Anásis de quinolonas en alimentos de origen animal mediante cromatografía líquida micelar

Tesis presentada por Khaled Tayeb-Cherif

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CERTIFIQUEN

Que la present Memòria, ***Análisis de quinolonas en alimentos de origen animal mediante cromatografía líquida micelar*** constitueix la tesi doctoral de:

KHALED TAYEB-CHERIF

Així mateix, certifiquen haver dirigit i supervisat les parts teòriques, metodològiques, instrumentals i aplicacions dels diferents treballs, així com també la seua redacció.

I perquè conste als efectes oportuns, signem la present.

Josep Esteve i Romero　　Samuel Carda Broch　　Juan Peris Vicente

Castelló de la Plana, 03 de Abril de 2017

A todos mis seres queridos. Destacar

a mis padres por su sabiduria, espiritu de lucha

y de trabajo transmitido

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Prólogo

El trabajo que se presenta en esta Memoria se enmarca dentro de una línea de investigación implementada por el grupo Química Bioanalítica (código 029 de la Oficina para la Cooperación en Investigación y Desarrollo Tecnológico), adscrito al Departamento de Química Física y Analítica de la Universitat Jaume I. Esta línea tiene como objetivo el desarrollo y validación de nuevos métodos de análisis en el campo de la segutidad alimentaria mediante el uso de disoluciones micelares como lixiviantes, solubilizantes y fases móviles en cromatografía líquida. Debido a su relevancia social, ha recibido apoyo en forma de financiación por parte de la Universitat Jaume I, a través del proyecto P1.1B2012-36: *Modificación de los mecanismos de retención a través de la introducción de equilibrios secundarios para la separación de compuestos básicos en cromatografía líquida de alta resolución*.

En la actualidad, mantener un buen estado de salud es prioritario para la mayor parte de la población. Esto se debe al incremento de la esperanza de vida y a un mayor conocimiento acerca de los diferentes factores que influyen en el estado físico. Sin duda, la alimentación se ha revelado como uno de los más importantes, por lo que la sociedad exige cada vez más la producción de alimentos más saludables, así como una mayor información y control acerca de su calidad y su composición química y biológica. Para ello, se han elaborado multitud de regulaciones y protocolos relativos a la seguridad alimentaria. Este concepto consiste en una serie de rutinas acerca de la preparación, manejo y almacenamiento de los alimentos, con el objetivo de maximizar sus propiedades nutracéuticas y evitar la presencia de sustancias que puedan ser dañinas para la salud. Para ello, se han desarrollado normativas de ámbito nacional e internacional, en las cuales se registran las sustancias y bacterias que son potencialmente nocivas o que son marcadores de un tratamiento inadecuado del alimento, y se fija la concentración residual máxima tolerable. Las normativas y regulaciones aplicadas son diferentes en cada país, y pueden variar enormemente de uno a otro. Su cumplimiento es responsabilidad del productor, distribuidor y los posibles intermediarios, mientras que a las agencias gubernamentales compete la verificación de su cumplimiento, a través de la inspección de los alimentos. Esto es especialmente importante

en el caso de productos importados y exportados, debido a la imposibilidad del receptor de controlar el alimento en origen y a la divergencia entre normativas entre países. El incumplimiento de la regulación puede acarrear graves consecuencias para los agentes involucrados, como la retirada del lote contaminado, multa, y una imagen negativa y el cierre del mercado para el productor y/o todo su país de orogen según sea la gravedad del caso.

La seguridad alimentaria es muy estricta en el caso de los productos de origen animal, de los cuales se tiene una imagen de poco saludables. Los compuestos químicos dañinos que se pueden encontrar son, mayoritariamente: algunos endógenos naturales, compuestos generados en situaciones de stress y putrefacción natural, aditivos potenciadores de sabor y conservantes, contaminantes ambientales incorporados en la cadena trófica y fármacos administrados para la prevención y tratamiento de enfermedades. Entre ellas, cabe destacar los antibióticos, cuya peligrosidad ha llegado al conocimiento del público debido al lanzamiento de varias alertas alimentarias por parte de la Organización Mundial de la Salud (OMS) y de la Organización para la Alimentación y la Agricultura (FAO).

Los antibióticos son compuestos sintéticos de bajo peso molecular, que tienen la capacidad, a bajas concentraciones, de inhibir el crecimiento de bacterias y microorganismos. Entre ellos, cabe destacar las quinolonas, que destacan por su capacidad de atacar diversas familias de patógenos y por su potente actividad farmacológica. Son ampliamente utilizadas como fármacos en Medicina, Veterinaria y Agricultura. En seres humanos, se prescriben para el tratamiento de infecciones de tipo urinario y respiratorio, entre otros. En las granjas industriales y en apicultura, se administran a los animales criados para consumo humano, mezclado en la comida o mediante inyecciones, para prevenir y tratar diversas enfermedades infecciosas y para acelerar su crecimiento. Debido a su excesivo uso, los alimentos derivados, como la miel y la carne, son susceptibles de contener cantidades residuales de estos fármacos antimicrobianos. El consumo de alimentos contaminados puede provocar reacciones alérgicas en individuos hipersensibles y eliminar microorganismos intestinales, pero el efecto más grave es que estimula el desarrollo de bacterias patógenas resistentes a los antibióticos. Esto no sólo afecta directamente al consumidor, sino que se puede transmitir al resto de la población, dando lugar a epidemias de infecciones que no pueden ser curadas por los antibióticos habituales. Esto representa un grave riesgo para la salud, reduce la vida útil de los antibióticos y obliga al desarrollo de fármacos más potentes. A parte de estos efectos adversos inmediados, pueden existir otros desconocidos a largo plazo. De hecho, numerosos

organismos internacionales recomiendan reducir o discontinuar su uso. Por todos estos motivos, queda más que justificado su control en muestras alimentarias de origen animal. En 2009, la Comisión Europea lanzó la normativa 37/2010, que regula la cantidad máxima admisible de cada antibiótico en los diferentes tipos de alimentos. Esta regulación debe ser aplicada por los alimentos producidos y consumidos en los estados miembros de la UE. Para garantizar el cumplimiento de la normativa, los alimentos deben ser analizados por laboratorios oficiales de control, los cuales deben disponer de métodos analíticos prácticos, fiables para la detección de estos antibióticos en miel y carne de diversos animales a los niveles máximos permitidos.

La categoría de quinolonas incluye numerosos compuestos. Cabe destacar el ácido oxolínico, flumequina (primera generación), enrofloxacino, danofloxacino, difloxacino, marbofloxacino, ciprofloxacina y sarafloxacina, (2ª generación). Todas ellas comparten un núcleo de quinolona, pero la gran cantidad de posibles sustituyentes hace que sus estructuras sean muy diversas. Excepto el ácido oxilínico, todas incluyen como mínimo un átomo de flúor, por lo que se denominan fluoroquinolonas. Existen en la bibliografía numerosos métodos para la determinación de estos compuestos en productos de origen animal mediante numerosas técnicas. Los más utilizados se basan en la lixiviación de la muestra con un disolvente orgánico, y resolución de los analitos mediante HPLC hidroorgánica convencional de fase reversa (RP). La disponibilidad de una herramienta (las disoluciones micelares) que posibilitan la mejora de los procedimientos experimentales, por parte del grupo de investigación, y el interés que despierta es estudio de la presencia de antibióticos en muestras alimentarias de origen animal, condujo a la propuesta de Tesis Doctoral, cuyos resultados se describen en la presente memoria.

Las disoluciones micelares son disoluciones acuosas de tensioactivo por encima de su concentración micelar crítica. Bajo estas condiciones, los monómeros de tensioactivos se organizan en agregados esféricos, las micelas, que se dispersan en la fase acuosa. El núcleo de la micela está formado por las cadenas carbonadas hidrófobas, que buscan aislarse del agua, mientras que los grupos polares, y eventualmente cargados, se sitúan en la superficie. La zona que incluye los carbonos más cercanos al grupo polar presenta una polaridad intermedia entre las dos zonas.

La cromatografía líquida micelar es una variante de la RP-HPLC que emplea disoluciones micelares como fases móviles, en lugar de las hidroorgánicas. Entre sus

características más destacables, se puede resaltar su elevada versatilidad, debido a la variedad de interacciones y equilibrios de reparto que se establecen por parte de los solutos entre la fase estacionaria, la fase móvil y la pseudofase micelar. Además, sus características de elución facilitan la resolución de analitos (cargados o neutros) con un elevado intervalo de hidrofobicidad, a partir de una única inyección en modo isocrático. En la mayor parte de las aplicaciones, se recurre a la adición de una baja cantidad de disolvente orgánico (que puede ser acetonitrilo, tetrahidrofurano o un monoalcohol de cadena corta), para acelerar la elución y aumentar la eficacia, lo que da lugar a fases móviles micelares híbridas. No obstante, la cantidad de disolvente orgánico es muy inferior a la utilizada en RP-HPLC hidroorgánica. Debido a la elevada reproducibilidad y estabilidad de la retención, ésta se puede modelizar con gran exactitud utilizando modelos matemáticos, para predecir los cambios en el factor de retención al modificar la composición de la fase móvil, a partir de los datos experimentales obtenidos a partir de pocos ensayos. Así pues, se simplifica en gran medida la optimización de las condiciones de separación, que se puede abordar desde una estrategia interpretativa.

En MLC, las propiedades de las disoluciones micelares también resultan útiles para la el tratamiento de muestras sólidas y fluidos viscosos previo a la inyección. La gran variedad de entornos que contiene (hidrófobos, polares, misceláneos y electrostáticos), permite a la micela interaccionar con compuestos o regiones moleculares con propiedades diversas. Esto dota a las disoluciones micelares de un gran poder extractante de los analitos desde matrices sólidas y partículas suspendidas, por una simple contacto y agitación. Por otra parte, las disoluciones micelares solubilizan macromoléculas biológicas, como proteínas, grasas y polisacáridos, ya que las micelas son capaces de unirse tanto a sus zonas lipofílicas como a las hidrofílicas. Por tanto, se pueden inyectar suspensiones sin que precipiten en la columna, y evitando que interaccionen con la fase estacionaria y con los solutos. Además, las micelas desplazan a los analitos, lo que resulta en una mayor recuperación. Por lo tanto, se mejora la etapa de lixiviación y se simplifica y reduce el tiempo requerido para la preparación de la muestra, ya que no se requieren etapas intermedias, además de reducir significativamente el uso de disolventes orgánicos. Todas estas características proporcionan importantes ventajas prácticas a la MLC sobre la RP-HPLC hidroorgánica, como la capacidad de procesar una gran cantidad de muestras por dia, elevado grado de automatización y la disminución del precio de los análisis. También se mejora la seguridad en el laboratorio y se reduce el impacto ambiental, al usar en general reactivos biodegradables e inocuos, y una cantidad baja

de disolventes tóxicos e inflamables.

Una vez desarrollado un método analítico, éste se debe validar, para establecer su intervalo de aplicación y demostrar la calidad de los datos experimentales. La validación consiste en la determinación de una serie de parámetros estadísticos: selectividad, linealidad, intervalo lineal, sensibilidad, límites de detección y cuantificación, exactitud, precisión, recuperación, robustez y estabilidad; que deben alcanzar un valor determinado de antemano. Dado que no se ha alcanzado un acuerdo global acerca de cómo llevar a cabo esta etapa, numerosos organismos y agencias internacionales han desarrollado diversas guías de validación. En cada una de ellas, se indica los analitos, muestras y zona geográfica en la que se aplica, los parámetros estudiados, cómo se determinan, y los criterios de aceptación. En el presente trabajo se utilizó la guía Decisión de la Comisión 2002/657/EC, que se aplica para el análisis de residuos de compuestos orgánicos en alimentos distribuidos en la Unión Europea, y es de obligada implementación en los laboratorios autorizados para el control oficial de residuos.

El grupo Química Bioanalítica tiene una larga trayectoria en el desarrollo y validación de procedimientos analíticos mediante cromatografía líquida micelar, la cual ha constituido la línea prioritaria de investigación. En un principio, los estudios se dirigieron a establecer los fundamentos de esta técnica innovadora, que destacaba por su versatilidad y posibilidad de la inyección directa de muestras complejas, y posteriormente, a la aplicación de la MLC al análisis clínico, alimentario y medioambiental. De hecho, la casi totalidad de publicaciones, Tesis Doctorales y comunicaciones están relacionados con esta temática.

En esta Tesis se utiliza la MLC para la cuantificación de 8 quinolonas en miel y carne de diversos animales (ovino, porcino, bovino, caprino, avícola, cunícola y equina). Se presentan cuatro métodos analíticos, 2 de ellos para miel y otros dos para carnes, y en cada uno de ellos se estudian cuatro quinolonas. Se optimizó el tratamiento de la muestra y las condiciones cromatográficas. Posteriormente, se validaron los métodos y se aplicaron a las correspondientes muestras alimentarias comerciales, para confirmar la ausencia de antibióticos, y determinar su conformidad con la regulación. Así pues, los trabajos desarrollados supondrán una ventaja en el ámbito de la seguridad alimentaria.

Esta memoria contiene un total de siete capítulos. En el primero, se detallan las caracteristicas de los antibióticos estudiados, de la cromatografía líquida micelar y del procedimiento de la validación. En el segundo, se exponen los objetivos de la Tesis. Del

tercero al sexto, se describen los analitos estudiados, la parte experimental, y se presentan y discuten los resultados obtenidos. Finalmente, en el séptimo capítulo se indican las conclusiones extraídos en la totalidad de los trabajos.

Al ser una memoria parcialmente redactada en inglés (Art. 24 de la *NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I* (Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)), debe contener un apartado relativamente largo en valenciano o castellano, que ha de formar parte de la encuadernación de la Tesis, y donde se incluyan necesariamente:

- Los objetivos generales y específicos de la la investigación
- Aportaciones originales
- Conclusiones obtenidas y futuras líneas de investigación.

Chapter 1

Introduction

1. Antibiotics studied

1.1 Description

Quinolones are a family of synthetic, broad spectrum antimicrobial agents with bactericidal activity, whose action is based on their anti-DNA activity. The parent of the group is nalidixic acid (Figure 1.1), discovered in 1962 by Lesher and co-workers. It was active against some Gram-negative bacteria and had limited usefulness because of its high protein binding and little half-life. Because of that, bacteria could develop a rapid resistance to this agent. In 1968, Kaminsky and Melfezer discovered oxolinic acid (Figure 1.1), which was lately approved by the US FDA (United States Food and Drug Administration). The first fluoroquinolones were widely used because of they were the only orally administered agents available for the treatment of serious infections caused by gram-negative organisms [1,2].

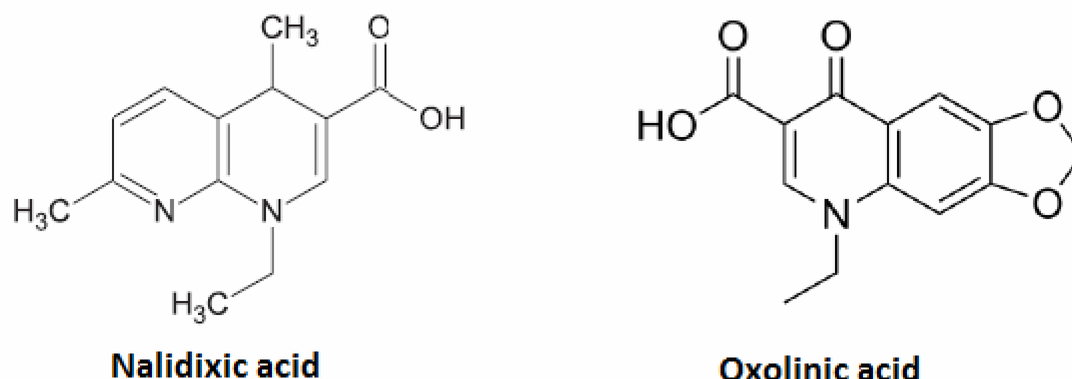

Figure 1.1. Structures of the two first quinolones.

Since then, extensive efforts have been undertaken for the development and to derive an array of drugs of this class [3]. A number of structure modifications to the quinolone nucleus have been performed to increase antimicrobial activity and to enhance the pharmacokinetic performance of these drugs. The general structure consists of a 1-substituted-1,4-oxopyridine-3-carboxylic moiety combined with either an aromatic or

heteroaromatic ring. Fluoroquinolones are quinolones with a fluorine atom at position 6 of the quinolone naphthyridine or benzoaxazine ring systems, and belong to the second generation of quinolones. Development of new antibiotics has been achieved from derivatives of known antimicrobial agents or by identification of novel agents active against previously unexploited targets. The most recent fluoroquinolones have a wider clinical use and a broader spectrum of antibacterial activity, including gram-positive and gram-negative aerobic and anaerobic organisms [2,4]. They are characterized by their greater effectiveness against bacterial activity, and are used in both human and veterinary medicine. In humans, they are used to treat an extensive range of infections, like sexually transmitted, urinary, gastrointestinal, respiratory and skin ones [5,6].

1.2 Relationship between structure and activity [7]

The 6-fluoroquinolones or quinolones (Figure 1.2) are a series of synthetic antibacterial agents derived from nalidixic acid and oxolinic acid.

Figure 1.2. General structure of the quinolones.

At position 1, they have a nitrogen in the bicyclical aromatic ring structure, with an alkyl group attached here, this side chain affects the potency of the drug. The first quinolones had an ethyl group as side chain linked to the nitrogen atom, but the substitution of the ethyl for cyclopropyl and difluorophenyl have resulted in an increase of the potency. The addition of some small groups at cyclopropyl, as fluorine, results in overall improved activity against gram-positive bacteria.

At position 3, the quinolones have a carboxylic acid which is required for

antimicrobial activity, this carboxylic acid is believed to be the portion of the pharmacophore that binds DNA gyrase of the bacterial cell and it is important that do not interfere with the stereochemistry of this area. It is the same with the keto group at position 4.

The fluorine atom at position 6 of the carboxylic acid nucleus enhances the efficacy of the quinolones against gram-negative pathogens and extends the activity's spectrum against gram-positive pathogens: a basic nitrogen-containing moiety increases the tissue penetration and reduces the central nervous system toxicity.

The evolution of the quinolones is based on the modifications of the basic structure at the position 7, which can produce the major changes in potency and alter the pharmacokinetics properties of the compound. Attachment of heterocyclic nitrogen containing rings results in better activity and in a modification of the pharmacokinetics of the compound.

Depending on the quinolone, the modification at position 8 of the aromatic ring affects the antibacterial activities particularly against anaerobes. A fluorine or chlorine atom at this position provides potentially active compounds. On the other hand, a methoxy group at this position confers a good anaerobic activity.

1.3 Mechanism of action

The fluoroquinolones are bactericidal, they inhibit the replication and transcription of bacterial DNA, which induce to the death of the cell. The fluoroquinolones also inhibit the activity of DNA gyrase, or topoisomerase II enzyme, and prevent the detachment of this enzyme from DNA. The topoisomerase II interacts with the DNA during the processes of replication and transcription. In these processes, enzymes called helicases uncoil the DNA double helix creating a tension in the remaining double helix, this tension must be relieved to continue the process. The topoisomerase II enzyme breaks both strands of the DNA chain, crossing the over and the resealing them. This action allows the relaxation of the supercoiled DNA. The fluoroquinolones have about 1000 fold selectivity towards bacterial topoisomerase over the enzyme of humans, because they are quite different from each other. The mechanism of action of the fluoroquinolones is shown in Figure 1.3.

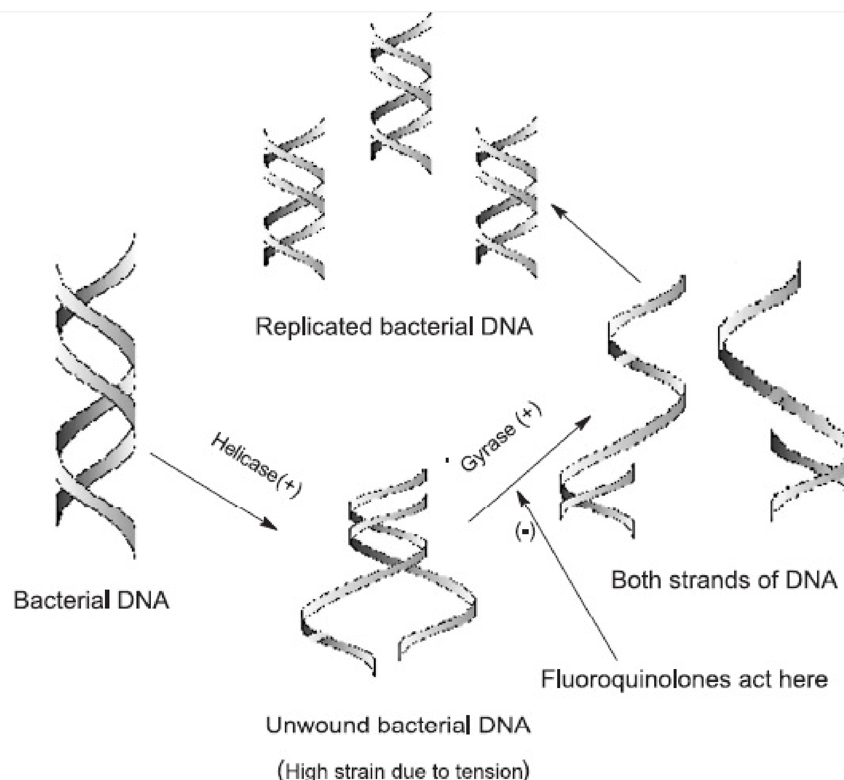

Figure 1.3. Mechanism of action of fluoroquinolones

Fluoroquinolones also inhibit the *in vitro* activities of topoisomerase IV. This enzyme has an important role in partitioning of chromosomal DNA during bacterial cell division and can be the primary target of fluoroquinolone activity in gram-positive bacteria. [1, 2,8]

1.4 Quinolones in animals

The use of fluoroquinolones has increased worldwide in intensive farming, because of the similarity of the bacterias affecting humans and animals. This can be explained by several factors. Firstly, they are broad spectrum antibiotics, so they can be used to treat a wide variety of diseases on intensive farms. Secondly, they can be also used as grow promoters and to homogenize the size of the food-producing animals. Therefore, they contribute to increase the profitability of the farm [9,10].

The abusive use of these antimicrobial drugs in veterinary in farming represent a strong threat to human health, as quinolone residues may remain in edible tissues. They can cause allergic reactions, toxicity, problems in fermented products, and to stimulate the

emergence quinolone-resistant pathogens. In this case, bacteria are passed from animals to humans through the food chain, then a reduction in the clinical efficacy of a human antimicrobial may be possible. This causes that some human versions of the drugs are not effective for the treatment of people infected by some bacteria, like Salmonella or Campylobacter [11,12]. The most used antimicrobials in animals are amifloxacin, benofloxacin, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin, oxolinic acid and sarafloxacin [11]. The antibiotics included in this work were ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, oxolinic acid, and sarafloxacin. The particularities of these compounds will be explained in the following chapters.

2. Micellar liquid chromatography

Micellar liquid chromatography (MLC) is an alternative to conventional reversed-phase liquid chromatography (RP-HPLC) with hydro-organic mobile phases. Almost three decades of experience have resulted in an increasing production of analytical applications. Current concern about the environment also reveals MLC as an interesting technique for "green" chemistry because it uses mobile phases containing 85% or more water. These micellar mobile phases have a low toxicity and are not producing hazardous wastes. The stationary phase is modified with an approximately constant amount of surfactant monomers, and the solubilizing capability of the mobile phase is altered by the presence of micelles, giving rise to a great variety of interactions (hydrophobic, ionic, and steric) with major implications in retention and selectivity. From its beginnings in 1980, the technique has evolved up to becoming a real alternative in some instances (and a complement in others) to classical RP-HPLC with aqueous-organic mixtures, owing to its peculiar features and unique advantages. The addition of an organic solvent to the mobile phase was, however, soon suggested in order to enhance the low efficiencies and weak elution strength associated with the mobile phases that contained only micelles.

2.1 Description

Micellar liquid chromatography (MLC), which uses mobile phases containing a surfactant above its critical micellar concentration (CMC), is an alternative to conventional reversed-phase liquid chromatography and provides a solution to the direct injection of physiological or food samples by solubilizing proteins (that are eluted together or shortly after the solvent front) [13,14,15]. The possibility of the direct injection of samples into the chromatograph simplifies and expedites treatment, which confers analytical procedures greater accuracy and a lower cost.

The versatility of MLC is due to the wide variety of interactions that are established among the eluted solutes, the stationary phase, the aqueous phase and micelles. Compounds with a wide range of polarities can be analyzed due to the MLC eluent characteristics. The presence of a surfactant modifies the interactions established inside the column and also reduces the necessary amount of organic solvent in the mobile phase, which can be recycled due to low evaporation. These characteristics are genuinely interesting given current concerns about reducing organic contaminant residues in laboratories [16].

MLC shares the basic components of RP-HPLC systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RP-HPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudophase) and the surrounding bulk water or aqueous-organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC. On the other hand, the stationary phase is modified by the adsorption of surfactant monomers, creating a structure similar to an open micelle, and reducing silanophilic interactions. With nonionic surfactants, only the polarity of the stationary phase changes, whereas with ionic surfactants, a net charge (positive or negative) appears on its surface with major implications [16].

2.2 Particularities of the Micellar Mobile Phase

Micelles provide hydrophobic and electrostatic (for ionic surfactants) sites of interaction. In the micelles, three sites of solubilization can be identified: the core (hydrophobic), the surface (hydrophilic), and the palisade layer (the region between the surfactant head groups and the core). Solutes associated to micelles experience a microenvironment that is different from that of bulk solvent [17].

Although pure micellar mobile phases are sometimes used, most separations in MLC are performed with hybrid micellar mobile phases in a buffered medium that contains micelles, surfactant monomers, molecules of organic solvent and water. The organic solvent decreases the polarity of the aqueous solution and alters the micelle structure. Although the separation mode is still predominantly micellar in nature, the micelle is perturbed by the organic solvent. This can change micellar parameters, such as the CMC and surfactant aggregation number. A high percentage of organic solvent can disrupt the micelle structure. The maximal allowable concentration depends on the type of organic solvent and surfactant [16,18].

2.2.1 Critical Micellar Concentration

A suitable surfactant for MLC should have a low CMC. A high CMC would imply operating at high surfactant concentration, which would result in viscous solutions, giving undesirable high system pressure and background noise in UV detectors. The selection is often limited to the following surfactants: the anionic sodium dodecyl sulphate (SDS), the cationic cetyltrimethylammonium bromide (CTAB), and the nonionic Brij-35, whose CMC (mol/L) are 8.2×10^{-3}, 9×10^{-4} and 9×10^{-5}, respectively [19,20]. It should also be taken into account that the CMC is strongly affected by the presence of an organic solvent. The changes are related to the modification of the structure of the micelle, which also induces, at least partially, the reduced retention in MLC [21].

2.2.2 Krafft Point

The Krafft point is defined for ionic surfactants as the temperature at which the solubility of a surfactant monomer becomes equal to the CMC [22]. Below the Krafft point temperature, the solubility is quite low and the solution appears to contain no micelles. Chromatographic work in MLC should be conducted above this temperature to avoid surfactant precipitation. This means that the Krafft point should be well below room temperature. The Krafft point for SDS and CTAB is around 15ºC and 20-25ºC, respectively [23,24]. Nonionic surfactants also have a specific temperature, that if exceeded, phase separation occurs, which is called the cloud point [20,25]. Chromatographic work with these surfactants should be conducted below this temperature (e.g., Brij-35, is nearly 100ºC for aqueous 1-6% solutions, whereas for Triton X-100 this value is 64ºC).

2.2.3 pH of the Mobile Phase

MLC employs the same packing materials as classical RP-HPLC, which, for conventional columns, have a limited working pH range of 2.5-7.5. Appropriate pH values depend on the nature of the analytes and the surfactant selected. The pH of the micellar mobile phase is commonly fixed with phosphoric or citric acid buffers [14,15]. For mobile phases containing SDS, potassium salts are not recommended as potassium dodecyl sulphate presents a high Krafft point and precipitates from aqueous solutions at room temperature [14].

2.2.4 Organic Solvents: Types and Concentration

The selection of the appropriate organic solvent modifier in MLC should consider the polarities of the analytes. For polar compounds, sufficiently short retention times (below 20 min) are obtained with 1-propanol, 2-propanol, or acetonitrile. For non-polar compounds or compounds with high affinity for the surfactant adsorbed on the stationary phase, stronger solvents as 1-butanol or 1-pentanol are needed [26]. However, it should be noted that the two latter alcohols give rise to microemulsion formation at sufficiently high concentrations [27]. In practice, the amount of organic solvent that can be added is limited by its solubility. It

should be noted that at high organic solvent concentrations, the micelles disaggregate and the mobile phase contains only free surfactant molecules. The organic solvent contents that preserve the integrity of micelles are below 15% for 1-propanol and acetonitrile, 10% for butanol, and 6% for pentanol [28]. These contents are low in comparison with those needed in classical RP-HPLC. The lower organic solvent consumption results in reduced cost and toxicity, which may become prominent for "green chemistry". Also, the stabilization of the organic solvent in the micellar media decreases the risk of evaporation. This means that micellar mobile phases can be preserved in the laboratory for a long time without significant changes in their composition.

2.3 Modified Stationary Phase

2.3.1 Surfactant Adsorption

The alkyl-bonded C18 is the stationary phase most widely used in MLC, but other columns can be selected (e.g., C8 and cyanopropyl). Alkyl-bonded phase columns are strongly modified when SDS, CTAB, or Brij-35 is incorporated into the mobile phase. Surfactant adsorption on the porous RP-HPLC packing affects drastically the chromatographic retention, owing to the change of diverse surface properties of the stationary phase (e.g., polarity, structure, pore volume, and surface area). Surfactant molecules coat the stationary phase pores, reducing appreciably their volume [29]. Ionic compounds are frequently added to micellar mobile phases for pH buffering and, eventually, ionic strength adjustment. Salt addition may change the amount of adsorbed ionic surfactant due to the reduction of both electrostatic repulsion and surfactant CMC, and the enhancement of hydrophobic interactions [30].

Surfactant coating masks the bonded-stationary phase. This means that a full similar coating would render the stationary phases all similar. Solid-state nuclear magnetic resonance studies for the most common used surfactant, SDS, reveal that the hydrophobic tail was found to be associated with the C18 alkyl-chain bonded to the silica stationary phase, the sulphate head group oriented away from the surface (Figure 1.4) [31]. This creates a negatively charged hydrophilic layer affecting the penetration depth of solutes into the bonded phase.

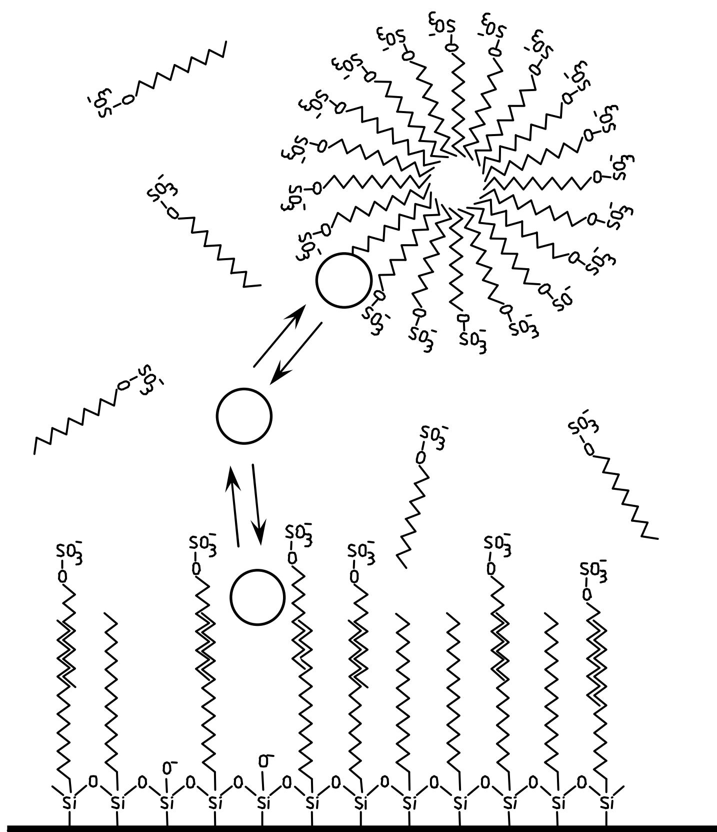

Figure 1.4. Solute environment in a chromatographic system using octadecyl-bonded phase, and mobile phase containing the anionic SDS. Equilibria between bulk solvent, micelle, and surfactant-modified stationary phase are depicted [16].

2.3.2 Effect of the Organic Solvent from the Mobile Phase

Organic solvents are added to micellar mobile phases to improve peak efficiencies and reduce retention times, giving rise to the so-called hybrid micellar mobile phases. Competition between alcohols and surfactant molecules for adsorption sites on the stationary phase explains the linear reduction in the amount of adsorbed surfactant with increasing concentration of alcohol in the mobile phase. Mobile phases rich in organic solvent can sweep completely the adsorbed surfactant molecules from the bonded phase [16,18].

2.4 Care of the Chromatographic System in MLC

2.4.1 Mobile Phase Saturation

Pure and hybrid micellar solutions contain high amounts of water (usually more than 90% v/v) and are able to dissolve small amounts of silica, which could produce serious column damage. This is especially critical at 30ºC and/or pH 6. For this reason, a saturating short column packed with 10 µm bare silica, or alternatively, the same packing as the analytical column, should be placed after the pump and before the injection valve to reduce pressure build-up [18].

2.4.2 Column Conditioning

A column for MLC is generally stored in 100% methanol. Before starting column conditioning, the solvent should be replaced by 100% water. For this operation, a low flow rate (≤0.5mL/min) should be selected at the beginning because of the high viscosity of the methanol-water mixture. Once the pressure decreases, the flow-rate may be raised. At least 30 column volumes of water are required to assure a complete organic solvent removing. Now, the system is ready to be flushed with the micellar mobile phase [18]. Different studies of column coating through surfactant breakthrough patterns have revealed that most surfactant adsorbs in less than one hour on the bonded stationary phase [30].

2.4.3 Mobile Phase Flushing

The micellar mobile phase should be continuously flushed through the system. If the chromatographic system is stopped during several hours, the micellar solution should not stay in contact with the bonded silica-based stationary phase to avoid surfactant precipitation. A static micellar mobile phase can also produce crystals around the pump plungers and seals. Such crystals may obstruct the system producing plugged connecting tubing and frits, seal failure, or scratched pistons. A micellar mobile phase can be kept inside the chromatographic system overnight if the pump is not off. This avoids daily cleaning and reequilibration.

To reduce the cost, the mobile phase can be recycled, reducing the flow-rate to a

minimal value (often 0.1-0.25 mL/min). However, it should be noted that in case of energy supply failure, column damage can occur. Mobile phase recycling is possible because of the low evaporation risk of organic solvents in hybrid micellar eluents. For the same reason, the micellar mobile phase can be recycled during the analysis, as long as a low number of injections are made [18].

2.4.4 Column Cleaning

In general, regeneration can be appropriately performed with methanol, where most surfactants are highly soluble [32]. The cleaning protocol comprises a two-step procedure that takes about half an hour. (i) First, the micellar mobile phase should be replaced by 100% pure water, by rinsing the chromatographic system with 10 to 20 column volumes of pure water. This step is necessary to avoid salt crystallization provoked by a brutal change from a buffered micellar mobile phase to 100% methanol. (ii) Next, water will be replaced by 100% methanol to remove the adsorbed surfactant on the stationary phase. The same caution commented under “column conditioning” about the initial use of a low flow-rate should be followed. To assure complete surfactant desorption, at least 10 column volumes of methanol should be passed through the column [18].

2.5 Solute-Micelle and Solute-Stationary Phase Interactions

The unique capabilities of micellar mobile phases are attributed to the ability of micelles to selectively compartmentalize and organize solutes at the molecular level. However, the association of the surfactant monomers to the bonded phase has deep implications with regard to retention and selectivity. The chromatographic behaviour in an RP-HPLC system of a solute eluted with a mobile phase containing a surfactant above the CMC can be explained by considering three phases: stationary phase, bulk solvent, and micellar pseudophase. Figure 1 illustrates the three-phase model. Solutes are separated on the basis of their differential partitioning between bulk solvent and micelles in the mobile phase or surfactant-coated stationary phase. For water-insoluble species, partitioning can also occur via direct transfer of solutes between the micellar pseudophase and the modified stationary phase (Figure 1.5) [16,18].

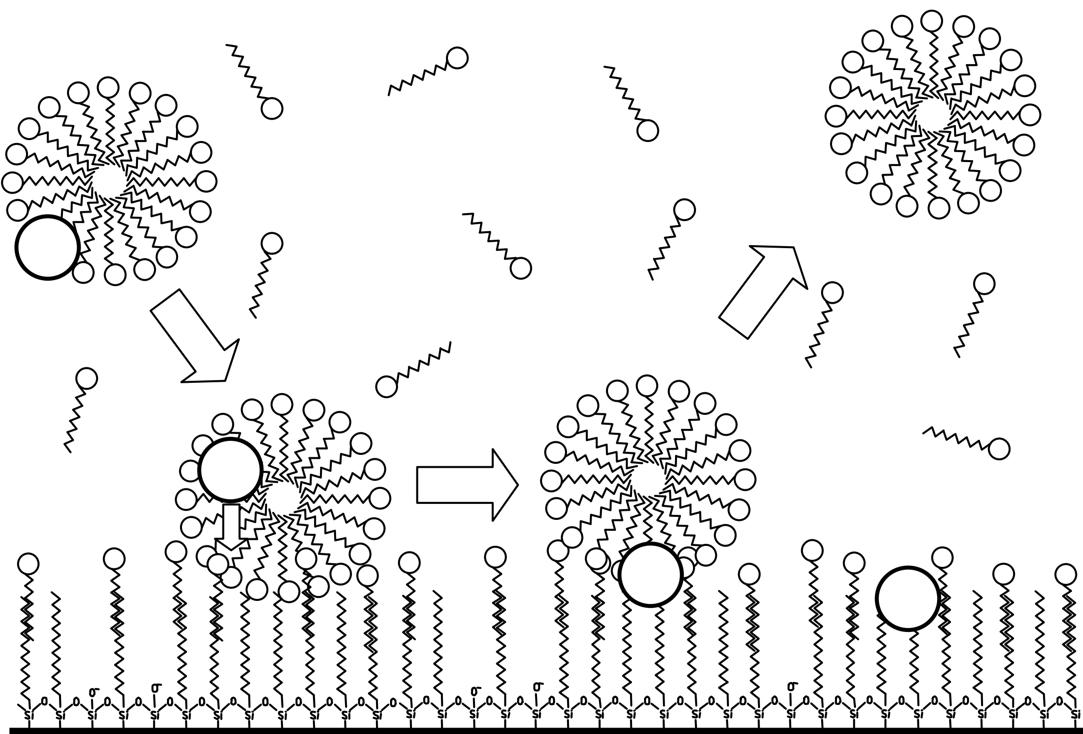

Figure 1.5. Direct transfer of highly hydrophobic solutes between micelle and surfactant-modified stationary phase [16].

The partitioning equilibria in MLC can be described by three coefficients: P_{WS} (partition between aqueous solvent and stationary phase), P_{WM} (between aqueous solvent and micelles), and P_{MS} (between micelles and stationary phase). The coefficients P_{WS} and P_{WM} account for the solute affinity to the stationary phase and micelles, respectively, and have opposite effects on solute retention: as P_{WS} increases, the retention increases, whereas as P_{WM} increases, the retention is reduced due to the stronger association to micelles. The retention behaviour depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with non-ionic and ionic surfactants and charged solutes eluted with nonionic surfactants will only be affected by nonpolar, dipole-dipole, and proton donor-acceptor interactions [33]. Besides these interactions, charged solutes will interact electrostatically with ionic surfactants (i.e., with the charged surfactant layer on the stationary phase and the charged outer layer of micelles). In any case, the steric factor can also be important [16].

With ionic surfactants, two situations are possible, according to the charges of solute and surfactant: repulsion or attraction (by both surfactant-modified stationary phase and

micelles). In the case of electrostatic repulsion, charged solutes cannot be retained by the stationary phase and elute at the dead volume, unless significant hydrophobic interaction with the modified bonded layer exists. In contrast, combined electrostatic attraction and hydrophobic interactions with the modified stationary phase may give rise to strong retention in MLC. Mixtures of polar and nonpolar solutes can be resolved, provided that an appropriate surfactant is chosen [16,18].

3. Validation

3.1 Description

The purpose of any analytical method is to provide consistent, reliable, and accurate data. For this reason, the performances and the limitations of the method, as well as the external influences which may modify these features, must be determined prior to its use. Validation plays a major role in achieving this goal [34,35]. The most accurate definition of validation is that provided by ISO 9000:2000 as the confirmation, by means of a thorough examination and obtaining realistic and unequivocal evidences, that the procedure is effectively applicable for its indented purpose [36].

Method validation is an important requirement in chemical analysis. Indeed, many important decisions are taken on the basis of the results: batch release or refusal, purchase of a specific product and trademark, prescription of a medical treatment, to permit the discharge of a water stream, the outcome of a trial, and so on. In all these cases, an incorrect value can lead to a wrong decision, with awful consequences for health, reputation, and economics. Besides, the cost of making these analyses is considerable, and on occasions, the decisions arising from the results may involve a significant disbursement. Thus, it is important to determine the correct value and be sure of its reliability. The analytical methods must be reliable enough to guarantee that any decision based on it will be taken with high confidence. For these reasons, the requirement for laboratories to use a validated method is now universally accepted [34,37-39].

Validation is the act of proving that any approach, strategy, experimental procedure, process, laboratory staff, instrumentation, reagents, and room conditions selected for the

method will function in a proper way under a fixed set of conditions. Besides, it can be used to individually evaluate the appropriateness of these factors [37]. The validation evaluates the range and conditions of applicability, and checks if every future measurement in routine analysis will provide a concentration of the analyte close enough to the true value [40]. In addition, it can also quantify the degree of coincidence of a measured concentration and the true value, by the calculation of the bias and the variability associated with the result [41]. Therefore, the validation verifies if the method is suitable to be used as a quality control tool and for research support [42]. It is an essential step in method development, which must be implemented by laboratories to prove they can produce analytical data with high reliability [43]. According to ISO/IEC 17025:2005, a laboratory must validate all the used methods. The methods will be separately validated for each matrix and working range, even dealing with the same analyte. A full validation is required when implementing a new method: in-house developed, taken from a bibliographic source, transferred from other laboratories, and reference one [34,42*].

3.2 Procedure

The validation consists in the determination of well-defined quality parameters: statistical (selectivity, specificity, linearity, calibration range, accuracy, precision, recovery, uncertainty, limit of detection, limit of quantification (LOQ), decision limit, detection capability, robustness, stability, system suitability, and comparison with other methods) [34,35,42*] and operating/economical (cross contamination, simplicity, analysis time, price per analysis, safety for laboratory staff, and environmental impact) [44,45]. The statistical validation parameters describe the performances and the limitations of the methods. The results from method validation evince the quality and consistency of the analytical results obtained in future determinations in incurred samples, whereas the operational/economic parameters appraise if the method can be used for routine analysis. The validation protocol is a set of directives detailing, for each parameter, the accurate meaning, the acceptance criteria, the experimental design, and the mathematical formula for its evaluation.

The procedure and the analytical requirements are not always the same, and must be individually established on the basis of the scope of the method, the analyte, the matrix,

possible interfering, the kind of the sample, the expected interval concentration, and the geographic zone. The validation parameters that have to be determined and the acceptance criteria should be completely specified before starting the development of the method, depending on the will of the customer, the local and international regulations and the scope of the analysis. A validation study must be conducted, as far as possible, considering all the effects that can be involved during the normal use of the method. Furthermore, the experiments must be performed, and the results must be taken, registered and processed to calculate the values of each studied parameter [46,47].

The fitness for purpose is the extent in which the performances of the method match the characteristics that have been agreed between the analyst and the end user of the results. If a method aims to reach a wider application, it must also meet the requirement of the government institutions and official analysis guide [48]. The final results of validation must be documented to be always available for consulting by laboratory staff, clients, and accreditation agencies, and ready to be transferred to other laboratories [49]. The method is considered validated, if the acceptance criteria are reached. Once a methodology is validated, it remains “validated” while applied in the same laboratory, under the same experimental conditions, and for the same matrix and analyte [50].

Many industry committees and regulatory agencies and individual researchers have published reviews and technical reports about validation strategies, quality assurance, and regulatory purposes [51]. Consequently, many validation guidelines, with different scopes, have been issued, describing the validation parameters to be studied, the way to determine each one, and their acceptance criteria. The different published documents agree about what type of studies should be done, but they show a great diversity in how the validation should be conducted [52]. A methodical understanding about all the aspects involving validation is essential to its correct implementation. The adequate guide must be carefully selected, on the basis of the geographic zone of application, the objective of the analysis, matrix and analyte [47].

3.3 The European Commission Decision 2002/657/EC guideline

The European Council proposed the guideline "European Commission Decision 2002/657/EC" in 2002, which provides legal directives to the laboratories to evaluate the chemical contamination of live animals and animal products for consumption, produced or exported to the EU, in order to ensure health and food safety [53]. Maximal residue limits (MRL) have been fixed by the same organization for many contaminants in the main foodstuff, as the permitted limit in compliant food sample. Other organics have been banned [54]. The guideline lays down rules for both sampling and validation of analytical methods (studied parameters and acceptance criteria), and describes the correct approach to deal with each matrix, instrumentation, and analyte, as well as the interpretation of the data. It has been proposed to uniform the procedures and performance criteria used by laboratories approved for the official residue control, in order to ensure the quality and comparability of the results. The guideline has the *status* of European law and its application is mandatory for these laboratories.

The European Commission Decision 2002/657/EC guideline is appropriate to analyze undesirable organic residues by chromatography or metallic elements by electrochemistry and atomic spectrometry (not here discussed), in matrices extracted from food products. The concentration of these contaminants is usually low ($\mu g\ kg^{-1}$). The laboratory has to report if the amount of the contaminant is under or over the maximum residue limit to accept or reject the batch, respectively. If an MRL has not been defined (prohibited substances), the sample is noncompliant if the analyte has been detected. In this case, the minimum required performance limits (MRPL) must be stated, for validation purposes, as the calibration curve of the method. Anyway, the accurate concentration can also be reported to complete the document. The guidance imposes the calculation of the specificity, trueness, ruggedness, stability, recovery, repeatability, within-laboratory reproducibility, decision limit ($CC\alpha$), detection capability ($CC\beta$), and calibration range. The limit of detection (LOD) and calibration range (lower and upper limits of quantification) have not been defined, and then the deffinition of the ICH Harmonized Tripartite Guideline [55] and European Medicines Agency [56], respectively, have been taken. To minimize the workload, the analytes must be studied in the same injection. The description and acceptance criteria are described below:

3.3.1 Selectivity/Specificity

The terms “selectivity” and “specificity” are interchangeably used [57]. They refer to the ability to produce a signal unequivocally due to the analyte, in the presence of possible interferences and under the instrumental conditions of the method. The identification test must be able to recognize the peak of the analyte among other peaks of the chromatogram, generated from other compounds or the instrumentation. Besides, it must discriminate between the analyte and closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents, *etc.*). A good selectivity is needed for both qualitative and quantitative purposes.

A sample must be analyzed using the suitable column, and the analyte should be eluted at a minimum of two times the dead time. A peak shall elute between ±5% of the retention time obtained by a standard solution to be assigned to the target analyte. The identification must be confirmed by comparing the characteristics of the spectrum of the sample peak with those of the standard peak. An appropriate number of representative blank samples ($n \geq 20$) must be analyzed. Blank samples, fortified with with substances that are likely to interfere with the identification and/or quantification of the analyte, may also be analyzed. In both cases, no peaks or baseline distortions should appear at the window time of the analyte.

3.3.2 Calibration range and linearity

The linearity is the ability of the chromatograph to produce a peak area for each analyte, which can be related to the concentration by a first-grade equation (the calibration curve) [58]. The linearity can be tested using spiked blank sample (preferably) [34,38] or standard solutions (in this case, the dilution/preconcentration coefficients must be considered) [48]. A minimum of six independent calibration points ($n=3$) containing increasing concentrations in the expected working range of the method, equally spaced, is recommended. The peak area measures are plotted *vs* the concentration to examine the linearity by visual appreciation. Furthermore, they are treated by statistics, like the least-square linear regression, to calculate the curve parameters: slope, y-intercept, (with their corresponding standard deviations) and determination coefficient (r^2). This last parameter

evaluates the goodness-of-fit of the experimental results with the calibration curve. The method is considered enough linear if $r^2 > 0.990$, and the residuals fit the trueness/recovery acceptance criteria (see 3.3.4) [34,50,59].

The lower and upper limit of quantification (LLOQ and ULOQ, respectively) are the minimal and maximal concentrations in which the analytical procedure provides quantitative results with a suitable level of linearity, accuracy, and precision, under the fixed acceptance criteria, respectively. They can be measured by testing samples at decreasing and increasing concentration until lost of linearity. However, the ULOQ may also be fixed to a lower value by the user. The calibration range is the interval between these concentrations. Consequently, the method can only be employed for samples containing concentrations inside this interval [34,38,51,56]. The calibration range must cover a range spanning the 50-150% of the MRL. For banned organic substances, the calibration range must be as low as possible, especially to low concentrations.

3.3.3 Sensitivity

The sensitivity is the ability to discriminate between small variations of the concentration of the analyte. In chromatographic analysis, it is calculated as the derivative of the peak area regarding the concentration, thus the slope of the calibration curve. It is also evaluated by the limit of detection and the limit of quantification [48].

The limit of detection (LOD) is a statistical value that establishes the minimal concentration that provides a signal that can be reliably differentiated from the background noise, with a specified significance level ($\alpha = 5\%$). Values under LOD are considered due to the background noise, and then the concentration is reported as "under LOD" or "not detected" (instead of zero or absent). The limit of quantification (LOQ) is the smallest amount in sample that can be quantified with enough reliability. In the region between the LOD and LOQ, the occurrence of the analyte is assessed, but the "found concentration" would have associated a too high uncertainty. Thus, the reported confidence interval would be uninformative. Thus, the result must be simply reported as “concentration between LOD and LOQ" [34,38,55].

The LOD and LOQ is measured using fortified samples. The LOD is usually measured by the 3 or 3.3s criterion, while LOQ is calculated by the 10 s criterion: 3 or 3.3

and 10, respectively, times the standard deviation of the blank (s_0), divided by the slope of the calibration curve. The s_0 can be taken by determined as: the standard deviation of 20 measurements of a blank sample fortified at the LLOQ, or using the curve calibration parameters: the standard deviations of the residuals or the standard deviation of the y-intercept (only if the calibration curve has been calculated using fortified samples) [55,59]. The LOD and LOQ must be enough under the MRL. For banned organic substances, the LOQ and LOQ must be as low as possible. The LOQ would be reasonably close to the LLOQ.

3.3.4 Trueness/Recovery

Trueness is the closeness between the average concentration obtained from a large series of tests (to minimize the effect the random errors) provided by the analytical assay (calculated from the peak area through the calibration curve) and the true value. It is expressed in terms of bias. The trueness must be measured using certified reference material (CRM), made of the a matrix similar to that analyzed, and with a know concentration of analyte. If they are not available, fortified blank samples can be used, but the parameter is named "recovery". In both cases, the matrix effect and losses during the sample preparation are already incorporated to the bias. However, unlike the analyte, the added element is not chemically bound in the real matrix and that therefore the results obtained by this approach have lesser validity than those achieved through the use of CRMs. If the calibration curve has been determined using standard solutions, loss of the analyte will result in low recoveries. Otherwise, they would have been incorporated in the slope. The tested samples and solutions must be different from that analyzed for the calibration [34,48].

The recovery depends on the concentration, and must be obtained at three concentrations, 0.5x, 1x and 1.5x MRL or 1x, 1.5x and 2x the MRPL, each one by six replicates. The fortified samples are analyzed, and the found concentration must be calculated. For each injection, the recovery is the quotient between the found concentration and the true value x 100. The average value is calculated for each level. The acceptance criteria depends on the level: for <1 $\mu g\ kg^{-1}$, 50-120%; between 1 and 10 $\mu g\ kg^{-1}$, 70-110%; and >10 $\mu g\ kg^{-1}$, 80-110%.

3.3.5 Precision

The precision (in chromatography) is defined as the closeness of agreement between the values of peak area obtained from several independent analyses of homogeneous aliquots. The precision is provided as dispersion or variability, and quantified through the RSD of the detector response. The variability depends on the concentration of the analyte. This parameter is determined using the same experimental assay as for recovery. Therefore, the matrix effect and losses during the pretreatment are also considered for the dispersion.

The precision may be measured in several ways, to distinguish the different sources of variance:

- Repeatability: from the data obtained under the same operating conditions, by the same worker, and repeated over a short period of time (within the same day). It corresponds to the bias and variability inherent to the procedure itself, and it is the minimal dispersion that can be obtained.
- Within-laboratory reproducibility: it represents the variability where the analyses are performed in different operational conditions (but in the same laboratory). The most informative is to evaluate the reproducibility by changing a single factor, to evaluate the its effect. The most usual in quality control is to compare the data obtained through long time intervals, to determine the influence of time in the quality of the results. In this case, repeatability studies are performed several days over a long period, and the RSD the obtained average peak area is calculated.

The RSD must be under that provided by the Horwitz equation: $2/3 \times 2^{(1-0.5\log C)}$ (min. 15.3 %), were C is the concentration of the analyte in g/g.

3.3.6 Accuracy

Accuracy means the closeness of agreement between a test result and the accepted reference value. It is not independently determined in the validation process, but *via* the trueness/recovery and precision. However, it can be determined for individual samples. For an incurred sample and CRM, it can be determined by comparing the result of the method by that obtained by a reference method or the know value, respectively. On many occasions, the accuracy taken as the trueness/recovery.

3.3.7 Decision limit

This criterion has been established specifically to evaluate the compliance of food batches. The decision limit (*CCα*) is applicable when the scope of the analysis is to check if the concentration of the analyte in the sample is ≤ or > an established permitted limit (in this case, the MRL), thus the sample must be accepted or rejected. If this value has not been defined, the decision limit is calculated considering a null concentration as "permitted limit."

The decision limit is defined as the "found concentration" above which it can be concluded that the analyte is over the permitted limit with a probability less than a fixed significance level (α) to obtain a false positive. If a sample containing the analyte exactly at the permitted limit is analyzed a large number of times, the inherent variability of the method will cause that half measures will provide a "found concentration" ≤ the permitted limit, and half measures will provide values > the permitted limit. In the first case, the laboratory will correctly accept the sample, whereas in the second case, the sample will be incorrectly rejected. Therefore, the maximal probability of a false negative is 50% (permitted limit = CCα at $\alpha = 50\%$). Considering that the consequences of the rejection would cause strong damages, this error probability is not acceptable for a reliable quality control laboratory. Therefore, the limit value to decide the suitability of a sample is switched to a higher value by reducing the α. Therefore, a sample containing the permitted limit would provide "found concentrations" over the CCα only the α % of the measures and under the *CCα* at $(1 - \alpha)$ % of the measures. The maximal probability of providing a false positive is reduced to α. A legal document would mark the CCα as limit found concentration in a compliant sample. The decision limit depends on the permitted limit, the variability of the measure, and α.

The CCα is determined as follows:

- If no MRL has been stated, α = 1 %, and the limit of decision equals the LOD.
- If an MRL has been stated, α = 5%: The MRL plus 1.64 the standard deviation obtained by analyzing ($n = 20$) a blank sample spiked at the MRL.

The decision limit must be as close to the MRL as possible, to reduce the probability of false positive.

3.3.8 Detection capability

The detection capability (CCβ) is the smallest content of the analyte (over the permitted limit) at which a method is able to detect truly contaminated samples with a β probability of a false compliant result. If a sample containing the CCα is analyzed by many replicates, the random errors would provoke that half measures provide a value over the CCα (correctly rejected), and half measures provide found concentrations under the CCα (incorrectly accepted). The probability of a false negative is 50%, thus the laboratory would provide false results in 50% of the analysis. With this result, the laboratory is not really able to identify as noncompliant a sample containing CCα with enough consistency (CCα = CCβ at $\beta = 50\%$). The "limit concentration in sample" from which the laboratory is really able to classify a sample as contaminated with sufficient reliability is switched to a higher value, by diminishing β. A sample containing CCβ would be measured as ≤CCα, the β % of replicates and as >CCα, the other $1 - \beta$ %. Thus, the maximal probability to make a false compliant result is reduced to β %. The laboratory must claim that it is able to detect contamination over the "detection capability," instead of over the permitted limit or the CCα. The detection capability will depend on the decision limit, the variability of the measurement, and β %. To avoid confusion, it must be stated that the CCα refers to the concentration obtained through the analysis, whereas the CCβ refers to the amount in the sample.

In routine practice, β = 5%, and CCβ is calculated as the CCα plus 1.64 times the standard deviation of a sample fortified at the CCα. The detection capability must be close to the CCα, to minimize the probability of a false negative.

3.3.9 Ruggedness

In a laboratory, the operational parameters (factors) rarely remain exactly at the values described in the method, and they always oscillate within a realistic range. Ruggedness minor changes, or simply ruggedness, is the ability of the method to remain unaffected by small but deliberate variations of the experimental conditions, likely to occur during the routine usage [55]. In chromatographic analysis, the robustness estimates the consistency of the main chromatographic parameters (retention time, and peak area), when internal experimental factors fluctuate from those described in the method, and provide an

indication of its stability during normal usage. Besides, the ruggedness evaluates, for selected factors, the range in which the modifications of the retention time and peak area are assumable, termed as "confidence interval" [60].

Experimental conditions related to all the steps of the analytical procedures, such as the sample preparation and chromatographic analysis, can be included in the study. The first phase consists in a thorough analysis of all the method and deciding which factors are expected to have higher variability and stronger influence on the final result. The main studied experimental conditions in HPLC methods are extraction time and volume, sampled volume, pH, temperature, flow rate, injection volume, composition of the mobile phase, detector conditions, and so on [60]. Once the factors to be studied have been selected, the minimal and maximal values among which the robustness has to be evaluated must be established. The oscillation range is usually symmetrically distributed around the optimized value. The deviation is taken depending on the expected variation, according to the uncertainty associated with its measure (pH ± 0.2, flow rate ±0.05 mL min^{-1}, surfactant concentration ± 0.05 M, *etc.*).

Ruggedness may be evaluated using a sequential approach, where each factor is evaluated one by one. Samples fortified at the MRL, or a concentration over the MRPL, are analyzed. The retention time and peak area are measured on the minimal, optimized, and maximal value of each parameter, maintaining the others constant. For each chromatographic response and analyte, the influence of the oscillation of the factors is concurrently calculated by the relative standard deviation of the measurements obtained in the three measures. If the variation of the analytical results is under a previously defined acceptance value, the parameter is stated as robust, and the studied range is considered as its confidence interval. This approach is quite simple and the results are easy to interpret, although the effect on the interactions between parameters are not considered.

3.3.10 Stability

The stability is defined as the ability of a sample to preserve its physicochemical properties, and especially the concentration of the analyte, after several times of storage under specific conditions. Stability assays are important to estimate the maximum allowed time span between sample collection and analysis. This is especially important when dealing

with food samples, which show an appreciable decay through time. Monitoring of the storage condition will form part of the normal laboratory accreditation system. A lack of stability will give rise to significant deviations in the outcome of the result of analysis.

The stability of the standards and the sample must be established by determining the decomposition kinetics of the analyte, measured as the reduction of its peak area through the time. Analyte decomposition should be determined under the most usual storage conditions, in order to determine the maximum storage time: in darkness at -20 °C, in darkness at -4 °C, in darkness at room temperature, and under light at room temperature. The stability should be studied in two chemical environments: standard solution and matrix, using incurred or spiked fortified samples, at the MRL levels, or a concentration over the MRPL. In all cases, the samples must be divided into a large number of aliquots and stored. Each fixed time (a day, two-days, a week, depending on the expected degradation kinetics), one aliquot is thawed and analyzed. The time required for the peak area to diminish up to a previously specified value is the maximal time of keeping.

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Chapter 2

Objectives

El objetivo de la investigación es el desarrollo de una varios procedimientos analíticos fiables, selectivos, sencillos, rápidos, prácticos, baratos y ecológicos, mediante MLC con detector de fluorescencia, para la detección de quinolonas potencialmente usadas en granjas y apicultura, en diversas clases de mieles y carnes. En primer lugar se considera la determinación de antibióticos, en dos grupos (ácido oxolínico, flumequina, marbofloxacina y enrofloxacina; danofloxacina, difloxacina, ciprofloxacina y sarafloxacina) en una amplia variedad de mieles, producidas en España y de países fuera de la UE. Al estar totalmente prohibidas en apicultura, se intentará alcanzar una elevado grado de sensibilidad. Posteriormente, se llevará a cabo la cuantificación de ácido oxolínico, danofloxacina, ciprofloxacina, y enrofloxacina en productos cárnicos procedentes de ganado vacuno y porcino, y la detección de flumequina, marbofloxacina, difloxacina y safafloxacina en carnes de ternera, cerdo, pollo, pavo, pato, cordero, cabra, conejo y caballo. Se pretende obtener un alto grado de fiabilidad de las medidas en el entorno del MRL para cada antibiótico y clase de muestra. En todos los casos, la información obtenida servirá para detectar el grado de contaminación y para distinguir entre las partidas de alimentos que cumplen y que incumplen la legistación europea, en relación a la presencia de antibióticos en productos alimentarios de origen animal. Por ello, resultarán útiles en el ámbito de la seguridad alimentaria.

Otro objetivo primordial es la validación de los procedimientos analíticos, a través de las directrices de la guía de validación "European Commission Decision 2002/657EC", que fue precisamente propuesta para el análisis de esta clase de muestras, producidas y distribuidas en la UE. Esta etapa es clave en el desarrollo del método, ya que sirve para demostrar la calidad analítica de los resultados y el rango de concentraciones en las cuales se puede aplicar. Esto resulta imprescindible para autorizar su uso en laboratorios acreditados de control de residuos en alimentos, debido a las consecuencias (económicas, de imagen, de salud y legales), de una incorrecta clasificación de las muestras. Posteriormente, se aplicarán, a muestras comerciales.

Los procedimientos deben ser implementables para el análisis rutinario de muestras alimentarias en laboratorios de control. Por ello, han de facilitar el procesamiento sucesivo de de un elevado número de muestras en un tiempo limitado. También tienen que ser sencillos de ejectuar, con pocas etapas experimentales y con la mínima intervención posible del operador. Se ha de incidir en la reducción del coste del análisis, ya que es interesante poder disminuir el presupuesto general del control de muestras sin que la calidad se vea afectada, y destinar el

excedente a otras tareas. Para ello, resulta interesante utilizar instrumentación económicos (teniendo en cuenta la adquisición, amortización y mantenimiento) y fungibles y consumibles que sean baratos y en baja cantidad. También resulta interesante minimizar la cantidad de reactivos y dissolventes tóxicos, volátiles e inflamables utilizados, para incrementar la seguridad en el trabajo en el laboratorio y restringir el impacto ambiental de su actividad.

Para alcanzar estos objetivos generales, se establecen los siguientes objetivos específicos, que son comunes en todos los trabajos:

- Búsqueda bibliográfica de los parámetros fisico-químicos de los antibióticos (pKa, hidrofobicidad, solubilidad, estructura, propiedades espectrofotométricas) y aspectos regulatorios (MRLs el los distintos alimentos).
- Establecer las condiciones cromatográficas generales (fase estacionaria, volumen de inyección) y de la fase móvil (pH, tensioactivo, disolvente orgánico, aditivos).
- Estudio del efecto de la concentración de tensioactivo y disolvente orgánico para cada antibiótico. Modelización de los parámetros de elución (factor de resolución, eficacia y asimetría) y de la resolución, y construcción de cromatogramas simulados.
- Optimización de la composición de la fase móvil (concentración de tensioactivo y de disolvente orgánico), para resolver las cuatro quinolonas, sin interferencias con compuestos de la matriz, en el mínimo tiempo de análisis utilizando el modo isocrático.
- Optimización de las condiciones de detección (longitudes de onda de excitación y emisión fluorescente).
- Optimización del ratio de dilución de la muestra líquida viscosa en disolución micelar.
- Optimización de las condiciones de lixiviación (modo y tiempo de agitación, proporción sólido/disolución micelar extractante).
- Determinación de los parámetros de validación indicados en la guía oficial: selectividad, curva de calibrado (límites mínimo y máximo de cuantificación), linearidad, límite de detección, exactitud, precisión, robustez y estabilidad. Los criterios de aceptación fueron los indicados en la guía y los requeridos para la detección de residuos de antibióticos.
- Aplicación a muestras obtenidos en comercios de alimentación locales.

Chapter 3

Analysis of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey using micellar liquid chromatography and validation according to the 2002/657/EC decision

Abstract

A reliable and sensitive method based on micellar liquid chromatography was optimized for the analysis of the fluoroquinolones danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey. The sample was 1 : 1 diluted in a 0.05 M sodium dodecyl sulfate solution buffered at pH = 3, thus avoiding an extraction step and the use of toxic chemicals. The fluoroquinolones were resolved in less than 25 min using a C18 column, without interference from the matrix. The mobile phase was a solution of 0.05 M sodium dodecyl sulfate, 1% 1-butanol and 0.5% triethylamine buffered at pH ¼ 3, running under isocratic mode at 1 mL min^{-1}. The excitation and emission wavelengths were 280 and 455 nm, respectively. The method was validated in accordance with the European Union Decision 2002/657/EC in terms of selectivity, sensitivity (limits of detection and quantification, 4 and 10 mg kg^{-1}, respectively), calibration range (10–200 mg kg^{-1}), linearity (r^2 > 0.9990), decision limit (4 mg kg^{-1}), detection capability (4.7-6.2 mg kg^{-1}), intra- and interday accuracy and precision (81.0-103.4% and <12.3%, respectively), and robustness (<8.5%). The method was applied to commercial honey samples purchased from a local supermarket.

1. Introduction

Fluoroquinolones are among the most important antibacterial agents and belong to the current arsenal of antibiotics developed against infections [1]. Therefore, these drugs are extensively used in the treatment of human and veterinary bacterial infections due to their effectiveness and broad spectrum of activity. In veterinary medicine, they are specically used as prophylactic agents to prevent respiratory diseases and bacterial infections in cattle, swine, broiler, turkey, and aquaculture [2]. They have been used as anti-infectious agents to treat foulbrood and nosemosis in bees [3].

The intensive use of FQ in live animals implies a potential danger for the population. It can stimulate the growth of mutated pathogens resistant to these quinolones, which can lately jump to humans. Besides, drug residues may persist in the edible products of animals, so that there is concern about the possibility of a continuous and long-term exposure of consumers to high levels of these compounds. As a result, they may unknowingly develop resistance to quinolones, and would be unaffected by future antibiotic treatments [2]. In the European Union (EU), the presence of these drugs in foodstuffs has been regulated through the Commission Regulation (EU) no. 37/2010, and maximum residue limits (MRLs) have been established for different food matrices of animal origin [4]. In honey, however, no MRLs have been defined for the fuoroquinolones danofloxacin (log Po/w = 0.14; pKa = 6.22/9.43) [5], difloxacin (log Po/w = 0.77; pKa = 5.66/7.24) [6,7], ciprofloxacin (log Po/w = 0.77; pKa = 6.09/8.09)[7,8] and sarafloxacin (log Po/w = 0.86; pKa = 4.12/6.78) [7], the structures of which are shown in Fig. 3.1. The use of fluoroquinolones is strictly forbidden, and, consequently, the presence of such residues and their metabolites in bee products must be considered as resulting from illegal beekeeping practices [4]. Thus, a honey sample is declared noncompliant if these compounds are detected, and then the corresponding batch would not be allowed to be distributed within the EU.

Honey is consumed worldwide, especially during breakfast, due to its nutritional and health benefits. It is also largely used in the food industry (bakery and cereal-based goods, baby foods, chocolate, *etc.*). Indeed, on a yearly basis, about 1.2 million tons of honey is produced worldwide and 400 000 tons is traded internationally [9]. In the last few years, the finding of antibiotics in this commodity has had a serious impact on both raw material

suppliers and food manufacturers, resulting in rejection and destruction of honey batches and affecting the reputation of the producers. Additionally, this has endangered the image of bee-derived products as healthy and clean. Recently, several fluoroquinolones have been found in honey originating from China, demonstrating that such broad spectrum antibiotics are used by some beekeepers [10]. Therefore, the development of screening methods to check the absence of danofloxacin (DAN), difloxacin (DIF), ciprofloxacin (CIP) and sarafloxacin (SAR) in honey before they are sent to markets is of the utmost importance to ensure that the batch complies with the EU regulation and to detect a possible threat to the consumers.

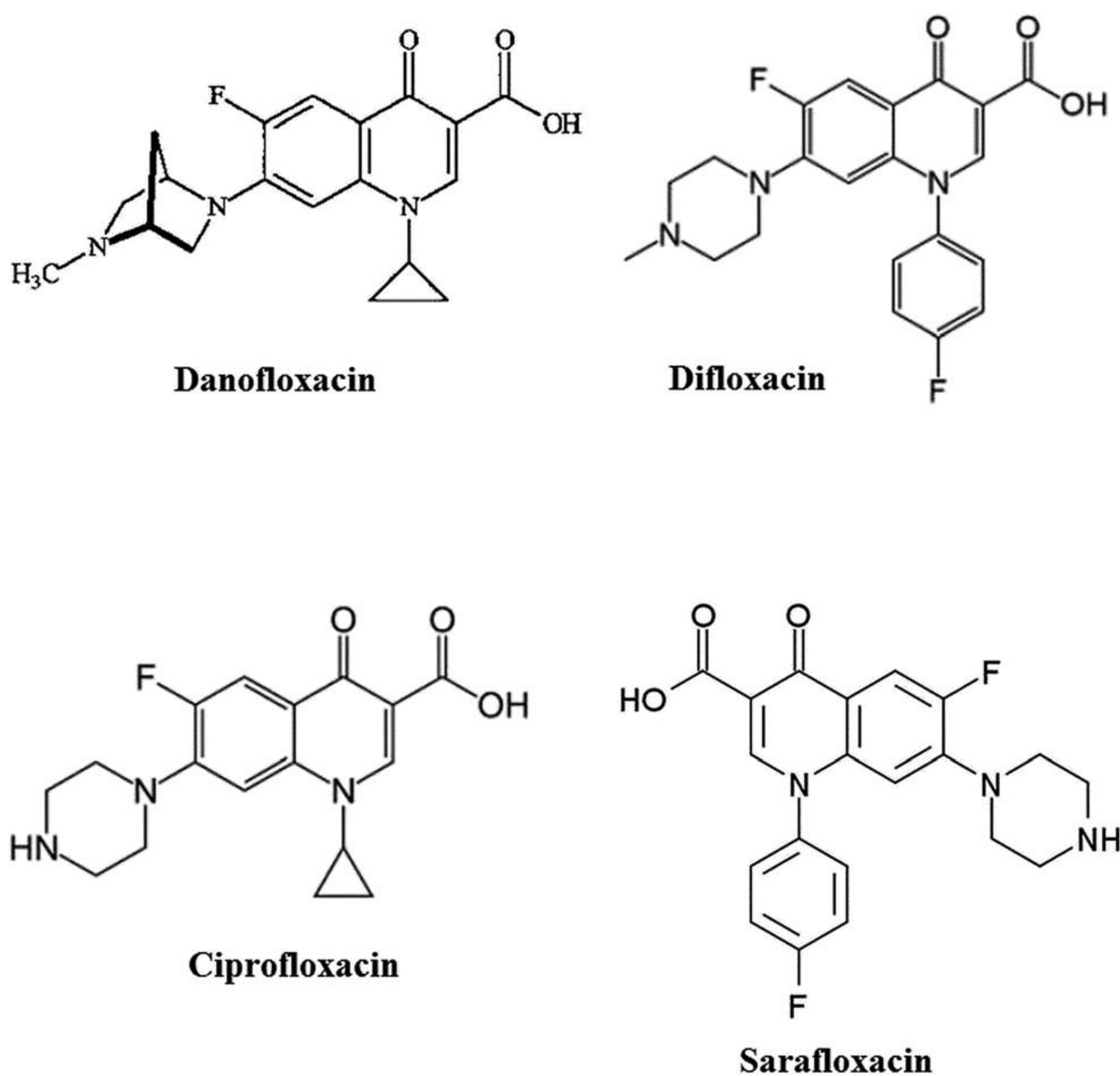

Figure 3.1. Structures of the studied fluoroquinolones.

Many methods based on separation techniques, such as capillary electrophoresis [11], thin layer chromatography, gas chromatography, and liquid chromatography [12] have been developed for the screening of fluoroquinolones in edible animal tissues. The latest generation of high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) equipment allows the multiresidue determination of these antibiotics in milk [13], tilapia [14] and honey [15-20]. However, this equipment is expensive and not all laboratories can afford it. Besides, due to the current situation of economic crisis, the trend points towards the development of inexpensive analytical procedures. Studies have been published on different liquid chromatographic methods based on fluorescence and UV-Visible absorbance detection of FQ in milk [13], chicken muscle and egg yolk [21], tissues of food-producing animals [22], eggs [23], feeds [24], livestock and marine products [25] and royal jelly [26]. However, only a few studies have been published about the analysis of quinolones in honey using LC-FLD [27,28]. Furthermore, most of the extraction procedures applied to analyze honey require clean-up procedures that are tedious and time-consuming, because of the viscosity and the presence of a large amount of sugars. The most usual methods are liquid/liquid [16,18,19] or solid/liquid [16,20,27,28] extraction, or precipitation of matrix compounds [17,18,20]. In some cases, several consecutive clean-up steps [16,18,20] or previous screening by microbiological methods [28] are required. The enlargement of the experimental procedure increases the probability of the loss of analytes, thus reducing the quality of the experimental results. Several authors have proposed the analysis of fluoroquinolones in honey by automated on-line sample purification, using turbulent flow chromatography coupled with LC-MS [15].

Micellar liquid chromatography (MLC), using mobile phases containing an aqueous solution of sodium dodecyl sulfate as the surfactant over the critical micellar concentration (CMC) and, eventually, a low amount of a short-chain alcohol, has been applied for the analysis of organic compounds in food [29]. Micellar solutions solubilize both polar and hydrophobic compounds. Thus, samples can be directly injected without the risk of precipitation into the column, thus shortening the experimental protocol. As a result, the analysis time, cost and environmental impact are lower than hydroorganic HPLC [30]. Besides, the chromatographic behavior of the analytes in micellar mobile phases is highly stable and reproducible, and can be related to the concentration of SDS and alcohol using several equations. Therefore, the composition of the mobile phase can be easily optimized by

testing a few mobile phases [31]. MLC has been successfully used to analyze the quinolones in fish from fisheries [32], eggs and milk [33].

The aim of this work was to develop an MLC procedure for the screening of DAN, DIF, CIP and SAR in honey. The analytical procedure must be reliable, simple, inexpensive and non-polluting, and useful for the routine analysis of honey samples. The method must be validated following the requirements of the EU Commission Decision 2002/657/EC regulation in terms of selectivity, linearity, decision limit, detection capability, accuracy, precision, and robustness [34]. The sensitivity was evaluated through the ICH Harmonized Tripartite Guideline [35]. The procedure developed would be applied to the analysis of the studied antibiotics in commercial honey samples.

2. Experimental

2.1 Standards and chemicals

The solid standards of danofloxacin (purity >99.9%), difloxacin (>99.8%) and sarafloxacin (>97.2%) were supplied by Fluka (Buchs, SG, Switzerland), whereas ciprofloxacin (>99.9%) was purchased from Sigma (St. Louis, MO, USA).

Sodium dodecyl sulfate (>99.9%) and sodium hydroxide (>99.0%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (reagent grade, 37%), triethylamine (>99.5%) and ethanol (HPLC grade) were bought from J. T. Baker (Deventer, the Netherlands). Sodium dihydrogen phosphate 1-hydrate (99%), 1-propanol, 1-butanol and 1-pentanol (HPLC grade) were obtained from Scharlab (Barcelona, Spain). Ultrapure water was in-lab generated from distilled water using an ultrapure water device (Millipore S.A.S., Molsheim, France).

2.2 Preparation of solutions and mobile phases

The mobile phases were prepared by weighing the adequate amount of SDS and sodium dihydrogen phosphate, and dissolving them in ultrapure water by shaking. The appropriate volume of triethylamine (TEA) was added to obtain a final concentration of 0.5% (v/v) and the pH was fixed to 3 by adding drops of HCl solutions. Furthermore, the organic

solvent was added to reach the desired proportion (%, v/v), and then ultrapure water was added up to the mark of the volumetric flask. Finally, the solution was ultrasonicated and filtered through a 0.45 mm nylon membrane with the aid of a vacuum pump.

Individual stock solutions of the studied antibiotics were prepared as follows: the adequate quantity of the solid standard was weighed and dissolved in few mLs of ethanol, and then filled up with a micellar solution of 0.05 M SDS at pH 3 (fixed with a phosphate buffer), to reach a final concentration of nearly 100 mg L^{-1}. The solution was ultrasonicated to assure complete solubilization. These solutions were stored at 4 ºC in darkness for 1 month. Working solutions were prepared by successive dilutions with the solution of 0.05 M SDS at pH 3. Working solutions containing the four fluoroquinolones were prepared by mixing the stock solutions. These solutions were kept at 4 ºC in darkness for 1 week.

2.3 Chromatographic instrumentation and conditions

The chromatographic system used for this study was a Series HP1100 supplied by Agilent Technologies (Palo Alto, CA, USA), equipped with an isocratic pump, an autosampler tray and a fluorescence detector. The stationary phase was in a reverse-phase C18 Kromasil column (150 x 4.6 mm; 5 mm particle size; 10 nm pore size) supplied by Scharlab. The mobile phase was an aqueous solution of 0.05 M SDS - 1% (v/v) 1-butanol - 0.5% (v/v) TEA at pH 3 running under isocratic mode at room temperature at 1 mL min^{-1}. The injection volume was 20 µL. The excitation and emission wavelengths were set at 280 and 455 nm, respectively. The Agilent Chemstation (Rev. B.03.01) software was used to control the HPLC instrumentation and to acquire chromatographic data. The obtained chromatograms were processed by the Michrom software [36] to measure the main chromatographic parameters: peak area (A), dead time (t_0, min), retention time (t_R, min), retention factor (*k*), efficiency (a number of theoretical plates, N) and asymmetry (B/A) [37]. The special care required for the chromatographic system when dealing with micellar mobile phases is described in [29].

2.4 Sample collection and processing

Twenty commercial honey samples were purchased from local supermarkets and kept in a fridge. The trademark, supplier and variety are indicated below:
- "Granja San Francisco" (Nutrexpa, Barcelona, Spain): multiflower, eucalyptus-lime, forest, and orange blossom.
- "Consum" (Reina Apícola Levantina, Alzira, Spain): multi-flower, rosemary, orange blossom, and eucalyptus.
- "El Brezal" (Mielso, Almazora, Spain): orange blossom, rosemary, multiflower, thyme, black eucalyptus, white eucalyptus, mountain (several mountain flowers), forest (honeydew), acacia, and Yucatan (Nahonal and Dzidzilche flowers).
- "El Quexigal" (El Quexigal, Cebreros, Spain): heather and lavender.

All the honey samples were manufactured in Spain except acacia honey and Yucatan honey, which were elaborated in Central Europe and Mexico, respectively.

The samples were taken out 30 min before analysis to warm up to room temperature. Then, 5 g were introduced into a 10 mL-volumetric flask, and filled up with a micellar solution of 0.05 M SDS at pH 3. The diluted solution was filtered through a 0.45-µm-Nylon membrane, placed into the vials and injected into the chromatographic system. The remaining solutions were not stored.

For spiked samples, the appropriate amount was injected into the honey, immediately before mixing with the micellar solution.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions (injection volume, 20 µL; flow-rate, 1 mL min^{-1}; surfactant, SDS; pH, 3; buffer, 0.01 M phosphate and addition of 0.5% of TEA) were taken from previously published papers about the analysis of difloxacin and sarafloxacin in fish flesh [32] and danofloxacin and difloxacin in eggs and milk [33]. These papers also recommend the use of hybrid mobile phases with a short-chained alcohol to obtain adequate retention times and peak shapes.

The composition of the mobile phase (concentration of SDS and the organic solvent) and the detection conditions were optimized. In all the optimization tests, a standard solution of DAN, DIF, CIP and SAR at 20 μg L^{-1} was used.

3.1.1 Selection of the alcohol for the mobile phase

Hybrid mobile phases containing 1-propanol, 1-butanol and 1-pentanol were tested. Using mobile phases with SDS/1-pentanol, the analytes were barely retained on the C18 column, and then they overlapped and were eluted too close to the dead time. Therefore, 1-butanol was selected, as mobile phases using SDS/1-butanol provides better peak shapes and less retention timesthan using SDS/1-propanol.

The studied range of SDS and 1-butanol amounts was between the minimum and the maximum concentration recommended for MLC, 0.05–0.15 M, and 1–7%, respectively. In order to evaluate the chromatographic behavior of each analyte, five mobile phases were tested, at the following SDS (M)/1-butanol (% v/v): 0.05–1; 0.05–7; 0.10–4; 0.15–1 and 0.15–7.30

The chromatographic parameters (t_0; t_R; k; N and B/A) were taken for each FQ and mobile phase, using the Michrom software [36]. The retention time and the efficiency decrease at higher concentrations of SDS, indicating that the FQ binds to the micelles. On the other hand, at higher concentrations of 1-butanol, the retention times diminish and the efficiency increases.

3.1.2 Optimization of the composition of the mobile phase

The concentration of SDS and 1-butanol were simultaneously optimized following an interpretative strategy, using a chemometrical approach. This mathematical model is based on equations that relate the chromatographic behaviour of the analytes with the composition of the mobile phase [31]. Thisapproach would be more effective and rapid than a sequential (one by one) optimization. Eqn (3.1) is used to describe the retention factor of the analyte, depending on the concentration of SDS ([M]) and 1-butanol (φ):

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+\dfrac{K_{AM}[M](1+K_{MD}\varphi)}{1+K_{AD}\varphi}} \tag{3.1}$$

K_{AS} and K_{AM} are the partition coefficients of the analyte between the bulk water and stationary phase and the micelle, respectively. K_{AD} and K_{MD} measure the relative variation of the analyte in the mobile phase and inside the micelles, because of the presence of the alcohol. K_{AM} and K_{AS} depend on the analyte and surfactant, whereas K_{AD} and K_{MD} depend on the analyte, the surfactant and the alcohol.

The peak shape is modelled by eqn (3.2) and can be used to calculate N and B/A. It considers that the distribution of the signal *h(t)* vs. elution time follows a modified normal (Gaussian) model, which maximum is at the retention time. The standard deviation is substituted by a linear equation:

$$h(t) = H_0 e^{-0.5\left(\dfrac{t-t_R}{s_0+s_1(t-t_R)}\right)^2} \tag{3.2}$$

H_0 represents the height at the retention time, and depends on the concentration and the fluorescence emission of the analyte. The constant s_0 is a measure of the peak width and s_1 constants quantify the distortion of the peak. The si constants depend on N and B/A, as well as the FQ and the mobile phase.

The chromatographic data obtained by the five mobile phases containing 1-butanol (see Section 3.1.1) were processed by the Michrom software [36] as “calibration levels” to fit eqns (3.1) and (3.2). Thus, the obtained equations are able to predict *k*; N; B/A and *h(t)* for the four fluoroquinolones in the range 0.05–0.15 M (SDS) and 1–7% (1-butanol) by interpolation.Combining these values, the software calculates the resolution (r_{ij}) of consecutive peaks following the valley-peak criterion, and the global resolution (Z) as the r_{ij} of the least resolved peak pair [38]. Besides, theoretical chromatograms can be drawn by the simultaneous plotting of the *h(t)* vs. time for the four analytes. Thus, the changes in the

chromatograms and chromatographic behaviour for each analyte, when the amount of SDS/butanol progressively varies, can be easily visualized.

The concentrations of SDS and 1-butanol were selected to obtain the maximum resolution between the studied antibiotics at the minimum analysis time. The optimal mobile phase was an aqueous solution of 0.05 M sodium dodecyl sulfate, 1% 1-butanol and 0.5% triethylamine buffered at pH = 3. Under these conditions, the analytes were completely resolved (Z = 0.998) in 25 min, and the peaks were nearly Gaussian. The chromatographic parameters (t_R; N; B/A) were: danofloxacin (15.5; 4201; 1.085), difloxacin (17.6; 1652; 1.012), ciprofloxacin (19.1; 1750; 0.985) and sarafloxacin (21.4; 3100; 1.047). As required by the 2002/657/EC regulation [34], the less retained compound was eluted more than two times the dead time. The errors in the predicted values for retention factors were <5%.

The use of a chemometric tool has allowed the optimization of the two parameters testing only five mobile phases, thus reducing time and effort. The optimized mobile phase has attractive advantages to apply the method for routine analysis. The use of isocratic mode removes the need of stabilization time between two injections, thus reducing the total time of analysis. As a result, the successive analysis of a large amount of samples is expedited and the analysis can be sold at a lower price. Besides, the optimized mobile phase contains harmless inorganic reagents and a minimal amount of organic solvents. This reduces the risk of the laboratory staff to handle toxic volatile solvents and the waste of toxic compounds to the environment.

3.1.3 Optimization of detection conditions

The studied fluoroquinolones show an intense fluorescence in micellar media [32,33]. However, the fluorescence properties can strongly vary depending on the chemical environment, and the spectral data from other mobile phases and matrices cannot be taken.

The excitation and emission spectra of the four drugs were obtained by analyzing a honey sample spiked with 40 mg kg^{-1} of each antibiotic, using the optimized chromatographic conditions. The maximum excitation/emission wavelengths (nm) were found to be similar for the studied analytes: danofloxacin, 280/450; difloxacin, 280/455; ciprofloxacin, 285/465, and sarafloxacin, 280/455, respectively. As the spectral data were similar for the studied fluoroquinolones, the detection conditions were set at intermediate values: 280/455. Under

these conditions, the four analytes are quantified close to their maximum signal-to-noise ratio and no changing of the detection wavelength during the run was needed.

3.2 Sample preparation

A honey sample was mixed with a micellar solution of 0.05 M SDS at pH 3 [32] in order to solubilize the saccharides and then obtain a liquid sample with low viscosity. Furthermore, the diluted sample must be filtered to avoid the injection of high particles and remaining aggregates. There is no risk of precipitation after the injection, because the compounds would remain in a micellar medium. The dilution ratio was optimized considering the need of avoiding an early obstruction of the filter before obtaining a volume sufficiently representative of the whole sample, but without excessively diminishing the sensitivity. Several dilution ratios were tested, by varying the amount of honey: 50:1; 20:1; 10:1; 5:1;1:1. In all cases, an aliquot of 2 mL was easily obtained without obstruction of the filter. Thus, 1:1 was selected to maximize the sensitivity.

A sample of multiflower honey (trademark “Consum” and manufactured in Spain), free of fluoroquinolones was analyzed using the optimized method (Fig. 3.2A). Several peaks were observed, but they elute before 10 min and do not interfere with the analytes.

The greatest advantage of this experimental procedure is the absence of extraction and clean-up steps, expediting it to dilution and filtration. Thus, the sample is quantitatively introduced in the chromatographic system. This simplified operating procedure reduces the probability of operator errors and strongly shortens the analysis time. As a consequence, the possible sources of variability and the risk of the loss of analytes are minimized, thus improving the reproducibility. Besides, analysis can be achieved using a small amount of innocuous reagents, without requiring specific instrumentation and large volumes of toxic organic solvents. This would improve the productivity of the laboratory, the safety of the laboratory staff and lessen the environmental impact of the analysis.

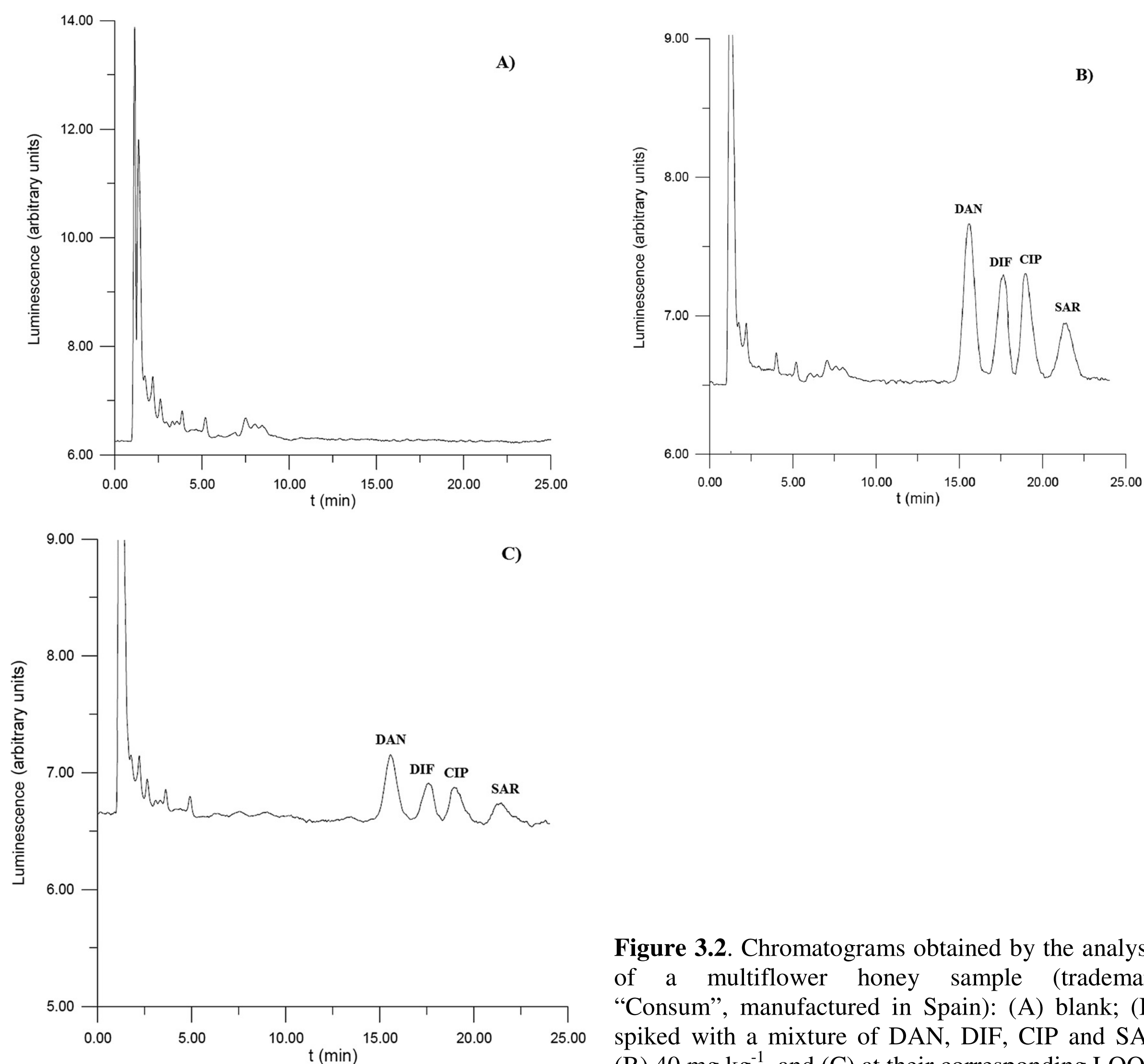

Figure 3.2. Chromatograms obtained by the analysis of a multiflower honey sample (trademark "Consum", manufactured in Spain): (A) blank; (B) spiked with a mixture of DAN, DIF, CIP and SAR (B) 40 mg kg^{-1}, and (C) at their corresponding LOQs.

3.3 Method validation

The method was validated following the directives of the EU Commission Decision 2002/657/EC [34]. The studied validation parameters were: selectivity, linearity, calibration range, crossover, intra- and interday accuracy and precision, decision limit, detection capability and robustness. The limits of detection and quantification were determined by the ICH Harmonized Tripartite Guideline [35], as the EU Commission Decision does not mention them. The whole validation was performed using spiked samples of multiflower honey (same as in Section 3.2), initially free of analytes. The concentrations refer to the w/w

amount of FQ in the honey sample, not in the injected aliquot.

3.3.1 Specificity

The specificity was studied by analyzing the twenty samples of honey described in Section 2.4. In all cases, several peaks were detected from the dead time to nearly 5 min, corresponding to the matrix endogenous compounds. No peaks were observed near the retention times of the analytes, and the baseline was quite stable at >10 min. Furthermore, the studied samples were spiked with 40 mg kg^{-1} of each FQ, and analyzed. The resulting chromatograms show similar profiles to the blanks, the only difference being the occurrence of the peaks from the analytes. No overlapping was observed between the analytes and the endogenous compounds. Therefore, the method is specific enough to unequivocally distinguish the analytes in a wide range of honey varieties.

As an example, chromatograms obtained before and after spiking a sample of multiflower honey (same as in Section 3.2) can be seen in Fig. 3.2A and B, respectively. Smaller peaks appear from the dead time to ≈10 min, sufficiently far from the elution times of the analytes. The difference between the retention time of the analytes in standard solution and in spiked samples was <2.0%, and the peak shape was similar.

3.3.2 Linearity and sensitivity

For calibration purposes, five solutions containing increasing concentrations (three replicates) of the four studied fluoroquinolones were analyzed in the 10–200 mg kg^{-1} range. The equation relating the peak area of each analyte and the concentration was adjusted using the least-squares linear regression, in order to calculate the slope and y-intercept. The goodness-of-fit of the data to the curve was evaluated through the determination coefficient. In order to consider the interday variability, five calibration curves were constructed in different days over a 3 months period, using new solutions each time. The average values can be seen in Table 3.1. An excellent linearity (r^2 > 0.9990) was obtained for danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in the considered range.

Table 3.1. Calibration parameters for the analytes (linear range = 10-200 mg kg^{-1})[a].

Compound	Slope	y-intercept	r^2	LOD/CC(α)	LOQ	CCβ
Danofloxacin	2.000 ± 0.004	24.0 ± 0.9	0.9991	4	10	5.5
Difloxacin	3.31 ± 0.03	- 15 ± 8	0.9990	4	10	5.2
Ciprofloxacin	2.64 ± 0.04	-11 ± 9	0.9995	4	10	6.2
Sarafloxacin	1.424 ± 0.008	-2.5 ± 1.7	0.9993	4	10	4.7

[a]n = 5; all concentrations in mg kg^{-1}.

The limits of detection (LOD) and quantification (LOQ) were calculated as the minimal concentration providing a chromatographic peak 3 or 10 times higher than the baseline noise, respectively [35]. The LOQ was taken as the minimal level of the calibration curve. The values are shown in Table 3.1. A chromatogram obtained by the analysis of a honey sample spiked at the LOQ for each analyte is shown in Fig. 3.2C. The low values prove that the method has enough sensitivity to detect low amounts of these fluoroquinolones in honey. These values are similar to those obtained using other HPLC-FLD-based methods: 4.4 μg kg^{-1} [27] and 7 μg kg^{-1} [28] using an easier sample preparation method.

3.3.3 Accuracy and precision

The intraday accuracy was calculated as the average value of the concentration measured by the method (6 successive analyses) and the true value, whereas the intraday precision was calculated as the relative standard deviation between the obtained peak areas by six successive injections of the same solution. The same solutions were used for accuracy and precision and, different from those used in calibration studies. The accuracy and precision of the method were determined for the four studied fluoroquinolones at 10, 20 and 40 μg kg^{-1}. The interday values were calculated as the average of five intraday measurements taken at several days during a three-months period. The solutions were remade each day. The results are shown in Table 3.2. The method was found quite accurate (81.0-103.4%) and precise (<12.3%). These values are in accordance with the European Commission Decision 2002/657/EC regulation, which accepts values within 80–110% for accuracy and <15% for precision [34].

Table 3.2. Intra- and inter-day accuracy and precision for the studied fluoroquinolones ([a]n = 6; [b]n= 5).

		Intra-day[a]		Inter-day[b]	
Fluoroquinolone	Concentration (ng/g)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)
Danofloxacin	10	92.7	2.2	95.6	3.4
	20	100.2	0.9	101.2	2.1
	40	100.0	1.9	98.5	1.8
Difloxacin	10	82.3	6.1	87.5	4.5
	20	102.3	6.9	98.5	6.4
	40	99.9	2.8	100.8	4.1
Ciprofloxacin	10	82.1	12.3	86.5	10.2
	20	85.2	3.4	90.5	5.3
	40	99.7	2.2	97.5	2.0
Sarafloxacin	10	81.0	4.9	83.8	5.2
	20	103.4	5.9	101.2	4.6
	40	99.9	3.8	98.6	2.5

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3.3.4 Decision limit and detection capability

The EU Commission Decision 2002/657/EC has introduced the determination of two validation parameters, the decision limit (CCα) and the detection capability (CCβ), which assess the critical concentrations (detected and really present) above which the method is able to distinguish a non-compliant sample, considering the method variability and the statistical risk of making a wrong decision. As no MRLs have been stated for the studied fluoroquinolones, the samples are non-compliant if the analytes are detected.

The CCα refers to the detected concentration above which it can be concluded that the sample is not compliant, with a probability of a to have a false positive. For compounds without MRL, $\alpha = 1\%$, and the CCα is taken as the limit of detection.

The detection capability (CCβ) is the smallest concentration of FQ in honey samples that can produce a non-compliant result with a maximal probability of b to make a false

negative. Considering $\beta = 5\%$, this value was calculated as the decision limit plus 1.64 times the standard deviation of a honey sample spiked at the CCα.

CCa and CCb values are shown in Table 3.1. According to the results, the method is able to notice non-compliant samples in honey batches even containing low concentrations of the studied antibiotics.

3.3.5 Robustness

The robustness was examined by measuring the changes in the retention time and peak area of each FQ, at small, but deliberate variations of the composition of the mobile phase (pH, SDS, 1-butanol, and TEA) and flow rate. These studies were performed using a processed honey sample spiked with 40 μg kg^{-1} of each analyte. The relative standard deviations of the retention time and peak area values, taken at: the optimal value, slightly over and slightly under (each one by three replicates), were calculated. Each parameter was separately studied, maintaining the other constant.

The retention time (<8.5%) and the peak area (<6.5%) are not significantly affected, when the above-mentioned parameters were modified. The concentration of TEA has the strongest influence on the retention of the analytes, compared to the other parameters. This coincides with that found in a previous paper [32]. Anyway, the method is robust enough to provide consistent results, when the experimental parameters oscillate within a realistic range.

3.4 Analysis of real samples

According to the results of the study, the method has been successfully validated following the EU Commission Decision 2002/657/EC, and then could be implemented in laboratories approved for the official residue control of these antimicrobial drugs in honey, or used as a test prior to sending honey batches to the EU market. Finally, the method was applied to the commercial honey samples described in Section 2.4. No significant differences were found in the chromatograms, and the studied fluoroquinolones were not detected.

4. Conclusions

The obtained results indicate that micellar liquid chromatography is an interesting alternative to analyze danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey. Despite the viscosity of the sample, it can be directly injected after simple dilution and filtration, thus avoiding tedious and time-consuming extraction procedures, reducing the global analysis time. The studied antibiotics have been eluted using an isocratic mobile phase, without interference from endogenous compounds of honey. The method was successfully validated following the requirements of the EU Commission Decision 2002/657/EC in terms of selectivity, calibration range, linearity, accuracy, precision, decision limit, detection capability and robustness. Besides, the method ensures that a honey sample declared as compliant has only up to µg kg^{-1} levels of FQ, due to the use of fluorescence detection. The method uses innocuous inorganic reagents and a low concentration of organic solvents, and then meets the requirements of "green chemistry". Besides, it facilitates the successive analysis of a high amount of samples, and it is relatively inexpensive, thus making it more advantageous. Therefore, the method is applicable to be used for routine analysis of residues of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey, in order to evaluate the suitability of the samples to be distributed with the European Union.

5. Acknowledgements

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Chapter 4

Use of micellar liquid chromatography to analyze oxolinic acid, flumequine, marbofloxacin and enrofloxacin in honey and validation according to the 2002/657/EC decision

Abstract

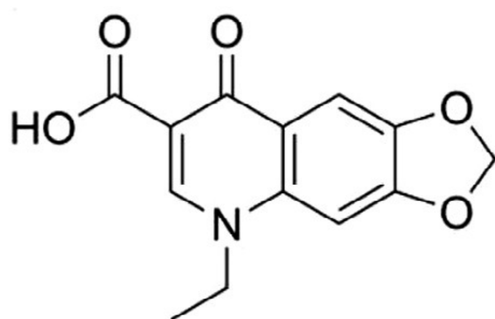
A micellar liquid chromatographic method was developed for the analysis of oxolinic acid, flumequine, marbofloxacin and enrofloxacin in honey. These quinolines are unethically used in beekeeping, and a zero-tolerance policy to antibiotic residues in honey has been stated by the European Union. The sample pretreatment was a 1:1 dilution with a 0.05 M SDS at pH 3 solution, filtration and direct injection, thus avoiding extraction steps. The quinolones were eluted without interferences using mobile phase of 0.05 M SDS/12.5% 1-propanol/0.5% triethylamine at pH 3, running at 1 mL/min under isocratic room through a C18 column. The analytes were detected by fluorescence. The method was successfully validated according to the requirements of the European Union Decision 2002/657/EC in terms of: specificity, linearity (r^2 > 0.995), limit of detection and decision limit (0.008–0.070 mg kg^{-1}), lower limit of quantification (0.02–0.2 mg kg^{-1}), detection capability (0.010–0.10 mg kg^{-1}), recovery (82.1–110.0%), precision (<9.4%), matrix effects, robustness (<10.4%), and stability. The procedure was applied to several commercial honey supplied by a local supermarket, and the studied antibiotics were not detected. Therefore, the method was rapid, simple, safe, eco friendly, reliable and useful for the routine analysis of honey samples.

1. Introduction

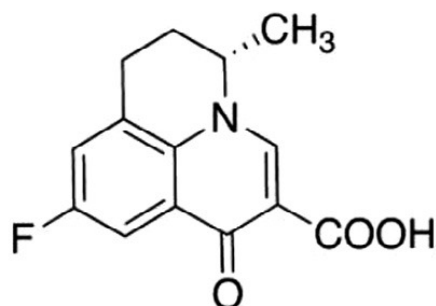
Honey is a natural and healthy foodstuff extremely appreciated since the Antiquity by its unique sweet taste and excellent nutraceutical benefits. It is highly caloric and provides instant energy due to its high amount of sugars (nearly 77%), and hold a strong antibiotic and antiseptic activity. Honey is widely consumed in breakfast and afternoon snack meal spread on bread and sweetener in baking, cooking, and hot beverages, and also used as additive in the food industry. It is also prescribed to palliate cough and sore throat, as well as several infections [1-3]. The European Union is the world main market of honey, with an annual consumption of 310 million of tones. The 40% is imported from non-EU countries, that makes an excellent opportunity for honey producers. Nowadays, the major suppliers are Argentina, Mexico and New Zealand [4].

Domesticated honey bees can be affected by diseases and pest, reducing production of honey and the profitability of the hives. The administration of the quinolones oxolinic acid (pKa = 6.8; log Po/w = 1.43), flumequine (pKa = 6.7; log Po/w = 2.41), marbofloxacin (pKa = 5.9/7.7; log Po/w = -2.92) and enrofloxacin (pKa = 6.03/8.3; log Po/w = 1.89) [5,6], which structures can be seen in Fig. 4.1, has been proposed to combat several bacterial infections, such as nosemosis and foulbrood, and as prophylactic agents [7]. These compounds are important broad-spectrum antibacterial agents largely prescribed in animals and humans to treat parasitic infections, because of their effectiveness. They have a high bioavailability and persist in edible tissues [8]. An extensive use of quinolones implies their persistence in bee products, posing in extreme danger the consumer. The hazards associated with the occurrence of these antibiotics in honey are allergies, toxic effects and the development of drug resistance strains of human pathogens [1,9]. During the last years, European government and citizens have been concerned by the danger due to the extensive use of these compounds in beekeeping. Therefore, the European Commission prohibited the use of antibiotics in apiculture for food and health safety reasons [2,10]. The EU Commission Regulation 37/2010, about the presence of drugs in foodstuff of animal origin, has not established maximum residue limits for oxolinic acid (OXO), flumequine (FLU), marbofloxacin (MARBO) and enrofloxacin (ENRO) in bee products, and then only honeys free of these antibiotics are allowed to be sold in the EU countries [11]. However, a worldwide

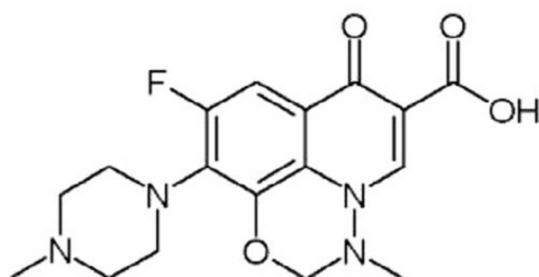
harmonization has not reached about this theme, so that these antimicrobial drugs are authorized for the treatment of honeybees in many countries outside the EU [12]. Besides, several EU beekeepers propose the use of quinolones long before honey collection [13]. Therefore, several surveillance systems have been established to evaluate the compliance of honey products with the EU regulation [1,4,12].



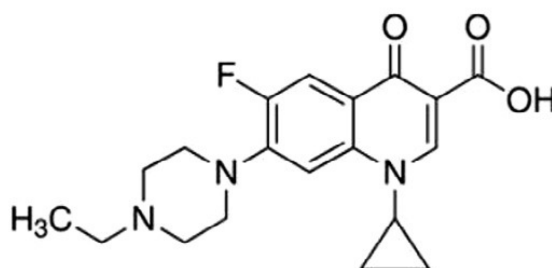
Oxolinic acid



Flumequine



Marbofloxacin



Enrofloxacin

Figure 4.1. Structure of the studied quinolones.

Recently, the occurrence of antimicrobial agents in several bee products has been noticed, especially ENRO, demonstrating that certain unethical beekeepers and honey traders do not comply with the EU regulation [1,2,14-16]. Antibiotic detection leads to the withdrawal of the corresponding batch, and has a serious impact on the reputation and the economy of the producer and its country, as well as that of the European distributor [12]. Besides, it defiles the image of bee products as natural and healthy [2,14]. In fact, the importation of honey from China, the world largest producer, was banned in 2002-2004, and

is nowadays barely accepted, because of the finding of antibiotics in several honey supplies [4]. Manufactures and distributors must analyze honey batches to verify the absence of quinolones and then have access to the EU market. Therefore, quality control laboratories need practical and reliable analytical methods to determine OXO, FLU, MARBO and ENRO in honey.

Two approaches are predominant for the analysis of these quinolones in honey: screening by enzyme immunoassays [17] and full quantification by liquid chromatography [3,10]. This last one is preferred, because it allows the determination of several compounds in one analytical run and provide more reliable quantitative results. Without doubt, LC-MS is currently the method-of-choice for the multiresidue analysis of OXO, FLU, MARBO, ENRO and other quinolones in honey [18-22]. However, mass spectrometry is an easy-to-contaminate and expensive instrumentation, both acquisition and maintenance, and only a limited number of laboratories can afford it. Therefore, several attempts have been undertaken to develop methods requiring more affordable detectors, such as UV-Visible absorbance diode array (DAD) [23] or fluorescence (FLD) [24]. This last option is preferable, because of its higher specificity and sensitivity. Moreover, honey is a viscous and complex matrix, as it contains several macromolecules potentially harmful for the column, such as sugars, pigments and phenolic compounds, which must be removed prior to the injection [3,10,25]. Even if several authors recommend the implementation of automated strategies for sample treatment [18,26], the most common protocols involve one or more off-line clean-up steps, such as matrix precipitation [3,14,19,20,23,24], as well as solid [3], either with magnetic particles [27], reverse phase [14,22], ion-exchange [22,28] or immunoaffinity [29] columns, and liquid [30] extraction. However, these procedures are time-consuming, cumbersome, and requires an exhaustive manipulation. These intermediate steps may have inadequate and variable recoveries, that may affect the reliability of the results. Besides, a large volume of toxic solvent and specific high-cost instrumentation are needed. Several studies have demonstrated that these drawbacks can be avoided using micellar liquid chromatography.

Micellar liquid chromatography coupled with fluorescence detection (MLC-FLD), using hybrid mobile phases of the anionic surfactant sodium dodecyl sulfate (SDS) and a short chained alcohol, has been proven as an interesting alternative to analyze quinolones in food samples, such as fish flesh [31], eggs and milk [32]. Micellar solutions solubilize

macromolecules and hydrophobic compounds, so that food samples diluted in a micellar environment can be injected without risk of precipitation, thus expediting the sample preparation [33]. Besides, the use of MLC leads to analytical methods cheaper, safer and more eco friendly if compared to hydroorganic HPLC [34].

The aim of the work was the development of a sensitive and reliable analytical method based on micellar liquid chromatography - fluorescence detection for the quantification of OXO, FLU, MARBO and ENRO in honey. The procedure should hold adequate practical performances and then be easy-to-handle, inexpensive, eco friendly, safe and useful for routine analysis. The method was in-house validated following the requirements of the EU Decision 2002/657/EC in terms of: specificity, calibration range, linearity, recovery, precision, decision limit, detection capability, matrix effects, robustness and stability [35]. The method was applied for the analysis of the studied quinolones in Spanish commercial honey in order to verify their compliance with the EU Regulation 37/2010 about the presence of drug residues in foodstuff from animal origin [11].

2. Experimental procedure

2.1 Apparatus and instrumentation

An analytical balance Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland) was used to weight the solid standards. The magnetic stirrer and the ultrasonic bath were purchased from Selecta (Barcelona, Spain). The pH measurements were performed using a Crison potentiometer (Model micropH 2001, Barcelona) equipped with a combined Ag/AgCl/glass electrode.

The chromatographic runs were carried out using a chromatograph HP1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, an autosampler tray, an injection system and a fluorescence detector, connected to a PC. The control of the instrumentation and the monitoring of the signal was performed by the ChemStation Software, version A.10.01 (Agilent Tech.). The chromatograms were processed using the Michrom software [36] to calculate the efficiency (N) and the asymmetry (B/A) of the peak corresponding to each analyte. The meaning of these parameters can be seen in [37].

2.2 Reagents and chemicals

The powdered standard of OXO (purity > 97.0%), FLU (>98.0%), MARBO (>98.0%) and ENRO (>98.0%) were bought from Sigma (St-Louis, MO, USA). Sodium dodecyl sulfate (>99.0%) was purchased from Merck (Darmstadt, Germany). Triethylamine (>99.5%), hydrochloric acid (reagent grade, 37.0%) and ethanol (HPLC grade) were obtained from J.T. Baker (Deventer, The Netherlands). Sodium dihydrogen phosphate monohydrate (>99.0%), 1-propanol, 1-butanol and 1-pentanol were purchased supplied by Scharlab (Barcelona, Spain). Ultrapure water was in-lab produced from deionized water using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France).

2.3 Solutions and mobile phases

Individual stock solutions containing 100 mg L^{-1} of each Q were prepared as follows: the appropriate amount of solid standard was weighted and solved in 5% of ethanol, then the volumetric flask was filled up with a solution of 0.05 M SDS at pH 3, and ultrasonicated to ensure the total solubilization. Working solutions containing the four quinolones were prepared by mixing the stock solutions. Further combined and individual working solutions were prepared by successive dilution of the stock solution in a solution of 0.05 M at pH 3. These solutions were stored in a fridge at +4 ºC in darkness. Working solutions were kept a maximum of one month.

In order to prepare the micellar solutions (both mobile phases and for sample dilution), the adequate quantity of SDS and NaH_2-PO_4.H_2O (final concentration 0.01 M) was solved in ultrapure water by stirring. Afterwards, the appropriate volume of triethylamine (TEA) was added to reach a final amount of 0.5% (v/v), if applicable, and then the pH was adjusted to 3 by adding drops of HCl solutions. Furthermore, the organic solvent was introduced to attain the desired proportion (if applicable), and the volumetric flask was filled up with ultrapure water, ultrasonicated and filtered with the aid of a vacuum pump through a 0.45-µm-Nylon membrane.

2.4 Chromatographic conditions

The stationary phase was in a Kromasil C18 column (Scharlab) with the following characteristics was used: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 μm; pore size, 10 nm. The mobile phase was an aqueous solution of 0.05 M SDS - 12.5% 1-propanol - 0.5% TEA, buffered with phosphate to pH 3, running at 1 mL/min at room temperature under isocratic mode. The following fluorescence detection program was run (emission/excitation wavelenght, nm): 0.0-8.0 min (240/400); 8.0-15.0 min (280/495). The injection volume was 20 μL. The special care required for the chromatographic equipment when dealing with micellar mobile phases was described in [33].

2.5 Sample collection

Commercial honey samples were purchased in local supermarkets and stored in a desiccator protected from light [3]. All the honey samples were produced and sold in Spain.

The following twenty honeys were taken to estimate the specificity, as the supplier has warranted the absence of quinolones:

- ‘‘Hacendado” (Apisol, Montroi, Spain): multi-flower.
- La Obrera (Primo Mendoza, Carlet, Spain): avocado pear, almond-tree, orange blossom, heather, lavender, chestnut, ilex, forest, eucalyptus, multi-flower, sunflower, lemon, mountain flowers, loquat, oak, rosemary, thyme.
- Luna de Miel (Tierra y Oro, Madrid, Spain): multi-flower honey mixed with royal jelly, multi-flower honey mixed with ginseng and multi-flower honey mixed with propolis.

The following 26 honey samples were analyzed to check the compliance with the EU Regulation 37/2010 in terms of the absence of Q residues [11]:

- ‘‘Carrefour” (Mielso, Almazora, Spain): multi-flower.
- Eroski (EROSKI S. Coop., Elorrio, Spain): multi-flower.
- El Colmenar de Valderromero (Buena miel, Alcarria, Spain): multi-flower, rosemary.
- Mel da Anta (Mieles Anta, Lugo, Spain): heather, chestnut, forest.
- Luna de Miel (Tierra y oro, Madrid, Spain): multi-flower, acacia, eucalyptus, rosemary, mountain, orange blossom.
- Mellarius (Buleo Miel, Minglanilla, Spain): rosemary, eucalyptus, orange blossom, heather

(Hoces del Cabriel, Spain).
- Miel Gozo-Gozoa (Eztikidetza, Galdames, Spain): multi-flower, heather, eucalyptus.
- ANAE (National Association of beekeepers, Ayora, Spain): multiflower, forest, lavender, eucalyptus, orange blossom, rosemary.

2.6 Sample processing

A quantity of 5 g of the honey sample and the appropriate volume of working standard solution (for spiked samples) were introduced in a 10 mL-volumetric flask, which was then filled up with a solution of 0.05 M SDS at pH 3. This diluted solution was filtered through a 0.45-μm-Nylon membrane, and the obtained solution was placed in the vial for injection. The remaining solutions were not stored.

3. Results and discussion

3.1 Optimization of the separation conditions

The primary chromatographic conditions were taken from previous papers about the analysis of OXO, FLU, and ENRO in fish flesh [31] and MARBO in milk and eggs [32]: injection volume, 20 μL; flow-rate, 1 mL/min; stationary phase, octadecyl-bonded silica (C18) column; and mobile phase containing: surfactant, SDS; buffer, phosphate; pH, 3 and triethylamine. Under these conditions, the studied quinolones were positively charged. The addition of a short-chained alcohol to the mobile phase has been used in previous works to improve the peak shape and reduce the retention time. Therefore, the concentration of SDS and the nature and proportion of the organic modifier, as well as the detection conditions, were optimized in this work. In all cases, the experiments were carried out using a working standard solution containing 0.2 mg L^{-1} of OXO, FLU, MARBO and ENRO.

Hybrid mobile phases containing SDS with 1-pentanol, 1-butanol or 1-propanol were tested. Using the largest alcohol, the studied quinolones were eluted too close to the dead time with a strong overlapping. Mobile phases of SDS/1-butanol provided useful retention times for FLU, MARBO and ENRO, but OXO was still eluted too close to the dead time. Therefore, these two alcohols were discarded, and 1-propanol was preferred to carry out the

optimization procedure.

3.1.1 Optimization of the SDS/1-propanol concentration

The composition of the mobile phase was selected to elute the four quinolones with a maximum separation in a minimum analysis time. The concentration of SDS and 1-propanol were simultaneously optimized using an interpretative strategy, assisted by chemometrics. This approach is based on several equations, which describes the chromatographic behavior of the analytes as a function of the concentration of SDS and 1-propanol. This model assumes that the acid and basic compounds are quantitatively in one form.

The retention behavior is predicted using a mechanistic model, that means the constants have a physico-chemical meaning. This model has been demonstrated to provide accurate results for moderately hydrophobic compounds resolved using hybrid mobile phases, in the following range of SDS/1-propanol: 0.05–0.15/2.5–12.5 [38]. The following equation is used to predict the retention factor:

$$k = \frac{K_{AS}\frac{1}{1+K_{AD}\varphi}}{1+\frac{K_{AM}[M](1+K_{MD}\varphi)}{1+K_{AD}\varphi}} \tag{4.1}$$

where [M] and φ are the concentration of SDS and the proportion of 1-propanol, respectively. K_{AS} and K_{AM} represent the partition coefficient of the quinolones between the stationary phase and the micelle, respectively, and the bulk water. K_{AD} and K_{MD} measure the variation of K_{AS} and K_{AM}, respectively, because of the decreasing of the polarity of the mobile phase caused by the presence of 1-propanol. Another equation is used to model the peak shape, and then the values of N and B/A [38].

A working standard solution of OXO, FLU, MARBO and ENRO was analyzed using five mobile phases, whose concentrations of SDS (M)/1-propanol (%) were selected using a full factorial design plus the central point: 0.05/2.5; 0.05/12.5; 0.01/7.5; 0.15/2.5 and 0.15/12.5. The experimental measurements of *k*, N and B/A were processed by the Michrom software [36] to adjust the modeling equations. Afterwards, these chromatographic parameters can be determined at intermediate values by interpolation. Besides,

the theoretical values of k; N and B/A of the studied Q calculated for a each SDS/1-propanol concentration, were combined to determine the paired-peak resolution (r_{ij}), by the valley-peak criterion, and the global resolution (Z), as the product of the three r_{ij} [38]. This information was also used to draw simulated chromatograms, and then the operator can visualize the changes of the chromatographic parameters when the composition of the mobile phase changes.

The retention factors of the four analytes increase at higher concentrations of SDS in the mobile phase. That means the quinolones positively interact with the micelles, probably by electrostatic attraction. As expected, the elution strength and the efficiency of the peaks augment at higher proportion of 1-propanol [38].

According to this model, the optimal mobile phase was an aqueous solution of 0.05 M SDS – 12.5% 1-propanol – 0.5% TEA at pH 3, which provides the maximal resolution (Z = 0.998) in the shortest analysis time (15 min), with a good peak shape. Under these conditions, the experimental values of the chromatographic parameters were (t_R; N; B/A): OXO (3.3; 2541; 1.14); FLU (7.2; 1985; 1.21); MARBO (9.1; 3520; 1.34) and ENRO (10.3; 2780; 1.09). The errors in the retention factors were <4%. The quinolones were completely resolved and the less retained compound was eluted over two times the dead time, as required by Decision 2002/657/EC [35].

The use of the mathematical approach has permitted the simultaneous optimization of two variables by testing only five mobile phases. This requires less effort and time than a sequential approach, because of the interaction between variables and the occurrence of local maxima of resolution. This mobile phase is able to resolve the four analytes in <15 min using isocratic mode, and then the column does not require to be equilibrated between two injections, as in gradient.

3.1.2 Optimization of the detection conditions

OXO, FLU, MARBO and ENRO are fluorescent in a micellar medium [31,32]. As the spectroscopic properties depend on the chemical environment, the detection conditions were optimized using a 0.4 mg kg^{-1} of each antibiotic in spiked honey samples analyzed under the optimized chromatographic conditions.

The excitation and emission spectra were registered at the corresponding retention

times, and the optimal wavelengths were selected by iteration to maximize the signal-to-noise ratio of the intensity of the emission. The experimental values (λecx/λem) were: OXO, 264/396; FLU, 240/370; MARBO, 300/488 and ENRO, 280/455. The detection conditions of OXO and MARBO were similar to those obtained to FLU and ENRO, respectively, and then each pair of analytes were quantified at intermediate values. Therefore, the signal was monitored at 240/400 to detect OXO and FLU, and then switched at 8.0 to 240/400 nm, to detect MARBO/ENRO. Hence, the antibiotics were quantified close to the optimal wavelengths with only one change of the detection wavelength during the chromatographic run.

3.2 Sample preparation

The honey samples were diluted using a micellar solution, in order to reduce the viscosity and solubilize the oligosaccharides and other non-water-soluble compounds, and filtered. Once injected, these compounds would remain solubilized, because the mobile phase is also a micellar solution.

The composition of the diluting solution was the same as the mobile phase, but without 1-propanol, in order to avoid possible unnoticed preconcentration due to the evaporation of the alcohol. Finally, the honey samples were diluted in a solution of 0.05 M at pH 3.

The dilution ratio was selected on the basis on decreasing the concentration of injected matrix, in order to diminish the intensity of the front of the chromatogram and enlarge the lifespan of the column, but without excessively diminish the sensitivity. A multi-flower honey (Hacendado) was 1:1-diluted and filtered. The obtained aliquot seemed quite clean and thin, and the front of the chromatogram was not very broad. Therefore, the dilution ratio was set to 1:1.

The main advantage of the procedure is its strong simplicity and the minimization of the operator intervention, as it only includes a single dilution stage, instead of time-consuming and cumbersome extraction or clean-up phases. Besides, this sample pretreatment can be carried out in a short time. The diluted sample is quantitatively injected into the chromatographic system, instead of undergone steps with variable recovery, thus reducing the probability of losing the analyte. The procedure involves less sources of variance, which

leads to a higher reproducibility.

3.3 Environmental, safety and economic aspects

The here-presented procedure uses biodegradable and harmless reagents, and only a minimal amount of toxic, flammable and volatile organic solvent: none in the sample preparation and only 12.5% in the mobile phase (less than typically used in hydroorganic HPLC, which can be up to 100%) [14]. Besides, the interaction of micelles and monomers with 1-propanol diminishes its volatility. Therefore, the handling of a low quantity of harmful chemical limits the possibility of intoxication by the operator, improving the workplace safety at the laboratory. Besides, the quantity of toxic waste is minimized, reducing the environmental impact of the analysis. This fits the current trend in the development of new analytical procedures [34].

The method only uses a low quantity of inexpensive chemicals and basic instrumentation, normally affordable by laboratories even with low economic power. Besides, the method allows the analysis of a large number of samples per day, which improves the productivity. Therefore, the analyses can be achieved at a low price.

3.4 Method validation

The developed analytical procedure was in-house validated following the guidelines of the EU Commission Decision 2002/657/EC, in order to check its reliability in the considered range [35]. This guide has been developed to validate of analytical methods applied to detect organic residues in animals and animal products commercialized for human consumption within the European Union. The following validation parameters were determined: specificity, calibration range, linearity, sensitivity, recovery, precision, decision limit, detection capability, robustness and stability. Unless specified, the experiments were carried out using spiked samples of multi-flower honey (Hacendado), initially free of antibiotics. The concentrations mean the proportion (w/w) of each quinolone in the unprocessed honey sample, not in injected aliquot.

3.4.1 Specificity

Twenty different kinds of honey (see Section 2.5) were analyzed using the optimized conditions, in order to check if the matrices contain compounds eluting near the studied quinolones. Furthermore, the same samples were spiked at 0.4 mg kg^{-1} of each quinolone, and analyzed. As an example, the chromatograms obtained by analysis of blank and spiked multi-flower honey (‘‘Hacendado”) samples can be seen in Fig. 4.2A and B, respectively.

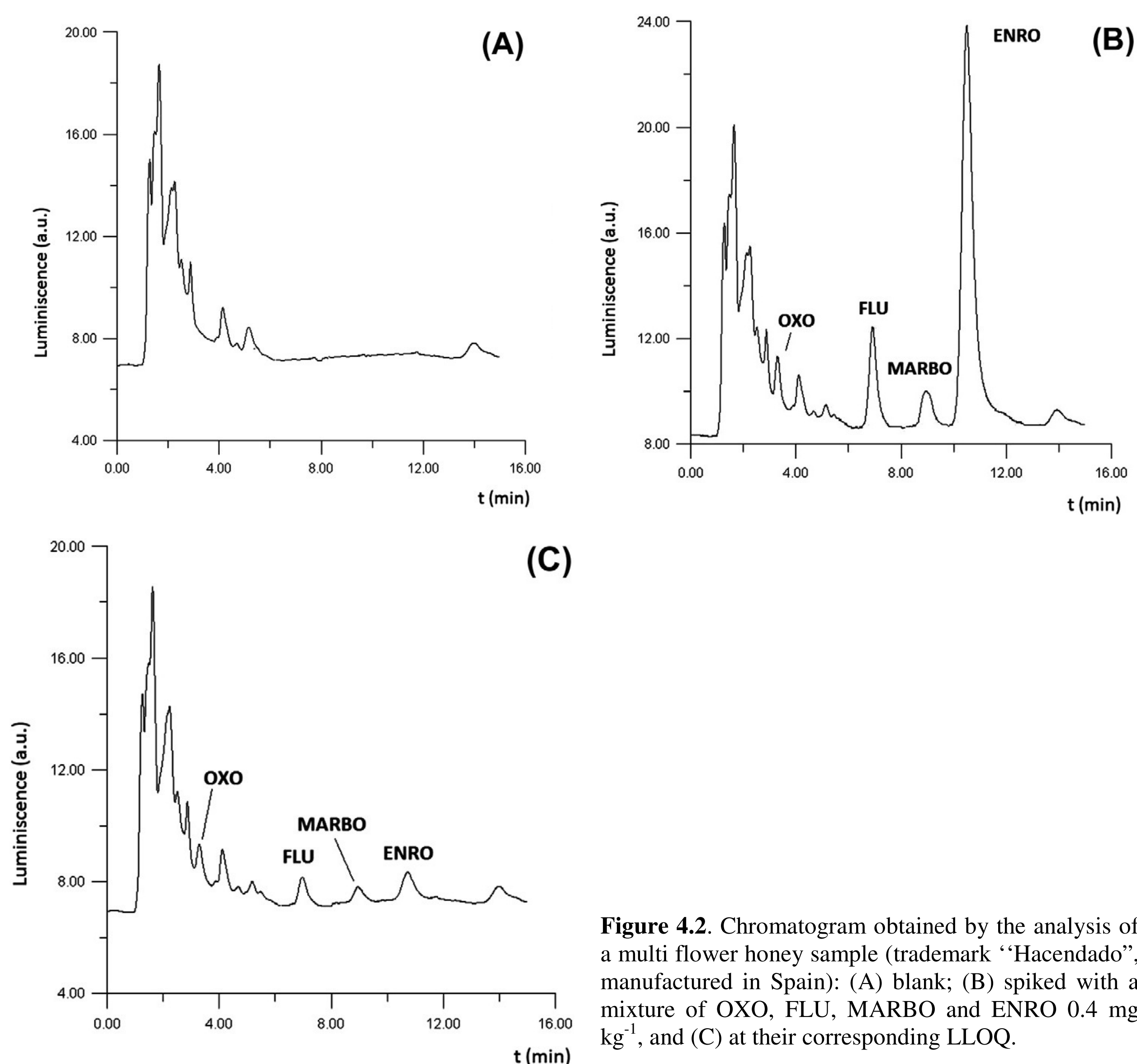


Figure 4.2. Chromatogram obtained by the analysis of a multi flower honey sample (trademark ‘‘Hacendado”, manufactured in Spain): (A) blank; (B) spiked with a mixture of OXO, FLU, MARBO and ENRO 0.4 mg kg^{-1}, and (C) at their corresponding LLOQ.

In all cases, the front of the chromatogram was observed from the dead time to 2.5 min. Several peaks can be seen up to nearly 5 min, but they were not eluted at the window time of OXO. The two peaks near OXO were effectively separated by more than the peak width at 10% of peak height. No peaks were detected near the other analytes.

The analytes show retention times (<2.0%) and peak shape similar to that obtained in the analysis of the working standard solution (Section 3.1.1), without overlapping with other compounds of the matrix. Besides, the maximum excitation and emission wavelengths were close to those measured in Section 3.2.

The procedure is enough specific to unequivocally recognize the studied antibiotics in a wide range of honey varieties. The use of fluorescence detection has contributed to this performance, because the substances with natural fluorescence is rather limited, if compared with UV–Visible detection.

3.4.2 Linearity and sensitivity

Several spiked samples containing increasing concentrations of the studied antimicrobial drugs up to 2 mg kg^{-1} were analyzed by triplicate. The average value of the peak area for each level (A) were plotted *vs.* the corresponding concentration (X). The slope, y-intercept and first-grade equation trendline A = f(X) were determined using the non-weighted least-square linear regression. The goodness-of-fit of the curve was evaluated through the determination coefficient. The obtained values are shown in Table 4.1.

Table 4.1. Calibration parameters for the studied antibiotics (concentrations in mg kg^{-1})

Quinolone	Slope	y-intercept	r^2	LOD/CCα	LLOQ	CCβ
Oxolinic acid	21.1 ± 0.6	3.9 ± 1.3	0.995	0.07	0.2	0.10
Flumequine	108.3 ± 2.0	9 ± 4	0.9990	0.04	0.1	0.06
Marbofloxacin	34.6 ± 2.2	2.3 ± 1.3	0.9990	0.06	0.2	0.08
Enrofloxacin	728 ± 19	27 ± 22	0.9993	0.008	0.02	0.010

n = 5

The lower limit of quantification (LLOQ) was the smallest concentration which can be measured with a precision and recovery in the range accepted by the guide (see Section

3.4.3). These values were taken as the lower level of the calibration curve. The chromatogram obtained by the analysis of a honey sample contain the corresponding LLOQ of each quinolone can be seen in the Fig. 4.2C. The limit of detection was set as the three times standard deviation of the blank (taken as the standard deviation of the residuals of the calibration curve) divided by the slope [39]. The results can be seen in Table 4.1.

A good linearity (r^2 > 0.997) was found in the considered range. Besides, a high sensitivity was achieved, especially for ENRO, indicating that the method is able to detect even low quantities of the studied quinolones in honey samples.

3.4.3 Recovery and precision

Both parameters were determined with the same experiments, at three concentration levels for each quinolone. The analyzed solutions were different to those injected for in the calibration studies.

Three honey samples were spiked at three different concentrations of antibiotics, and processed. The resulting solutions were successively sixfold analyzed. The repeatability was the relative standard deviation (RSD) of the peak areas, whereas the intraday recovery was the quotient between the average of the concentration provided by the method and the true value. This same approach was repeated five different days over a 2-months period, by preparing each time new spiked honey samples. The interday recovery was the average value of the five intraday recoveries, while the within-laboratory reproducibility was the RSD of the average of the six peak areas measured each day. The results are shown in the Table 4.2.

The obtained values were in the accepted range indicated by European Commision Decision 2002/657/EC regulation for recovery (82.4-110.0%, maximal deviation accepted: 80–110%) and precision (<9.4%; maximal variation of the signal 10.7%) [35]. Therefore, the reliability of the method is sufficient to quantify these analytes in honey to evaluate its safety for the consumer and compliance with the EU regulations.

Table 4.2. Intra- and inter-day accuracy and precision for the studied quinolones.

		Intra-day[a]		Inter-day[b]	
Quinolone	Concentration (mg kg^{-1})	Recovery (%)	Repeatability (RSD, %)	Recovery (%)	Within-laboratory reproducibility (RSD, %)
Oxolinic acid	0.2	107.4	8.9	98.6	6.8
	0.4	106.8	1.1	105.6	3.4
	1.0	104.7	2.0	103.6	2.3
Flumequine	0.1	108.2	0.4	108.4	5.3
	0.4	106.7	3.5	100.6	2.7
	1.0	92.5	3.2	93.7	3.2
Marbofloxacin	0.2	103.9	4.9	106.7	5.4
	0.4	103.3	3.6	97.8	9.4
	1.0	90.8	4.8	95.3	8.6
Enrofloxacin	0.02	82.1	1.4	82.4	1.8
	0.10	95.3	1.0	93.2	5.9
	0.40	110.0	0.8	97.6	3.2

[a]n=6; [b]n = 5

3.4.4 Matrix effect

In order to evaluate if the matrix compounds affect quantitative results provided by the method, a working solution of 0.2 mg L^{-1} and a honey sample spiked at 0.4 mg kg^{-1} of each analyte were analyzed by triplicate. In both cases, the injected aliquot contain 0.2 mg L^{-1} of each quinolone, the only difference is the presence of the substances of the honey. No significant differences in peak area were found. This indicate that the honey macromolecules barely interact with the analytes, maybe due to their preferential interaction with the micelles.

3.4.5 Decision limit and detection capability

These parameters were introduced by the EU Commission Decision 2002/657/EC, in order to determine the critical concentrations (measured and present of the sample) from which the method is able to distinguish a non-compliance sample. They are calculated from the variability of the method and the probability to make a wrong decision. As no permitted limit has been fixed, a honey batch would be considered non-compliant if the analytes are detected.

The decision limit (CCα) is the found concentration above which it can be asserted that the sample is non-compliant, with a probability of 1% of making a false decision. It is quantified as 3 times the standard deviation of the blank, and then was set as the LOD. The detection capability (CCβ) is the lower true concentration of Q in a honey sample, whose analysis has a maximal probability to return a concentration under the CCα (due to the random errors), and then be incorrectly classified as compliant, is 5%. It was calculated as the CCα plus 1.64 times the standard deviation obtained by the analysis of a honey sample spiked at the CCα (n = 20). According to the results (Table 4.1), the method is able to distinguish noncompliant samples even at low concentrations of quinolones.

3.4.6 Robustness

The composition of the mobile phase hardly ever equals to the indented values, it often oscillates inside a realistic range, due to the random errors during the preparation. The robustness evaluates the extend in which these variations can affect the chromatographic results.

The change in the elution power and the sensitivity were examined at slight, but deliberate modifications of the concentration of the main components of the mobile phase: SDS, 1-propanol, TEA and pH. The considered ranges of variation were those we judge that can occur in a normal situation at the laboratory (SDS, 0.049-0.051 M; 1-butanol, 12-13%; TEA, 0.45-0.55% and pH, 2.9-3.1). For each chromatographic condition, the RSD of the retention time and the peak area measured at the optimal value, and the minimum and maximum values of the studied range (each one by triplicate) were calculated, maintaining

the others constant. This study were carried out using a multi-flower honey sample spiked at 0.4 mg kg^{-1} of each antibiotic.

The oscillations of the composition of the mobile phase show no significant influence in the retention time (<10.4%) and peak area (<5.3%). Therefore, the method is enough robust to allow the correct identification and quantification of the four quinolones when the experimental chromatographic conditions vary in a short range.

3.4.7 Stability

The possible degradation of the quinolones was studied in three situations: in working solution, in a stored honey sample, and in a processed sample at room temperature (to examine if the time lapsed between the preparation and the injection is relevant). In the first case, a working solution containing 0.2 mg L^{-1} of each quinolone was taken. The other two studies were performed using honey samples (initially free of analytes) spiked at 0.4 mg kg^{-1} for OXO, FLU, MARBO and ENRO.

- Working solution: 30 aliquots of the working solution were stored in the fridge, and each day, one was thawed and analyzed. No significant diminishing of the peak area was observed for the four analyzed during 30 days. Therefore, the working solutions were kept a maximum of one month.
- Storage conditions: 16 samples of the same honey were spiked. One was immediately analyzed and the others 15 were stored in the desiccator protected from light. Every day (during 15 days), one of these samples was taken, analyzed and thrown away. No significant diminishing of the peak area for the four quinolones through time was noticed for this period. Therefore, an individual sample of honey can be kept in a dry and dark place for two weeks prior analysis without providing an incorrect value of analyte concentration.
- Stability of processed samples: A spiked honey sample was analyzed, and the remaining processed sample was kept at room temperature. Each hour, one aliquot was taken and analyzed, up to 8 h. The peak areas of the four antibiotics were found rather similar in all the analyses. Therefore, it was deduced that the time lapsed between the sample preparation and the injection, within the same working day, has no significant influence on the concentration.

3.5 Analysis of real samples

The demonstrated performances of the method and the results obtained in the validation indicate that the analytical procedure is useful for routine analysis and can be implemented in quality control laboratories which aim to control the occurrence of OXO, FLU, MARBO and ENRO residues in honey batches.

The method was applied to the commercial honey samples, described in Section 2.5, purchased from local supermarkets. The quinolones OXO, FLU, MARBO and ENRO were not detected. Therefore, all these honey batches were found compliant with the EU Regulation 37/2010 [11]. The chromatograms were similar to those obtained from the blank samples in Section 3.4.1.

4. Conclusions

Micellar liquid chromatography has been proven a useful technique to develop an analytical procedure for the detection of oxolinic acid, flumequine, marbofloxacin and enrofoxacin in honey. Its main advantage is the possibility of direct injection, after a simple dilution, resulting in an interesting simplification of the sample pretreatment, compared to the long, tedious, and variable recovery extraction steps normally applied in hydroorganic-HPLC. The four analytes were resolved without interferences from the honey matrix using an usual column and a hybrid mobile phase, optimized by a low effort by the use of chemometrics, running at isocratic mode in <15 min. The method was validated following the guidelines of the EU Commission Decision 2002/657/EC. Adequate values for the following validation parameters were obtained: specificity, linearity, calibration range, recovery, precision, robustness and stability. According the values of decision limit and detection capability, honey samples containing more than 0.01–0.1 mg kg^{-1} of quinolones can be classified as non-compliant. Besides, the matrix does not significantly affect the results. The method uses only a low amount of toxic chemicals, and then has an insignificant impact on the environment and the health of the laboratory staff. Moreover, the analyses can be performed at low price, and a large amount of samples per day can be studied. These performances make the method useful for the routine analysis of the four studied quinolones in honey in laboratories dedicated to the residue control in food samples sold within the EU.

5. Acknowledgements and Conflict of interest disclosure

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The authors state that there is no financial/commercial conflict of interest.

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Chapter 5

Determination of oxolinic acid, danofloxacin, ciprofoxacin and enrofloxacin in porcine and bovine meat by micellar liquid chromatography with fluorescence detection

Abstract

A method was developed for the determination of oxolinic acid, danofloxacin, ciprofloxacin and enrofloxacin by micellar liquid chromatography – fluorescence detection in commercial porcine and bovine meat. The samples were ultrasonicated in a micellar solution, free of organic solvent, to extract the analytes, and the supernatant was directly injected. The quinolones were resolved in <22 min using a mobile phase of 0.05 M SDS – 7.5% 1-propanol – 0.5% triethylamine buffered at pH 3, running through a C18 column at 1 mL/min using isocratic mode. The method was validated by the in terms of: selectivity, calibration range (0.01–0.05 to 0.5 mg kg^{-1}), linearity (r^2 > 0.9998), recovery (89.3–105.1%), precision (<8.3%), decision limit (<12% over the maximum residue limit), detection capability (<21% over the maximum residue limit), ruggedness (<5.6%) and stability. The procedure was rapid, eco-friendly, safe and easy-to-handle.

1. Introduction

Quinolones are a family of synthetic and broad spectrum antimicrobial agents with bactericidal activity. They act by preventing the bacterial cell growth by inhibition of DNA replication, recombination and repair. Quinolones have a high bioavailability, good tissue penetration, long half-lives, high efficacy, and low incidence of adverse effects. Because of these characteristics, they are largely used against a wide range of parasitic diseases in humans and animals [1,2].

The quinolones oxolinic acid (OXO), danofloxacin (DANO) and enrofloxacin (ENRO) are widely administered in farms to cattle and swine, either orally or in injectable solutions, to prevent and remedy several respiratory and gastrointestinal infections, as well as to promote growing [1,3-6]. Ciprofloxacin (CIPRO) is not approved for veterinary uses, but it can occur as a metabolite of enrofloxacin [6] (Their structures can be seen in Fig. 5.1). This limits the mortality of the animals, improves feed efficiency, and stimulates uniformity between the animals, thus increasing the economic benefits [7]. However, their extensive use has also serious negative effects and represents a public health danger. It may promote the emergence of zoonotic quinolone-resistant pathogens in the food-producing animals which can lately be transmitted to the population by direct contact or through the food chain [4,8,9]. In addition, sub-therapeutic amounts of quinolones can persist in edible tissues and be unintentionally long-term ingested by the consumer. This can stimulate the development of endogenous drugresistance bacteria strains in the human microbiota, and induce allergic reactions and toxic effects. Besides, the resistance genes can be transferred to endogenous or exogenous bacterias, which may propagate to other organisms [4,8,10]. As a result, there is a risk of increasing mobility and mortality in the population because of the loss of effectiveness of antibacterial therapies [9,10].

Nowadays, there is a great concern among citizens, governments and international agencies about the indiscriminate use of antibiotics in food-producing animals, mainly by the increasing prevalence of failure of antibiotic treatments [4,7]. Therefore, the European Union (EU) and the World Health organization (WHO) have recommended discontinuing the use of antimicrobial agents in cattle and swine stockraising [11,12]. In order to ensure food safety and minimize the risks to human health, the EU has established maximum residue limits

(MRLs) for OXO, DANO, CIPRO + ENRO in beef (0.1; 0.2 and 0.1 mg kg^{-1}, respectively) and swine (0.1; 0.1 and 0.1, respectively) meat produced or sold in the EU [13]. In order to verify the compliance of producers and traders with this policy, control laboratories requires practical and reliable levels for the quantification of oxolonic acid, danofloxacin, ciprofloxacin and enrofloxacin in porcine and bovine edible tissues at these regulatory levels.

Many analytical methods based on reverse phase liquid chromatography have been developed for the multiresidue screening of antimicrobials in samples from animal tissues, mainly because of its ability to analyze several analytes in a single run. LC coupled to MS has been largely used to simultaneously determine OXO, DANO, CIPRO and ENRO in cattle [14] and swine [15-17] flesh samples, because its excellent analytical performances. However, this is an expensive instrumentation, considering both acquisition and maintenance, easy-to-contaminate, and requires highly specialized operator, and then only a few laboratories can afford it [18]. Therefore, several authors have proposed several procedures to simultaneously determine these quinolones in bovine [19-21] and porcine [15,19-23] edible tissues by LC coupled to absorbance [19,22,23] and fluorescence (FLD) [15,20,21] detection. This last one is preferable, as it offers the maximum performance-to-price ratio. However, these methods use mobile phases with high proportions of toxic organic solvents (up to 62%) and programmed as a gradient.

A complex, careful and multistep sample preparation is often required for the analysis of porcine and bovine flesh by HPLC. Firstly, the quinolones must be extracted in a liquid phase, by mixing the sample with an hydroorganic solution, followed by automatic stirring [16,22,23], vortexing [14], homogenization by strong crushing [17,24], ultrasonication [15,19], accelerated solvent extraction [25] and microwave assisted extraction [22]. The obtained supernatant must be further purified to remove aggregates, particles and non-water soluble macromolecules, also extracted from the tissue, in order to avoid damaging the chromatographic system. The most usual are filtration [14], and solid-phase, with a C18 [19,21], C8-cationic [17], N-vinylpyrrolidone and divinylbenzene [25], immunoaffinity [16], hydroxylated polystyrene-divinylbenzene [15,22], metalchelate affinity [20] or molecular imprinted polymer [23] coating, dispersive liquid-liquid micro-, and dispersive micro-solid-phase [24] extraction. These sample pretreatments are cumbersome, time-consuming, as well as costly and specific chemicals (including large proportions of toxic solvents) and laboratory material. Besides, the long manipulation increases the probability of loss of the quinolones

(either by incomplete recovery or chemical change), thus increasing the systematic error and the uncertainty of the results. For these reasons, internal standard is often required [14,15,17,19,22]. Several authors have proposed a simple one-step procedure to extract melamine and quinolones kidney tissue [26] and fish flesh [27], respectively, using an acidic micellar solution of SDS, without using hazardous reagents, by ultrasonication, with high sample throughput.

Micellar liquid chromatography (MLC)-FLD, using sodium dodecyl sulfate (SDS) as a surfactant, a short-chain alcohol and triethylamine (TEA), has been proposed for the determination of quinolones in food matrices [27-29]. Micelles strongly interacts with proteins, fats and other macromolecules. Therefore, they are easily solubilized in a micellar solution, and then the suspension obtained from the solid/liquid extraction of animal edible tissues can be directly injected, after decantation and filtration, without risk of damaging the column. Besides, matrix compounds are barely retained and rarely interfere with the analytes. This avoids the needing of an extra purification step, and then expedite the sample preparation [26]. Otherwise, the surfactant modifies the stationary phase, which acquires a negative charge, and introduce a new environment dispersed in the bulk mobile phase, the micellar pseudophase. Therefore, the retention mechanism is ruled by three equilibria. This increases the versatility of MLC, and allows the separation of compounds with a different hydrophobicity in the same run using an isocratic mode, and a maximum of 12.5% of organic solvent. The high reproducibility and the stability of the retention behavior allows its modelling, as a function of the mobile phase composition using a chemometric approach. Besides, the fluorescence is enhanced in organized environments. In addition, micellar solutions are less toxic, non-flammable, biodegradable, easy-to-handle, and relatively inexpensive in comparison to aqueous-organic ones [18].

The aim of the work was the development of a practical and reliable method for the determination of OXO, DANO, CIPRO and ENRO by MLC-FLD in porcine and bovine meat. Its main application would be to establish if a commercial batch represents a danger to the consumer health, according to the EU Regulation. Therefore, it must be able to quantify the analytes under their respective MRLs, and be rapid, inexpensive, eco-friendly, safe and easy-to-handle. The procedure should be validated by the directive of the EU Decision 2002/657/EC in terms of: selectivity, calibration range, linearity, sensitivity, recovery, precision, decision limit, detection capability, robustness and stability [30], in order to

measure its analytical performances. The method should be applied to several commercial samples of swine and pork meat to evaluate its suitability for routine analysis.

2. Experimental procedure

2.1 Standards, reagents and apparatus

Solid standards of oxolinic acid (OXO, purity >97%), enrofloxacin (ENRO,>98%), ciprofloxacin (CIPRO,>98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and danofloxacin (DANO,>93.5%) was bought from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Sodium dodecyl sulfate (>99%), methanol, ethanol, 1-butanol (HPLC grade) and sodium dihydrogen phosphate monohydrate (99%) were obtained from Scharlab (Barcelona, Spain). Sodium hydroxide (99%) and 1-propanol (HPLC grade) came from Merck (Darmstadt, Germany). Hydrochloride acid (37%) and triethylamine (99.8%) were bought from J.T. Baker (Deventer, The Netherlands). Ultrapure water was in-lab generated from deionized water (provided as tap water by the university) using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France).

The solid standards were weighted using an analytical balance Metter-Toledo (Greifensee, Switzerland). The pH measures were taken using a GLP 22 potentiometer equipped with a combined Ag/AgCl/glass electrode (Crison, Barcelona, Spain). An ultrasonic bath Ultrasons-H (Selecta, Abrera, Spain) was used to achieve the solubilization of the solutes. The filters were 0.45-µm-Nylon membrane (Micron Separations, Westboro, MA, USA).

2.2 Preparation of solutions and mobile phases

Individual stock solutions of each quinolone (100 mg L^{-1}) were prepared by solving the adequate weight of the solid standard in 5% ethanol. Furthermore, the flask was filled up with a micellar solution of 0.05 MSDS at pH 3 (0.01 Mphosphate buffer) and ultrasonicated for 5 min. Working solutions were obtained by successive dilutions of these stock solutions in the same micellar solution. All the standard solutions were stored at 4ºC a maximum of two months. Before use, these standard solutions were thawed for 30 min, in order to

redissolve the crystals of SDS formed overnight.

The micellar solutions and mobile phases were prepared by weighing the appropriate amount of SDS and $NaH_2PO_4.H_2O$ and solving them in ultrapure water. Furthermore, the adequate volume of triethylamine was added, and the pH was adjusted by adding drops of HCl or NaOH solutions. Afterwards, the organic solvent was added to reach the desired proportion, and then the flask was filled up with ultrapure water. Finally, the solution was ultrasonicated for 5 min to achieve solubilization, and filtered with the aid of a vacuum pump.

2.3 Chromatographic instrumentation and conditions

The analyses were performed using a chromatograph HP1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, an autosampler and a fluorescence detector (FLD), connected to a PC. The ChemStation (Rev.A.10.01) software was used to control the instrumentation and acquire the signals. The registered chromatograms were processed by Michrom Software [31] to measure the dead time (t_0), retention time (t_R), efficiency (N) and asymmetry factor (B/A) [18].

The mobile phase was an aqueous solution of 0.05 M SDS - 7.5% 1-propanol - 0.5% triethylamine buffered at pH 3 with 0.01 M phosphate. This mobile phase run at 1 mL/min under isocratic mode. The stationary phase was in a column Kromasil C18 (150 x 4.6 mm; 5 µm particle size; 10 nm pore size), supplied by Scharlab. The injection volume was 20 µL. An excitation/emission (nm) wavelength program was applied for fluorescence detection: 0-8.0 min, 260/366; 8.0-22.0 min, 280/455. The injected solutions (both standard and processed samples) were filtered before introduction into the vials. The instrumentation care required when working with micellar mobile phases has been described in [32].

2.4 Sample treatment

Commercial samples of porcine and bovine meat were purchased at a local supermarket, and stored in a freezer at -20ºC a maximum of two months. Meat samples were thawed for 30 min and finely ground using a mincer model MZ10 (Petra Electric, Burgau, Germany) at 5000 rpm for 10 min. Afterwards, 5 g of meat were mixed with 50 mL of a 0.05

M SDS - pH 3 solution. The flasks were shaken for 1 h using a magnetic stirrer, and then ultrasonicated for 15 min. Finally, the supernatant was filtered with the aid of a vacuum pump and placed in the autosampler vials.

For spiked samples, the appropriate volume of a standard solution of the quinolone is added to the minced meat. The sample was stored overnight at room temperature to ensure the evaporation of the solvent and the interaction of the antimicrobial with the matrix. Therefore, these added samples imitate those ‘‘naturally” contaminated [26]. Furthermore, the extraction continues as indicated above.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions were taken from previously published procedures about the analysis of quinolones in fish flesh [27] and honey [28,29]: injection volume, stationary phase, flow-rate, running mode, surfactant, organic solvent, 1-propanol and the addition of triethylamine.

The parameters to-be-optimized in this work were the concentrations of the main components of the hybrid mobile phase (pH, SDS, 1-propanol and TEA) and the detection conditions. The study was performed using a standard solution containing 0.01 mg L^{-1} of OXO, DANO, CIPRO and ENRO.

3.1.1 Selection of the pH

The pH of the mobile phase is an important parameter, as the four quinolones show several weak acid/alkaline groups in their structure (Fig. 5.1). The pKa of the acidic COOH moiety is 6.8; 6.1; 5.9 and 5.9 for OXO, DANO, CIPRO and ENRO, and the pKa of the basic Piperazine N is 8.6; 8.2 and 7.7 for DANO, CIPRO and ENRO, respectively [33,34].

Depending on the pH, DANO, CIPRO and ENRO may be anionic, zwitterionic or cationic, whereas OXO can be neutral or cationic [34]. Hence, the pH of the mobile phase was selected in order to maintain the analytes quantitatively under one acid/basic form, in order to improve the robustness of the retention mechanism [35]. Besides, they would be

positively charged, in order to favour their interaction with the anionic sulfate groups situated at the outer layer of the modified stationary phase and the surface of the micelles [36]. The study was restricted to the working pH of silica-based C18 columns, 2.5–7.5, in order to enlarge the column lifespan [37].

According to the structures (Fig. 5.1) and dissociation constants, the pH must be under 4, in order to ensure that all the groups would be quantitatively in the acidic form. However, a lower pH allows the partial protonation of the free silanols (pKa ≈ 4-5), thus reducing their interaction with the polar and charged analytes [37]. Finally, pH 3 was selected. Under these conditions, the charge of OXO, DANO, CIPRO and ENRO were 0, +1, +1 and +1, respectively.

3.1.2 Effect of SDS and 1-propanol concentrations on the chromatographic behavior

According to their moderate hydrophobicity (log Po/w of 1.43; 1.20; 0.65 and 1.89 for OXO, DANO, CIPRO and ENRO, as indicated in [33], the use of hybrid micellar mobile phases of 1-propanol is recommended to reach a manageable retention in a C18 column [38]. The effect of the concentrations of SDS and 1-propanol was evaluated using a full factorial design plus the central point. The -1 and +1 points were the minimum and the maximum concentrations of SDS and 1-propanol recommended for MLC, 0.05-0.15 M and 2.5-12.5%, respectively [18]. Therefore, the four studied quinolones were analyzed by five mobile phases containing SDS (M)/1-propanol (%) - 0.5% triethylamine - 0.01 M phosphate buffered at pH 3: 0.05/2.5; 0.05/12.5; 0.10/7.5; 0.015/2.5 and 0.15/12.5.

For all the mobile phases, OXO was significantly less retained than the other ones, because of its neutral charge. The elution order of the other cationic quinolones was not maintained. Otherwise, the elution order of the quinolones, even those with the same charge, does not match the order of hydrophobicity. According to these results, the electrostatic interaction between the analytes and the stationary phase play a major role in the retention.

The antimicrobial showed a binding behavior with the micelles, as the retention and the efficiency decreased at increasing values of SDS in the mobile phase. This was probably due to the electrostatic attraction between the analytes and the micelles, rather than a hydrophobic interaction. On the other hand, at higher amounts of 1-propanol, the retention and the broadness of the peaks diminished, as usual in RP-HPLC [18].

3.1.3 Optimization of SDS and 1-propanol concentration

The optimal concentrations of SDS and 1-propanol were selected using an interpretative strategy assisted by chemometrics. The model is based on several mechanistic equations, valid for moderately hydrophobic compounds quantitatively in one acid/basic form, that describe the chromatographic behavior of each analyte as a function of the SDS and 1-propanol concentration [28]. These equations have been demonstrated to be useful for moderately hydrophobic compounds, quantitatively in one acid/alkaline form [38].

For each quinolone, the experimental values of retention time, efficiency and asymmetry obtained by the factorial design were processed by Michrom software [31] to adjust the model (the measured dead time of the chromatographic system was 1.00 min). The software can predict the values of k, N, B/A of each quinolone, the individual and global resolution for each pair and the mixture, respectively, for concentrations of SDS and 1-propanol in the 0.05-0.15 M and 2.5-12.5% ranges, respectively, by interpolation, by testing only five mobile phases, thus reducing time and effort. The software is also able to draw simulated chromatograms, in order to visualize the changes of the chromatographic parameters when the SDS and 1-propanol concentrations vary, which strongly facilitate the selection of the optimal mobile phase composition.

Under the criterion maximum resolution-minimum analysis time, the optimal mobile phase was 0.05 M SDS - 7.5% 1-propanol - 0.5% TEA 0.01 M phosphate buffered at pH 3. The theoretical values of the global resolution and retention time for ENRO were 0.99997 and 18.7 min, respectively. A mixture of the four studied quinolones (0.01 mg L^{-1}) was analyzed using the optimal mobile phase. The experimental values of (t_R; N and B/A) were: OXO, (5.5; 1415; 1.4); DANO, (14.1; 5284; 1.1); CIPRO, (16.1; 2235; 1.3) and ENRO (18.7; 1284; 1.5). The error in the prediction of the retention factors was <5%.

3.1.4 Optimization of TEA volumetric fraction

Cationic analytes may interact with the anionic free silanols of the surface of the silica particles, resulting in peak broadening and tailing. To reduce this effect, triethylamine was added to the mobile phase as sacrificial base. At pH 3, trimethylamine is protonated and blocks the silanol groups [37]. The influence of triethylamine was evaluated by analyzing a

mixture of the studied antibiotic using the optimal mobile phase, at several values of TEA (%, v/v): 0; 0.25; 0.5; 1; 1.5.

Retention time was not significantly affected by the changes in TEA proportion. The efficiency and asymmetry ameliorate when the proportion of TEA increased from 0 to 0.5%, and remain nearly constant above this value. Otherwise, an augmentation of the system pressure at higher concentrations of TEA was noticed, probably by the reduction of the volume pore and the viscosity of this additive. Therefore, 0.5% v/v was selected for the analysis.

3.1.5 Optimization of the detection conditions

Fluorescence detection was selected, due to its higher selectivity and sensitivity. As the quinolones show natural fluorescence, no derivatization is required. The maximum excitation/emission (nm) wavelengths were taken from previous published papers related to the chromatographic determination of these quinolones using hybrid micellar mobile phases: OXO, 260/366 nm [27]; DANO, CIPRO [28] and ENRO, 280 and 455 [29].

The monitored excitation/emission (nm) wavelengths were programmed in time to detect each quinolone at its optimal value: 0.0-8.0 min, 260/366 and 8.0-20.0, 280/455. The switching time was applied long before the elution time of DANO to ensure the stabilization of the zero. No abrupt variation of the baseline level and noise was observed.

3.2. Optimization of the sample preparation

The sample preparation was based on that described in [27] about the extraction of quinolones from fish flesh using a pure micellar solution. The sample/supernatant (Sa/Su) ratio (w/v) was optimized for bovine and porcine meat to maximize the recovery. The investigation was performed using quinolone-free samples of bovine and porcine meat, and the results were similar in both cases.

The tested sample/supernatant (w/v) ratios were: 1/1; 1/2; 1/5; 1/10; 1/20 and 1/50. For 1/1 and 1/2, it was observed that the mixture between the micellar solution and the sample formed a viscous paste, which was not able to be stirred, and then they were directly discarded. In the other cases, a reasonable volume of filtrate was obtained before the

obstruction of the filter. The recovery was directly evaluated as the peak area of the quinolone times the Sa/Su. The extraction capacity increased from 1/5 to 1/10, and remained nearly constant from 1/10 to 1/50. We considered that the mobility of meat particles is reduced at Sa/Su > 1/10, thus limiting the contact between the two phases and then the transfer of the quinolones from the meat to the supernatant. No advantages in the extraction yield were obtained at Sa/Su under 1/10, and then this value was selected to excessively diminish the sensitivity.

3.3 Method validation

The procedure was in-lab validated by the guidelines of the European Commission Decision 2002/657/EC [30]. The evaluated parameters were: selectivity, calibration range, linearity, sensitivity, recovery, precision, decision limit (CCα), detection capability (CCβ), ruggedness and stability.

3.3.1 Selectivity

The selectivity by the analysis of blank samples of porcine and bovine meat, before and after spiking with 0.1 mg kg^{-1} OXO, DANO, CIPRO and ENRO, under the optimized conditions, and comparing the obtained chromatograms.

Similar results were obtained for both porcine and bovine meat. In the blank samples, the front of the chromatogram was observed from the dead time to 3.5 min. Besides, no peak was observed at and near to (±2.00 min) the window time of the analytes. The chromatograms of the spiked samples showed similar shape plus the peaks of the quinolones, which do not overlap with matrix peaks (Fig. 5.1). In addition, the retention times and the excitation/emission spectra were similar to those obtained by the analysis of the standard solution. Therefore, the method is enough specific to unequivocally recognize the studied quinolones in porcine and bovine meat.

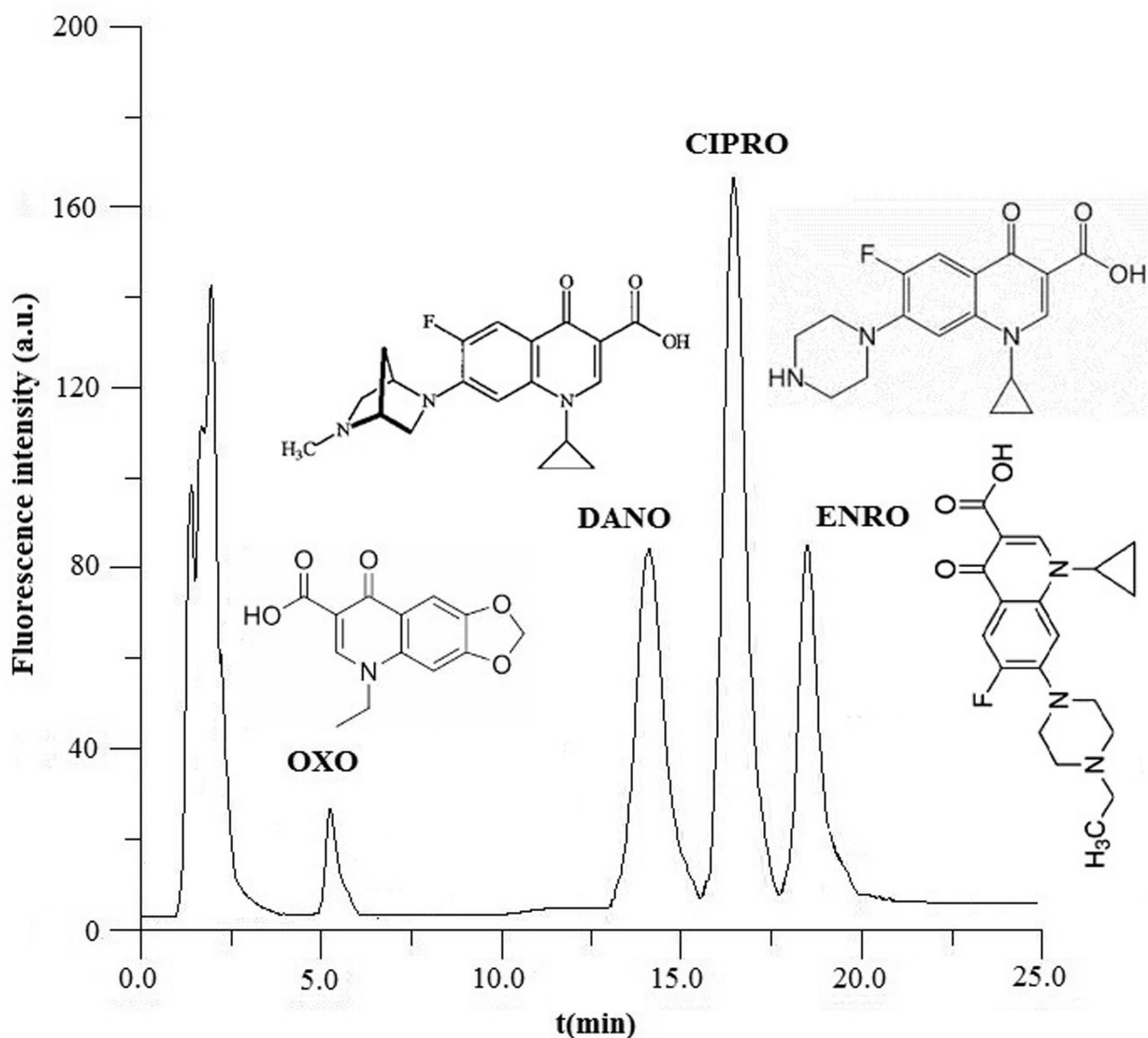

Figure 5.1. Chromatograms obtained by the analysis of a sample of porcine meat spiked at 0.1 mg kg^{-1} of each quinolone. The structure of each antimicrobial is also shown..

3.3.2 Calibration range, linearity and sensitivity

Standard solutions containing increasing concentrations of the quinolones were analyzed by triplicate, with a 1/10 dilution, to include the effect of the sample preparation. Therefore, the concentrations directly refer to mg kg^{-1} of the quinolone in meat samples. The calibration ranges were: OXO, 0.05-0.5 mg kg^{-1}; DANO, 0.03-0.5 mg kg^{-1}; CIPRO, 0.01-0.5 mg kg^{-1} and ENRO, 0.02-0.5 mg kg^{-1}. For each quinolone, the peak area was plotted v.s. the corresponding concentration, by least-square linear regression [39]. The slope, y-intercept and the determination coefficients (r^2) can be seen in Table 5.1.

Table 5.1. Calibration curves and sensitivity of the method (concentrations in mg kg^{-1})

Quinolone	Slope	y-intercept	r^2	LOD	LOQ
Oxolinic acid	104.3 ± 0.9	2±8	0.9998	0.015	0.05
Danofloxacin	1486±12	-10±30	0.99990	0.010	0.03
Ciprofloxacin	1727±1	24±19	0.99990	0.003	0.01
Enrofloxacin	818±2	17±4	0.99990	0.007	0.02

The limit of detection (LOD) is the lowest concentration in the matrix, which provides a signal clearly differentiable from the baseline noise. The limit of quantification (LOQ) is the lowest concentration which can be measured with adequate recovery and precision. These values were calculated by the 3 s and 10 s criterion, respectively: 3 and 10 times the standard deviation of the blank (taken as the standard deviation of the residuals), divided by the sensitivity (slope of the calibration curve) [39]. The results can be seen in Table 5.1. A chromatogram obtained from the analysis of a spiked sample of porcine meat is shown in Fig. 5.2.

A good linearity (r^2 > 0.9998) was obtained in all cases. The reached sensitivity indicates that the method is able to detect these quinolones in commercial samples. The method can be used to detect concentrations under the MRL stated by the EU regulation for each quinolone and kind of sample.

3.3.3 Recovery and precision

These parameters were determined in spiked samples, in both repeatability and within-laboratory reproducibility conditions.

Samples of porcine and bovine meat were spiked at 0.5x, 1x and 1.5x the MRL stated by the EU, and analyzed six times by successive injection. For each case, the recovery was calculated as the quotient between the found quinolones concentration and the true value measured by six successive injections. Recovery and precision under repeatability conditions were calculated as the average and the coefficient of variation (CV) of these individual recoveries. This protocol was performed five different days over a two-months period, using renewed samples. The recovery and precision under within-laboratory reproducibility conditions were determined as the average and the CV of the five repeatability recovery obtained. The results can be seen in Table 5.2.

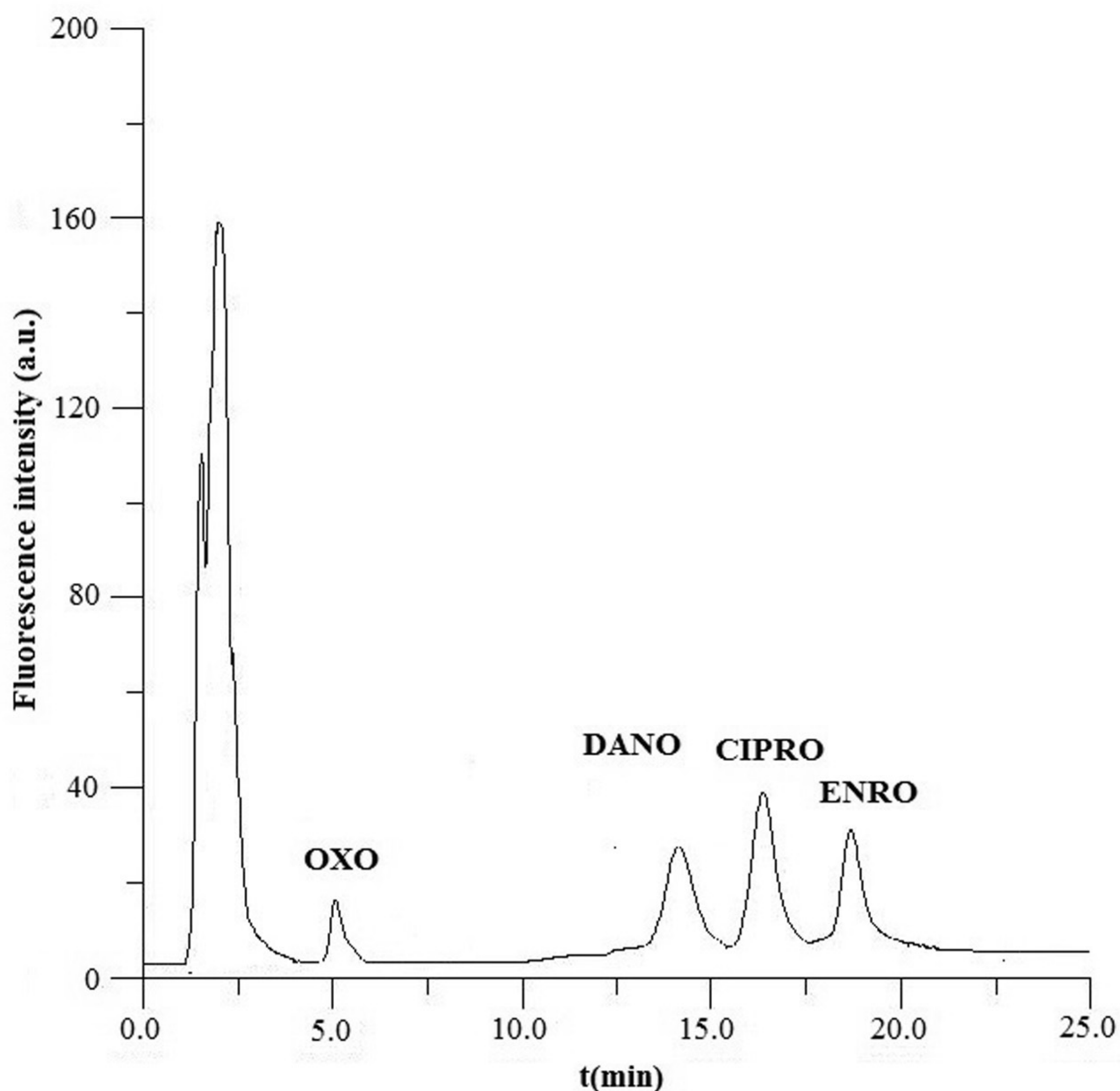

Figure 5.2. Chromatograms obtained from a sample of porcine meat spiked at their corresponding LOQ.

For all the studied quinolones, matrices and concentration levels, the values of recovery (89.3–105.1%) and coefficient of variation (<8.3%) were in agreement with the side those required by the regulation (80–110%). The coefficients of variation (CV < 8.3%) were under the value stated by the regulation (15.3%). Therefore, the method provides reliable quantitative information about the analytes around the MRL levels. This was possible by the polar, anionic and hydrophobic sites of the micelles, which increase the interaction of the studied antibiotics with the micelles and promote the transfer to the liquid phase.

Table 5.2. Recovery/precision measured in repeatability and within-laboratory reproducibility conditions (%/CV, %).

		Bovine meat		Porcine meat	
Quinolone	Fortified amount	Repetability[a]	Within-laboratory reproducibility[b]	Repetability[a]	Within-laboratory reproducibility[b]
OXO	0.5xMRL	92.2/6.0	93.1/5.8	91.5/7.4	89.9/7.4
	MRL	93.3/8.1	94.5/7.6	97.8/4.1	96.5/5.3
	1.5xMRL	98.4/2.3	99.2/2.0	96.8/1.1	97.4/2.0
DANO	0.5xMRL	89.3/2.2	90.5/2.4	91.8/5.2	95.5/6.9
	MRL	104.5/1.8	104.1/1.1	101.2/3.8	102.8/5.1
	1.5xMRL	99.9/3.1	99.1/2.7	98.7/3.2	100.9/4.1
CIPRO	0.5xMRL	105.1/3.7	100.3/0.6	103.8/6.8	102.4/5.4
	MRL	98.5/3.4	100.5/1.5	99.4/2.9	98.7/3.2
	1.5xMRL	100.0/2.8	102.5/1.0	98.4/3.9	99.0/2.4
ENRO	0.5xMRL	101.6/7.1	95.7/8.3	98.4/5.0	99.5/4.5
	MRL	100.2/4.6	103.8/6.3	97.4/3.8	96.9/5.8
	1.5xMRL	100.0/2.2	99.9/3.3	101.5/2.4	100.8/3.1

[a]n = 6; [b]n= 5

3.3.4 Decision limit and detection capability

The meaning of these parameters can be found in [40]. These parameters were separately determined for each kind of meat (bovine and porcine) and quinolone.

Twenty blank samples were fortified at the corresponding MRL of each quinolone and analyzed. The CCα was calculated as the MRL plus 1.64 times the standard deviation of the measurements. Afterwards, twenty blank samples spiked at the MRL were analyzed. The CCβ was calculated as the CCα plus 1.64 times the standard deviations of these measurements [30]. The results can be seen in the Table 5.3.

In order to reduce the probability of a false positive to <5%, the samples with a found concentration of CCα, instead of the MRL, would be classified as ‘‘compliant”. Considering the closeness between these two parameters, the probability of a false negative is very low. Samples containing quinolones between the MRL and CCβ have >5% of being incorrectly classified as ‘‘non-compliant” due to the random errors. As the MRLs are similar to the CCβs, the concentration interval at which a contaminate sample would elude the control is

very narrow. In fact, the results indicate that the random error has a low relevance in the decision of accepting or rejecting a sample.

Table 5.3. Decision limit and detection capacity for each quinoline in bovine and porcine meat (concentrations in mg kg^{-1}).

	Bovine meat		Porcine meat	
	CCα	CCβ	CCa	CCβ
OXO	112	121	107	113
DANO	206	213	107	115
CIPRO	105	113	105	115
ENRO	108	119	106	113

3.3.5 Ruggedness

The effects of small variations in the concentrations of the main components of the mobile phase in the instrumental response (retention time and peak area) using a Youden approach with 4 factors [30]. The studied ranges were: SDS, 0.04-0.06 M (factor A); 1-propanol, 7.3-7.7% (factor B); TEA, 0.4-0.6% (factor C) and pH 2.8-3.2 (factor D). The study was performed using a standard solution of 0.1 mg L^{-1} OXO, DANO, CIPRO and ENRO; 1/10 diluted. The standard deviations of the difference were compared with that obtained by the analysis of the same solution using the optimal conditions (n = 4).

The maximal difference observed in the retention time and peak area for the four studied analytes were <4.8% and <5.6%. In addition, the standard deviation of the differences is similar to that obtained at the optimal conditions for the two parameters. Therefore, the method is enough robust to be unaffected by changes in the composition of the mobile phase in the considered ranges.

3.3.6 Stability

The possible degradation of the analytes in standard solutions and meat samples at their usual storage conditions was examined.

A fresh standard solution of 0.5 mg L^{-1} (1/10 diluted) was prepared in a solution of

0.05 M SDS at pH 3. It was stored at 4ºC during two months. Each day, one aliquot was taken and analyzed. No significant diminution in the peak area was noticed, and no degradation products were detected. Therefore, the standard solutions can be used for two months without introducing a systematic error.

Several samples were fortified at 0.5 mg kg^{-1} and stored in a freezer at +20ºC. A sample was analyzed each day during a two-months period. The peak areas corresponding to the quinolones remain nearly constant, and no other peaks were observed. Therefore, the quinolones do not undergo a significant degradation in the meat during this period.

3.4 Analysis of real samples

In order to demonstrate the usefulness of the method for routine analysis, it was applied to several samples or porcine and bovine meat from animals raised on a farm, purchased from a local supermarket. The studied quinolones were not found in any samples, indicating that their ingestion does not represent a risk for the consumer.

The samples were analyzed in a single day, as many samples can be simultaneously processed, using basic laboratory material and instrumentation and low amount of chemicals. Besides, the use of isocratic mode allowed to perform successive injection without stabilization time.

4. Conclusions

MLC-FLD has been demonstrated as a suitable technique for the determination of OXO, DANO, CIPRO and ENRO in bovine and porcine commercial meat samples, at an affordable price, which is reasonably interesting in the current context of economic crisis. We consider it as an interesting alternative for laboratories of public agencies and the agro-food industry to evaluate the compliance of the meat samples with the EU Regulation 37/2010 in terms of antibiotic occurrence, before launching to the market.

The main feature of the method was the strong simplification of the sample preparation. The analytes were extracted by simple shaking and ultrasonication with a minimal participation of the operator, despite of the complexity of the matrix, due to the particular properties of micellar media. Besides, the supernatant does not need any treatment

before injection. Therefore, the sample pretreatment is simple, and does not use specific reagents or internal standards. This reduces significantly the potential sources of variance, and the probability of loss of the analyte, thus obtaining a high sample throughput.

The analytes were eluted in <22 min using a typical C18 column and a hybrid mobile phase running under isocratic mode, which composition was deduced from few experiments. In addition, the effect of each component of the mobile phase was investigated by empirical studies and explained from their structures.

The method was successfully validated following the guidelines of the EU commission Decision 2002/657/EC, and hold enough sensitivity and analytical performance to reliably distinguish samples with quinolones at concentrations in a large interval, including the corresponding MRLs. This was due to the characteristics of the sample preparation, the reproducibility of MLC, and the use of fluorescence detection. Besides, the method was found to be applied for routine analysis in laboratories receiving a high number of samples per day.

The procedure is relatively safe for the laboratory staff and ecofriendly. Indeed, the prepared micellar solutions use small amounts of innocuous and biodegradable reagents. No toxic, flammable and volatile organic solvent is used in the sample treatment, and only a low proportion (<13%, less than required in hydroorganic RP-HPLC) is added in the mobile phase. Besides, the interaction of 1-propanol with SDS-micelles even reduces its evaporation rate. Therefore, the operator is barely exposed to toxic chemicals and the waste contains a minimum proportion of pollutants.

5. Acknowledgements and Conflict of interest disclosure

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The authors declare that they do not have any financial/commercial conflict of interest.

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Chapter 6

Validation of a procedure to quantify oxolinic acid, danofloxacin, ciprofloxacin and enrofloxacin in selected meats by micellar liquid chromatography according to EU Commission Decision 2002/657/EC

Abstract

The suitability of an analytical method to determine oxolinic acid, danofloxacin, ciprofloxacin and enrofloxacin in edible tissues, based on micellar liquid chromatography coupled with fluorescence detection, to be applied in chicken, turkey, duck, lamb, goat, rabbit and horse muscle is described. The method was fully matrix-matched in-lab revalidated, for each antimicrobial drug and meat, following the guidelines of the EU Commission Decision 2002/657/EC. The permitted limits were the maximum residue limits stated by the EU Commission Regulation 37/2010. The results obtained for the studied validation parameters were in agreement with the guidelines: selectivity (the antibiotics were resolved without interferences), linearity (r^2>0.995), limit of detection (0.004-0.02 mg kg^{-1}), limits of quantification (0.01-0.05 mg kg^{-1}), calibration range (up to 0.5 mg kg^{-1}), recovery (89.5-105.0%), precision (<8.3%), decision limit, detection capability, ruggedness, stability and application to incurred samples. Therefore, the method was found able to provide reliable concentrations with low uncertainty in a large interval, including the respective maximum residue limits, and then is useful to find out prohibited contaminated samples. The method does not require to be adapted for these matrices, and then it maintains its interesting advantages: short-time, eco-friendly, safe, inexpensive, easy-to-conduct, scarce manipulation, and useful for routine analysis.

1. Introduction

Oxolinic acid (OXO), danofloxacin (DANO), enrofloxacin (ENRO) and its main metabolite ciprofloxacin (CIPRO) are synthetic antibiotics belonging to the quinolone group. These antimicrobial drugs have received great attention in medicine and veterinary practice to treat parasitic infections because of their pharmacological characteristics: good oral and intravenous bioavailability, tolerability for handling, diffusion throughout the tissues, effectiveness, broad-spectrum activity, tolerable side effects, and favourable pharmacokinetics [1]. In industrial husbandry, they are administered to the food producing animals as prophylactic and therapeutic agent, and increase the animal mass. However, their widespread administration and misuse may leave residues of antibiotics in edible tissues. The medium and long-term exposure of consumers to low concentration of these compounds is a public health concern, related to allergy reactions, weakening for intestinal flora, carcinogenesis, mutagenesis, teratogenesis and the emergence of quinolone-resistant bacterial strains and human antibiotic resistance. This may provoke an outbreak of infections, which do not respond to the current antibiotic arsenal. Zoonotic bacteria may also acquire the immunity to the antimicrobial drugs in the living food-producing animal, and be lately transferred to other living animals and to the population through the food chain or by direct contact. Besides, they can reach appreciable concentrations in the environment, and then disturb the ecological equilibrium [2,3]. In order to avoid these negative effects, several practices have been suggested to producers to rationalize the use of antibiotics, such as the prescription of therapeutic doses only to ill or risk animal until the desired clinical response under the supervision of a veterinary, and enlarge the withdrawal period [4].

Maximum residue limits (MRL) of OXO, DANO, and ENRO + CIPRO in muscle tissues of poultry, lamb (0.1; 0.2; and 0.1 mg kg^{-1}, respectively), goat, rabbit and horse (0.1; 0.1 and 0.1 mg kg^{-1}, respectively) have been established by the EU (Commission Regulation 37/2010), in order to ensure the safety of produced and served livestock products to consumers and minimize the risks for human health [5]. In order to verify the compliance with the regulation and evaluate the quality of food supplies, sample monitoring programs must be implemented by the agro-food industry and public agencies. Therefore, food control laboratories require practical, reliable, cost-effective, multi-class and enough sensitive

analytical methods to detect the quinolones in muscle tissues of food-producing animals at the regulatory levels [6,7].

The multiresidue determination of OXO, DANO, ENRO and CIPRO in edible tissues of farm animals have been mainly undertaken using liquid chromatography (LC) methods, because of their selectivity and ability to measure many analytes in one run. The safety of poultry meat has been largely studied by LC-MS [6,8-14], LC-ultraviolet absorbance detection (UV) [8,9,11,15] and LC-fluorescence detection (FLD) [7,16-19]. Comparatively, the bibliography about the analysis of ovine (LC-UV [20], LC-MS [12], LC-FLD [7]) muscle tissues is rather limited. However, reverse phase-HPLC has not been previously used to simultaneously determine these antimicrobial drugs in caprine, equine and rabbit meat. Among these detectors, fluorescence offers the highest selectivity-, sensitivity- and simplicity-to-cost ratio. The chromatographic separation is performed using mobile phases containing high proportions of organic solvents (up to 45%), running under gradient mode.

Efficient and sample-throughput solid-to-liquid extraction is required to isolate the antibiotics from the matrix [20]. As OXO, DANO, CIPRO and ENRO are soluble in both aqueous (acidic and basic) solutions and in polar organic solvents [20], pure buffered aqueous solutions [6,16-19], dichloromethane [15], acetonitrile [7,14], methanol [20] or a water/polar organic solvent mixture (20-90% organic solvent) [8-13], either at acidic [6,8-10,13,14,20] or neutral pH [7,11,12,15-19], have been used as extracting solutions. Then, the more usual leaching protocols (applied to minced meat samples) involve 15 min-stand [16-19], shaking [8,11,15,19], homogenization [7,9] ultrasonication [8,12,20], or vortexing [6,7,10-14,16-18,20] steps, and the obtained supernatant is separated by centrifugation. On occasions, several and duplicate steps are required. However, substantial amounts of endogenous compounds, like proteins, fats, carbohydrates, vitamins, minerals, electrolytes, metabolites and other small nutrients [21], may also be incorporated into the extracting solution, solved, as colloid particles or as aggregates. These may disturb the analysis [17], by interaction with the analyte, coelution, increasing baseline noise or precipitating into the column, and then must be removed before injection, and then elaborate purifications before the chromatographic analysis are needed. The most usual are solid phase extraction [6-9,12,15,16,18-20], liquid/liquid extraction [15], QuEChERS [10,14], cleanup using an immunoaffinity column [11,17], which require the evaporation of the eluate and

reconstitution of the dry residue, and on-line turbulent flow chromatography [13]. This long succession of intermediate operations complicates the sample preparation, results in an excessive and tedious manipulation, enlarges the analysis and augments the probability of loss of the analyte, which may affect the final recovery and variability. For these reasons, an internal standard is sometimes used [8-11,13-15,20]. Besides, high volumes of organic solvents and specific laboratory devices are employed.

Several authors have reported a promising alternative procedure based on micellar liquid chromatography, to determine organic compounds in flesh samples. Indeed, acidic micellar solutions of the anionic surfactant sodium dodecyl sulfate (SDS) were used as extracting solutions and mobile phases (in this last case, with a low proportion of organic solvent and, eventually, with a sacrificial base). The leaching was carried out by shaking and/or ultrasonication (without centrifugation), and the supernatant was directly injected [22,23]. We have developed an analytical procedure to determine OXO, DANO, CIPRO and ENRO in pork and beef meat, applying this strategy, using FLD [24]. A high recovery was obtained, thanks to the binding of the antibiotics with the micelles, their intrinsic solubility in water, and the direct and quantitative injection of the supernatant. This approach avoids the main drawbacks of sample preparation for HPLC analysis, at a reasonable cost. Its validation by the EU Commission Decision 2002/657/EC, a guideline especially devoted to the determination of organic contaminants in foodstuff [25], demonstrate the reliability of the results around the MRL values [24]. As a general rule, a method should be revalidated to be applied to a different, although similar, matrix that has been taken in the original validation, in order to ensure the applicability of the method in a new matrix [26].

The aim of the paper is to establish a fast, simple, ecological, inexpensive and convenient procedure for the determination of oxolinic acid, danofloxacin, ciprofloxacin and enrofloxacin in poultry, ovine, caprine, rabbit and equine meat, to verify the compliance of the meat samples with EU Commission Regulation 37/2010 [5]. Therefore, we study the suitability of the method detailed in [24], which has been demonstrated to hold these characteristics, to samples of chicken, turkey, duck, lamb, goat rabbit and horse muscle tissue. It would be mandatory to calculate the respective quantification parameters, corroborate the appropriateness of the leaching process, and evaluate the possible effect of the endogenous compounds, which may be different as for beef/pork meat. Besides, it must be verified that the calibration range covers the respective MRLs. To achieve these goals, we

propose a matrix-matched revalidation in these matrices, by the guidelines of the EU Commission Decision 2002/657/EC [25] and the ICH Harmonised Tripartite Guideline [27], taking the MRLs as permitted limits. The reliability of the method should be finally checked by the analysis of incurred samples.

2. Experimental procedure

2.1 Preparation of solutions and mobile phases

A description of the standards, chemicals and general laboratory apparatus used in this study can be found in [24]. The structures and physico-chemical properties of the studied quinolones have been detailed in [24].

The micellar solutions were prepared by weighing the appropriate amount of SDS and NaH2PO4.H2O and solving them in ultrapure water. The adequate volume of triethylamine was added, and the pH was set to 3 by adding drops of HCl solutions. Furthermore, 1-propanol was added to attain the desired proportion, and the flask was filled up with ultrapure water, ultrasonicated for 5 min, and filtered with the aid of a vacuum pump.

The stock solutions of the antibiotics were prepared by weighing the adequate quantity of the solid and solving it in 5% ethanol, and topped to the final volume with a micellar solution of 0.05 M-SDS at pH 3 (0.01 M phosphate buffer). This solution was ultrasonicated for 5 min. The working solutions were prepared by successive dilutions of the stock solutions in the same micellar solutions. The standard solutions were kept in the dark at +4ºC. The solutions were thawed for 30 min at room temperature before use, in order to solve the SDS crystals formed overnight.

2.2 Chromatographic conditions

The chromatographic system was an HP1100 (Agilent Technologies, Palo Alto, CA, USA) consisting of several modules (isocratic pump, degasser, autosampler, column, fluorescence detector) connected in series. The column was a Kromasil C18 (150 x 4.6 mm; 5 µm; 10 nm pore size, supplied by Scharlab (Barcelona, Spain). The control of the instrumentation and the registration and processing of the signals was performed by the

software ChemStation (Rev.A.10.01) (Agilent).

The conditions were the same as exposed in [24]. The injection volume was 20 µL. The mobile phase was an aqueous solution of 0.05 M SDS - 7.5% 1-propanol - 0.5% triethylamine buffered at pH 3 with 0.01 M phosphate salt, running under isocratic mode at 1 mL min^{-1} without controlling the temperature. The detection was carried out by measuring the fluorescence intensity at these excitation/emission wavelengths (nm): 0.0-8.0 min, 260/366; 8.0-22.0 min, 280/455. All the injected solutions were filtered when introduced in the vials. The special care and the cleaning protocol required when dealing with micellar mobile phases are detailed in [28].

2.3 Sample treatment

Samples of chicken, turkey, duck, sheep, goat, rabbit and horse muscle tissues were purchased in several local retail butcher shops, finely ground a mincer (Model MZ10, Petra Electric, Burgau, Germany) at 5000 rpm for 5 min, and stored in a freezer at -20ºC.

The mixed matrix was prepared was follows: 10 g of each kind of muscle were introduced in a glass beaker and hand-blended. Afterwards, the entire mixture was ground for 10 min, to reach a reasonable homogenization degree [24].

For the fortification, the appropriate volume of a quinolone(s) standard solution was injected to the minced meat. The sample was stored at room temperature overnight to evaporate the solvent and stimulate the chemical binding of the antibiotics with the matrix. These fortified samples reproduce those "naturally" contaminated [23].

The extracting solution (50 mL) was mixed with 5 g of the minced meat (blank, spiked or incurred), shaken for 1 h using a magnetic stirrer (C-MAG HS 7 IKA Werke GmbH & Co. KG, Staufen im Breisgau, Germany) and ultrasonicated for 15 min. Afterwards, the obtained supernantant was separated from the precipitated matrix by filtration with the aid of a vacuum pump, and introduced in the chromatographic vials [24].

3. Results and discussion

The method was revalidated to consider the adequacy of the method to chicken, turkey, duck, lamb, goat, rabbit and horse muscle tissues. A full *in-lab* matrix-matched revalidation was developed, following the guidelines of the EU Commission Decision 2002/657/EC in terms of: selectivity, matrix effect, trueness, precision, decision limit (CCα), detection capability (CCβ), ruggedness and stability [25]. The limits of detection (LOD) and quantification (LOQ), linearity and calibration range, were investigated by the ICH Harmonised Tripartite Guideline [27]. A practical maximum residue limit of 0.1 mg kg^{-1} was considered for CIPRO and ENRO separately, instead of for the sum.

3.1 Selectivity

To check the ability of the method to recognize analytes, the following analysis were performed: a calibration standard solution of 0.01 mg L^{-1} OXO, DANO, CIPRO and ENRO, blank samples of chicken, turkey, duck, lamb, goat, rabbit and horse, free of analytes, and these same samples after fortification with each quinolone at their respective MRL.

The retention times (min) obtained by the analysis of the standard solution were: OXO, 5.6; DANO, 14.0; CIPRO, 15.9 and ENRO 18.4. The peak width at 10% was <2 min, and then the peaks do not overlap. The less retained compound was eluted at > 2 times the void time (nearly 1.00 min).

Similar results were achieved for all the studied meats, due to the similarity of the chemical composition, in terms of endogenous compounds detectable by MLC-FLD. A broad band appeared from the dead time to nearly 4.0 min, no peak was found at the window time (retention time ±2.00 min) of each antimicrobial drug, and the baseline was quite stable.

The chromatograms of the fortified samples exhibited similar shape as the blank ones, plus the peaks of the quinolones. The retention times (difference <2.3%), the shape and the fluorescence excitation/emission spectra of each chromatographic peak were comparable to those obtained from the calibration standards. Besides, no overlapping was noticed between the peaks of the analytes and those of the matrices. A chromatogram obtained from the analysis of the fortified chicken meat samples can be seen in the Figure 6.1.

The analytical procedure is sufficiently selective to unambiguously identify OXO,

DANO, CIPRO and ENRO in the studied meats.

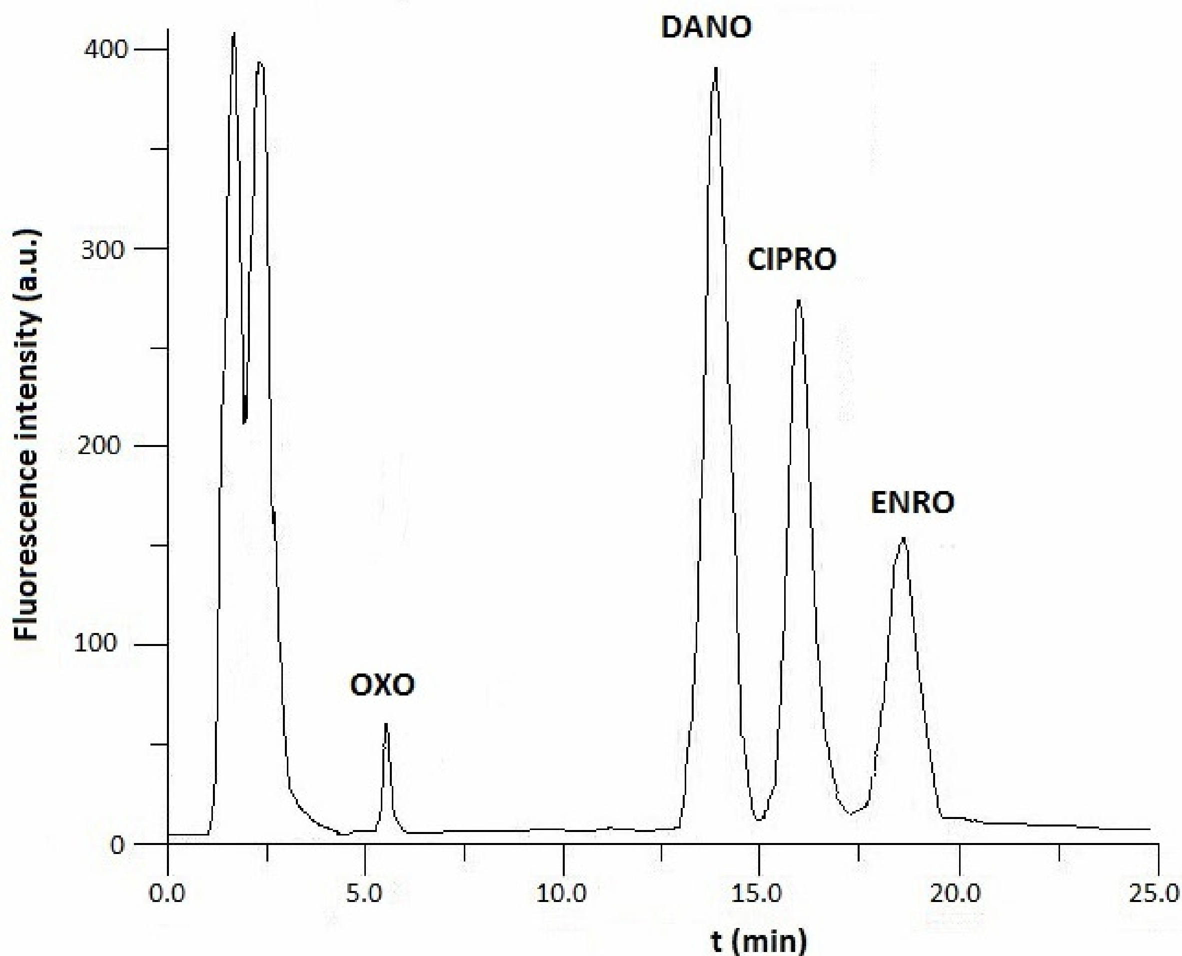

Figure 6.1. Chromatograms obtained by the analysis of a sample of chicken meat fortified at the corresponding MRL of each quinolone: OXO, 0.1 mg kg^{-1}; DANO, 0.2 mg kg^{-1}; CIPRO, 0.1 mg kg^{-1} and ENRO 0.1 mg kg^{-1}.

3.2 Matrix effect

The effect of the endogenous compounds extracted from the matrices on the chromatographic quantification was studied. The obtained supernatants were spiked at 0.01 mg L^{-1} of each antibiotic, and analyzed. The values of the peak areas were similar to those obtained from the calibration standards indicated in 3.1. Therefore, the matrix effect can be

considered negligible. Indeed, antibiotics barely interact with the endogenous compounds, in part due to the binding of the main ones (proteins and fats) with the micelles [22,24].

3.3 Calibration range and linearity

The disposal of one calibration curve (per antibiotic) for the seven studied matrix makes the method more useful and versatile for a food control laboratory, instead of having one curve per animal meat. This would permit the analysis of samples from different meats in the same analytical run, and then it would be more adaptable to the day-to-day needs of the laboratory. This approach can be performed because of the insignificance of the matrix effect (section 3.2). Therefore, the calibration was carried out using the mixed matrix (section 2.3), made of equivalent amounts of the studied kinds of meat.

Several samples of the mixed matrix were fortified with increasing concentration of OXO, DANO, CIPRO and ENRO (up to 0.5 mg kg^{-1}) and analyzed. For each quinolone, the peak area was plotted vs the corresponding concentration, and was found linear at a first glance. The calibration parameters (slope, y-intercept and determination coefficient, r^2) were calculated taking the concentration interval LOQ to 0.5 mg kg^{-1} [26,29].

The LOD is the quantity in sample, which provide a peak clearly above the baseline noise. The LOQ refers to the smallest quantity in a matrix that can be quantified with enough reliability. They were calculated by the 3.3 and 10 s criterion, respectively: 3.3 or 10 times the deviation standard of the blank divided by the sensitivity. These parameters were taken from the calibration curve of each antibiotic: the standard deviation of the residuals and the slope, respectively [27,29]. The LOQ was taken as the lower level of the calibration range. As the LOQ and the parameters of the calibration curve are interdependent, an iteration strategy was applied. The final results are shown in Table 6.1.

Table 6.1. Calibration curves and sensitivity of the method (concentrations in mg kg^{-1})

Quinolone	Slope	y-intercept	r^2	LOD	LOQ
Oxolinic acid	110.5 ± 1.9	13±7	0.998	0.02	0.05
Danofloxacin	1521±20	-9±15	0.9990	0.012	0.03
Ciprofloxacin	1687±5	18±20	0.995	0.004	0.01
Enrofloxacin	832±7	13±8	0.9997	0.009	0.02

n = 7

In all cases, an adequate linearity (r^2 > 0.995) was accomplished. The sensitivity and calibration range largely covers the MRL for the four studied antimicrobial drugs. Therefore, the method shows enough sensitivity and calibration range to reliably differentiate compliant and non-compliant samples.

3.4 Recovery and precision

These validation parameters (repeatability and within-laboratory conditions) were independently measured in each matrix at 0.5x; 1x and 1.5xMRL.

- Repeatability: For each level and meat, free-antibiotic samples were fortified and analyzed six times, successively and within the same day. The quinolones were quantified for each injection. The recovery was calculated as the average found concentration divided by the true value, whereas the precision was the relative standard deviation (RSD) of the six measurements.
- Within-laboratory reproducibility: the stability of the results through the time was considered. The above referred practice was repeated five different days in a three-months period. The recovery and the precision were established as the average/know concentration and the RSD of the five average found concentrations, respectively. The results are shown in Table 6.2 for OXO and DANO and Table 6.3 for CIPRO and ENRO.

The precision (<8.3%) and the recovery (89.5-105.0%) fit the requirement criteria stated by the guideline (<9.0%, 80-110%, respectively). Therefore, the quantitative determinations are enough trustworthy around the MRL values.

Table 6.2. Recovery (%)/precision (RSD, %) measured in repeatability and within-laboratory reproducibility conditions (%/RSD, %) for OXO and DANO.

		OXO		DANO	
Meat	Fortified amount	Repeatability[a]	Within-laboratory reproducibility[b]	Repeatability[a]	Within-laboratory reproducibility[b]
Chicken	0.5xMRL	92.5/4.9	93.9/6.2	93.5/3.2	92.8/5.2
	MRL	95.8/4.8	96.4/3.4	98.5/2.6	99.0/3.5
	1.5xMRL	97.8/3.5	98.9/3.0	101.2/1.9	102.2/2.3
Turkey	0.5xMRL	91.8/6.1	90.9/5.8	95.5/4.5	94.5/5.3
	MRL	93.8/4.0	94.1/3.5	102.3/3.4	103.2/4.4
	1.5xMRL	96.5/2.9	95.6/2.5	98.5/1.9	97.8/2.5
Duck	0.5xMRL	90.2/7.5	91.5/7.1	94.8/5.4	93.5/3.9
	MRL	94.2/6.2	95.8/4.6	98.7/4.3	99.8/4.9
	1.5xMRL	97.7/4.2	98.8/3.9	102.1/3.8	101.8/2.4
Sheep	0.5xMRL	91.8/6.5	92.8/4.9	96.8/6.6	95.8/7.0
	MRL	95.4/5.4	94.5/6.0	102.5/4.3	103.5/5.2
	1.5xMRL	98.8/3.5	97.9/4.1	98.9/3.6	97.8/4.3
Goat	0.5xMRL	93.4/5.9	92.9/4.8	95.4/8.1	94.8/7.7
	MRL	97.3/4.3	97.2/5.2	97.7/6.5	98.9/6.0
	1.5xMRL	101.5/2.6	100.6/3.7	96.8/4.4	96.1/5.1
Rabbit	0.5xMRL	89.5/6.5	89.9/7.5	94.2/5.9	93.9/6.8
	MRL	92.7/4.8	91.4/6.3	95.2/6.0	96.4/6.5
	1.5xMRL	95.9/3.4	94.2/4.0	96.9/4.8	97.8/4.0
Horse	0.5xMRL	90.9/7.0	91.5/6.7	95.8/4.9	96.8/5.4
	MRL	93.8/6.5	94.2/5.7	103.5/5.2	104.5/6.9
	1.5xMRL	96.8/5.0	97.4/4.8	98.9/3.8	99.5/2.7

[a]n = 6; [b]n= 5

Table 6.3. Recovery (%)/precision (RSD, %) measured in repeatability and within-laboratory reproducibility conditions for CIPRO and ENRO.

		CIPRO		ENRO	
Meat	Fortified amount	Repeatability[a]	Within-laboratory reproducibility[b]	Repeatability[a]	Within-laboratory reproducibility[b]
Chicken	0.5xMRL	104.1/6.5	103.0/5.9	97.8/4.8	98.8/5.2
	MRL	102.1/4.2	101.5/3.4	98.5/2.9	97.8/3.6
	1.5xMRL	99.5/2.1	99.0/1.5	100.5/1.2	101.0/2.5
Turkey	0.5xMRL	105.0/5.7	104.3/6.8	96.8/6.0	97.8/4.5
	MRL	103.5/4.1	102.5/3.5	102.5/4.9	101.0/3.8
	1.5xMRL	102.3/3.5	102.0/2.6	98.8/3.7	99.5/3.2
Duck	0.5xMRL	102.9/5.8	101.5/5.1	97.0/5.7	96.5/5.0
	MRL	98.9/6.5	99.0/5.3	101.5/3.8	102.3/4.2
	1.5xMRL	97.8/4.0	98.5/2.7	99.8/1.4	100.5/2.3
Sheep	0.5xMRL	103.5/5.8	102.5/4.5	96.5/6.7	97.1/6.0
	MRL	98.5/4.4	98.9/3.7	98.7/4.5	98.4/4.2
	1.5xMRL	102.3/3.9	101.9/3.1	101.2/2.6	100.5/3.3
Goat	0.5xMRL	104.8/8.1	105.0/7.9	97.5/4.0	96.5/4.5
	MRL	104.5/5.6	103.9/5.5	101.7/3.8	102.1/4.1
	1.5xMRL	103.2/4.9	102.9/4.0	99.8/1.7	99.4/2.4
Rabbit	0.5xMRL	104.3/7.1	103.8/6.5	96.4/6.4	95.8/5.9
	MRL	101.5/6.7	100.9/3.2	98.5/4.9	99.0/5.1
	1.5xMRL	97.8/3.9	97.5/2.5	101.0/2.7	100.9/3.0
Horse	0.5xMRL	104.8/8.3	104.9/8.0	97.5/5.7	97.0/6.0
	MRL	102.2/6.6	103.8/5.9	99.0/4.0	98.4/3.6
	1.5xMRL	100.5/3.8	101.0/4.2	102.8/3.2	102.0/2.7

[a]n = 6; [b]n= 5

3.5 Decision limit

A sample containing exactly the MRL of an antibiotic may provide found concentrations above the MRL, and then be falsely classified as non-compliant, a 50% of the measures, due to the random errors. This is a too high ratio, considering the economic and prestige damages caused by the withdrawal of a supposedly contaminated meat batch. The decision limit (CCα) to claim a sample as "non-compliant" is moved to a higher value, which would be overcome a maximum of 5% of the measurements (for samples containing the MRL). Therefore, the probability of a false positive is <5%. However, the negative aspect of this approach is that the probability of accepting a contaminated sample is augmented, and even more at increasing values of CCα [26]. For each antibiotic and kind of meat, the CCα is defined as the MRL plus 1.64 times the standard deviation of the found concentrations obtained from the analysis of 20 samples fortified at the corresponding MRLs [25]. The values can be seen in Table 6.4.

In all cases, the values are close to the MRL (<11%), and then the probability of making a wrong classification, due to the random errors is relatively low.

Table 6.4. Decision limit/detection capacity for each quinolone in the studied meats (concentrations in μg kg^{-1}).

Meat	OXO	DANO	CIPRO	ENRO
Chicken	108/113	208/220	107/113	104/110
Turkey	106/112	211/227	107/113	108/115
Duck	110/117	214/231	111/120	106/114
Sheep	108/119	214/233	107/114	107/115
Goat	107/116	110/121	110/120	106/114
Rabbit	107/117	109/121	111/117	108/117
Horse	110/120	109/122	111/122	106/113

3.6 Detection capability

By the way, a sample containing an antibiotic at the decision limit (thus contaminated) would be quantified as <CCα the 50% of the analysis, and then accepted, because of uncertainty of the measurements. Therefore, the laboratory cannot honestly claim that it is able to detect non-compliant samples from the CCα. The true detection capability (CCβ) is defined as the amount in a sample, which would provide found concentrations under the CCα, only 5% of the quantifications. Therefore, the maximal probability of a false negative is 5%. In this case, the method is unable to detect contaminated samples in the interval MRL-CCβ. Thus, higher the detection capability, the ability of the method to detect non-compliant samples decreases [26]. For each antimicrobial drug and kind of muscle tissue, the CCβ was calculated as the CCα plus 1.64 the standard deviation calculated by the quantification of twenty samples spiked at the CCα [25]. The values can be seen in Table 6.4.

The CCβ was relatively close to the MRL (<22%), and then the concentration interval that the method cannot reliably identify as non-compliant is relatively narrow.

3.7 Ruggedness

The influence of the slight oscillations of the experimental and operating conditions on the main instrumental response (found concentration and retention time) was examined through a Youden approach [25]. Seven experimental parameters can be studied by a fractional factorial design. In each experiment, 4 factors are set at their highest values and the other 4 at their minimal value, and only eight different combinations must be assayed to set the effect of each factor (without interactions). This is less than required by one-per-one strategy and the testing of all the possible combinations.

The experimental parameters more related to the handling of the operator were investigated. The studied ranges were those can usually occur in the normal handling of the laboratory equipment: concentration of SDS, 0.045-0.055 M (A); 1-propanol 7.3-7.7% (B); TEA, 0.4-0.6% in the mobile phase (C); pH, 2.8-3.2 (D) of the mobile phase; SDS, 0.045-0.055 M (E); pH, 2.8-3.2 (F) in the extracting solution and sample/supernatant ratio, 4.5/50-5.5/50 (G). In each measurement, a mixed matrix (section 2.3) fortified at 0.1 mg kg^{-1} of each

quinolone was analyzed.

For the two considered instrumental responses, the calculated differences do not show outstanding dissimilarities, and the standard deviation of the difference was close to that obtained by the analysis of the same sample using the optimal conditions (n=4). Therefore, these factors do not exert a significant influence on the retention time and quantification, in the studied intervals.

3.8 Stability

The possible decomposition of the four studied antimicrobial drugs and the formation of possible interfering compounds (by decay of endogenous compounds or the antibiotics themselves) through time was investigated, to establish the maximal keeping time. The applied storage conditions were those usually employed for meat in dwellings, retail stores and laboratories, in a freezer at -20ºC.

Free-antibiotic muscle samples of chicken, turkey, duck, lamb, goat, rabbit and horse were fortified at the MRL of each quinolone. They were stored as above indicated and analyzed each three days (including the day zero) for 20 weeks. No significant diminishing of the peak area corresponding to each analyte, and no additional peaks were observed in the chromatograms. Therefore, the meat samples can be stored during the studied time span in the laboratory freezer without losing analytical performance.

3.9 Analysis of incurred samples

The adequacy of the method for routine analysis was evaluated in incurred samples of chicken, turkey, duck, lamb, goat, rabbit and equine meat (five of each one), purchased from local supermarkets and retail butchers. The entire set of samples was processed in the same day by a single operator, because many can be simultaneously treated. A hand-manipulation is only required in the preparation of the mixtures meat/extracting solutions, as the leaching steps (stirring and ultrasonication), are automated.

The studied quinolones were not detected in the studied samples, thus indicating that they comply with the regulation and then they do not represent a threat to consumer health.

4. Conclusions

The results obtained by the revalidation were, for all parameters, in agreement with the requirements of the EU Commission Decision 2002/657/EC. Therefore, this MLC-FLD procedure is suitable for the quantification of OXO, DANO, CIPRO and ENRO in muscle tissues of chicken, turkey, duck, lamb, goat, rabbit and horse in a wide interval around their corresponding MRL levels, in addition to pork an beef meat. No modification of the analytical method was required, in spite of the change of the matrix. This was because of the physico-chemical similarities between these edible tissues and the ability of the micellar environment to reduce the matrix effect. As for [24], the good analytical performances were because of the appropriateness of the sample pretreatment (yielding of the extraction and direct injection of the supernatant), the stability of MLC and the sensitivity of fluorescence. In addition, the method retains its excellent practical performances: eco-friendly, safe, inexpensive, easy-to-handle, semi-automated, and applicable to the analysis of many samples per day. Thus, the method can be implemented for routine analysis in food control laboratories of public agencies and producers to verify the compliance of the meat samples with the EU Regulation 37/2010.

5. Acknowledgements and Conflict of interest disclosure

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Chapter 7

Development and validation of a micellar liquid chromatograpic method to determine flumequine, marbofloxacin, difloxacin and sarafloxacin in the most consumed meats

Abstract

Some antibiotics have been quantified by micellar liquid chromatography in porcine, bovine, poultry, ovine, caprine, rabbit and equine meat. The analytes were recovered by ultrasound-assisted leaching in a pure micellar solution, which was directly injected. The fluoroquinolones were resolved in <19 min using a C18 column, with an isocratic mobile phase of 0.05 M sodium dodecyl sulphate - 8 % 1-butanol - 0.5 % triethylamine buffered at pH 3, and detected by fluorescence. The limits of quantification (0.01-0.05 mg kg^{-1}) were below the maximum residue limits stated by EU Regulation 37/2010 (0.15-0.4 mg kg^{-1}). The method was validated following the EU Commission Decision 2002/657/EC, in terms of: selectivity, linearity, calibration range, recovery (83.9 to 107.8%), precision (<9.4%), decision limit, detection capability, ruggedness and stability. The main practical advantages were: simplicity, low-cost, eco-friendliness, safety, and requiring a minimal manipulation. Therefore, this procedure was found useful for routine analysis in surveillance programs.

1. Introduction

Meat is a highly-appreciated foodstuff due to their taste and elevated content of proteins, fats, vitamins, minerals and micronutrients, which must be included in a balanced diet and are essential for growth. In the last years, the consumption of meat has increased worldwide, because of the augment of the population, urbanization and income, although it has remained stable at a high level in developed countries [1,2]. The production of meat is an important economic activity in the EU, because of its high production, consumption and trading. The production of pork (22.6 million tons), beef (7.7 million tons) and poultry (12.6 million tons) meat is directed to the inner market (110 %, slightly above 100% and 104% of self-sufficiency rate, respectively) and the exportation, mainly to Russia and East Asia [3]. Although 0.92 tons of sheepmeat and goatmeat are annually produced, the EU is a net importer (88 % of self-sufficiency rate), mainly from New Zealand and Australia [4]. The production of other kind of meats, such as rabbit (0.6 million tons) and horse (62.8 million tons) has also reached a high economic relevance [5,6]. Most of these animals are reared in farms at higher stocking densities and fed with a manufactured feed to reduce the high production costs, and maintain an affordable retail price. However, this practice stimulates the incidence and propagation of infectious diseases among the animals, thus increasing their morbidity and mortality and affecting the productivity of the farm [7].

Fluoroquinolones are synthetic broad-spectrum antimicrobials and have a significant post-antibiotic effects against gram positive and negative bacteria. Among them, flumequine (FLU), marbofloxacin (MARBO), difloxacin (DIF) and its main metabolite sarafloxacin (SAR) are widely prescribed in medical and veterinary practice against a wide range of diseases originated by bacterial infections [8]. Their structure and properties can be seen in Figure 7.1 and Table 7.1 (respectively) [9,10]. In farms, antimicrobial drugs are administered, either orally or in injected, to the food-producing animals as prophylactic and curative agents, to safeguard their welfare, as well as to promote growing [11]. However, their indiscriminate use has resulted in the occurrence of antibiotic residues in edible tissues. The unnoticed exposure to sub-therapeutic amounts has been associated with severe long-term health problems for consumers, such as hazardous effects, allergies and the emergence of fluoroquinolone-resistant human pathogens [12,13]. This stimulates the boost of infectious

epidemics, that cannot be treated by the current antibacterial arsenal, and may provoke serious consequences for individual patients and increase the costs of medical care [11].

Nowadays, there is a worldwide concern among population and international agencies about the potential risks originated by the abusive use of floroquinolones [11]. Therefore, several governments have established regulations and actions to avoid the misuse of antibiotics in animal farming [14]. Within the frame of its policy to protect human health and keep the image of European meat as healthy and high-quality, the EU has set maximum residue limits (MRLs) for FLU, MARBO and DIF in muscle tissue of several animals, produced and distributed in its area (EU Regulation 37/2010) [15]. No MRL has been established for SAR, but its residue would not be higher than that of DIF (Table 7.1). Their monitoring is necessary to verify the compliance with the regulation and ensure food safety.

Table 7.1. Characteristics and MRL (mg kg^{-1}) of the studied fluoroquinolones [9,10,15].

Antibiotic	Flumequine	Marbofloxacin	Difloxacin	Sarafloxacin
pKa COOH group (acidic)	6.4	5.7	5.7	5.6
pKa N-piperazynil moiety (basic)	---------	8.0	7.2	8.2
Log Po/w	2.3	-2.9	1.3	1.1
MRL in porcine and bovine meat	0.2	0.15	0.4	0.4[a]
MRL in poultry meat	0.4	0.15[b]	0.3	0.3[a]
MRL in ovine meat	0.2	0.15[b]	0.3	0.3[a]
MRL in caprine meat	0.2	0.15[b]	0.4	0.4[a]
MRL in rabbit and horse meat	0.1	0.15[b]	0.3	0.3[a]

[a]No regulatory MRL. Practical MRL same as for DIF.
[b]No regulatory MRL. Practical MRL same as for porcine and bovine meat.

Several multiresidue methods have been developed for the determination of fluoroquinolones in animal muscle tissues using microbiological tests [16], immunoassay [17], electrophoresis [18] and reverse phase high performance liquid chromatography (RP-HPLC) [19]. This last one is the technique-of-choice by its higher versatility and selectivity. Several HPLC methods have been developed for the analysis of FLU, MARBO, DIF and SAR in porcine, bovine, ovine and poultry meat. In general, they require a careful multistep sample preparation [19]. Firstly, the antimicrobials must be extracted by leaching using a solvent (aqueous [20-22] or hydroorganic [12,23-28]), by simple mixing [20], vortexing [12,21,22], shaking [23-26,28,29], ultrasound-assisted [23,28] or microwave-assisted [24],

eventually followed by centrifugation [12,20-26,28,29]. Sometimes, several successive extraction steps are even required. Afterwards, the supernatant is often purified before injection to avoid the introduction of particles, proteins, macromolecules, or other small endogenous compounds, which may be harmful for the column and/or overlap with the analytes, by solid phase extraction using a C18 [20], hydrophilic-lipophilic [21,23,29] or hydroxylated polystyrene-divinylbenzene [24,25], immunoaffinity [22] or metalchelate affinity [27] coating, liquid/liquid extraction [12,28] or QuEChERS [26] extraction. These procedures enlarge the time, effort, economic and laboratory resources, and amount of toxic chemicals required for the analysis. Besides, they provide variable recoveries and increase the sources of variance of the method. Finally, the drugs are separated in a polystyrene-divinylbenzene [28], C8 [25] or C18 [12,20,21,23,24,26,29] columns, a mobile phase with a high concentration of organic solvent (up to 100 %), usually programmed as a gradient [12,20-26,28,29], and detected by mass spectrometry [20,21,23,25,26], UV-Visible absorbance [24,25] or fluorescence [12,21,22,27,28,29]. This last one is preferred because of its higher analytical performance-per-cost ratio. However, at our knowledge, no HPLC method has been published about the analysis of these antibiotics in caprine, rabbit or horse meat.

Liquid chromatography with acidic hybrid mobile phases, using sodium dodecyl sulphate (SDS) as surfactant and triethylamine (TEA) as sacrificial base, has been proven as an interesting alternative to the determination of quinolones in food [30-32]. Micellar solutions are able to solubilize compounds within a large range of molecular mass, hydrophobicity and charge. Therefore, proteins and other non-water soluble compounds are harmless eluted at the front of the chromatogram, and does not interfere with less retained analytes. This avoids the injection of aqueous suspensions without cleanup after a simple filtration, thus simplifying the sample pretreatment [33]. Besides, the negative layer on the stationary phase and the presence of the micellar pseudophase increase the versatility and the reproducibility of the retention mechanism, and allows the resolution of a mixture of cationic and neutral drugs with different hydrophobicities in the same run using a mobile phase containing <12.5% of organic solvents working under isocratic mode. In addition, the fluorescence is enhanced in organized environments [34]. Ultrasound-assisted leaching using acidic pure micellar solutions has been also used to extract fluoroquinolones from the flesh with a high yielding [30]).

The aim of the work was the development of an analytical method for the screening of flumequine, marbofloxacin, difoloxacin and sarafloxacin in edible muscle from several animals (pork, beef, chicken, turkey, duck, sheep, goat, rabbit and horse) using micellar liquid chromatography - fluorescence detection. It must be appropriate for quality control to verify the compliance of commercial samples with the EU Regulation 37/2010 [15]. Therefore, it should be practical, easy-to-handle, safe, environmentally friendly, inexpensive and sensitive enough to provide consistent values close to the maximum residue limits for each fluoroquinolone. The analytical performances of the method were verified by validation through the guidelines of EU Commission Decision 2002/657/EC [35]. The suitability of the method for routine analysis would be demonstrated by the analysis of incurred samples from retail stores.

2. Materials and Methods

2.1 Standards and chemicals

Solid standards of FLU (purity>98%), MARBO (>98%), DIF (>99.8%) and SAR (>97.2 %) were obtained from Sigma (St-Louis, MO, USA). SDS (>99.0%) was supplied as a powder by Merck (Darmstadt, Germany). Sodium dihydrogen phosphate monohydrate (>99.0%), 1-propanol, 1-butanol and 1-pentanol (HPLC grade) were bought from Scharlab (Barcelona, Spain). Hydrochloric acid (37.0 %), ethanol (HPLC grade) and trimethylamine (>99.5 %) were purchased from J.T. Baker (Deventer, The Netherlands). Ultrapure water was in-lab produced from deionized water (supplied by the University as tap water) using an ultrapure generator device Simplicity UV (Millipore S.A.S., Molsheim, France).

2.2 Preparation of solutions

Micellar solutions were prepared by weighting the appropriate amount of SDS and $NaH_2PO_4.H_2O$, and solving them in ultrapure water using a magnetic stirrer. The adequate amount of trimethylamine was added, and then the pH was set to 3 by adding drops of HCl solutions. Furthermore, the organic solvent was added to reach the selected proportion, and the flask was filled-up with ultrapure water. Finally, the solution was ultrasonicated for 5 min

to achieve solubilization and filtered through a 0.45-µm-Nylon membrane filter (Micron Separations, Westboro, MA, USA) placed on a Büchner funnel, with the aid of a vacuum pump.

Individual solutions of each fluoroquinolone (100 mg L^{-1}) were prepared by solving the adequate amount of the powdered standard and solving it in 5 % of ethanol in a volumetric flask, and then a solution of 0.05 M SDS buffered with phosphate salt 0.01 M at pH 3 was added up to the mark. These solutions were ultrasonicated for 5 min to assure the complete solubilization. Working solutions were prepared by successive dilutions of the stock solutions in the same micellar solution. All the standard solutions were kept at +4ºC a maximum of two months.

2.3 Chromatographic conditions

The chromatograph was an HP1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, a 20-µL loop, an autosampler and a fluorescence detector. The control of the instrumentation and the registration of the signal was performed using the Chemstation Rev.A.10.01 (Agilent Technologies) software. The efficiency (N) was calculated as indicated in [36], using the half-peak width obtained by the software. The dead time (t_0) and retention time (t_R) were directly taken from the chromatogram. The asymmetry was evaluated by visual appreciation.

The stationary phase was in a C18 Kromasil column (Scharlab) with the following characteristics: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 µm; pore size, 10 nm). The mobile phase was an aqueous solution of 0.05 M SDS - 8 % 1-butanol - 0.5 % triethylamine, buffered at pH 3 with 0.01 M phosphate salt, running at 1 mL min^{-1} under isocratic mode. The detection was performed by fluorescence, and the excitation/emission wavelengths (nm) were programmed in-time as follows: 0.0-8.5 min, 240/370; 8.5-11.5, 300/488; 11.5-20, 280/455. The solutions were filtered through a 0.45-µm-Nylon membrane filter before introduction into the vials. The special care required with the chromatographic instrumentation when dealing with micellar mobile phases (change of mobile phase, cleaning before switching off, *etc.*) has been detailed in [33].

2.4 Sample processing

Samples of pork, beef, chicken, turkey, duck, sheep, goat, rabbit and horse meat were bought from a local supermarket, finely minced and stored at -20ºC in a freezer for a maximum of two months. Before processing, sample meat was thawed for 30 min at room temperature.

The samples were leached to recover the antimicrobial drugs. Thus, 5 g of meat were mixed with 50 mL of a 0.05-M SDS solution buffered at pH 3. The obtained solutions were placed in an Erlenmeyer flask, shaken using a magnetic stirrer for 1 h, and ultrasonicated for 15 min. Finally, the supernatant was taken by decantation and filtered through a 0.45-µm-Nylon membrane filter using a Büchner funnel, with the aid of a vacuum pump. This supernatant was immediately injected or kept at +4ºC in the fridge a maximum of two months, until analysis.

For spiked samples, the appropriate volume of the standard solution was injected in the minced meat. Furthermore, the sample was kept overnight at room temperature to provoke the slow vaporization of the solvent and the incorporation of the antibiotic to the matrix. Therefore, these fortified samples adequately imitate those biologically contaminated [37]. Afterwards, the analytes were extracted as indicated above.

Before the analysis, the stored solutions (standard or supernatant) were warmed at room temperature for 30 min to dissolve the crystals of SDS formed overnight.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

The main separation conditions were taken from other methods devoted to the determination of fluoroquinolones in honey [31,32] and fish flesh [30], which have provided adequate results: stationary phase, C18; flow rate, 1 mL min^{-1} under isocratic mode; surfactant, SDS; required organic solvent, 1-propanol or 1-butanol; pH, 3 and 0.5 % triethylamine. In this work, we optimize the composition of the hybrid micellar mobile phase (concentration of SDS, and the nature and concentration of the organic solvent) and the detection conditions, in order to resolve a mixture of FLU, MARBO, DIF and SAR with a

good peak shape, at the minimum analysis time. The studies were performed using a standard solution containing 0.02 mg L^{-1} of each fluoroquinolone.

According to the previous studies, these antimicrobials show a binding behaviour with the micelles, and then the retention times and the efficiency decrease at higher concentrations of SDS. Indeed, depending on their hydrophobicity and charge, they have the possibility to interact with the polar, anionic and hydrophobic sites of the micelles [34]. In order to maximize the efficiency, the concentration was set to the minimal value recommended for MLC: 0.05 M.

The pure micellar mobile phase provided too long analysis times and broad peaks. In order to avoid it, the addition of 1-propanol (2.5 to 12.5 %) or 1-butanol (1 to 10 %) was tested [34]. In both cases, lower retention times and higher efficiencies were obtained. This effect was higher using 1-butanol than using 1-propanol, and augmented at increasing concentrations of alcohol. Sarafloxacin was too retained using 1-propanol, even at larger proportions, and then it was discarded. Using 1-butanol, a proportion of 8 % provided the maximal resolution at the minimal analysis time. The less retained peak was flumequine ($t_R \approx$ 7.3 min), enough far from the front of the chromatogram. Adequate efficiencies and low tailings were obtained for the four fluoroquinolones.

A standard solution of the four quinolones was analyzed using the optimized mobile phase: 0.05 M SDS - 8 % v/v 1-butanol - 0.5 % v/v triethylamine, buffered at pH 3 with 0.01 M phosphate salt. The obtained values of (t_R; N) were: flumequine, (7.3 min; 3842); marbofloxacin, (10.2; 2985), difloxacin (13.6; 4580) and sarafloxacin (16.9; 3214). The analytes were adequately resolved. According to the retention time of the first eluting fluoroquinolone, no overlapping with the front of the chromatogram or the less retained compounds of the matrix is expected.

The antibiotics were resolved using a mobile phase containing a less proportion of toxic, volatile and flammable solvent (<8.5 %), than usually required in hydroorganic HPLC (up to 100 %). Besides, the interaction with SDS even reduced its volatility. The mobile phase works under isocratic mode, which improves the baseline stability, the reproducibility of the results and enlarges the column lifespan. Besides, a reequilibration time is not needed between two successive injections, thus reducing the analysis time per sample [38].

3.2 Detection conditions

Fluorescence was selected as a detection technique due to its higher selectivity and sensitivity than absorbance, and lower cost than mass spectrometry. A derivatization was not required, because the studied fluoroquinolones show natural fluorescence. As the spectrophotometric properties of the fluorophore depends on the chemical environment, the excitation/emission wavelengths (nm) of maximal emitted intensity were chosen from several methods about the analysis of these antimicrobials using similar mobile phases: FLU, 240/370; MARBO, 300/488 [32]; DIF and SAR, 280/455 [31].

In order to maximize the sensitivity, the detector was programmed to detect each fluoroquinolone at its optimal excitation/emission wavelengths. At the beginning of the chromatography run, the signal was monitored at 240/370. Once flumequine has been eluted (8.5 min), the detection wavelengths turned into 300/488, until the complete elution of marbofloxacin (11.5 min). From this point to the end of the chromatograms, the signal was registered at 280/455. The baseline noise was similar for the three sets of wavelengths, and no sudden oscillation of the baseline was observed at the wavelength changes.

3.3 Sample preparation

The sample preparation was based on the leaching described in [30]: extraction of the fluoroquinolones from the flesh to a solvent (1/10, w/v) by shaking, followed by filtration of the supernatant and direct injection. Several solvents (methanol and 0.05 M SDS at pH 3) were tested and the duration of the stirring were optimized. The studies were performed using a sample of porcine meat spiked at 0.2 mg kg^{-1} of each antibiotic. The recoveries were compared considering the area of the corresponding chromatographic peaks.

A at glance, it can be observed that, the micellar solutions contain a larger particles, and then it must be ultrasonicated for 15 min to reduce their size to favour the filtration. The chromatographic peaks were sharper using the micellar solution, although the recoveries were similar with both solvents. The use of methanol was discarded, because the volume of organic solvent handled and wasted would be too high, and it can partially vaporize during the processing, thus providing variable and falsely enhanced recoveries.

Several stirring times, from 10 min to 3 h were tested. The recovery strongly

increased from 0 min to 30 min, augments at a low rate to 60 min, and does not show significant variations beyond this value. Therefore, the stirring time was fixed at 60 min.

The sample preparation was easy-to-handle, as it only includes a simple leaching and the direct injection of the supernatant. Time-consuming and cumbersome cleanup steps are not needed and no reactions are involved. The used reagents are accessible, stable, innocuous and biodegradable, and no toxic organic solvent was required. Therefore, the loss of analyte, either by incomplete recuperation or by chemical change, and the risk of contamination of the sample are reduced, thus enhancing the reliability of the procedure. Besides, several samples can be simultaneously processed by the same operator, which is an interesting practical feature.

3.4 Method Validation

The procedure was in-lab validated following the guidelines of the European Commission Decision 2002/657/EC in terms of selectivity, calibration range, linearity, recovery, precision, sensitivity, decision limit (CCα), detection capability (CCβ), ruggedness and stability [35].

3.4.1 Selectivity

Free-fluoroquinolone samples of each studied meat were analyzed by the developed method. The front of the chromatogram cover from the dead time to 2.5 min, and other small peaks were observed, but far from the window time ± 2.0 min of the studied antibiotics. The chromatograms obtained from all of them were similar.

The same samples were fortified to 0.2 mg kg^{-1} FLU, MARBO, DIF and SAR, and analyzed. The chromatogram obtained from the spiked porcine meat sample can be seen in Figure 7.1. In all cases, peaks corresponding to the four antibiotics appeared at similar retention times (<2 %) and peak areas (<4 %) to those obtained by the analysis of a standard solution. The excitation and emission wavelength were taken, and the wavelengths of maximal emitted fluorescence were the same as those indicated in Section 3.2. These results prove the absence of matrix effect. Besides, no overlapping with meat compounds was observed.

The high selectivity of the method was reached because of the low retention of the proteins, fats and other macromolecules, because their strong interaction of the micelles; and the specificity of fluorescence, which reduces the number of potential interfering compounds.

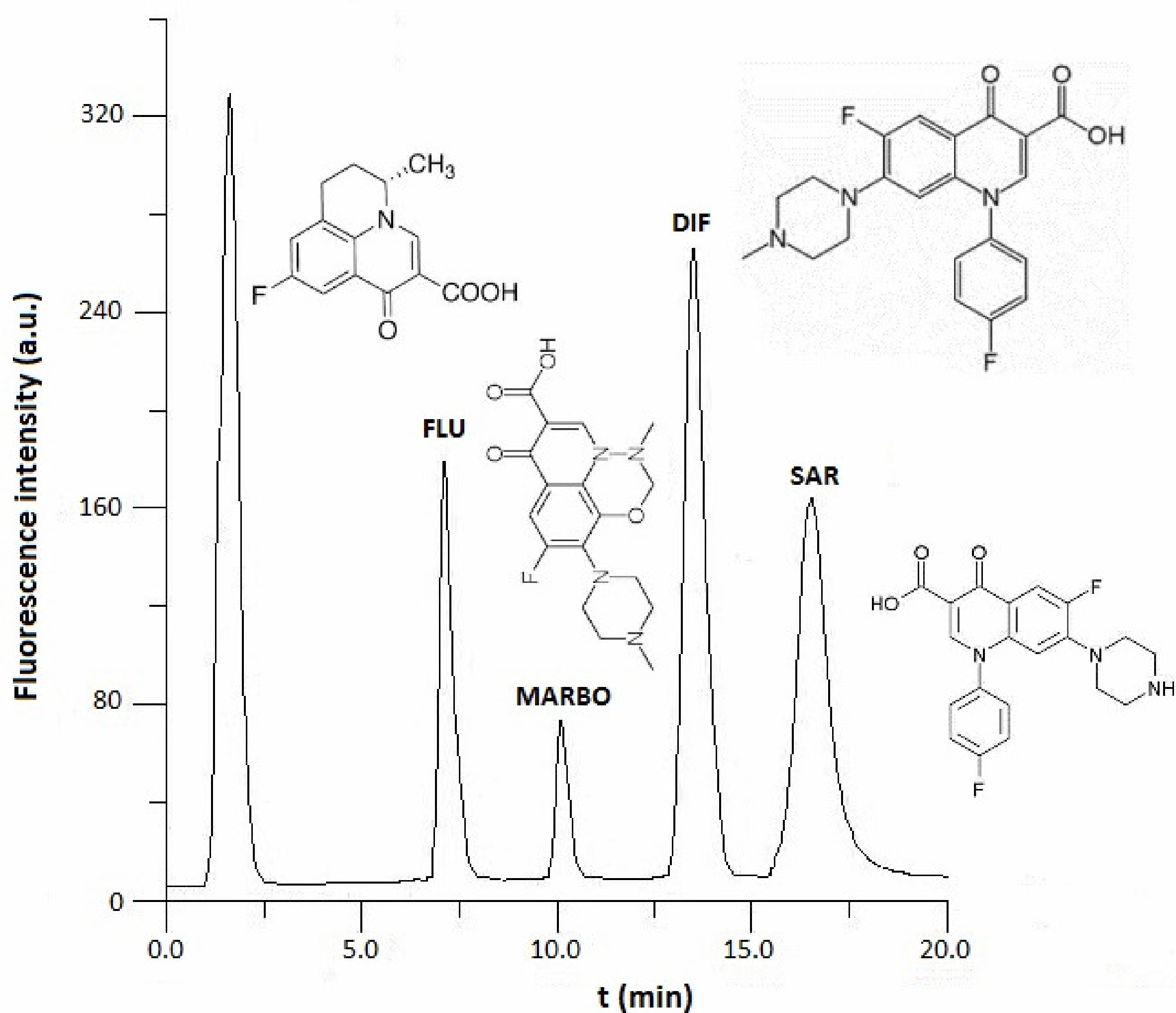

Figure 7.1. Chromatograms obtained by the analysis of a sample of porcine meat spiked at 0.2 mg kg^{-1} of each quinolone. The structure of each antimicrobial is also shown.

3.4.2 Calibration range and linearity

Standard solutions containing increasing concentrations (up to 0.8 mg L^{-1}) of the studied fluoroquinolones were 1/10 diluted, to include the dilution caused by the transfer of the analytes from the meat to the supernatant, and analyzed by triplicate. Therefore, the quantitative values refer to concentrations in meat, not in the injected solution. The average peak area was related to the corresponding concentration by a first-grade equation by least-square linear regression [39]. The slope, y-intercept and determination coefficients can be seen in the Table 7.2.

Table 7.2. Calibration curves and sensitivity of the method (concentrations in mg kg^{-1}).

Antibiotic	Slope	y-intercept	r^2	LOD	LOQ
FLU	524±3	-2±5	0.9998	0.015	0.05
MARBO	172.9±0.8	3±4	0.9997	0.03	0.1
DIF	2448±5	14±9	0.9996	0.003	0.01
SAR	1055±7	-12±15	0.9994	0.015	0.05

The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times the standard deviation of the blank divided by the sensitivity [39]. The calibration range was from LOQ to 0.8 mg kg^{-1}. The results can be seen in the Table 7.2. The chromatogram obtained from the analysis of a porcine meat sample spiked with the studied antibiotics at their corresponding LOQ can be seen in Fig. 7.2.

A satisfactory linearity was reached, according to the high goodness of fit of the regression ($r^2 > 0.9994$). For each fluoroquinolone, the calibration ranges cover the maximum residue limits in porcine and bovine muscle, mainly thanks to the high sensitivity of fluorescence detection.

3.4.3 Recovery and precision

These parameters were determined under repeatability and within laboratory reproducibility conditions. Each level, fluoroquinolone and kind of meat were separately investigated.

For the repeatability measurements, blank samples of porcine and bovine meat were fortified with each fluoroquinolone at 0.5x; 1x and 1.5x the corresponding MRL (the lowest concentration evaluated for MARBO was 0.1 mg kg^{-1}, as the 0.5xMRL falls under LOQ). The processed samples were analyzed by six successive injections. The recovery was calculated as the average of the concentrations provided by the calibration curve minus the true value, divided by the true value, while the precision was the relative standard deviation of the six peak areas. For the within laboratory reproducibility studies, the same protocol was performed five separate days over a three-months period, by renewing the fortified samples. The recovery was the average of the five average found concentrations measured each day minus the true value, divided by the fortified concentration, whereas the precision was the relative standard deviation (RSD) of the five average values of the peak areas obtained each day. The results are shown in Table 7.3 (for flumequine and marbofloxacin) and in Table 7.4 (for difloxacin and sarafloxacin).

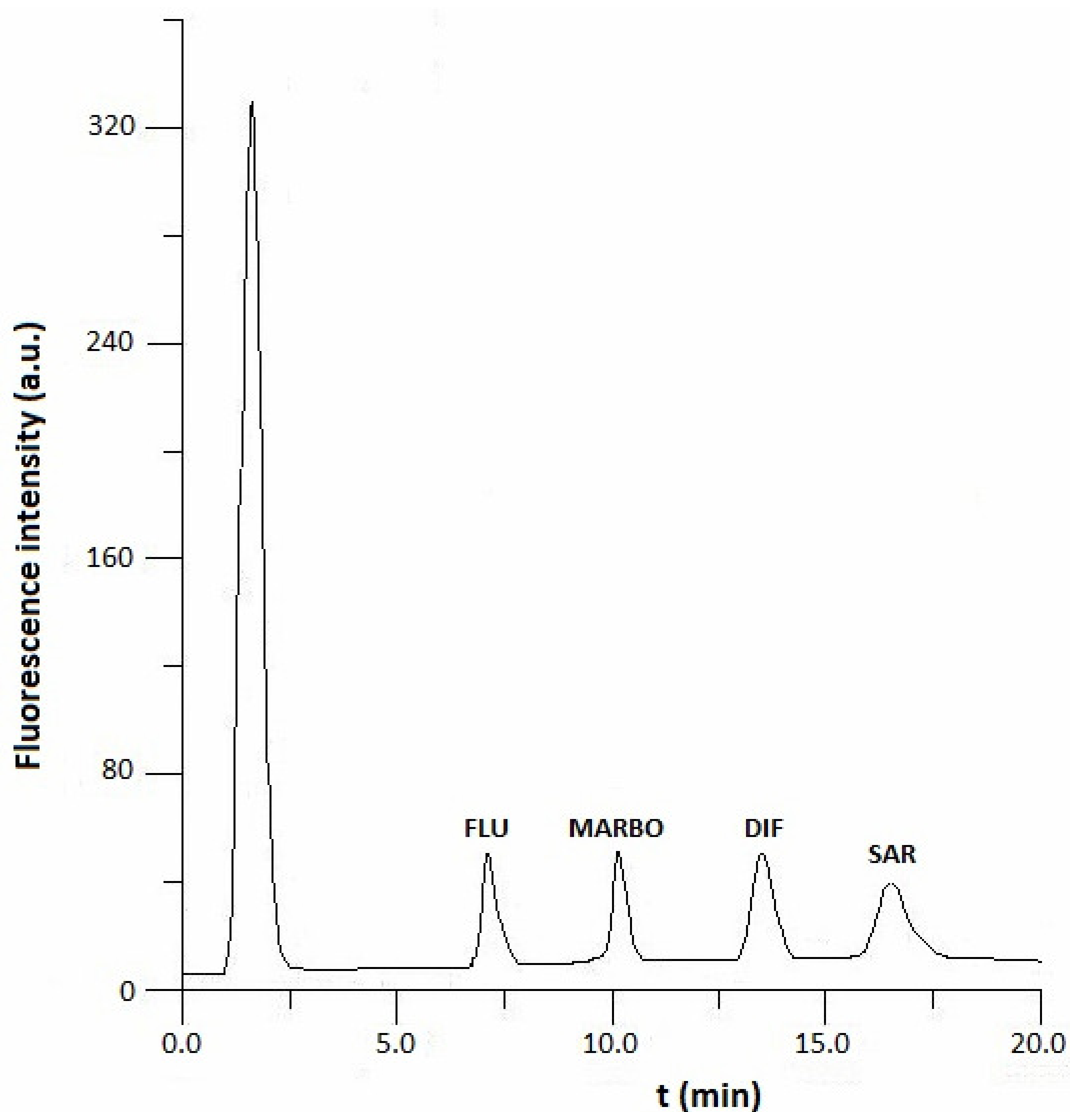

Figure 7.2. Chromatogram obtained from a sample of porcine meat spiked at their corresponding LOQ.

The values of the extraction throughput (83.9 to +107.8 %) and variability (RSD <9.4%) provided by the procedure were adequate for the studied levels, analytes, and matrices, and fulfil the requirements stated by the validation guideline (from -20 to +10 % and <12 %, respectively) by the EU guidelines. This demonstrated the high and stable yielding of the leaching step, and the advantages of the direct injection of the supernatant.

Table 7.3. Recovery/precision measured in repeatability and within-laboratory reproducibility conditions (%/RSD, %) for FLU and MARBO.

		FLU		MARBO[a]	
Meat	Fortified amount	Repeatability[b]	Within-laboratory reproducibility [c]	Repeatability[b]	Within-laboratory reproducibility [c]
Pork	0.5xMRL	107.8/9.0	106.4/7.5	83.9/8.2	84.8/8.4
	MRL	105.8/5.5	104.4/6.3	90.8/7.3	89.7/7.1
	1.5xMRL	101.8/4.1	102.4/3.0	97.7/4.2	96.8/4.7
Beef	0.5xMRL	104.1/6.8	103.8/7.9	85.8/7.5	86.5/9.4
	MRL	102.4/5.1	101.2/4.6	90.5/6.8	91.8/7.7
	1.5xMRL	97.2/3.5	98.9/2.7	96.1/4.1	97.5/5.5
Chicken	0.5xMRL	105.2/5.8	104.2/6.1	84.6/8.5	85.2/9.3
	MRL	102.1/3.9	105.5/3.4	91.5/6.9	92.5/7.0
	1.5xMRL	101.0/1.9	100.9/2.8	96.0/4.2	96.8/5.2
Turkey	0.5xMRL	104.5/4.2	103.9/3.8	87.6/7.5	88.2/7.4
	MRL	98.0/4.2	98.1/4.1	93.2/5.1	93.0/6.4
	1.5xMRL	98.5/3.3	101.0/2.1	96.2/2.9	96.0/3.5
Duck	0.5xMRL	104.8/5.4	104.0/4.8	86.2/8.0	87.6/7.1
	MRL	102.0/3.1	102.5/3.0	91.6/6.8	91.9/7.0
	1.5xMRL	101.1/2.8	101.8/2.1	95.9/3.9	95.2/3.4
Sheep	0.5xMRL	104.1/4.1	103.5/3.4	86.7/6.9	85.2/7.4
	MRL	97.2/3.9	98.3/2.5	90.2/8.1	91.6/7.8
	1.5xMRL	100.9/2.5	101.0/1.9	94.6/4.5	95.2/4.9
Goat	0.5xMRL	104.8/5.3	104.0/4.2	87.2/7.9	89.0/8.4
	MRL	103.8/4.1	103.5/2.7	93.1/5.8	93.9/6.7
	1.5xMRL	102.0/3.4	102.2/1.9	96.4/5.1	95.8/4.7
Rabbit	0.5xMRL	106.9/8.7	107.5/7.8	85.5/8.5	86.2/7.4
	MRL	105.0/3.9	104.5/4.2	91.6/7.8	91.0/8.1
	1.5xMRL	103.9/2.7	103.0/3.8	94.8/5.5	93.8/5.7
Horse	0.5xMRL	107.2/8.1	106.8/7.9	87.2/8.3	88.0/7.9
	MRL	104.9/6.8	104.5/5.5	92.2/7.1	92.0/7.3
	1.5xMRL	104.0/3.4	103.5/4.2	95.1/6.1	95.6/5.4

[a]0.1 mg kg^{-1} instead of 0.5xMRL; [b]n = 6; [c]n = 5

Table 7.4. Recovery/precision measured in repeatability and within-laboratory reproducibility conditions (%/RSD, %) for DIF and SAR.

		DIF		SAR	
Meat	Fortified amount	Repeatability[a]	Within-laboratory reproducibility [b]	Repeatability[a]	Within-laboratory reproducibility [b]
Pork	0.5xMRL	105.8/7.2	104.5/6.5	93.3/5.8	93.0/4.5
	MRL	101.9/3.9	100.2/3.2	96.4/3.8	97.9/2.8
	1.5xMRL	103.5/0.8	102.2/1.9	101.2/1.4	99.8/2.1
Beef	0.5xMRL	105.0/5.7	103.8/6.6	92.8/5.8	92.0/6.8
	MRL	98.4/3.4	99.5/2.5	95.5/3.6	96.6/3.3
	1.5xMRL	99.7/2.4	98.6/1.7	97.1/2.2	98.2/2.5
Chicken	0.5xMRL	105.5/6.8	103.4/5.3	94.8/4.6	93.2/7.1
	MRL	102.0/3.8	101.5/2.8	96.3/2.9	96.0/3.8
	1.5xMRL	100.9/2.4	99.2/1.5	98.3/3.4	98.0/3.0
Turkey	0.5xMRL	104.5/4.0	104.2/4.4	95.9/5.4	95.2/6.0
	MRL	98.9/2.8	99.1/2.0	97.2/3.8	97.0/4.1
	1.5xMRL	97.9/3.1	98.5/2.7	99.2/2.0	89.9/2.9
Duck	0.5xMRL	105.1/6.5	104.9/5.4	97.1/4.5	96.8/4.1
	MRL	100.9/1.8	101.5/2.4	100.8/1.9	100.0/2.8
	1.5xMRL	101.9/2.5	102.9/3.2	101.2/2.9	101.9/2.4
Sheep	0.5xMRL	103.1/5.9	103.9/4.5	96.1/5.9	96.0/6.8
	MRL	103.5/4.2	104.0/3.8	97.3/3.5	97.4/4.5
	1.5xMRL	102.9/3.4	102.5/3.0	98.3/2.5	98.2/3.5
Goat	0.5xMRL	104.0/3.9	103.8/4.9	94.2/4.6	94.5/4.0
	MRL	99.1/4.1	98.9/3.8	97.0/3.9	96.5/4.0
	1.5xMRL	96.5/2.9	97.1/2.4	98.8/2.4	98.0/3.1
Rabbit	0.5xMRL	103.9/4.2	104.1/5.0	95.2/5.9	96.1/6.0
	MRL	100.8/1.9	99.7/3.9	98.1/3.4	97.1/4.0
	1.5xMRL	102.1/3.5	100.9/2.9	100.5/2.8	99.5/2.1
Horse	0.5xMRL	105.1/3.5	104.5/2.9	95.0/3.5	95.8/4.2
	MRL	97.5/5.4	98.5/3.2	97.1/4.6	96.5/3.8
	1.5xMRL	100.4/3.9	100.5/2.8	99.6/3.3	99.0/2.5

[a]n = 6; [b]n = 5

3.4.4 Decision limit and detection capability

These parameters have been proposed by the EU Commission Decision 2002/657/EC, in order to consider the disturbance in the recognition of compliant and non-compliant samples, because of the uncertainty of the quantitative measurements. These parameters have been described in [35]. In brief, the decision limit is the minimal found concentration resulting in a rejection, with a reduced probability (<5%) of making a wrong decision. However, this increases the probability to accept a contaminated sample. The CCα is the minimal concentration in a sample that the method is able to classify as non-compliant with a certainty of >95%.

CCα and CCβ were separately measured for each kind of meat and fluoroquinolone. The decision limit was the MRL plus 1.64 times the standard deviation obtained by the analysis of a muscle piece spiked at the MRL (n=20). The detection capability was the CCα plus 1.64 times the standard deviation obtained by the analysis of a sample fortified at the CCα [35]. The results can be seen in Table 7.5.

For the studied meats and antimicrobials, the decision limits (<13% over MRL) and the detection capabilities (<27% over MRL) were close to the MRL. Therefore, the probability to obtain a result, leading to the acceptance of a potential non-compliant sample is relatively low. Besides, the concentration range at which the method is unable to correctly classify a contaminated meat sample is quite narrow. Therefore, random errors would provoke a false decision only in a few situations.

Table 7.5. Decision limit/detection capacity for each quinolone in the studied meats (concentrations in mg kg^{-1}).

Meat	FLU	MARBO	DIF	SAR
Pork	0.22/0.24	0.17/0.18	0.43/0.45	0.42/0.44
Beef	0.22/0.23	0.17/0.18	0.42/0.44	0.42/0.44
Chicken	0.43/0.45	0.17/0.18	0.32/0.33	0.31/0.33
Turkey	0.43/0.46	0.16/0.18	0.31/0.32	0.32/0.34
Duck	0.42/0.44	0.17/0.18	0.31/0.32	0.32/0.33
Sheep	0.21/0.22	0.17/0.19	0.32/0.34	0.32/0.34
Goat	0.21/0.22	0.16/0.18	0.43/0.45	0.42/0.45
Rabbit	0.11/0.11	0.17/0.19	0.31/0.33	0.32/0.34
Horse	0.11/0.12	0.17/0.19	0.33/0.35	0.32/0.33

3.4.5 Ruggedness

The changes in the retention and sensitivity caused by small variations of the experimental conditions was examined, in the range that can occur in the normal laboratory practice, using a Youden approach [35]. The ruggedness was separately studied for each fluoroquinolone, and instrumental response (retention time and peak area), using a standard solution of 0.02 mg L^{-1} of FLU, MARBO, DIF and SAR.

The considered factors and their intervals were: SDS, 0.045-0.055 M (A); 1-butanol proportion, 7.8-8.2 % (B); pH, 2.8-3.2 (C); TEA, 0.45-0.55 % (D); flow-rate, 0.98-1.02 mL min^{-1} (E); excitation wavelength; optimal value ±5 nm (F) and emission wavelength: optimal value ±5 nm (G). The standard deviation of the method was determined under within-laboratory reproducibility using the optimal instrumental conditions, as indicated in Section 3.4.2, but using the standard solution.

For both peak area and retention time, the differences obtained for each factor were similar. Besides, these differences and the standard deviation of the differences were slightly over the standard deviation obtained under optimal conditions. Therefore, the method is enough robust to be unaffected by the modifications of the instrumental conditions in the considered ranges, mainly because of the reproducibility of MLC.

3.4.6 Stability

The degradation of the fluoroquinolones in the standard solutions and in the studied muscle tissues was investigated at their common storage conditions (as indicated in Section 2), in order to corroborate the adequacy of the selected storage time.

A standard solution of MRL/10 mg L^{-1} of each fluoroquinolone was stored in a fridge and analyzed each day. The peak areas remained nearly constant for two months, and no other peaks appeared in the chromatogram.

Samples of each studied meat were fortified at their respective MRLs of the studied antimicrobials and kept in a freezer. On the day 0 and each week, a sample was analyzed. The concentration of the antibiotics does not undergo a significant declining after two months, and no degradation products were observed.

The fluoroquinolones remain stable in both micellar standard solution at +4ºC and in meat at -20ºC, in the darkness, for at least two months. The standard solutions were discarded after two months, and samples meats can be stored during this period until analysis.

3.5 Analysis of real samples

The developed method was used to determine the quantity of FLU, MARBO, DIF and SAR in incurred samples from pig, beef, chicken, turkey, duck, sheep, goat, rabbit and horse meat (five samples each one) purchased from a local supermarket, in order to evaluate its applicability for routine analysis. Fluoroquinolone residues were not detected in any sample, and then they can be sold without risk for the population.

A single operator was able to analyzed the whole set of samples in one day. Indeed, the meat pieces were simultaneously processed in < 2 h, and the total chromatographic sequence takes nearly 14.5 h. The participation of the operator was restrained to the preparation of the solutions, mixtures, filtration, control of the instrumentation and apparatus, as well as the supervision of the whole process, as the other tasks (stirring, ultrasonication, injection and chromatographic separation) were fully automated.

The procedure is able to study a large number of samples per day, using basic laboratory instrumentation and material, and a low amount of chemicals. Besides, the method

does not suppose a risk for the health of the operator or the environment, because of the limited toxicity of the prepared solutions. In addition, this allows the reduction of the costs for waste segregation and treatment. Therefore, the analyses were performed at a reasonable price. These practical features make the developed method useful for routine analysis.

4. Conclusions

The determination of residues of FLU, MARBO, DIF and SAR in the most consumed meats can be reliably performed by micellar liquid chromatography - fluorescence detection. The designed procedure reached a high sample throughput with an easy-to-handle pretreatment and a minimal participation of the operator, in spite of the complexity of the matrix. Besides, it was eco-friendly, safe for the laboratory staff, relatively inexpensive and useful for routine analysis. These can be considered the main advantages of the procedure. The analytical quality (selectivity, calibration range, linearity, recovery, precision, decision limit, detection capability, robustness and stability) was thoroughly evaluated following the guidelines of the EU Commission Decision 2002/657/EC, with satisfactory results. It was observed that the method provides consistent quantitative values around the maximum residue limits (0.15-0.4 mg kg^{-1}). The remarkable analytical and practical performances were reached mainly by the specific properties of micellar solutions. Therefore, this analytical method is a suitable alternative for quality-control laboratories to evaluate the compliance of commercial edible animal muscle samples with the EU regulation 37/2010, regarding to the occurrence of the antimicrobials flumequine, marbofloxacin, difloxacin and sarafloxacin.

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The authors state that there is no conflict of interest.

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Chapter 8

General Conclusions

La presente memoria describe el desarrollo y validación de diversas estrategias analíticas para la determinación de ocho fármacos antimicrobianos de uso veterinario pertenecientes al grupo de las quinolonas (ácido oxolínico, flumequina, danofloxacina, marbofloxacina, difloxacina, sarafloxacina, enrofloxacina y ciprofloxacina) en alimentos de origen animal: miel y carne procedente de ganado porcino, bovino, avícola, ovino, caprino, cunícula y equino, mediante cromatografía líquida micelar, utilizando dodecil sulfato sódico como tensioactivo, con detección mediante fluorescencia. Estos antibióticos se utilizan, en ocasiones de forma indiscriminada, en la producción intensiva de los animales para consumo humano, pero representan un riesgo para la población, por lo que se debe evitar su presencia en los alimentos producidos. También se determinaron y se discutieron sus cualidades analíticas y prácticas: sencillez, baja manipulación, semi-automatización, mejora de la seguridad en el laboratorio, bajo impacto medioambiental, reducción del precio del análisis, fiabilidad de los resultados en un amplio intervalo de concentraciones, adecuada sensibilidad y posibilidad de analizar una gran cantidad de muestras en poco tiempo. De forma general, se concluyó que los procedimientos analíticos se pueden utilizar en análisis rutinario para evaluar el cumplimiento de los lotes de alimentos de carne i mieles con la normativa impuesta por la UE, en relación a a presencia de cantidades residuales de fármacos de uso veterirnario (*Regulación de la Comisión Europea 37/2010*). Por lo tanto, representan una avance interesante en relación a un ámbito de gran importancia social como es la seguridad alimentaria.

Debido a la semejanza entre los ocho compuestos, no se analizaron simultáneamente, sino distribuidos en dos grupos de cuatro. También se utilizó un tratamiento de muestra específico para cada matriz (miel y carne), debido a su diferente estado físico (líquido viscoso y sólido compacto, respectivamente). En primer lugar, se propusieron dos métodos para la cuantificación de danofloxacina, difloxacina, ciprofloxacina y sarafloxacina (Q1) y ácido oxolínico, flumequina, marbofloxacina y enrofloxacina (Q2) en miel. Posteriormente, se desarrollaros dos procedimientos analíticos para la determinación de ácido oxolínico, danofloxacina, ciprofloxacina y enrofloxacina (Q3) y flumequina, marbofloxacina, difloxacina y sarafloxacina (Q4) en tejido muscular comestible de cerdo, ternera, pollo, pavo, pato, oveja, cabra, conejo y caballo. Se pudo emplear un pretratamiento común para todas estas clases de carne.

La principal ventaja de los métodos propuestos fue la simplificación de la etapa de preparación de muestra (la más crítica en métodos basados en técnicas separativas), gracias a la capacidad de las disoluciones micelares para solubilizar compuestos de diversa naturaleza, incluyendo macromoléculas y/o sustancias poco o insolubles en agua, en matrices complejas de origen animal, como proteínas, grasas, carbohidratos, vitaminas, electrolitos, metabolitos, y otras moléculas pequeñas. Las muestras de miel se diluyeron en una disolución 0.05 M SDS a pH 3 en proporción 1:1. Esta proporción fue suficiente para obtener una disolución fluida, a pesar de la viscosidad inicial de la muestra. El tratamiento de la muestra de carne fue más complejo. Tras ser finalmente triturada, fue necesaria una lixiviación, mediante agitación (60 min) e ultrasonicación (15 min), en una disolución micelar de 0.05 M SDS a pH 3, en proporción 1/10 w/v. Los parámetros de la extracción sólido a líquido se optimizaron para maximizar la proporción de antibiótico extraído, evitando un tiempo de análisis y dilución excesiva. El poder solubilizante de las disoluciones micelares, facilitó la recuperación de las quinolonas, así como de otros compuestos de la matriz, algunos de ellos en la disolución y otros como partículas. Tanto el sobrenadante como la miel diluida, se filtraron sin obtrucción, por lo que el filtrado obtenido fue representativo de la muestra inicial. Además, se inyectaron directamente en el sistema cromatográfico, lo que elimina la necesidad de un patrón interno. Como la fase móvil también era una disolución micelar, las sustancias de la matriz siguieron solubilizados y no precipitaron en la columna. Por lo tanto, no fueron necesarios largos y tediosos procedimientos de extracción de analitos y/o purificación de la muestra, las cuales se requieren normalmente HPLC hidroorgánica para evitar la introducción en la columna de sustancias que puedan precipitar en la columna o interferir en la señal. Por ello, se redujo el número de etapas intermedias, la manipulación por parte del operador y la cantidad de reactivos utilizados. Consecuentemente, se disminuyó la probabilidad de pérdida del analito, ya sea por una recuperación ineficiente o cambio quimico-físico, durante la preparación de la muestra, y se facilitó el estudio simultáneo de una gran cantidad de muestras.

La composición de la fase móvil fue seleccionada para maximizar la resolución entre los analitos, evitar el solapamiento con la matriz y minimizar la duración de la carrera cromatográfica. En todos los casos, se utilizaron columnas apolares C18, y fases móviles micelares híbridas tamponadas a pH 3 con sales de fosfato y 0.5 % de trietilamina como base sacrificial, circulando en modo isocrático a 1 mL/min. Las concentraciones de tensioactivo y alcohol se optimizaron (de forma independiente para cada uno de los cuatro combinaciones

de antibióticos estudiadas) mediante una estrategia interpretativa, basada en el uso de ecuaciones que permiten modelizar con gran exactitud el comportamiento cromatográfico de los analitos (parámetros cromatográficos individuales, así como la resolución por pares y global de cada analito) a partir de la composición de la fase móvil), gracias a la estabilidad y reproducibilidad del mecanismo de retención en MLC. Estos datos se pueden visualizar a partir de la representación de cromatogramas simulados, lo que facilita su interpretación. Para ello, se realizó un estudio completo del comportamiento cromatográfico de las quinolonas, en fases móviles de SDS (0.05-0.15 M) con 1-propanol (2.5-12.5%) o 1-butanol (1-10%). Las cantidades ensayadas se seleccionaron según un diseño factorial con cinco puntos: cuatro con las combinaciones de las cantidades máximos y mínimos recomendados en MLC para el tensioactivo y cada alcohol, y el quinto con los valores centrales. Los valores experimentales de factor de rentención, eficacia y asimetría se utilizaron para ajustar el modelo matemático, el cual se pudo emplear para valores intermedios de SDS y disolvente orgánico. Esta estrategia redujo en gran medida tiempo y esfuerzo requeridos para el estudio simultáneo de estos dos factores dentro de un amplio rango de concentraciones, ya que no fue necesario probar cada una de las combinaciones de tensioactivo y alcohol, sino únicamente cinco. Además, se pudo evaluar un amplio intervalo de concentraciones. Una vez seleccionados los valores de SDS y alcohol, se optimizó la concentración de trietilamina (0.0-1.5%), mediante la evaluación directa de su efecto en el tiempo de retención, eficacia y asimetría. Para cada grupo de quinolonas y matriz, la fase móvil utilizada y el tiempo total de análisis cromatográfico fue el siguiente:

- DANO, DIF, CIPRO y SAR en miel: 0.05 M SDS - 1% 1-butanol; < 25 min.
- OXO, FLU, MARBO y ENRO en miel: 0.05 M SDS - 12.5% 1-propanol; <13 min.
- OXO, DANO, CIPRO y ENRO en carne: 0.05 M SDS - 7.5% 1-propanol; < 22 min.
- FLU, MARBO, DIF y SAR en carne: 0.05 M SDS - 8 % 1-butanol; <22 min.

En todos los casos, el comportamiento de la matriz se estudió mediante el análisis de muestras no contaminadas. Se observó una banda intensa entre el tiempo muerto y antes de la elución del primer analito, tras la cual no se apreciaron picos alrededor de los tiempos de retención correspondientes a cada antibiótico. Los compuestos de la matriz, principalmente proteínas, grasas y carbohidratos, se encuentran unidos preferentemente con las micelas de la fase móvil, por lo que apenas interaccionan con la fase estacionaria o las quinolonas. Así

pues, el uso de MLC también permite evitar la interferencia (*via* solapamiento cromatográfico y efecto matriz) entre la miel y los extractos de carne matriz en la etapa de análisis cromatográfico, a pesar de su complejidad.

Para la detección se seleccionó la fluorescencia ya que esta técnica ofrece un elevado grado de selectividad y sensibilidad, a un coste relativamente bajo. Como los antibióticos presentan fluorescencia natural, no fue necesaria ninguna etapa de derivarización. Para seleccionar las longitudes de onda de excitación y emisión, para la monitorización de la señal, se midieron los correspondientes espectros durante un análisis cromatográfico, con las condiciones cromatográficas ya optimizadas, para tener en cuenta el efecto del entorno organizado. Para la cuantificación de cada antibiótico, se seleccionó el par de longitudes de onda (nm) que incrementaba la relación señal/ruido:

Q1: 280/455 nm

Q2: 0.0-8.0 min (240/400) para OXO y FLU; 8.0-15.0 min (280/495) para MARBO y ENRO.

Q3: 0-8.0 min, 260/366 para OXO; 8.0-22.0 min, 280/455 para DANO, CIPRO y ENRO.

Q4: 0.0-8.5 min, 240/370 para FLU; 8.5-11.5, 300/488 para MARBO y DIF; 11.5-20, 280/455 para SAR.

Los cambios de condiciones de detección dentro de un cromatograma se aplicaron lejos de cada pico y no afectaron significativamente a la estabilitat o a la anchura de línea base.

Estos resultados muestran que la MLC se puede efectivamente utilizar para resolver e identificar los principales antibióticos en las muestras alimentarias de origen animal estudiadas. La elevada versatilidad de la MLC, debido a la variedad de entornos y equilibrios de reparto (fase estacionaria modificada con monómeros de tensioactivo, fase móvil y micela) y de puntos de interacción en la fase estacionaria y la micela (polar, hidrofóbica y aniónica), facilita el estudio de cada grupo de quinolonas, en una única carrera cromatográfica. La adición del disolvente orgánico permite incrementar el poder de elución y la eficacia de la fase móvil, mientras que la adición de trietilamina normaliza la forma de los picos. El uso del modo isocrático elimina la necesidad de un tiempo de acondicionamiente entre dos inyecciones, aumenta la estabilidad de la línea base y ofrece un entorno menos agresivo para la fase estacionaria.

La última etapa, y la más importante, en el desarrollo de los métodos fue su validación, para verificar su calidad analítica y aportar más rigurosidad al estudio. Se realizó

directamente en matrices fortificada, a partir de las directrices de la *EU Commission Decision 2002/657EC*, la cual se redactó específicamente para la determinación de contaminantes orgánicos en muestras alimentarias de origen animal, y es de obligado cumplimento para los laboratorios autorizados para el control oficial de residuos, y la *ICH Harmonized Tripartite Guideline*. Para el caso de la miel, la normativa prohibe totalmente el uso de antibióticos en apicultura y marca un límite permitido de cero, por lo que se intentó alcanzar la máxima sensibilidad. En el caso de la carne, la normativa impone límites máximos permitidos para cada antibiótico y clase (0.1-0.4 mg kg^{-1}), por lo que el estudio se centró alrededor de dichos valores. Los parámetros estadísticos evaluados y los resultados se describen a continuacion, y cumplieron en todos los casos los requisitos de aceptación: selectividad (se demostró que todos los analitos se podían detectar sin interferencias en la etapa de separación cromatográfica), límite de detección (0.004-0.07 mg kg^{-1}), límite de cuantificación (0.01-0.2 mg kg^{-1}), intervalo lineal (desde el LOQ hasta valores no alcanzables en la práctica), linealidad (r^2>0.995), límite de decisión (LOD para la miel y <13% sobre el límite permitido para la carne), capacidad de detección (<55% y <27% sobre el LOQ para la miel y el límite permitido para la carne), recuperación (82.1–110.0%), precisión (<12.3%), efecto matriz (no se observó), robustez (proporciona respuestas analíticas prácticamente invariantes frente a pequeños cambios en las condiciones experimentales) y estabilidad (no se observó degradación de los analitos ni disoluciones estándar en dos meses ni en matriz en dos semanas, bajo sus condiciones habituales de almacentamiento). Estos resultados muestran que los métodos proporcionan valores cuantitativos fiables alrededor de los límites permitidos por la legislación y dentro de un intervalo amplio de concentraciones. Se obtuvieron valores aceptables de recuperación y de reproducibilidad, debido a la sencillez y eficacia del tratamiento de muestra, a la introducción cuantitativa de la muestra diluida o del sobrenadante en la columna y a la reducción de las fuentes de varianza. Los valores elevados de sensibilidad se alcanzaron gracias a las características del detector y a la disminución del ratio de dilución de la muestra aplicado en el pretratamiento. Finalmente, se utilizaron para el análisis de una gran cantidad de mieles y carnes, procedentes de comercios minoristas locales.

Considerando tanto el tratamiento de muestra y la separación cromatográfica, cabe resaltar que el análisis se puede realizar empleando intrumentación, material y reactivos analíticos baratos, robustos, de uso general y fácilmente accesibles. Además el procedimiento

experimental es sencillo y relativamente rápido. Por otra parte, se observa que las muestras se pueden almacenar durante un tiempo prudencial antes de su análisis i que se pueden procesar un número elevado de muestras en poco timepo, lo que resulta útil si se reciben más muestras de las que se pueden analizar en un momento dado. Los reactivos principales (SDS y sales de fosfato) son biodegradables e inocuos, y se empleó una proporción mínima (<13%) de disolventes orgánicos tóxicos, inflamables y volátiles. Así pues, se redujo en todo los posible el manejo y vertido de disoluciones peligrosas para la salud y el medio ambiente. Esto sigue la tendencia actual de la Química Analítica y las exigencias de la sociedad.

Anexo 1. Aportaciones originales

Los estudios descritos en la presente memoria se han realizado gracias a la financiación recibida por parte de la Universitat Jaume I (P1.1B2012-36), la cual consideró que su importancia y relevancia científica y social les hacían merecedores de su apoyo. La difusión de los resultados se realizó a través de la publicación de varios artículos y comunicaciones en revistas y congresos científicos de primer nivel, lo que muestra la calidad del mismo.

Artículos en revistas de investigación científica

1. Tayeb Cherif K, Peris-Vicente J, Carda-Broch S, Esteve-Romero J (2015) Analysis of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey using micellar liquid chromatography and validation according to the 2002/657/EC decision. *Anal. Methods* 7, 6165–6172.
2. Tayeb Cherif K, Peris-Vicente J, Carda-Broch S, Esteve-Romero J (2016) Use of micellar liquid chromatography to analyze oxolinic acid, flumequine, marbofloxacin and enrofloxacin in honey and validation according to the 2002/657/EC decision. *Food Chem.* 202, 316-323.
3. Terrado-Campos D, Tayeb-Cherif K, Peris-Vicente J, Carda-Broch S, Esteve-Romero J (2017) Determination of oxolinic acid, danofloxacin, ciprofloxacin, and enrofloxacin in porcine and bovine meat by micellar liquid chromatography with fluorescence detection. *Food Chem.* 221, 1277-1284.
4. Peris-Vicente J, Tayeb-Cherif K, Carda-Broch S, Esteve-Romero J (2017) Validation of a procedure to quantify oxolinic acid, danofloxacin, ciprofloxacin and enrofloxacin in selected meats by micellar liquid chromatography according to the EU Commission Decision 2002/657/EC. *Electrophoresis*, Aceptado.
5. Peris-Vicente J, Terrado-Campos D, Albiol-Chiva J, Tayeb-Cherif K, Carda-Broch S, Esteve-Romero J (2017) Development and validation of a micellar liquid chromatographic method to determine flumequine, marbofloxacin, difloxacin and sarafloxacin in the most

consumed meats. *J Food Eng.* enviado.

Comunicaciones en congresos internacionales de investigación científica

39th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2013 - Amsterdam). 16-20 de Junio de 2013; Amsterdam, Países Bajos.
1) "Determination of Fungicides in Seed, Formulation, Plant Material, Soil and Water Samples" (CMTR20_TU; p. 320) Mourya SK, Durgabanshi A, Esteve-Romero J, Bose D, J. Peris-Vicente, Carda-Broch S, Tayeb-Cherif K

*20th International Symposium on Separation Science (*20th ISSS*).* 30 de Agosto - 2 de Septiembre de 2014; Praga, República Checa.
Publicación: “Book of proceedings of the 20th International Symposium on Separation Science", (Editores = A. Horna, P. Jandera) Ed. Radanal, Pardubice, Czech Repulik, 2014. ISBN: 978-80-7395-777-3
2) "Micellar liquid chromatography: an interesting tool for determination of biological samples in bioanalytical chemistry" (P66; p. 121) Esteve-Romero J, Carda-Broch S, Peris-Vicente J, Roca-Genovés P, Tayeb-Cherif K, Romero-Cano R, Monferrer-Pons L
3) "Determination of lipophilicity by high performance liquid chromatography" (P10; p. 65) Peris-Vicente J, Esteve-Romero J, Raviolo MA, Villarreal-Traver M, Tayeb-Cherif K, Carda-Broch S

14th International Nutrition and Diagnostics Conference (14th INDC). 02-05 de Septiembre de 2014; Praga, República Checa.
- Publicación: “Book of proceedings of the 14th International Nutrition and Diagnostics Conference", (Editor = A. Horna), Ed. Radanal, Pardubice, Czech Repulik, 2014. ISBN: 978-80-7395-776-6
4) "Quantification of antibiotics in milk and egg samples by micellar liquid chromatography" (P125; p. 182) Esteve-Romero J Rambla-Alegre M, Carda-Broch S, Peris-Vicente J, Villarreal-Traver M, Tayeb-Cherif K
5) "Determination of antibiotics in fish grown in fisheries using micellar liquid

chromatography" (P126; p. 183) Esteve-Romero J, Peris-Vicente J, Rambla-Alegre M, Carda-Broch S, Tayeb-Cherif K, Garrido-Cano I, Álvarez-Rodriguez L

European Symposium on the Practical Applications of Analytical Technologies in the iopharmaceutical Industry (ATEurope 2016). 15-28 de Marzo de 2016; Viena, Austria.

6) "Relationship Between Tamoxifen/Endoxifen Concentration Ratio and CYP2D6 Genotipe in Men and Women" (LB-03c) Albiol-Chiva J, Roca-Genovés P, Ochoa-Aranda E, Esteve-Romero J, Peris-Vicente J, Tayeb-Cherif K, García García A

7) "Development and Validation of a Method to Detect Eight Fluoroquinolones in Honey Using Micellar Liquid Chromatography - Fluorescence Detection" (LB-03d) Albiol-Chiva J, Tayeb-Cherif K, Peris-Vicente J, Roca-Genovés P, Esteve-Romero J, Carda-Broch S

8) "Use of Micellar Liquid Chromatography to Quantify Several Quinolones in Porcine and Bovine Flesh" (LB-03g) Albiol-Chiva J, Tayeb-Cherif K, Carda-Broch S, Roca-Genovés P, Esteve-Romero J, Peris-Vicente J

9) "Determination of Antibiotics in Pharmaceuticals and Physiological Samples by Micellar Liquid Chromatography" (LB-03j) Albiol-Chiva J, Carda-Broch S, Tayeb-Cherif K, García-García A, Peris-Vicente J, Roca-Genovés P, Esteve-Romero J

Anexo 2. Futuras líneas de investigación

El grupo de Química Bioanalítica tiene como línea de investigación el desarrollo y validación de métodos analíticos aplicables en el campo de la seguridad alimentaria. El objetivo principal es presentar procedimientos alternativos con interesantes ventajas prácticas en el análisis de rutina: sencillez, rapidez, respetuoso con la seguridad laboral, bajo impacto ambiental, reducido coste, al alcance de cualquier laboratorio y aplicables al análisis sucesivo de gran cantidad de muestras, manteniendo la fiabilidad de los datos cualitativos y cuantitativos proporcionados. Esta temática presenta un gran interés, ya que sigue las actuales tendencias en el ámbito de la Química Analítica. Tengo previsto continuar mi colaboración dentro de esta línea.

En un futuro tengo previsto ampliar el estudio detallado en esta memoria a otras quinolonas y otros antibióticos de uso veterinario. También se pretende la optimización y validació de métodos para determinar las quinolonas en otros tejidos y fluidos biológicos extraídos directamente de los animales, para evaluar su exposición global y el tiempo requerido para su total eliminación del organismo. Esto se puede aplicar para sugerir la implementación de medidas preventivas en le etapa de producción que eviten la presencia de los antióticos en los alimentos. Asimismo tengo pensado participar en la determinación de estos compuestos en el medioambiente, para estudiar el efecto del excesivo uso de fármacos antimicrobianos sobre la naturaleza. Por último, se pretende estudiar la determinación de los antibióticos mediante el uso de disoluciones micelares mixtas, donde el disolvente orgánico es sustituido por otro tensioactivo no-contaminante. Para ello, será necesario evaluar el comportamiento cromatográfico de los analitos en este medio, realizar estudios teóricos sobre el mecanismo de retención y el desarrollo de ecuaciones y diseños factoriales para su modelización. Así pues, se dispondría de una colección de métodos anlíticos completamente ecológicos y seguros.

Anexo 3. Aceptación de los coautores de las publicaciones que integran la tesis, de que el doctorando presenta el trabajo como Tesis y la renuncia explícita de éstos a presentar los como parte fr otra Tesis Doctoral (según el Art. 23 de la NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I, Aprobada por el Consejo de Gobierno núm. 19 del 26 de enero de 2012)

Josep Esteve Romero, director de la presente Tesis, declara que los coautores de las publicaciones que se presentan en esta memoria, y que paso a enumerar: Samuel Carda Broch y Juan Peris Vicente no utilizarán el material que aquí se incluye para formar parte de otras Tesis. Y para que conste donde convenga, firmo la presente.

Josep Esteve Romero

Juan Peris Vicente

Samuel Carda Broch

Universitat Jaume I, 03 de Abril de 2017

Anexo 4. Abreviaturas y acrónimos

Quinolonas y compuestos relacionados

CIP, CIPRO: ciprofloxacina

DAN, DANO: danofloxacina

DIF: difloxacina

DNA: ácido desoxirribonucleido/*deoxyribonucleic acid*

ENRO: enrofloxacina

FLU: flumequina

MARBO: marbofloxacina

OXO: ácido oxolínico

SAR: sarafloxacina

Reactivos de laboratorio

CTAB: Bromur d'hexadeciltrimetilamoni/*cetyltrimethylammonium bromide*

HCl: Ácido clorhídrico

$NaH_2PO_4.H_2O$: Dihidrogenofosfato de sodio monohidratado

NaOH: Hidróxido de sodio

SDS: dodecilsulfato sódico/*Sodium Dodecyl Sulfate*

TEA: trietilamina

Instrumentación

C8: octasilano

C18: octadecilsilano

DAD: detección de matriz de diodos/*diode array detection*

FLD: detección por fluorescencia

HPLC o LC: cromatografía líquida de alta resolución/*High Performance Liquid Chromatography*

MLC: cromatografia líquida micelar/*Micellar Liquid Chromatography*

MS: espectrometría de masas/mass spectrometry

MS-MS: espectrómetría de masas en tándem

RP: fase inversa/*reverse phase*

UV: ultravioleta

Asociaciones y Organismos

ICH: Conferencia Internacional de Harmonización/*Internacional Conference on Harmonization*

IEC: Comisión Electrotécnica Internacional/ *International Electrotechnical Commission*

ISO: Organización Internacional de Normalización/*International Organization for Standardization*

FAO: Organización para la Alimentación y la Agricultura/*Food and Agriculture Organization*

FDA: Administración de Alimentos y Medicamentos/*Food and Drug Administration*

OMS/WHO: Organización Mundial de la Salud/*World Health Organization*

UE/EU: Unión Europea/*European Union*

Parámetros cromatográficos

A: área de pico/peak area

B/A: Factor de asimetría

φ: proporción de disolvente orgánico (v/v)

k: factor de retención o factor de capacidad

K_{AS}: Constante de equilibrio de reparto del analito entre el agua pura y la fase estacionaria multiplicado por el volumen de la fase estacionaria, dividido por el volumen muerto

K_{AM}, P_{MS}: Constante de equilibrio de reparto del analito entre el agua pura y la micela

K_{AD}: medida de la variación de concentración del analito en la fase acuosa debido a la adición del disolvente orgánico.

K_{MD}: medida de la variación de concentración del analito en la micela a causa de la adición del disolvente orgánico.

$h(t)$: señal del cromatograma

H_0: altura del pico al tiempo de retención

N: número de platos teóricos (eficacia)

P_{MS}: Constante de reparto del analito entre la micela y la fase estacionaria

P_{WS}: Constante de reparto del analito entre el agua pura y la fase estacionaria

r_{ij}: resolución por pares de pico

s_0; s_1...: parámetros de ajuste del perfil de pico

t_0: tiempo muerto

t_R: tiempo de retención

Z: resolución global

Parámetros químicos

CMC: concentración micelar crítica/*critical micellar concentration*

Ka: constante de desprotonación de un ácido

Po/w : coeficiente de reparto octanol-aigua/*octanol-water partition coefficient*

rpm: revoluciones por minuto

Sa/Su: proporción muestra/sobrenadante / *sample/supernatant ratio*

Parámetros de validación y regulatorios

CCα: límite de decisión/*decision limit*

CCβ: capacidad de detección/*detection capability*

CRM: material de referencia certificado/*certified reference material*

CV: coeficiente de variación/*coefficent of variation*

LOD: límite de detección/*Limit of Detection*

LOQ: límit de quantificació/*Limit of Quantitation*

LLOQ: límite mínimo de cuantificación/*lower limit of quantification*

ULOQ: límite máximo de cuantificación/*upper limit of quantification*

MRL: límite máximo de residuo/*maximum residue limit*

MRPL: Límite mínimo de funcionamiento exigido/*minimum required performance limit*

r^2: coeficiente de determinación/*determination coefficient*

RSD: desviación estándard relativa/*Relative Standard Derivation*