconazole, cyromazine, deltamethrin, diazinon, (2,4-dichlorophenoxy)acetic acid (2,4-D), dichlorprop, dichlorvos, difenoconazole, epoxiconazole, etofenprox, famoxadone, fenpropidin, fenpropimorph, fenthion, fenthion-sulfone, fenthion-sulfoxide, fenvalerate, esfenvalerate, fipronil, fipronil-sulfone, flonicamid, fluazifop-p-butyl, fluazifop acid, fluoxastrobin, fluquinconazole, fluroxypyr, flusilazole, flutolanil, haloxyfop, haloxyfop-R methyl ester, hexythiazox, indoxacarb, ioxynil, ivermectin, lambda-cyhalothrin, malathion, malaoxon, metaflumizone, methidathion, nicotine, nitarsone, parathion-methyl, paraoxon-methyl, permethrin, phosmet, phoxim, picloram, pirimiphos-methyl, prochloraz, profenofos, propargite, prothioconazole, pyrazophos, pyridate, quinoxyfen, rotenone, roxarsone, spinosyn A, spinosyn D, spiroxamine, tau-fluvalinate, tebuconazole, tepraloxydim, tetrachlorfenvinphos, tetraconazole, thiabendazole, thiacloprid, thiophanate-methyl, topramezone, triazophos and pyridate metabolite, were used to prepare intermediate solutions. Those solutions and different solutions of fenthion-oxon, fenthion-oxon-sulfone, fhenthion-oxon-sulfoxide and phosmet-oxon were used for preparation of a stock standard solution of 0.333 mg/l including all the pesticides, which was kept in the freezer at -80 °C.

VDs stock standard solutions for each family of compounds were prepared in MeOH, as follows: 10 mg/l for Qns (marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid and flumequine); 100 mg/l for tetracyclines (chlortetracycline, doxycycline, oxytetracycline, tetracycline); 50 mg/l for sulfonamides (sulfachloropyridazine, sulfadiazine, sulfadimethoxine,

sulfadimidine, sulfadoxine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfametoxypyridazine, sulfamonomethoxine, sulfamoxole, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole and dapsone); 5 mg/l for coccidiostats (halofuginone, robenidine, diclazuril, 4,4-dinitrocarbanilide (DNC), decoquinate, semduramicin, monensin, salinomycin, narasin, maduramicin, lasalocid, ponazuril, toltrazuril, amprolium, flubendazol, meticlorpindol and clazuril); 40 mg/l for non-steroidal anti-inflammatory drugs (NSAIDS: carprofen, diclofenac, phenylbutazone (FBZ), fenbufen, firocoxib, flufenamic acid, flunixin, indoprofen, ketoprofen, 4-methylaminoantipyrine (MAA), meclofenamic acid, meloxicam, naproxen, niflumic acid, OH-flunixin, oxy-FBZ, piroxicam, propyphenazone, tolfenamic acid, tolmetin and vedaprofen); 50 mg/l for macrolides (tylosin-tartrate, tiamulin-fumarate, lincomycin, tilmicosin, valnemulin, erythromycin, spiramicin, josamicin, tulathromycin, aivlosin and pirlimycin). Finally all of them were combined to get a 2 mg/l stock standard solution containing all VDs. All the reference standards, VDs and pesticides, were purchased from commercial suppliers.

To prepare EDTA-McIlvain buffer, 74.4 g Na_2EDTA was dissolved in a mixture of 500 ml of 0.1 M citric acid solution and 280 ml of 0.2 M phosphate buffer ($Na_2HPO_4 \cdot 2\ H_2O$); the solution was diluted to 2 l with water and pH was tested to be 4.

6.2.3. *Samples*

Different chicken meat samples were used for method optimization and validation. Poultry meat samples were crushed and keep in the freezer at -20 ºC. They were left to reach room temperature one hour before analysis. Then, portions of 2 g were place in 12 ml tubes for sample treatment.

6.2.4. *Instrumentation*

LC analyses were performed in an Accela HPLC system coupled to an Exactive single-stage Orbitrap MS (Thermo Fisher Scientific, San Jose, Ca, USA). The UHPLC system consists of a degasser, a quaternary pump, an autosampler and a column oven. Separation was performed on a reversed-phase C18 column (Atlantis T3 C18 100mm×3mm, 3 µm; Waters, Milford, Ma, USA) and data were recorded using Xcalibur 2.2 software (Thermo Fisher Scientific).

6.2.5. *Chromatographic and MS conditions*

The chromatographic separation was adapted from some works previously developed in the laboratory for VDs [31,32] and pesticide analysis, among other compounds [33]. Mobile phase consisted of water (solvent A), and MeOH (solvent B), both of them with 2 mM ammonium formiate and 0.016% of formic acid. The gradient profile started at 100% A until 1 min; then it went to 55% A in 2 min and then to 100% B in 6 min and kept until 20 min; finally the gradient was switched to 100% A in 0.5 min and left equilibrated until 25 min. The flow rate was set to 0.3 ml/min and the oven temperature to 35 °C. These chromatographic conditions make possible to have all the analytes distributed along the chromatogram.

Data were acquired between m/z 100 and 1100, the resolving power was set to 50,000 full width at half maximum (FWHM) at m/z 200, resulting in a scan time of 0.5 s. The MS operated with a heated electrospray interface (HESI) in positive and negative ionization using the operation parameters indicated in table 6.1.

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Table 6.1. Operation parameters for MS detection.

HESI source	**Positive**	**Negative**
Sheath gas flow rate	19	19
Aux gas flow rate	7	7
Sweep gas flow rate	0	0
Spray voltage (kV)	2.80	2.00
Capillary temp. (°C)	360	360
Capillary voltage (V)	30	-27.50
Tube lens voltage (V)	80	-80
Skimmer voltage (V)	16	-14.00
Heater temp (°C)	300	300

6.2.6. *Sample treatment*

A very simple and fast extraction method was used to extract the analytes from the meat. Chicken meat was crushed and homogenized; portions of 2 g were placed in 12 ml tubes and spiked at different concentration levels using the working standard solutions. Before addition of extraction solvent, sample were left stand for 15 min (equilibration time). After this time 1 ml of EDTA-McIlvain buffer were added and tubes were vortexed for 10 s to homogenize the mixture. Then, 5 ml of AcN were added and the samples were vortexed for 10 s, to get better homogenization, and shaken head over head for 15 min. After centrifugation (3500 rpm, 15 min), 2.5 ml of supernatant was transferred to a second tube and the solvent was evaporated to dryness at 55 °C under a stream of nitrogen. Finally, the residue was re-dissolved in 200 µl of MeOH. The final extracts were kept in the freezer at -20 °C until analysis. Before injection all vials were centrifuged and transferred to a second vial to avoid that the precipitated residue could reach the chromatographic system.

6.3 *Results and discussion*

6.3.1. *Database elaboration and data processing*

First of all, a database including all the parameters related to the molecular masses of each compound was set up and subsequently used to construct extracted ion chromatograms out of the raw data file to study each compound. A characterization of the analytes must be performed including at least: retention time of each analyte and exact mass of the compound and the most common adducts that could be formed at each polarity ($[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$ or $[M-H]^-$); additionally isotopic pattern using e.g. Bromine (^{79}Br and ^{81}Br), Chlorine (^{35}Cl and ^{37}Cl) and Carbon (^{12}C and ^{13}C) atoms can be established to perform a confirmation tool. The most intense adduct ion, $[M+H]^+$ in most of the cases, was selected for the confirmation of the identity of the compounds. Results are presented in table 6.2.

Detection of analytes was based on peaks, corresponding to the quasi-molecular ion present in the extracted ion chromatogram fulfilling the requirements of LC methods in Commission Decision 2002/657/EC [14] and SANCO 12495/2011 [30] that is: an exact mass deviation tolerance of ±5 ppm, and a retention time tolerance ±2.5% from the retention time obtained from a matrix-matched standard. Xcalibur software was employed.

Table 6.2. Database of studied compounds, including the possible adduct.The most intense detected adduct is labeled in blue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	Screening detection limit (SDL) (µg/kg)	MRL in chicken (µg/kg)
Quinolones	Marbofloxacin	$C_{17}H_{19}FN_4O_4$	362.1390	363.1463	380.1729	385.1283	361.1318	5.47	10	-
	Norfloxacin	$C_{16}H_{18}FN_3O_3$	319.1332	320.1405	337.167	342.1224	318.1259	5.72	10	-
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.1332	332.1405	349.1671	354.1225	330.126	5.76	10	100
	Danofloxacin	$C_{19}H_{20}FN_3O_3$	357.1489	358.1561	375.1827	380.1381	356.1416	5.76	10	200
	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.1645	360.1718	377.1984	382.1538	358.1573	5.82	10	100
	Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	385.1238	386.131	403.1576	408.113	384.1165	6.04	10	-
	Difloxacin	$C_{21}H_{19}F_2N_3O_3$	399.1394	400.1467	417.1733	422.1287	398.1322	6.06	10	300
	Oxolinic Acid	$C_{13}H_{11}NO_5$	261.0637	262.071	279.0976	284.053	260.0565	7.8	10	-
	Nalidixic Acid	$C_{12}H_{12}N_2O_3$	232.0848	233.092	250.1186	255.074	231.0775	8.63	10	-
	Flumequine	$C_{14}H_{12}FNO_3$	261.0801	262.0874	279.114	284.0694	260.0729	8.8	10	400
Tetracyclines	Chloortetracycline	$C_{22}H_{23}ClN_2O_8$	478.1143	479.1215	496.1481	501.1035	477.107	7.25	50	100
	Doxycycline	$C_{22}H_{24}N2O_8$	444.1533	445.1605	462.1871	467.1425	443.146	7.65	10	100
	Oxytetracycline	$C_{22}H2_4N_2O_9$	460.1482	461.1554	478.182	483.1374	459.1409	6.37	50	100
	Tetracycline	$C_{22}H_{24}N_2O_8$	444.1533	445.1605	462.1871	467.1425	443.146	6.24	10	100

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Sulfonamides	Sulfacloropyridazine	$C_{10}H_9ClN_4O_2S$	284.0135	285.0207	302.0473	307.0027	283.0062	6.49	10	100
	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.0524	251.0597	268.0863	273.0417	249.0452	5.54	10	100
	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	310.0736	311.0808	328.1074	333.0628	309.0663	7.35	10	100
	Sulfadimidine	$C_{12}H_{14}N_4O_2S$	278.0837	279.091	296.1176	301.073	277.0765	6.25	10	100
	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	310.0736	311.0808	328.1074	333.0628	309.0663	6.67	10	100
	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264.0681	265.0753	282.1019	287.0573	263.0608	5.9	10	100
	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	270.0245	271.0318	288.0584	293.0138	269.0173	6.08	10	100
	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.0521	254.0594	271.086	276.0414	252.0449	6.46	10	100
	Sulfamethoxypyridazine	$C_{11}H1_2N_4O_3S$	280.0630	281.0703	298.0969	303.0523	279.0558	6.64	10	100
	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	280.0630	281.0703	298.0969	303.0523	279.0558	6.25	10	100
	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	267.0678	268.075	285.1016	290.057	266.0605	5.97	10	100
	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.0572	250.0644	267.091	272.0464	248.0499	5.74	10	100
	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	300.0681	301.0753	318.1019	323.0573	299.0608	7.53	10	100
	Sulfathiazole	$C_9H_9N_3O_2S_2$	255.0136	256.0209	273.0475	278.0029	254.0064	5.56	10	100
	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	267.0678	268.075	285.1016	290.057	266.0605	6.6	10	100
	Dapson	$H_4N_2C_{12}H_8SO_2$	248.0619	249.0692	266.0958	271.0512	247.0547	5.97	10	Forbidden

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Coccidiostats	Halofuginone	$C_{16}H_{17}BrClN_3O_3$	413.0142	414.0214	431.048	436.0034	412.0069	7.42	10	-
	Robenidine	$C_{15}H_{13}Cl_2N_5$	333.0548	334.0621	351.0887	356.0441	332.0475	9.47	10	-
	Diclazuril	$C_{17}H_9Cl_3N_4O_2$	405.9791	406.9864	424.013	428.9684	404.9719	10.61	10	-
	DNC	$C_{13}H_{10}N_4O_5$	302.0651	303.0724	320.099	325.0544	301.0579	10.36	10	-
	Decoquinate	$C_{24}H_{35}NO_5$	417.2515	418.2588	435.2854	440.2408	416.2443	12.8	10	-
	Semduramicin	$C_{45}H_{76}O_{16}$	872.5133	873.5206	890.5472	895.5026	871.5061	13.27	50	-
	Monensin	$C_{36}H_{62}O_{11}$	670.4292	671.4365	688.4631	693.4185	669.422	13.77	10	-
	Salinomycin	$C_{42}H_{70}O_{11}$	750.4918	751.4991	768.5257	773.4811	749.4846	15.11	50	-
	Narasin	$C_{43}H_{72}O_{11}$	764.5075	765.5147	782.5413	787.4967	763.5002	16.17	≥ 100	-
	Maduramicin	$C_{47}H_{80}O_{17}$	916.5395	917.5468	934.5734	939.5288	915.5323	15.22	50	20
	Lasalocid	$C_{34}H_{54}O_8$	590.3819	591.3891	608.4157	613.3711	589.3746	13.98	10	-
	Ponazuril	$C_{18}H_{14}F_3N_3O_6S$	457.0555	458.0628	475.0894	480.0448	456.0483	9.87	10	100
	Toltrazuril	$C_{18}H_{14}F_3N_3O_4S$	425.0657	426.073	443.0995	448.0549	424.0584	10.76	10	-
	Amprolium	$C_{14}H_{18}N_4$	242.1531	243.1604	260.187	265.1424	241.1459	4.46	10	50
	Flubendazol	$C_{16}H_{12}FN_3O_3$	313.0863	314.0935	331.1201	336.0755	312.079	9.46	10	-
	Meticlorpindol	$C_7H_7Cl_2NO$	190.9905	191.9977	209.0243	213.9797	189.9832	6.39	10	-
	Clazuril	$C_{17}H_{10}Cl_2N_4O_2$	372.0181	373.0253	390.0519	395.0073	371.0108	10.06	10	-

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
NSAID	Carprofen	$C_{15}H_{12}ClNO_2$	273.0557	274.0629	291.0895	296.0449	272.0484	10.6	50	-
	Diclofenac	$C_{14}H_{11}Cl_2NO_2$	295.0167	296.0239	313.0505	318.0059	294.0094	10.74	10	-
	FBZ (Phenylbutazone)	$C_{19}H_{20}N_2O_2$	308.1525	309.1597	326.1863	331.1417	307.1452	10.25	50	-
	Fenbufen	$C_{16}H_{14}O_3$	254.0943	255.1015	272.1281	277.0835	253.087	9.88	10	-
	Firocoxib	$C_{17}H_{20}O_5S$	336.1031	337.1104	354.137	359.0924	335.0959	8.88	10	10
	Flufenamic acid	$C_{14}H_{10}F_3NO_2$	281.0664	282.0736	299.1002	304.0556	280.0591	11.23	50	-
	Flunixin	$C_{14}H_{11}F_3N_2O_2$	296.0773	297.0845	314.1111	319.0665	295.07	10.61	10	-
	Indoprofen	$C_{17}H_{15}NO_3$	281.1052	282.1124	299.139	304.0944	280.0979	9.19	10	-
	Ketoprofen	$C_{16}H_{14}O_3$	254.0943	255.1015	272.1281	277.0835	253.087	9.57	10	-
	MAA	$C_{12}H_{15}N_3O$	217.1215	218.1288	235.1554	240.1108	216.1143	5.85	10	-
	Meclofenamic acid	$C_{14}H_{11}Cl_2NO_2$	295.0167	296.0239	313.0505	318.0059	294.0094	11.21	10	-
	Meloxicam	$C_{14}H_{13}N_3O_4S_2$	351.0347	352.042	369.0686	374.024	350.0275	9.4	10	-
	Naproxen	$C_{14}H_{14}O_3$	230.0943	231.1015	248.1281	253.0835	229.087	9.85	50	-
	Niflumic acid	$C_{13}H_9F_3N_2O_2$	282.0616	283.0689	300.0955	305.0509	281.0544	10.74	10	-
	OH-flunixin	$C_{14}H_{11}F_3N_2O_3$	312.0722	313.0794	330.106	335.0614	311.0649	10.14	10	-
	Oxy-FBZ	$C_{19}H_{20}N_2O_3$	324.1474	325.1546	342.1812	347.1366	323.1401	9.29	≥ 100	-
	Piroxicam	$C_{15}H_{13}N_3O_4S$	331.0627	332.0699	349.0965	354.0519	330.0554	8.98	10	-

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
NSAID	Propyphenazone	$C_{14}H_{18}N_2O$	230.1419	231.1492	248.1758	253.1312	229.1347	8.93	10	-
	Tolfenamic acid	$C_{14}H_{12}ClNO_2$	261.0557	262.0629	279.0895	284.0449	260.0484	11.71	50	-
	Tolmetin	$C_{15}H_{15}NO_3$	257.1052	258.1124	275.139	280.0944	256.0979	9.54	10	-
	Vedaprofen	$C1_9H_{22}O_2$	282.1620	283.1692	300.1958	305.1512	281.1547	12.12	10	-
Macrolides	Tylosin	$C_{46}H_{77}NO_{17}$	915.5191	916.5264	933.553	938.5084	914.5119	8.17	10	100
	Tiamulin	$C_{28}H_{47}NO_4S$	493.3226	494.3298	511.3564	516.3118	492.3153	8.04	10	100
	Lincomycin	$C_{18}H_{34}N_2O_6S$	406.2138	407.221	424.2476	429.203	405.2065	5.47	10	100
	Tilmicosin	$C_{46}H_{80}N_2O_{13}$	868.5660	869.5733	886.5999	891.5553	867.5588	7	10	75
	Valnemulin	$C_{31}H5_2N_2O_5S$	564.3597	565.3669	582.3935	587.3489	563.3524	9.08	10	-
	Erythromycin	$C_{37}H_{67}NO_{13}$	733.4612	734.4685	751.4951	756.4505	732.454	8.3	10	200
	Spiramicin	$C_{43}H_{74}N_2O_{14}$	842.5140	843.5213	860.5479	865.5033	841.5068	6.16	100	200
	Josamicin	$C_{42}H_{69}NO_{15}$	827.4667	828.474	845.5006	850.456	826.4595	8.88	10	-
	Tulathromycin	$C_{41}H_{79}N_3O_{12}$	805.5664	806.5736	823.6002	828.5556	804.5591	5.38	10	-
	Aivlosin	$C_{53}H_{87}NO_{19}$	1041.5872	1042.5945	1059.6211	1064.5765	1040.58	9.39	10	-
	Pirlimycin	$C_{17}H_{31}ClN_2O_5S$	410.1642	411.1715	428.1981	433.1535	409.157	7.28	10	-

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Pesticides	Aminopyralid	$C_6H_4Cl_2N_2O2$	205.965	206.9722	223.9988	228.9542	204.9577	4.94	≥ 100	10
	Asulam	$C_8H_{10}N_2O_4S$	230.0361	231.0434	248.07	253.0254	229.0289	5.63	50	100
	Bifenthrin	$C_{23}H_{22}ClF_3O_2$	422.126	423.1333	440.1599	445.1153	421.1188	13.6	≥ 100	50
	Bixafen	$C_{18}H_{12}Cl_2F_3N_3O$	413.031	414.0382	431.0648	436.0202	412.0237	10.6	10	20
	Boscalid	$C_{18}H_{12}Cl_2N_2O$	342.0327	343.0399	360.0665	365.0219	341.0254	9.97	10	50
	Bromoxynil	$Br_2C_6H_2(OH)CN$	274.8581	275.8654	292.892	297.8474	273.8509	9.19	10	50
	Carbaryl	$C_{10}H_7CO_2NHCH_3$	201.079	202.0862	219.1128	224.0682	200.0717	8.78	10	50
	Carbosulfan	$C_{20}H_{32}N_2O_3S$	380.2134	381.2206	398.2472	403.2026	379.2061	13.4	≥ 100	50
	Chlorpropham	$C_{10}H_{12}NO_2Cl$	213.0557	214.0629	231.0895	236.0449	212.0484	10.3	≥ 100	50
	Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	348.9263	349.9335	366.9601	371.9155	347.919	12.11	50	50
	Chlorpyrifos-methyl	$C_7H_7Cl_3NO_3PS$	320.895	321.9022	338.9288	343.8842	319.8877	11.39	100	50
	Cypermethrin	$C_{22}H_{19}Cl_2NO_3$	415.0742	416.0814	433.108	438.0634	414.0669	12.2	≥ 100	100
	Cyproconazole	$C_{15}H_{18}ClN_3O$	291.1138	292.1211	309.1477	314.1031	290.1066	10.41	10	50
	Cyromazine	$C_6H_{10}N_6$	166.0967	167.1039	184.1305	189.0859	165.0894	4.75	10	50
	Deltamethrin	$C_{22}H_{19}Br_2NO_3$	502.9732	503.9804	521.007	525.9624	501.9659	12.39	≥ 100	100
	Diazinon	$C_{12}H_{21}N_2O_3PS$	304.1011	305.1083	322.1349	327.0903	303.0938	11.06	10	50
	2,4 D	$Cl_2C_8H_6O_3$	219.9694	220.9767	238.0033	242.9587	218.9621	9.36	10	-
	Dichlorprop	$C_9H_8Cl_2O_3$	233.985	234.9923	252.0189	256.9743	232.9778	9.96	10	50

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Pesticides	Dichlorvos	$C_4H_7Cl_2O_4P$	219.9459	220.9532	237.9798	242.9352	218.9387	8.52	50	-
	Difenoconazole	$C_{19}H_{17}Cl_2N_3O_3$	405.0647	406.0719	423.0985	428.0539	404.0574	11.21	10	100
	Epoxiconazole	$C_{17}H_{13}ClFN_3O$	329.0731	330.0804	347.107	352.0624	328.0659	10.53	10	10
	Etofenprox	$C_{25}H_{28}O_3$	376.2038	377.2111	394.2377	399.1931	375.1966	13.75	10	10
	Famoxadone	$C_{22}H_{18}N_2O_4$	374.1267	375.1339	392.1605	397.1159	373.1194	10.77	50	50
	Fenpropidin	$C_{19}H_{31}N$	273.2456	274.2529	291.2795	296.2349	272.2384	8.66	10	20
	Fenpropimorph	$C_{20}H_{33}NO$	303.2562	304.2635	321.2901	326.2455	302.249	9.68	10	10
	Fenthion	$C_{10}H_{15}O_3PS_2$	278.02	279.0273	296.0539	301.0093	277.0128	10.03	10	50
	Fenthion-oxon	$C_{10}H_{15}O_4PS$	262.0429	263.0501	280.0767	285.0321	261.0356	9.63	10	50
	Fenthion-oxon-sulfone	$C_{10}H_{15}O_6PS$	294.0327	295.0399	312.0665	317.0219	293.0254	7.08	10	50
	Fenthion-oxon-sulfoxide	$C_{10}H_{15}O_5PS$	278.0378	279.045	296.0716	301.027	277.0305	6.91	10	50
	Fenthion-sulfone	$C_{10}H_{15}O_5PS_2$	310.0099	311.0171	328.0437	332.9991	309.0026	8.75	10	50
	Fenthion-sulfoxide	$C_{10}H_{15}O_4PS_2$	294.0149	295.0222	312.0488	317.0042	293.0077	8.6	10	50
	Fenvalerate	$C_{25}H_{22}ClNO_3$	419.1288	420.1361	437.1627	442.1181	418.1216	12.5	≥ 100	20
	Esfenvalerate	$C_{25}H_{22}ClNO_3$	419.1288	420.1361	437.1627	442.1181	418.1216	12.5	≥ 100	20
	Fipronil	$C_{12}H_4Cl_2F_6N_4OS$	435.9387	436.946	453.9726	458.928	434.9314	10.48	10	10
	Fipronil sulfone	$C_{12}H_4Cl_2F_6N_4O_2S$	451.9336	452.9409	469.9675	474.9229	450.9263	10.67	10	-
	Flonicamid	$C_9H_6F_3N_3O$	229.0463	230.0535	247.0801	252.0355	228.039	4.86	50	30

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Pesticides	Fluazifop-P-butyl	$C_{19}H_{20}F_3NO_4$	383.1344	384.1417	401.1683	406.1237	382.1272	11.5	10	50
	Fluazifop acid	$C_{15}H_{12}F_3NO_4$	327.0718	328.0791	345.1057	350.0611	326.0646	9.88	10	50
	Fluoxastrobin	$C_{21}H1_6ClFN_4O_5$	458.0793	459.0866	476.1132	481.0686	457.0721	10.2	10	50
	Fluquinconazole	$C_{16}H_8Cl_2FN_5O$	375.009	376.0162	393.0428	397.9982	374.0017	10.4	10	20
	Fluroxypyr	$C_7H_5Cl_2FN_2O_3$	253.9661	254.9734	272.0000	276.9554	252.9589	8.21	100	50
	Flusilazole	$C_{16}H_{15}F_2N_3Si$	315.1003	316.1076	333.1342	338.0896	314.0931	10.56	10	20
	Flutolanil	$C_{17}H_{16}F_3NO_2$	323.1133	324.1206	341.1472	346.1026	322.1061	10.01	10	50
	Haloxyfop	$C_{15}H_{11}ClF_3NO_4$	361.0329	362.0401	379.0667	384.0221	360.0256	10.75	10	10
	Haloxyfop-Rmethyl ester	$C_{16}H_{13}ClF_3NO_4$	375.0485	376.0558	393.0824	398.0378	374.0413	11.24	10	10
	Hexythiazox	$C_{17}H_{21}ClN_2O_2S$	352.1012	353.1085	370.1351	375.0905	351.094	12.12	10	50
	Indoxacarb	$C_{22}H_{17}ClF_3N_3O_7$	527.0707	528.078	545.1046	550.06	526.0635	11.07	10	300
	Ioxynil	$C_7H_3I_2NO$	370.8304	371.8377	388.8643	393.8197	369.8231	9.65	10	50
	Ivermectin	$C_{48}H_{72}O_{14}$	872.4922	873.4995	890.5261	895.4815	871.485	13.15	≥ 100	-
	Lambda-Cyhalothrin	$C_{23}H_{19}ClF_3NO_3$	449.1006	450.1078	467.1344	472.0898	448.0933	12.06	≥ 100	20
	Malathion	$C_{10}H_{19}O_6PS_2$	330.0361	331.0433	348.0699	353.0253	329.0288	10.12	10	20
	Malaoxon	$C_{10}H_{19}O_7PS$	314.0589	315.0662	332.0928	337.0482	313.0517	8.53	10	-
	Metaflumizone	$C_{24}H_{16}F_6N_4O_2$	506.1177	507.125	524.1516	529.107	505.1105	11.35	10	20
	Methidathion	$C_6H_{11}N_2O_4PS_3$	301.9619	302.9691	319.9957	324.9511	300.9546	9.64	10	20

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Pesticides	Nicotine	$C_{10}H_{14}N_2$	162.1157	163.1229	180.1495	185.1049	161.1084	2.06	≥ 100	-
	Nitarsone	$C_6H_6AsNO_5$	75.925	247.9535	264.98	269.9354	245.9389	5.78	≥ 100	-
	Parathion-methyl	$C_8H_{10}NO_5PS$	263.0017	264.009	281.0356	285.991	261.9945	8.35	50	20
	Paraoxon-methyl	$C_8H_{10}NO_6P$	247.0246	248.0318	265.0584	270.0138	246.0173	9.76	≥ 100	-
	Permethrin	$C_{21}H_{20}Cl_2O_3$	390.0789	391.0862	408.1128	413.0682	389.0717	13.45	≥ 100	50
	Phosmet	$C_{11}H_{12}NO_4PS_2$	316.9945	318.0018	335.0284	339.9838	315.9873	9.7	10	100
	Phosmet oxon	$C_{11}H_{12}NO_5PS$	301.0174	302.0246	319.0512	324.0066	300.0101	7.94	10	-
	Phoxim	$C_{12}H_{15}N_2O_3PS$	298.0541	299.0614	316.088	321.0434	297.0469	10.99	10	25
	Picloram	$C_6H_3Cl_3N_2O_2$	239.926	240.9333	257.9599	262.9153	238.9188	6.47	50	20
	Pirimiphos-methyl	$C_9H_{16}N_3O_3PS$	277.065	278.0723	295.0989	300.0543	276.0577	10.85	10	50
	Prochloraz	$C_{15}H1_6Cl_3N_3O_2$	375.0308	376.0381	393.0647	398.0201	374.0236	11.13	10	100
	Profenofos	$C_{11}H_{15}BrClO_3PS$	371.9351	372.9424	389.969	394.9244	370.9279	11.66	10	50
	Propargite	$C_{19}H_{26}O_4S$	350.1552	351.1624	368.189	373.1444	349.1479	12.14	10	100
	Prothioconazole	$C_{14}H_{15}Cl_2N_3OS$	343.0313	344.0385	361.0651	366.0205	342.024	11.07	10	50
	Pyrazophos	$C_{14}H_{20}N_3O_5PS$	373.0861	374.0934	391.12	396.0754	372.0789	11.25	10	20
	Pyridate	$C_{19}H_{23}ClN_2O_2S$	378.1169	379.1241	396.1507	401.1061	377.1096	13.24	≥ 100	50
	Pyridate metabolite	$C_{10}H_7ClN_2O$	206.0247	207.0319	224.0585	229.0139	205.0174	8.27	10	50
	Quinoxyfen	$C_{15}H_8Cl_2FNO$	306.9967	308.0039	325.0305	329.9859	305.9894	12.22	10	200

Table 6.2 Continue.

Family	Analyte	Molecular formula	Exact mass	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$	RT (min)	SDL (µg/kg)	MRL in chicken (µg/kg)
Pesticides	Rotenone	$C_{23}H_{22}O_6$	394.1416	395.1489	412.1755	417.1309	393.1344	10.61	10	10
	Roxarsone	$C_6H_6O_6NAs$	262.9406	263.9483	280.9749	285.9303	261.9338	5.43	≥ 100	-
	Spinosyn A	$C_{41}H_{65}NO_{10}$	731.4608	732.4681	749.4947	754.4501	730.4536	10.32	10	-
	Spinosyn D	$C_{42}H_{67}NO_{10}$	745.4765	746.4837	763.5103	768.4657	744.4692	10.61	10	200
	Spiroxamine	$C_{18}H_{35}NO_2$	297.2668	298.274	315.3006	320.256	296.2595	9.07	10	50
	Tau-Fluvalinate	$C_{26}H_{22}ClF_3N_2O_3$	502.1271	503.1344	520.161	525.1164	501.1199	12.62	≥ 100	10
	Tebuconazole	$C_{16}H_{22}ClN_3O$	307.1451	308.1524	325.179	330.1344	306.1379	10.91	10	100
	Tepraloxydim	$C_{17}H_{24}ClNO_4$	341.1394	342.1466	359.1732	364.1286	340.1321	10.42	10	500
	Tetrachlorfenvinphos	$C_{10}H_9Cl_4O_4P$	363.8993	364.9065	381.9331	386.8885	362.892	10.72	10	-
	Tetraconazole	$C_{13}H_{11}Cl_2F_4N_3O$	371.0215	372.0288	389.0554	394.0108	370.0143	10.32	10	20
	Thiabendazole	$C_{10}H7N_3S$	201.0361	202.0433	219.0699	224.0253	200.0288	7.58	10	100
	Thiacloprid	$C_{10}H_9ClN_4S$	252.0236	253.0309	270.0575	275.0129	251.0164	7.47	10	50
	Thiophanate-methyl	$C_{12}H_{14}N_4O_4S_2$	342.0456	343.0529	360.0795	365.0349	341.0384	8.43	10	50
	Topramezone	$C_{16}H_{17}N_3O_5S$	363.0889	364.0961	381.1227	386.0781	362.0816	6.61	10	10
	Triazophos	$C_{12}H_{16}N_3O_3PS$	313.065	314.0723	331.0989	336.0543	312.0577	10.25	10	10

6.3.2. *LC optimization*

A representative group of 166 analytes (79 VDs and 87 pesticides), were detected using standard solutions (100 µg/l and 50 µg/l for VDs and pesticides, respectively). A set of 155 were measured in ESI positive mode and 11 in negative mode. As starting point, a mobile phase consisted of water (solvent A) and MeOH (solvent B), both of them in presence of 2 mM ammonium formiate and 20 µl/l of formic acid. However, as previously reported [31,32], a higher quantity of acid in the mobile phase is necessary in order to overcome some problems such as bad peak shapes for some VDs as quinolones, coccidiostats or macrolides. For such a reason, 160 µl/l of formic acid was added to the mobile phase, giving some examples of the improvement obtained in figure 6.1.

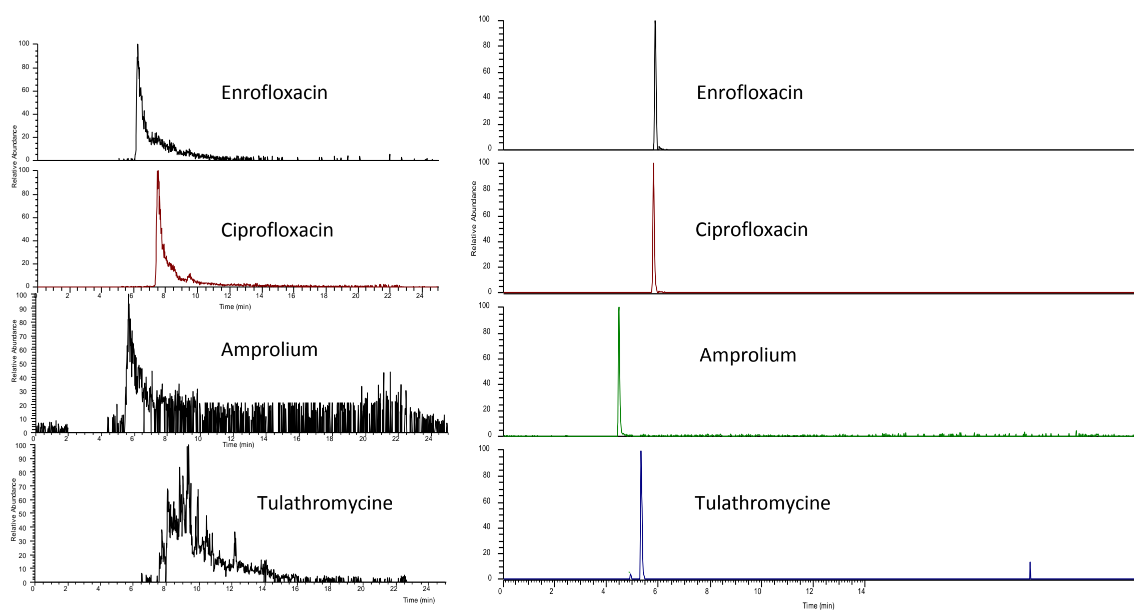

Figure 6.1. Comparison of peaks obtained for several compounds with 20 µl/l (left) and 160 µl/l (right) formic acid added to mobile phase.

6.3.3. *Optimization of sample treatment*

The aim of the sample treatment optimization was to get the cleaner extract in order to have the lower ion suppression and the higher sensitivity for most of the analytes.

The sample treatment initially used was based on previous experiences in the analysis of VDs and is shown in figure 6.2.

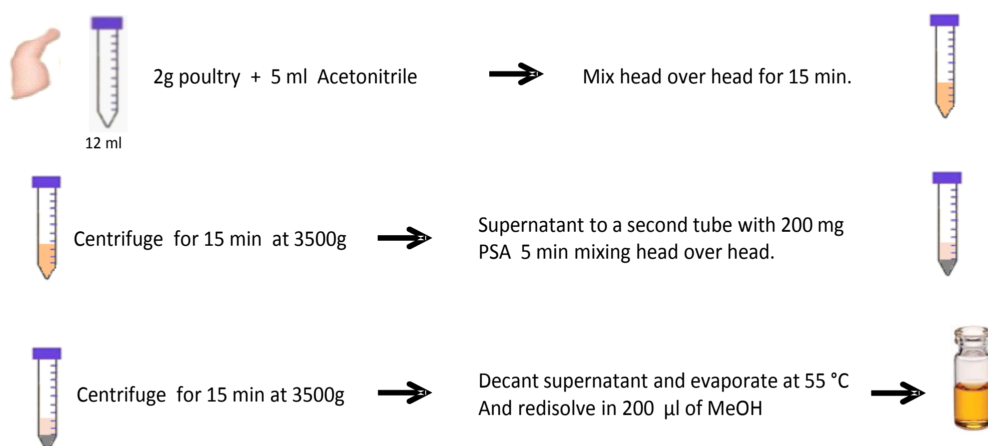

Figure 6.2. Initial sample treatment.

Twenty four compounds were chosen for sample treatment optimization, in order to have a representative group of compounds and to provide evidence that acceptable performance is achieved for all of them; the selection was as follow: 12 VDs, two from each family (chlortetracycline, oxytetracycline, marbofloxacin, enrofloxacin, sulfadiazin, sulfamethoxazole, halofuginone, decoquinate, diclofenac, meloxicam, tiamulin and erythromycin) and 12 pesticides distributed along the chromatogram so as to have compounds with different polarities (cyromazine, dichlorvos, carbaryl, malathion, boscalid, fluazifop-P-butyl, thiabendazole, hexythiazox, propargite, quinoxyfen, pyridate and carbosulfan). Optimization of sample treatment included the following aspects: (1) effect of “volume of evaporated extract”, to get the highest signal for each compound;

(2) addition of EDTA as chelating agent; (3) use of PSA, to get cleaner extracts with good recoveries.

Along this study, tetracyclines were considered as a priority group, as they are one of the most important and applied group of antibiotic agents used in livestock and poultry production, and in addition to the antibiotic use, tetracyclines are also applied as growth-stimulants [34,35], although this practice was forbidden in EU by EC Regulation No 1831/2003 [36]. Thus, one of the main targets of the optimization was to obtain the highest recoveries for these relevant compounds.

6.3.3.1. Volume of evaporated extract

Working with LC-MS a potential problem is that different concentrations of matrix may exhibit matrix effects of variable magnitude. So the potential for matrix effects to occur was considered with the purpose of reach good sensibility for most of the VDs and pesticides. It is known that ion suppression effect is less important when sample extract volume decreases, due to the lower quantity of matrix component in the final extract. However, a lower signal is obtained for all the compounds when the extract volume decreases (reduction of the amount of analyte). So, spiked chicken meat samples were extracted following the previously described procedure and, after extraction and centrifugation, different volumes of final extract (0.5, 1, 2.5 and 5 ml) were evaporated and re-dissolved to study the matrix effect and test out sensitivity.

The results showed that:

- No signal was obtained for the studied tetracyclines from spiked samples; however, signal was obtained when the final extract of a blank sample was

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[34] I. Chopra, M. Roberts, Microbiol. Mol. Biol. Rev., 65 (2001) 232.

[35] A.R. Shalaby, N.A. Salama, S.H. Abou-Raya, W.H. Emam, F.M. Mehaya, Food Chemistry, 124 (2011) 1660.

[36] Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union L 268, 18-10-2003.

spiked. That means that these compounds are not extracted from the sample or are lost during the clean-up step, although their detection in the presence of matrix is possible.

- The matrix effect, ion suppression or signal enhancement (ion suppression < 0%), in the analysis has been studied by comparing the responses of spiked extracts obtained after sample treatment (matrix matched standards) with those of standard solutions (solvent standards), as follows:

$$\textit{Ion suppression (\%)} = (1\text{-}MMR / SSR) \cdot 100$$

MMR = Response of matrix matched standards

SSR = Response of solvent standards

- Thus, an ion suppression of 100% means that no signal was obtained in presence of matrix, which supposes a very high matrix effect. On the contrary, ion suppressions lower than 0% means that higher signal is obtained in presence of matrix than in the solvent standard. Results are indicated in table 6.3.

Table 6.3. Effect of the extract volume on ion suppression on the twenty-four selected analytes (12 VDs and 12 pesticides).

Sample Volume	Ion suppression (%)	Veterinary drugs		Pesticides		All analytes	
		N° of compounds	% of 12	N° of compounds	% of 12	N° of compounds	% of 24
0.5	< 0%	3	25.0	1	8.3	4	16.7
0.5	0-20 %	4	33.3	2	16.7	6	25.0
0.5	20-40 %	2	16.7	4	33.3	6	25.0
0.5	40-70 %	3	25.0	3	25.0	6	25.0
0.5	70-100 %	0	0.0	2	16.7	2	8.3
1	< 0%	3	25.0	1	8.3	4	16.7
1	0-20 %	1	8.3	0	0.0	1	4.2
1	20-40 %	4	33.3	1	8.3	5	20.8
1	40-70 %	2	16.7	7	58.3	9	37.5
1	70-100 %	2	16.7	3	25.0	5	20.8
2.5	< 0%	0	0.0	1	8.3	1	4.2
2.5	0-20 %	2	16.7	0	0.0	2	8.3
2.5	20-40 %	1	8.3	0	0.0	1	4.2
2.5	40-70 %	5	41.7	6	50.0	11	45.8
2.5	70-100 %	4	33.3	5	41.7	9	37.5
5	< 0%	0	0.0	1	8.3	1	4.2
5	0-20 %	0	0.0	0	0.0	0	0.0
5	20-40 %	2	16.7	0	0.0	2	8.3
5	40-70 %	7	58.3	4	33.3	11	45.8
5	70-100 %	3	25.0	7	58.3	10	41.7

As it can be seen in table 6.3., higher ion suppression was produced when a higher volume of extract was used. When 0.5 ml was employed, limited matrix effects were observed. Nevertheless, this volume provided the lowest signals among the studied volumes and, as a consequence, it was not possible to reach low detection limits. On the other side, when 5 ml of extract was used, the highest ion suppression was observed; the signal decreased significantly for some of the compounds, resulting in even lower signals than those

obtained for a lower volume of extract, as 2.5 ml. This effect was very significant for hexythiazox and propargite as it can be observed in figure 6.3.

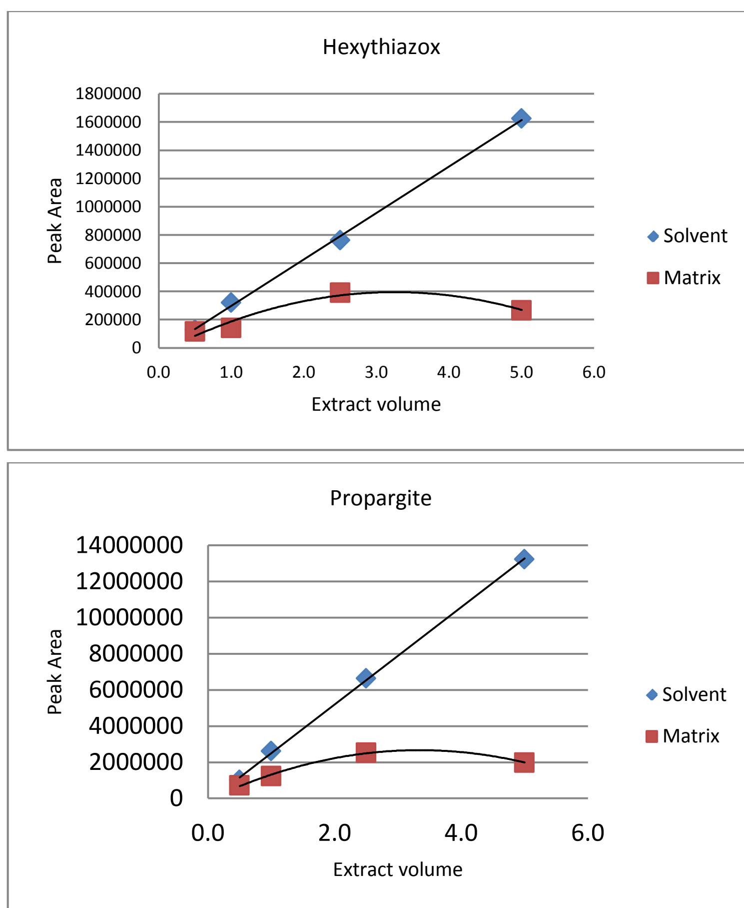

Figure 6.3.Representation of signal vs. volume of extract for matrix matched standards and solvent standards with the same concentration.

– Additionally, recoveries of the selected target analytes were studied in order to know if significant differences were found when the volume of extract changes. The results obtained are shown in table 6.4.

Table 6.4. Effect of the extract volume on the recovery of the twenty-four selected analytes (12 VDs and 12 pesticides).

Sample Volume	Recovery	Veterinary drugs		Pesticides		All analytes	
		N° of compounds	% of 12	N° of compounds	% of 12	N° of compounds	% of 24
0.5	0-20 %	2	16.7	2	16.7	4	16.7
0.5	20-40 %	0	0.0	1	8.3	1	4.2
0.5	40-70 %	1	8.3	0	0.0	1	4.2
0.5	70-120 %	9	75.0	9	75.0	18	75.0
0.5	>120 %	0	0.0	0	0.0	0	0.0
1	0-20 %	2	16.7	3	25.0	5	20.8
1	20-40 %	0	0.0	0	0.0	0	0.0
1	40-70 %	3	25.0	3	25.0	6	25.0
1	70-120 %	7	58.3	6	50.0	13	54.2
1	>120 %	0	0.0	0	0.0	0	0.0
2.5	0-20 %	2	16.7	2	16.7	4	16.7
2.5	20-40 %	0	0.0	0	0.0	0	0.0
2.5	40-70 %	3	25.0	1	8.3	4	16.7
2.5	70-120 %	7	58.3	9	75.0	16	66.7
2.5	>120 %	0	0.0	0	0.0	0	0.0
5	0-20 %	2	16.7	2	16.7	4	16.7
5	20-40 %	1	8.3	0	0.0	1	4.2
5	40-70 %	8	66.7	3	25.0	11	45.8
5	70-120 %	1	8.3	5	41.7	6	25.0
5	>120 %	0	0.0	2	16.7	2	8.3

When 0.5 ml was used the signal was too low in many cases, as commented before. Thus, in order to increase the signal for all the compounds a higher volume of extract was required.

Taking into account all the results, from the ion suppression study and recoveries, finally 2.5 ml of extract was selected as a compromise between recoveries and signals.

6.3.3.2. Addition of EDTA

As it has been indicated before, the analysis of tetracyclines is of special interest when chicken meat analysis is carried out, since they are one of the most used antibiotics in livestock and poultry production. Due to the fact that no signal was obtained for tetracyclines after sample treatment in the previous experience, a more detailed study was carried out. It is well known that tetracyclines rapidly form strong complexes with different cations (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+} ,...) [37], and this could be the reason of their low recoveries from sample matrix. A strategy to release tetracyclines from their metallic complexes is the study of the addition of a chelating agent as EDTA to the matrix. With this purpose, the four tetracyclines included in the studied analytes were considered: chlortetracycline, doxycycline, oxytetracycline, and tetracycline together with a mixture of the other 10 VDs and 12 pesticides selected for the sample treatment study. Thus, different volumes (0, 100, 500 and 1000 µl) of EDTA-McIlvain buffer solution were added to the sample before the extraction solvent (AcN) addition. It is important to note that a very different mixture of sample with the extraction solvent was observed when the EDTA aqueous solution was added. In these cases, mixture of sample and solvent was much better and meat was very well dispersed into the solvent, especially when 500 and 1000 µl were used. Nevertheless, when no aqueous solution was added poultry sample was more like a sticky piece of meat.

Results of recoveries using different volumes of EDTA-McIlvain buffer are shown in table 6.5 for the selected analytes.

[37] J. Zhu, D.D. Snow, D.A. Cassada, S.J. Monson, R.F. Spalding, J. Chromatogr. A, 908 (2001) 177.

Table 6.5. Recoveries of studied compounds using different volumes of EDTA-McIlvain buffer solution.

		Tetracyclines	**Veterinary drugs**		**Pesticides**		**All analytes**	
Volume of EDTA (µl)	**Recovery**	**N° of compounds**	**N° of compounds**	**% of 10**	**N° of compounds**	**% of 12**	**N° of compounds**	**% of 26**
0	0-20 %	4	0	0	3	25	7	26.9
0	20-40 %	0	0	0	0	0	0	0
0	40-70 %	0	3	30	3	25	6	23.1
0	70-120 %	0	7	70	6	50	13	50.0
0	>120 %	0	0	0	0	0	0	0
100	0-20 %	4	0	0	2	16.7	6	23.1
100	20-40 %	0	1	10	0	0	1	3.8
100	40-70 %	0	4	40	1	8.3	5	19.2
100	70-120 %	0	5	50	9	75	14	53.8
100	>120 %	0	0	0	0	0	0	0
500	0-20 %	4	0	0	2	16.7	6	23.1
500	20-40 %	0	0	0	0	0	0	0
500	40-70 %	0	6	60	5	41.7	11	42.3
500	70-120 %	0	4	40	4	33.3	8	30.8
500	>120 %	0	0	0	1	8.3	1	3.8
1000	0-20 %	1	0	0	2	16.7	3	11.5
1000	20-40 %	1	0	0	0	0	1	3.8
1000	40-70 %	2	5	50	5	41.7	12	46.2
1000	70-120 %	0	5	50	5	41.7	10	38.5
1000	>120 %	0	0	0	0	0	0	0

No signals (or very low signals) were obtained for tetracyclines with volumes of EDTA lower than 500 µl. Nevertheless, after addition of 1000 µl of EDTA three of the four tetracyclines studied were detected with recoveries between 20-70%. Thus, a volume of 1000 µl was selected as optimum volume of EDTA-McIlvain buffer solution to obtain higher signals for tetracyclines, with a limited effect for the others VDs and pesticides. Higher volumes of EDTA were not tested in order to minimize the volume of water in the extraction solvent, as it reduced the extraction of non-polar analytes.

6.3.3.3. Use of PSA in the clean-up step

The effect of PSA in the clean-up was studied in order to know if the analytes, specially the acidic ones, were affected by PSA in the presence of water in the extraction solvent.

Different results were found for tetracyclines when PSA was not included in the sample treatment: higher signals were obtained for chlortetracycline, oxytetracycline and doxycline when PSA was not added and no change was obtained for tetracycline (see figure 6.4). Diverse results were obtained for the others VDs and for pesticides; in some cases slightly lower signals were observed without PSA, but still high enough for their sensitive determination. Thus, in order to get higher signals for tetracyclines, addition of PSA was discarded.

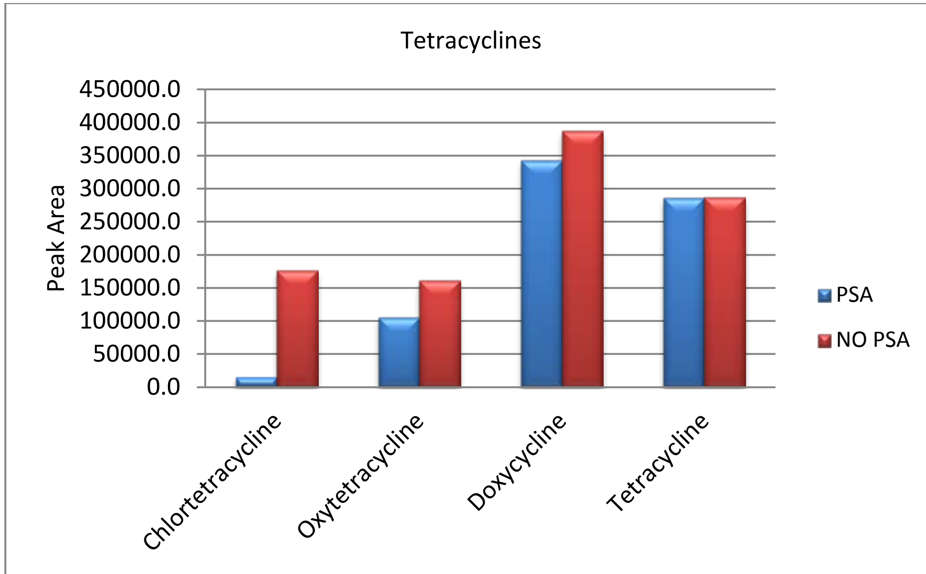

Figure 6.4. Signals obtained for tetracyclines with and without addition of PSA in the clean-up step.

After optimization, the final sample treatment consists in only the extraction step, evaporation of extract and re-dissolution in 200 µl of MeOH (see figure 6.5)

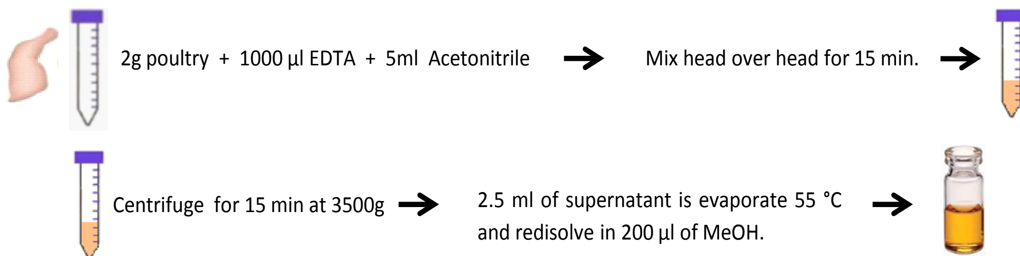

Figure 6.5. Optimized sample treatment.

6.4 *Validation of the screening method*

The validation of multiresidue qualitative screening methods is focused on detectability. So, it is necessary to establish a "screening detection limit" (SDL), defined as the lowest concentration for which it has been demonstrated that a certain analyte can be detected (not necessarily meeting unequivocal identification criteria) in at least 95% of the samples (i.e. a false-negative rate of 5% is accepted) [30]. Following the indications of SANCO 12495/2011, a basic validation of a qualitative method should involve the analysis of at least 20 samples spiked at the anticipated SDL. Thus, in this study 20 different real chicken meat samples were analysed (control samples) before and after be spiked at three different levels: 10, 50 and 100 μg/kg, which allows the determination at the MRLs established by EU for almost all the analytes, taking into account that the lower MRL for the studied compounds corresponds to 10 μg/kg (see table 6.2). In this way, the occurrence of one or no false-compliant results following the analysis of 20 samples at a certain spiked concentration level is sufficient to demonstrate that an analyte can be detected in at least 95% of the samples at this SDL.

After analysis and data processing, the number of false positives (a result wrongly indicating that the analyte concentration exceeds a specified value) and false negatives (a result wrongly indicating that the analyte concentration does not exceed a specified value) were studied using different classification criteria. Based on the simplest criterion, a sample was considered as negative when the mass deviation (mass error) was higher than ±5 ppm, when the retention time deviation was higher than ±2.5% of the one obtained for solvent standards or simply when no signal was observed at the expected retention time [14,30].

However, in order to reduce the number of false positives, an additional parameter was incorporated, based on the establishment of response thresholds (or cut-off values) [28]. Thus, five different thresholds based on absolute responses were selected, as follows: minimum peak areas of (a) 0 (the less restrictive, mentioned above); (b) 5000;

(c) 10000; (d) 20000; (e) 50000 units. However, since using these absolute thresholds it is obvious that the lesser sensitive analytes can be harmed because smaller peaks can be discarded, an additional individual relative threshold (f) was defined as ½ SDL (half of the lower signal obtained for each analyte in any of the 20 samples for the screening detection limit, SDL, 10, 50 or 100 μg/kg).

The percentage of false positives was obtained from the analysis of the 20 control samples, while the percentage of false negatives was calculated from the 20 spiked samples at three different levels (10, 50 or 100 μg/kg), all of them studied at each threshold. As it was expected, the study showed a different number of false positives and negatives depending on the threshold considered. An important reduction of false positives was obtained when the threshold level was increased. However, this supposed a significant increase in the number of false negatives, because a higher number of signals coming from the studied analytes were discarded.

Another option for reduction of false positives is the use of an additional criteria for identification, which can be achieved through additional mass spectrometric information, for example evaluation of full scan spectra, additional accurate mass (fragment) ions, additional product ions (in MS/MS), or accurate mass product ions. Also if the isotope ratio of the ion(s), or the chromatographic profile of isomers of the analyte is highly characteristic it may provide sufficient evidence. The applicability of different second ions (another adduct, an isotope or a fragment) as confirmation criteria are suggested to be regarded as guidance criteria for identification in SANCO 12495/2011 [30] and have been previously reported [28]. In this work, the most abundant isotope as second diagnostic ion was selected. Therefore, isotopic pattern was studied for all positives samples in order to confirm the results by using the most abundant isotope ion, that is, characteristic atoms such as Bromine (^{79}Br and ^{81}Br), Chlorine (^{35}Cl and ^{37}Cl) and Carbon (^{12}C and $1^{3}C$) as additional confirmation criteria (see example of one isotopic pattern in figure 6.8).

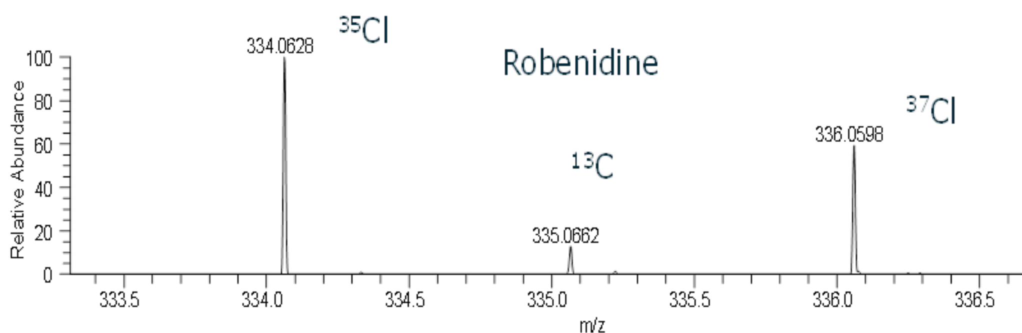

Figure 6.8. Example of Isotope pattern for robenidine.

The peak corresponding to the isotope ion was considered as a “real peak” when the difference between retention time of isotope and main adduct was lower than 1% and the intensity ratio between the main adduct and the isotope was similar to the one obtained for the spiked samples.

Following the previous criteria, the results obtained for the study of false positives and negatives for VDs and pesticides were as follows:

Veterinary drugs:

- Seventy nine VDs were studied by this screening method: 73 were measured in ESI positive mode and 6 in ESI negative mode; that means 1580 possibilities (79 analytes × 20 samples) of having a false positive for VDs. When the less restrictive absolute threshold was used (Area > 0), a total of 90 positives was observed, while the more restrictive absolute threshold (Area > 50000), showed only 12. Also, when the individual relative threshold for each compound was used (Area >½ SDL) the number of false positives was reduced to 6. The results obtained (expressed as percentages) with each criterion are shown in figure 6.6.

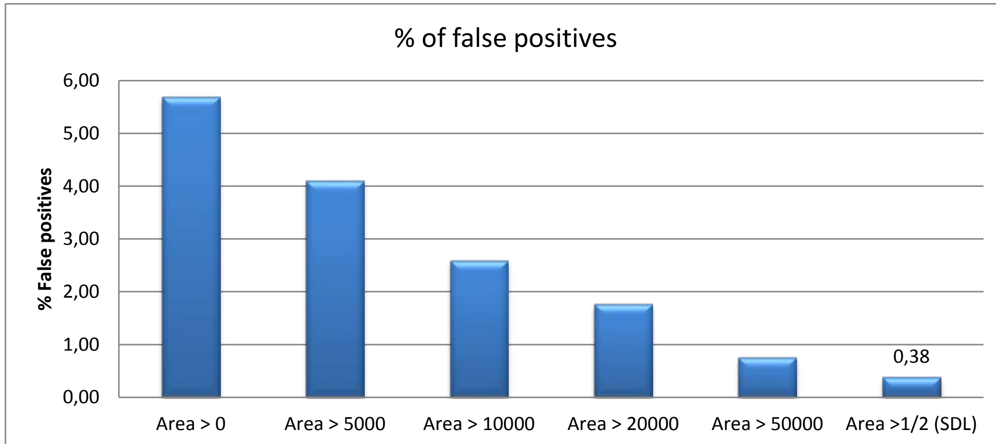

Figure 6.6. Percentage of false positives of VDs found with each decision threshold.

On the other hand, the number of false negatives was established at each spiked concentration level, and it increased when the decision threshold involved higher areas, as can be observed in figure 6.7.

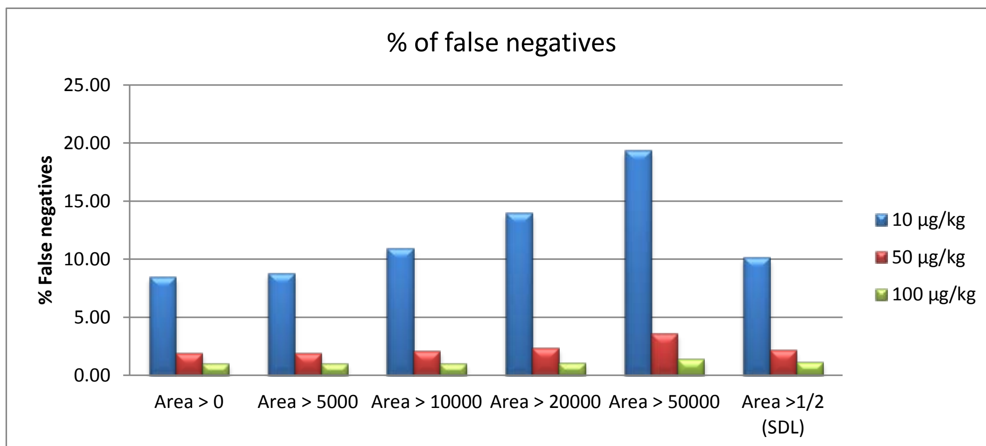

Figure 6.7. Percentage of false negatives of VDs found with each decision threshold.

Subsequently, the percentages of VDs detectable in chicken meat with 95% confidence were calculated for each threshold. Thus, a VDs was not included in this classification if more than 5% of the studied samples were considered as negatives

(that is, it could not be detected at 95% confidence at that concentration if more than 1 of 20 samples were not compliant). The results are shown in table 6.7.

Table 6.7. Percentage of VDs detectable with 95% confidence (less than a 5% of false negatives).

Decision threshold	10 μg/kg	50 μg/kg	100 μg/kg
Area > 0	84	94	97
Area > 5000	82	94	97
Area > 10000	82	94	97
Area > 20000	78	92	97
Area > 50000	72	91	97
Area >½ (SDL)	81	94	97

Among the different used criteria “Area > ½ (SDL)” (specific for each compound) shows the best balance between number of false positives and negatives, and number of VDs detectable with 95% confidence.

In addition, false positives observed when the less restrictive threshold was used (peak area > 0) were further studied through the search of an additional diagnostic ion such as an isotope ion. Using this criteria (as described above) three positives samples, for tulathromycine, tetracycline and DNC were confirmed. In all the other cases where a positive was studied (amprolium, danofloxacin, sulfapyridine, difloxacin, sarafloxacin, sulfadimidine, sulfametoxazole, pyrlimicin, sulfaquinoxalin, doxycycline, tiamulin, nalidixic acid, flumequine, josamicin, propyphenazone, piroxicam, valnemulin, tolmetin, naproxen and salinomycin), the confirmation was not possible because no peak corresponding to the isotopic pattern of these compounds was found.

Pesticides:

- In the case of pesticides, the number of studied compounds was 87. From them 81 were detected in ESI positive mode and 6 in ESI negative mode. The number of studied blank samples was 20, which means 1740 possibilities (87 analytes × 20 samples) of having a false positive. Following the same criteria as for VDs, the percentages of false positives found are shown in figure 6.9. When the less restrictive threshold was used (peak area > 0) a total of 6 positives was observed, while the most restrictive threshold (peak area > 50000) and the specific threshold for each compound did not show any positive.

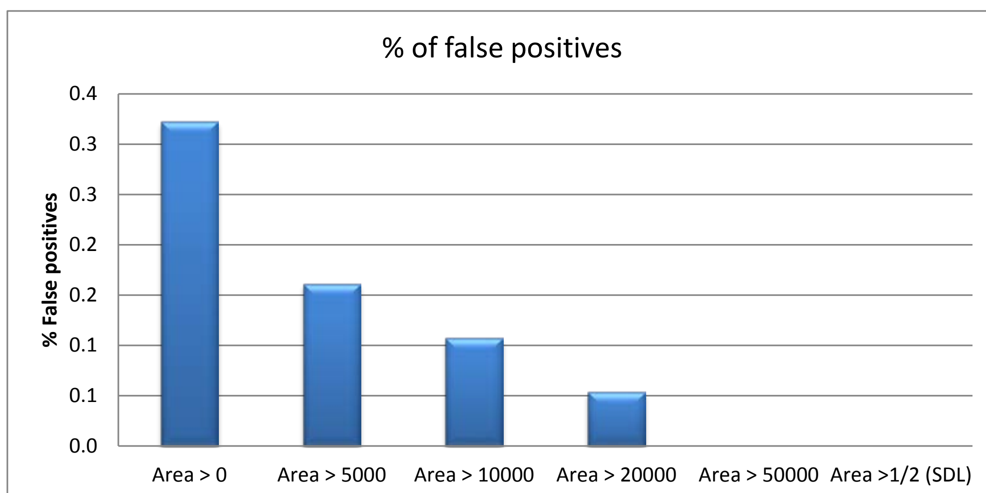

Figure 6.9. Percentage of false positives of pesticides found with each decision threshold.

On the other hand, the number of false negatives increased when the area taken as decision threshold was higher, as can be observed in figure 6.10. As can be observed, the number of false positives was lower and the number of false negatives higher than for VDs; this fact was due to the lower sensitivity observed for some pesticides, mainly belonging to the pyretroid family.

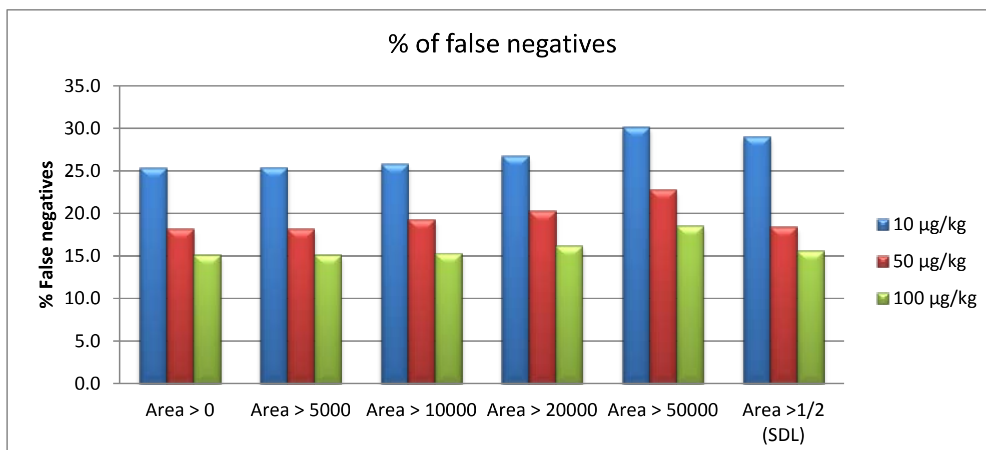

Figure 6.10. Percentage of false negatives of pesticides found with decision threshold.

As in the case of VDs, the relative individual criterion for each pesticide (Area > ½ SDL) shows the lower number of false positives and negatives among the different criteria used.

The percentages of pesticides detectable in chicken meat samples (following the same criterion as for VDs) with 95% confidence, using the different threshold are shown in table 6.8.

Table 6.8. Percentage of pesticides detectable with 95% confidence (less than a 5% of false negatives).

Decision threshold	**10 µg/kg**	**50 µg/kg**	**100 µg/kg**
Area > 0	70	78	80
Area > 5000	70	78	80
Area > 10000	69	77	80
Area > 20000	69	75	80
Area > 50000	63	74	79
Area > ½ (SDL)	66	77	80

Pesticides isotopic pattern was also studied to have another confirmation criterion when classifying a sample as positive, following the same criteria as for VDs. Although positive results for fenpropimorph, tetraconazole, rotenone, 2,4-D and

etofenprox were obtained in some samples when the less restrictive threshold was considered, the confirmation was not possible because no peak corresponding with the most intense isotope ion of these compounds was found.

In general, isotope ion detection is difficult due to the lack of sensitivity of ions without characteristic atoms like Cl, Br or S, and with low numbers or carbons in their structure. In the case of brominated and polichlorinated compounds sensitivity can reach to be similar to the quasi-molecular ion.

As a summary of this validation study, we can conclude that:

- SDL for the 147 detected compounds were 10, 50 y 100 µg/kg for 127, 17 y 3, respectively. That means that 86% of the compounds can be detected at 10 µg/kg after sample treatment, satisfying MRL established by EU (see table 6.2) for most of the studied compounds.
- The best compromise between false positives and negatives, when a response threshold has been used to classify them, was achieved when the relative individual criteria for each analyte (Area > ½ SDL) was used to classify obtained results. As a summary, table 6.9. shows the results obtained for all the compounds.

Table 6.9. Comparison of thresholds in terms of false positives and detected compounds.

	VDs	% of VDs detected with 95% confidence			Pesticides	% of Pesticides detected with 95% confidence		
	False positives	**10 µg/kg**	**50 µg/kg**	**100 µg/kg**	**False positives**	**10 µg/kg**	**50 µg/kg**	**100 µg/kg**
Area > 0	90	84	94	97	6	70	78	80
Area > 5000	65	82	94	97	3	70	78	80
Area > 10000	41	82	94	97	2	69	77	80
Area > 20000	28	78	92	97	1	69	75	80
Area > 50000	12	72	91	97	0	63	74	79
Area > ½ SDL	6	81	94	97	0	66	77	80

- When an additional ion is studied, isotope ion in this case, the number of false positives was reduced from 90 to 3 in case of VDs and from 6 to 0 in the case of pesticides. However, due to the lack of sensitivity of the most abundant isotope for many compounds, alternative additional ions, like fragment ions, could help to reach a higher degree of confirmation.
- 147 compounds could be detected with 95% confidence in presence of matrix from a total of 166:
    - In the case of VDs, following the established criteria, only 2 compounds from 79 (OxyFBZ and Narasin) were not detectable at any concentration level.
    - In the case of pesticides, 17 compounds (10 of them from the pyretroid family) were not detectable at any concentration level after sample treatment; however, they were detectable when standard solutions were analysed. This could be due to a very strong matrix effect.
    - Carbosulfan and pyridate were not detected but their metabolites (carbofuran and pyridate metabolite, respectively) were detected.

Maybe the reason was that they are degraded very fast in samples so it is not possible to find them after sample treatment. So, this method cannot be employed for detection of carbosulfan or pyridate but it could confirm their absence, if their metabolites are not detected. Although carbofuran was not included in the group of selected compounds, it was studied later thanks to the possibility of achieved retrospective analysis of untargeted compounds.

- Paraoxon methyl showed peaks at the different spiked concentrations but its retention time was not stable and peak area changed without any relation with concentration, so finally no peaks have been considered coming from paraoxon methyl.
- Very small signal or no signal was obtained for dichlorvos, because it is a very volatile compound and maybe it was lost in the evaporation step.

6.5 *Conclusions*

LC-HRMS has shown to be a very useful technique for multiresidue screening of VDs and pesticides in chicken meat, using a generic sample treatment. The studies carried out have shown that the amount of matrix in the final extract has an important influence in efficiency, in terms of recovery and sensitivity of the method. Furthermore, addition of EDTA was necessary to detect tetracyclines. On the other hand, some pesticides that were not detectable in sample matrix, most of them pyretroids, would need a different sample treatment since they were not detectable even at the highest concentration levels.

When an absolute response threshold is used, less sensitive compounds could be harmed since smaller peaks can be discarded, an acceptable percentage (<5%) of false negatives results for 81% of VDs and 66% of pesticides and the best compromise

between false positives and negatives was observed when the relative individual threshold (Area > ½ SDL) was considered.

Despite using HRMS, an additional identification criterion is recommended for a higher degree of confirmation. Search of the most abundant isotope ion or molecular fragment, working in MS/MS mode, is a very interesting way of obtaining more qualifiers, but that means an important additional effort. In the latter case, molecular fragment exact mass must be calculated, which means that fragmentation pattern must be well known to identify every molecular fragment. So, study of isolated molecule fragmentation is necessary due to some compounds or matrix component can co-elute and the fragments used for their confirmation must be characteristic of each one. Therefore, the establishment of a molecular fragment database would be very interesting to get an unequivocal identification in future works. In this work the use of most abundant isotope ions has proven to be useful for false positive reduction without any impact in the number of false negatives, although other criteria could be more appropriate for the less sensitive compounds.

The use of HRMS technique has shown the following advantages:

- A large number of compounds can be simultaneously analyzed using a generic extraction method and generic conditions of analysis.
- Only one injection is needed to provide enough information for screening and confirmation purposes.
- Retrospective analysis for the determination of untargeted compounds can be performed.
- Several criteria such as isotopic pattern and fragments can be used for confirmation purposes.

The main disadvantage of this work was the consumed time for manual data processing, which decreased enormously the sample throughput, due to every peak was checked manually and individually; in this sense, the use of automated data

processing software, as ToxID from Thermo Scientific, would imply a faster treatment of the data. Including in the same data processing file the main adducts and isotopes, a qualifier could be obtained in the same time that data processing is achieved, meaning a great saving of time. Currently, this program is being tested and the results are being compared with those obtained by manual integration.

The publication of this work is preparation.

Final Conclusions

In this Thesis different methods for determination of contaminants, Qns and others residues, in food and environmental samples have been developed. Different analytical techniques have been evaluated, both miniaturized,as CE and capillary HPLC, and non-miniaturized as UHPLC. They had been coupled to different detection systems of great sensibility and selectivity as LIF, MS/MS and HRMS. That has contributed to increase the number and improvement of analytical methods for food and water quality control. In addition, different sample treatments have been assessed as an alternative to those previously reported in order to improve efficiency and sample throughput.

The assessment of these analytical techniques (LC and CE) coupled to the mentioned detection systems, has shown their usefulness on allowing the sensitive determination of Qns and other residues. Moreover, the advantages and drawbacks of each methodology have been emphasized, founding significant differences among them:

- CE methods implied lower consumption of solvent and reactive, especially organics solvent, than chromatographic methods. Moreover CE consumables meant lower cost than LC ones.

- Both CE and UHPLC showed a very high efficiency, specially the last one, in comparison to capillary HPLC.

- CE methods supposed longer analysis time (more than 20 min to determine 6 Qns), than those involving LC. Thus, 7 Qns were determined in 15 min and 19 Qns in less than 5 minusing capillary HPLC and UHPLC, respectively.

- Concerning to detection methods, although LIF is a very selective technique, a good resolution is mandatory in order to quantify the Qns, whereas the use of MS allowed the unequivocal identification of compounds with similar retention times.

- In terms of sensitivity, LIF showed much lower LOQs than conventional fluorescence in comparison with other reported works. On the other side

MS/MS allowed also very low LOQs, in the same order than those obtained using LIF.

- Moreover, HRMS provided very good LOD, not only for Qns, but for almost all the pharmaceuticals and pesticides included in the multirresidue analysis of chicken meat. Although triple quadrupole MS/MS has proven be a powerful tool for confirmation analysis, HRMS has shown some advantages. For example: it make possible to determine a much higher number of analytes in a single run, it does not need an exhaustive optimization previous to the analysis as in the case of triple quadrupole, and untargeted analysis can be carried out.

Another target of this Thesis has been the study of alternative sample treatments, in order to increase selectivity and sensibility. So, specifics sample treatment, as molecularly imprinted polymers, and generics sample treatments, as SPE or QuEChERS, for Qns analysis have been assessed.

- Thus, when SPE was used to analyze water samples by CE-LIF, a high preconcentration factor, along with a sensitive detection, made possible reaching very low LODs. On the other hand, the use of QuEChERS did not allow such a high preconcentration, as this treatment involved a dilution of the sample. In the same way, MIPs did not allow use very high sample extract and high preconcentration factor could not be achieved.
- In terms of selectivity, it is clear that MISPE is the most selective sample treatment since the used MIPs were specifically designed for Qns extraction. When MISPE was used together with CE-LIF,very cleanselectroferograms were obtained, and no peaks were found comigrating with Qns. However, some interfering peaks were found when MISPE was used together with capillary HPLC. On the other side, QuEChERS allowed analyze a higher number of analytes together with

capillary HPLC although dirtier extracts were obtained, as can be observed in chapter 3.

- QuEChERS-based methodologies have shown to be ideal when very selective detection techniques, as LIF and MS/MS, were used, especially when it is necessary to determine a high number of analytes, as in multirresidue methods.

As a summary, the most remarkably analytical characteristics of the developed methods are shown in table C1.

Table C1. Comparison of methods developed in this Thesis.

Analytes	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LOD
Ofloxacin, lomefloxacin, norfloxacin, danofloxacin, enrofloxacin, sarafloxacin	Well and consume water	SPE (Oasis HLB and Strata X)	CE-LIF (BGE: 125 mM phosphoric acid separation buffer at pH 2.8, with 36 % methanol)	25	0.30 – 1.90 ng/l
Ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin	Milk and Kidney	MIPSPE	E-LIF (BGE: 125 mM phosphoric acid separation buffer at pH 2.8, with 36 % methanol)	25	0.17– 0.98 µg/kg for milk 1.10 to 10.5 µg/kg for kidney
Ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacindifloxacin, oxolinic acid, flumequine	Milk	MIPSPE and QuEChERS	Capillary HPLC-LIF. Mobile phase: A- 10 mM aqueous citric acid solution (pH 4.75) B- 10 mM citric acid in Acetonitrile	15	0.40 – 60 µg/kg
Marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, flumequine,	Honey Royal jelly and propoli	QuEChERS	UHPLC – MS/MS. Mobile phase: A-0.02% aqueous formic acid solution B- Acetonitrile	5	0.2 – 4.1 µg/kg

Continue table C1

Analyte	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LOD
Marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, flumequine, oxolinic acid, norfloxacin, cinoxacin, pefloxacin, nalidixic acid, pipemidic acid, enoxacin, lomefloxacin, fleroxacin, orbifloxacin, moxifloxacin	Wellwater	Liquid-Liquid extraction	UHPLC – MS/MS Mobile phase: A-0.02% aqueous formic acid solution B- Acetonitrile	5	10 – 90 ng/l
77 Pharmaceutical and 70 pesticides	Chicken meat	Solid-liquid extraction with acetonitrile	HPLC - HRMS Mobile phase: A-Water 2 mM ammonium formiate and 0.016 % formic acid B- MeOH 2 mM ammonium formiate and 0.016 % formic acid	25	10 – 100 µg/kg

En esta Tesis se han desarrollado diferentes métodos para la determinación de contaminantes, fundamentalmente Qns pero también otros residuos, en alimentos y muestras medioambientales. Para ello se han evaluado diferentes técnicas instrumentales separativas, tanto miniaturizadas (como CE y HPLC-capilar) como de ultra resolución (UHPLC) acopladas a diferentes sistemas de detección de gran sensibilidad y selectividad como LIF, MS/MS y HRMS. Con ello se ha contribuido al aumento y la mejora de los métodos de control de la calidad de alimentos y aguas. Además se han estudiado y propuesto metodologías de tratamientos de muestra alternativas a las ya existentes que permiten aumentar la eficacia y el rendimiento de los análisis.

El estudio de las diferentes técnicas utilizadas, LC y CE, acopladas a los métodos de detección indicados, ha mostrado que son útiles y permiten llevar a cabo la determinación sensible de las Qns y de otros residuos. Aunque se han encontrado importantes diferencias que se comentan a continuación:

- Los métodos de CE implicaron un menor consumo de reactivos y disolventes, especialmente disolventes orgánicos, que los métodos cromatográficos. Además los consumibles de la CE supusieron un coste inferior a los de HPLC.
- Tanto la CE como la UHPLC han mostrado poseer una elevada eficacia, especialmente esta última, en comparación al método de HPLC capilar.
- Se ha encontrado que los métodos de análisis de CE eran más largos (más de 20 minutos para determinar 6 Qns), que los desarrollados con LC. Así, cuando se utilizó la HPLC capilar se pudieron determinar 7 Qns en 15 minutos y cuando se utilizó la UHPLC se determinaron 19 Qns en menos de 5 minutos.
- En cuanto a los métodos de detección, aunque la detección LIF es altamente selectiva, el obligatoria una buena resolución de las Qns a determinar para una

adecuada cuantificación, mientras que la MS ha permitido diferenciar e identificar compuestos con igual tiempo de retención.

- En cuanto a la sensibilidad, hay que señalar que LIF permitió alcanzar unos LOQ más bajos si comparamos con trabajos publicados anteriormente. Por otra parte la MS/MS también ha permitido alcanzar unos LOD muy bajos del mismo orden que los encontrados para LIF.

- Así mismo, el uso de la HRMS ha permitido alcanzar muy buenos límites de detección, no solo para Qns, si no para la mayoría de los fármacos y pesticidas incluidos en el estudio multirresiduo. Aunque la espectrometría de masas de triple cuadrupolo ha demostrado ser tremendamente útil en los métodos de confirmación, la HRMS ha mostrado tener algunas ventajas respecto a esta. Por ejemplo permite el análisis de un número de compuestos muchísimo mayor, no necesita de una optimización instrumental previa tan exhaustiva como el triple cuadrupolo y permite el análisis de desconocidos.

Otro de los objetivos planteados fue el estudio de tratamientos de muestra alternativos para evaluar la selectividad y sensibilidad de los mismos. Por ello se han evaluado desde métodos muy específicos para el análisis de Qns como es el uso de MIPs hasta métodos generales como son la SPE o los QuEChERS, para el análisis de Qns:

- Así, cuando se empleó la extracción en fase sólida para el análisis de muestras de agua mediante CE-LIF se alcanzaron LOQs muy bajos ya que se puede conseguir un factor de preconcentración muy elevado y la detección era muy sensible. En cambio, el uso de métodos basados en la metodología QuEChERS (extracción líquido-líquido o sólido-líquido) no permitió tan elevada preconcentración ya que el tratamiento implica la dilución de la muestra. Algo similar ocurre cuando se utilizaron los MIPs para el tratamiento de muestras de alimentos: no fue posible utilizar extractos de muestra muy elevadas y la preconcentración no pudo ser muy elevada.

- En cuanto a la selectividad de cada tratamiento, parece claro que los MIPs empleados son los más selectivos ya que están diseñados especialmente para extraer exclusivamente las Qns de las posibles matrices. Cuando se utilizaron junto con CE-LIF se encontraron electroferogramas muy limpios sin que aparecieran picos comigrando con ninguna de las Qns estudiadas. Sin embargo, sí se encontraron sustancias coeluyendo con algunas Qns estudiadas cuando se llevó a cabo el análisis mediante HPLC capilar–LIF. Por el contrario, los QuEChERS permitieron el análisis de un mayor número de compuestos con esta última misma técnica a pesar de que los extractos eran menos limpios, como puede observarse en los cromatogramas correspondientes (ver capitulo 3).
- Los tratamientos basados en los QuEChERS se han mostrado como idealea cuando se utilizan métodos de detección muy selectivos como son LIF y MS/MS. Especialmente es así cuando se quieren estudiar gran número de compuestos de naturaleza muy diferente como es el caso de los análisis multirresiduo.

En la tabla C1, y para acompañar las conclusiones anteriores, se pueden observar las características más notables de cada uno de los métodos desarrollados en esta Tesis.