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EVALUACIÓN DEL POTENCIAL DE LA ELECTROFORESIS CAPILAR PARA SU IMPLANTACIÓN EN LOS LABORATORIOS AGROALIMENTARIOS

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TITULO: *EVALUACIÓN DEL POTENCIAL DE LA ELECTROFORESIS CAPILAR PARA SU IMPLANTACIÓN EN LOS LABORATORIOS AGROALIMENTARIOS*

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Trabajo presentado para aspirar al Grado de Doctor en Ciencias

LA DOCTORANDA

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CERTIFICAN: Que la Tesis Doctoral ***"EVALUACIÓN DEL POTENCIAL DE LA ELECTROFORESIS CAPILAR PARA SU IMPLANTACIÓN EN LOS LABORATORIOS AGROALIMENTARIOS"*** ha sido desarrollada en los Laboratorios del Departamento de Química Analítica de la Universidad de Córdoba, así como en el Laboratorio de Instrumentación Analítica y Laboratorio de Bioquímica de la Unidad de Investigación en Ciencia y Tecnología de los Alimentos de la Universidad del Zulia, y que a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en la ciudad de Córdoba, a 11 de julio de 2013.

Miguel Valcárcel Cases ***Lourdes Arce Jiménez***

Roberto Bauza Fermín

ÍNDICE

OBJETIVO

En los últimos años, la Electroforesis Capilar(CE) ha presentado un importante desarrollo en la determinación de diversos analitos en alimentos, sin embargo, las técnicas electroforéticas tienen todavía cierta resistencia a implantarse de forma rutinaria en los laboratorios agroalimentarios, posiblemente debido a dos grandes limitaciones de la técnica: baja sensibilidad y por los problemas ocasionados por la matriz cuando se analizan muestras complejas. Estas limitaciones hacen que las etapas previas de tratamiento de muestra, procesos de *clean-up* y de preconcentración, jueguen un papel fundamental en el análisis electroforético, ya que la superación de estas limitaciones pudiera llevar a la completa implantación de los sistemas electroforéticos en algunos laboratorios de rutina.

El objetivo general de esta Tesis Doctoral es evaluar el potencial de laCEpara su implantación en los laboratorios agroalimentarios. En este sentido, se plantean diversos objetivos específicos que se describen a continuación.

En primer lugar, se presenta una revisón exhaustiva y crítica sobre la literatura existente relacionada con la aplicación de la CEpara la determinación de analitos de distinta naturaleza en el ámbito agroalimentario y se plantea una reflexión sobre el empleo de muestras fortificadas y/o reales para la validación de métodos.

El segundo objetivo específico de este trabajo es demostrar la importancia de la etapa del tratamiento de muestra dentro del proceso analítico, cuando se emplea la CE como técnica de separación. Para ello, se presenta un análisis crítico sobre la dificultad de extraer y preconcentrar compuestos minoritarios a nivel de trazas, como residuos de antibióticos pertenecientes al grupo de las penicilinas (PENs),presentes en matrices complejas como muestras de leche.

Finalmente, se presentan dos alternativas de tratamientos de muestras para determinar fluoroquinolonas (FQs)–otra familia de antibióticos– en leche bovina y caprina, medianteCE y Cromatografía Líquida de Alta Resolución (HPLC). En ambos casos, se plantean procedimientos sencillos basados principalmente en el empleode la extracción en fase sólida. Todo esto con el fin de proponer métodos atractivos para que sean implantados en los laboratorios agroalimentarios de rutina.

CAPÍTULO I

Introducción

Electroforesis capilar (CE)

1.1 Generalidades

La CE es una técnica que permite la separación, identificación y cuantificación de diferentes analitos en el interior de un capilar, el cual se llena con una disolución amortiguadora apropiada bajola influencia de un campo eléctrico[1].

La separación de los analitos se produce como consecuencia de la acción combinada de la migración electroforéticas de moléculas cargadas en la solución que van en dirección a un electrodo de carga opuesta, y del flujo electrosmótico ocasionado por la pared interna cargada del capilar y el potencial aplicado, de tal manera que todas las moléculas se ven arrastradas al cátodo (polaridad normal) donde se realiza la detección [2, 3].El proceso de separación electroforética se lleva a cabo en un sistema similar al indicado en la Fig. 1.

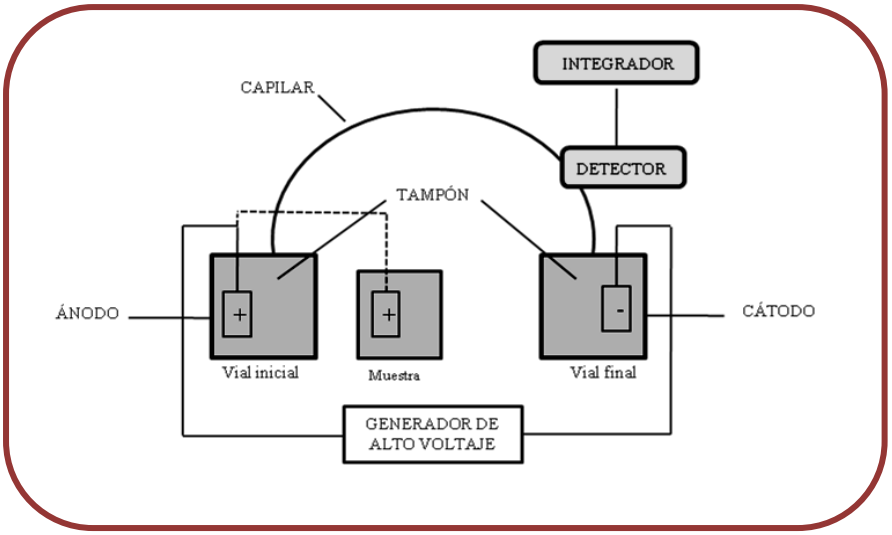

Fig. 1. *Esquema básico de un sistema CE. Adaptado de C. Cruces-Blanco (1998) [4]*

Como se muestra en la Fig. 1,el capilar está en contacto con unos viales(inicial y final), los cuales a su vez contienen los electrodos que se encuentran conectados a un generador de alto voltaje. Dicho capilar se llena con una disolución tampón que recibe el nombre de electrolito de fondo o BGE (por sus siglas en inglés: background electrolyte) y constituye el medio de conducción de la corriente eléctrica. El BGE debe ser una disolución tampón adecuada a la muestra. La muestra se inyecta en el interior del capilar reemplazando el vial inicial por el vial que contiene la muestra y posteriormente, el vial de entrada vuelve a colocarse para aplicar la diferencia de potencial. La separación tiene lugar a lo largo del tiempo.

1.2 Fenómenos de migración

Los analitos o iones con carga positiva (cationes) migraran hacia el cátodo y los iones con carga negativa (aniones) migraran hacia el ánodo. La velocidad de la migración dependerá de las relaciones carga/tamaño, es decir, un ión pequeño migrará más rápido que otro más grande de la misma carga. De igual forma, un ión con alta carga migrará más rápido que uno con carga más pequeña, si son del mismo tamaño[2, 4]. Esta migración o separación de los analitos en el interior del capilar se rige por dos fenómenos, que tienen lugar simultáneamente: la electromigración y la electroósmosis.

1.2.1 Electromigración

Bajo la influencia de un campo eléctrico, cada uno de los analitos contenidos en la muestra migrará a través de la disolución tampón que se encuentra dentro del capilar a distinta velocidad. A esta velocidad se le denomina *velocidad electroforética*.

La separación de los analitos ocurre debido a la diferencia en sus velocidades electroforéticas y éstas a su vez dependen de la carga y tamaño de cada analito. Por lo tanto, cuanto mayor sea la relación carga/tamaño, mayor será la movilidad electroforética.

Para medir la velocidad y la movilidad electroforética es necesario conocer el tiempo que tarda un analito en migrar desde el punto de inyección hasta el detector. Este tiempo se denomina *tiempo de*

migración (t_m) y se refiere al tiempo que tarda un analito en moverse desde el principio del capilar hasta la ventana del detector [4].

1.2.2 Electroósmosis

La electroósmosis, también conocida como *electroendoósmosis*, es un fenómeno de los procesos de separación electroforética, que consiste en el movimiento relativo de un líquido con respecto a una superficie cargada, bajo la acción de un campo eléctrico. Este movimiento es lo que se conoce con el nombre de flujo electroosmótico (EOF) y es un fenómeno que se produce siempre que se aplica un campo eléctrico a un sistema líquido que esté en contacto directo con una superficie cargada, como ocurre en la CE [4].

1.3Sistema de inyección

Los modos de inyección en CE son inyección hidrodinámica e inyección electrocinética, siendo la inyección hidrodinámica la más empleada.

La muestra puede inyectarse hidrodinámicamente de tres formas: a) por aplicación de presión en el extremo de inyección del capilar;b) haciendo vacío en el extremo contrario al de inyección;c) o por efecto sifón, al elevar el vial de muestra respecto al vial de la solución tampón situado en el otro extremo de inyección. La forma de inyección hidrodinámica más empleada es aquella donde se aplica presión en la entrada.

En la inyección electrocinética, el vial de la muestra reemplaza el vial de la solución tampón en el extremo de inyección del capilar y seguidamente, se aplica una diferencia de potencial entre los extremos del capilar durante un tiempo determinado. Los analitos se introducen en el capilar por el efecto conjunto de su migración electroforética y el EOF, por lo que cada analito será inyectado en distinta cantidad, de tal manera que los más móviles entrarán en mayor proporción [2].

1.4 Modos de CE

La CE puede llevarse a cabo por distintos modos, a continuación se presentan brevemente los modos electroforéticos más utilizados y referenciados en la literatura.

1.4.1 Electroforesis Capilar de Zona (CZE)

La CZE (llamada así por sus siglas en inglés) es la modalidad más utilizada a causa de su simplicidad operacional y elevado poder de separación. Este modo electroforético está basado en la separación de los analitossegún su relación carga/tamaño.

En esta técnica la composición del tampón es constante manteniendo su fuerza iónica y pH en todo el capilar durante el tiempo que dura la separación. El potencial aplicado hace que los diferentes componentes iónicos de la muestra (aniones, cationes y/o analitos neutros) migren según su propia movilidad y se separen en zonas que puedan estar completamente resueltas o parcialmente solapadas.Mediante CZE es posible separar y analizar una gran variedad de moléculas pequeñas [5].

1.4.2 Cromatografía Electrocinética Micelar (MEKC)

Este método, que combina la cromatografía y la CE, permite la separación de moléculas no cargadas.Para ello, es necesario añadir un elemento tensioactivo, como por ejemplo dodecil sulfato sódico (SDS), en concentraciones lo suficientemente grandes como para que forme micelas.

Las micelas se forman en solución acuosa cuando la concentración de una sustancia iónica que tiene una cola de una larga cadena de hidrocarburos se incrementa por encima de cierto valor denominado *concentración crítica micelar*.Lasmicelas constituyen una segunda fase estable que es capaz de alojar compuestos no polares en el interior hidrocarbonado de las partículas y, por lo tanto “solubiliza” compuestos no polares [5]

1.4.3 Isotacoforesis

El nombre deriva de la separación electroforética de las bandas que migran todas a igual velocidad.

La mezcla de analitos es colocada entre dos disoluciones electrolíticas con iones de diferente movilidad, uno rápido llamado ión líder o inicial y otro más lento llamado ión terminal.

Para la separación de un catión, el electrolito inicial puede contener un catión de elevada movilidad como el ión hidrógeno, mientras que el terminal puede contener un ión que sea más lento que el que se desea separar. Por esta razón, el electrolito líder siempre debe ser colocado para migrar hacia el cátodo y el terminal debe migrar hacia el ánodo [6].

1.4.4 Electroforesis Capilar por Isoelectroenfoque

Este procedimiento está destinado a la separación de componentes anfotéricos de una mezcla en un gradiente de pH continuo y estable que se extiende desde bajo pH en el ánodo y elevado en el cátodo.

La obtención de un gradiente de pH estable y continuo se logra empleando anfolitos obtenidos por la unión de poliaminas y ácidos orgánicos, formando uniones poliamínicas y policarboxílicas que en un ámbito de protones los ceden o incorporan a las moléculas, lo que actúa como un estabilizador de pH.

Cuando a un medio con anfolitos se le aplica un campo eléctrico, los anfolitos migran hacia un punto isoeléctrico, generando un gradiente estable de pH. Cuando al gradiente de pH se le introduce una proteína en ese gradiente de anfolitos, cada zona intercambia protones con la muestra proteica, generando una separación isoeléctrica conocida como electroenfocado o *electrofocusing*[7].

1.5 Aplicaciones de CE

Desde la creación de la CE a principios de 1980,esta técnica ha venido incrementado su popularidad por las diversas ventajas que ofrececomo: alta

eficacia, rapidez de las separaciones, bajo coste de análisis, uso de pequeños volúmenes de muestra y bajo consumo de reactivos [1, 8].

Actualmente, el uso de esta técnica se ha expandido en diversos campos de aplicación, como el área farmacéutica y clínica, donde se emplea de forma rutinaria. En particular, parael análisis de alimentosesta técnicapuede ofrecerventajasinteresantessobre las técnicascromatográficas, debido a su mayorsimplicidady eficiencia. Sin embargo, la CE tiene cierta resistencia a implantarse de forma significativa en los laboratorios agroalimentarios de rutina, debido a los diversos inconvenientes que se presentan cuando se pretende separar analitos presentes en bajas concentraciones en muestras de alimentos complejas. Las principales limitaciones que presenta esta técnica son su baja sensibilidad, en parte por los pequeños volúmenes (nanolitros) de muestra que se introducen en el capilar [9, 10]y los problemas ocasionados por la matriz cuando se analizan muestras complejas [11], ya que debe existir compatibilidad entre el extracto obtenido al finalizar el tratamiento de la muestra y el sistema electroforético. También la robustez de la técnica ha representado un punto de divergencia en la comunidad científica

Con el fin de minimizar estos inconvenientes, se han desarrollados diferentes estrategias que permiten mejorar la sensibilidad de la técnica [12], sin embargo, es necesario considerarla etapa de preparación de muestra como una parteclave para evitaro reducir al mínimolas dificultades que se presentan en el análisis de alimentos y el efecto sobrela calidadde los resultados analíticos. La CE podrá implantarse en los laboratorios agroalimentarios de rutina cuando existan diferentes alternativas para superar estos inconvenientes.

Para suministrar al lector una idea global sobre la situación actual del uso de la CE en el análisis de alimentos, a continuación se presenta una revisión donde se aborda las diferentes empresas encargadas de la comercialización de los equipos de CE y la situación actual del mercado relacionado con esta tecnología. Además se presenta un resumen sobre las aplicaciones clásicas de esta técnica en los laboratorios de análisis de alimentos y se describe brevementealgunas nuevas tendencias y aplicaciones avanzadasen el campo agroalimentario.

Como se verá más adelante, la mayoría de los trabajos realizados para demostrar el potencial de la CE en el análisis de alimentos, utilizan muestras fortificadas o enriquecidas. Por otro lado, se observa la carencia de una discusión centrada en la complejidad de las matrices de alimentos y los inconvenientes que se pudieran presentar para extraer los analitos a partir de muestras reales.

La revisión llevada a cabo en este trabajo permitió tener una visión general actualizada sobre la CE en el análisis de alimentos y así plantear los diferentes trabajos experimentales presentados en esta memoria.

REFERENCIAS

[1] Y. Picó, Food toxicants analysis: techniques, strategies and developments. Elsevier, Amsterdam, 2007.

[2] R. Kuhn, S. Hoffstetter-Kuhn, Capillary Electrophoresis. Principles and practice. Springer-Verlag, Berlín, 1993.

[3] S. Li, Capillary Electrophoresis. Principles, practice and applications. Elsevier, Amsterdam, 1992.

[4] C. Cruces-Blanco, Electroforesis Capilar, Universidad de Almería, Servicio de Publicaciones, Almería, 1998.

[5] D.A. Skoog, F.J. Holler, S.R. Crouch, Principios de análisis instrumental. México, D. F., Cengage Learning Editores, sexta edición, 2008.

[6] F.M. Evaerest, J.L. Beckers, E.M. Verheggen,Isotachophoresis, theory, instrumentation and applications. Elsevier Publishing Company. 1976.

[7] H. Svensson, Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients, Acta Chem. Scad. 16 (1962) 546-466.

[8] B.M. Simonet, A. Ríos, M. Valcarcél, Enhancing sensitivity in Capillary Electrophoresis, Trends. Anal. Chem. 22 (2003) 605-614.

[9] G. Hempel, Strategies to improve the sensitivity in Capillary Electrophoresis for the analysis of drugs in biological fluids,Electrophoresis 21 (2000) 691-698.

[10] C. Horstkötter, E. Jiménez-Lozano, D. Barrón, J. Barbosa, G. Blaschke,Determination of residues of enrofloxacin and its metabolite ciprofloxacin in chicken muscle by Capillary Electrophoresis using laser-induced fluorescence detection,Electrophoresis 23 (2002)3078-3083.

[11] J. Hernández-Borges, T.M. Borges-Miquel, M.A. Rodriguez-Delgado, A.Cifuentes, Sample treatments prior to Capillary Electrophoresis-mas spectrometry, J. Chromatogr. A1153 (2007)214-226.

[12] S. Almeda, L. Arce, M. Valcarcel,The more and less common approaches to enhancing sensitivity in Capillary Electrophoresis,Curr. Anal. Chem. 6 (2010) 126-143.

ELECTROPHORESIS

Electrophoresis 2011, 32, 1379–1393

THIRTY YEARS OF CAPILLARY ELECTROPHORESIS IN FOOD ANALYSIS LABORATORIES: POTENTIAL APPLICATIONS

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Abstract

CE has generated considerable interest in the research community since instruments were introduced by different trading companies in the 1990s. Nowadays, CE is popular due to its simplicity, speed, highly efficient separations and minimal solvent and reagent consumption; it can also be included as a useful technique in the nanotechnology field and it covers a wide range of specific applications in different fields (chemical, pharmaceutical, genetic, clinical, food and environmental). CE has been very well evaluated in research laboratories for several years, and different new approaches to improve sensitivity (one of the main drawbacks of CE) and robustness have been proposed. However, this technique is still not well accepted in routine laboratories for food analysis. Researching in data bases, it is easy to find several electrophoretic methods to determine different groups of analytes and sometimes they are compared in terms of sensitivity, selectivity, precision and applicability with other separation techniques. Although these papers frequently prove the potential of this methodology in spiked samples, it is not common to find a discussion of the well-known complexity of the matrices to extract analytes from the sample and/or to study the interferences in the target analytes. Summarizing, the majority of CE scientific papers focus primarily on the effects upon the separation of the analytes while ignoring their behavior if these analytes are presented in real samples.

Keywords:CE / Food analysis /Research laboratories /Routine laboratories /Routine methods.

1. Introduction

When CE was first introduced, it was seen as a revolutionary technique. Now, it is a well-established technique in analytical research laboratories worldwide. CE offers highly efficient separations, short analysis times, simplicity, precision, easy automation and low costs (for labor, solvent volumes, waste disposal, stationary phases, e.g. chiral separations) and possible nanoliter sample amounts when compared with other separation techniques. CE is robust and generates almost no waste disposal. The major strength of CE, however, is that the basic separation principles are different from those of HPLC and GC. Therefore, CE and HPLC used together make a powerful combination. CE offers the advantage that several separation modes can be run on a single instrument. This makes CE a very versatile techniquefor a broad range of applications and separation challenges. The scope of CE application in food analysis is, in general, identical to that of HPLC, and users must often choose between the two techniques; however, nowadays with the worldwide shortage of acetonitrile (ACN), the most commonly used in HPLC solvent, and a renewed focus on green separation technologies, the use of CE technique would be more appropriate, in some particular cases. CE should be considered first when dealing with highly polar, charged or chiral analytes and it is a technique with tremendous potential to solve different separation problems especially in life sciences. CE is extensively used in the comprehensive characterization of macromolecules used in biologic as well as in proteomic or metabolomic studies.

Despite the many excellent technical reviews found in the literature on CE related to food analysis [1-8], there is still a need for more specific critical evaluations on the determination of analytes present in different matrices. CE-real sample analysis or CE-routine analyses are still not well studied. With this situation, it will be very difficult to transfer the CE analytical methodologies to routine laboratories. CE is still regarded with suspicion by scientists and particularly by industrial companies. This apparently inexplicable situation could be justified by the following:

(i) The lack of sound electrophoretic experience of the majority of the workers from industrial companies, who traditionally use chromatographic and spectroscopic techniques. Indeed, GC and

HPLC apparently offer solutions to almost all the analytical problems.

(ii) The amount of scientific bibliography related to CE. About 39000 scientific articles can be retrieved from the database ‘‘ISI Web of Knowledge’’ using the keyword ‘‘Capillary Electrophoresis’’ up to May 2010. This number is significantly lower compared with the scientific articles related to liquid chromatography (more than 100000).

Since the introduction of commercial CE systems over 20 years ago, separation mechanisms have become more clearly understood. However, it is important to think in terms of the ‘‘CE world’’ when troubleshooting CE methods rather than using conventional ‘‘chromatography-mode thinking’’ [9]. In 1989, Beckman Instruments introduced the first fully automated CE instrument to the scientific community. At that time, CE demonstrated exceptional resolving of selected compounds, but the new technology lacked a track record of applications. The subsequent application of automated CE to real-world separation problems has propelled the advancement of this technology to the robust analyzers dedicated to some specific uses today. CE will be well established in routine laboratories as a scientific study in detail of how to extract analytes present in real samples before analysis by CE. Sample preparations are almost always carried out off-line in CE analysis. There are a number of interesting approaches [10–12] using different analytical strategies to extract analytes from complex samples but sometimes these procedures are time-consuming and they are not validated by using a repre- sentative number of samples (containing the analytes of interest). In 2006, some members of our research group presented an interesting paper to support the transfer of advances from CE research laboratories to routine laboratories. They focused their research on the integration of sample treatment devices into commercial CE equipment [13]. In 2005, Castañeda et al. [14] reviewed some analytical approaches to demonstrate the analytical usefulness of CE in routine food analysis.

In the work presented here, we have evaluated (i) the CE companies and current market status for CE technology, (ii) the classical use of CE in routine food analysis and (iii) a brief tendency of advanced CE applications in food analysis.

Author methodology for CE current status analysis and forecast projections made in this work encompassed different data sources (mainly our professional experience based on 15 years working with CE, ISI Web of Knowledge database and internet). Based on interaction with industry stakeholders and experts, we analyzed the potential of CE technology, product segments, end-users and different electrophoresis applications. The authors have interactedwith electrophoresis manufacturers specializing in different product segments to obtain data for this study. In addition, data were also compiled from governmental and other public research data sources.

2. CE companies and market status

While 10 years ago there were a number of manufacturers of CE instruments, these days the market is dominated by Agilent Technologies' CE System (www.agilent.com, 2009) and Beckman-Coulter's CE System (www.beckman.com, 2010). Both companies provide different kinds of detection units, including mainly diode arrays (DAD), fluorescence and mass spectrometry (MS).

Integration of CE onto a microchip is the first critical step to produce a fully integrated and automated analysis system. Microchip capillary array electrophoresis analyzers provide rapid high-throughput separation of samples and can increase workflow and reduce costs. The microchip CE format is also important because it facilitates electrophoretic analysis of submicroliter to nanoliter sample volumes. Agilent is pioneering the ''Lab-on-a-Chip'' system, which uses the same principles of CE but in a microchannel on a 22cm-sized chip. Tiny electrodes at the ends of the channels generate the electric force. The lab-on-a-chip is all based on CE theory. The driving force within a capillary and in a chip is an electric field that produces an electro-osmotic flow. The Agilent 2100 Bioanalyzer lab-on-a-chip system can be used for protein sizing and quantitation, RNA and DNA detection and quantitation, and apoptosis, among other applications.

Beckman has different types of equipments; as an example, we can mention the ''ProteomeLAb PA800.'' It is a system configured especially for particular uses, such as proteomic application, quality control, glycoproteins or DNA. Another category of CE systems is based on capillary gel electrophoresis (CGE).

This has become a dominant method for DNA sequencing and genotyping applications, particularly in high-throughput, automated systems, such as those used for the Human Genome Project. Beckman has a dedicated system for CGE – the ‘‘GenomeLab GeXP,’’ which supports an array of eight capillaries. Agilent and Beckman have the capabilities to perform CGE using a polyacrylamide gel matrix on their standard CE systems.

Besides the two companies (Agilent and Beckman) that manufacture CE equipment that can be used for very different applications (‘‘open equipment’’), there are other companies that offer CGE systems to resolve specific problems (‘‘closed equipment’’). The two big players in this end of the CE spectrum, however, are Amersham Biosciences (www.amershambiosciences.com, 2010) and Applied Biosystems (www.appliedbiosystems.com, 2010). For example, the latter company has been a pioneer in the field of genetic analysis by offering systems to address the expansion of genetic analysis applications and the evolving needs of today’s research environment. DNA sequencing by CE is a key technology in a number of experimental workflows inthe laboratory of life science. A deep description of this kind of equipment is out of the scope of this work. Two other manufacturers of CE instruments (hydrodynamic closed system) are Recman-laboratory systems (www.recman.cz, 2010) and Villa Labeco (www.villalabeco.sk, 2010).

We have studied the current status of the worldwide electrophoresis industry to assess its growth potential in the near future. We were particularly interested in understanding the reason why the current market scenario for CE technology are only research, clinical and pharmaceutical laboratories. In addition, we were interested in analyzing the electrophoresis food industry from end-users perspectives.

CE was initially regarded as an analytical separation tool for proteins and peptides. Its characteristics imply that biomacromolecules theoretically should derive the biggest profit from this technique. However, it has turned out that the applications have spread into many more areas than just the bioscience area. According to different sources, nowadays there are around 200 different pharmaceutical companies using CE in their routine laboratories. Although still mainly in use in R&D laboratories, the technique is definitely migrating toward controlled analytical laboratories such as QA/QC and products testing labs (e.g.

forensic labs, determination of drugs abuse, explosives analysis, to name a few). This indicates that the technique does offer unique benefits and can expect a sustained growth in the future [15]. But we have also confirmed that the potential of CE in food routine laboratories is still testimonial.

The state of the CE market today is very difficult to establish, as this type of information is not easily available to us. But we have tried to research this topic and we can highlight the following comments, which are only indicative for the scientific community. When the first commercial instruments became available in 1990, the market was estimated to be several million dollars in size. In 1994, the market size reached over 50 million dollars. According to ‘‘Market Research Reports and Technical Publications’’ (www.bccresearch.com, 2009), a new technical market research report titled ‘‘Electrophoresis technology: global markets,’’ the global market for CE technology was around $456 million in 2008. This is expected to increase to over $600 million by the end of 2013.

3. Applying CE technology to real-world applications

Although CE technology may be applied to many different types of research, it has gained its reputation from the study of molecules that have traditionally been difficult to separate by HPLC. CE excels in the determination of ions when rapid results are desired and has become the predominant technique for the determination of both basic and chiral pharmaceuticals. This technology is making its mark in biotechnology, replacing traditional electrophoresis for the characterization and determination of macromolecules such as proteins and carbohydrates, and it promises to be avaluable tool in the characterization challenges posed by proteome-wide analysis. CE technology has also served to accelerate the accumulation of genome-level knowledge by automating DNA sequencing and genotyping [www.beckman.com, 2010].

CE is used across a wide range of analytical chemistry and biochemistry applications, and it has been applied to different fields (see Fig. 1A). More than 1000 articles were found in the database “ISI Web of Knowledge” using the keywords “Capillary Electrophoresis” combined with “chemical”, “genetic”, “pharmaceutical”, “clinic”, “food” or “environmental,” up to May 2010. But for other applications, CE has been overshadowed by liquid chromatography. In food analysis in the early 1990s, CE was thought as a replacement for liquid

chromatography but it never took off. Among other reasons, we can justify this fact due to the following:

(i) The cost of a CE equipment compared with a similar separation technique such as HPLC. At this moment, the price of aCE equipment is 20–30% higher than the price of a HPLC.

(ii) The lack of understanding of the chemistry behind it discourages users.

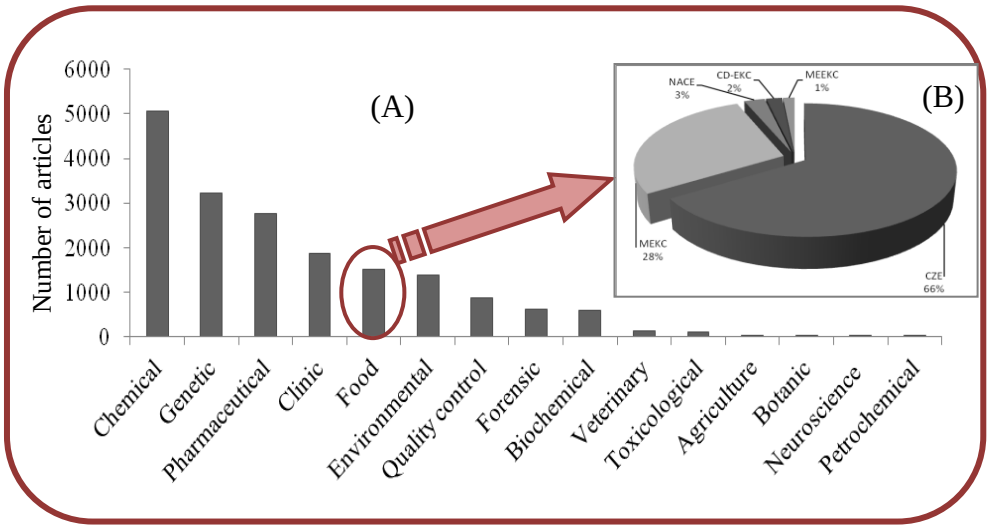

Fig. 1.*(A) Search papers concerning CE method categorized by field of application. (B) Percentage distribution of different CE mode in food analysis. According to the database "ISI Web of Knowledge" (up to May 2010).*

In food analysis, capillary zone electrophoresis (CZE) is the most commonly employed separation mode in CE (66%), as shown in Fig. 1B, due to its (i) versatility and ease of operation, (ii) separation of analytes based on the differences of their electrophoretic mobilities, which are related to their charge density at a given pH, and (iii) because the direction and velocity of analytes are determined by both electrophoretic mobility and electroosmotic flow (EOF).

Researchers groups that persevered with CE methods (instead of using HPLC method) reaped benefits, with cheap operating costs and reproducible

assays. Notice that CE can be used for a variety of different separations, from simple organic and inorganic anions and cations, to biological macromolecules including DNA, proteins and carbohydrates (see Fig. 2). The highest number of articles (over 13000) were found using the keywords "CE" and "ions" (including anions, cations, organic ions and/or inorganic ions), since they can be easily separated by this technique, even with equivalent results and in lesser time than those whose separation was obtained with ion chromatography (the technique traditionally used for this purpose). On the other hand, notice that ions (e.g. metal ions) can be used as additives in order to improve the selectivity of some CE methodology, so this figure is not necessarily representative of the number of scientific articles published to determine these analytes by CE.

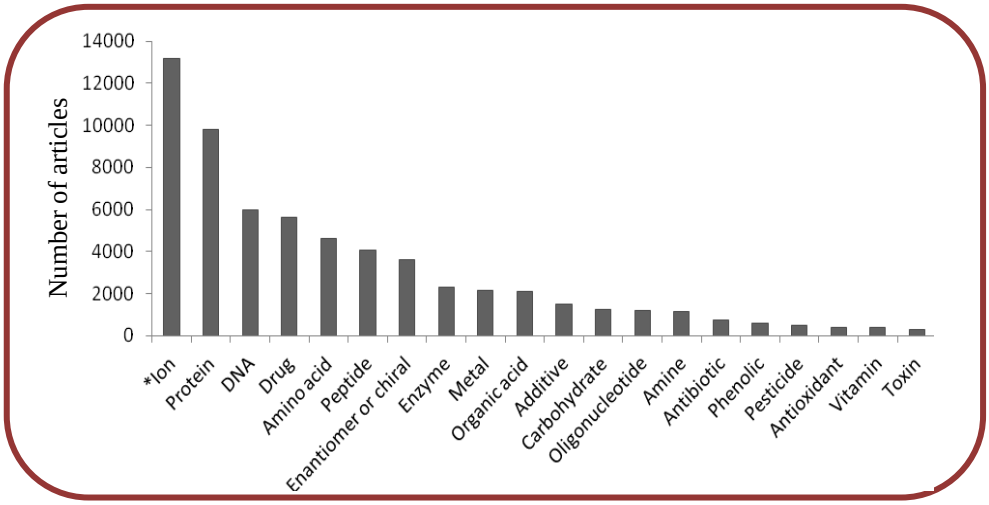

Fig. 2.*Search papers concerning CE method using the name of different groups of analytes. According to the database "ISI Web of Knowledge" (up to May 2010).* [*] *The terms ion, anion, cation, inorganic ion and organic ion were included.*

Figure 2 shows that besides ion determination, the main application of CE remains in the determination of proteins, DNA, drugs, aminoacids, peptides and enantiomers (more than 3500 scientific articles published in each case); however, there is a growing interest in placing the practice of CE in the determination of

other analytes such as enzymes, metals, organic acids, additives, carbohydrates, oligonucleotides, amines and antibiotics, where it can be observed that each day there is an increase in the number of published papers.

In our opinion, this amount of bibliography can be used to demonstrate the potential of CE in these areas, but these findings cannot be directly extrapolated to routine laboratories. In connection with this fact, one question may arise for the readers: should researchers go on and publish more and more articles detailing new electrophoretic methods to separate different groups of analytes? Our answer to this question is yes, but we should also not ignore the attempt to show where CE can be useful and publish methods with suitable analytical properties for routine tasks. If we do not make special efforts in this line of work in the near future, we will see that the CE is forgotten for the routine analysis. Another important aspect is the participation of manufacturers of CE equipments in the analytical process. Manufacturers should become more involved with researchers and those potential laboratories for routine analysis that provide training and promote the advantages of CE methods.

Authors have been in contact with different companies that sell CE equipment in different countries such as United Kingdom, Japan and Spain and we can confirm that CE is useful technique for the pharmaceutical industry and some analysis carried out in hospitals. In UK, CE is a very populartechnique in routine and quality control laboratories of the pharmaceutical industry. In addition, in Japan, the Japanese police use CE instruments for forensic analysis and Japanese brewing companies switched all their ion chromatography testing to CE methods. Finally, we can confirm that in Spain very few companies are using CE in other different sectors other than agro food, clinical or pharmaceutical routine laboratories. We have also found some CE equipment in public forensic and military laboratories.

As can be seen, the data shown in Figs. 1A and 2 are not comparable with the number of companies that are nowadays using CE as a routine technique (in particularly in food analysis). Readers may ask another question: is there any reason to justify this fact? From our particular point of view, we think that there is too much literature to demonstrate the high potential of this technique, but there are very few applications in which we could find a deep validation (using real samples) of the proposed CE methodologies.

Researchers are encouraged to demonstrate in which field and applications CE could be an alternative to other separation technique in the near future. At this moment, we agree that CE is a useful technique for the pharmaceutical industry and some analysis carried out in hospitals. But we have to keep studying the CE literature already published and we have to clarify which of these applications could be used in routine laboratories.

CE methodologies have been used for the determination of different analytes present in different types of food samples as shown in Fig. 3 (e.g. milk, kidney, fruit, wine, tea, fish, juice and chicken, among others). Although there are scientific articles showing the potential of electrophoretic methods using real samples [4, 7, 16–19], unfortunately, it is not always possible to find papers in which the authors have demonstrated the potential of the CE methodologies using samples in which the analytes are in native form and consequently the interaction matrix–analyte is unpredictable. In most cases, spiked samples are used to demonstrate this relative potential.

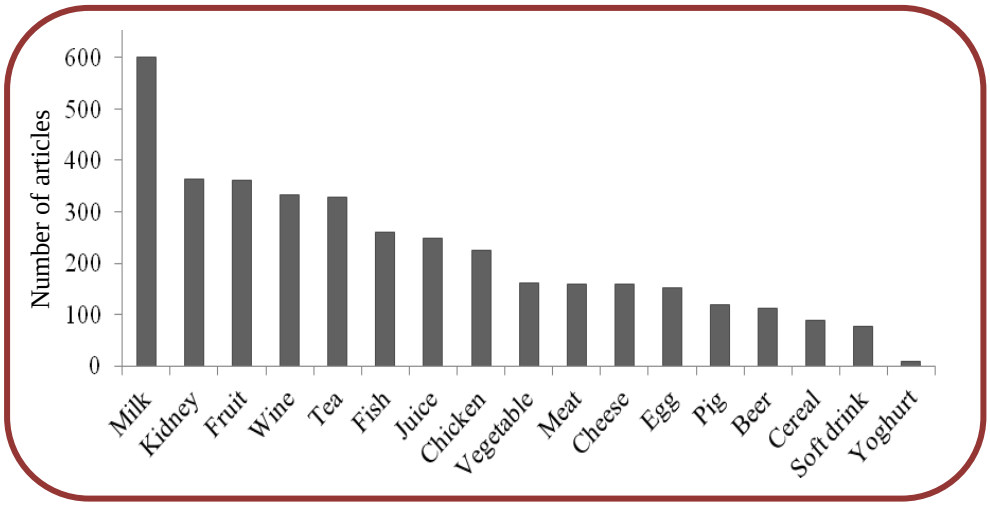

Fig. 3.*Search papers concerning CE methods using different type of food matrices.According to the database "ISI Web of Knowledge" (up to May 2010).*

4. Classical applications of CE in routine food analysis

CE has been applied to a wide range of important areas of food analysis and is rapidly being established as an alternative technique to chromatographic methods including HPLC and GC within routine analysis and research laboratories [20]. Although the low reproducibility, sensitivity and robustness are the main drawbacks of CE compared with GC or HPLC, this technique has expanded their use in the food analysis field enabling the determination of a wide variety of compounds with different intentions. Especially an increasing number of applications in food analysis have been observed by using CE-MS showing interesting prospects for its application to solve emerging analytical problems. In this regard, it is expected that new technological advances, as well as novel instrument configurations would make this technique more robust and useful for routine food analysis [17].

Several CE methods for food analysis have been developed in the last years and in some cases have been suggested for its possible potential application in routine analysis (see Tables 1–4); however, there is no guarantee that these methods can be directly applied in routine food analysis, since some relevant parameters should be revalidated or verified after method transfer [21]. The papers shown in these tables were found in the database ‘‘ISI Web of Knowledge’’ using the keywords ‘‘Capillary Electrophoresis or CE”, “food” and “routine analysis or routine laboratories or routine methods”. Additionally, a scientific paper of each category was selected as an example to be potentially used in routine food laboratories in the near future. Without intending to provide a detailed description of each one of the methodology selected, in these tables, there are comments upon some analytical features such as background electrolyte (BGE) composition, sample treatment, number of samples analyzed, LOD and recoveries values are included.

The information included in these tables has been classified according to its usefulness in four classical types of routine food analysis: (i) food quantitative analysis, (ii) process monitoring, (iii) food authenticity or adulteration and (iv) legal requirements.

4.1 Determination of different nutrients in food samples

CE is now used for the separation, identification and quantitative analysis of many compounds contained in food products, since the use of this technique in food control laboratories can essentially facilitate the verification of the declared and real composition of food products.

CE could be a standard technique in routine laboratories (in the near future) if analytical chemists made the effort of establishing fully validated and transferable analytical methods. Special attention must be given to robustness, especially for methods that will ultimately be transferred from a development/research laboratory to quality control or routine sites. Other factors required for using CE methods as routine are as follows: representative sampling, reduction of errors by careful sample handling, proper use of reference standards, use of qualified instrumentation as installation qualification (IQ), operational qualification (OQ), performance qualification (PQ) and a system suitability test (similar to that applied to HPLC methods).

Table 1 shows some of the methodologies published in the last 10 years [22-36] in the analysis of different food components by CE. Although all the methods shown in this table proved some properties that enhance its possibleapplications in routine analysis because of its exhibit speed, low laboriousness and low running cost, in general, the number of samples analyzed is low and some of the methods proposed do not indicate important analytical parameters as the limit of detection or recovery values.

As it can be seen in Table 1, one of the applications highlighted by the number of published scientific articles (more of 70 papers up to May 2010) is the determination of catechins in tea. In this case, Bonoli et al. [25]used micellar electrokinetic chromatography (MEKC) for the determination of catechins in green tea and the separation conditions were compared to that of HPLC and showed that CE offered several advantages in relation to the time of analysis and sensitivity. In this instance, seven tea catechins and gallic acid were detected in green tea extractswithin 4.5 min.A comparative study between HPLC and CE was also performed by Lee and Ong [26] which used CE for the determination of chiral catechins and theaflavins in green an

Table 1.CE applications for the determination of different nutrients in food samples.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Amino acid (L-theanine)	Tea	27	CE-Isotachophoresis (ITP)	0.01 Mhydrochloric acid (HCl), 0.02 mMtris(hydroxymethyl)aminomethano (TRIS), 0.05% 2-hydroxyethyl Cellulose (HEC), pH 8.1	Extraction in boiling water	7	0.7 mg/L	97-101	[22]
Amino acids (L-and D-carnitine)	Dietary food supplements (drinks, biscuits, tablets, and capsules)	5	Cyclodextrin electrokinetic chromatography (CD-EKC)	0.5 M Ammonium formate buffer, 0.2% (m/v) succ-γ-CD (4 succinyl groups/CD ring), pH 2.5	Homogenized and diluted (drinks). Homogenized and four consecutive water extractions (biscuits, tablets, and capsules)	22	10 ng/mL	85-102	[23]
Carbohydrates (fructose, glucose, galactose, lactose, and sucrose)	Powdered milk and yogurt	5	CZE	15 mM Sorbate, 0.3 mMcetyl trimethyl ammonium bromide (CTAB), 55 mM sodium hydroxide (NaOH)	Liquid-Liquid Extraction (LLE)	9	15-36 µg/mL	93-109	[24]
Carbohydrates (fructose, glucose, maltose, maltotriose, and sucrose)	Cereals flakes	8	CZE	15 mM Sorbate, 0.2 mM CTAB, 35 mM NaOH	LLE	6	16-31 µg/mL	89-106	
Catechins	Green tea	74	MEKC	3 Parts of 20 mM potassium dihydrogenphosphate (KH_2PO_4), 1 part of 50 mM sodium tetraborate ($Na_2B_4O_7$) and 2 parts of 200 mMsodium dodecyl sulphate (SDS), pH 7.0	Dilution	n.i.	1.2-5.1 x 10^{-3} µg/mL	n.i.	[25]
Catechins and theaflavins	Green and black teas	79	CD-EKC	200 mM Boric acid (pH 7.2), 10 mM KH_2PO_4 (pH 4.2), 4.5 m*M* of β-CD and 27.5% (v/v) of CAN	LLE	10	0.5 µg/mL	n.i.	[26]
Cholesterol	Egg yolk and milk	3	Non-aqueous capillary electrophoresis (NACE)	100 mM Sodium acetate-acetic acid in methanol (MetOH)	LLE and saponification	2	5 µg/mL	n.i.	[27]
Flavonoid aglycones	Propolis, *Ginkgo biloba*, red wine, orange peel, orange pulp	5	MEKC	25 mM SDS, 25 mM sodium cholate, pH 7.0	Different extraction procedures according to the matrix sample (LLE, acid hydrolysis)	5	1.2-4 µg/mL	85-100	[28]
Folic acid	Instant fried noodles	3	CZE	8 mM Phosphate, 12 mM borate, 5% MetOH, pH 9.5	Enzymatic extraction	n.i.	5.3 mg/L	96-103	[29]
Iodine and bromine	Tomato leaves, salt and seaweed samples	6	CZE	10 mM TRIS buffer adjusted by 0.1 M HCl to pH 8.0	Microwave-assisted extraction	3	20-50 ng/mL	94-105	[30]
Phenolic compounds	Lentils, black beans and almond peels	1	MEKC	50 mM acetic acid/ sodium acetate, 100 mM SDS, pH 5.0	Maceration, centrifugation and dilution	3	n.i.	n.i.	[31]
	Exotic fruits	41	CZE	50 mM Tetraborate buffer, 7.5% (v/v) MetOH, pH 9.2	LLE followed by alkaline hydrolysis	3	1.3 µg/mL	81-115	[32]
Proteins	Soybean–rice biscuits and breads	59	CZE	80 mM Borate buffer, 20% v/v ACN, pH 8.5	LLE	7	0.4 mg/mL	94-106	[33]
Riboflavin	Non-alcoholic beverages and green tea	2	CE-laser induced fluorescence (LIF)	20 mMDisodium hydrogenorthophosphate (Na_2HPO_4)-sodium phosphate monobasic (NaH_2PO_4), 10 mM borate, pH 9.0	Dilution, centrifugation and filtration	6	3.0 nM	92.4-109.4	[34]
	Beer	5	MEKC	100 mM$Na_2B_4O_7$, pH 8.2	Filtration. On-line sample concentration	12	480, 20 and 1 ng/mL	n.i.	[35]
Water-soluble vitamins (thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, pantothenic acid, nicotinamide and cobalamin)	Soft drinks	4	MEKC	50 mM Borate, 25 mM SDS, pH 8.5	Dilution and filtration	5	0.06-2.0 µg/mL	41-103	[36]

n.i.: not indicated; LOD: limit of detection; R: recovery

blackteas. The time of analysis in CE was three times faster; however, it is five times less sensitive than HPLC, which has limits of detection of 0.05 and 0.5 mg mL^{-1} for catechins and theaflavins, respectively.Similarly, other authors [37] also compared HPLC and CE and concluded that MEKC was more time efficient than HPLC.

On the other hand, a large number of papers compared the results obtained by CE with HPLC analysis on the same samples, in order to demonstrate the confidence, reliability and robustness of the CE methodology. This comparison serves to validate or to confirm the results obtained by CE and also shows that CE presents in some cases identical HPLC applications. In some of these cases, the use of CE technology could be even better than HPLC.

4.2 Process monitoring

The study of the modifications of compounds present in food that can happen during the manufacture, processing or storage is also of utmost importance in Food Science and Technology. In fact, unwanted reactions could easily occur due to interactions between food ingredients with themselves or with other components during the relatively aggressive processes that are commonly applied to foods (e.g. sterilization, packaging, storage and cooking). Several applications of CE to the monitoring of components throughout a food production process have been published [38–44]. The main features of such applications are summarized in Table 2.

In this area, for example, the determination of organic acids in beverage samples is important because they influence the organoleptic properties, monitoring the fermentation process as well as the stability and microbiological control of the products. A method developed by Mato et al. [43] has been applied to several beverage samples with only a simple dilution and filtration treatment of the sample. The proposed method is fast because the separation time decreases two, four or even six times the separation times ofthe earlier reported CE methods. It is also simple and cheap due to a low consumption of chemicals and samples. All of these

reasons allow for the method to be considered adequate for routine analysis of organic acids in beverage samples. At this point, it is important to remark that the number of real samples analyzed in this work is low (only 6) and that it would be desirable to demonstrate that the method also works well in a larger number of samples.

4.3 Food authenticity or adulteration

The authenticity of food is currently of great concern for researchers, consumers, industries and policymakers. The detection of food adulteration needs highly selective and sensitive analytical methods that are also simple and cost-effective. CE can meet these requirements by offering high-resolution separations at a minimal cost in terms of sample size, reagent consumption and operator time. Also, it is desirable that studies involving the demonstration of food authenticity or adulteration include a large number of samples to be analyzed to validate the methodology. In this context, only in two of the analytical methodologies that are shown in Table 3 [45–55], the number of samples analyzed is significantly high (over 50 samples) [47, 50].

A methodology that was tested for the analysis of 56 real samples was a fast and reliable CE method for the determination of (E)-10-hydroxy-2-decenoic acid (10-HDA) in royal jelly. This method was developed and compared with HPLC. The two methods were applied in the quantification of 10-HDA in pure royal jelly samples of different geographical origin. This study demonstrated that CE gives comparable performances to HPLC in terms of analytical results, efficiency, sensitivity and time of analysis without employing any EOF, pH or organic modifiers. A high instrumental repeatability, a lower solvent consumption and the use of aqueous solution as BGE make CZE an effective alternative to HPLC for accessing the quality of royal jelly and royal jelly-based preparations in routine analyses [47].

4.4 Legal requirements

Nowadays, foodstuffs is produced and distributed in a global market leading to stringent legislation and regulation for food quality and safety in order to protect consumers and ensure fair trade. Regulatory agencies such as the European Food Safety Authority (EFSA) and the Food and Drug

Table 2. Examples of CE applications in food process monitoring.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Acrylamide	French fries	2	Microemulsion electrokinetic chromatography (MEEKC)	0.8% m/v *n*-Amyl alcohol, 3.3% m/v SDS, 6.6% m/v 1-butanol, and 89.3% m/v, 40 mM phosphate buffer, pH 6.5	Filtration and solid phase extraction (SPE)	4	0.7 µg/mL	84	[38]
Anthocyanins	Wine and wine musts	28	CZE	200 mM Borate-ammonium, pH 9.0	SPE	4	4-10 mg/L	n.i.	[39]
Flavonoids	Beans and soybean	8	CZE	50 mM Ammonium acetate (CH_3COONH_4), 20% v/v MetOH, pH 10.5	LLE and acid hydrolysis	4	0.25-1.0 mg/L	n.i.	[40]
Furosine	Breakfast cereals	3	CZE	50 mM Phosphate, pH 7.0	SPE	8	0.2 mg/L	n.i.	[41]
Melamine	Milk	17	CZE	20 mM Phosphate, pH 9.0	Disposable microfluidic device (avoided the need of sample pretreatment)	n.i.	0.23 µg/mL	82	[42]
Organic acids	Wine and juice	24	CZE	7.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 2.5 mMtetradecyltrimethylammonium hydroxide (TTAOH), 0.24 mMcalcium chloride ($CaCl_2$), pH 6.4	Dilution and filtration	6	0.01 - 0.9 mg/L	94.7-103.4	[43]
Sulfite	Wine	6	CZE-ITP	15 mMSuccinate, 0,2% w/v methylhydroxyethylcellulose, pH 4,0	Column-coupling (CC) chip	8	60 µg/L	90	[44]

n.i.: not indicated; LOD: limit of detection; R: recovery

Table 3. Some analytical methodologies for food authentication.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Aspartame, saccharine, acesulfame, alitame, benzoic and sorbic acid	Soft drinks, cordials, tomato sauce, marmalade jam and table-top sweeteners	17	MEKC	0.05 M Sodium deoxycholate, 0.01 MKH_2PO_4, 0.01 $MNa_2B_4O_7$, pH 8.6	Dilution and filtration	13	n.i.	94-112	[45]
DNA	Basmati rice (*Oryza sativa*)	18	CE-based microsatellite multiplex assay	n.i.	Four procedures were tested: CTAB method, modified CTAB method, Nucleon phytopure DNA extraction kit (Amersham Biosciences) and Qiagen DNeasy plant mini kit	18	1%	n.i.	[46]
(E)-10-hydroxy-2-decenoic acid (10-HDA)	Royal jelly	1	CZE	50 mM Tetraborate, pH 9.4	Dilution, centrifugation and clean-up treatment	56	0.002 mg/mL	86.7-94.3	[47]
Inorganic metal cations	Orange juice	15	CZE	5 mM UVCat-I, 8 mM α-hydroxyisobutyric acid, pH 4.4	Dilution without filtration	n.i.	5 ppm	n.i.	[48]
Organic acids (citric, isocitric, malic and tartaric acids)	Orange juice	19	CZE	200 mM Phosphate, pH 7.5	Dilution and filtration	10	2-9 mg/L	97-104	[49]
Organic acids (succinic, malic, tartaric, citric, acetic and lactic acids)	White wine	64	CZE	180 mNa_2HPO_4, 0.5mM CTAB and 10% MetOH, pH 7.5.	Filtration	56	n.i.	n.i.	[50]
Proteins	Cow, goat and ewe cheeses	26	CZE	50 mM Iminodiacetic acid (pH 2.30), 0.5% HEC, 0.1% or 10% Tween 20 and 6 M urea, pH 3.1	Centrifugation and dilution	9	1.1-2.1%	n.i	[51]
	Ovine and caprine milk	39	CZE	1 M Formic acid (HCOOH), pH 1.9	LLE	9	5%	n.i.	[52]
	Smoked paprika	2	CZE	8.75 mM Phosphate, 20.6 mM tetraborate, pH 9.0	n.i.	15	5-10% (w/w)	n.i.	[53]
	Soybeans	34	CZE	80 mM Borate, 20% v/v ACN, pH 8.5	LLE	19	n.i.	n.i.	[54]
Vanilla flavours	Real vanilla	2	CE microchips with electrochemical detection	20 mM Borate, pH 9.5	Maceration or dilution and filtration	5	0.09-0.31 mM	90	[55]

n.i.: not indicated; LOD: limit of detection; R: reco

Administration (FDA) require the availability of analysis methods that have a comprehensive contaminant scope in order to provide the data for risk assessment, the establishment of maximum residue limits (MRLs) as well as the development and execution of monitoring plans. In this regard, different CE methods for international regulations on food contaminants and residues have been published. The main features of some applications in this field are summarized in Table 4. In general, although the methods listed in this table [56–82] include simple sample treatment and low LOD, CE has not been able to replace any official methods using HPLC and GC as separation techniques.

CE has been applied to thedetermination of pesticides in water samples in several occasions; however, it is not frequent to find a CE method that can provide the simultaneous analysis of a large number of pesticides at the required maximum residue limit values. Recently, Ravelo- Perez et al. [72] proposed the development of a new analytical strategy that combines MEKC-UV analysis with SPE as an off-line preconcentration technique, and reversed-electrode polarity stacking mode (REPSM) as on-line stacking procedure for the simultaneous separation and ultrasensitive determination of 12 pesticides in mineral, stagnant and tap waters. However, GC and HPLC continue being the suggested techniques by official international organizations. Similarly, the determination of biogenic amines in different foodstuffs has been reported by CE. In this study, it was found that some of the methods that were suggested for routine application were used to analyze a low number of samples (e.g. n=12) [55–58]. Nowadays, HPLC is the most popular technique to determine biogenic amines in different food samples.

5. CE advanced applications in food analysis

CE methods have already been shown to provide important contributions for different omics approaches, namely (i) new research on food functions via nutrigenomics or nutrigenetics approaches, (ii) development of new transgenic food using molecular tools and (iii) the metabolomic study of food toward compounds profiling, among other applications.

Table 4.CE applications in legal requirements.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Biogenic amines	Beer	12	CZE	50 mM $Na_2B_4O_7$, 20% acetone, pH 9.3	Derivatization	12	5-198.3 µg/L	n.i.	[56]
	Red and white wine	13	CZE	1 MHCOOH, pH 2.0	Dilution and filtration	7	10 ng/mL	n.i.	[57]
	Salami, cheese, wine and beer	56	CZE	15 mM Histidine, 5 mM adipic acid, 0.1 mMethylenediaminetetraacetic acid (EDTA), 1.5 mM sulphuric acid, 50% MetOH, 0.1% HEC, pH 5.8	Dilution (liquid samples). Extraction, filtration and dilution (solid samples)	10	2-5 µmol/L	86-107	[58]
	Fish	39	MEKC	30 mM Boric acid, 20% v/v ACN, 25 mM SDS, pH 9.3	Derivatization	3	0.25-2.5 nmol/L	92-101	[59]
β-lactam	Milk	13	CZE	175 mM TRIS, 20% ethanol, pH 8.0	LLE, SPE and on-line preconcentration (LVSS)	9	2-10 µg/L	86-93	[60]
DNA	Maize	53	CGE-LIF	20 mM TRIS, 10 mM phosphoric acid (H_3PO_4), 2mM EDTA and 4.5% HEC, pH 7.3	CTAB method	6	1%	n.i.	[61]
			CGE-LIF/ CGE-UV	20 mM TRIS, 10 mM orthophosphoric acid, 2 mM EDTA and 4% HEC, pH 7.3	Modified CTAB method	2	1%	n.i.	[62]
Endotoxin from *Bacillus thuringensis*	Maize	1	CEIA-LIF	0.02 M Tricine, pH 8.0	Extraction buffer (Tris-borate buffer, pH 7.5), agitation and centrifugation	n.i.	0.5 nM (33 µg/L)	62-96	[63]
Fungicides	Fruit juices	3	MEKC	10 mM Phosphate, 30 mM SDS, 6.5% v/v 2-propanol, 0.7% v/v isobutyl alcohol, pH 7.8	Homogenization, evaporation and dilution	3	0.7-10.4 µg/L	82-103	[64]

n.i.: not indicated;LOD: limit of detection; R: Recovery

Table 4. Continued.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Herbicides	Potato, carrot, lettuce, zucchini, runnerbeans, oranges and wheat	6	NACE	0.0075 MPerchloric acid ($HClO_4$), 0.04 M SDS in 30:70 v/v ACN/MetOH	Pressurized liquid extraction and SPE	6	10–15 µg/kg	93-116	[65]
Melamine	Milk powder	10	CZE	500 mMHCOOH in 50% ACN	Extraction with dichloromethane and ACN	n.i.	0.06–0.5 mg/kg	96-100	[66]
	Grain, animal tissue, dairy products and eggs	4	CZE	30 mM $Na_2B_4O_7$, pH 9.3	Extraction with 1% trichloroaceti acid while 0.03 g sodium deoxycholate and SPE	7	0.25-0.5 mg/Kg	94-102	[67]
Metabolic profiles of genetically modified organisms (GMOs)	Maize	2	CE-TOF-MS	5% HCOOH, pH 1.9	Extraction with MetOH:water (50:50) in ultrasonic bath and centrifugation	6	n.i.	n.i.	[68]
Neurotoxin C and bacterial 16S sequences in DNA from *Clostridium botulinum*	*Clostridium botulinum* strain	7	CGE-LIF	20 mM TRIS, 9.5 mM ortophosphoric acid, 2 mM EDTA, and 4.5% HEC, pH 7.3.	DNA fragments from PCR amplication reactions were obtained	6	7 x 10^{-5}µg/mL	n.i.	[69]
Parabens, sorbic acid, benzoic acid, and dehydroacetic acid	Soft drinks, soy sauces and wines	29	MEEKC	0.1 M NaOH, 7.5 mM$Na_2B_4O_7$, pH 9.5	SPE	9	n.i.	82.3-115.3	[70]
Penicillins	Chicken	3	CZE	60 mM CH_3COONH_4, pH 6.0	LLE and SPE	11	8-12 µg/Kg	n.i.	[71]
Pesticides	Water	283	MEKC	100 mMNa2B4O7, 30 mM SDS, 6% v/v 1-propanol, pH 8.5	SPME and REPSM	3	64 ng/L	21-112	[72]

n.i.: not indicated;LOD: limit of detection; R: Recovery

Table 4. Continued.

Type of Analytes	Matrix	Number of papers found in the literature (analyte + matrix + CE)	Method used as an example in this category						
			CE mode	BGE composite	Sample treatment	Number of samples analyzed	LOD	%R	Ref.
Primary aromatic amines and melamine	Milk powders and pet feeds	11	CZE	80 mM H_3PO_4-TRIS, pH 2.65	LLE with ACN	13	0.4- 0.6 µg/L	92-107.1	[73]
Quinolones	Bovine raw milk	3	CZE	70 mMCH_3COONH_4, pH 9.1	SPE without protein precipitation	n.i.	6 µg/L	81-110	[74]
Sodium tripolyphosphate	Raw pork meat and meat products	1	CZE	10 mM Succinic acid, 15 mM β-alanine, 0.1% HEC, pH 8.0	On-line pre-concentration	5	0.80 mg P_2O_5/dm^3	97.4-98.3	[75]
Sorbate and benzoate	Soft drinks and tea	9	CZE	25 mM TRIS, 12.5 mM 2-hydroxyisobutyric acid, pH 8.1	Dilution	13	0.3-0.9 mg/L	97.9-105	[76]
Sulfonamides	Chicken, beef tissue and liver	8	CZE	30-60 mM Phosphate buffer, pH 5.5-8.5	SPE	n.i.	3.7-6.0 µg/Kg	83.3-94.5	[77]
	Chicken and pig edible tissues	9	CZE	40 mM $Na_2B_4O_7$, 25 mM KH_2PO_4, pH 6.2	Sample clean-up and pre-concentration	14	4.4 x 10^{-9}- 1.7 x 10^{-7} g/mL	81-92	[78]
	Meat	17	CZE	35 mM Phosphate, pH 6.5	LLE and SPE	12	5–10 lg/kg	81-97	[79]
	Milk	21	CZE	50 mMCH_3COONH_4, pH 8.5	Precipitate proteins (MetOH) and SPE	3	0.6-1.0 ng/mL	89-96	[80]
	Pork meat	3	CZE	50 mMCH_3COONH_4, pH 4.16	Homogenized with diatomaceous and PLE. Extraction with hot water and SPE	50	1.56 – 12.5 ng/g	76-98	[81]
Zein protein fractions	Maize	12	CE-MS	ACN/isopropanol/ HCOOH /water (40:20:2:38 v/v)	Extraction with ACN/2-mercaptoethanol/ water buffer (60:5:35 v/v), agitation, centrifugation and precipitation	6	n.i.	n.i.	[82]

n.i.: not indicated;LOD: limit of detection; R: Recovery

CGE method with laser-induced fluorescence (CGE-LIF), CGE-UV, CE-based immunoassays with LIF (CEIA-LIF), CE-MS, microchips-CE and chiral-CE have all been used in advanced applications. In this context, the huge potential of CGE-LIF and/or CGE-UV for DNA separation has been reported [61, 62]. CGE-LIF has also been used for the detection of toxin C producing *Clostridium botulinum* strains [69]. CEIA-LIF is among the more interesting applications of affinity CE and has recently been described for the determination of the Cry1Ab endotoxin from *Bacillus thuringensis* [63]. CE-MS has been applied for metabolomics studies of transgenic maize [68] and the complex zein protein fractions from maize [82]. Microchips-CE has been used for the real detection and quantitative determination of target flavors in selected vanilla samples [55]. The use of microchips-CE and their new instrumental developments are also expected to find important applications in the food analysis domain in the near future. Chemical reactions can be conducted by moving picoliters of fluid from different reservoirs opening a great possibility for replication and analysis of DNA [83]. In addition, the different chiral-CE methods used to study and characterize foods and food compounds have been recently reviewed [16].

In the near future, it is expected that nanotechnology will provide revolutionary improvements in terms of sensitivity and selectivity in capillary electromigration techniques that could be implemented in food analysis. However, although thefuture ofCEin advanced applicationsispromising, it isclearthatthe economic power of food industry laboratories are notcomparablewith otherlaboratories of the pharmaceutical industryorclinical ones, so that the incorporation of analysis that are less common and more expensive accounts for additional difficulties.

6. Concluding remarks

Several publications exist related to the determination of different analytes, which could potentially be present in food or agriculture samples. Some of these attractive methods should be assessed better by studying interaction analytes (native form) with matrix. The success of any electrophoretic methodology depends on the previous sample treatment carried out before CE separation. These treatments should be compatible with the real samples that are analyzed and the final medium (in which the analytes are dissolved) should be compatible with the

capillary. Strong acid, basic medium or even organic solvents are sometimesincompatible with the buffer separation. As far as we know, there are no papers in scientific literature focusing on full validation (including inter-lab studies), which are highly desirable in order to fully demonstrate the possibilities of the use of this technique in routine food analysis.

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7. References

[1] Frazier, R., Papadopoulou, A., *Electrophoresis* 2003, *24*, 4095-4105.

[2] Simó, C., Barbas, C., Cifuentes, A., *Electrophoresis* 2005, *26*, 1306-1318.

[3] Cifuentes, A., *Electrophoresis* 2006, *27*, 283-303.

[4] García-Cañas, V., Cifuentes, A., *Electrophoresis* 2007, *28*, 4013-4030.

[5] Font, G., Ruiz, M. J., Fernández, M., Picó, Y., *Electrophoresis* 2008, *29*, 2059-2078.

[6] García-Cañas, V., Cifuentes, A., *Electrophoresis* 2008, *29*, 294-309.

[7] Ravelo-Pérez, L. M., Asensio-Ramos, M., Hernández-Borges, J., Rodríguez-Delgado, M. A., *Electrophoresis* 2009, *30*, 1624-1646.

[8] Rodríguez-Robledo, V., Smyth, W., *Electrophoresis* 2009, *30*, 1647-1660.

[9] Shmitt-Kopplin, Ph., Menzinger, F., Freitag, D., *LC GC Europe* 2001.

[10] Almeda, S., Arce, L., Valcárcel, M., *Current Analytical Chemistry* 2010, *6*, 126-143.

[11] Arce, L., Nozal, L., Simonet, B., Ríos, A., Valcárcel, M., *Trends in Analytical Chemistry* 2009, *28*, 842-853.

[12] Valcárcel, M., Ríos, A., Arce, L., *J. Chromatogr. A* 2001, *924*, 3-30.

[13] Santos, B., Simonet, B. M., Ríos, A., Valcárcel, M., *Trends Anal. Chem.* 2006, *25*, 968-976.

[14] Castañeda, G., Rodriguez-Flores, J., Ríos, A., *J. Sep. Sci.* 2005, *28*, 915-924.

[15] Strohmeier, F., *Hewlett-Packard Journal* 1995.

[16] Herrero, M., Simó, C., García-Cañas, V., Fanali, S., Cifuentes, A., *Electrophoresis* 2010, *31*, 2106-2114.

[17] Herrero, M., García-Cañas, V., Simó, C., Cifuentes, A., *Electrophoresis* 2010, *31*, 205-228.

[18] Asensio-Ramos, M., Hernández-Borges, J., Rocco, A., Fanali, S., *J. Sep. Sci.* 2009, *32*, 3764-3800.

[19] Herrero, M., Ibáñez, E., Cifuentes, A., *J. Sep. Sci.* 2005, *28*, 883-897.

[20] Cheung, R. H. F., Marriott, P. J., Small, D. M., *Electrophoresis* 2007, *28*, 3390-3413.

[21] Watzig, H., Degenhardt, M., Kunkel, A., *Electrophoresis* 1998, *19*, 2695-2752.

[22] Kvasnicka, F., Kratka, J., *Central Eur. J. Chem.* 2006, *4*, 216-222.

[23] Sánchez-Hernández, L., Castro-Puyana, M., García-Ruiz, C., Crego, A. L., Marina, M. L., *Food Chem.* 2010, *120*, 921-928.

[24] Jager, A. V., Tonin, F. G., Tavares, M. F. M., *J. Sep. Sci.* 2007, *30*, 586-594.

[25] Bonoli, M., Pelillo, M., Toschi, T. G., Lercker, G., *Food Chem.* 2003, *81*, 631-638.

[26] Lee, B. L., Ong, C. N., *J. Chromatogr. A* 2000, *881*, 439-447.

[27] Xu, X. H., Li, R. K., Chen, J., Chen, P., Ling, X. Y., Rao, P. F., *J. Chromatogr. B* 2002, *768*, 369-373.

[28] Herrero-Martínez, J. M., Oumada, F. Z., Roses, M., Bosch, E., Rafols, C., *J. Sep. Sci.* 2007, *30*, 2493-2500.

[29] Su, A. K., Chang, Y. S., Lin, C. H., *Talanta* 2004, *64*, 970-974.

[30] Chen, J. H., Wang, K. E., Jiang, S. J. *Electrophoresis* 2007, 28, 4227-4232.

[31] Cifuentes, A., Bartolomé, B., Gómez-Cordovés, C., *Electrophoresis* 2001, *22*, 1561-1567.

[32] Fukuji, T. S., Tonin, F. G., Tavares, M. F. M., *J. Pharm. Biomed. Anal.* 2010, *51*, 430-438.

[33] García-Ruiz, C., García, M. A., García, M. C., Marina, M. L., *Electrophoresis* 2006, *27*, 452-460.

[34] Hu, L., Yang, X., Wang, C., Yuan, H., Xiao, D., *J. Chromatogr. B* 2007, *856*, 245-251.

[35] Lino, C. M., Silva, L. J. G., Pena, A. S., *Química Nova* 2006, *29*, 293-299.

[36] Schreiner, M., Razzazi, E., Luf, W., *Nahrung-Food* 2003, *47*, 243-247.

[37] Barroso, M. B., Werken, G. V. D., *J. High Resolut. Chromatogr* 1999, *22*, 225-230.

[38] Bermudo, E., Ruiz-Calero, V., Puignou, L., Galceran, M., *Electrophoresis* 2004, *25*, 3257-3262.

[39] Bednar, P., Papouskova, B., Muller, L., Bartak, P., Stávek, J., Pavlousek, P., Lemr, K., *J. Sep. Sci.* 2005, *28*, 1291-1299.

[40] Dinelli, G., Aloisio, I., Bonetti, A., Marotti, L., Cifuentes, A., *J. Sep. Sci.* 2007, *30*, 604-611.

[41] Delgado-Andrade, C., Rufian-Henares, J. A., Morales, F. J., *Eur. Food Res. Technol.* 2005, *221*, 707-711.

[42] Zhai, C., Qiang, W., Sheng, J., Lei, J. P., Ju, H. X., *J. Chromatogr. A* 2010, *1217*, 785-789.

[43] Mato, I., Huidobro, J. F., Simal-Lozano, J., Sancho, M. T., *Anal. Chim. Acta* 2006, *565*, 190-197.

[44] Masar, M., Dankova, M., Olvecka, E., Stachurova, A., Kaniansky, D., Stanislawski, B., *J. Chromatogr. A* 2004, *1026*, 31-39.

[45] Thompson, C. O., Trenerry, V. C., Kemmery, B., *J. Chromatogr. A* 1995, *694*, 507-514.

[46] Archak, S., Lakshminarayanareddy, V., Nagaraju, J., *Electrophoresis* 2007, *28*, 2396-2405.

[47] Ferioli, F., Marcazzan, G. L., Carboni, M. F., *J. Sep. Sci.* 2007, *30*, 1061-1069.

[48] Weston, A., Brown, P. R., Heckenberg, A. L., Jandik, P., Jones, W. R., *J. Chromatogr.* 1992, *602*, 249-256.

[49] Saavedra, L., García, A., Barbas, C., *J. Chromatogr. A* 2000, *881*, 395-401.

[50] Garrido-Delgado, R., Lopez-Vidal, S., Arce, L., Valcarcel, M., *J. Sep. Sci.* 2009, *32*, 3809-3816.

[51] Herrero-Martínez, J. M., Simo-Alfonso, E. F., Ramis-Ramos, G., Gelfi, C., Righetti, P. G., *J. Chromatogr. A* 2000, *878*, 261-271.

[52] Muller, L., Bartak, P., Bednar, P., Frysova, I., Sevcik, J., Lemr, K., *Electrophoresis* 2008, *29*, 2088-2093.

[53] Hernández, A., Martín, A., Aranda, E., Bartolome, T., Cordoba, M. D., *J. Agric. Food Chem.* 2006, *54*, 4141-4147.

[54] García-Ruiz, C., García, M. C., Cifuentes, A., Marina, M. L., *Electrophoresis* 2007, *28*, 2314-2323.

[55] Avila, M., González, M. C., Zougagh, M., Escarpa, A., Ríos, A., *Electrophoresis* 2007, *28*, 4233-4239.

[56] Cortacero-Ramirez, S., Arraez-Roman, D., Segura-Carretero, A., Fernández-Gutierrez, A., *Food Chem.* 2007, *100*, 383-389.

[57] Simó, C., Moreno-Arribas, M. V., Cifuentes, A., *J. Chromatogr. A* 2008, *1195*, 150-156.

[58] Kvasnicka, F., Voldrich, M., *J. Chromatogr. A* 2006, *1103*, 145-149.

[59] Cao, L., Wang, H., Ma, M., Zhang, H., *Electrophoresis* 2006, *27*, 827-836.

[60] Bailón-Pérez, M. I., García-Campaña, A. M., Cruces-Blanco, C., Del Olmo Iruela, M., *Electrophoresis* 2007, *28*, 4082-4090.

[61] García-Cañas, V., Mondello, M., Cifuentes, A., *Electrophoresis* 2010, *31*, 2249-2259.

[62] García-Cañas, V., González, R., Cifuentes, A., *J. Sep. Sci.* 2002, *25*, 577-583.

[63] Giovannoli, C., Anfossi, L., Baggiani, C., Giraudi, G., *Anal Bioanal Chem* 2008, *392*, 385-393.

[64] Molina, M., Silva, M., *Electrophoresis* 2000, *21*, 3625-3633.

[65] Carabias-Martínez, R., Rodríguez-Gonzalo, E., Miranda-Cruz, E., Dominguez-Alvarez, J., Hernández-Méndez, J., *Electrophoresis* 2007, *28*, 3606-3616.

[66] Klampfl, C. W., Andersen, L., Haunschmidt, M., Himmelsbach, M., Buchberger, W., *Electrophoresis* 2009, *30*, 1743-1746.

[67] Meng, L., Shen, G. J., Hou, X. L., Wang, L.L., *Chromatographia.* 2009, *70*, 991-994.

[68] León, C., Rodríguez-Meizoso, I., Lucio, M., García-Cañas, V., Ibáñez, E., Schmitt-Kopplin, P., Cifuentes, A., *J. Chromatogr. A*, 2009, *1216*, 7314-7323.

[69] Sánchez-Hernández, L., Cifuentes, A., Jiménez, B., Mateo, R., González, R., *Eur. Food. Res. Technol.* 2008, *227*, 495-502.

[70] Huang, H. Y., Chuang, C. L., Chiu, C. W., Ye, J. M., *Food Chem.* 2005, *89*, 315-322.

[71] Bailón-Pérez, M. I., García-Campaña, A. M., Del Olmo Iruela, M., Cruces-Blanco, C., García, L. G., *Electrophoresis* 2009, *30*, 1708-1717.

[72] Ravelo-Pérez, L. M., Hernández-Borges, J., Cifuentes, A., Rodríguez-Delgado, M. A., *Electrophoresis* 2007, *28*, 1805-1814.

[73] Wang, X., Chen, Y., *J. Chromatogr. A* 2009, *1216*, 7324-7328.

[74] Lara, F. J., García-Campaña, A. M., Ales-Barrero, F., Bosque-Sendra, J. M., García-Ayuso, L. E., *Anal. Chem.* 2006, *78*, 7665-7673.

[75] Jastrzebska, A., *Talanta* 2006, *69*, 1018-1024.

[76] Costa, A. C. O., Perfeito, L. D., Tavares, M. F. M., Micke, G. A., *J. Chromatogr. A* 2008, *1204*, 123-127.

[77] Farooq, M. U., Su, P., Yang, Y., *Chromatographia.* 2009, *69*, 1107-1111.

[78] Chu, Q. C., Zhang, D. L., Wang, J. Y., Ye, J., *J. Sci. Food Agric.* 2009, *89*, 2498-2504.

[79] Fuh, M. R. S., Chu, S. Y., *Anal. Chim. Acta* 2003, *499*, 215-221.

[80] Santos, B., Lista, A., Simonet, B. M., Ríos, A., Valcárcel, M., *Electrophoresis* 2005, *26*, 1567-1575.

[81] Font, G., Juan-García, A., Picó, Y., *J. Chromatogr. A* 2007, *1159*, 233-241.

[82] Erny, G.L., Marina, M.L., Cifuentes, A., *Electrophoresis*2007, *28*, 4192-4201.

[83] Khandurina, J., McKnight, T. E., Jacobson, S. C., Waters, L. C., Foote, R. S., Ramsey, J. M., *Anal Chem*. 2000, *72*, 2995–3000.

CAPÍTULO II

Herramientas analíticas

En este Capítulo se resumen y describen brevemente las herramientas analíticas usadas en los diferentes trabajos experimentales incluidos en esta Memoria, entre las que se encuentran: estándares, reactivos, muestras, instrumentación, aparatos y materiales.

ESTÁNDARES Y REACTIVOS

A continuación se mencionan los estándares y reactivos usados durante los diferentes trabajos experimentales.Todos los estándares usados fueron de alta pureza analítica (>99%) y estuvieron almacenados bajo las condiciones especificadas por cada proveedor.

Familia	Analito(s)	Casa comercial
Penicilinas	Amoxicilina, ampicilina, cloxacilina, oxacilina y penicilina G	Sigma-Aldrich
Antiinflamatorio no esteroideo	Naproxeno	Sigma-Aldrich
Fluroquinolonas	Ciprofloxacina, enrofloxacina y lomefloxacina	Sigma-Aldrich y Lab Zhejang Phar-maceutical

Los reactivos empleados para el desarrollo de esta tesis doctoral se resumen a continuación:

Tipo de reactivo	Nombre del reactivo	Casa comercial
Disolvente orgánico	Metanol	Panreac y J.T. Baker
	Acetonitrilo	Burdick & Jackson y Merck
	Etanol	Merck
Ácidos	Ácido fosfórico	Merck
	Ácido clorhidrico	Merck
	Ácido acético	Sigma y Fluka Riedel-de-Haën
	Ácido trifluoroacético	J.T. Baker
Bases	Hidróxido de sodio	Panreac
Sales	Tetraborato de sodio	Merck
	Fosfato diácido de sodio	Merck
	Sulfato de sodio	Merck
Tensioactivos	Dodecil sulfato de sodio	Sigma

Otros reactivos:

- El agua usada para la preparación de las distintas disoluciones fue agua ultrapura, obtenida mediante un sistema de purificación Milli-Q (Millipore, Bedford, MA, USA).

MUESTRAS

Para la realización de los trabajos experimentales presentados en esta Memoria se han analizado diferentes tipos de muestras de leche de origen animal, las cuales se resumen a continuación.

Tipo de muestra	Sub-tipo	Condición del animal	Origen	Conservación
Leche cruda	Bovina	- Sano	Granja de Córdoba	Recipientes estériles de plástico a -18ºC
	Caprina	- Sano - Tratamiento con enrofloxacina	Centro experimental de Producción animal de la Universidad del Zulia	Recipientes estériles de plástico a -4ºC

INSTRUMENTACIÓN

La parte experimental de esta Tesis Doctoral se realizó usando principalmente un equipo de CE, aunque también se usó otra técnica analíticacomo la cromatografía líquida de alta resolución (HPLC).

1. Electroforesis capilar

Se utilizó un equipo P/ACE MDQ (Beckman), dotado con un detector ultravioleta visible de diodos en fila (DAD). Dicho equipo está conectado a un microprocesador para su control y la adquisición de datos. El procesamiento de datos se llevó a cabo mediante el software 32Karat (Beckman).En la Figura 1 se muestra una fotografía del equipo CE-DAD usado.

Fig. 1. *Equipo comercial de CE P/ACE MDQ*

Los componentes principales del equipo empleado son: lámpara de deuterio, capilar de sílice fundida de 75 µm de diámetro y 60.2 cm de longitud total, electrodos de platino, viales y un automuestreador. En la Figura 2 se muestra el cartucho que permite insertar el capilar en el equipo.

Fig.2*. Cartucho que permite la inserción del capilar al equipo CE comercial.*

2. Cromatógrafo líquido de alta resolución

En esta Tesis Doctoral también se empleó un cromatógrafo líquido serie 1200 (Agilent Technologies, Palo Alto, CA, USA), equipado con una bomba cuaternaria (G1311A) empleada para el bombeo de la fase móvil, un desgasificador en línea (G1323A), un automuestreador (G1313A) programable y un detector de fluorescencia (G1321A). El software empleado para el control del instrumento, adquisición y análisis de datos fue ChemStation de la misma casa comercial.En la Figura 3 se presenta la imagen del equipo HPLC usado.

Fig. 3. *Equipo comercial de HPLC*

Para la separación cromatográfica de los analitos se empleó una columna Chromolith RP-18e (100 mm x 4.6 mm), conectada a una pre-columna RP-18, ambas suministradas por Merck (Barcelona, España).

APARATOS Y MATERIALES

En este apartado se enumeran los aparatos y materiales más empleados en el desarrollo de esta Tesis Doctoral:

- Balanza de precisión Explorer OHAUS
- Balanza analítica Scaltec, modelo SBA33
- Vortex Heidolph REAX top
- Vortex Fisher Scientific
- Centrífuga J. P. Selecta, modelo Centronic- BL II
- Centrífuga Hettich, modelo 32R
- pH metro Crison, modelo pH 2000
- Placa calefactora para viales (ECO 16 Thermorreactor, Velp Scientifica, Usmate, Italia)
- Sistema de obtención de agua ultrapura Milli-Q (Millipore, Bedford, MA)
- Micropipetas (LABMATE, PZ HTL, Warsaw, Polonia)
- Material de vidrio de laboratorio clase A
- Cartuchos HLB (60 mg, 3 cm^3; Waters, Milford, MA, USA)
- Cartuchos HLB (500 mg, 12 cm^3; Waters, Milford, MA, USA)
- Cartuchos Bond Elut C18 (500 mg; Varian, Harbor City, CA, USA)
- Cartuchos Strata X-Phenomenex (Torrance, CA, USA)
- Cartuchos Extract-Clean con octadecilo (C18), etilo (C2), ciclohexilo (CH) and fenilo (PH) (100 mg, 1.5 mL; Alltech Co., USA)

- Tubos de extracción QuEChERS conteniendo 4 g $MgSO_4$, 1 g NaCl, 1 g citrato de sodio y 0,5 g citrato de sodio sesquihidrato(Agilent Technologies Inc., Wilmington, DE, USA)

- Tubos de dispersión QuEChERS de 15 mL conteniendo 150 mg C18, 150 mg amina primaria secundaria (PSA) y 900 mg $MgSO_4$ (Agilent Technologies Inc., Wilmington, DE, USA)

- Filtros de Nylon, 0.45μm

- Gas nitrógeno

CAPÍTULO III:

Importancia del tratamiento de muestra para la determinación de analitos minoritarios en matrices complejas por Electroforesis Capilar

INTRODUCCIÓN

La presencia de residuos de antibióticos en alimentos de origen animal, es un tema que amerita cada vez mayor importancia, ya que representa uno de los principales problemas que afecta actualmente a la seguridad alimentaria. El uso de antibióticos en animales, cuyos productos o subproductos estén destinados al consumo humano, se encuentra regulado según lo señalado por la Unión Europea (UE) en el Reglamento 2377/90/EC [1], debido a que la ingesta de estos compuestos puede resultar perjudicial para la salud del consumidor [2, 3].

Además de los efectos tóxicos que se pueden presentar en el humano, el consumo de alimentos con residuos de antibióticos puede originar procesos alérgicos y resistencia bacteriana, así como también ocasionar inconvenientes, desde el punto de vista tecnológico, ya que durante la elaboración de algunos alimentos, como productos lácteos derivados (queso y yogur), se requiere la adición de cultivos iniciadores o microorganismos que produzcan características organolépticas deseables y ante la presencia de residuos de antibióticos, el desarrollo de dichos microorganismos puede ser inhibido [4].

Actualmente, para la medicina humana y veterinaria el grupo más importante de antibióticos está representado por los β-lactámicos, que a su vez incluye a las PENs y las cefalosporinas. De estos antibióticos, las PENs han sido ampliamente utilizadas desde hace más de 80 años y representan más de un tercio de la producción total de antibióticos [5, 6].El principal uso de las PENs en la medicina veterinaria es contra los patógenos causantes de mastitis, una enfermedad que provoca importantes pérdidas económicas a la industria láctea [7].Esta situación amerita que los laboratorios agroalimentarios de rutina dispongan de métodos sencillos y rápidos para determinar antibióticos como PENs en alimentos de consumo masivo como leche.

Aunque se han optimizado diferentes métodos analíticos para determinar PENs en leche usando distintas técnicas de separación, como HPLC y CE, la principal dificultad se presenta en la etapa de tratamiento de la muestra, donde se debe lograr extraer estos analitos (presentes generalmente en muy baja concentración) contenidos en una matriz compleja como la leche. Otro factor que

debe ser considerado, es que el extracto obtenido durante el tratamiento de la muestradebe ser adecuadosegún la técnica de separación seleccionada.

Debido a que esta etapa constituye la principal fuente de error ydificulta su aplicación en un laboratorio de rutina, en este capítulo se presentan y discuten los diversos factores que influyen en el proceso de extracción de las PENs, asi como el proceso de limpieza de la muestra o eliminación de interferencias de la matriz, ya que como se ha mencionado antes, la etapa de tratamiento de la muestra pudiera representar el cuello de botella en la determinación de PENs en leche.

En vista de que la literatura carece de estudios que ofrezcan una guía práctica a los laboratorios de rutina, sobre las ventajas e inconvenientes de los tratamientos de muestras publicados para la extracción de PENs en leche, así como la necesidad de generar extractos compatibles para ser analizados por CE, en este capítulo se presenta una estudio sistemático sobre diferentes condiciones electroforéticas (composición y pH del BGE) que han sido empleadas para la determinación de PENs por CE. También se evalúan diversos tratamientos de muestras usados para extaer PENs en leche y finalmente se presentan dos nuevas estrategias analíticas para la determinación de estos analitos en muestras de leche por CE.

REFERENCIAS

[1] Council Regulation (EEC). 1990. N° 2377/90 of 26 June 1990, laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Commun. L 224, 20.

[2] M. Mitchell, Enrofloxacin. Therapeutic review. J. Exot. Pet Med. 15 (2006) 66-69.

[3] R. Stahlmann, Clinical toxicological aspects of fluoroquinolonas. Toxicol. lett. 127 (2002) 269-277.

[4] M.D. Marazuela, M.C. Moreno-Bondi,Multiresidue determination of fluoroquinolonas in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection. J Chromatogr. A. 1034 (2004) 25-32

[5] W.M.A Niessen, Analysis of antibiotics by liquid chromatography mass spectrometry, J. Chromatogr. A 812 (1998) 53–75.

[6] C.K. Fagerquist, A.R. Lightfield, Confirmatory analysis of beta-lactam antibiotics in kidney tissue by liquid chromatography/electrospray ionization selective reaction monitoring ion trap tandem mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 660–671.

[7] M.I Bailón–Pérez, A.M. García–Campaña, M. Del Olmo Iruela, C. Cruces-Blanco, Trace determination of β-lactam antibiotics in environmental aqueous samples using off-line and on-line preconcentration in capillary electrophoresis, J. Chromatogr. A 1185 (2008) 273–280.

DETERMINATION OF PENICILLINS IN MILK OF ANIMAL ORIGIN BY CAPILLARY ELECTROPHORESIS: IS SAMPLE TREATMENT THE BOTTLENECK FOR ROUTINE LABORATORIES?

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Abstract

Capillary electrophoresis (CE) is now being used increasingly, not only for research purposes but also in order to be used in routine analysis. However, this goal is difficult to achieve when the determination of analytes at very low concentrations in complex food samples is required, such as the determination of penicillins (PENs) in milk of animal origin. To our knowledge, today there are no papers devoted to the presentation of all the difficulties and disadvantages founds in daily practice related to sample treatment for the determination of PENs in milk by CE. This work does not attempt to present a new revision of the main applications of CE for the determination of PENs in different types of samples, but rather to show that the weak point of the methods proposed by different authors for the determination of PENs in milk samples could be in the sample treatment and it is not due the lack of robustness of the CE technique. Also, this review presents some problems and drawbacks that can occur during the sample treatment and method development, based on our experience. Clearly the most important error source is associated with the sample processing steps, since it must ensure the best extraction and preconcentration of analytes and to obtain extracts compatible with the separation technique. On the other hand, the use of laborious procedures can lead to loss certain amount of analyte in the different steps. It is noteworthy that a drastic simplification in the sample preparation process can reduce the sensitivity of the method, but it could be a favorable factor, which contributes to obtain high recovery values. As in all the methodological developments in routine analysis, only the comprehensive consideration of all these factors will ensure satisfactory results.

Keywords: Capillary electrophoresis / Milk / Penicillins / Research laboratories / Routine laboratories / Sample treatment

1. Introduction
2. CE methodologies for the determination of PENs
3. Analytical methodologies for determination of PENs in milk samples by CE
   3.1. Milk sample preparation for determination of PENs
   3.2. Practical considerations on the treatment of milk samples for extraction of antibiotics
4. Conclusions

1. Introduction

The determination of antibiotics in food samples is a critical point not only for their quality control, but also from the viewpoint of public health since it may lead to the presence of the drugs and their metabolites in foodstuffs. These substances may cause antibiotic-resistant bacteria (resulting in infections more difficult to treat), allergic reactions in humans, or they may be directly toxic [1]. Separation of antibiotics is mainly carried out by HPLC, though the favorable characteristics of CE have permitted an increase in the number of studies using this separation technique. This fact is corroborated by the more of 1200 papers published during the last years, and some reviews on CE-antibiotic provide a broad knowledge of the present state-of-the-art [1,2].

The most important group of antibiotics for human and veterinary medicine is represented by β-lactam, including penicillins (PENs) and cephalosporins, which have been widely used as antimicrobial drugs for more than 80 years [3]. The main use of these antibiotics in the dairy industry is to combat the pathogens causing mastitis, a disease which leads to significant economic losses [4]. On the other hand, PENs represent more than one-third of the total antibiotic production [5].With the worldwide use of PENs comes the need for tighter controls. To ensure human food safety, many countries such as the United States and the European Union (EU) have set a definitive maximum residue limits (MRLs) in food products. Thus, analytical methods need to be developed to confirm the presence of these compounds below the MRL level.

Analytical methods for determination and screening of PENs have been widely developed in recent years by different research groups [6]. An interesting review of the monitoring of PENs in food samples by CE recently published shows potentials applications of CE for detection and quantification for PENs [7]. Nevertheless, the number of CE routine applications to real food samples is limited because several problems remain associated to be solved, such as: (i) very small sample volume required for CE analysis (at the nanoliter level), which can negatively impact the precision (ii) low sensitivity, due to the low volume loadability of the capillary in which the detection is performed continuously [8], and (iii) compatibility between the sample and features of the CE equipment [9].

For these reasons, new approaches to improve sensitivity, selectivity and robustness have been proposed. Figure 1 illustrates this situation.

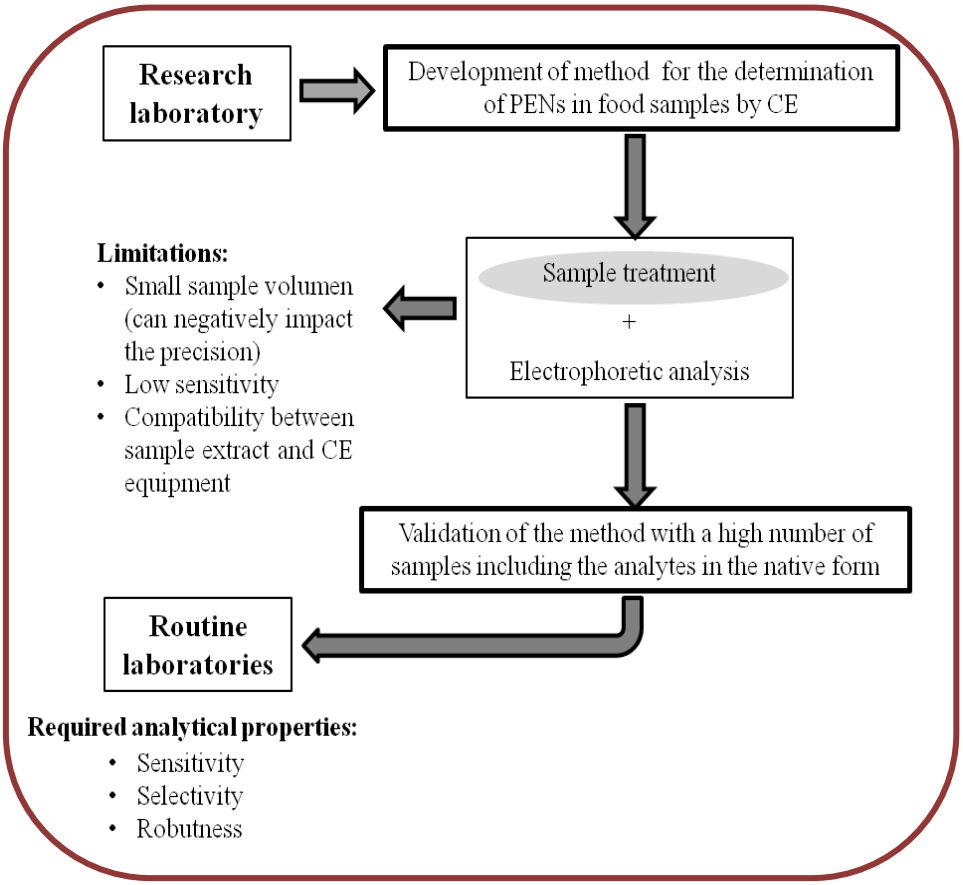

Fig. 1. *Limitations of sample treatment and required analytical properties by the routine laboratories in the determination of PENs in food samples by CE.*

It is known that CE suffers from limited sensitivity when using UV detection due to the short optical path length when in-capillary detection is employed. Due to this fact, other commonly detection modes have been used for antibiotic determination by CE such as laser induced fluorescence (LIF),

electrochemical detection (ED), chemiluminescence (CL), electrochemiluminescence (ECL), and mass spectrometry (MS) detection that provide a better sensitivity compared with classical UV–Vis detection. Aside, other more novel detectors have also been applied such as contactless conductivity detection (C^4D) or potential gradient detection (PGD). Finally, CE in chip format has also attracted interest in recent years and there are several studies employing chip-based microfluidic systems for the determination of antibiotics [1]. However, for the determination of PENs the most common detector used are UV and MS, due to its structure and chemical behavior.

Although there are a number of interesting methods to demonstrate the analytical usefulness to expanding the use of CE in the determination of PENs, so far have raised few analytical strategies (only five) to extract these analytes from milk samples. This could contribute that this technique is still not well accepted in food routine laboratories for the determination of antibiotics nowadays.

The main drawback found in the determination of PENs in complex samples, such as milk, can be seen in the extraction of these analytes from the matrix. This step can be the bottleneck in a routine analytical method. In most cases, different pretreatment steps in order to extract and preconcentrate the analytes are required. Particularly, during food analysis the matrix components can disturb CE separations through the action of saline constituents, macromolecules, and other major compounds characterizing the wide variety of matrices. Moreover another problem is the presence of particulate matter, which can easily clog the CE system [10], for these reasons food samples sometimes need more complex treatments prior to their analysis by CE.

The aim of this work is not limited to present a new review of the use of CE methods for the determination of PENs in milk samples, but is intended to show that the weak point of the different methods published, by diverse authors since many years, is the treatment of the sample and not some analytical properties of the technique, such as the robustness among others.

2. CE methodologies for the determination of PENs

Several research groups worldwide have developed different methods in the last years in order to determinate the presence of PENs in food samples. These

existing methods vary in reliability, rapidity in obtaining results and cost of analysis, but most of them can be grouped into four main categories: (i) microbiological approaches based on bacterial growth inhibition, (ii) biosensors, (iii) immunochemical techniques, and (iv) chromatographic or electrophoretic methods. The advantages and drawbacks of these techniques, and specific aspects of the determination of PENs, have been discussed in a review [11].

The analytical methods for the determination of PENs accepted by the EU (Commission decision 2002/657/EC) are based on chromatographic techniques and/or analytical molecular spectrometry. However, the EU has stated that regulatory laboratories must find the best analytical techniques for the determination of pharmacological substances and therefore it is likely that other methods will have a place in future, if their efficiency can be proven [12].

Although HPLC is mainly used for the determination of PENs by separation techniques, CE is being increasingly employed due to its favorable characteristics (high efficiency, simplicity, short analysis time and low consumption of samples and reagents). In addition, CE is being used in routine analysis pharmaceutical and clinical fields, because it allows obtaining appropriate analytical characteristics and good quantitative results. The determination of PENs by CE is mainly included in two different working modes: (i) capillary zone electrophoresis (CZE) where a separation buffer without or with additives is used for the determination of ionic antibiotics based on their different electrophoretic mobilities, and (ii) micellar electrokinetic chromatography (MEKC) where a micellar system (surfactant at a concentration higher than its *critical micelle concentration*) is added to the separation buffer to perform the separation of neutral and/or ionic antibiotics based on the generation of a pseudostationary phase in which analyte partition takes place [13]. As shown in Figure 2, CZE (46%) and MEKC (36%) have been the separation mode preferred. Nevertheless, the use of m*icroemulsion* electrokinetic capillary chromatography (MEEKC) (11%), cyclodextrin *electrokinetic* chromatography (CD–EKC) (3.5%) and non-aqueous capillary electrophoresis (NACE) (3.5%) modes have also been reported for the determination of PENs.

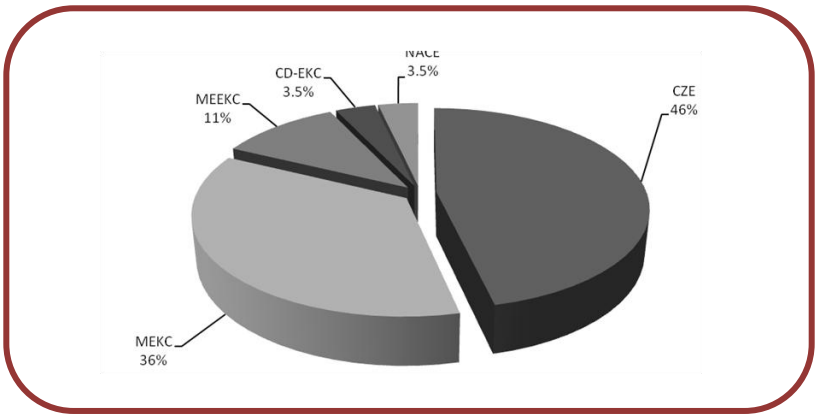

Fig. 2. *Percentage distribution of different CE mode for the determination of PENs in different matrices. According to the database ''ISI Web of Knowledge'' (up to May 2013).*

During recent years, several methods have already been described for determination of PENs residues by CE [14–39]. Table 1 shows in chronological order (from the most recent to oldest) the CE experimental conditions such as the background electrolyte (BGE) composition, capillary conditioning, temperature, pressure and time of injection, voltage, detection system, analysis time and CE instrument used in each method published. As it can be seen in this Table, most PENs included in this review have been separated using borate and/or phosphate at different pH. 25 different methodologies have been found in the literature to separate PENs, half of them using sodium dodecyl sulfate (SDS) micelles in the buffer solutions. Also shows that most of the authors describe the capillary conditions used for washing (before and after electrophoretic analysis), ensuring that it could influence the accuracy of the results. On the other hand, UV detection was the most popular detector employed for the determination of PENs by CE, although MS was also employed. Despite of the high number of different buffers used until now there is a lack of critical revision in which new users could find the strength of each method published to select the best suitable buffers for a specific separation of PENs.

Table 2 summarizes the application of the developed methods covering different fields, such as pharmaceutical, environmental, food, clinical, among others. This table shows the applied separation mode CE and sample preparation requirements needed in each case. The papers shown in the tables were found in the database ‘‘ISI Web of Knowledge’’ using the keywords ‘‘capillary electrophoresis or CE or micellar electrokinetic capillary chromatography or MEKC or electrokinetic capillary chromatography or EKC” and ‘‘β-lactam or penicillins”. As can be seen, the largest number of works have been done in the pharmaceutical field. This is consistent with some studies that indicate that CE is a well-established and frequently used technique in the pharmaceutical industry. In this field, efforts for sample pre-treatment are usually uncomplicated, the precision is good and the sample-throughput is high [40]. Notice that the main type of sample used for determination of PENs by CE are pharmaceutical preparations, drug and others commercial pharmaceutical products following by milk samples (see Figure 3).

3. Analytical methodologies for determination of PENs in milk samples by CE

CE methodologies have been proposed for determination of different PENs in food samples, as water, milk and animal tissues (see Table 2). To our knowledge, CE has not been very extensively applied to the determination of PENs in milk samples [15,17,21,22,31], this may be due to it is quite difficult because of the complexity of the biological matrix and the low level of concentration of these compounds in milk samples. Normally, a large sample size may be needed to obtain the necessary sensitivity, aspect which generates no drawback when it comes milk sample. When analyzing for trace and ultra-trace levels of known contaminants, increased method selectivity (that reduces potential matrix interferences) can help provide the sensitivity required to determine the analytes of interest.

Only five CE methodologies were reported for the determination of PENs in milk, the separation modes employed including CZE, MEKC, and CD–EKC, as shown in Table 2. Different methodologies were developed using CZE. Tian et al. [15] developed a CE method for the simultaneous determination of penicillin intermediate and PENs in milk, including 6-amino-penicillanic acid (6–APA),

Table 1. Summary of proposed CE methodologies for the determination of PENs.

Analyte(s)	BGE composition		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
OXA, PEN V, PEN G, NAF, AMP and AMX	5% SDS, 80% 1-butanol, 15% sodium acetate	8.0	1 min 0.1 M NaOH, 2 min water and 5 min running buffer	n.i	37.5	50 mbar/ 3 s	-29	UV-vis	9	HP^{3D}CE system (Agilent Technologies)	[14]
6-APA, PEN G, AMP and AMX	40 mM Potassium dihydrogen phosphate, 20 mM borax solution	7.8	n.i	n.i	30	n.i	28	n.i	4.5	HP^{3D} CE system (Agilent Technologies)	[15]
NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	60 mM Ammonium acetate	6.0	3 min water, 3 min 0.1 M NaOH, 3 min water, and 5 min running buffer (N_2 pressure, 7 bar)	1 min running buffer (N_2 pressure, 7 bar)	30	50 mbar/ 80 s	30	Tandem MS (MS/MS)	n.i.	HP^{3D} CE system (Agilent Technologies)	[16]
NAF, DCLX, AMP, OXA, PEN V, CLX, PEN G, and AMX	50 mM Phosphate 89.27%, SDS 2.21%, 2-propanol 7.71%, propylene glycol monomethylether acetate 0.81%	2.0	5 min 0.1 M NaOH and 5 min running buffer	n.i	30	50 mbar/ 3 s	-20	UV-vis	7	HP^{3D}CE system (Agilent Technologies)	[18]
PEN G	30 mM Sodium tetraborate	9.2	10 min 100 mM NaOH 5 min water and 10 min running buffer	5 min running buffer	20	1 psi/ 1-5 s	15	DAD	30	P/ACE MDQ CE system (Beckman-Coulter)	[19]
NAF, CLX, OXA, DCLX, AMP, AMX, and PEN G	175 mM Tris buffer with 20% ethanol	8.0	3 min 0.1 M NaOH, 3 min, water and 5 min running buffer (N_2 pressure, 7 bar)	1 min 0.1 M NaOH, 1 min water and 2 min running buffer (N_2 pressure, 7 bar)	30	7 bar/ 1 min (LVSS)	-20 and 25	DAD	30	HP^{3D}CE system (Agilent Technologies)	[21]

OXA: oxacillin; PEN V: penicillin V; PEN G: penicillin G; NAF: nafcillin; AMP: ampicillin; AMX: amoxicillin; 6-APA: 6-amino penicillanic acid; DCLX: dicloxacillin; CLX: cloxacillin; PIP: piperacillin; SDS: sodium dodecyl sulfate; ACN: acetonitrile; n.i.: not indicated

Table 1. Continued.

Analyte(s)	BGE composition		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
AMP, AMX, CLX, PEN G, tetracycline and chloramphenicol	2.7 mM Potassium dihydrogen-phosphate, 4.3 mM sodium tetraborate	8.0	n.i.	n.i.	25	0.5 psi/ 3 s	18	DAD	15	P/ACE MDQ CE system (Beckman-Coulter)	[22]
CLX, DCLX, OXA, PEN G, PEN V, AMP, NAF, PIP, and AMX	26 mM Sodium tetraborate, 100 mM SDS	8.5	2 min 0.1 M NaOH, 2 min H_2O Milli-Q and 2 min running buffer	n.i.	30	50 mbar/ 5 s	20	DAD	22	HP^{3D} capillary electrophoresis system (Agilent Technologies)	[23]
AMX, DCLX, NAF, PEN V, PEN G, OXA, CLX and AMP	0.5% Ethyl acetate, 1.2% 1-butanol, 2% Brij 35, 10% 2-butanol, 86.3% 10 mM borate	10.0	8 min 0.1 M NaOH, 8 min, water and 10 min microemulsion solution	2 min microemulsion solution	25	50 mbar/ 5 s	10	DAD	12	HP3D CE system (Agilent Technologies)	[24]
PEN V and related substances	Phosphate–borate buffer with 69 mM SDS and 12.5 mM pentanesulfonic acid sodium salt	6.3	n.i.	n.i.	25	10 s	15	UV	n.i.	Waters Quanta 4000 CE system	[25]
PEN V, AMX, DCLX, NAF, PEN G, OXA, CLX and AMP	20 mM Sodium tetraborate, 60 mM SDS	8.0	2 min 0.1 M NaOH, 2 min, water and 2 min running buffer	2 min water	25	50 mbar/ 10 s	15	DAD	17	HP^{3D} CE system (Agilent Technologies)	[26]
AMP	40 mM Phosphate-borate, 75 mM SDS	7.5	1 min water, 1 min 0.1 M NaOH, 1 min water, and 5 min running buffer	n.i.	25	0.5 psi/ 6 s	18	DAD	n.i.	P/ACE MDQ CE system (Beckman-Coulter)	[27]

OXA: oxacillin; PEN V: penicillin V; PEN G: penicillin G; NAF: nafcillin; AMP: ampicillin; AMX: amoxicillin; 6-APA: 6-amino penicillanic acid; DCLX: dicloxacillin; CLX: cloxacillin; PIP: piperacillin; SDS: sodium dodecyl sulfate; ACN: acetonitrile; n.i.: not indicated

Table 1. Continued.

Analyte(s)	BGE composition		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
AMX, AMP, PEN Gsodium salt, PEN G-procaine salt, PEN G-benzathine salt, OXA, PEN V and CLX	40 mM Sodium tetraborate, 100 mM SDS	8.5	2 min 0.1 M NaOH, 2 min H_2O Milli-Q and 2 min running buffer	n.i.	20	10 s	10	DAD	33	P/ACE MDQ CE system (Beckman-Coulter)	[28]
Benzylpenicillin, procaine, benzathine and clemizole	3.12 g/L Disodium hydrogenphosphate, 7.64 g/L sodium tetraborate, 14.4 g/L SDS	8.7	n.i.	n.i.	25	10 s	18	UV	15	Waters Quanta 4000 CE system	[29]
Procaine, dihydrostreptomycin and PEN G	80 mM Sodium tetraborate decahydrate	8.0	5 min 0.1 M KOH, 5 min water and 10 min running buffer	n.i.	35	10 s	15	UV	10	Waters Quanta 4000 CE system	[30]
OXA, CLX and DCLX	50 mM Phosphoric acid, 5.2 mM 2-hydroxypropyl-beta-cyclodextrin	3.6	n.i.	2 min 0.2 M NaOH, 2 min water, 5 min 0.2 M HCl, 5 min running buffer	25	3.0 psi/ 50 s	-30	DAD	19	P/ACE MDQ CE system (Beckman-Coulter)	[31]
PEN V and its related substances	20 mM Ammonium Acetate, 20 mM ammonium acetate in ACN/MeOH 60/40 v/v	6.5	n.i	n.i	25	50 mbar/ 3 s	-20	UV and ESI-MS	n.i	HP3D CE system (Agilent Technologies)	[32]
PEN V, clofibric acid, naproxen, bezafibrate, carbamazepine,diclofenac, ibuprofen, mefenamic acid and paracetamol	20 mM Ammonium acetate	5.1	n.i.	3 min running buffer	n.i	5 kPa/0.3 min	20	MS	20	Crystal 310 CE instrument (Thermo CE)	[33]

OXA: oxacillin; PEN V: penicillin V; PEN G: penicillin G; NAF: nafcillin; AMP: ampicillin; AMX: amoxicillin; 6-APA: 6-amino penicillanic acid; DCLX: dicloxacillin; CLX: cloxacillin; PIP: piperacillin; SDS: sodium dodecyl sulfate; ACN: acetonitrile; n.i.: not indicated

Table 1. Continued.

Analyte(s)	BGE composite		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
AMX	20 mM Sodium tetraborate	9.0	15 min 0.1 M NaOH, 15 min H_2O Milli-Q and 10 min running buffer	2 min water and 3 min running buffer	30	100 mbar/ 1.8 s	15	UV	12	Prince CE System (Lauer, Emmen, Holland)	[34]
AMX and its potential impurities	70 mM Sodium dihydrogenphosphate, 125 mM SDS 5% ACN	6.0	5 min running buffer	n.i.	25	4 s	15	UV	20	Spectraphoresis 500 Equipment (Thermo, USA)	[35]
PEN G, 6-APA and phenyl acetic acid	30 mM Tetraborate	9.2	n.i.	n.i.	30	12.7 cmHg/ 1 s	15	UV	5	Model 270A CE system (Applied Biosystems)	[36]
PEN V and its related substances	40 mM Sodium dihydrogenphosphate, 100 mM SDS	7.0	5 min running buffer	n.i.	25	5170 Pa/ 5-20 s	15	UV	25	Spectraphoresis 1000 (Thermo, USA)	[37]
OXA, AMP, PIP, PEN G, PEN V, CLX, DCLX, cephapirin and NAF	20 mM Sodium tetraborate, 75 mM SDS	8.5	n.i.	n.i.	25	50 mbar/ 3.6 s	15	UV	20	Crystal 310 CE instrument (Thermo CE)	[38]
PEN G	10mM Sodium dihydrogenphosphate, 6 mM sodium tetraborate	9.0	n.i.	n.i.	n.i.	10 s	30	UV	10	n.i.	[39]

OXA: oxacillin; PEN V: penicillin V; PEN G: penicillin G; NAF: nafcillin; AMP: ampicillin; AMX: amoxicillin; 6-APA: 6-amino penicillanic acid; DCLX: dicloxacillin; CLX: cloxacillin; PIP: piperacillin; SDS: sodium dodecyl sulfate; ACN: acetonitrile; n.i.: not indicated

Table 2. Analytical determination of PENs by CE in different matrices.

Field of application	Matrix	Analyte(s)	CE mode	Sample treatment	Ref.
Pharmaceutical	Pharmaceutical preparations, drug and others commercial pharmaceutical products	NAF, DCLX, AMP, OXA, PEN V, CLX, PEN G, and AMX	MEEKC	Mixed with water and sonicated. The resulting clear liquid was filtered and diluted with a phosphate buffer of pH 2 or 8	[18]
		CLX, DCLX, OXA, PEN G, PEN V, AMP, NAF, PIP, AMX	MEKC	Dissolved in water in an ultrasonic and filtered	[23]
		AMX, DCLX, NAF, PEN V, PEN G, OXA, CLX and AMP	MEEKC	Dissolved in water and filtered	[24]
		PEN V and related substances	MEKC	n.i.	[25]
		PEN V, AMX, DCLX, NAF, PEN G, OXA, CLX and AMP	MEKC	n.i.	[26]
		AMP	MEKC	Solutions were sonicated for 3 min and filtered	[27]
		Benzylpenicillin, procaine, benzathine and clemizole	MEKC	Dissolved in water	[29]
		Procaine, dihydrostreptomycin and PEN G	CZE	Dissolved in water	[30]
		AMX and its potential impurities	MEKC	n.i.	[35]
		PEN G, 6-APA and phenyl acetic acid	CZE	n.i.	[36]
		PEN V and its related substances	MEKC	n.i	[37]
		OXA, AMP, PIP, PEN G, PEN V, CLX, DCLX, cephapirin and NAF	MEKC	n.i.	[38]

NAF: nafcillin; DCLX: dicloxacillin; AMP: ampicillin; OXA: oxacillin; PEN V: penicillin V; CLX: cloxacillin; PEN G: penicillin G; AMX: amoxicillin; PIP: piperacillin; 6-APA: 6-amino penicillanic acid; n.i.: not indicated

Table 2. Continued.

Field of application	Matrix	Analyte(s)	CE mode	Sample treatment	Ref.
Environmental / Food	Water (waste, well, river, surface and potable water)	NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	CZE	Extraction with ACN, preconcentration and cleanup with SPE (HLB and Alumina N cartridge)	[16]
		AMX, AMP, PEN G-sodium salt, PEN G-procaine salt, PEN G-benzathine salt, OXA, PEN V, and CLX	MEKC	Filtration	[28]
		PEN V, clofibric acid, naproxen, bezafibrate, carbamazepine, diclofenac, ibuprofen, mefenamic acid and paracetamol	CZE	LLE and SPE	[33]
Food	Milk	6-APA, AMX, AMP and PEN G	CZE	n.i	[15]
		AMP, AMX, PEN V and cephalexin	MEKC	Protein precipitationand SPE	[17]
		NAF, CLX, OXA, DCLX, AMP, AMX, and PEN G	CZE	Solvent extraction with ACN and SPE (HLB and Alumina N cartridge)for cleanup and preconcentration, in combination with LVSS (in-line preconcentration)	[21]
		AMP, AMX, CLX, PEN G, tetracycline and chloramphenicol	CZE	Protein precipitation with TCA and SPE (C18)	[22]
		OXA, CLX and DCLX	CD-EKC	Extraction with ethyl acetate and large-volume stacking using the electroosmotic flow pump (LVSEP)	[31]
	Animal tissue (porcine organs,chicken muscles, meat and fish)	OXA, PEN V, PEN G, NAF, AMP and AMX	MEEKC	Extraction with ACN and n-hexane. SPE with C18.	[14]
		NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	CZE	Extraction with ACN and preconcentration and cleanup with SPE (HLB and Alumina N cartridge)	[16]
		AMX, AMP, OXA, and PEN V	CZE	ACN (extraction and protein precipitation) and cleanup with SPE (C18)	[20]
Clinical	Biological fluid (urine, blood, plasma, gastric contents and amniotic fluid)	PEN G	CZE	SPE (C18)	[19]
		AMX	CZE	SPE (C18)	[34]
		PEN G	CZE	Purification by centrifugation and DEAE cellulose treatment of the stomach contents (diluted with pH 9 phosphate-borate buffer)	[39]
Other	Fermentation broth	PEN V and its related substances	CZE/NACE	n.i.	[32]

NAF: nafcillin; DCLX: dicloxacillin; AMP: ampicillin; OXA: oxacillin; PEN V: penicillin V; CLX: cloxacillin; PEN G: penicillin G; AMX: amoxicillin; PIP: piperacillin; 6-APA: 6-amino penicillanic acid; n.i.: not indicated

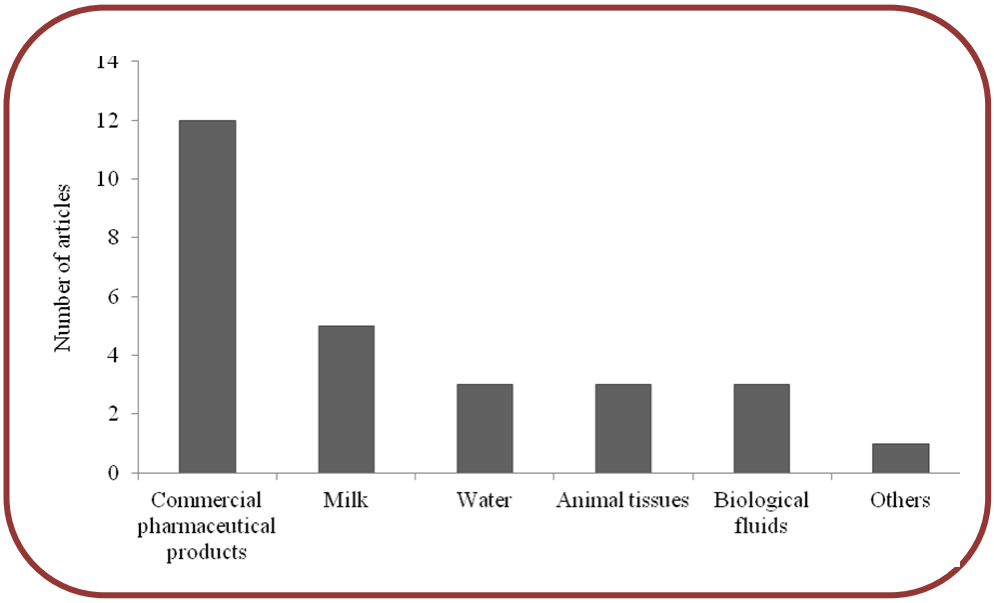

Fig. 3.*Number of papers concerning CE methods for determination of PENs in different type of matrices. According to the database ''ISI Web of Knowledge'' (up to May 2013).*

ampicillin (AMP), amoxicillin (AMX) and penicillin G (PEN G). The four PENs were baseline separated within 4.5 min with the running buffer of 40 mM potassium dihydrogen phosphate and 20 mM borax solution at pH 7.8. The average recoveries at three fortification levels were in the range of 85–97% with acceptable relative standard deviations (RSDs) of 1–9%.

A different method was used for the simultaneous determination of seven PENs in fortified milk samples in less than 30 min using 175 mM Tris at pH 8.0 with 20% ethanol and UV detection at 220 nm. To improve the sensitivity of the method, this study combined the use of SPE with capillary stacking preconcentration methodology, such as large volume sample stacking (LVSS) injection. This protocol enabled to obtain limits of detection (LODs) ranging from 2 to 10 μg L^{-1}, which are below the MRLs regulated in the EU directive for milk

and satisfactory recoveries for bovine raw milk (86–93%), bovine skimmed milk (88–93%), and goat raw milk (87–91%) [21].

Finally, a CZE method has been proposed for the simultaneous detection of AMX, AMP, cloxacillin (CLX) and PEN G in spiked milk samples. The CE analysis time was 15 min. Quantification of AMX was not possible because of the low recovery indices, which were mainly due to an inefficient SPE extraction procedure. Recovery indices were largely influenced by the chosen SPE cartridges, which were not adequate for the extraction of more polar antibiotics like AMX. Regardless of AMX, average of recoveries of all antibiotics was over 72%. The LODs were between 0.48–1.09 μg $mL^{-1}$and the LOQs were between 1.59–3.64 μg mL^{-1} [22].

MEKC modality was also used for the separation of AMP, AMX and penicillin V (PEN V) in spiked milk samples using a phosphate buffer containing SDS. The LODs were 0.16–0.20 mg L^{-1} and the average recoveries of PENs from milk were over 70% for all of them except AMX [17]. Due to these compounds are neutral or weakly ionic molecules, MEKC is often the mode of CE used to separate them.

With regards to CD–EKC, the use of CDs and their derivatives have been applied in CE for the separation of isoxazolylpenicillins. Zhu et al. [31] developed a method for the determination of CLX, oxacillin (OXA) and dicloxacillin (DCX) in milk samples. This method comprises large-volume sample stacking using the electroosmotic flow (EOF) pump (LVSEP), separation using 2-hydroxypropyl-β-cyclodextrin (HP–β–CD) as selective complex-forming background electrolyte additive, and direct UV detection. The LOD obtained to all the analytes was 2 μg L^{-1}. In this case, the milk samples also were spiked with the isoxazolylpenicillins.

In all the studies mentioned above, the type of sample used was spiked milk. Although the availability of real samples often be a difficult task, to demonstrate the suitability of the proposed method is desirable that the analytes in the samples are present in their native forms. Another important aspect to note is that in some cases, the methods proposed LOD and LOQ above MRLs (ppm level), which indicates the difficulty of being able to obtain a better sensitivity of the method.

3.1. Milk sample preparation for determination of PENs

Sample preparation, in an analytical process, usually intended to achieve the following objectives: (i) to get analytes dissolved in a smaller size of the matrix, (ii) reduction or elimination of organic solvents, (iii) generic extraction procedures for multiclass compounds, (iv) integration of several preparation steps into one, (v) potential for automation and/or high-throughput determination [41]. In this regard, the determination of trace analytes (as PENs) in samples by CE-based analytical techniques usually requires their prior extraction from the matrix and preconcentration [42, 43]. Sample treatment and preconcentration is a crucial part of chemical analysis and in a sense has become the bottleneck of the whole analytical process [43].

Although SPE and liquid-liquid extraction (LLE) continue to be the most widely used extraction and concentration techniques, milk samples often contain a large number of matrix components that may co-elute with the analytes and disturb the quantitative analysis, There is a growing search for time and labor saving sample pretreatment methods which aim at the reduction of the matrix content and the enrichment of the target analytes. Also, they are expected more eco-friendly capable of using smaller amounts of solvents and sample as well as ideally involving as few operations as possible in order to minimize potential errors and shorten analysis times. Thus, some cleanup/concentration methodologies such as solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), matrix solid-phase dispersion (MSPD), hollow fiber (HF) extraction, supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), cloud point extraction (CPE), and dispersive liquid-liquid microextraction (DLLME) have demonstrated effective for preconcentration purposes and there by allow significant improvement for the separation, identification and quantitation by CE [41].

In the field of food analysis, for the determination of antibiotic residues by CE, different procedures have been propose to improve the preconcentration of analytes and cleanup process from different matrices. Typically, the extraction of PENs from milk samples includes various steps, such as protein precipitation, extraction and preconcentration, prior to CE analysis. Figure 4 show a schematic diagram of common procedures used for the determination of PENs in milk samples by CE. Current methods for the pretreatment of milk

samples first involve a protein precipitation step using various protein-precipitating reagents, e.g., trichloroacetic acid (TCA) [22] or acetonitrile (ACN) [21]. SPE using C18, Alumina N or Oasis HLB polymeric sorbent (which contains lipophilic divinylbenzene units and more hydrophilic N-vinylpyrrolidone units) have been used for a second clean up step and preconcentration of the analytes [17,21,22].

Recently, new methodologies have been proposed for the treatment of milk samples containing antibiotics using the commercially available molecularly imprinted polymers (MIPs) as sorbent for SPE (MISPE). MIPs are synthetic materials with artificially generated recognition sites able to specifically capture target molecules, so there are materials with higher selectivity and can provide cleaner sample extracts and easier process than usual SPE sorbents. Some of these materials have been synthesized in several laboratories. Particularly, the preparation of a MIP specific to two cephalosporins–a subclass of β-lactam antibiotics– as template for the imprinted polymer synthesis has been made by Quesada et al. [44]. In this paper, the MIP demonstrates useful cross-selectivity and being able to extract three structurally related compounds from complex samples, such as milk, with satisfactory recoveries in these preliminary experiments. The results of this study were evaluated by using HPLC with DAD detection.

Others strategies, as QuEChERS (standing for Quick, Easy, Cheap, Effective, Rugged and Safe) and dispersive extraction by QuEChERS in MSPD format have been applied for the treatment of milk samples containing antibiotics. The QuEChERS methodology presents some advantages, over SPE and other traditional methods of extraction, such as its simplicity, minimum steps, and effectiveness for cleaning-up complex samples. The original procedure involves initial SPE of the sample with ACN, followed by liquid-liquid partitioning by the addition of anhydrous magnesium sulphate and sodium chloride. Removal of water and cleanup are performed simultaneously on an aliquot of the ACN extract with dispersive SPE using $MgSO_4$ and primary secondary amine sorbent [45]. This methodology has been extensively use for extraction of pesticide residues in fruits and vegetables [46,47], and recently, it also has been applied in the determination of antibiotic residues in different food samples such as animal tissue [48,49], eggs [50] and milk [51,52].

Till now modified QuEChERS sample preparation procedure is widely employed. A modified MSPD procedure was applied for the extraction and clean up procedure of PENs and amphenicols in milk using a mixture of Strata by Phenomenex and QuEChERS as a sorbent [53]. Since milk is with no doubt a complex matrix requiring a sophisticated sample preparation to isolate target analytes, advantage of all benefits from combination of ultrasonic-assisted MSPD–QuEChERS method have been applied for milk sample preparation by HPLC analysis. Moreover sonication enhances recovery by providing an efficient contact between the solid and the extractant, yielding higher recovery rates of the target analytes [54]. To the best of our knowledge, QuEChERS methodology has not been applied for obtaining extracts of milk samples, containing PENs to be determined by CE.

3.2. Practical considerations on the treatment of milk samples for extraction of antibiotics

To achieve the correct development of the full analytical process is necessary to consider as an aspect of paramount importance the compatibility between the extract obtained in the sample pretreatment and the buffer used in the CE separation. Figure 4 illustrates the importance of this fact. Keep in mind that the sample treatment necessary to determine PENs in milk by HPLC could not necessarily be applied directly to use CE as separation technique, as it will be necessary to demonstrate the compatibility of the extract obtained by electrophoretic system.

The determination of analytes in complex matrix, as milk samples, by CE-based analytical techniques usually requires numerous steps for the extraction of the analytes from the matrix, and the application of preconcentration strategies coupled or uncoupled to the electrophoretic separation, as shown in Figure 4. For a routine laboratory is desirable a sample pretreatment simple and fast, because it decreases the number of steps before the sample analysis and also reduces the uncertainty or error leading to the loss of analyte. To clean extracts and prevent loss of analytes must find the combination of steps necessary for this purpose.

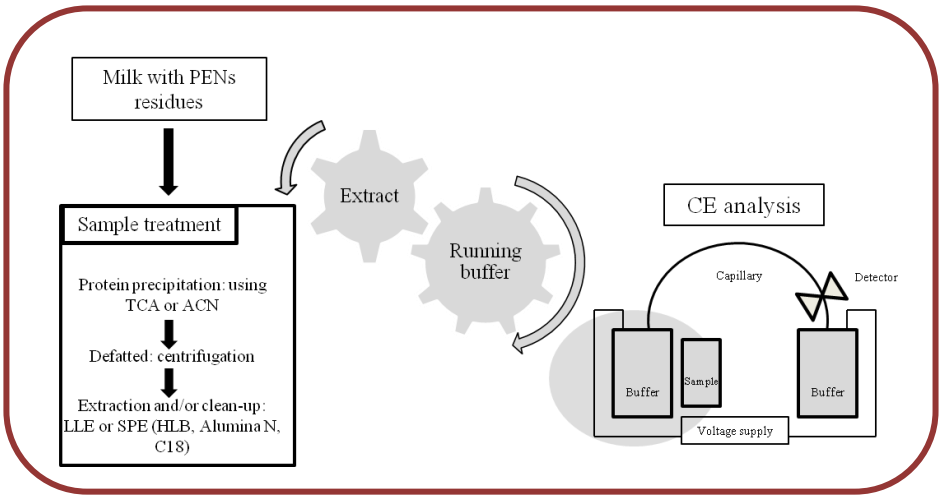

Fig. 4. *Compatibility between the sample pretreatment and CE analysis.*

Taking into account the requirements imposed by EU Directive in relation to the low MRLs for all antibiotics in foods of animal origin, including milk, and considering the limited sensitivity usually inherent to CE methods using UV–Vis detection, different strategies for the (off-line and/or in-line) preconcentration have shown to be useful for determining analytes at low concentrations.

The use of off-line SPE is probably the most widely used sample pretreatment procedure prior to CE for the preconcentration of analytes, however it should control all aspects that could influence the sorption and desorption stage of the analytes in the sorbent used. When considering an extraction process it should be considered the influence of the natural pH of the medium in which the analytes are presented since many times the optimization of the extraction process has been carried out with standard solutions and when this methodology is applied to natural samples it does not work due to difference pH between samples and standards.

Also different strategies have been used for the in-line preconcentration of PENs in milk samples, including LVSS, also called "stacking matrix removal" [21] and LVSEP [31]. Although LVSS is an effective option to concentrate the analytes, it only works with low-conductivity matrices. In fact, high-conductivity matrices (as milk) require laborious pretreatment process, involving various stages of extraction and cleanup of the matrix prior to LVSS–CE. Vera-Candioti et al. [55] showed that when applying LVSS, high milk conductivity interfered with the elimination of the matrix, making it impossible to apply this method for preconcentration of antibiotic residues in milk and their quantitation by CE.

4. Conclusions

CE is a useful and real alternative to chromatographic methods for monitoring of PENs residues in milk samples of animal origin. Different modes of CE have been used, mainly CZE and MEKC, to determine a great variety of these compounds. Although direct UV–detection is the most popular system employed, the lack of sensitivity inherent to CE with this mode of detection, the low levels of these compounds expected in the food samples and, the requirements of the legislation in relation to the MRLs permitted in foods for safe consumption, has involved the development of different strategies to improve the sensitivity of CE–UV. Sample treatment is the first and most important step of the analytical process and it is the bottleneck of the determination of PENs in milk samples by CE. In this sense, different methodologies have been proposed for sample treatment, including sample clean up and (off-line and/or in-line) preconcentration of the analytes. SPE has been extensively used as off-line preconcentration for this purpose. New extraction systems to determination of PENs, for example MISPE and QuEChERS, have also been satisfactorily employed, with high efficiency, however QuEChERS has not been using for determination of PENs in milk by CE. In-line preconcentration procedures (LVSS and LVSEP) have also been recently applied in this field introducing a very large sample volume, with the objective of improving sensitivity, however for the successful implementation of this strategy requires low conductivity extracts so the milk sample must be submitted to rigorous cleanup. Although, there are five research papers demonstrating the determination of PENs in milk samples, as far as we are concerned, there is a lack of research articles that demonstrates how to extract PENs from milk samples and obtain extracts compatible with CE-UV.

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References

[1] V. Pérez–Fernández, E. Domínguez–Vega, A. Crego, M.A. García, M.L Marina, Recent advances in the analysis of antibiotics by CE and CEC, Electrophoresis 33 (2012) 127–146.

[2] M. Castro–Puyana, A. Crego, M.L. Marina, Recent advances in the analysis of antibiotics by CE and CEC, Electrophoresis 31 (2010) 229–250.

[3] W.M.A Niessen, Analysis of antibiotics by liquid chromatography mass spectrometry, J. Chromatogr. A 812 (1998) 53–75.

[4] M.I Bailón–Pérez, A.M. García–Campaña, M. Del Olmo Iruela, C. Cruces-Blanco, Trace determination of β-lactam antibiotics in environmental aqueous samples using off-line and on-line preconcentration in capillary electrophoresis, J. Chromatogr. A 1185 (2008) 273–280.

[5] C.K. Fagerquist, A.R. Lightfield, Confirmatory analysis of beta-lactam antibiotics in kidney tissue by liquid chromatography/electrospray ionization selective reaction monitoring ion trap tandem mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 660–671.

[6] R. Babington, S. Matas, M.P. Marco, R. Galve, Current bioanalytical methods for detection of penicillins,Anal. Bioanal.Chem. 403 (2012) 1549–1566.

[7] A.M. García-Campaña, L. Gámiz-Gracia, F.J Lara, M. Del Olmo Iruela, C. Cruces-Blanco, Applications of capillary electrophoresis to the determination of antibiotics in food and environmental samples, Anal. Bioanal. Chem. 395 (2009) 967–986.

[8] B.M. Simonet, A. Ríos, M. Valcárcel, Enhancing sensitivity in capillary electrophoresis,Trends Anal. Chem. 22 (2003) 605–614.

[9] G. Castañeda, J. Rodriguez-Flores, A. Ríos, Analytical approaches to expanding the use of capillary electrophoresis in routine food analysis, J. Sep. Sci. 28 (2005) 915–924.

[10] J.R. Veraart, H. Lingeman, U.A.T. Brinkman, Coupling of biological sample handling and capillary electrophoresis,J. Chromatogr. A 856 (1999) 483–514.

[11] L. Kantiani, M. Farré, D. Barceló, Analytical methodologies for the detection of beta-lactam antibiotics in milk and feed samples,Trends in Analytical Chemistry, 28 (2009) 729–744.

[12] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Communities, 2002.

[13] C. García-Ruiz, M.L. Marina, Recent advances in the analysis of antibiotics by capillary electrophoresis,Electrophoresis 27 (2006) 266–282.

[14] H.Y. Huang, W.L. Liu, B. Singco, S.H. Hsieh, Y.H.Shih,On-line concentration sample stacking coupled with water-in-oil microemulsion electrokinetic chromatography,J. Chromatogr. A 1218 (2011) 7663–7669.

[15] C. Tian, H. Tan, L. Gao, H. Shen, K. Qi, Determination of penicillin intermediate and three penicillins in milk by high performance capillary electrophoresis,Chinese J. Chromatogr. 29 (2011) 1128–1132.

[16] M.I. Bailón-Pérez, A.M. García-Campaña, M. Del Olmo Iruela, C. Cruces-Blanco, L.G. García, Multiresidue determination of penicillins in environmental waters and chicken muscle samples by means of capillary electrophoresis-tandem mass spectrometry,Electrophoresis 30 (2009) 1708–1717.

[17] Q. Zhang, N. Ye, X. Gu, X. Hao, N. Liu, Simultaneous determination of four beta-lactam antibiotics in milk by solid-phase extraction-micellar electrokinetic chromatography,Chinese J. Chromatogr. 26 (2008) 682–686.

[18] H.Y. Huang, S.H. Hsieh, Sample stacking for the analysis of penicillins by microemulsion electrokinetic chromatography,Electrophoresis 29 (2008) 3905–3915.

[19] A. Thomas, O.K. Upoma, J.A. Inman, A.K. Kaul, J.H. Beeson, K.P. Roberts, Quantification of penicillin G during labor and delivery by capillary electrophoresis, J. Biochem. Biophys. Methods 70 (2008) 992–998.

[20] A. Juan-García, G. Font, Y. Picó, Simultaneous determination of different classes of antibiotics in fish and livestock by CE-MS,Electrophoresis 28 (2007) 4180–4191.

[21] M.I. Bailón-Pérez, A.M. García-Campaña, C. Cruces-Blanco, M. Del Olmo Iruela, Large-volume sample stacking for the analysis of seven β-lactam antibiotics in milk samples of different origins by CZE, Electrophoresis 28 (2007) 4082–4090.

[22] S.M. Santos, M. Henriques, A.C. Duarte, V.I. Esteves, Development and application of a capillary electrophoresis based method for the simultaneous

screening of six antibiotics in spiked milk samples,Talanta 71 (2007) 731–737.

[23] M.I. Bailón-Pérez, L. Cuadros-Rodríguez, C. Cruces-Blanco, Analysis of different beta-lactams antibiotics in pharmaceutical preparations using micellar electrokinetic capillary chromatography, J. Pharm. Biomed. Anal. 43 (2007) 746–752.

[24] P. Puig, F. Borrull, C. Aguilar, M.J. Calull, Sample stacking for the analysis of penicillins by microemulsion electrokinetic capillary chromatography Chromatogr. B-Analytical Technol. Biomed. Life Sci. 831 (2006) 196–204.

[25] Pajchel, G., Michalska, K., Tyski, S., Analysis of phenoxymethylpenicillin potassium by capillary electrophoresis, J. Chromatogr. A 1087 (2005) 197–202.

[26] P. Puig, F. Borrull, M.J. Calull, C. Aguilar, Sample stacking for the analysis of eight penicillin antibiotics by micellar electrokinetic capillary chromatography, Electrophoresis 26 (2005) 954–961.

[27] M. Dolezalova, B. Kunteova, R. Jobanek, Determination of the purity of ampicillin by micellar electrokinetic chromatography and reversed phase liquid chromatography on a monolithic silica column,J. Sep. Sci. 27 (2004) 560–568.

[28] L. Nozal, L. Arce, A. Ríos, M. Valcárcel, Development of a screening method for analytical control of antibiotic residues by micellar electrokinetic capillary chromatography,Anal. Chim. Acta 523 (2004) 21–28.

[29] G. Pajchel, K. Michalska, S. Tyski, Application of capillary electrophoresis to the determination of various benzylpenicillin salts, J. Chromatogr. A 1032 (2004) 265–272.

[30] K. Michalska, G. Pajchel, S. Tyski, Capillary electrophoresis method for simultaneous determination of penicillin G, procaine and dihydrostreptomycin in veterinary drugs,J. Chromatogr. B 800 (2004) 203–209.

[31] Z. Zhu, L. Zhang, A. Marimuthu, Z. Yang, Large-volume sample stacking combined with separation by 2-hydroxypropyl-b-cyclodextrin for analysis of isoxyzolylpenicillins by capillary electrophoresis, Electrophoresis 24 (2003) 3089–3096.

[32] E.F. Hilder, C.W. Klampfl, W. Buchberger, P.R. Haddad, Comparison of aqueous and non aqueous carrier electrolytes for the separation of penicillin

V and related substances by capillary electrophoresis with UV and mass spectrometric detection,Electrophoresis 23 (2002) 414–420.

[33] W. Ahrer, E. Scherwenk, W. Buchberger, Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry,J. Chromatogr. A 910 (2001) 69–78.

[34] M. Hernández, F. Borrull, M.J. Calull, Determination of amoxicillin in plasma samples by capillary electrophoresis,J. Chromatogr. B 731 (1999) 309–315.

[35] Y.M. Li, A. Van Schepdael, Y. Zhu, E. Roets, J. Hoogmartens, Development and validation of amoxicillin determination by micellar electrokinetic capillary chromatography,J. Chromatogr. A 812 (1998) 227–236.

[36] H.B. Wan, J. Liu, K.C. Ang, S.F.Y. Li, Application of capillary electrophoresis to process monitoring in the manufacturing of semisynthetic penicillins, Talanta 45 (1998) 663–671.

[37] Y.H. Zhu, A. Van Schepdael, E. Roets, J. Hoogmartens, Micellar electrokinetic capillary chromatography for the separation of phenoxymethylpenicillin and related substances,J. Chromatogr. A 781 (1997) 417–422.

[38] M.E.P. Hows, D. Perrett, J. Kay, Optimisation of a simultaneous separation of sulphonamides, dihydrofolate reductase inhibitors and beta-lactam antibiotics by capillary electrophoresis, J. Chromatogr. A 768 (1997) 97–104.

[39] S. Arrowood, A.M. Hoyt, M.J. Sepaniak, Monitoring of benzylpenicillin decomposition in gastric contents by capillary zone electrophoresis, J. Chromatogr-Byomedical Applications 583 (1992) 105-110.

[40] C. Cianciulli, H. Wätzig, Analytical instrument qualification in capillary electrophoresis,Electrophoresis 33 (2012) 1499–1508.

[41] Y. Wen, J. Li, J. Ma, L. Chen, Recent advances in enrichment techniques for trace analysis in capillary electrophoresis, Electrophoresis 33 (2012) 2933–2952.

[42] S. Almeda, L. Arce, M. Valcárcel, The more and less common approaches to enhancing sensitivity in capillary electrophoresis, Curr. Anal. Chem. 6 (2010) 126–143.

[43] Y. Chen, Z.P. Guo, X.Y. Wang, C.G. Qiu, Sample preparation,J. Chromatogr. A 1184 (2008) 191–219.

[44] C. Quesada-Molina, B. Claude, A.M. Garcia-Campaña, M. Del Olmo Iruela, P. Morin, Convenient solid phase extraction of cephalosporins in milk using a molecularly imprinted polymer Food Chem. 135 (2012) 775–779.

[45] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J Schenck, Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce J. AOAC Int. 86 (2003) 412–431.

[46] S.J. Lehotay, K.A. Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska, E. Hoh, N. Leepipatpiboon, Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables,J. Chromatogr. A 1217 (2010) 2548–2560.

[47] H.G.J. Mol, P. Zomer, M. De Koning, Qualitative aspects and validation of a screening method for pesticides in vegetables and fruits based on liquid chromatography coupled to full scan high resolution (Orbitrap) mass spectrometry, Anal. Bioanal. Chem. 403 (2012) 2891–2908.

[48] R. Perez-Burgos, E.M.Grzelak, G. Gokce, J. Saurina, J.Barbosa, D. Barron, QuEChERS methodologies as an alternative to solid phase extraction (SPE) for the determination and characterization of residues of cephalosporins in beef muscle using LC-MS/MS, J. Chromatogr. B-Analytical Technologies In The Biomedical And Life Sciences 899 (2012) 57–65.

[49] G. Stubbings, T. Bigwood, The development and validation of a multiclass liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for the determination of veterinary drug residues in animal tissue using a QuEChERS (QUick, Easy, CHeap, Effective, Rugged and Safe) approach,Anal. Chim. Acta 637 (2009) 68–78.

[50] A. Garrido-Frenich, M.M. Aguilera-Luiz, J.L. Martínez-Vidal, R. Romero-González, Comparison of several extraction techniques for multiclass analysis of veterinary drugs in eggs using ultra-high pressure liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta 661 (2010) 150–160.

[51] M. Lombardo-Aguei, L. Gamiz-Gracia, C. Cruces-Blanco, A.M. García-Campana, Comparison of different sample treatments for the analysis of

quinolones in milk by capillary-liquid chromatography with laser induced fluorescence detection,J. Chromatogr. A 1218 (2011) 4966–4971.

[52] J.L. Martínez-Vidal, A. Garrido-Frenich, M.M. Aguilera-Luiz, R. Romero-González, Development of fast screening methods for the analysis of veterinary drug residues in milk by liquid chromatography-triple quadrupole mass spectrometry, Anal. Bioanal. Chem. 397 (2010) 2777–2790.

[53] E.G. Karageorgou, V.F. Samanidou, Development and validation according to European Union Decision 2002/657/EC of an HPLC-DAD method for milk multi-residue analysis of penicillins and amphenicols based on dispersive extraction by QuEChERS in MSPD format, J. Sep. Sci. 34 (2011) 1893–1901.

[54] E.G. Karageorgou, V.F. Samanidou, Application of ultrasound-assisted matrix solid-phase dispersion extraction to the HPLC confirmatory determination of cephalosporin residues in milk,J. Sep. Sci. 33 (2010) 2862–2871.

[55] L. Vera-Candioti, A.C. Olivieri, H.C Goicoechea, Development of a novel strategy for preconcentration of antibiotic residues in milk and their quantitation by capillary electrophoresis, Talanta 82 (2010) 213–221.

EVALUATION OF DIFFERENT STRATEGIES TO EXTRACT AND PRECONCENTRATE PENICILLINS PRESENT IN MILK PRIOR DETERMINATION BY CAPILLARY ELECTROPHORESIS

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Abstract

One of the main problems concerning the determination of residues of penicillins (PENs) in complex matrix, as milk samples of animal origin, is the sample treatment. The aim of this work was to evaluate the influence of different background electrolyte (BGE) composition and pH, found in the literature, in the determination of PENs by capillary electrophoresis (CE) and different sample treatments for the determination of PENs in bovine milk samples by CE. Off-line preconcentration, as classical solid-phase extraction (SPE) and QuEChERS (namely quick, easy, cheap, effective, rugged and safe) methodology and in-line preconcentration strategies, as large volume sample stacking (LVSS), were applied. In general, the milk sample treatment included protein precipitation prior to preconcentration. For this purpose, hydrochloric acid (HCl) and trichloroacetic acid (TCA) was mainly used. To evaluate the SPE steps, several commercial sorbents (Oasis HLB, Bond Elut C18 and Strata-X) were studied. In order to provide useful information enabling the determination of these analytes in routine laboratories, the strengthsand weaknessesof each of the sample treatments tested are presented. In this case, due to the maximum residue limits (MRLs) established for these analytes in milk samples, very clean extracts and with a low conductivity is necessary to apply additional preconcentration strategy (LVSS) in the CE proposed method.

Keywords: Capillary electrophoresis / Penicillins / Milk / Sample treatment/ QuEChERS / SPE.

1. Introduction

Nowadays the foods are produced and distributed in a global market which requires stringent legislation and regulation for food quality and safety in order to protect consumers and ensure fair trade. Regulatory agency as European Food Safety Authority (EFSA) and Food and Drugs Administration (FDA) require the availability of analysis methods in order to provide the data for risk assessment, the establishment of maximum residue limits (MRLs) and the development and execution of monitoring plans.

Regulatory requirements for veterinary drug in food, as penicillins (PENs) residues in milk, are fairly stringent. Rapid screening tests are used to determine whether to accept or reject tanker loads of milk. For this reason, there is a need for sensitive confirmatory tests that can be used to assess accuracy of the screening tests. Also, the MRLs of these substances in foodstuffs of animal origin are established in 2377/90/EEC regulation being 4 μg L^{-1} for amoxicillin (AMX), ampicillin (AMP), and penicillin G (PEN G) and, 30 μg L^{-1} for cloxacillin (CLX) and oxacillin (OXA) [1].

Analytical methods for detecting PENs and their levels in milk samples have been widely developed in recent years. Liquid chromatography coupled with different detection systems are the technique most commonly used for this purpose, however capillary electrophoresis (CE) is becoming a useful alternative technique in this field. CE is a separative analytical technique which is widely accepted due to its ability to simultaneously determine different analytes with both high efficiency and resolution, low consumption of samples and electrolytes, and short analysis times. The physicochemical properties of PENs, their ionizable nature and multiple ionization sites, make these compounds highly suitable for electrophoresis determination.

The determination of PENs by CE is mainly included in two different working modes: (i) capillary zone electrophoresis (CZE), and (ii) micellar electrokinetic chromatography (MEKC). Now there are a high number of different buffers used to determination of PENs, however, in the literature there is a lack of systematic study on the influence of different background electrolyte (BGE) composition in the separation of these analytes.

In the food analysis field, there are few CE methods for the determination of PENs in milk [2-6] and animal tissues [7-9]. This may be due to it is quite difficult to measure PENs in food because of the complexity of the biological matrix and the extremely low concentration of these compounds. Most of the works related with the separation of penicillin mixtures are applied to the determination of these compounds in commercial pharmaceutical products [10-21]. Another examples are related with the determination of PENs in matrices of environmental impact as water [8, 22-23], or in biological fluid samples [24-26].

In a complex matrix as milk, the sample treatment is still the major bottleneck in the analytical procedure. The determination of PENs in milk is a difficult task, due to its high protein and fat content, which often interfere in analytical procedures. Moreover, the analytes are often present at low concentration in these samples. In this case, it is essential to have an effective extraction and clean up steps to improve the selectivity of sample treatment and preconcentration step to improve the sensitivity of the method.

Different strategies for the extraction and preconcentration of PENs in milk samples have been used, such as liquid-liquid extraction (LLE) [27] and solid-phase extraction (SPE) [3-5]. The use of off-line SPE is probably the most widely used sample pretreatment procedure prior to CE determination. So far, different sorbents, such as C18, hydrophilic-lipophilic balance (HLB) and Alumina N have been used.

Recently, new methodologies have been proposed for the treatment of milk samples containing antibiotics, as PENs, by HPLC. Among them, QuEChERS (quick, easy, cheap, effective, rugged and safe) in matrix solid phase dispersion (MSPD) format with Strata X sorbent [28] is used. QuEChERS -an attractive method for sample preparation procedure- was introduced by Anastassiades et al. [29] in 2003 and was promising to provide a fast and reliable way to determine the target antibiotics in milk. The original procedure involves initial SPE of the sample with acetonitrile (ACN), followed by liquid–liquid partitioning by the addition of anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride. Removal of water and clean up are performed simultaneously on an aliquot of the ACN extract with dispersive SPE using $MgSO_4$ and primary secondary amine sorbent. The QuEChERS procedure has some advantages because it simplifies and reduces the time taken for the extraction and clean up processes. However, so farno

studieswere found where QuEChERSmethodology was usedas sample treatment in the determination ofPENs in milk by CE.

Although there are,at least, fivework for determination of PENs in milk samples by CE [2-6], as far as weknow, in the literature, there are no studiesthatpresentclear guidance for routine laboratories showing the strengthsand weaknesses of the different sample treatments already published. It can be confirmed that there is a lack of sample treatment protocol that generate extracts compatible with CE analysis. In this paper, a systematic evaluation of different BGE composition and pH, found in the literature for the determination of PENs by CE and different sample treatment procedures used for extracting PENs from milk samples prior CE analysis with UV-Vis detection is presented. In this context, some strategies of preconcentration, as classical SPE, QuEChERS and large volume sample stacking (LVSS) have been evaluated, in order to provide useful information for routine laboratories on the difficulty of determining these analytes in this type of sample.

2. Material and methods

2.1 Reagent and materials

All chemicals and solvents were of analytical grade.Sodium tetraborate ($Na_2B_4O_7$), sodium dihydrogenphosphate (NaH_2PO_4), hydrochloric acid (HCl), and ACN were purchased from Merck (Darmstad, Germany); sodium hydroxide (NaOH), trichloroacetic acid (TCA) and methanol (MeOH) were obtained from Panreac (Barcelona, Spain); and sodium docedyl sulphate (SDS) was purchased from Sigma. AMX, AMP, CLX, OXA, PEN G, and Naproxen (I.S) were obtained from Sigma (St. Louis, MO).

Individual stock solutions containing a 100 μg mL^{-1}concentration of each penicillin were prepared in water and stored at 4°C prior to use. Under such conditions, they were stable for at least 2 months. Working solutions (containing all PENs) were prepared daily by diluting the stock solutions in water. All water used was purified by passage through a Milli-Q system from Millipore (Bedford, MA).

The SPE cartridges used in this study were: Oasis hydrophilic-lipophilic balance (HLB) cartridge (500 mg, 12 cm^3; Waters, Milford, MA, USA), Bond Elut C18 (500 mg; Varian, Harbor City, CA, USA), Strata X-Phenomenex

(Torrance, CA, USA). Kits SampliQ QuEChERS (kindly supplied by Agilent Technologies Inc., Wilmington, DE, USA) consisted on extraction tubes (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive tubes (150 mg C18, 150 mg primary secondary amine (PSA) and 900 mg $MgSO_4$).

2.2 Electrophoretic conditions

P/ACE MDQ CE System from Beckman (Palo Alto, CA, USA) equipped with a DAD were used for the separation and quantification of PENs. Electrophoresis experiments were performed in a 60.2 cm x 75 μm id, uncoated fused-silica capillary (Beckman Coulter) with an optical path length of 220 mm and an effective length of 50 cm.

The BGE used was 35 mM of sodium tetraborate and 75 mM of SDS adjusted at pH 8.5. Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min, water for 5 min and separation buffer for 15 min. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, water for 5 min and separation buffer for 15 min; between runs the capillary was rinsed with water for 1 min, 0.1 M NaOH for 2 min, water for 1 min and separation buffer for 5 min.These solutions were filtered through a 0.45 μm nylon membrane before analysis.

2.3 Extraction procedure

Before the extraction procedure, milk samples were treated as follows: 50 mL aliquots of raw bovine milk were spiked with different aliquots of stock standard solution of the PENs studied: AMX, AMP, CLX, OXA and PEN G (structures shown in Fig. 1). Samples were shaken on a vortex mixer for 30 s and then allowed to stand for at least 20 min, to enable sufficient equilibrium with the milk matrix. Also, raw bovine milk samples from cows treated with PENs were used.

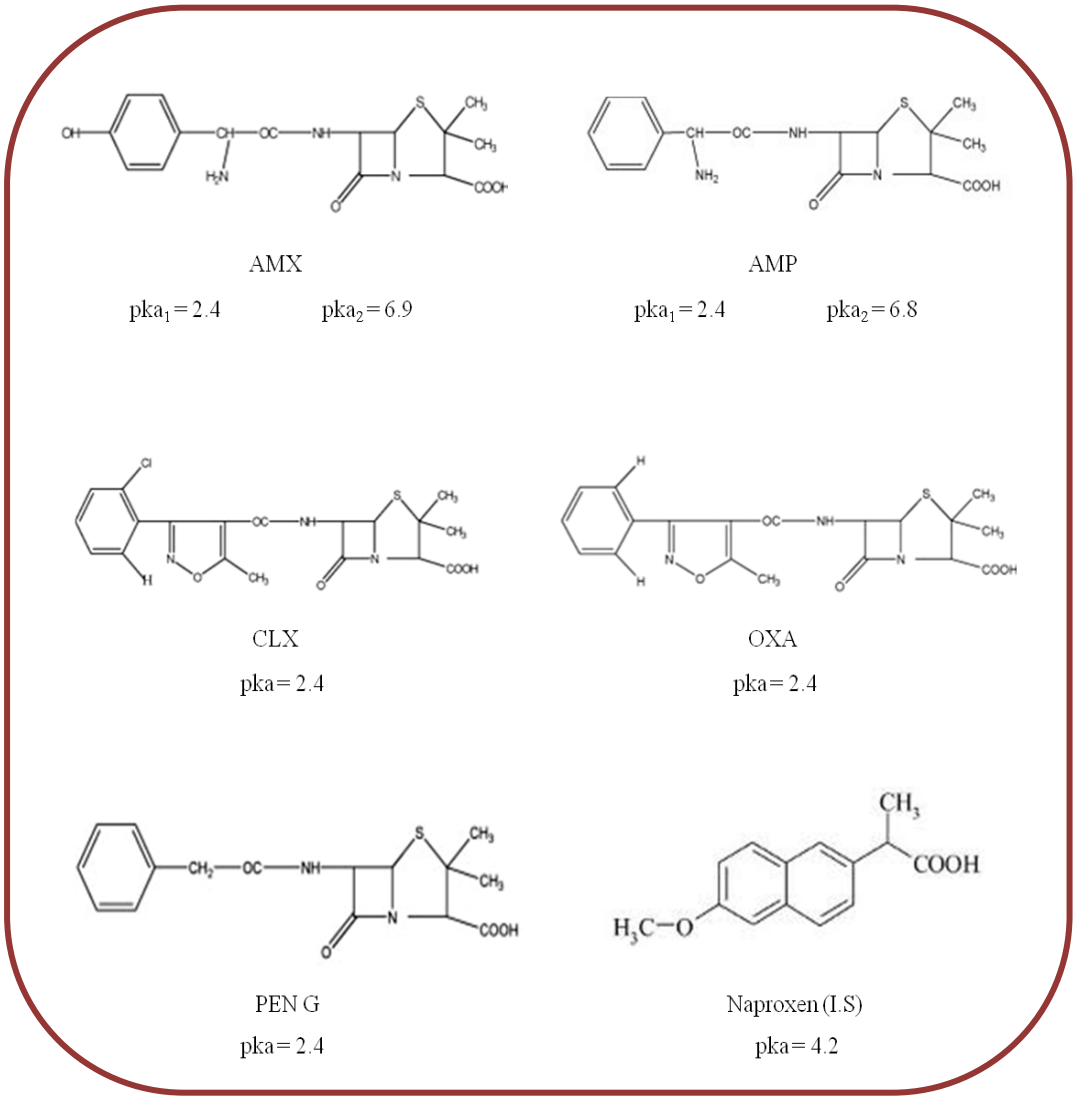

Fig. 1.*Chemical structures and pKa values of the studied PENs*

Two different methodologies for extraction and off-line preconcentration of analytes presents in milk samples were evaluated: classical SPE format and QuEChERS (Fig. 2).

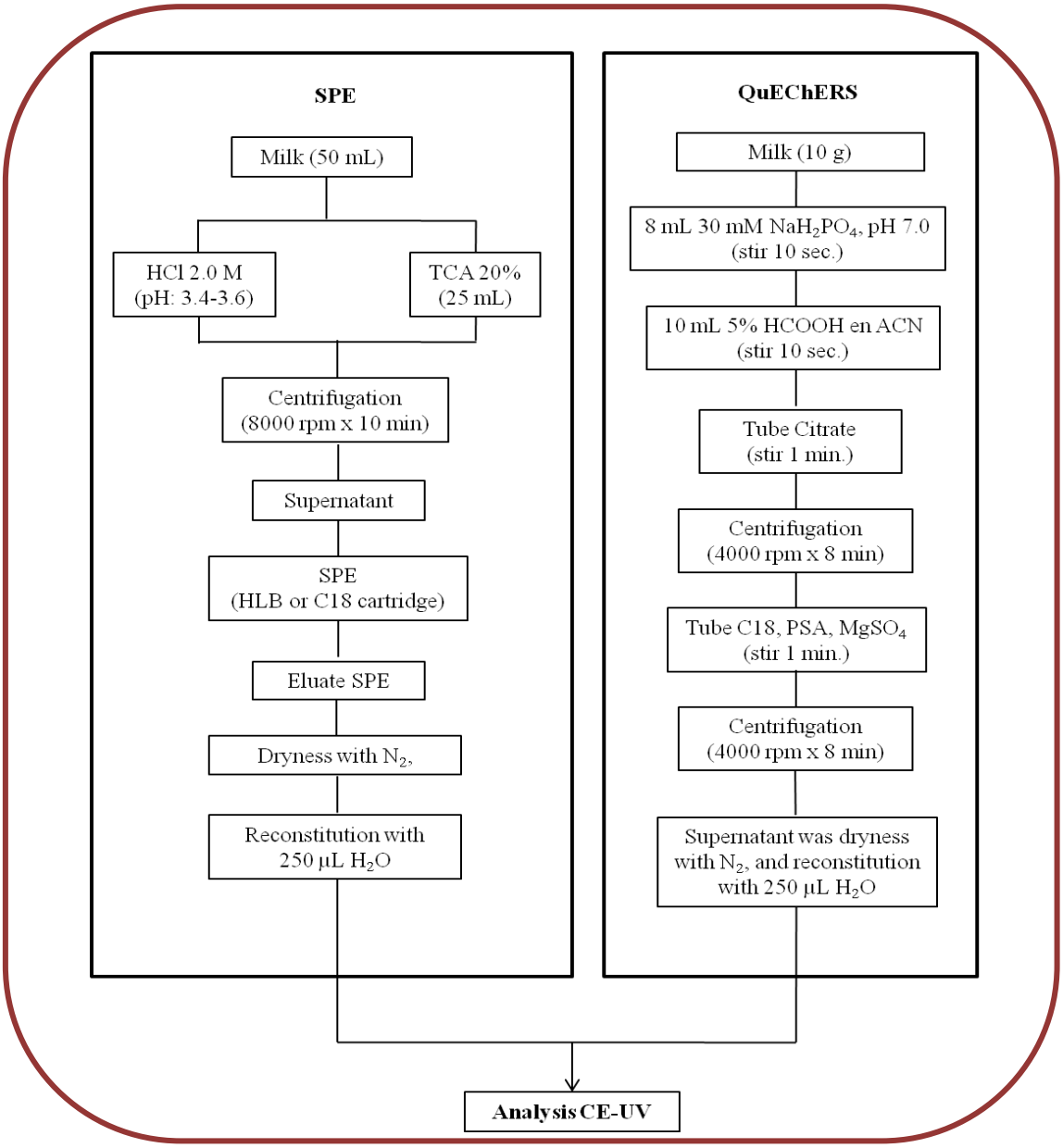

Fig. 2. *Different sample treatments (SPE and QuEChERS methodology) for the determination of PENs applied in this study.*

2.3.1 SPE

All the SPE experiments were performed at room temperature. A study related to the recoveries of the PENs using standard solutions was made in order to get an idea of the most advantageous sorbents for these analytes, testing 3 types of sorbents: HLB, C18 and Strata-X. Then the optimum sorbent was used to extract PENs in milk samples.

Different deproteination procedures used HCl 2 M or TCA 20% were tested prior to SPE. The milk sample (50 mL) was deproteinated by adding 2.0 M HCl to decrease the pH to 3.4–3.6 using a pH meter (Crison model pH 2000) or by adding 25 mL of 20% aqueous TCA. Thus the extract obtained was defatted by centrifugation (J. P. Selecta, Barcelona, Spain) at 8000 rpm for 10 min. Finally the extract was passed through a SPE cartridge. It was previously conditioned with 2 mL MeOH followed by 2 mL water. After rinsing with 2 mL water, the PENs were eluted with 1 mL MeOH and -1 mL ACN successively. The collected eluate was evaporated to dryness using a stream of nitrogen at 40°C. The residue was resuspended in 250 µL of water and analyzed by CE system.

2.3.2 QuEChERS

The QuEChERS procedure was adapted from that described by Agilent Technologies for the determination of quinolones in bovine liver [30]. Samples of 10 g of milk were spiked at different concentration levels of PENs using the working standard solutions. They were placed into 50 mL centrifuge tubes and homogenized in vortex. Then 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was added, shaking by hand for 10 s. Subsequently, 10 mL of 5% formic acid in ACN was added to the tube, shaking by hand for 10 s. Agilent SampliQ QuEChERS extraction tubes ($MgSO_4$, NaCl, sodium citrate, and disodium citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 4000 rpm for 8 min and 4 mL of the upper ACN layer was transferred to another tube containing the dispersive SPE (C18, PSA and $MgSO_4$) and

stirred in vortex for 1 min. The tube was centrifuged at 4000 rpm for 8 min. Then, all supernatant was transferred to a vial, dried at 40°C under a stream of nitrogen and reconstituted with 250 µL of water and analyzed by CE system.

2.4 LVSS procedure

Standard solutions containing the PENs were loaded for 270 s into the electrophoretic system so that the whole capillary was filled with the sample solution. Water was used as the sample solvent to produce a low-conductivity analyte matrix. A negative voltage (-20 kV) was then applied and the sample stacking started. Reverse polarity was applied for a time of 2.1 min. A positive voltage (20 kV) was then applied to separate the compounds.

3. Results and discussion

In this work, systematic study on the influence of different BGE composition and pH, found in the literature, for the determination of PENs by CE is presented. Also different procedures to extract and preconcentrate AMX, AMP, CLX, OXA and PEN G present in bovine milk have been evaluated. Off-line preconcentration strategies (classical SPE and QuEChERS) and in-line (as, LVSS) were applied. In order to provide useful information enabling the determination of these analytes in routine laboratories, the advantages and disadvantages of each of the sample treatments tested are here discussed.

3.1 Selection of the appropriate instrumental CE variables

All the preliminary studies were focused on the optimization of the experimental parameters affecting the CE separation of the target compounds by using UV-Vis detection. The UV-Vis spectra of the analysis were registered choosing a wavelength of 210 nm with a bandwidth of 8 nm for monitoring the selected PENs. To optimize the separation, the influence of the running buffer nature, its concentration and the pH were studied. 20 different running buffer found in the literature for the determination of PENs were initially tested (see Table 1). The different works have beenordered in thetableaccording to

thepHof the running buffer. CZE and MEKC modality were mainly used and basic pH was dominant over acid pH. The best resultswereobtainedby usinga mixture of sodium tetraborate and SDS as surfactant (MEKC mode). In general, this modality allows the separation of neutral and/or ionic antibiotics.

The influence of the concentration of sodium tetraborate (20–40 mM) and the concentration of SDS (60–100 mM) were investigated. The concentrations of sodium tetraborate and SDS are parameters with a more significant influence on sensitivity. A concentration of 35 mM of sodium tetraborate and 75 mM of SDS increased the area of all peaks, obtaining also an adequate electric current (below 110 μA). Due to this fact, both concentrations were selected for the separation of the PENs. The influence of buffer pH was also studied. The pH of the running electrolyte is one of the critical factors in resolution due to its impact on EOF in a fused-silica capillary, and the possible effect on solute charge altering relative migrations. The effect of pH value was investigated over the range of 7.5-8.5. From our experimental results, we can conclude that the pH of the buffer solution affects the resolution of the PENs studied. The best results were achieved at a pH of 8.5. With this buffer it was carried out the separation of the PENs tested (AMX, AMP, CLX, OXA, and PEN G), in less than 11 min, as it is shown in Fig. 3. Theseresults are consistent withseveral studies found in the literaturethat employ a similar BGE composition andpH, as it can be seen inthe works thatare highlighted in the Table 1.

A voltage of 15 kV was applied as optimum so as to achieve a good compromise between the running time, the resolution and the electric current. The effect of the temperature on the separation was investigated in the range of 20-30°C, lower values did not provide an adequate resolution for all the analytes. A capillary temperature of 25°C was selected as optimum. The figures of merit corresponding to PENs studied are shown in Table 2.

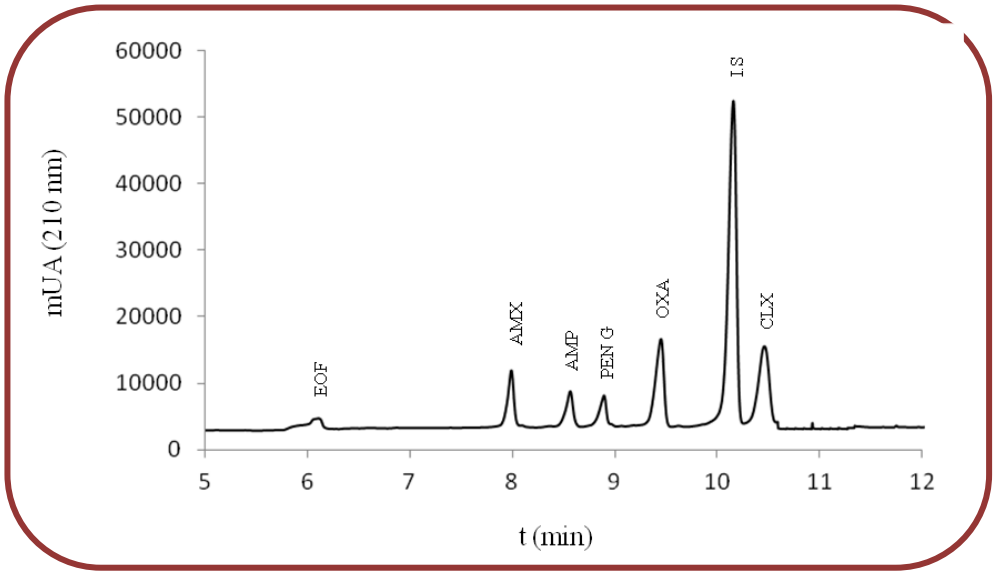

Fig. 3. *Electropherogram corresponding to standard solution mixture of five PENs (5 mg L^{-1} in ultra-pure water). AMX: amoxicillin; AMP: ampicillin; PEN G: penicillin G; OXA: oxacillin; CLX: cloxacillin; I.S: naproxen (2.5 mg L^{-1}). Experimental CE conditions were: 35 mM sodium tetraborate and 75 mM SDS, pH 8.5, separation voltage 20 kV, temperature 25 °C, hydrodynamic injection (applying 0.5 psi 10 s) and detection at 210 nm.*

The electrophoretic method was validated directly with standard solutions of PENs. In order to establish the standard calibration curve, solutions containing PENs were prepared at six concentration levels. Table 2 summarizes the LODs obtained with this methodology. LODs were determined by calculating three times the SD of the intercept divided by slope. With the CE using UV-Vis detection was notpossible to obtainLODsbelowMRLs establishedby legislation.

Table 1. BGE composition of the CE methods used previously for the separation of PENs

Analytes	BGE composition	pH	Ref.
AMX, AMP, CLX, OXA, and PEN G	50 mM Phosphoric acid and 5.2 mM 2-hydroxypropylbeta-cyclodextrin	3.6	[6]
	20 mM Ammonium acetate	5.1	[23]
	60 mM Ammonium acetate	6.0	[8]
	70 mM Sodium dihydrogenphosphate, 125 mM SDS, 5% ACN	6.0	[18]
	20 mM Ammonium acetate and 20 mM ammonium acetate in ACN/MeOH(60/40 v/v)	6.5	[35]
	40 mM Sodium dihydrogenphosphate and 100 mM SDS	7.0	[20]
	40 mM Phosphate-borate and 75 mM SDS	7.5	[15]
	175 mM Tris buffer with 20% ethanol	8.0	[4]
	2.7×10^{-2} M Potassium dihydrogenphosphate and 4.3×10^{-2} M sodium tetraborate	8.0	[5]
	60 mM Ammonium acetate with 10% of MeOH	8.0	[9]
	20 mM Sodium tetraborate with 60 mM SDS	8.0	[14]
	80 mM Sodium tetraborate decahydrate	8.0	[17]
	26 mM Sodium tetraborate with 100 mM SDS	8.5	[11]
	20 mM Sodium tetraborate and 75 mM SDS	8.5	[21]
	40 mM Sodium tetraborate and 100 mM SDS	8.5	[22]
	3.12 g/L Disodium hydrogenphosphate, 7.64 g/L sodium tetraborate and 14.4 g/L SDS	8.7	[16]
	20 mM Sodium tetraborate	9.0	[25]
	10 mM Sodium dihydrogenphosphate, 6 mM sodium tetraborate	9.0	[26]
	30 mM Tetraborate	9.2	[19]
	30 mM Sodium tetraborate	9.2	[24]

Table 2. Calibration curves with CE method for the determination of PENs

Analyte	$y = ax + b$	R^2	$S_{y/x}$	LOD	LOQ
AMX*	a = 7.4 x10^{-5} ± 1.7 x10^{-6} b = -0.06271 ± 0.00559	0.992	0.010	226.6	755.4
AMP*	a = 6.4 x10^{-5} ± 1.6 x10^{-6} b = -0.04563 ± 0.00518	0.991	0.009	242.8	809.4
CLX*	a = 1.9 x10^{-4} ± 7.2 x10^{-6} b = -0.19161 ± 0.02322	0.981	0.041	366.6	1222.1
OXA*	a = 1.3 x10^{-4} ± 3.5 x10^{-6} b = -0.09466 ± 0.01134	0.990	0.020	261.7	872.3
PEN G*	a = 5.7 x10^{-5} ± 1.8 x10^{-6} b = -0.02521 ± 0.00604	0.985	0.011	317.9	1059.6

*The values were obtained with respect to the internal standard (Naproxen). Concentration of penicillins in µg kg^{-1}; y: absorbance; *a:* slope; *b:* intercept; R^2*:* correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.

3.2 Optimization of LVSS

LVSS was applied to increase the analyte concentration prior to their separation. Significant parameters that influence the lvss such as injection time, voltage and time reverse polarity, and voltage normal polarity. To optimize the injection time, different values (90, 180, 210, 270 and 360 s) were used in each experience. These values were estimated, considered the diameter and length of the capillary and the applied pressure. We adopted an injection time of 270 s, since no gain in preconcentration factor was obtained by using longer times. The negative voltage was studied in the interval of -15 kV to -25 kV. The stacking voltage was kept at –20 kV in this step for 2.1 min, and a voltage of 20 kV applied for MEKC analysis. Electropherogram corresponding to a mixture of the selected PENs, after the application of the LVSS procedure, is shown in Fig. 4, where a significant increase in the signal scale is observed.

The statistic parameters calculated and the performance characteristics of the LVSS-CE method are presented in Table 3. The LOD obtained for the analytes in aqueous solution was up to 29 times lower when LVSS was used compared with CE.

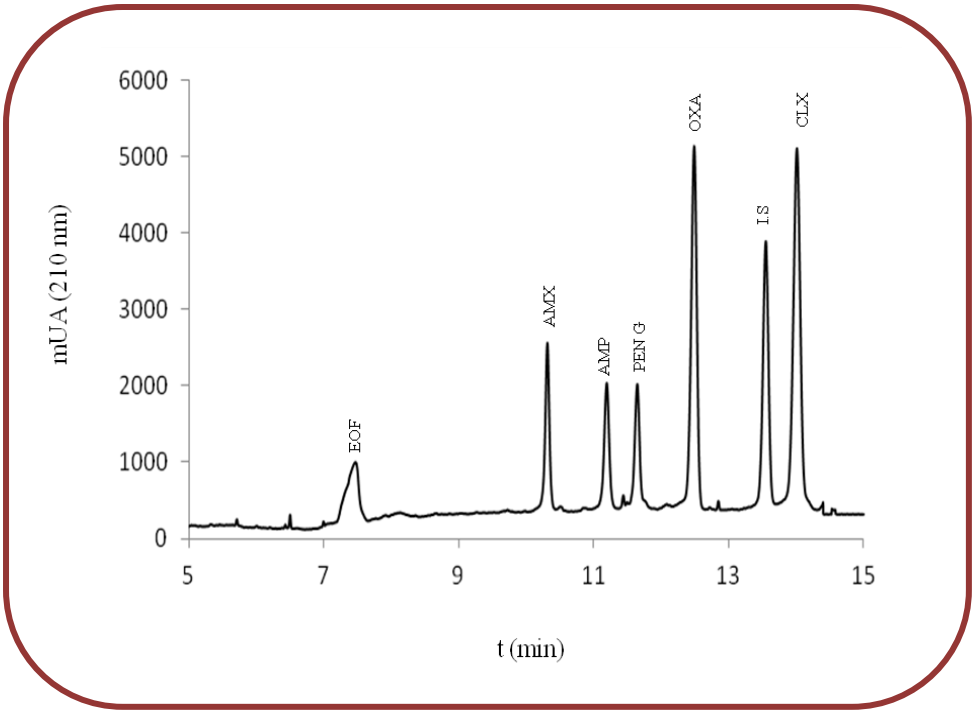

Fig. 4.*Electropherogram corresponding to standard solution mixture of five PENs (1 mg L^{-1} in ultra-pure water). AMX: amoxicillin; AMP: ampicillin; PEN G: penicillin G; OXA: oxacillin; CLX: cloxacillin; I.S: naproxen (0.1 mg L^{-1}). Experimental LVSS-CE conditions were: 35 mM sodium tetraborate and 75 mM SDS, pH 8.5, hydrodynamic injection (applying 0.5 psi 270 s), separation voltage (reverse polarity) -20 kV, time voltage in reverse polarity 2.1 min, separation voltage (normal polarity) 20 kV, temperature 25 °C and detection at 210 nm.*

Table 3. Calibration curves with LVSS-CE method for the determination of PENs

Analyte	$y = ax + b$	R^2	$S_{y/x}$	LOD	LOQ
AMX*	a = 0.00082 ± 0.00002 b = 0.02662 ± 0.01159	0.990	0.029	42.4	141.3
AMP*	a = 0.00094 ± 0.00001 b = -0.01796 ± 0.00483	0.997	0.013	15.4	51.3
CLX*	a = 0.00283 ± 0.00006 b = 0.04472 ± 0.03986	0.992	0.092	42.2	140.8
OXA*	a = 0.00228 ± 0.00001 b = -0.02367 ± 0.00694	0.998	0.022	9.1	30.4
PEN G*	a = 0.00101 ± 0.00001 b = -0.01677 ± 0.00545	0.996	0.015	16.1	53.9

*The values were obtained with respect to the internal standard (Naproxen).
Concentration of penicillins in µg kg^{-1}; y: absorbance; *a:* slope; *b:* intercept; R^2*:* correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.

3.3 Off-line preconcentration SPE

Three SPE sorbents like (Oasis HLB, C18 and Strata-X) were tested.Oasis HLB provided higher recoveries for AMX, AMP and PEN G and C18 sorbent for CLX and OXA, as shown in Fig. 5. The statistic parameters calculated and the performance characteristics of the SPE-CE method are presented using HLB (see Table 4). With this preconcentration method was reachedMRLsestablished forPENsstudied. Electropherogram corresponding to a mixture of the selected PENs, after the application of the SPE procedure with HLB is shown in Fig. 6. SPEallowedbetterLODfor differentanalytesstudied in aqueous matrix compared with CE and LVSS-CE, as shown the Table 5.

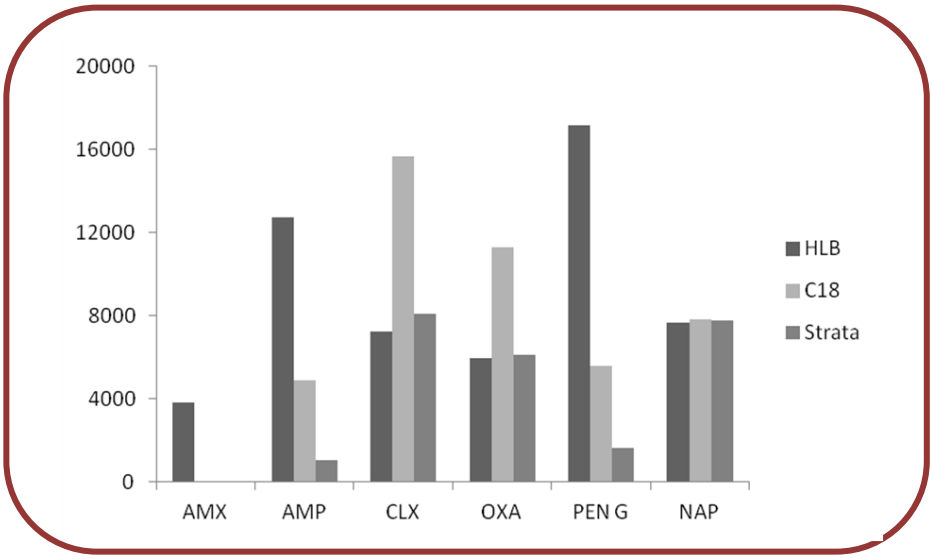

Fig. 5. *Peak area corresponding PENs studied obtained using different types of sorbents.*

Table 4. Calibration curves with SPE-CE method using HLB for the determination of PENs

Analyte	$y = ax + b$	R^2	$S_{y/x}$	LOD	LOQ
AMX*	a = 0.00148 ± 0.00004 b = 0.00217 ± 0.00158	0.991	0.003	3.2	10.7
AMP *	a = 0.00763 ± 0.00015 b = 0.00631 ± 0.00617	0.995	0.014	2.4	8.1
CLX*	a = 0.00132 ± 0.00002 b = 0.00743 ± 0.00148	0.994	0.003	3.3	11.2
OXA*	a = 0.00115 ± 0.00002 b = 0.00812 ± 0.00162	0.990	0.041	4.2	14.1
PEN G*	a = 0.01126 ± 0.00025 b = -0.00317 ± 0.01010	0.994	0.023	2.7	9.0

*The values were obtained with respect to the internal standard (Naproxen). Concentration of penicillins in µg kg^{-1}; y: absorbance; *a:* slope; *b:* intercept; R^2*:* correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.

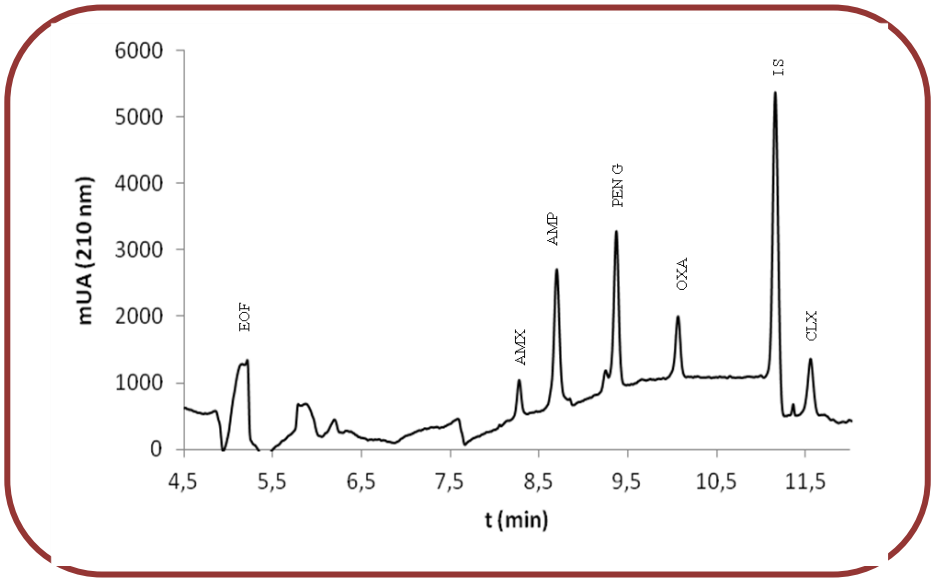

Fig. 6.*Electropherogram corresponding to standard solution of five PENs (25 µg L^{-1} in ultra-pure water) using SPE-CE method with HLB cartridge. AMX: amoxicillin; AMP: ampicillin; PEN G: penicillin G; OXA: oxacillin; CLX: cloxacillin; I.S: naproxen (25 µg L^{-1}). Experimental conditions were the same as in Fig. 3.*

Table 5. LOD using different methodologies

Analyte	CE	LVSS/CE	SPE**/CE	MRLs
AMX*	226.6	42.4	3.2	4.0
AMP*	242.8	15.4	2.4	4.0
CLX*	366.6	42.2	3.3	30.0
OXA*	261.7	9.1	4.2	30.0
PEN G*	317.9	16.1	2.7	4.0

*The analytes were dissolved in water in all cases
** HLB cartridge was used for SPE
Concentration of PENs in µg kg^{-1}; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G; LVSS: large volume sample stacking; MRLs: maximum residue limits.

3.4 Determination of PENs in real samples

Before conducting the SPE procedures to milk samples, a protein precipitation step was required. Different organic solvents, inorganic salts or strong acids have been currently used to precipitate proteins [31], in our case,the precipitating agentmost appropriatetoavoid compatibility problems between the extract and the electrophoreticsystemare theacids. In this study, HCl removed more interference and has a low dilution effect compared with TCA. HCl was added to reduce the pH among the range 3.4–3.6 and to remove the proteins present in milk sample (casein, mainly). Subsequently, the sample was centrifuged to remove precipitated proteins and the fatty material in the sample. HClhas been used ina previous studyconducted byour research group as deproteinizationagent on determination oforganic acids in goat milk samplesby CE [32]. This sample treatment allows partial removal of matrix interferences, making only thedetermination of AMP and PEN G in milk samples, as shown in Fig. 7.

Also, the QuEChERS procedure described by Agilent technologies for the determination of quinolones in bovine liver [30] has been adapted in this work for bovine milk samples. In our case, the final reconstitution step consisted on 250 μL of water. Figure 8 shows an electropherogram of bovine milk sample (solid line) and spiked bovine milk sample (dashed line) treated following the QuEChERS procedure using UV-visible detection at optimum method conditions. As can be observed, all examined analytes were resolved from matrix, except for AMP. An interference peak was found co-migrating with CLX. With this sampletreatment, AMX, PEN G and OXA can be detectedin milk samples.

Finally LVSS was used to preconcentrate the extract obtained from real samples (bovine milk) after the different sample treatments were applied. Notice that LVSS requires extracts with very low conductivity to obtain the best focusing of the analytes. For this reason was impossible toimplement this strategyin the milk matrix, in others samples (as farm waste water) couldbe aneffectivepreconcentrationstrategy. To increase the sensitivity of the CE method other detector, such as mass spectrometry (MS) or fluorescence (FL) should be tested.

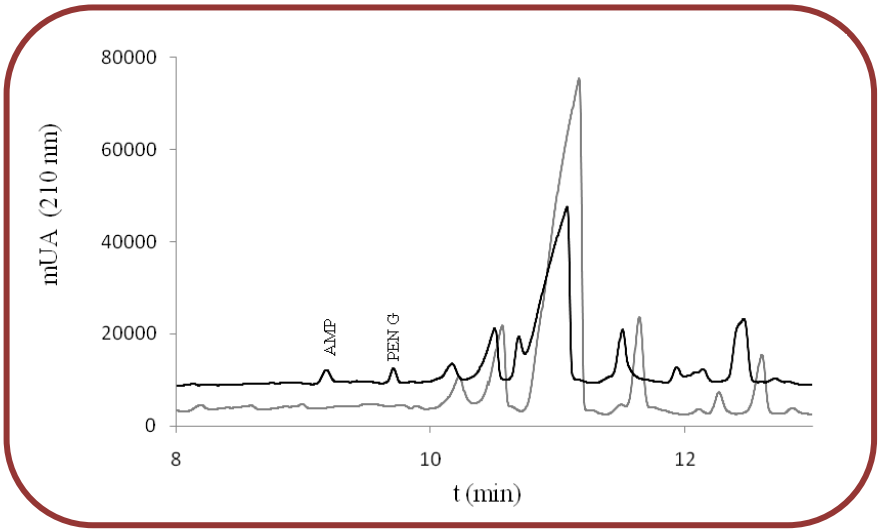

Fig. 7.*Electropherogram corresponding to blank and spiked milk sample with PENs using SPE-CE method with HLB cartridge. AMP: ampicillin; PEN G: penicillin G. Experimental conditions were the same as in Fig. 3.*

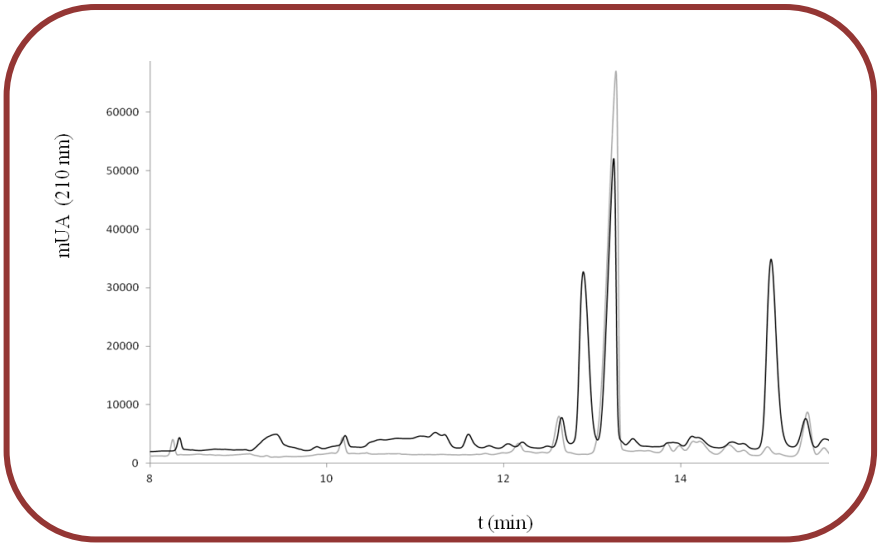

Fig. 8. *Electropherogram corresponding to blank and spiked milk sample with PENs at 10 mg L^{-1} extracted with QuEChERS and analyzed by CE method: AMX: amoxicillin; PEN G: penicillin G; OXA: oxacillin; CLX: cloxacillin. Experimental conditions were the same as in Fig. 3.*

4. Concluding remarks

Two different analytical strategies that combine MEKC-UV analysis with SPE or QuEChERS procedure, as off-line preconcentration technique for the determination of PENs in milk samples were presented. Deproteination with HCl followed by SPE or QuEChERS procedureproved to be efficient for removing matrix interferences, showing higherselectivity than otherprocedures evaluated. The sample treatment with SPE allowed the determination of AMP and PEN G, while QuEChERS procedure can be used for the determination of AMX, PEN G and OXA. The sample treatments propose are useful to reduce the number of interferences present in the milk samples but not to achieve the LODs required by the actual legislation. The sensitivity of the method can be increased by using MS and FL detector. These sample treatments are not suitable procedures to determine residues of PENs in milk samples, nevertheless these treatments could be useful to determine other antibiotics (such as fluoroquinolones) present in milk samples.

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5. References

[1] Establishment of Maximum Residues levels of Veterinary Medical Products in Foodstuffs of Animal Origin. European Community Council Regulation 2377/90. Off. J. Eur. Commun. 1990, L224, 1.

[2] C. Tian, H. Tan, L. Gao, H. Shen, K. Qi, Chinese J. Chromatogr. 29 (2011) 1128.

[3] Q. Zhang, N. Ye, X. Gu, X. Hao, N. Liu, Chinese J. Chromatogr. 26 (2008) 682.

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

[5] S.M. Santos, M. Henriques, A.C. Duarte, V.I. Esteves, Talanta 71 (2007) 731.

[6] Z. Zhu, L. Zhang, A. Marimuthu, Z. Yang, Electrophoresis 24 (2003) 3089.

[7] H.Y. Huang, W.L. Liu, B. Singco, S.H. Hsieh, Y.H. Shih, J. Chromatogr. A 1218 (2011) 7663.

[8] M.I. Bailón-Pérez, A.M. García-Campaña, M. Del Olmo Iruela, C. Cruces-Blanco, L.G. García, Electrophoresis 30 (2009) 1708.

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

[10] H.Y. Huang, S.H. Hsieh, Electrophoresis 29 (2008) 3905.

[11] M.I. Bailón-Pérez, L. Cuadros-Rodríguez, C. Cruces-Blanco, J. Pharm. Biomed. Anal. 43 (2007) 746.

[12] P. Puig, F. Borrull, C. Aguilar, M.J. Calull, Chromatogr. B-Analytical Technol. Biomed. Life Sci. 831 (2006) 196.

[13] G. Pajchel, K. Michalska, S. Tyski, J. Chromatogr. A 1087 (2005) 197.

[14] P. Puig, F. Borrull, M.J. Calull, C. Aguilar, Electrophoresis 26 (2005) 954.

[15] M. Dolezalova, B. Kunteova, R. Jobanek, J. Sep. Sci. 27 (2004) 560.

[16] G. Pajchel, K. Michalska, S. Tyski, J. Chromatogr. A 1032 (2004) 265.

[17] K. Michalska, G. Pajchel, S. Tyski, J. Chromatogr. B 800 (2004) 203.

[18] Y.M. Li, A. Van Schepdael, Y. Zhu, E. Roets, J. Hoogmartens, J. Chromatogr. A 812 (1998) 227.

[19] H.B. Wan, J. Liu, K.C. Ang, S.F.Y. Li, Talanta 45 (1998) 663.

[20] Y.X. Zhu, A. Van Schepdael, E. Roets, J. Hoogmartens, J. Chromatogr. A 781 (1997) 417.

[21] M.E.P Hows, D. Perrett, J. Kay, J. Chromatogr. A 768 (1997) 97.

[22] L. Nozal, L. Arce, A. Ríos, M. Valcárcel, Anal. Chim. Acta 523 (2004) 21.

[23] W. Ahrer, E. Scherwenk, W. Buchberger, J. Chromatogr. A 910 (2001) 69.

[24] A. Thomas, O.K. Upoma, J.A. Inman, A.K. Kaul, J.H. Beeson, K.P. Roberts, J. Biochem. Biophys. Methods 70 (2008) 992.

[25] M. Hernández, F. Borrull, M.J. Calull, J. Chromatogr. B 731 (1999) 309.

[26] S. Arrowood, A.M. Hoyt, M.J. Sepaniak, J. Chromatogr-Byomedical Applications 583 (1992) 110.

[27] L. Vera-Candioti, A.C. Olivieri, H.C. Goicoechea, Talanta 82 (2010) 213.

[28] E.G. Karageorgou, V.F. Samanidou, J. Sep. Sci. 34 (2011) 1893.

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

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

[31] M.A. Zezzi, Trends in sample preparation, Nova Science Publishers, New York, 2006.

[32] A. Carpio, V. Rodríguez-Estévez, M. Sánchez Rodríguez, L. Arce, M. Valcárcel, Electrophoresis 31 (2010) 2211.

CAPÍTULO IV:

Determinación de fluoroquinolonas en leche mediante CE y HPLC usando SPE como sistema de preconcentración off-line

INTRODUCCIÓN

Dentro de los antibióticos ampliamente empleados tanto en animales como en humanos se encuentran las quinolonas, cuya actividad bactericida ha sido mejorada o ampliada mediante la adición de uno o varios átomos de flúor, dando lugar a las llamadas fluorquinolonas o fluoroquinolonas (FQs) [1]. Una de las FQs más ampliamente usada en la actualidad es la enrofloxacina (ENR)o 1-ciclopropil-6-fluoro-1,4-dihidro-4-oxo-7-[4-etil-1-piperazinil]-3-quinolona ácido carboxílico, desarrollada exclusivamente para uso veterinario como herramienta terapéutica en el control y tratamiento de enfermedades infecciosas bacterianas como mastitis, infecciones gastrointestinales, respiratorias y del aparato urinario, causadas por bacterias Gram-negativas y Gram positivas. La ENR al catabolizarse parcialmente origina ciprofloxacina (CIP), la cual también posee actividad farmacológica [2, 3].

La ENR suele ser empleada en rumiantes, especialmente en ganado bovino y caprino. Este antibiótico cuando seadministra por vía intravenosa tiene una amplia distribución, con una importante llegada a los tejidos, tal como lo demuestra su amplio volumen de distribución. Tras su administración intramuscular, el antibiótico también muestra una rápida y completa absorción, con una biodisponibilidad cercana al 100%. Sin embargo, por cualquiera de las vías de administración (intravenosa e intramuscular) se logra una importante conversión de ENR a CIP por metabolismo hepático [4].

Debido a que la ENR y CIP son excretados del animal después de su administración, se debe esperar un lapso de tiempo (período de supresión o retiro) para que ocurra la desasimilación de éstos. Particularmente, la leche constituye una de las principales vías de excreción de las FQs, por lo que, en aquellos casos donde este tiempo de retiro no se cumpla, puede ocurrir la presencia de residuos de fluoroquinolonas en leche [3].

Cabe destacar que la presencia de FQs en leche representa un riesgo para la salud del humano, especialmente para la población infantil, por ser sus principales consumidores. En general, los antibióticos pertenecientes al grupo de las quinolonas, pueden causar efectos adversos específicos a nivel de los cartílagos de crecimiento en niños, también se ha observado que favorecen el desarrollo de artropatía en jóvenes, sin embargo el mecanismo por el cual se produce este efecto aún permanece sin aclarar. Los hallazgos patológicos, macroscópicos y microscópicos, en perros y ratas de laboratorio enfrentados a quinolonas, son de

agrupación de condrocitos y erosiones en el cartílago de crecimiento de las articulaciones que soportan el mayor peso del animal. Por otra parte, se ha determinado que pueden afectar diversos sistemas, provocando alteraciones a nivel gastrointestinal, renal, cardiovascular, sistema nervioso central, ocular, así como también alteraciones en la espermatogénesis, mutagenicidad y fotosensibilidad [5, 6]. Adicionalmente, el consumo de alimentos con residuos de FQs puede originar procesos alérgicos y resistencia bacteriana, así como también ocasionar inconvenientes en el campo de la tecnología de alimentos [7].

Esta situación apremia para que los laboratorios agroalimentarios de rutina dispongan de métodos sencillos y rápidos para determinar FQs en muestras complejas de alimentos como leche. Se han optimizado un número significativo de métodos analíticos para determinar estos antibióticos usando distintas técnicas de separación, principalmente HPLC y CE, empleando diferentes detectores (fotometría y fluorescencia mayoritariamente). Sin embargo, para la extracción de FQs en leche es necesario realizar un pretratamiento de la muestra que origine un extracto compatible con la técnica de separación seleccionada. Esta etapa de extracción constituye la principal fuente de error y dificulta su aplicación en un laboratorio de rutina. Un método optimizado para extraer FQs en leche de vaca cruda por HPLC no siempre será compatible si esos mismos antibióticos se quieren determinar en leche de cabra cruda o pasteurizada o viceversa. Existen suficientes estudios que refieren a la extracción en fase sólida (SPE) como etapa inicial para extraer las FQs de la leche. En general, se deben precipitar las proteínas antes de pasar la leche por el cartucho seleccionado, aunque este paso no siempre es necesario. Por otro lado, tampoco esta estandarizado cual es el mejor sorbente para extraer este tipo de analitos.

En este capítulo se presentan dos tratamientos de muestras, basados en SPE, para la extracción de ENR y su metabolito ciprofloxacina CIP en leche de vaca y cabra, empleando CE y HPLC, respectivamente. Para la determinación de FQs en leche de vaca cruda por CE, se realizó un proceso de desproteinización con HCl 2 M, seguido por SPE (con cartuchos HLB). El método propuesto mostró recuperaciones entre 89% y 97% para CIP y entre 93% y 98% para ENR. La precisión del método se evaluó en términos de repetitividad y reproducibilidad. Un segundo procedimiento se propuso para la determinación de ENR y CIP en leche de cabra cruda por HPLC. En este caso, el tratamiento de muestra se basó en un procedimiento sencillo y directo de SPE, sin previa precipitación de proteínas. Para

ello se evaluaron diferentes sorbentes para la SPE como octadecilo (C18), etilo (C2), ciclohexilo (CH) y fenilo (PH). Las mejores recuperaciones se obtuvieron en cartuchos C18. La preparación de la muestra por este método produce extractos completamente libre de interferencias con recuperaciones de hasta 99,7% para ENR y 95,9% para CIP. Elmétodo validadose aplicóa muestrasreales deleche provenientes de cabra tratadas con ENR. En ambos métodos, los límites de detección se encontraron por debajo de los límites máximos de residuos establecidos por organismos oficiales para estas FQs en leche [8, 9]. Los métodos propuestos presentan diversas ventajas en cuanto a preparación de la muestra, simplicidad de la etapa de extracción, reducción del uso de solventes y bajo coste, por lo que representan una alternativa atractiva para el monitoreo de ENR y CIP en leche en los laboratorios agroalimentarios de rutina.

REFERENCIAS

[1] R.C. Owens, P.G. Ambrose, Clinical use of the fluoroquinolonas, Med. Clin. North Am. 84 (2000) 1447-1469.

[2] M. Mitchell, Enrofloxacin. Therapeutic review. J. Exot. Pet Med. 15 (2006) 66-69.

[3] G.S. Rao, S. Ramesh, A.H. Ahmad, H.C. Tripathi, L.D. Sharma, J.K. MaliK, Disposition kinetics of enrofloxacina and ciprofloxacin following intravenous administration of enrofloxacina in goats, Small Rum. Res. 44 (2002) 9-15.

[4] L. Ambros, L. Montoya, V. Kreil, S. Waxman Dova, G. Albarellos, M. Rebuelto, R. Hallu, M.I. San Andres Larrea, Farmacocinética de la enrofloxacina y su metabolito ciprofloxacina en cabras. Rev. Argent. Prod. Anim. 27 (2007) 329-345.

[5] L. Otero, N. Mestorino, J. Errecalde,Enrofloxacina una fluoroquinolona de uso exclusivo en veterinaria. Parte II: Farmacocinética y Toxicidad. Analecta Veterinaria. 21 (2001): 42-49.

[6] R. Stahlmann, Clinical toxicological aspects of fluoroquinolonas. Toxicol. lett. 127 (2002) 269-277.

[7] M.D. Marazuela, M.C. Moreno-Bondi,Multiresidue determination of fluoroquinolonas in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection. J Chromatogr. A. 1034 (2004)25-32.

[8] Commission regulation No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in

foodstuffs of animal origin. Commission of the European communities. Off. J. Eur. Commun. L15 (2010) 1.

[9] Title 21 (Food and Drugs). Code of Federal Regulation. U.S. Food and Drug Administration (FDA). Vol. 6, Part 556 (2006) Revised April 1.

ELECTROPHORESIS

Electrophoresis 2012, 33, 2978–2986

AN EASY SAMPLE TREATMENT FOR THE DETERMINATION OF ENROFLOXACIN AND CIPROFLOXACIN RESIDUES IN RAW BOVINE MILK BY CAPILLARY ELECTROPHORESIS

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Abstract

An easy, selective, and sensitive method has been developed for the determination of enrofloxacin (ENR) and its main active metabolite, ciprofloxacin (CIP), in raw bovine milk using CE with UV detection at 268 nm. Milk samples were prepared by a clean-up/extractionprocedurebasedonproteinprecipitationwithhydrochlorideacidfollowed by being defatted by centrifugation and SPE using a hydrophilic-lipophilic balance cartridge. Optimum separation was obtained using a 50 mM phosphoric acid at pH 8.4 and the total electrophoretic run time was 6 min. Sample preparation by this method yielded clean extracts with quantitative and consistent mean recoveries from 89 to 97% for CIP and from 93 to 98% for ENR.LODs obtained were lower to the maximum residue limits for these fluoroquinolones. The precision of the ensuing method is acceptable; thus, the RSD for peak area and migration time was less than 8.5 and 0.5% for CIP and 9.9 and 0.9% for ENR, respectively. The results showed that the proposed method was efficient showing good recoveries, sensitivity, and precision for the studied compounds and could be satisfactorily applied in routine analysis for the monitoring of ENR and CIP residues in milk, due to its ruggedness and feasibility demonstrated.

Keywords: CE / Fluoroquinolones / Milk / Routine analysis /Sample treatment

1. Introduction

Fluoroquinolones (FQs) are synthetic antibacterial compounds used in humans and in food-producing animals for treatment of a variety of bacterial infections. Some FQs have been developed specifically for veterinary practice, which is the case of enrofloxacin (ENR), while others like ciprofloxacin (CIP) are restricted to human treatment [1]. However, in several animal species, ENR is deethylated to its primary metabolite CIP, and both ENR and CIP are found in the edible products of animals receiving ENR [2].

The use of ENR in lactating breeding animals may leave residues of ENR and CIP in milk. The widespread usage of antimicrobials may be responsible for the promotion of resistant strains of bacteria. Other problems related to the misuse of antibiotics are as follow: (i) they can produce allergic hypersensitivity reaction in some people; (ii) fermentation processes, such as the cheese or yoghurt elaboration, could fail; and (iii) the presence of antibiotics could hide the existence of pathogens in foodstuffs when bacteriological analyses are carried out [3]. Besides this, the high stability of FQs represents a significant risk to human health because the residues of these antibiotics can remain in milk after heat treatment and, therefore, can reach the dairy industry and consumers [4].

Consequently, it is necessary to control/monitor residual levels of these compounds, in order to meet regulatory requirements and especially to protect the consumer and the environment. For these reasons, both the Commission of the European Community [5] and the United States Food and Drug Administration [6] have established maximum residue limits (MRLs) of 0.1 mg kg^{-1} for ENR and CIP in milk. These low MRLs require the development of highly sensitive and selective methods for their monitorization in routine laboratories.

Traditionally, HPLC has been the most widely used technique for the determination of FQs in milk [7-11]. However, during the last years, CE has also been proposed for the determination of these compounds [3, 12-19] as an alternative technique (see Table 1). Compared with HPLC, CE has the advantages of high separation efficiency, short analysis time, ease of automation, small amount of sample and solvent consumption and low cost per analysis. In addition, CE can separate compounds in highly polar and water soluble matrices that have been traditionally difficult to handle by chromatographic techniques [20].

The application of CE in routine quality control laboratories of different fields have increased in the last years. Nowadays, CE methods have been incorporated into routine quality control testing in pharmaceutical, forensic and clinical laboratories. Although some analytical approaches to demonstrate the analytical usefulness of CE in food analysis have been presented [21, 22], this technique is still not well accepted in routine laboratories.

The majority of the studies found in the literature agree that one of the most difficult steps in antibiotic determination is the extraction and clean-up of the drug from the milk sample. FQs are commonly extracted from milk -a complex matrix due to its high protein and fat content- with two or more of the following procedures: (i) elimination of fat milk and/or a protein precipitation step (ii) analyte liquid-liquid extraction with organic solvents, and (iii) analyte SPE. However, the use of laborious procedures may cause lower recoveries and the difficulty of reproducing the optimized CE method in routine laboratories.

In the last few years, an extraction method named QuEChERS (quick, easy, cheap, effective, rugged and safe) has shown its usefulness in the determination of residues in food. QuEChERS methodology involves two steps: an extraction step based on partitioning via salting-out extraction, and a dispersive SPE step. The QuEChERS procedure described by Agilent technologies has been adapted for the determination of FQs in milk samples by capillary-liquid chromatography with laser induced fluorescence detection [11].

Another important aspect to consider is the transfer of CE methods. Many CE methods are now in routine use across a number of regulated industries following successful method transfer exercises. Santos et al. [23] presented an interesting paper to support the transfer of advances from CE research laboratories to routine laboratories.

In this study, we applied several strategies to extract FQs and/or clean-up milk sample (some of them found in the literature) and the strengths and/or weaknesses observed are here presented. The purpose of this study was to propose an easy and simple sample treatment for the extraction of ENR and CIP in raw bovine milk. The method validated involved clean-up and preconcentration procedures, based on protein precipitation followed by SPE, prior to CE analysis.

Table 1. Some analytical features of the 8 methodologies found in the literature for the determination of FQs in bovine milk by CE

Number of FQs determined in each sample	Number of samples analyzed	Sample treatment	BGE composition	Detector	LOD	Ref.
8	18	Two-step SPE procedure: Oasis MAX and HLB cartridges (without protein precipitation)	70 mM Ammonium acetate, pH 9.1	MS/MS	6 µg/kg	[3]
4	18	LLE	30 mM Tris and 4 mM phosphoric acid, pH 8.9	PGD	23-65 µg/L	[12]
4	6	Precipitation of protein and extraction with McIlvane buffer. SPE with Oasis HLB cartridge	40 mM $Na_2B_4O_7$ - 42 mM H_3BO_3- 28 mM NaH_2PO_4, pH 9.2	DAD	13.3-19.8 µg/kg	[13]
2	3	LLE and SPE with Oasis HLB cartridge	15 mM Phosphate, pH 8.5	ECL	10-15 µg/L	[14]
2	100	LLE and SPE with Oasis HLB cartridge	80 mM Ammonium acetate, pH 4.6	MS/MS	8 µg/kg	[15]
1	6	Samples were deproteinized by adding methanol, centrifuged and filtered	Sodium tetraborate, pH 10.0	UV	1000 µg/L	[16]
4	30	LLE and SPE with MIP	125 mM Phosphoric acid, pH 2.8	LIF	0.17-0.98 µg/kg	[17]
1	4	Protein precipitation, LLE and SPE with Oasis HLB cartridge	19.35 M Sodium borate, pH 9.5	DAD	170 µg/L	[18]
7	20	Protein precipitation, magnetic SPE	40 mM phosphate, pH 8.1	DAD	9-12 µg/L	[19]

LLE: liquid–liquid extraction; MIP: molecularlyimprinted polymer; PGD: potential gradient detection; ECL: electrochemiluminescence.

2. Experimental

2.1 Reagent and standards

All reagents used were of analytical grade. Phosphoric acid (H_3PO_4) and hydrochloric acid (HCl) were purchased from Merck (Darmstad, Germany), sodium hydroxide (NaOH) and methanol (MeOH) were obtained from Panreac (Barcelona, Spain), and acetic acid was purchased from Sigma (St. Louis, MO).

FQs standards (Lomefloxacin (LOM), CIP and ENR) were supplied by Sigma (St. Louis, MO). Ultrapure water (Milli-Q system, Millipore, Bedford, MA, USA) was used throughout the work.

Individual standardsolutions of LOM (internal standard), CIP and ENR at a concentration of 100 mg L^{-1} were prepared in 50 mM acetic acid, and stored in the refrigerator at 4°C. Under such conditions, they were stable for at least 2 months. Working solutions (containing LOM, CIP and ENR) were prepared daily in the range of 0.025-1.0 mg L^{-1} by appropriate dilution of the stock solutions.

2.2 Electrophoretic method

P/ACE MDQ CE System from Beckman (Palo Alto, CA, USA) equipped with a DAD were used for the separation and quantification of FQs. Electrophoresis experiments were performed in fused-silica capillary (Beckman Coulter) of 75 μm inner diameter, 60.2 cm total length and 50 cm effective separation length.

The BGE used was a 50 mM of phosphoric acid adjusted at pH 8.4 with 1.0 M NaOH. Before the first use, the capillary was conditioned by rinsing with 1.0 M HCl for 5 min, water for 1 min, 0.1 M NaOH for 10 min, water for 1 min and separation buffer for 15 min. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, water for 5 min and separation buffer for 10 min. Before each analysis, the capillary was rinsed with water, 0.1 M NaOH and water for 1 min each one and separation buffer for 3 min. All solutions were filtered through a 0.45 μm membrane filter of Nylon. The FQs were detected with a DAD at the adequate wavelength (268 nm). The injection was done hydrodynamically at a pressure of 0.5 psi for 5 s. Capillary temperature was 25°C and separation voltage was 25 kV.

2.3 Preparation of milk samples for analysis

Raw bovine milk samples (obtained from a local farm in Cordoba, Spain) were used in this study and they were frozen at -18°C until their analysis. A volume of 25 mL of defrosted milk was spiked with known variable amounts of

the analytes. The mixture was allowed to stand for 20 min at room temperature to allow the total interaction between the FQs and milk sample. Firstly, the milk sample was deproteinated by adding 2.6 mL of 2.0 M HCl to decrease the pH to 3.0-3.5 using a pH meter (Crison model pH 2000). Thus the extract obtained was defatted by centrifugation (J. P. Selecta, Barcelona, Spain) at 8000 rpm for 8 min. Finally, 10 mL of the extract, previously filtered through a 0.45 mm membrane filter of Nylon, was passed through an Oasis hydrophilic-lipophilic balance cartridge (HLB; 60 mg, 3 cm^3; Waters, Milford, MA, USA). It was previously conditioned with 2 mL MeOH followed by 2 mL water. After rinsing with 2 mL water, the FQs were eluted with 2 mL MeOH. The collected eluate was evaporated to dryness using a stream of nitrogen at room temperature. The residue was re-suspended in 300 μL of Milli-Q water and analyzed by CE system.

3. Results and discussion

The primary aim of this work was to develop an analytical method for the determination of ENR and CIP in raw bovine milk by CE-UV, involving minimal pre-treatment samples based in a simple protein precipitation, defatted and SPE. Because the main difficulty in the development of an analytical method for a complex matrix -as raw bovine milk- is the presence of interferences, so that various strategies to extract CIP and ENR and/or clean-up of milk sample were examined. In this work, an electrophoretic method has been optimized and validated and the whole procedure could be used in a routine food laboratory.

3.1. Optimization of the electrophoretic method

The BGE is an important factor for the separation of FQs. According to the characteristics of these analytes (see Figure 1), adequate separation between FQs can be achieved with basic buffers. The effect of several buffers at basicpH used by previous authors to separate FQs in biological matrix were here evaluated: 40 mM sodium borate decahydrated, 42 mM boric acid and 28 mM sodium diacid phosphate at pH 9.2 [13]; 70 mM ammonium acetate at pH 9.1 [3]; and, 50 mM of phosphoric acid at pH 8.4 [24]. The 50 mM phosphoric acid separation buffer with a pH value of 8.4 was selected for separation of CIP and ENR because better sensitivity and selectivity in the electrophoretic separation

were obtained. Figure 2 shows the electropherograms obtained by using these buffers.

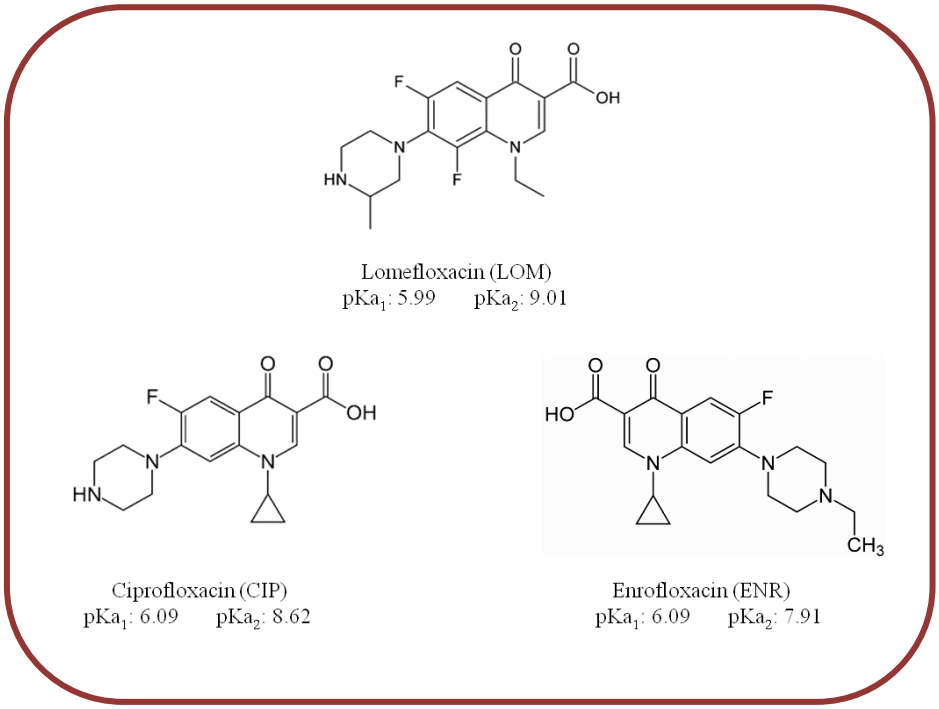

Fig.1.*Chemical structures, pKa values of the studied FQs: LOM (I.S), CIP and ENR.*

The temperature and voltage of the separation was established at 25ºC and 25 kV since a good resolution of the analytes was obtained in less time and with an acceptable current (below 160 µA). Therefore, using these buffer conditions and according to the structure and pKa values of the FQs [25], the analytes migrated towards the detection window in less than 6 min due to the EOF. Figure 3 shows an electropherogram (solid line) using UV-visible detection of a standard of FQs (LOM, CIP and ENR) at 1 mg L^{-1}.

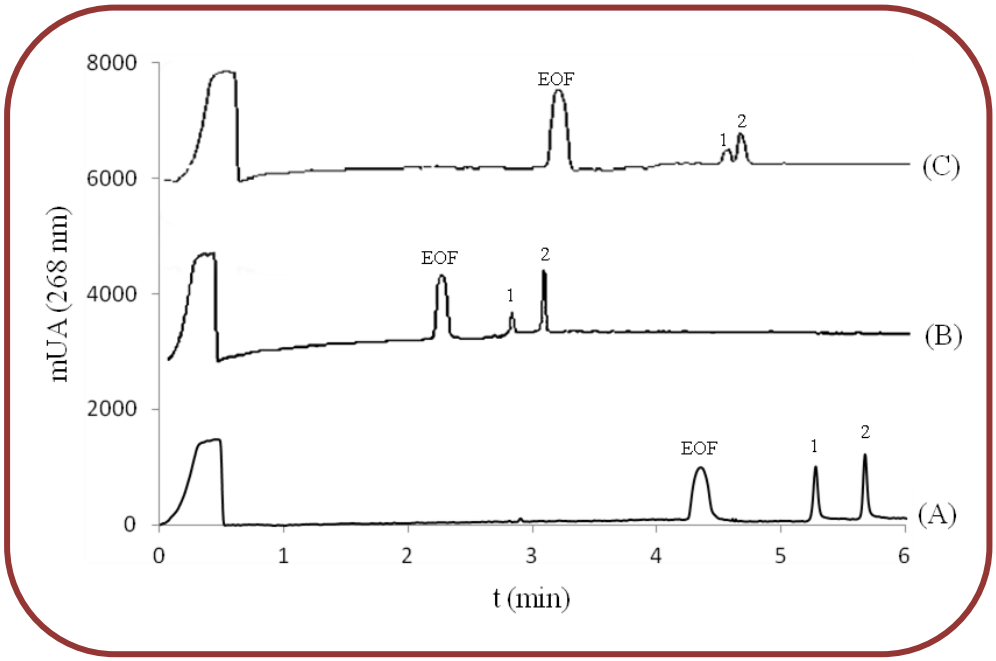

Fig. 2. *Electropherograms showing the effect of the separation buffer on the separation standard solution of FQs at 5 mg* L^{-1} *(1, CIP; 2, ENR). (A) Buffer consisting of 50 mM phosphoric acid, pH 8.4; (B) Buffer consisting of 70 mM ammonium acetate, pH 9.1; (C) 40 mM* $Na_2B_4O_7$ *- 42 mM* H_3BO_3 *- 28 mM* NaH_2PO_4*, pH 9.2*

3.2. Extraction and preconcentration of the analytes in milk samples

Various methodologies can been found in the literature for extraction of CIP and ENR in milk by CE. The traditional strategies for extracting the antibiotics were based on the removal of milk proteins with acidic solutions (buffer McIlvane/EDTA solution [13], HCl [26]) or organic solvents (dichloromethane [24], ACN [27] and ethanol [28]) followed by clean-up with SPE. From a theoretical point of view, any of them could be suitable for routine food laboratories since there is a lack of critical studies comparing the existing methodologies. It is possible to confirm that although all methods are potentially optimal, not all can be easily reproduced.

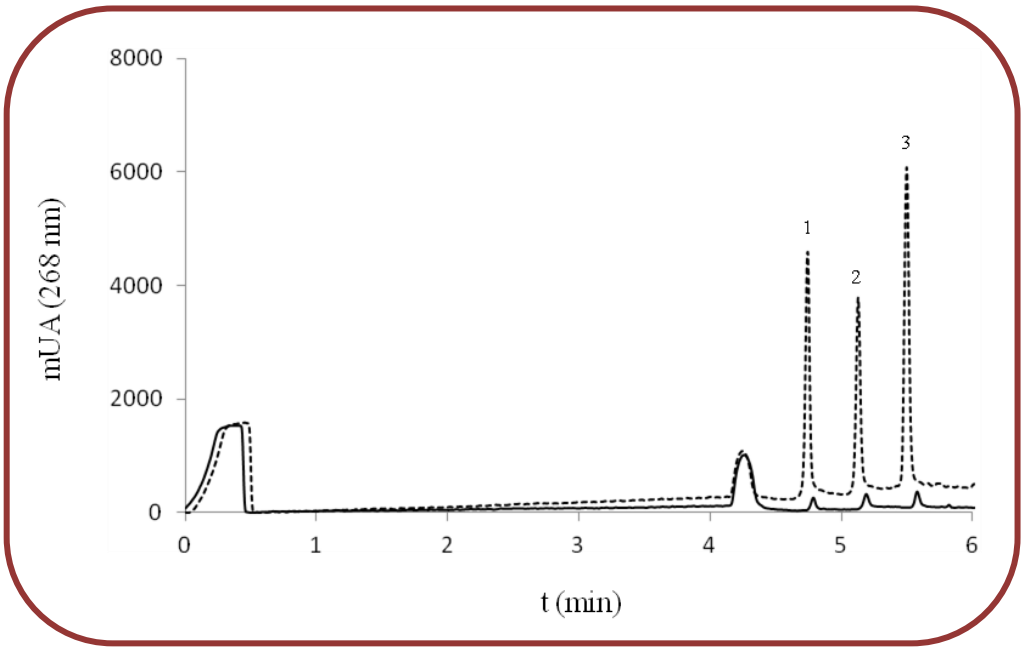

Fig.3.*Electropherograms corresponding to standard solution of FQs at 1 mg L^{-1} (1, LOM (I.S); 2, CIP; 3, ENR) before (solid line) and after of SPE (dashed line). Buffer consisting of 50 mM phosphoric acid, pH 8.4; voltage applied, 25 kV (normal polarity); UV detection at 268 nm.*

Because the milk matrix contains compounds such asproteins, lactose, and inorganic ions, the success of the extraction procedure depended on the effective deproteinization and washing steps [13], however the main analytical difficulty during the pretreatment of the milk samples was the co-extraction of the fat and/or protein and analytes.

In the present work, different strategies were applied to extract and preconcentrate the analytes prior to the analysis by CE. Some attempts were made to extract CIP and ENR directly from milk with SPE, without prior protein precipitation. The effectiveness of different extracting agents (buffer McIlvane/EDTA solution, dichloromethane, ACN and ethanol) for extraction and clean-up of CIP and ENR in bovine milk samples were also investigated. The main strength and/or weakness observedduring this investigation are shown

in Table 2. We should note that effective separation and identification of the FQs in raw bovine milk by CE-UV is impossible unless appropriate steps are taken to preconcentrate the analytes and remove interferences from the matrix.Finally, simple protein precipitation was performed using 2.6 mL of 2 M HCl. This acid was added to reduce the pH among the range 3.0-3.5 and to remove the proteins present in milk sample (casein, mainly). Subsequently, the sample was centrifuged to remove precipitated proteins and the fatty material in the sample. The extract was filtered, obtaining a clear solution. Additional clean-up and preconcentration procedure of the analytes extracted from the milk sample was included using HLB cartridge (see Figure 4).

3.2.1 Optimization of the SPE

In the literature, many sorbents and different conditions, washing and elution steps, in SPE have been proposed to improve the clean-up and preconcentration of antibiotics from food, biological tissues and water [24]. From previous studies [10, 18], the best results in the extraction of FQs in milk are obtained when polymeric sorbents are used. The Oasis HLB cartridge contain a polymeric macroporous poly[divinylbenzene-co-N-vinylpyrrolidone] that exhibits both hydrophilic and lipophilic retention characteristics retaining both polar and nonpolar compounds. The retention of FQs on the HLB cartridge has been attributed to hydrogen bonding between the piperazinyl amine group of the FQs and the carbonyl on the vinylpyrrolidone of the HLB sorbent [29]. In this study, HLB cartridges were used for subsequent extractions.

In order to obtain the maximum recovery of the analytes, the extraction conditions were optimized. The parameters evaluated for the optimization of the SPE procedure were: sample volume and composition and volume of the eluting solution. In order to ensure a high preconcentration factor, we examined the effect of the sample volume in the range of 8 at 15 mL of a standard solution containing 1 mg L^{-1} of FQs. The maximum acceptable volume for 60 mg cartridges was found to be 10 mL. To optimize the elution step, several composition and volumes of eluting solution were tested. A volume of 2 mL of MeOH was used for the elution of FQs from the HLB

cartridge. The preconcentration factor found under these conditions was between 12 and 11 for CIP and ENR, respectively (see Fig. 3).

Table 2. Different strategies applied in the present work for the extraction of FQs and/or clean-up of milk sample

Sample treatment		Ref.	Strength and/or weakness observed experimentally by us
Procedure	**Extracting agent**		
LLE and SPE	McIlvane buffer and HLB cartridge	[13]	Simple clean-up system, but protein precipitation was incomplete and low recoveries for all FQs.
LLE and SPE	Dichloromethane and HLB cartridge	[24]	Inefficient extracting agent. Low recoveries for the FQs.
Deproteinization and Ultrafiltration	HCl and Amicon-10 microfilter	[26]	The LODs obtained for all FQs were higher than the MRLs established for these antibiotics
LLE and SPE	ACN and HLB cartridge	[27]	Numerous peaks caused by extraction solvent interfering with the target analytes resolution
LLE and SPE	Ethanol-1% acetic acid (99:1) and HLB cartridge	[28]	Inefficient extraction in LLE process due to strong emulsification of the milk
SPE[a)]	HLB cartridge	[c)]	It removes a lot of interfering substances present in the milk sample. In some cases the cartridge was obstructed
Deproteinization and SPE[a), b)]	HCl and HLB cartridge	[c)]	The LODs obtained for all FQs were lower than the MRLs established for these antibiotics

a) New procedures applied in this study.
b) Procedure selected.
c) Procedure has not been referenced before in the bibliography.
LLE, liquid–liquid extraction.

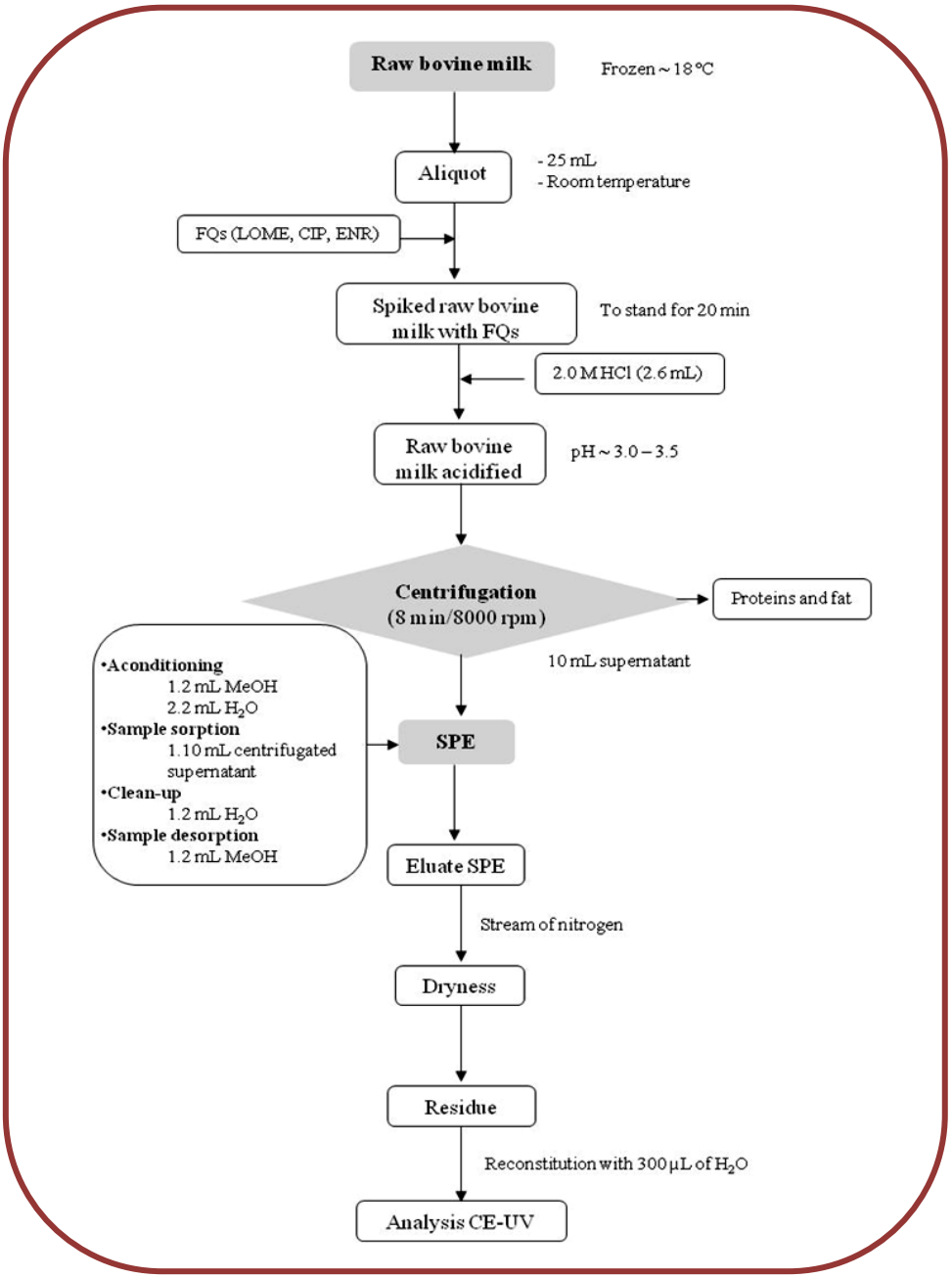

Fig. 4. *Sequence of sample clean-up and extraction of CIP and ENR from raw bovine milk samples.*

The electropherogram of the extract from spiked milk samples showed the presence of non-identified endogenous peaks at different migration times with high signals, which interfered with the detection and quantification of the FQs, mainly CIP. Moreover, the repeatability of the extraction process was low, and changes in the migration times between runs and peak broadening were observed, making this methodology unsuitable. Therefore, before conducting the SPE procedure to milk samples, we applied a protein precipitation step with HCl to remove potentially interfering compounds from the matrix sample. For it, 2 M HCl was used to remove proteins by precipitation and the fat was eliminated by centrifugation. This precipitating agent was chosen because it removes interferences and has a low dilution effect.

The method proposed was highly selective, sensitive and also offering high-resolution separations at a minimal cost in terms of sample size, reagent consumption, and operator time. Figure 5 shows an electropherogram of raw bovine milk sample or blank (solid line) and raw spiked bovine milk sample at 0.25 mg kg^{-1} concentration level (dashed line) using UV-visible detection at optimum method conditions.

Once the extraction process was optimized and the suitability with the CE method demonstrated, the method was validated using raw bovine milk samples fortified with several levels of FQs stock standard solution.

3.3 Validation of the method

The final goal of the validation of an analytical method is to ensure that every future measurement in routine analysis will be close enough to the unknown true value for the content of the analyte in the sample [30]. Analytical methods need to be validated or revalidated before their introduction into routine use whenever (i) the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix), and (ii) the method is changed and the change is outside the original scope of the method [31]. The analytical method was validated according with the International Conference on Harmonization (ICH) [32], FDA [33] and Commission Decision 2002/657/EC [34] in terms of linearity,

decision limit, detection capability, LOD, LOQ, selectivity, precision, accuracy (by means of recovery studies) and ruggedness.

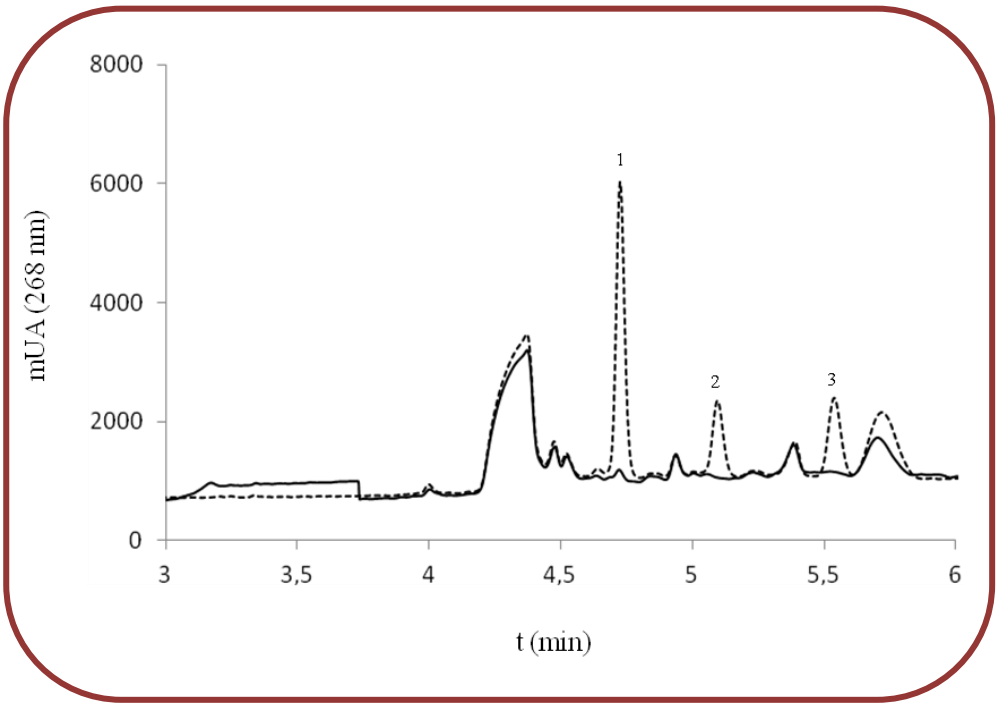

Fig. 5. *Electropherograms of the blank sample (solid line) and spiked milk sample (dashed line) with FQs (1, LOM, 1 mg kg^{-1} (I.S); 2, CIP, 0.25 mg kg^{-1}; 3, ENR, at 0.25 mg kg^{-1}), precipitated with 2 M HCl and extracted with Oasis HLB cartridges. Buffer consisting of 50 mM phosphoric acid, pH 8.4; voltage applied, 25 kV (normal polarity); UV detection at 268 nm.*

First, the calibration curves were calculated by linear regression, plotting the response factor (peak area analyte/internal standard peak area) as a function of analyte concentration. The equations of calibration curves obtained based on three replicate measurements of standard solution are shown in Table 3. In order to evaluate matrix effects, raw bovine milk sample was used as matrix. Six concentration levels of CIP and ENR were prepared over the concentrationrange 0.05-0.5 mg kg^{-1}and spiked before sample treatment. Then

they were subjected to the analytical procedure and analyzed by triplicate for each concentration level. In all cases, 1 mg kg^{-1}LOM was added as IS. This compound is a fluoroquinolone (FQ) only applied for human use, which has been selected as IS because it presents a satisfactory stability and purity and its use is forbidden in veterinary medicine. The application of the IS can improve significantly the quantitative performance of the method in terms of precision, linearity and recovery data.

Table 3. Calibration curves with off-line SPE and CE for the determination of CIP and ENR in milk

Concentration of FQs in mg kg−1; y, absorbance; *a*, slope; *b*, intercept; *R2*, correlation coefficient.

Analyte	Range of concentration tested	$y = ax + b$	R^2	LOD	LOQ	CCα	CCβ
CIP[a)]	0.05-0.5	a = 1.351 ± 0.051 b = -0.031 ± 0.015	0.983	0.03	0.1	0,11	0,13
ENR[a)]	0.05-0.5	a = 1.795 ± 0.039 b = 0.0016 ± 0.011	0.991	0.02	0.06	0,12	0,17

[a)] The values were obtained with respect to the internal standard (LOM).

The LOD and LOQ were calculated as three and ten times the standard deviation of the intercept among the slope, respectively. LODs obtained were lower than the MRLs legislated to FQs [5], as shown in the Table 3.

The decision limit (CCα) is the limit at and above which it can be concluded with an error probability of that a sample is non-compliant. The detection capability (CCβ) means the smallest content of a substance that may be detected, identified, and/or quantified in a sample with an error probability of β [34].To determine the CCα, 20 blank samples of raw bovine milk were spiked with each FQ at MRL concentration (0.1 mg kg^{-1} for CIP and ENR). CCα is equal to the concentration at the permitted limit plus 1.64 times the corresponding SD. CCβ was calculated as CCα plus 1.64 times the corresponding SD. Table 3 summarizes the CCα and CCβ values for CIP and ENR in bovine milk samples.

The selectivity indicates the ability of the method to accurately measure the analyte response in the presence of potentially interfering sample components. With the aim of verifying that the FQs peaks correspond to the pure compounds, a comparison between the electropherogram of a milk sample spiked with 0.25 mg kg^{-1} of each FQ and the electropherogram of the blank sample after the extraction procedure was performed (see Figure 5). As a result, the separation of FQs from other peaks present in the sample matrix was satisfactory. Although in some of the samples analyzed, the presence of a small peak that interferes with the CIP peak was observed. This interference was evaluated in terms of concentration and it can be confirmed that the area of this unknown peak corresponds to 0.017 mg kg^{-1} of CIP being this value insignificant for quantization purpose. Moreover, four other FQs (danofloxacin, marbofloxacin, difloxacin and flumequine) were analyzed using the proposed method and all analytes (including LOME, CIP and ENR) were separated from selective way. Therefore this method could be used for the determination of these seven FQs.

The precision of the method has been evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by means of repetitive application of the whole procedure by using spiked raw milk samples (0.1 mg kg^{-1}). The intraday precision was assessed on the same day by means of repetitive application (six times) of the SPE procedure (experimental replicates) and each sample was injected by triplicate (instrumental replicates). Intermediate precision was assessed with a similar procedure, but the samples were analyzed in three consecutive days. The results obtained for the precision of the full method and electrophoretic method, expressed as %RSD of peak areas and migration times, are summarized in Table 4. As can be observed, very good results were obtained in all cases.

Accuracy was evaluated computing recoveries by using the standard addition method. Known amounts of CIP and ENR stock standard solutions were added to raw bovine milk samples at two concentration levels (0.1 mg $kg^{-1}$and 0.2 mg kg^{-1}). In all cases, each level of concentration was tested in triplicate analysis, and each sample injected three times. In order to evaluate possible interferents, blank samples were submitted to the proposed method and no matrix peaks were found co-migrating with the analytes. The calculated

recoveries are shown in Table 5. As can be seen, the method provides good trueness in terms of recovery (from 89% to 97% and from 93% and 98% for CIP and ENR, respectively) and precision.

Table 4. Precision study of the electrophoretic method and the full method (including precipitation procedure and SPE before CE)for the determination of CIP and ENR in milk.

Analyte	Intraday precision (*n*=6, %RSD)		Interday precision (*n*=9, %RSD)	
	Migration time	*Peak area*	*Migration time*	*Peak area*
Electrophoretic method				
CIP [a)]	0.4	6.1	1.7	5.8
ENR [a)]	0.6	7.5	3.0	8.1
Full method (precipitation+SPE + CE)				
CIP [a)]	0.5	8.5	2.1	11.5
ENR [a)]	0.9	9.9	3.2	13.2

[a)]The values of %RSD were obtained with respect to internal standard (LOM)

Table 5. Values of recoveries obtained in different raw bovine milk samples for different levels of concentration

Analyte	Concentration added (mg kg^{-1})	Concentration found (mg kg^{-1})	% Recovery
CIP	0.1	0.084	89 ± 4
		0.091	
		0.092	
	0.2	0.212	97 ± 9
		0.175	
		0.198	
ENR	0.1	0.101	98 ± 4
		0.094	
		0.100	
	0.2	0.176	93 ± 8
		0.181	
		0.206	

Finally, the ruggedness evaluates the constancy of the results when external factors such as analyst, instruments, laboratories, reagents, days are varied deliberately [31]. Considering the instrumental transfer problems in CE methods, the effects of different analysts, reagents, and analysis days, on the responses migration time, peak areas, separation selectivity and resolutions, were examined. The relative values from migration time and peak area after analyzing a milk sample fortified with 0.1 mg $kg^{-1}$of CIP and ENR by two different analyst, reagents, analysis days (procedure 1 and 2) and also different instruments (procedure 3) are summarized in Table 6. The results show that there are statistically significant differences in the relative values from peak area of CIP and relative values from migration time of ENR applying a test ANOVA to a confidence level to 99%. These results were statistically different due to the variance of the instrument. Therefore if only the procedures 1 and 2 are compared, statistically significant differences were not observed since the analyses were performed in the same instrument but with different analyst, reagents and analysis days.

Table 6. Ruggedness study of the method proposed for the determination of CIP and ENR at 0.1 mg kg^{-1} added to a milk sample.

Procedure	CIP		ENR	
	Migration time	*Peak area*	*Migration time*	*Peak area*
1	1.072±0.018	0.458±0.135	1.150±0.029	0.378±0.110
2	1.090±0.006	0.386±0.105	1.173±0.009	0.354±0.023
3	1.0708±0.0005	0.213±0.013	1.132±0.004	0.404±0.030

The values of peak area and migration time were obtained with respect to I.S (LOM). Each procedure involved different analyst, reagents and day of analysis. Procedure 3 also involved different instrument. ANOVA´s test was applied to a 99% of confidence level. CIP: ciprofloxacin; ENR: enrofloxacin

4. Concluding remarks

In this work, we applied various methodologies found in the literature for the extraction of FQs in milk samples; however, we have not achieved optimum results with any of these methodologies and observe it more difficult in those methodologies that include complex procedures. In order to present a reproducible procedure and easy to implement in routine laboratories for determination of drug in food, we have presented a fast, simple, sensitive and selective CE-UV method for the determination of ENR and it main metabolite CIP in raw bovine milk. Previously to the CE analysis, a deproteinization defatted and SPE procedure for extraction, off-line preconcentration and sample clean-up were used. The extraction procedure is quick, effective and cheap showing high sample throughput. The LOD was lower than the MRLs regulated by the European Union for these compounds in milk.

The developed method could be satisfactorily applied as a routine procedure to identify and quantify CIP and ENR in laboratories of food quality and safety control and also for the monitoring of these residues in milk, due to its ruggedness and feasibility.

Acknowledgments

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5. References

[1] Brown, S. A., *J. Vet. Pharmacol. Therap.*, 1996, *19*, 1-14.

[2] Fu, L. X., Jiang, Z. G., Ding, H. Z., Liu, Y. H., *J. Vet. Pharmacol. Therap.*, 2008, *31*, 240-245.

[3] Lara, F. J., García-Campaña, A. M., Alés-Barrero, F., Bosque-Sendra, J. M., García-Ayuso, L. E., *Anal. Chem.*2006, *78*, 7665-7673.

[4] Roca, M., Castillo, M., Marti, P., Althaus, R. L., Molina, M. P., *J. Agric. Food Chem.* 2010, *58*, 5427-5431.

[5] Commission of the European communities, Commission regulation No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, *Off. J. Eur. Commun.* 2010, *L15*, 1-72.

[6] U.S. Food and Drug Administration (FDA). Code of Federal Regulation, 2006. Title 21 (Food and Drugs), Vol. 6, Part 556. Revised April 1.

[7] Cinquina, A.L., Roberti, P., Giannetti, L., Longo, F., Draisci, R., Fagiolo, A., Brizioli, N. R., *J. Chromatogr. A* 2003, *987*, 221-226.

[8] Marazuela, M. D., Moreno-Bondi, M. C., *J. Chromatogr. A* 2004, *1034*, 25-32.

[9] Idowu, O. R., Peggins, J. O., *J. Pharm. Biomed. Anal.* 2004, *35*, 143-153.

[10] Hermo, M. P., Nemutlu, E., Kir, S., Barrón, D., Barbosa, J., *Anal. Chim. Acta* 2008, *613*, 98-107.

[11] Lombardo-Agüí, M., Gámiz-Gracia, L., Cruces-Blanco, C., García-Campaña, A. M., *J. Chromatogr. A* 2011, *1218*, 4966-4971.

[12] Yongxia, F., Xiao, G., Shuo, L., Weidong, Q., *Electrophoresis*2007, *28*, 4101-4107.

[13] Sun, H., Zhao, W., He, P., *Chromatographia* 2008, *68*, 425-429.

[14] Zhou, X., Xing, D., Zhu, D., Tang, Y., Jia, L., *Talanta* 2008, *75*, 1300-1306.

[15] Blasco, C., Picó, Y., Andreu, V., *Electrophoresis*2009, *30*, 1698–1707.

[16] Solangi, A. R., Memon, S. Q., Mallah, A., Khuhawar, M. Y., Bhanger, M. I., *Biomed. Chromatogr.*2009, *23*, 1007-1013.

[17] Lombardo-Agüí, M., García-Campaña, A. M., Gámiz-Gracia, L., Cruces-Blanco, C., *J. Chromatogr. A* 2010, *1217*, 2237-2242.

[18] Vera-Candioti, L., Olivieri, A. C., Goicoechea, H. C., *Talanta* 2010, *82*, 213-221.

[19] Ibarra, I. S., Rodríguez, J. A., Santos, E. M., Miranda, J. M., *Electrophoresis* 2012, *33*, 2041-2048.

[20] Picó, Y., Food Toxicants Analysis: Techniques, Strategies and Developments, Elsevier, Amsterdam, 2007, pp 561-597.

[21] Castañeda, G., Rodríguez-Flores, J., Ríos, A., *J. Sep. Sci.* 2005, *28*, 915-924.

[22] Piñero, M. Y., Bauza, R., Arce, L., *Electrophoresis* 2011, *32*, 1379-1393.

[23] Santos, B., Simonet, B. M., Ríos, A., Valcárcel, M., *Trends Anal. Chem.* 2006, *25*, 968-976.

[24] Jiménez-Lozano, E., Roy, D., Barrón, D., Barbosa, J., *Electrophoresis* 2004, *25*, 65-73.

[25] Jiménez-Lozano, E., Marqués, I., Barrón, D., Beltrán, J. L., Barbosa, J., *Anal. Chim. Acta* 2002, *464*, 37-45.

[26] Carpio, A., Rodríguez-Estevéz, V., Sánchez Rodríguez, M., Arce, L., Valcárcel, M., *Electrophoresis* 2010, *31*, 2211-2217.

[27] Bailón-Pérez, M. I., García-Campaña, A. M., Cruces-Blanco, C., Del Olmo Iruela, M., *Electrophoresis* 2007, *28*, 4082-4090.

[28] San Martin, A., Canon, H., Iraguen, D., *Arch. Med. Vet.* 2001, *33*, 115-124.

[29] Phonkeng, N., Burakham, R., *Chromatographia* 2012, *75*, 233-239.

[30] Hubert, Ph., Nguyen-Huu, J. J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., Compagnon, P. A., Dewé, W., Feinberg, M., Lallier, M., Laurentie, M., Mercier, N., Muzard, G., Nivet, C., Valat, L., *STP Pharma Pratiques* 2003, *13*, 101-138.

[31] Rambla-Alegre, M., Esteve-Romero, J., Carda-Broch, S., *J. Chromatogr. A* 2012, *1232*, 101-109.

[32] ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2(R1), Geneva, Switzerland, 2005, http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

[33] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2001, Rockville, USA,http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf

[34] Commission of the European Communities, Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of the results, *Off. J. Eur. Comun.* 2002, *L221*, 8-36.


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SIMPLE SAMPLE TREATMENT FOR THE DETERMINATION OF ENROFLOXACIN AND CIPROFLOXACIN IN RAW GOAT MILK

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Abstract

A simple procedure of SPE, without previous protein precipitation, for the determination of enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP) in goat milk was developed. Several sorbents, as octadecyl (C18), ethyl (C2), cyclohexyl (CH) and phenyl (PH), were tested for SPE before HPLC with fluorescence detection (FD) determination. Loading parameters which affect the extraction procedure such as breakthrough volume, and composition/volume of the eluting solution were studied. Better recoveries and optimal cleanup efficiency were obtained with C18 cartridge using 5 mL of H_2O (containing 2% TFA)/acetonitrile (ACN)/methanol (MeOH)/ (77:15:8, v/v/v) as eluting solution. The sample preparation by this method yielded completely clean extracts with recoveries up to 99.7% for ENR and 95.9% for CIP. The total chromatographic run time was 6 min with retention times of 5.32 and 4.25 min for ENR and CIP, respectively. The analytical response was linear over the concentration range from 10 to 50 μg L^{-1} for ENR and CIP, with correlation coefficients (*r*) of 0.998 (ENR) and 0.997 (CIP). The validated method was applied to real samples of goat milk where the analytes (ENR and CIP) were natively in the matrix, since they came from animals that were treated with ENR. The use of SPE in the sample treatment, as a single and rapid step, proves to be a valuable alternative for the majority of the studies found in the literature that include laborious procedures to the extraction and cleanup of ENR and CIP from milk samples.

Keywords: ciprofloxacin, enrofloxacin, goat milk, SPE, HPLC.

1. Introduction

The use of fluoroquinolones (FQs) —one of the most useful classes of antimicrobial agents used in human and animal medicine today— has recently been regulated because their residues may persist in edible animal products and facilitate the development of drug-resistant bacterial strains or allergies as a result. In food technology, the presence of FQs in milk can alter fermentation processes during production of dairy derivative products such as cheese and yogurt, which requires the addition of microorganisms [1]. For these reasons, the use of FQs on food-producing animals has been regulated or banned by various government bodies in the USA [2], European Union [3], Japan [4], and the Republic of China [5]. These organizations have established maximum residue limits (MRLs) for FQs in bovine, ovine and caprine milk, and also in other food products, in their respective territories.

Enrofloxacin (ENR) is currently the most widely used fluoroquinolone in veterinary medicine for the treatment of pulmonary, urinary and digestive infections [6]. ENR is de-ethylated to its primary metabolite, ciprofloxacin (CIP), and both are found in the edible products from goats receiving ENR [7]. This has raised the need to develop effective analytical methodologies for the determination of these FQs below the MRLs level.

Several methods for the determination of FQs residues in various types of milk samples have to date been reported. Many use HPLC [8-16] or CE [1, 17-23] in combination with different detection systems. The favorable features of capillary electrophoresis (CE) have boosted its use as the separation technique of choice for the determination of antibiotics. However, determining trace analytes by HPLC or CE usually requires prior extraction from the matrix and preconcentration. Although some traditional extraction methods continue to be the most widely used, a large number of matrix components may co-elute with the analytes and disturb quantitative analysis. There is growing research into time- and labor-saving sample pretreatment methods to facilitate reduction of matrix contents and enrichment with the target analytes.

Sample treatments for determining FQs in milk are especially complex because the analytes are present in a large volume of an aqueous matrix consisting

of highly concentrated proteins, lipoproteins, lipids, vitamins, salts and a number of other compounds that may be chemically similar to the target analytes. Moreover, the analytes are often present at low concentrations [24].

Most existing analytical methods for the determination of FQs have been validated with bovine milk samples. To our knowledge, only three studies on the determination of FQs in goat milk by HPLC have so far been reported [9, 14, 15]. The procedure commonly used for extraction and cleanup of FQs from goat milk involves protein precipitation with trichloroacetic acid (TCA) [9, 14, 15]. This acid has also been used with organic solvents such as methanol (MeOH), followed by SPE [9, 14] or liquid–liquid extraction (LLE) with acetonitrile (ACN) and hexane [15]. SPE has gained increasing popularity since its inception and is currently held as the leading sample preparation method. In fact, SPE affords efficient extraction of analytes without interference from endogenous compounds, which leads to increased recoveries [25]. Hydrophilic–lipophilic balance (HLB) [14] and octadecyl-silica-based (C18) cartridges [9] are the most commonly used to extract ENR and CIP from goat milk.

In this work, we developed a new strategy for extracting and determining ENR and CIP in unspiked raw goat milk based on a direct SPE procedure requiring no protein precipitation. This approach is expeditious –it involves minimal sample pretreatment– and economical, and provides acceptable recoveries and cleanup efficiency together with limits of detection, below of the MRLs established. The greatest strength of this approach is that it enables the analysis of milk samples from ENR–treated goats, where the analytes (ENR and its metabolite CIP) are present in their native forms. This is a substantial contribution to demonstrating the potential of the method proposed for use in routine food analyses. Unlike existing methods [9, 14, 15], which focus primarily on the effects upon separation of the analytes but ignore their behavior when present in real samples, ours can be applied to unspiked samples.

2. Experimental

2.1. Reagent and standards

All reagents used were analytical grade. ACN 99.9% was supplied by Burdick & Jackson; MeOH 99.9% and TFA 99.9% were obtained from J. T. Baker and

acetic acid (HAc) was purchased from Fluka Riedel-de-Haën. ENR (99.3%) and CIP (99.5%) were obtained from Lab Zhejang Pharmaceutical.

Individual stock solutions containing a 100 μg mL^{-1} concentration of each analyte (ENR and CIP) in water were prepared and stored at 4 °C in the dark prior to use. Working standard solutions were prepared on a daily basis by appropriately diluting the stock solutions to 10, 20, 30, 40 and 50 μg L^{-1} with purified water.

2.2. Apparatus

Analyses were performed on an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), an online degasser (G1323A), an autosampler injector (G1313A), and an online-connected fluorescence detector (FD) (G1321A). The ChemStation for LC 3D software package, also from Agilent, was used to govern the instrument, and to acquire and process data. A Fisher Scientific vortex-mixer, Scaltec SBA33 Balance and a Hettich 32R centrifuge were used in order to perform the extractions.

2.3. Chromatographic conditions

Chromatographic separation of the FQs was achieved on a Chromolith Performance RP-18e column (100 mm x 4.6 mm i.d., 5 μm particle size) protected with an RP-18 pre-column, both from Merck. The mobile phase consisted of H_2O (containing 4% HAc)/ACN/MeOH (84:8:8, v/v/v), in isocratic mode. The flow-rate was 1.0 mL min^{-1}, the injected volume 20 μL and the column temperature 30 °C. The FD was set at an excitation wavelength of 280 nm and an emission wavelength of 448 nm.

2.4. Goat milk samples

Milk samples were obtained from six goats of the Nubian-Alpine breed at the Center for Experimental Animal Production (CEPA) of the Faculty of Veterinary of the University of Zulia (Zulia State, Venezuela). The goats were non-pregnant and healthy based on their clinical history, and physical and clinical examination.

Two types of samples were collected, namely: (i) antibiotic-free milk (control milk) obtained during the first milking of the day, and (ii) milk containing ENR and CIP residues. The latter samples were obtained 6 h after intramuscular administration of ENR at 7.5 mg kg^{-1} to ensure the presence of ENR and CIP in the milk. All samples were collected in sterile 100 mL plastic screw-top containers, placed on ice for transfer to the laboratory and stored at –4 °C until analysis.

2.5. Extraction and cleanup of milk samples

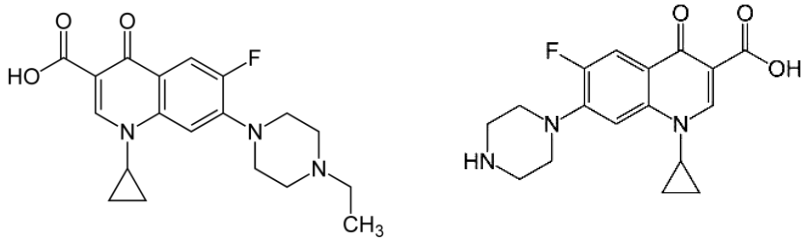
The reversed-phase sorbents studied for SPE included octadecyl (C18), ethyl (C2), cyclohexyl (CH) and phenyl (PH). All were obtained as Extract-Clean 100 mg (1.5 mL) cartridges from Alltech (Nicholasville, KY, USA).

Three different procedures were examined for extraction and cleanup of the raw goat milk samples, namely: deproteination (DP) (procedure 1), DP followed by SPE (procedure 2), and SPE without DP (procedure 3).For the first procedure, a 1 mL sample aliquot was placed in a 15 mL polypropylene tube and supplied with 5 mL of extracting solution (absolute ethanol/H_2O containing 1% HAc, 99:1 v/v) and 0.8 g of sodium sulfate. The mixture was vortexed vigorously for 15 s and centrifuged at 3000 rpm for 5 min. Then, the supernatant was transferred to another polypropylene tube and the sediment re-extracted with another 5 mL of extracting solution. The two supernatants were combined, centrifuged at 3000 rpm for 5 min and filtered for injection into the HPLC system.

The supernatant obtained after milk DP with absolute ethanol/H_2O containing 1% HAc (99:1, v/v) (procedure 2) or 1 mL of milk sample heated briefly at 45 °C to reduce viscosity (procedure 3) was passed through different Extract-Clean cartridges (100 mg, 1.5 mL; Alltech, Nicholasville, KY, USA)containing various sorbents (C18, C2, CH and PH). The SPE cartridges were conditioned with 3 mL of MeOH and 3 mL of H_2O. After sample percolation, each cartridge was washed with 3 mL of H_2O each (1 mL x 3). Finally, FQs were eluted with 5 mL of H_2O (containing 2% TFA)/ACN/MeOH/ (77:15:8, v/v/v). In this procedure, SPE was used mainly to extract analytes and cleanup samples rather than for preconcentration. All SPE runs were performed at room temperature.

3. Results and discussion

The aim of this work was to develop a rapid and efficient sample treatment for the determination of ENR and CIP (Fig. 1) in goat milk involving minimal pretreatment of the samples with SPE but no DP. The ensuing SPE-HPLC-FD method was optimized and validated for use in routine food analyses.



Enrofloxacin (ENR) Ciprofloxacin (CIP)

Fig. 1.*Chemical structures of the target FQs.*

3.1. Optimization of chromatographic separation

Standard solutions of ENR and CIP were used to optimize the chromatographic separation. Aqueous HAc solution, ACN and MeOH were selected as solvents for separating the FQs by HPLC. The use of an acid in the mobile phase was necessary to ensure removal of carboxylate ion and protonated nitrogen molecules. The aqueous mobile phase containing 4% HAc gave the higher signals and best peak shapes. The FQs were separated in less than 6 min, in the following elution sequence: CIP (t_R = 4.25 min) and ENR (t_R = 5.32 min). The sample concentration was calculated by comparing peak area with an external calibration curve spanning the concentration range of 10–50 μg L^{-1}.

3.2. Extraction of FQs from milk samples

Three different methods were used to extract FQs from the goat milk samples. Procedure 1 was based on a sample treatment proposed by San Martin et al. [8] to extract ENR and CIP from bovine milk with DP. This method was adapted to our specific purpose; however, it was found inefficient to cleanup such a complex matrix as raw goat milk (the samples were not rendered acceptably free from matrix interferences). In fact, the chromatogram for an extract of spiked goat milk samples exhibited tall peaks corresponding to unknown endogenous substances which interfered with the determination of CIP. This was probably due to the increased contents of fatty acids (butyric, caproic, caprylic and capric acid) of goat milk relative to bovine milk [26]. For this reason, FQs in goat milk cannot be determined with the procedure of San Martin et al [8], which was in fact originally developed for bovine milk samples.

In subsequent tests, DP with absolute ethanol/H_2O containing 1% HAc (99:1, v/v) followed by SPE was assessed. The DP–SPE combination (procedure 2) slightly improved recoveries (particularly that of CIP); however, it considerably increased analysis times and hence propagation of uncertainties.

Direct SPE without DP (procedure 3) was then studied. Initially, the main problem with direct SPE was the high viscosity of goat milk, which precluded direct application to the cartridges. This shortcoming was overcome by warming the samples at 45 °C to reduced their viscosity and facilitate their loading onto the sorption cartridges as a result. Warming the sample avoided not only viscosity and dispersion problems (irreversible sorption) of the matrix on the sorbent surface, but also potential thermal degradation of the analytes [27]. Procedure 3 was therefore selected on the grounds of its simplicity and suitability for coupling to HPLC. The results obtained confirmed the potential usefulness of this procedure for routine analytical laboratories, where analysis times and costs are two complementary analytical properties to be considered in implementing new methods.

3.2.1. Optimization of the SPE procedure

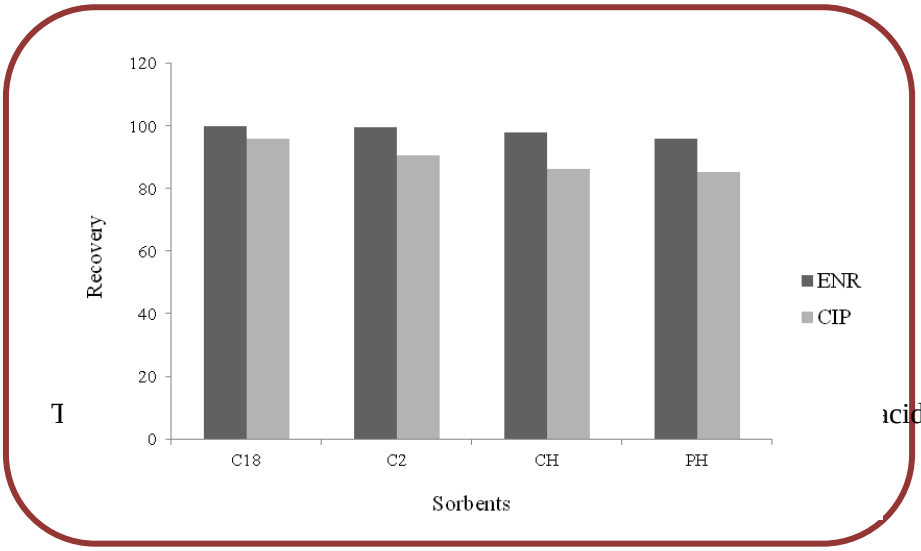
As noted earlier, SPE was optimized by using procedure 3 (i.e. bypassing the goat milk samples through the cartridges directly after gentle warming). The

parameters evaluated to optimize the procedure in terms of FQ recoveries were (i) the type of sorbent material, (ii) the sample breakthrough volume, and (iii) the composition and volume of the eluent.

Preliminary tests with a standard aqueous mixture without acidification used at 10 μg L^{-1} as loading sample and different sorbents with a variable carbonaceous loading (% carbon as bonded phase) [28] including C18 (12%), CH (12%),C2 (4.8%), and PH were performed. Figure 2 shows the recoveries obtained from a standard mixture of ENR and CIP. In general, recoveries decreased in the sorbent sequence C18 > C2 > CH > PH for both analytes, i.e. with increasing polarity of the sorbent (C18 is less polar than PH).The FQs were loaded at about pH 7.0 onto the sorbents. Therefore, both analytes were present in uncharged (protonated forms with no net charge, HFQ^0, in equilibrium with their zwitterionic forms), which facilitated absorption onto the sorbent. Because the nonpolar character of the primary interaction between the analytes and sorbents is relevant, the nature of the interactions between absorbed molecules and solid surfaces can be deemed primarily nonspecific (that is, as dispersion forces). The fact that recoveries with the sorbents with a low loading weight of carbon (e.g. C2) exceeded 85% indicates that C2 cartridges can be potential candidates for the intended purpose, but further study is required for confirmation. No reference to the use of C2, CH or PH cartridges for ENR and CIP in goat milk was found in the literature. In this work, we chose to use C18 on the grounds of the high recoveries obtained (99.72% for ENR and 95.94% for CIP at a 10 μg kg^{-1} concentration level), which is highly consistent with the results for widely reported FQs in milk of animal origin [9].

The influence of the breakthrough sample volume for the SPE cartridges was also assessed by applying loaded sample volumes of 1–12 mL of 10 μg L^{-1} standard solutions (equivalent to 0.01–0.12 μg of analyte) to C18 cartridges in order to identify potential losses of the analytes. In general, the C18 packing (100 mg sorbent) adsorbent up to 0.1 μg of CIP and ENR, respectively. With milk, a volume of 1 mL was passed through the SPE cartridge as a compromise between the analyte concentrations potentially present in the samples and the proportion of concomitant in the milk potentially occluding and deactivating the sorbent. Diluting the sample or

increasing the amount of sorbent used in the SPE cartridge is recommended to quantify FQs above their MRLs in raw goat milk.



T ıcid–base properties) and elution st

Fig. 2.*Recoveries from a 10 µg* L^{-1} *standard solution of ENR and CIP as obtained withdifferentsorbent materials.*

Using TFA instead of HAc led to increased recoveries. Finally, the influence of the TFA concentration (2, 4 or 15%) on analyte recovery was examined and the highest recoveries of both analytes found to be obtained with 2% TFA in the aqueous phase, where the amino group in TFA retained its cationic form and the molecules were more hydrophilic and hence easier to elute from the cartridges. The optimum eluted volume to ensure complete extraction of the analytes was also determined. Several tests were performed by adding a known amount of the target analytes to goat milk samples. Separate elution runs were performed by adding 1, 2, 3, 4, 5 or 6 mL of extraction solvent [H_2O containing 2% TFA/ACN/MeOH/ (77:15:8, v/v/v)], which was collected in different containers. A volume of 5 mL of extraction solvent was used to elute the analytes from the C18 cartridge. Note that the volume used for desorption (5 mL) was greater than the sample volume loaded onto the cartridge (1 mL), so SPE was mainly used to cleanup samples rather than for preconcentration.

3.3. Validation of the method

The whole analytical method was validated in compliance with the analytical performance parameters required for method validation, which include linearity, limit of detection (LOD), limit of quantitation (LOQ), repeatability, intermediate precision, and trueness, via recovery test. In all cases, a blank sample was analyzed in parallel to check whether either analyte was already present in the goat milk or if some interference might co-migrate with the analytes.

The analytical response was linear over the concentration range 10–50 µg L^{-1} for ENR and CIP, with correlation coefficients (*r*) of 0.998 (ENR) and 0.997 (CIP). Calibration curves were constructed by plotting peak areas against increasing concentrations of the FQs in the raw goat milk (10, 20, 30, 40 and 50 µg kg^{-1} for each analyte).Table 1 shows the linear regression data obtained.

Table 1. Calibration curves and figures of merit of the determination of ENR and CIP in goat milk by SPE–HPLC.

y: fluorescence signal; *a*: slope; *b*: intercept; R^2: correlation coefficient; $S_{y/x}$: regression standard deviation.

Analyte	Concentration range studied (µg kg^{-1})	$y = ax + b$	R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
CIP	10–50	a = 0.0219 ± 0.0004 b = 0.0065 ± 0.0148	0.998	0.014	2.02	6.75
ENR	10–50	a = 0.0311 ± 0.0007 b = 0.1066 ± 0.0237	0.997	0.022	2.28	7.62

LOD and LOQ were calculated as the signal-to-noise ratios of 3 and 10 times the standard deviation of the intercept divided by the slope of a graph obtained from raw goat milk samples spiked with variable concentrations of the analytes. LOD was 2.02 µg kg^{-1} for CIP and 2.28 µg kg^{-1} for ENR, and LOQ

was 6.75 μg kg^{-1} for CIP and 7.62 μg kg^{-1} for ENR. As can be seen, all LODs and LOQs were lower than the MRLs for the analytes.

The precision of the method was evaluated in terms of repeatability and intermediate precision. Repeatability (intraday precision) was assessed on the same day by repeating the SPE procedure six times on raw goat milk samples spiked at three different concentration levels (10, 30, and 50 μg kg^{-1}) and injecting each sample in triplicate. Intermediate (interday) precision was assessed for three consecutive days by subjecting samples to the same procedure as for repeatability, using three samples each day. The results expressed, as RSD% for relative peak areas, are shown in Table 2. As can be observed, the precision was acceptable in all cases.

The accuracy of the sample treatment was assessed in terms of recovery as determined at three different analyte concentrations: 10, 30 and 50 μg kg^{-1}. A comparison of the recovery values obtained from a standard mixture and spiked goat milk samples revealed that the proposed treatment is accuracy. Table 3 summarizes the experimental ENR and CIP recoveries from goat milk samples spiked at three different levels. Recoveries decreased with increasing analyte concentration. This finding was repeated in the precision tests. One possible reason is that an increased overloaded mass may have led to premature breakthrough in the sorbent (100 mg in an Extra-Clean cartridge) above 50 μg kg^{-1} and caused losses of the analytes by effect of their curved sorption isotherm. The effect was especially marked with CIP, possibly because of its increased hydrophilicity and the resulting decreased retention on a nonpolar sorbent. As can be seen, the method provided acceptable trueness values in terms of recovery (96% for ENR and 78% for CIP). Fig. 3 shows a chromatogram for a raw goat milk sample spiked with 50 μg kg^{-1} concentration and processed with the proposed SPE–HPLC–FD method under optimum conditions. As can be seen, three different elution peaks were observed before those for the target analytes; however, CIP and ENR were well enough resolved for identification and quantitation purposes.

Table 2. Precision of the proposed method carried out using goat milk analyzed by SPE-HPLC.

Analyte	Intraday precision (*n*=6, %RSD)		
	10 µg kg^{-1}	30 µg kg^{-1}	50 µg kg^{-1}
CIP	0.27	0.14	0.10
ENR	0.29	0.05	0.03
	Interday precision (*n*=9, %RSD)		
	10 µg kg^{-1}	**30 µg kg^{-1}**	**50 µg kg^{-1}**
CIP	0.80	0.50	0.26
ENR	0.94	0.78	0.54

Table 3. Recoveries from goat milk samples spiked with the analytes at different concentrations levels as obtained by using C18 SPE cartridges.

Analyte	Concentration added (µg kg^{-1})	Concentration found (µg kg^{-1})	Mean recovery (%)
CIP	10	9.6	78
	30	22.9	
	50	30.4	
ENR	10	9.9	96
	30	28.6	
	50	46.6	

3.4. Analysis of goat milk samples

A screening test was performed before the proposed method was used to determine the analytes in goat milk samples. For this purpose, different raw goat milk samples obtained from local farmer were analyzed by following the proposed procedure. The samples were collected from six goats treated with ENR in order to ensure that their milk would contain the analytes in their native forms. Both ENR and its main metabolite CIP were detected after intravenous

administration of ENR, which is consistent with previous findings of Ambros et al. [29] on the pharmacokinetics of these FQs in goats. As can be seen from Fig. 4, both analytes were determined free of interferences in the six milk samples.

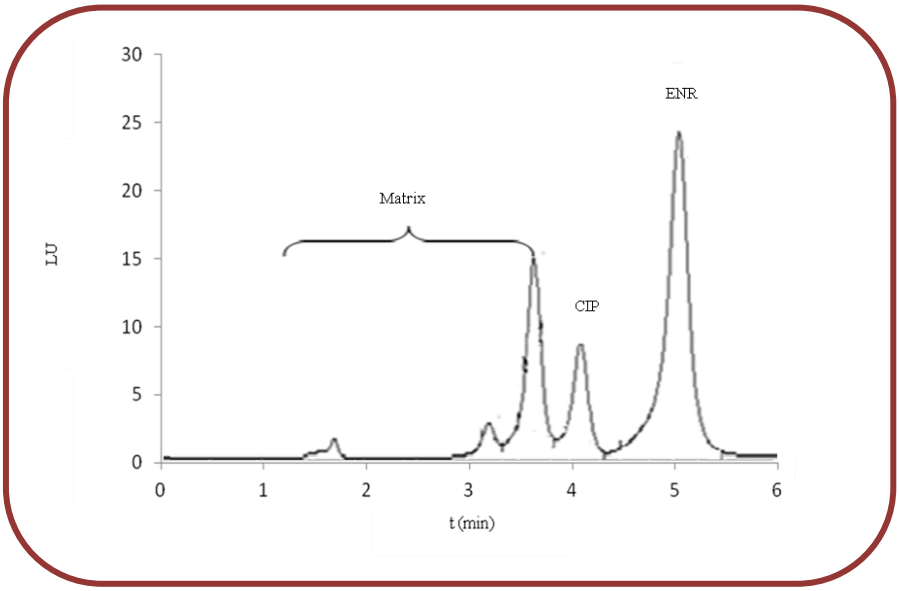


Fig. 3.*SPE–HPLC–FD chromatogram for a raw goat milk sample spiked with 50 µg* L^{-1} *concentration of ENR and CIP.Mobile phase:* H_2O *(containing 4% HAc)/ACN/MeOH (84:8:8, v/v/v). Flow rate: 1 mL* min^{-1}*. Detection:* λ_{exc} *= 280nm,* λ_{em} *= 448 nm.*

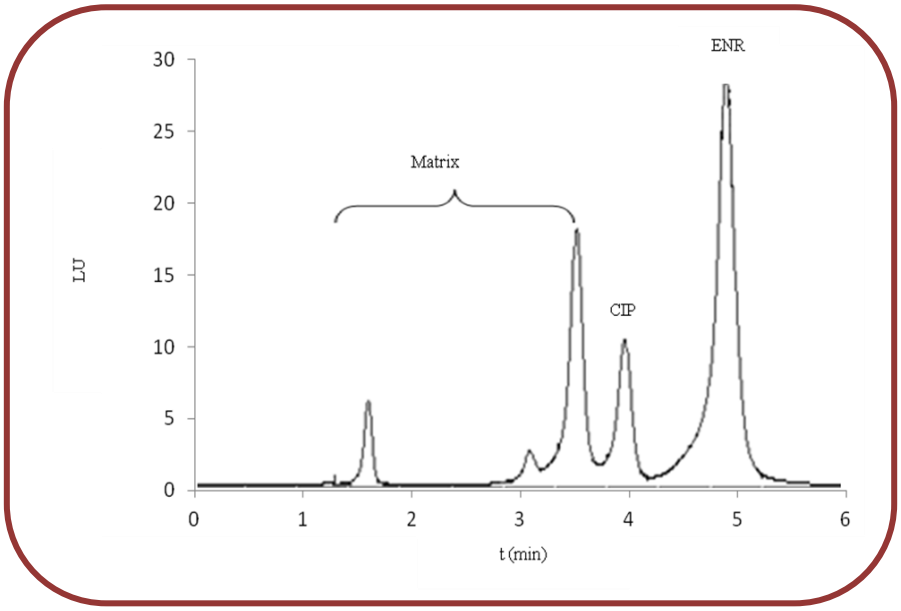


Fig. 4.*SPE–HPLC–FD chromatogram for a raw goat milk sample containing residues of native ENR and CIP. Mobile phase: H_2O (containing 4% HAc)/ACN/MeOH (84:8:8, v/v/v). Flow rate: 1 mL min^{-1}. Detection: λ_{exc} = 280 nm, λ_{em} = 448 nm.*

4. Conclusions

This paper reports the first HPLC method using a simple SPE procedure involving no protein precipitation for the extraction of ENR and CIP from goat milk samples. The proposed method has practical environmental and economical advantages in terms of sample preparation time, simplicity, reduced solvent consumption and cost. It is particularly suitable for routine applications requiring a high sample throughput. The method was validated in terms of linearity, precision, and recovery, all of which testify to its usefulness as an analytical tool for the quality control of dairy products. It was applied to real samples of milk obtained from ENR-treated goats, where it confirmed the presence of ENR and its main metabolite CIP.

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The authors have declared no conflict of interest.

References

[1] F.J. Lara, A.M. García-Campaña, F. Áles-Barrero, J.M. Bosque-Sendra, L.E. García-Ayuso, Multiresidue method for the determination of quinolone antibiotics in bovine raw milk by capillary electrophoresis–tandem mass spectrometry, Anal. Chem. 78 (2006) 7665-7673.

[2] U.S. Food and Drug Administration (FDA). Code of Federal Regulation, title 21 (Food and Drugs), Vol 6, Part 530: Extralabel drug use in animals. Revised April 1, 2012.

[3] E.U. Council Regulation No. 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, Off. J. Eur. Commun. L 224 (1990) 1–20.

[4] The Positive List System for Agricultural Chemical Residues in Foods, Department of Food Safety, Ministry of Health, Labour and Welfare, June 2006.

[5] Ministry of Agriculture of the People's Republic of China No. 235/2002.

[6] E.M.E.A (European Medicines Agency). Veterinary Medicines and Inspections, Committee for medicinal products for veterinary use (CVMP) reflection paper on the use of fluoroquinolones in food-producing animals in the European Union: development of resistance and impact on human and animal health, EMEA/CVMP/SAGAM/184651. 2005.

[7] D.J. Dimitrova, L.D. Lashev, S.G. Yanev, B. Pandova, Pharmcacokinetics of enrofloxacin in turkeys, Res. Vet. Sci. 82 (2007) 392–397.

[8] A. San Martin A, H. Canon, D. Iraguen, A fast assay for the simultaneous determination of enrofloxacin and ciprofloxacin in milk by high performance liquid chromatography, Arch. Med. Vet. 33 (2001) 115–124.

[9] A.L. Cinquina, P. Roberti, L. Giannetti, F. Longo, R. Draisci, A. Fagiolo, N.R. Brizioli, Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatography with diode-array detection – Optimization and validation, J. Chromatogr. A 987 (2003) 221–226.

[10] M. Hassouan, O. Ballesteros, J. Vilchez, A. Zafra, A. Navalon, Simple multiresidue determination of fluoroquinolones in bovine milk by liquid chromatography with fluorescence detection, Anal. Lett. 40(2007) 779–791.

[11] E. Rodriguez, M.C. Moreno-Bondi, M.D. Marazuela, Development and validation of a solid-phase extraction method coupled to liquid chromatography with fluorescence detection for the determination of fluoroquinolone residues in powdered infant formulae Application to the analysis of samples from the Spanish and Latin American market,J. Chromatogr. A 1209 (2008) 136–144.

[12] M.P Heymo, E. Nemutlu, S. Kir, D. Barron, J. Barbosa, Improved determination of quinolones in milk at their MRL levels using LC-UV, LC-FD, LC-MS and LC-MS/MS and validation in line with regulation 2002/657/EC, Anal. Chim. Acta 613 (2008) 98–107.

[13] Q.F. Tang, T.T. Yang, X.M. Tan, J.B. Luo, Simultaneous determination of fluoroquinolone antibiotic residues in milk sample by solid-phase extraction–liquid chromatography–tandem mass spectrometry, J. Agric. Food Chem. 57(2009) 4535–4539.

[14] A. Herrera-Herrera, J. Hernández-Borges, M.A. Rodríguez-Delgado, Fluoroquinolone antibiotic determination in bovine, ovine and caprine milk using solid-phase extraction and high-performance liquid chromatography–fluorescence detection with ionic liquids as mobile phase additives, J. Chromatogr. A 1216 (2009) 7281–7287.

[15] H.H Chung, J.B. Lee,Y.H. Chung, K.G. Lee, Analysis of sulfonamide and quinolone antibiotic residues in Korean milk using microbial assays and high performance liquid chromatography, Food Chem. 113 (2009) 297–301.

[16] M.M. Zheng, R. Gong, X. Zhao, Y.Q. Feng, Selective sample pretreatment by molecularly imprinted polymer monolith for the analysis of fluoroquinolones from milk samples, J. Chromatogr. A 1217 (2010) 2075–2081.

[17] H. Sun, W. Zhao, P. He, Effective separation and simultaneous determination of four fluoroquinolones in milk by CE with SPE,Chromatographia 68(2008) 425–429.

[18] X. Zhou, D. Xing, D. Zhu, Y. Tang, L. Jia, Development and application of a capillary electrophoresis-electrochemiluminescent method for the analysis of enrofloxacin and its metabolite ciprofloxacin in milk, Talanta 75(2008) 1300–1306.

[19] C. Blasco, Y. Picó, V. Andreu, Analytical method for simultaneous determination of pesticide and veterinary drug residues in milk by CE–MS, Electrophoresis 30 (2009) 1698–1707.

[20] M. Lombardo-Agüí, A.M. García-Campaña, L. Gámiz-Gracia, C. Cruces-Blanco, Laser induced fluorescence coupled to capillary electrophoresis for the determination of fluoroquinolones in foods of animal origin using molecularly imprinted polymers, J. Chromatogr. A 1217(2010) 2237–2242.

[21] L. Vera-Candioti, A.C. Olivieri, H.C. Goicoechea, Development of a novel strategy for preconcentration of antibiotic residues in milk and their quantitation by capillary electrophoresis,Talanta 82(2010) 213–221.

[22] I.S. Ibarra, J.A. Rodríguez, E.M. Santos, J.M. Miranda, Determination of quinolones in milk samples using a combination of magnetic solid-phase extraction and capillary electrophoresis,Electrophoresis 33(2012) 2041–2048.

[23] M.Y. Piñero, R. Garrido-Delgado, R. Bauza, L. Arce, M. Valcárcel, Easy sample treatment for the determination of enrofloxacin and ciprofloxacin residues in raw bovine milk by capillary electrophoresis, Electrophoresis 33 (2012) 2978–2986.

[24] R.W. Fedeniuk, P.J. Shand, Theory and methodology of antibiotic extraction from biomatrices, J. Chromatogr. A 812(1998) 3–15.

[25] J. Sousa, G. Alves, A. Fortuna, A. Falcao, Analytical methods for determination of new fluoroquinolones in biological matrices and pharmaceutical formulations by liquid chromatography: a review, Anal. Bioanal. Chem. 403 (2012) 93–129.

[26] J. Faria, A. Garcia, M. Allara, A. Garcia, Y. Olivares, G. Rios, Algunas características fisíco–químicas y microbiológicas de la leche de cabra producida en Quisiro, Rev. Fac. Agron. 16 (1999) 99–106.

[27] S. Bogialli, G.D. Áscenzo, A. Di Corsia, A. Lagana, S. Nicolardi, A simple and rapid assay based on hot water extraction and liquid chromatography-tandem mass spectrometry for monitoring quinolone residues in bovine milk, Food Chem. 108 (2008) 354–360.

[28] E. M. Thurman, M.S. Mills, Solid–Phase Extraction. Wiley & Sons, inc., Volume 147, New York, 1998.

[29] L. Ambros, L. Montoya, V. Kreil, S. Waxman Dova, G. Albarellos, M. Rebuelto, R. Hallu, M.I. San Andrés Larrea, Farmacocinética de la

CAPÍTULO V:

Análisis final

CONCLUSIONES

En esta Tesis Doctoral se evaluó el potencial de la CE como técnica de separación útil para su implantación en los laboratorios agroalimentarios. Las conclusiones más relevantes generadas en los diferentes trabajos de investigación incluidos en esta memoria se muestran a continuación:

(1) En primer lugar, se ha presentado una revisión sobre las diferentes aplicaciones de la CE para la determinación de diversos analitos de interés en el ámbito agroalimentario.

En este trabajo, se evidenció que esta técnica de separación ha sido ampliamente evaluada, a lo largo de más de 30 años. En los distintos trabajos publicados se han propuesto diferentes enfoques para mejorar uno de los principales inconvenientes de la CE, como es la sensibilidad y robustez. Sin embargo, la CE no se emplea en la actualidad en los laboratorios de rutina para análisis de alimentos. Uno de los principales factores que pudiera contribuir a este hecho es que, la mayoría de los trabajos de investigación, encontrados en diferentes bases de datos, se han centrado en demostrar el potencial de la CE en muestras fortificadas o enriquecidas, y pocas veces se han aplicado los métodos optimizados a muestras reales, por este motivo la literatura carece de estudios del comportamiento de los analitos en la matriz real.

(2) Por otro lado, se ha demostrado la importancia de la etapa de pretratamiento de la muestra como un factor fundamental que debe garantizar la extracción de los analitos y limpieza de la muestra, así como la obtención de un extracto compatible con las condiciones electroforéticos empleadas.

En este sentido, se ha realizado un análisis crítico sobre los diferentes problemas e inconvenientes que se presentan durante el tratamiento de muestras complejas, como leche cruda de origen animal, para la determinación de compuestos minoritarios como residuos de penicilinas, basados en nuestra experiencia.

La etapa de tratamiento de muestra, es ahora mismo, el cuello de botella para la determinación de penicilinas en leche por CE. En este sentido, se han propuesto diferentes estrategias analíticas que combinan el análisis mediante MEKC-UV con métodos de preconcentración *off-line*, como SPE y QuEChERS. En este trabajo, también se ha demostrado que otras estrategias de preconcentración *in-line*, como LVSS, no son apropiadas para ser aplicadas a extractos provenientes de leche, los cuales se caracterizan por presentar una alta conductividad.

(3) Por último, en cuanto a la determinación de fluoroquinolonas en leche mediante HPLC y CE, se ha demostrado que no todos los tratamientos de muestras para extraer estos antibióticos a partir de muestras de leche, son compatibles con las distintas técnicas de separación y detectores disponibles para la determinación de estos analitos. Como ejemplo, se presenta un tratamiento de muestra simple que fue compatible con HPLC-FD, pero no así con CE-DAD. Para éste ultimo caso, fue necesario emplear un paso adicional en el tratamiento de la muestra que permitiera la precipitación de proteínas y contribuyera con la eliminación de otros interferentes presentes en la matriz. Sin embargo, ambos métodos presentan diversas ventajas en cuanto a preparación de la muestra, simplicidad de la etapa de extracción, reducción del uso de solventes y bajo coste, por lo que representan una alternativa atractiva para el monitoreo de fluoroquinolonas en leche en los laboratorios agroalimentarios de rutina.

ANEXO:

Producción científica

ARTÍCULOS CIENTÍFICOS

1. **Thirty years of capillary Electrophoresis in food analysis laboratories: potential applications**
   Maria-Ysabel Piñero, Roberto Bauza, Lourdes Arce
   Electrophoresis 2011, 32, 1379–1393
   Factor de impacto: 3.261

2. **Easy sample treatment for the determination of enrofloxacin and ciprofloxacin residues in raw bovine milk by capillary electrophoresis**
   Maria-Ysabel Piñero, Rocío Garrido-Delgado, Roberto Bauza, Lourdes Arce, Miguel Valcárcel
   Electrophoresis 2012, 33, 2978–2986
   Factor de impacto: 3.261

3. **Simple sample treatment for the determination of enrofloxacin and ciprofloxacin in raw goat milk**
   Maria-Ysabel Piñero, Mairy Fuenmayor, Roberto Bauza, Lourdes Arce, Miguel Valcárcel
   Microchemical Journal, aceptado el 25 de junio de 2013
   Factor de impacto:2.879

4. **Determination of penicillins in milk of animal origin by capillary electrophoresis: sample treatment is the bottleneck for routine laboratories?**
   Maria-Ysabel Piñero, Roberto Bauza, Lourdes Arce, Miguel Valcárcel
   Talanta, enviado julio 2013.

5. Evaluation of different strategies to extract and preconcentrate penicillins in milk samples by capillary electrophoresis

Maria-Ysabel Piñero, Roberto Bauza, Lourdes Arce, Miguel Valcárcel

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Electrophoresis 2011, 32, 1379–1393

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Review

Thirty years of capillary electrophoresis in food analysis laboratories: Potential applications

CE has generated considerable interest in the research community since instruments were introduced by different trading companies in the 1990s. Nowadays, CE is popular due to its simplicity, speed, highly efficient separations and minimal solvent and reagent consumption; it can also be included as a useful technique in the nanotechnology field and it covers a wide range of specific applications in different fields (chemical, pharmaceutical, genetic, clinical, food and environmental). CE has been very well evaluated in research laboratories for several years, and different new approaches to improve sensitivity (one of the main drawbacks of CE) and robustness have been proposed. However, this technique is still not well accepted in routine laboratories for food analysis. Researching in data bases, it is easy to find several electrophoretic methods to determine different groups of analytes and sometimes they are compared in terms of sensitivity, selectivity, precision and applicability with other separation techniques. Although these papers frequently prove the potential of this methodology in spiked samples, it is not common to find a discussion of the well-known complexity of the matrices to extract analytes from the sample and/or to study the interferences in the target analytes. Summarizing, the majority of CE scientific papers focus primarily on the effects upon the separation of the analytes while ignoring their behavior if these analytes are presented in real samples.

Keywords:
CE / Food analysis / Research laboratories / Routine laboratories / Routine methods
DOI 10.1002/elps.201000541

1 Introduction

When CE was first introduced, it was seen as a revolutionary technique. Now, it is a well-established technique in analytical research laboratories worldwide. CE offers highly efficient separations, short analysis times, simplicity, precision, easy automation and low costs (for labor, solvent volumes, waste disposal, stationary phases, e.g. chiral separations) and possible nanoliter sample amounts when compared with other separation techniques. CE is robust and generates almost no waste disposal. The major strength of CE, however, is that the basic separation principles are different from those of HPLC and GC. Therefore, CE and HPLC used together make a powerful combination. CE offers the advantage that several separation modes can be run on a single instrument. This makes CE a very versatile technique for a broad range of applications and separation challenges. The scope of CE application in food analysis is, in general, identical to that of HPLC, and users must often choose between the two techniques; however, nowadays with the worldwide shortage of acetonitrile (ACN), the most commonly used in HPLC solvent, and a renewed focus on green separation technologies, the use of CE technique would be more appropriate, in some particular cases. CE should be considered first when dealing with highly polar, charged or chiral analytes and it is a technique with tremendous potential to solve different separation problems especially in life sciences. CE is extensively used in the comprehensive characterization of macromolecules used in biologic as well as in proteomic or metabolomic studies.

Despite the many excellent technical reviews found in the literature on CE related to food analysis [1–8], there is still a need for more specific critical evaluations on the determination of analytes present in different matrices. CE-real sample analysis or CE-routine analyses are still not well studied. With this situation, it will be very difficult to transfer the CE analytical methodologies to routine laboratories. CE is still regarded with suspicion by scientists and

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Research Article

Easy sample treatment for the determination of enrofloxacin and ciprofloxacin residues in raw bovine milk by capillary electrophoresis

An easy, selective, and sensitive method has been developed for the determination of enrofloxacin (ENR) and its main active metabolite, ciprofloxacin (CIP), in raw bovine milk using CE with UV detection at 268 nm. Milk samples were prepared by a clean-up/extraction procedure based on protein precipitation with hydrochloride acid followed by being defatted by centrifugation and SPE using a hydrophilic-lipophilic balance cartridge. Optimum separation was obtained using a 50 mM phosphoric acid at pH 8.4 and the total electrophoretic run time was 6 min. Sample preparation by this method yielded clean extracts with quantitative and consistent mean recoveries from 89 to 97% for CIP and from 93 to 98% for ENR. LODs obtained were lower to the maximum residue limits for these fluoroquinolones. The precision of the ensuing method is acceptable; thus, the RSD for peak area and migration time was less than 8.5 and 0.5% for CIP and 9.9 and 0.9% for ENR, respectively. The results showed that the proposed method was efficient showing good recoveries, sensitivity, and precision for the studied compounds and could be satisfactorily applied in routine analysis for the monitoring of ENR and CIP residues in milk, due to its ruggedness and feasibility demonstrated.

Keywords:
CE / Fluoroquinolones / Milk / Routine analysis / Sample treatment
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1 Introduction

Fluoroquinolones (FQs) are synthetic antibacterial compounds used in humans and in food-producing animals for treatment of a variety of bacterial infections. Some FQs have been developed specifically for veterinary practice, which is the case of enrofloxacin (ENR), while others like ciprofloxacin (CIP) are restricted to human treatment [1]. However, in several animal species, ENR is deethylated to its primary metabolite CIP, and both ENR and CIP are found in the edible products of animals receiving ENR [2].

The use of ENR in lactating breeding animals may leave residues of ENR and CIP in milk. The widespread usage of antimicrobials may be responsible for the promotion of resistant strains of bacteria. Other problems related to the misuse of antibiotics are as follow: (i) they can produce allergic hypersensitivity reaction in some people; (ii) fermentation processes, such as the cheese or yoghurt elaboration, could fail; and (iii) the presence of antibiotics could hide the existence of pathogens in foodstuffs when bacteriological analyses are carried out [3]. Besides this, the high stability of FQs represents a significant risk to human health because the residues of these antibiotics can remain in milk after heat treatment and, therefore, can reach the dairy industry and consumers [4].

Consequently, it is necessary to control/monitor residual levels of these compounds, in order to meet regulatory requirements and especially to protect the consumer and the environment. For these reasons, both the Commission of the European Community [5] and the United States Food and Drug Administration [6] have established maximum residue limits (MRLs) of 0.1 mg kg^{-1} for ENR and CIP in milk. These low MRLs require the development of highly sensitive and selective methods for their monitorization in routine laboratories.

Traditionally, HPLC has been the most widely used technique for the determination of FQs in milk [7–11]. However, during the last years, CE has also been proposed for the determination of these compounds [3, 12–19] as an alternative technique (see Table 1). Compared with HPLC, CE has the advantages of high separation efficiency, short analysis time,

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Abbreviations: CCα, decision limit; CCβ, detection capability; CIP, ciprofloxacin; ENR, enrofloxacin; FQ, fluoroquinolone; LOM, lomefloxacin; MRL, maximum residue limit; QuEChERS, quick, easy, cheap, effective, rugged, safe

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A simple sample treatment for the determination of enrofloxacin and ciprofloxacin in raw goat milk

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ABSTRACT

A solid-phase extraction (SPE) procedure requiring no prior protein precipitation for the determination of enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP) in goat milk with fluorescence detection (FD) was developed. Octadecyl (C18), ethyl (C2), cyclohexyl (CH) and phenyl (PH) sorbents were initially tested for this purpose. The influence of the loading variables affecting the extraction efficiency (viz. breakthrough volume and eluent composition/volume) was examined. Using C18 cartridges in combination with 5 mL of H_2O (containing 2% TFA)/acetonitrile (ACN)/methanol (MeOH)/(77:15:8, v/v/v) as eluent was found to provide the best recoveries and highest efficiency. This sample preparation method provided absolutely clean extracts with recoveries up to 99.7% for ENR and 95.9% for CIP. The overall chromatographic running time was 6 min, and the retention times for ENR and CIP were 5.32 and 4.25 min, respectively. The analytical response was linear over the concentration range from 10 to 50 μg L^{-1} for ENR and CIP, with a correlation coefficient (r) of 0.998 for ENR and 0.997 for CIP. The method was validated and applied to real goat milk samples where the analytes (ENR and CIP) were natively present in the matrix since they were obtained from ENR-treated animals. Using SPE to treat the samples expedites the process in relation to most available methods for the same purpose, which involve time-consuming extraction and clean up of ENR and CIP from milk samples.

1. Introduction

The use of fluoroquinolones (FQs)—one of the most useful classes of antimicrobial agents used in human and animal medicine today—has recently been regulated because their residues may persist in edible animal products and facilitate the development of drug-resistant bacterial strains or allergies as a result. In food technology, the presence of FQs in milk can alter fermentation processes during production of dairy derivative products such as cheese and yogurt, which requires the addition of microorganisms [1]. For these reasons, the use of FQs on food-producing animals has been regulated or banned by various government bodies in the USA [2], European Union [3], Japan [4], and the Republic of China [5]. These organizations have established maximum residue limits (MRLs) for FQs in bovine, ovine and caprine milk, and also in other food products, in their respective territories.

Enrofloxacin (ENR) is currently the most widely used fluoroquinolone in veterinary medicine for the treatment of pulmonary, urinary and digestive infections [6]. ENR is de-ethylated to its primary metabolite, ciprofloxacin (CIP), and both are found in the edible products from goats receiving ENR [7]. This has raised the need to develop effective analytical methodologies for the determination of these FQs below the MRLs level.

Several methods for the determination of FQs residues in various types of milk samples have to date been reported. Many use HPLC [8–16] or CE [1,17–23] in combination with different detection systems. The favorable features of capillary electrophoresis (CE) have boosted its use as the separation technique of choice for the determination of antibiotics. However, determining trace analytes by HPLC or CE usually requires prior extraction from the matrix and preconcentration. Although some traditional extraction methods continue to be the most widely used, a large number of matrix components may co-elute with the analytes and disturb quantitative analysis. There is growing research into time- and labor-saving sample pretreatment methods to facilitate reduction of matrix contents and enrichment with the target analytes.

Sample treatments for determining FQs in milk are especially complex because the analytes are present in a large volume of an aqueous matrix consisting of highly concentrated proteins, lipoproteins, lipids, vitamins, salts and a number of other compounds that may be chemically similar to the target analytes. Moreover, the analytes are often present at low concentrations [24].

Most existing analytical methods for the determination of FQs have been validated with bovine milk samples. To our knowledge, only three studies on the determination of FQs in goat milk by HPLC

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COMUNICACIONES A CONGRESOS

1. **15thLatin American Symposium on Biotechnology, Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis and Microchip Technology (Seville, Spain, 2009)**
   Oral presentation:*"30 Years of CE in research laboratories: is it time to find CE established in routine laboratories?*
   Arce L., Piñero, M. Y., Valcárcel, M.

2. **LX Convención Anual ASOVAC (Ciudad Bolívar, Venezuela, 2010)**
   Póster:*"Evaluación de la selectividad cromatográfica en la separación de enrofloxacina y ciprofloxacina en leche de cabra"*
   Fuenmayor, M., Bauza, R., Piñero, M. Y.

3. **X Congreso Venezolano de Química (Caracas, Venezuela 2011)**
   Póster:*"Evaluación del tratamiento de muestra en la determinación de fluoroquinolonas en leche por electroforesis capilar"*
   Piñero, M. Y.Bauza, R.,Arce L., Valcárcel, M.

4. **XVIII Reunión de la SEQA (Jaén, España 2013)**
   Póster:*"Determinación de fluoroquinolonas en leche mediante técnicas analíticas de separación usando extracción en fase sólida"*
   Piñero, M. Y. Bauza, R., Arce L., Valcárcel, M.

5. ***ITP 2013: 20th International Symposium on Electro- an Liquid Phase-separation Technique (Tenerife, España 2013)***
   Póster:" Evaluation of different strategies to extract and preconcentrate penicillins present in milk prior determination by capillary electrophoresis"
   Piñero, M. Y., Jurado, N., Bauza, R., Arce L., Valcárcel, M.