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DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA EL CONTROL DE CONTAMINANTES Y RESIDUOS EN LECHE Y PRODUCTOS LÁCTEOS

TESIS DOCTORAL

Ahmed Mohammed Hamed Mahmoud

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DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA EL CONTROL DE CONTAMINANTES Y RESIDUOS EN LECHE Y PRODUCTOS LÁCTEOS

DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE MONITORING OF CONTAMINANTS AND RESIDUES IN MILK AND DAIRY PRODUCTS

Por

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DEPARTAMENTO DE QUÍMICA ANALÍTICA

UNIVERSIDAD DE GRANADA

MEMORIA presentada para aspirar al Grado de Doctor en Ciencias

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Dña. Ana María García Campaña, Catedrática del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada, Directora del Departamento y responsable del grupo de investigación "Calidad en Química Analítica, Alimentaria, Ambiental y Clínica" (FQM-302).

CERTIFICA:

Que el trabajo que se presenta en esta **TESIS DOCTORAL**, con el título de **"DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE MONITORING OF CONTAMINANTS AND RESIDUES IN MILK AND DAIRY PRODUCTS"**, ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de la profesora Dª Laura Gámiz Gracia, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y reúne todos los requisitos para poder optar al Grado de Doctor.

Granada a de de 2017

El doctorando Ahmed Mohammed Hamed Mahmoud y las directoras de la tesis Dª. Laura Gámiz Gracia y Dª Ana M. García Campaña y garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de las directoras de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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OBJETIVES

The purpose of this thesis was to develop new strategies of sample treatment combined with the use of advances and miniaturized analytical separation techniques to control regulated chemical hazards (contaminants and residues) in milk, dairy products and vegetable milks.

Thus, the general objective was to validate new analytical methods (within the "Green Analytical Chemistry" framework) for the determination of compounds belonging to different groups that have been classified by the European Food Safety Authority (EFSA) as hazardous chemicals, i.e., mycotoxins, pesticides and antibiotics. It should be noted that this Thesis was developed as a continuation and expansion of the research lines previously developed by the group in the field of quality and safety in food.

The Thesis focused the attention mainly in different families of food contaminants widely used in veterinary medicine for cows (such as, aminoglycosides, quinolones and tetracyclines), in agriculture as pesticides (carbamates) and natural contaminants, such as mycotoxins (aflatoxins and *Fusarium* toxins).

The specific objectives of this work were as follow:

- Evaluation of a method for the sensitive, simple, and rapid determination of the five aflatoxins regulated by European legislation (aflatoxin B1, B2, G1, G2 and M1) in different yogurt based on the simultaneous precipitation of protein and extraction by dispersive liquid-liquid microextraction followed by HPLC-PI-FLD. This objective was proposed considering the scarce analytical methods for determination of these contaminants in yogurt, and to expand the applicability of miniaturized extraction methods.

- Development of a sensitive and rapid UHPLC-MS/MS method coupled with QuEChERS-based extraction for the determination of *Fusarium* mycotoxins in different types of vegetable milk, such as oat, soya, rice and birdseed, and application to commercial samples. Although these cereal-based samples are susceptible of mycotoxin contamination, but little attention has been paid on this topic.

- Evaluation of a new sorbent (Z-Sep+) for the QuEChERS extraction and clean-up of 28 carbamates in a dairy product of high-fat content, such as cheese (Gorgonzola, Camembert and Roquefort) and their determination by UHPLC-MS/MS. Considering the recent development of this new sorbent for clean-up of matrices of high-fat content, it was considered interesting to test its applicability on these dairy products.

- Validation of a method for routine analysis of 11 aminoglycoside residues in different types of milk and milk-based functional foods, using a recently commercially available molecularly imprinted polymer for selective solid phase extraction of the compounds. Moreover, considering the high polarity of these compounds, hydrophilic interaction chromatography (HILIC) was used for their UHPLC-MS/MS determination, as an alternative to ion-pair chromatography.

- Development of a new analytical method based on capillary electrophoresis and high resolution mass spectrometry (CE-Q-TOF-MS/MS) as an alternative to LC-MS/MS for the identification and simultaneous quantification of fifteen antibiotics (7 quinolones and 8 tetracyclines) in different milk samples. In addition, a new solid phase extraction sorbent (Oasis HLB PRiME) was tested as a simple and efficient sample treatment.

OBJETIVOS

El propósito de esta Tesis Doctoral ha sido el desarrollo de nuevas estrategias analíticas para el tratamiento de muestra combinadas con el empleo de técnicas separativas avanzadas y miniaturizadas para el control de agentes químicos de riesgo (contaminantes y residuos) en leche, productos lácteos y leches vegetales.

Así, como objetivo general se planteó la validación de métodos analíticos (enmarcados en la "Química Analítica Verde") para la determinación de compuestos pertenecientes a diversos grupos de compuestos clasificados por la Agencia Europea de Seguridad Alimentaria (EFSA) como agentes químicos de riesgo, como son las micotoxinas, residuos de plaguicidas y residuos de antibióticos. Cabe destacar que esta Tesis Se ha desarrollo como continuación y ampliación de la línea desarrollada por el grupo de investigación sobre calidad y seguridad alimentaria.

La Tesis ha centrado su atención en diferentes familias de contaminantes, como residuos de antibióticos ampliamente empleados en veterinaria (aminoglicósidos, quinolonas y tetraciclinas), residuos de plaguicidas empleados en agricultura (como los carbamatos) y contaminantes naturales como las micotoxinas (centrándonos en las aflatoxinas y toxinas de *Fusarium*).

Como objetivos específicos de esta Tesis, cabe destacar:

- Evaluación de un método sensible, simple y rápido para la determinación de las cinco aflatoxinas reguladas por la legislación europea (aflatoxinas B1, B2, G1, G2 y M1) en diferentes tipos de yogur, basado en la precipitación de las proteínas y simultánea extracción de los compuestos mediante microextracción líquido-líquido dispersiva. Como método de

determinación se escogió HPLC-FLD. Este objetivo se propuso dado las escasas aportaciones analíticas existentes para la determinación de estos contaminantes en este tipo de matrices, así como para expandir la aplicabilidad de los tratamientos de muestra miniaturizados.

- Desarrollo y validación de un método sensible y rápido empleando UHPLC-MS/MS y extracción basada en QuEChERS para la determinación de toxinas de *Fusarium* en diferentes tipos de leches vegetales (avena, arroz, soja y alpiste) y su aplicación en el análisis de muestras comerciales. Aunque estas muestras son susceptibles de contaminación por micotoxinas, han sido poco estudiadas en este aspecto.

- Evaluación de un Nuevo sorbente (Z-Sep+) en el tratamiento de muestra basado en el método QuEChERS, para la determinación mediante UHPLC-MS/MS de 28 carbamatos en productos lácteos de alto contenido graso, como son quesos Gorgonzola, Camembert y Roquefort. Teniendo en cuenta la reciente disponibilidad de este tipo de sorbentes, recomendados para la limpieza de matrices grasas, se consideró interesante estudiar su aplicabilidad en derivados lácteos de alto contenido graso.

- Validación de un método para el análisis de rutina de residuos de 11 aminoglicósidos en diferentes tipos de leche y leches enriquecidas, empleando para el tratamiento de muestra extracción en fase sólida con un nuevo sorbente consistente en polímeros de impronta molecular, altamente selectivo. Además, dada la alta polaridad de estos compuestos, se propuso el empleo de cromatografía de interacción hidrofílica (HILIC) en un sistema UHPLC-MS/MS como alternativa a la cromatografía de pares

iónicos, empleada usualmente para la determinación de estos compuestos.

- Desarrollo de un nuevo método analítico basado en electroforesis capilar con espectrometría de masas de alta resolución (CE-Q-TOF-MS/MS) como alternativa a los métodos basados en LC-MS/MS para la identificación y cuantificación simultánea de cuantificación of 15 antibióticos (7 quinolonas y 8 tetraciclinas) en diferentes tipos de leche y leches enriquecidas. Además, se estudió el empleo de un nuevo sorbente de extracción en fase sólida (Oasis HLB PRiME) como tratamiento más simple y efectivo que los usualmente empleados con otros sorbentes.

SUMMARY

Milk and dairy products are very rich in nutrients and thus provide an ideal growth environment for food-borne pathogens. Moreover, milk and dairy products can also contain chemical hazards and residues mainly introduced through the environment, animal feedstuffs, animal husbandry and industry practices.

We focused in this Thesis on chemical hazards which include contaminants (such as mycotoxins) and residues of other chemicals (as pesticides or antibiotics) that are used or added during the animal production or manufacturing processes, such as veterinary drugs and pesticides.

In addition, considering the increasingly consume of vegetable milks (most of them based on cereals), this commodity has been included in the study.

Mycotoxins are highly toxic secondary metabolites produced by certain fungi that grow on agricultural products. Ingestion, inhalation or skin absorption of mycotoxins can cause illness or even death in both humans and animals. The presence of mycotoxins in milk is a topic of great interest, since milk is an important food for adults and children. Given the variety of mycotoxins that may occur in the diet of animals, the number of studies related to the transfer of these compounds to milk and especially to dairy products is very limited. It would also be possible to find mycotoxins in vegetable-derived milk. Studies on mycotoxins in these matrixes are scarce, although the products of origin (soy, oat, rice, etc.) may be contaminated.

Milk production has an effect on the environment, and otherwise, the environment can have an effect on milk production through environmental contaminants such as pesticides, extensively used for agricultural activities, which may lead to residues in milk. Among the different families of pesticides, carbamates are commonly used as insecticides, and their presence in foods

could have adverse health effects, as they have high acute toxicity. Thus, their presence on milk and dairy products as a consequence of feeding the animals with contaminated food or water is a matter of concern.

With regard to veterinary antibiotics, the presence of residues in foods of animal origin and specially milk and dairy products, may have adverse health effects. The development of antibiotic resistance in bacteria has long been attributed to the overuse of antimicrobials in human medicine but the relationship between the agricultural use of antimicrobials and the antibacterial resistance in humans is also the subject of much concern.

Referring to these problems and taking into account the last technical advances in terms of efficiency and miniaturization, different separation techniques, such as high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC) and also capillary electrophoresis (CE) have been assessed, coupled to detection techniques such as fluorescent detection (FLD), tandem mass spectrometry (MS/MS) and high resolution mass spectrometry (Q-TOF). In addition, alternative sample treatments have been proposed, making possible an increased efficiency and sample throughput.

As a summary, the Thesis compiles the following works:

- In the first chapter, HPLC-FLD has been proposed for the analysis of five mycotoxins (aflatoxin M_1, B_1, B_2, G_1 and G_2) in different types of yogurt (natural, skim and liquid), using dispersive liquid-liquid microextraction as an alternative sample treatment, scarcely explored for these compounds.
- In the second chapter, a multi-mycotoxin method by UHPLC-MS/MS to determine seven *Fusarium* toxins (fumonisin B1, fumonisin B2, HT-2 and

T-2 toxin, zearalenone, deoxynivalenol, and fusarenon-X) was carried out. In this case, different types of vegetable milks (oat, soya, rice and birdseed milks) were analyzed. The sample treatment consisted of a QuEChERS-based extraction with no further clean-up.

- In the third chapter, a simple and efficient method for the determination of twenty eight carbamates in high-fat cheeses was carried out. The methodology was based on a QuEChERS procedure using a new sorbent (Z-Sep+) followed by UHPLC-MS/MS determination. The method has been validated in different kinds of cheese (Gorgonzola, Roquefort, and Camembert).
- In the fourth chapter, an analytical method for the determination of eleven aminoglycosides in different types of milk and milk-based functional products has been optimized and validated. A hydrophilic interaction chromatography (HILIC) column was proposed for the separation of analytes by UHPLC–MS/MS. Also, a molecularly imprinted polymer has been used for the solid phase extraction of the analytes, in order to achieve high selectivity in the sample treatment.
- In the last chapter, a new analytical method based on CE-MS/MS for the identification and simultaneous quantification of fifteen antibiotics (seven quinolones and eight tetracyclines) in milk samples has been validated. Detection using an Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) was used. A solid-phase extraction method using the new Oasis HLB PRiME cartridge was applied for clean-up. This work was developed in the research group of Prof. Antonio Molina (University of Jaen).

RESUMEN

La leche y los productos lácteos son una importante Fuente de nutrientes y, por ello también proporcionan un ambiente idóneo para el crecimiento de microorganismos patógenos. Además, la lecho y sus derivados son susceptibles de contaminación por parte de agentes químicos y de residuos introducidos a través del ambiente, piensos, ganadería o prácticas industriales.

Esta Tesis Doctoral está enfocada al estudio de riesgos químicos que incluyen, entre otros, contaminantes naturales (como las micotoxinas) y residuos empleados o añadidos durante los procesos de ganadería o producción animal, como los antibióticos veterinarios o los plaguicidas.

Además, considerando el gran incremento del consumo de productos alternativos a la leche, como las leches de origen vegetal (muchas de ellas a base de cereales), estas muestras también se han incluido en el estudio.

Respecto a los contaminantes estudios, la primera familia son las micotoxinas, metabolitos secundarios altamente tóxicos producidos por algunos hongos que crecen en productos agrícolas, especialmente cereales. Su ingesta, inhalación o absorción por la piel puede causar diversas enfermedades (incluido cáncer) o incluso la muerte, tanto en humanos como en animales. La presencia de micotoxinas en leche, como consecuencia de la alimentación del animal con piensos contaminados, es por tanto un tema de gran interés, ya que la leche es un producto de alto consumo, especialmente en poblaciones sensibles como los niños. Dada la gran variedad de micotoxinas que pueden estar presentes en la dieta de los animales, el número de estudios relativo a su transferencia hacia la leche y, especialmente a los productos lácteos, es aun relativamente limitado. Además, dado que la presencia de micotoxinas en cereales (arroz, soja, avena, etc) es bastante frecuente, sería posible encontrar estos

contaminantes en leches vegetales. No obstante, esta matriz ha sido muy poco estudiada es este sentido.

Las condiciones ambientales pueden tener efectos negativos sobre la leche, dado que los contaminantes ambientales, tales como plaguicidas empleados en la agricultura, pueden pasar a la cadena alimenticia a través de la ingesta de piensos o aguas contaminados, pasando a la leche. Entre las diversas familias de plaguicidas, los carbamatos son frecuentemente empleados como insecticidas, y su presencia en alimentos derivados de animales puede suponer un riesgo para el consumidor, dada su toxicidad aguda. Es por ello, que la presencia de residuos de carbamatos en leche y productos lácteos es un tema de gran interés.

En lo que respecta a los antibióticos de uso veterinario, la presencia de sus residuos en alimentos de origen animal (como leche y derivados), puede dar lugar a efectos adversos. El desarrollo de resistencia bacteriana se ha atribuido mayormente al abuso del empleo de antibióticos en medicina, pero también está causado por el empleo de antibióticos en la ganadería, que pasarían a los humanos a través de la cadena alimentaria.

Teniendo en cuenta estos problemas, en esta Tesis Doctoral se han propuesto diversos métodos analíticos para la determinación de contaminantes en leche, productos lácteos y leches vegetales, aprovechando los últimos avances de las técnicas separativas en términos de eficacia y miniaturización, diversas técnicas analíticas como cromatografía líquida de alta resolución (HPLC) y de ultra-resolución (UHPLC) así como la electroforesis capilar (CE) acopladas con diversos sistemas de detección como fluorescencia, espectrometría de masas en tándem (MS/MS) y de alta resolución (Q-TOF). Además, se han empleado

tratamientos de muestra alternativos, con objeto de simplificar esta etapa, incrementando la eficacia y minimizando el consumo de disolventes.

En resumen, la Tesis Doctoral comprende los siguientes trabajos:

- En el primer capítulo, se propone la determinación mediante HPLC-FLD de cinco micotoxinas (aflatoxina M_1, B_1, B_2, G_1 y G_2) en diferentes muestras de yogurt (natural, desnatado y líquido), empleando microextracción líquido-líquido dispersiva como tratamiento de muestra, escasamente empleado para estos compuestos.
- En el segundo capítulo, se ha empleado un método basado en UHPLC-MS/MS para determinar siete toxinas de *Fusarium* (fumonisina B1, fumonisina B2, toxinas HT-2 y T-2, zearalenona, deoxinivalenol, y fusarenon-X) en diversas leches vegetales (avena, soja, arroz y alpiste). El tratamiento de muestra consistió en una extracción basada en el método QuEChERS, que no requirió limpieza adicional.
- En el tercer capítulo, se propuso un método sencillo y eficaz para la determinación de veintiocho carbamatos en muestras de queso con alto contenido graso. La metodología propuesta se basó en un tratamiento de muestra empleando QuEChERS con un nuevo sorbente (Z-Sep+) seguido de una determinación mediante UHPLC. El método se validó en distintos tipos de queso (Gorgonzola, Roquefort, y Camembert).
- En el cuarto capítulo, se llevó a cabo la validación de un método para la determinación de once aminoglicósidos en diferentes tipos de leche y alimentos funcionales lácteos (leches enriquecidas). En este caso, se propuso el empleo de una columna de interacción hidrofílica (HILIC)

para la determinación de los compuestos mediante UHPLC–MS/MS. Además, el tratamiento de muestra se basó en extracción en fase sólida empleando polímeros de impronta molecular, altamente selectivos.

- En el último capítulo, se ha validado un método analítico basado en CE y MS de alta resolución, como es quadrupolo-tiempo de vuelo (Q-TOF), para la identificación y cuantificación de quince antibióticos (siete quinolonas y ocho tetraciclinas) en diferentes muestras de leche. Como tratamiento de muestra se propuso la extracción en fase sólida empleando un sorbente de reciente comercialización (Oasis HLB PRiME). Este trabajo se desarrolló en los laboratorios del grupo de investigación del Prof. Antonio Molina (Universidad de Jaén).

INTRODUCTION

I.1. Milk and related products

Milk and dairy products are nutrient-dense foods supplying energy and significant amounts of proteins and micronutrients. They are commonly consumed by people of all age groups, especially children. Accordingly, milk is very important in human nutrition, being one of the major nutrient sources thanks to its biochemical complexity, providing all essential amino acids. Confirmation of these nutritive advantages is the extensive and constant consumption of milk and dairy products in many countries [1,2].

Although it has not been possible to establish the exact date at which milk from various species was used to nourish humans, a good estimate would be consider that as soon as animals were domesticated (about 9000 BC), their milk, in a variety of forms, was used as food for humans [3]. Cow milk is the main milk type used for human consumption corresponding to 83% of the world milk production, then buffalo milk by 13%, goat milk with 2%, sheep milk with 1% and finally camel milk with 0.3% [4]. Many factors contribute to milk consumption [5], including beliefs, situations and sensory evaluation. Thus, the average milk consumption (as fluid milk and processed products) per person varies widely. Figure I.1 shows the cow milk production around the world in 2016. Nevertheless, due to globalization and migration processes these trends are changing, a factor that needs to be considered by dairy industry. Moreover, milk is processed into an assortment of dairy products such as cream, butter, yogurt, kefir, ice cream, condensed milk, powdered milk and cheese. Focusing on Europe, the usage of milk consumption has varied greatly. For example, in some countries like Finland, Norway and Sweden there is a high consumption of fluid milk, while in France and Italy cheeses dominate milk consumption [6].

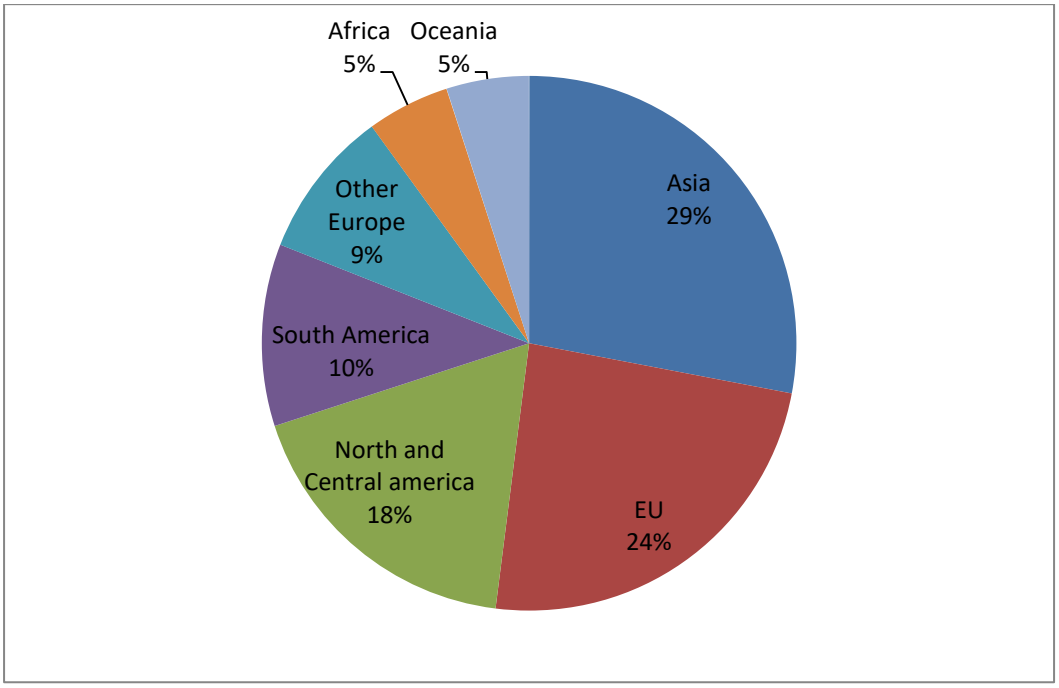

Figure I.1. Cow milk production in 2016 (share per region). Reproduced from [7].

I.1.1 Vegetables milks

Nowadays, the interest in products alternative to milk is increasing. This fact can be related to the raising number of people affected by lactose intolerance, a disorder that increases with aging, and that is especially important in some regions such as Asia, where it is estimated that affects more than 80% of the population. Moreover, the growing trend of following vegetarian diets or avoiding consumption of dairy products has led to the marketing of so-called "vegetable milks" or "plant milks", obtained from ingredients like rice, oats, almonds, coconuts or soy. Although the resulting liquid is called milk no matter which product it comes from, the nutritional composition varies according to the basic ingredient [8].

I.2 Food Contaminants

Occurrence of chemical hazards and contaminants in our food from various sources is a fact. They cause a health concern, resulting in rigorous regulations of their levels by some national governments and also internationally by the *Codex Alimentarius* Commission. Thus, in spite of the important nutritional value of milk, it can contain chemical hazards and contaminants, mainly introduced through the environment, animal feedstuffs, animal husbandry and industry pursuit [9]. A food-safety hazard is defined as "a biological, chemical or physical agent in a food, or condition of food with the potential to cause an adverse health effect" [10]. Considering biological hazards, milk and dairy products are very rich in nutrients and thus provide an ideal growth environment for many microorganisms. This implicates spoilage organisms in milk, some strains of which can survive pasteurization and grow at refrigeration temperatures. In addition, milk can be a potentially effective source of food-borne pathogens, whose presence is determined by the health of the dairy herd, quality of the raw milk, milking and pre-storage conditions, available storage facilities and technologies, and hygiene of the animals, environment and workers. Referring to physical hazards, they usually are foreign materials unintentionally introduced to food products, due to accidental contamination or poor handling practices (e.g.: pieces of metal, glass or wood, insects, stones, soil, dirt…) that are hazardous to the consumer. These contaminants can be transfer to the food product at any stage of production.

The source of chemical hazards varies and can include air, soil, water, substances used in animal husbandry practices and animal feedstuffs [11]. Thus, chemical hazards include contaminants (heavy metals, radionuclides, persistent priority pollutants as polychlorinated biphenyls or dioxins, and

mycotoxins), residues of pesticides (as a consequence of feeding the animal with contaminated feedstuff), residues of veterinary drugs (as a consequence of drug administration to the animal) and some chemicals which are used or added during the manufacturing processes, such as substances migrating from packaging materials (e.g. isopropyl thioxanthone and bisphenol A).

Thus, analysis of relevant chemical contaminants is an important factor of food safety testing programs to ensure consumer safety and compliance with regulatory limits [12]. Modern, accurate and sensitive analytical techniques can measure known chemical contaminants at low concentration levels in complex food matrices. In addition, they may also help to discover and identify new or unforeseen chemical contaminants.

In this Doctoral Thesis, we will focus our interest on the study of three families of chemical hazards that can be found in milk, dairy products or vegetables milks, namely mycotoxins, residues of pesticides, and residues of antibiotics. Consequently, the following sections will be devoted to these compounds.

I.2.1 Mycotoxins

I.2.1.1 Definition and classification of mycotoxins

The name mycotoxin is a collection of the Greek word for fungus “mykes” in addition Latin word “toxicum” meaning poison. This term is usually reserved for the relatively small (MW ~700), toxic chemical products formed as secondary metabolites by a few filamentous fungi that readily settle down crops in the field or after harvest. Figure I.2 shows the most common species of fungus producing mycotoxins.

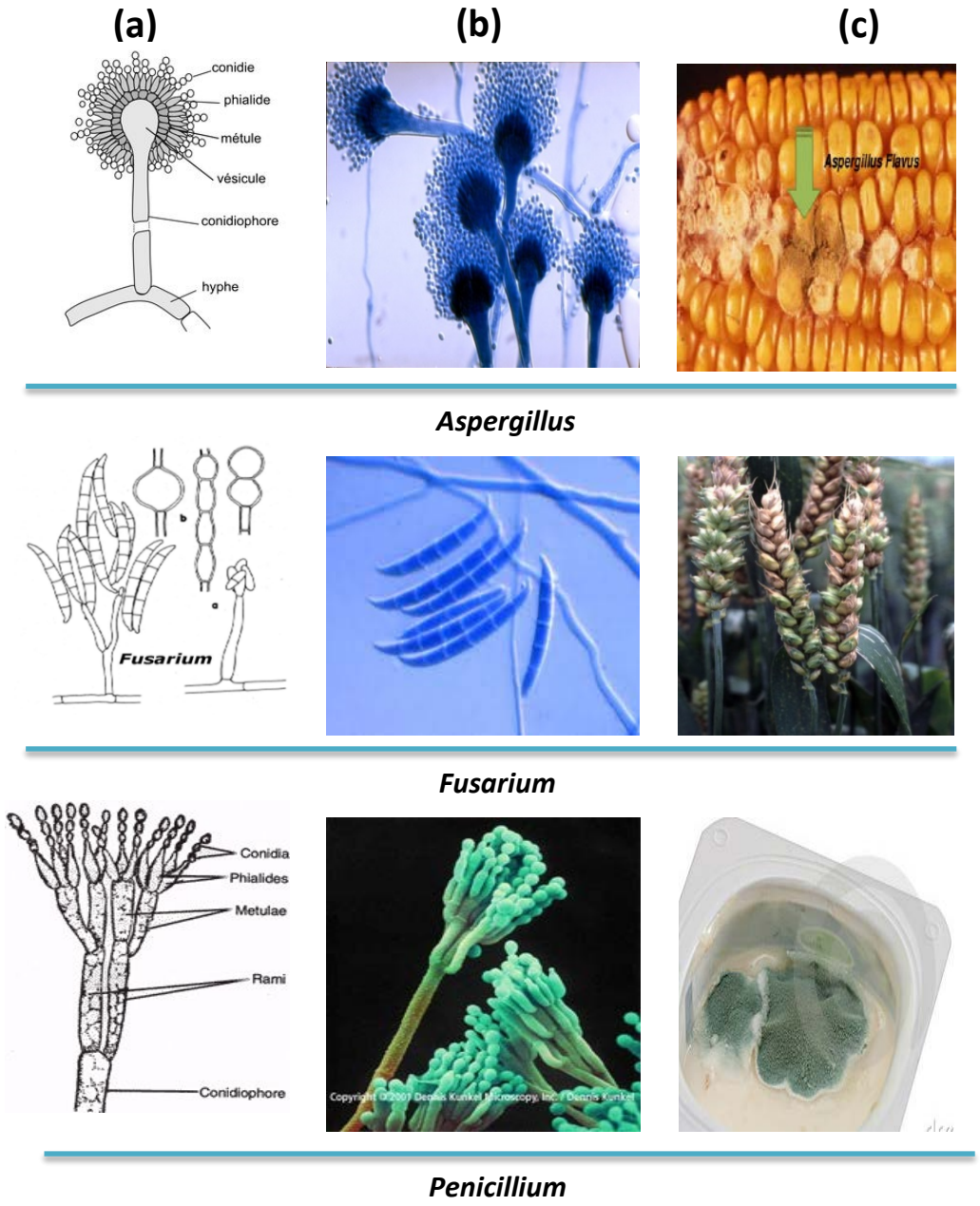

Figure I.2. Diagram of common species of fungus producing mycotoxins (a); with their microscopic image (b); in different types of food (c). Adapted from [13,14,15].

These compounds show a potential threat to both human and also animal health via the ingestion of different contaminated food products [16,17]. Until now, more than 300 mycotoxins have been identified worldwide.

According to Food and Agriculture Organization (FAO) it is estimated that about 25% of the world's food crops are contaminated with mycotoxins [18]. Factors such as prolonged drought, high temperatures, substrate composition, storage time and storage conditions play an important role in fungal growth and the synthesis of mycotoxins [19]. Thus, the most important factors affecting mycotoxin contamination are biological and environmental while harvesting, storage and distribution-processing conditions of agricultural products are also crucial [20].

Although the production of mycotoxins is not a continuous process, it should be assumed that if a mold exists in the environment and can produce toxins, the mycotoxins can be present in the food. Furthermore, the fungus may be absent, but the toxin may be present and active [21].

Concerning their stability, mycotoxins are stable in most food processing systems. However, some studies suggest that some treatments such as aqueous cooking and steeping reduce mycotoxin concentrations. Roasting and extrusion cooking at high temperatures (above 150 °C) appear to decrease mycotoxin concentrations [22].

Mycotoxicosis is the toxic effect of mycotoxins on animal and human health. The exposure to mycotoxins can happen through the ingestion of contaminated foodstuff, while dermal exposure results in slow and insignificant absorption. The harmful effect (acute or chronic) depends on the concentration and the time of exposure to the toxin, the body weight, age and nutritional status of the individual, environmental factors (farm management) and other

harmful dietary effects, and the presence of several mycotoxins in the same matrix (synergistic effects) [23,24]. Thus, some studies reported that aflatoxin B1 (AFB1) and fumonisin B1 (FB1) interacted synergistically in the cancer initiation and promotion, depending on intake conditions.

Their acute toxicity and the incidence of certain types of cancer related to some mycotoxins have drawn attention to the feed and food safety [25]. Thus, the European Commission has established maximum levels for some mycotoxins (aflatoxins B1, B2, G1, G2 and M1, ochratoxin A, patulin, deoxynivalenol, fumonisins, T-2 and HT-2 toxins and zearalenone) in several foodstuff by Regulation 1881/2006 [26] and subsequent modifications, as well as several recommendations for other toxins.

One of the main foods susceptible of mycotoxin contamination is cereals and their related products, which may be mixtures of various raw materials. Simultaneous occurrence of different mycotoxins has been reported in baby foods, breakfast cereals, flours and bread [27,28].

However, food contamination by mycotoxins is not only a human health care; it also causes economic losses to farmers due to mycotoxin adverse effects that cause poor animal productivity. Mycotoxin existence in material used in the production of animal feed (such as grains and silage) susceptible to mycotoxin contamination is a matter of concern and a way of introduction of mycotoxins into human diet through the food chain [29]. Some studies suggest that the presence of mycotoxins in dairy cow plasma promote the possibility that these toxins could be carried over into the cow milk [30]. Moreover, though the rumens are supposed to be a barrier against mycotoxin contamination, the analysis of milk samples explains that the carry-over of mycotoxins into the

milk is possible in some cases [31]. Figure I.3 shows how the mycotoxins reach to the humans through food chain, causing adverse effects.

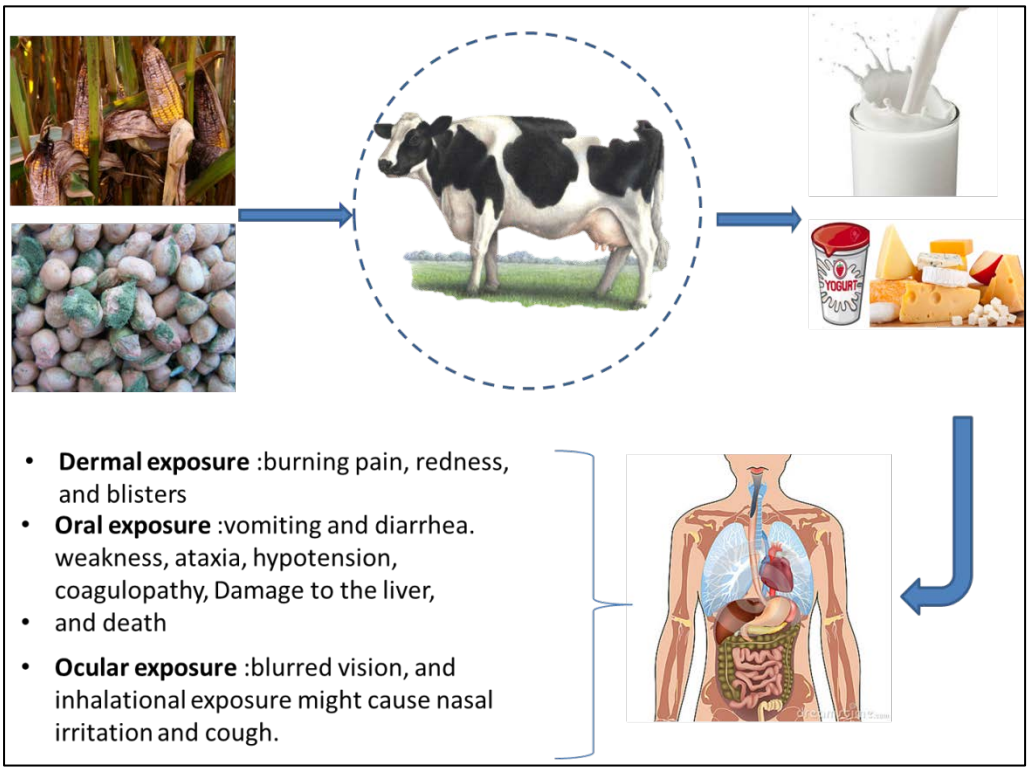

Figure I.3. Presence of mycotoxins in milk and introduction into the food chain. Adapted from [32,33].

In this Thesis two main groups of mycotoxins (aflatoxins and *Fusarium* toxins) have been considered due to their importance and occurrence in milk and cereal-based products. A brief summary of the characteristics of each group will be shown below.

I.2.1.2 Aflatoxins

Aflatoxins are considered as the most dangerous mycotoxins because of their occurrence, toxicological effects and their effects on human well-being and crop trade [34]. The main mycotoxins are AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). 'B' and 'G' refer to the blue and green

fluorescent colors produced by these toxins under UV light during the thin layer chromatography plate visualization.

Chemically, aflatoxins are difuranocoumarin derivatives produced primarily by *Aspergillus*, *Fusarium* and *Penicillium* molds, which are especially found in areas with hot and humid climates [35]. AFB1 and AFB2 are mainly produced by the most frequent *Aspergillus flavus*, while AFB1, AFB2, AFG1 and AFG2 are produced by *Aspergillus parasiticus* [36,37]. Other *Aspergillus* species such us *A. bombycis, A. ochraceoroseus, A. nomius, A. pseudotamarii, A. tamarii, A. foetidus* and *A. oryzae* are known to produce aflatoxins but their toxicological significance is low [38,39].

Aflatoxins are highly toxic compounds (ranking in order of AFB1 > AFG1 > AFB2 > AFG2 for toxicity) causing acute or chronic liver disease, immunosuppressive, hepatotoxic, mutagenic, teratogenic, and are considered as human carcinogen (group 1) by the International Agency for Research on Cancer (IARC) [40,41]. On the other hand, exposure to aflatoxins in animals results in impairment of liver function and also reduce the food intake, which might also explain the reduced milk production in dairy cattle exposed to aflatoxins [42].

The main food products affected by aflatoxin contamination can be divided into the following categories: 1) Cereals (primary production and processed products) and small grains such as wheat, barley and rice; 2) Milk and dairy products i.e. butter, yogurt; 3) Nuts and dried fruits; 4) Feeds; and 5) Other i.e. olives, olive oil, bee pollen. Considering the possible contamination of milk by aflatoxins, although the rumen is assumed to act as a filter for mycotoxin contamination, the analysis of milk samples reported that the carry-over of mycotoxins into the milk happens and is a matter of concern [43]. Moreover, two metabolic products are produced in the animal rumen and secreted in milk

of animals fed with contaminated feed: aflatoxin M1 (AFM1), the hydroxylated metabolite of AFB1 and aflatoxin M2 (AFM2, considered less toxic), the hydroxylated metabolite of AFB2 [44]. In fact, the concern for mycotoxin contamination in dairy products began in the 1960s, with the 1st reported case of contamination by AFM1 [21 por encima de]. AFM1 can be found in animal milk within 12-24 h after the first ingestion of AFB1 (coming from contaminated animal feedstuff) and can last up to 3 days after the last ingestion of the mycotoxin. Some studies have demonstrated that a concentration of 20 μ kg^{-1} of AFB1 in the total mixed ration dry matter of lactating dairy cattle will result in AFM1 levels in milk less than the limit of the Food and Drug Administration (FDA) which is 0.5 μkg^{-1} [45]. In humans, AFM1 can also be present in milk from nursing mothers who consumed a diet contaminated with AFB1 [46]. Moreover, aflatoxins are thermostable so they can persist in dairy products and fermented food despite the heat treatments like pasteurization and sterilization. In this way, milk and derived products show a great potential for the introduction of aflatoxins in human nutrition and considering the high consume of milk and dairy products, this poses a potential risk for human health [47,48].

I.2.1.3 Fusarium toxins

They are toxins produced by more than 50 species of *Fusarium* mainly occurring in the grain of developing cereals such as wheat, maize and others [49,50]. They include different mycotoxins, those produced mainly by *Fusarium verticilloides* (fumonisins), and those produced mainly by *Fusarium graminearum*, which includes the so-called estrogenic mycotoxins (being the most important zearalenone and zearalenol) and non-estrogenic mycotoxins or trichothecenes (such as deoxinivalenol, nivalenol and T2 and HT2 toxins).

Fumonisins

Fumonisins (FBs) were discovered in 1988 through their isolation in cultures of *Fusarium verticilloides* (formerly *Fusarium moniliforme*) [51]. They are mainly found in maize, but have also been reported in rice products [52]. Animal and human health problems associated with these mycotoxins are mainly related with the consumption of contaminated maize or its derivatives [53]. Eighteen different FBs have been isolated and identified depending on their hydroxyl groups. The most toxigenic and predominant molecular form is FB1, which, together with fumonisin B2 (FB2), comprise about 70% of all fumonisin found in nature and food [54,55,56]. FB1 is included by the IARC in Group 2B (possible carcinogen to humans) [40]. As a result, European Commission has recommended a provisional maximum tolerable daily intake (PMTDI) for FB1, FB2 and fumonisin B3, alone or in combination, of 2 mg kg^{-1} b.w. per day [57].

Zearalenone

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin common in temperate and warm countries and is a frequent contaminant of cereal crops worldwide, including corn but also, to a lesser extent barley, oats, wheat, sorghum, sesame, millet, and rice [58]. Moreover, indirectly contaminated products such as milk, meat, and eggs from animals that have consumed contaminated feeds are other sources for human ZEN uptake [59]. Some toxic effects have been reported caused by ZEN, such as stimulate adverse liver lesions with subsequent development of hepatocarcinoma [60], and haematotoxic effects in rats [61].

Trichothecenes

Trichothecenes comprise the largest group of mycotoxins. More than 150 trichothecene mycotoxins have been identified so far and they are ubiquitous in cereals of moderate climate areas. They are divided into different groups: Type A, including diacetoxyscirpenol, neosolaniol and the highly toxic HT-2 and T-2 toxins, and Type B, including deoxynivalenol (DON), 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol (NIV) and fusarenon X (F-X) [62]. Type A causes vomiting, diarrhea, leukopenia, necrotic lesions and hemorrhage, while Type B causes food refusal and vomiting, kidney problems and immunosuppression. Moreover, trichothecenes have high toxicity at the subcellular, cellular, and organic system level. They can stop the synthesis of the proteins by their effect on ribosomes to interfere with protein synthesis and covalently bond to sulfhydryl groups [63].

- ✓ **T-2 and HT-2 toxins** are especially prevalent in oats, and high levels have been found in Europe [64]. Moreover, it has been reported that levels in raw grains were higher than in grain products for human consumption, suggesting that processing applied to grains results in lower T-2 toxin and HT-2 toxin concentrations [65]. T-2 toxin and HT-2 toxin are toxic to all animal species as well as to humans. Historical cases of human intoxications associated with the consumption of overwintered, moldy grains are described as Alimentary Toxic Aleukia (ATA), characterized by sepsis and hemorrhages and a general pancytopenia [66].
- ✓ **DON**, known as vomitoxin is linked with acute gastrointestinal adverse effects such as vomiting (emesis) both in animals and humans. The main impacts of long-term dietary exposure of different animals to DON are weight gain suppression, anorexia and altered nutritional efficiency [67,68].

The high incidence of DON in cereals [69] support the need for studies to evaluate the effect of this mycotoxin and its degradation products in different samples for human consumption.

- ✓ Concerning **NIV**, its highest mean in food, feed and unprocessed grains were observed in oats, maize, barley and wheat and products thereof. Higher concentrations were observed in grains without processing compared to grains for human consumption. Normal cooking conditions appear to have little influence on the reduction of NIV concentrations in the contaminated raw materials. NIV, is unstable under high temperatures (> 150 °C) and alkaline conditions, and the rate of degradation increases by increased time and/or temperature conditions [70]. The scientific committee on food (SCF) (2000) noted that the general toxicity and immunotoxicity/haematotoxicity of NIV are considered as the critical effects. They observed from long term studies in mice that these effects are similar to those of other thrichothecenes [68].
- ✓ **FX** is one of the 12,13-epoxytrichothecenes which naturally occurs in agricultural commodities like wheat and barley. FX has been observed to occur frequently with DON in agricultural products. FX has some negative health effect such as: a rise in blood pressure and a decrease in respiratory rate but induced no significant change in heart beat rate [71,72].

I.2.1.4 Legislation on mycotoxins

Since the discovery of aflatoxins in the 1960s, the toxicity of mycotoxins has led many countries to establish regulations for their control in foods intended for human or animal consumption [73]. Moreover, according to recent studies, mycotoxin contamination of animal feeds is a frequent issue around whole Europe, sometimes even above allowed or recommended levels [74].

According to the EU regulatory framework minimization of the exposure to aflatoxins (the most dangerous mycotoxins and one of the group studied in this Thesis) is based on establishing maximum levels of aflatoxins in different foodstuffs (4-10 μg kg^{-1} total aflatoxins) and feed products exceeding the maximum levels should not be placed on the EU market. For instance, EU has established maximum levels in cereals and cereal products intended for direct human consumption at 2 μg kg^{-1}, and 4 μg kg^{-1} for AFB1 and the sum of aflatoxins, respectively, and a maximum level of 0.05 μg kg^{-1} of AFM1 in milk and milk for the manufacture of milk-based products [26,75]. Acceptable limits worldwide on aflatoxin levels range between 5 and 20 μg kg^{-1} [76]. Thus, compared to other regions of the world, EU has the most extensive and most detailed regulations governing aflatoxin presence in food and feed.

Concerning other mycotoxins, such as *Fusarium* toxins, the European Commission has established limits for DON, ZEN, FBs (sum of FB1 and FB2) in cereals and their products intended for human consumption, while admissible levels of T-2 and HT-2 toxins in cereals are under discussion [26,57], although a recommendation has already been established for T-2 and HT-2 toxins in cereal and cereal products [77]. Moreover, contamination of feed by some mycotoxins is also under consideration [78].

As for the requirements of the methods of sampling and analysis for control the mycotoxins in food, these are laid down in Regulation (EC) No. 401/2006 [79], modified by Regulation (EU) 178/2010 for some commodities [80]. Thus, laboratories can select any method of their choice provided which meets the criteria established by this regulation.

I.2.2 Pesticides

According to FAO, A pesticide is any substance or mixtures of substances, natural or synthetic, formulated to control or expel any pest that competes with humans for food, destroys property, and spreads disease. The term pest includes insects, weeds, mammals, and microbes, among others [81]. Nowadays, due to the possible toxic effects of pesticides on human health and on the environment, there are rigorous regulations for their registration and use all over the world, especially in developed countries. Generally, the toxicity refers to the ability of a substance which can produce adverse effects. These negative effects may range from slight symptoms such as headaches to severe symptoms like coma, convulsions, or death [82].

There are different groups of pesticides according to their type of use and also according to their chemical structure. Depending on their effects, the main pesticide groups are herbicides, used to kill weeds and other plants growing in places where they are unwanted; insecticides, utilized to kill insects and other arthropods; and fungicides, used to kill fungi. According to their chemical structure, different families can be considered, being the most important ones: organochlorines, organophosphates, carbamates, dithiocarbamates, carboxylic acid derivatives, substituted ureas, triazines, pyretroids and neem products [83]

In this Thesis, the study was focused on determination of carbamates (CRBs).

I.2.2.1 Carbamates

Carbamates (CRB) are a family of pesticides extensively used for agricultural activities. The first CRB, carbaryl, was discovered in 1956, and has been the most extensively used CRB. They are derived from carbamic acid (NH_2COOH). A

carbamate group, carbamate ester (e.g., ethyl carbamate), and carbamic acids are functional groups which are inter-related structurally and often they are interconverted chemically. They are mainly used as insecticides, inhibiting the growing of the insects by inhibition of cholinesterase enzymes, affecting nerve impulse transmission [84].

CRBs are a highly polar and soluble in water and show thermal instability. CRBs can be absorbed continuously, ingested, inhaled, or injected. Though most patients quickly become symptomatic, the onset and severity of symptoms depend on the specific compound, amount, route of exposure, and average of metabolic degradation [85]. As stated before, CRB pesticides inhibit cholinesterase enzymes, and nerve impulses could not be transmitted normally. This can paralyze the nervous system, and it may cause the death, usually from respiratory failure [86,87]. Possible neurological complications are:

- Intermediate syndrome – proximal muscle weakness, respiratory muscle weakness and facial muscle weakness, which usually occurs days to a few weeks after the poisoning.
- Delayed neuropathy – distal muscle weakness, usually occurs weeks to months after the poisoning.

Thus, their presence in food is a matter of concern.

The environment can have an effect on milk production through environmental contaminants such as pesticides, which may lead to residues in milk. A dairy cow may be exposed to pesticides via the air it breathes, the water which it drinks, and the forage that it consumes. In this way, pesticides may appear in milk due to several reasonable causes [88]. Fig.I.4 shows the transfer of pesticides through food chain.

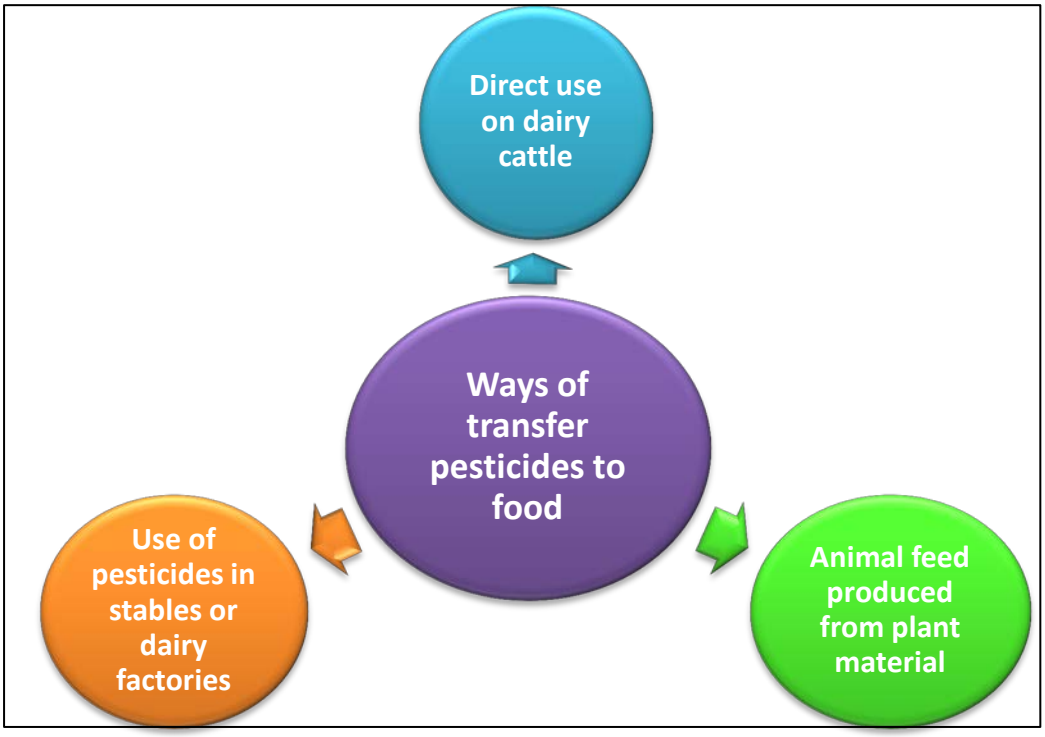

Figure I.4. Diagram of pesticides transfer to food.

Moreover, the presence of pesticides in milk depends on different factors, such as the properties and stability of the pesticide, its metabolism in animals and its way of application or intake. Depending on the type and properties of the pesticide, the residue is excreted through urine, adsorbed in the adipose tissue, or excreted into the milk. Water-soluble compounds will be mainly eliminated through urine and feces. However, fat-soluble compounds will be eliminated at most through milk, resulting in the gradual contamination of dairy products [89,90]. Moreover, raw milk undergoes various processing treatments before consumption and is consumed as different dairy products. These processing can comprise pasteurization, sterilization, concentration, separation of fat, fermentation, coagulation, and drying. During these processes, pesticides can

be transferred from one phase to another or degraded totally or partially to other compounds that may be more or less toxic than the parent compound. Evidence of the presence of pesticides in milk and dairy products has been extensively reported [91,92].

I.2.2.2 Legislation on pesticide residues in food

In order to protect consumers and animal health and to facilitate trade by setting common standards, several international organizations such as the EU by Regulation (EC) No. 396/2005 [93] and subsequent amendments, have set up maximum residue limits (MRLs) in different types of food (including milk and some milk-based products) that cover a large number of pesticides including some CRBs. Moreover, a recent paper summarizes the development of principles and methods applied within the program of the FAO/WHO *Codex Alimentarius* during the past 50 years for the safety assessment of pesticide residues in food and feed and [94].

Regarding determination of pesticides, EU has established a guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed, compiling the required performance characteristic of analytical methodologies used for determination, identification and confirmation of pesticides [95].

I.2.3 Antibiotics

I.2.3.1 Definition and classification of antibiotics

An antibiotic, according to the Merriam-Webster dictionary, is defined as “a substance produced by or a semisynthetic substance derived from a microorganism and can inhibit or kill the other microorganism” in dilute

solution [96]. This definition would encompass things like natural penicillin (a product of a mold) and ampicillin (a semisynthetic derivative of penicillin); exclude entirely synthetic factors such as sulfonamides and quinolones; and leave in a limbo drugs like chloramphenicol which, although initially discovered as a product of soil bacteria, it is now produced entirely by chemical synthesis.

A general definition from Wikipedia states that “antibiotics are a type of antimicrobial utilize in the treatment or prevention of bacterial infection”, whereas “antimicrobial” is simply “an agent that can kills microorganisms or inhibits their growth”, which would also include antiseptics and disinfectants [97].

Antibiotics are generally classified depending on their mechanism of action, their chemical structure, or spectrum of activity [98]. Table I.1 shows the classes of antibiotics and their properties [99].

Table I.1. Classes of antibiotics and their properties.

Chemical class	Biological source	Spectrum (effective against)	Mode of action
Beta-lactams (penicillins and Cephalosporin)	*Penicillium notatum* and *Cephalosporium* species	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Semi synthetic beta-lactams		Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Carboxypenems	*Streptomyces cattleya*	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Aminoglycosides	*Streptomyces griseus*	Gram-positive and Gram-negative bacteria	Inhibits translation (protein synthesis)
	Micromonospora species	Gram-positive and Gram-negative bacteria esp. *Pseudomonas*	Inhibits translation (protein synthesis)
Glycopeptides	*Amycolatopsis orientalis* (formerly designated *Nocardia orientalis*)	Gram-positive bacteria, esp. *Staphylococcus aureus*	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	*Streptomyces lincolnensis*	Gram-positive and Gram-negative bacteria esp.	Inhibits translation (protein synthesis)
Macrolides	*Streptomyces erythreus*	Gram-positive bacteria, Gram-negative bacteria not enterics, *Neisseria, Legionella, Mycoplasma*	Inhibit translation (protein synthesis)
Tetracyclines	*Streptomyces* species	Gram-positive and Gram-negative bacteria, *Rickettsias*	Inhibit translation (protein synthesis)
Quinolones	Synthetic	Mainly Gram-negative bacteria	Inhibits DNA replication
Fluoroquinolones	Synthetic	Gram-negative and some Gram-positive bacteria (*Bacillus anthracis*)	Inhibits DNA replication
Growth factor Analogs	Synthetic	Gram-positive and Gram-negative bacteria	Inhibits folic acid metabolism (anti-folate)

To protect consumer health and to ensure high quality of produced milk, the EU has established MRLs for drugs residues (including antibiotics) in milk [100] which are not permitted for use as growth promoters in the EU.

FDA regulates antibiotics purposed for use in humans or in animals. In food-producing animals, FDA-approved uses of antibiotics include:

- Treatment for sick animals;
- Control for the disease of a group of animals when some of the animals are sick;
- Prevention of disease for a group of healthy animals which are at risk of becoming sick; and

Since 2003, FDA has estimated the potential human health impact of using antibiotics in animal products as part of the animal drug approval process, using information and analyses from Centers for Disease Control and prevention (CDC) and others. FDA has provided guidance that reinforces the judicious use of antibiotics in animal products, recommending that antibiotics important for human health be limited to uses in food-producing animals that are important to assure animal health; and involve veterinary oversight or consultation [101]

Incorrect utilization of drugs in veterinary practice or if the dragging time for the treated cows has not been respected, have as a consequence that antibiotic residues can persist in edible tissues and milk. Thus, antibiotic residues are among the most frequent inhibitory compound found in milk, having undesirable effects on milk quality, milk technological properties, dairy products quality, and human health problems. The amount of antibiotic excreted into milk may vary from eight to 80 % of the dose; usually it averages about 50% [102]. It is extremely important to check the presence of antibiotic residues in milk because a little amount of the people is violently allergic to

antibiotics and extremely little doses can be fatal. Other people have allergy to small amounts of drugs that cause mild reactions that can be uncomfortable. Moreover, a continued low-level intake of drugs from food could result in a buildup of antibiotic resistance for organisms in humans who are resistant to the drug.

On the other hand, antibiotic residues in milk that is used to produce fermented products such as yogurt and ripening cheese can interfere with the fermentation process by inhibition of dairy starter microorganisms such as desired lactic acid bacteria using in the manufacturing process, causing important economic losses [103]. In a survey of the reason of slow acid production by cheese starters in the UK, some 28% of respondents attributed these problems to antibiotics [102].

Main veterinary drugs which are used today include lactams, sulfonamides, tetracyclines, aminoglycosides, chloramphenicol, macrolides and quinolones [104]. In this Thesis we have focus the study on three families of antibiotics (aminoglycosides, quinolones and tetracyclines) that will be commented in brief.

I.2.3.2 Aminoglycosides

Aminoglycosides (AGs) are broad-spectrum antibiotics produced by various species of *Streptomyces* and *Micromonospora* bacteria which have bactericidal activity for some Gram-positive and many Gram-negative organisms [105,106]. Their general structure is characterized by several aminosugars linked by glycosidic bonds to an aminocyclitol component [107].The most commonly used AGs are gentamicins C, neomycin B, dihydrostreptomycin and streptomycin. Others may include apramycin and spectinomycin [108].

AGs have been extensively employed in animal husbandry against the bacterial infections or growth inhibition, and are usually administered through intramuscular injection or intramammary infusion [109]. Due to their toxicity and possible antibiotic resistance, considerable attention has been paid to the potential human health risk, as AGs are known to cause ototoxic damage, nephrotoxicity (kidney damage), and encephalopathy [110,111].

Monitoring for AGs residues in food commodities is important because these drugs tend to accumulate in the kidney as they are generally excreted through the urinary tract. In addition, some residues of these compounds have been found in milk from some lactating cows [112].

I.2.3.3 Quinolones

Quinolones (QNs) are a group of synthetic antibacterial compounds highly active against a wide range of Gram-negative and Gram-positive bacteria. QNs are among the most important groups of antibiotics, whose activity is depending on the inhibition of bacterial DNA synthesis [113]. They have been extensively used in veterinary medicine to treat various diseases, but mainly for the treatment and prevention of cattle mastitis, causing residues in food staff which produce mild to moderate adverse reaction in humans, as allergic reactions or antibiotic resistance. Common adverse effects include gastrointestinal effects such as nausea, vomiting, diarrhea, headache and insomnia [114].

The QNs most commonly used in veterinary medicine (which have been studied in this Thesis) are danofloxacin (DAN), ciprofloxacin (CIP), marbofloxacin (MAR), enrofloxacin (ENR), difloxacin (DIF), sarafloxacin (SAR), oxalinic acid (OXA), and flumequine (FLU).

I.2.3.4 Tetracyclines

Tetracyclines (TCs) are a group of broad-spectrum antibiotics against a variety of both Gram-positive and Gram-negative bacteria, used for medical purposes as well as animal husbandry. They are considered a clinically relevant group of antibiotics, though dissemination of tolerance and resistance determinants have limited their use. Furthermore, they are both easy to administer, effective through oral dosing via water and feed, and inexpensive [115,116]. Their extensive applications might result in TCs residues remaining in food products, such as milk and its products, meat and honey, which may motivate allergic reactions in some hypersensitive individuals, liver damage, yellowing of teeth and gastrointestinal troubles. The rate of metabolic process of TCs in dairy cows has been estimated 25-75% and a significant amount of the administrated TCs are excreted in bovine milk. A recent review has covered the most important aspects regarding regulation, analytical methods, bacterial resistance, and environmental and health implications of the presence of TCs in food [117].

TCs included in this Thesis are: methacycline (METH), doxycycline (DOXY), tetracycline(TC), 4-epitetracycline (4-epiTC), minocycline (MIN), demeclocycline (DMC) and chlortetracycline (CTC).

I.2.3.5 Legislation on antibiotic residues in food

In order to protect humans from harmful effects of drug residues in milk, due to their toxicity and possible antibiotic resistance, considerable attention has been paid to the potential adverse effects on human health. In this sense, regulatory agencies and government authorities establish MRLs for antibiotics in food worldwide [118].

The needs to enhance the quality of food items and safety led the EU to establish a number of regulations to control food hygiene in 2004 [119], specifically for food of animal origin. These regulations establish the hygiene-health parameters to evaluate in the raw milk of these animal species, and the different ways to control residues of medicines for veterinary use and certain contaminants. The European Commission, as defined by the directives from Council Regulation (EEC) 470/2009 and Commission Regulation 37/2010 have set MRLs for a variety of veterinary drugs in animal products [100,120].

In addition, technical guidelines and performance characteristics, such as detection level, selectivity, and specificity for residue control are described in the Commission Decision 2002/657/EC, including additional requirements for confirmatory methods by introducing the concept of identification points (IPs) in order to achieve unambiguous identification of the monitored legislated residues [121].

EU established provisional MRL of AGs in edible tissues, fat, milk and eggs. The world health organization (WHO) and the FAO have set standards for acceptable daily intake (ADI) and also for MRLs in foods.

Referring to QNs, EU has set MRLs of antibiotics in foodstuffs of animal products by means of the Commission Regulation 37/2010. Eight QNs have been included in this regulation, named danofloxacin (DAN), sarafloxacin(SAR) and its metabolite difloxacin (DIF), enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP), flumequine (FLU), marbofloxacin(MAR) and oxolinic acid (OXO). In case of milk samples, MRL has not been setting for SAR while DIF and OXO are prohibited in animals from which milk is produced for human consumption. On the other hand, the EU has adopted a MRL of 100 μg kg^{-1} for tetracycline antibiotics in foodstuffs of animal origin [120].

I.3 Analytical methods for determination of contaminants in food.

The basic assumption underlying any technique for determining trace of contaminants is that it should guarantee true and precise results, also providing low limits of detection for a wide spectrum of components. Moreover, such a methodology should also enable selective determination of analytes, and be applicable with small amounts of solvents, being environmentally friendly.

In our study, several instrumental techniques and sample treatments have been proposed for determination of mycotoxins, pesticides and antibiotics in milk, dairy products and vegetable milks.

Recent advances in the analysis of residues at ultra-trace concentrations in food are due to the implementation of liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS). This technique has become the most powerful analytical tool for organic compound determination at μg kg^{-1}, or even ng kg^{-1}, level providing the sensitivity, selectivity and specificity needed to meet legislation [122]. Also, the well-known advantages of ultra-high performance LC (UHPLC) make it a good alternative to conventional LC for the determination of contaminants [123].

Concerning analytical methods for determination of mycotoxins, recent reviews have been published about this topic [124,125,126]. Thin-layer chromatography (TLC) was one of the first methods used to detect aflatoxins in agricultural products. Usually immunological methods i.e. enzyme linked immunosorbent assays (ELISA) are used for monitoring purposes while several methods based gas chromatography (GC) are being used as well. However, applications of LC-MS and LC-MS/MS for multimycotoxin determination are becoming increasingly relevant, allowing the determination of up to 100

mycotoxins in a single run, or the determination of mycotoxins simultaneously with other contaminants [127].

Considering pesticide determination, a number of analytical methods designed to determine multiple pesticide residues have been developed since the last century and have greatly contributed to agricultural productivity. In relation to instrumental analysis, high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is one of the most powerful techniques for pesticide residue analysis. This technique provides excellent detectability/selectivity and discriminates analyte and matrix signal more efficiently than gas chromatography coupled with tandem mass spectrometry (GC–MS/MS), besides its capacity to detect different chemical groups of analytes simultaneously, including highly polar and thermally labile pesticides [122,128,129,130].

The development of analytical methods for determination of antibiotics in food has also been a matter of concern, as reflected in the number of review articles devoted to this topic, including revisions on determination of the families of antibiotic covered in this Thesis [107,116,131,132,133].

Another recent strategy for the analysis of polar compounds, including pesticides and pharmaceutical residues in food, is the use of hydrophilic interaction liquid chromatography (HILIC) as an alternative to both normal- and reversed-phase chromatography [134,135].

Besides the extensively application of LC in this area, capillary electrophoresis (CE) has also been proposed as an alternative powerful separation technique with fast analysis speed, high separation efficiency, simplicity, low cost, short analysis time and low reagent consumption. Most of the recent advances in CE are focused on improving sensitivity, as this is its main limitation to be used in

trace analysis [136]. Although this technique is effective for many applications, its use is much extended in the field of drug analysis [137,138,139].

I.4 Sample treatment for determination of contaminants in milk and related products.

Sample treatment is the first and maybe the most important step in analysis. Moreover, both the isolation of the analytes from the matrix and their pre-concentration are usually required aspects of determination of trace contaminants. Also, it is frequently required to clean-up extracts of difficult matrices, such as milk and its products, high in fat and proteins, and such ingredients may cause interactions in the analytical process.

Most methods for the analysis of contaminants and residues in food have some disadvantages, including high solvent consumption, tedious clean-up steps which require more time for analysis, and high costs. Current trend in sample treatment implies miniaturization and saving of solvents, in agreement with the principles of Green Chemistry. Following this trends, in this Thesis we have proposed different methods of sample treatment for determination of trace of contaminants and residues in milk and related products.

I.4.1 Dispersive liquid – liquid microextraction

Dispersive liquid-liquid microextraction (DLLME) is a relatively new sample-preparation method offering high enrichment factors from low amount of samples. It has found wide acceptance due to its several advantages, including low cost, simplicity and also ease of method development, which made it useful to virtually all analytical laboratories [140]. In DLLME, extraction of analytes happens in dispersion of the extracting solvent made in water. To facilitate dispersion, a second solvent (the dispersing solvent) is used. The extraction process consists of two steps: (1) the mixture of extracting and

dispersing solvents is rapidly injected to a water sample. Dispersion is formed and facilitates fast extraction of analytes from the water sample. (2) The dispersion is discarding by centrifugation and the extracting solvent, containing analytes is taken for analysis with a microsyringe.

Several requirements have to be met to set sample isolation using DLLME. The dispersing solvent should be fully miscible with the aqueous phase. Usually some solvents like acetone, acetonitrile and methanol are used for this objective. The extracting solvent has to fulfill several requirements. It has to have potential for extracting analytes. Also, it has to be miscible in the dispersing solvent while its solubility in water has to be very low. Finally, the density of the extracting solvent has to differ greatly from the density of water to enable phase separation [141]. Fig.I.5 shows the steps involved in a DLLME procedure.

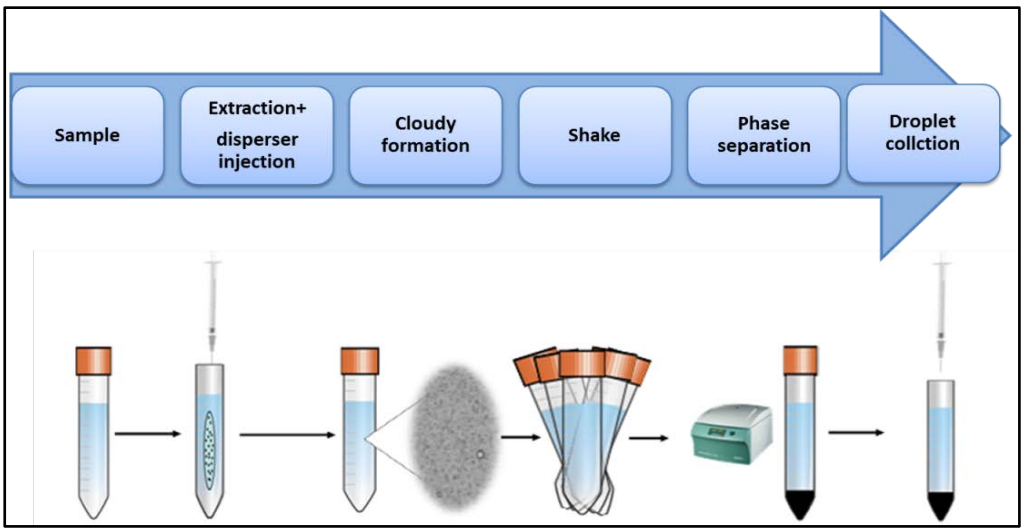

Figure I.5. Steps involved in a DLLME procedure.

Since the first application of DLLME by Rezaee et al [142], DLLME has obtained widespread acceptance because it has some advantages as a simple, fast, and

miniaturized sample preparation technique [143,144]. Moreover, an important number of applications of DLLME for determination of contaminants in different food matrices have been reported [145,146,147,148,149,150], some of them compiled in review articles [151,152,153]. Moreover, a recent review about the application of DLLME to the analysis of milk and dairy products has been reported [154].

This extraction technique has been proposed in this Thesis for the determination of aflatoxins in yogurt samples.

I.4.2 Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)

One specific method which has become increasingly popular is the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method which was created to ease the rapid screening of high amount of food and agricultural samples for pesticide residues. It involves microscale extraction using a very small amount of acetonitrile, followed by a dispersive solid-phase extraction (d-SPE) [155]. Fig.I.6 shows the steps of QuEChERS method.

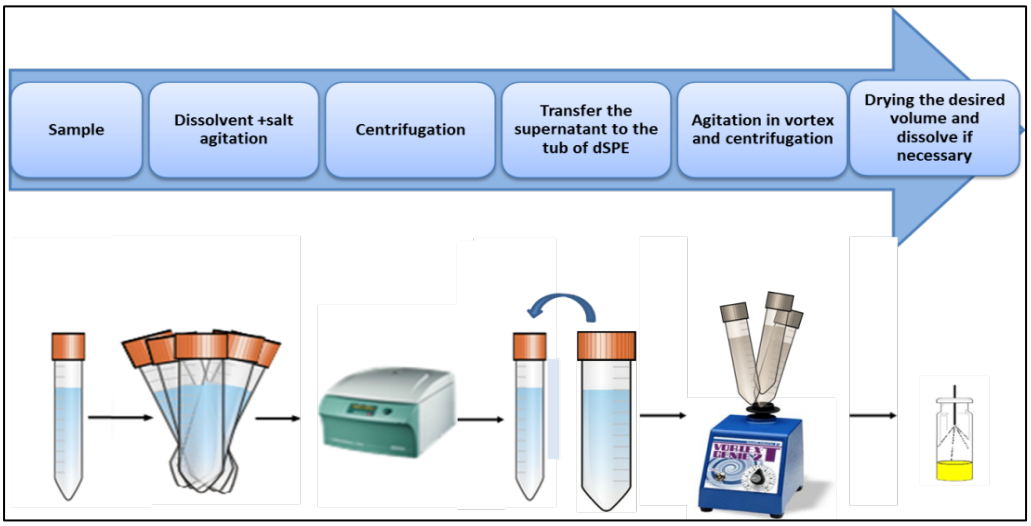

Figure I.6. Scheme of the QuEChERS method

During extraction, magnesium sulphate is used to reduce the water content and enhance the partitioning of different analytes into an organic layer, while sodium acetate is used to dissolve the fat globules. Sometimes, this first step of extraction/ partitioning is enough for analysis. However, frequently a second clean-up step is required. In this d-SPE step, different sorbents can be used for clean-up, each one exhibiting unique characteristics related to retention and selectivity. For instance, C18 enhances non-polar interactions, while primary secondary amine (PSA) can be used to remove compounds such as fatty acids, organic acids, and several sugars, and are especially good for removing matrix co-extractants, which can interfere in the determination. A recent sorbent is Z-Sep+, a silica carrier coated with zirconium dioxide and octadecylsilane groups [156], being a good sorbent of carboxylic acids. The presence of a double bond in the carboxylic acid molecule can improve adsorption. Furthermore, dicarboxylic acids adsorb more strongly than monocarboxylic acids because more stable structures are formed [157]. In a recent experiment examining the clean-up of food extracts prior to pesticide residue analysis, the Z-Sep+ sorbent showed improved clean-up over PSA and C18 [158].

This methodology has been the target of different revision papers [159,160].

In this Thesis, a QuEChERS-extraction was proposed for determination of *Fusarium* toxins in vegetables milk, while a Z-Sep+ was selected as a sorbent for determination of CRB residues in high-fat cheese using QuEChERS method as alternative sample treatment.

I.4.3 Solid phase extraction

Solid phase extraction (SPE) is an important technology for separation and purification which based on liquid chromatography theory. The technology has been widely used in separation and enrichment of organics or inorganics

[161].In SPE, the sample is passed through a cartridge or a packed column which filled with a solid sorbent where the analytes can be absorbed and then eluted with an organic solvent. The first SPE materials were silica-based and were modified with C18, C8, phenyl, CH, CN, or NH2 groups. However, silicabased materials present several disadvantages, such as instability at extreme pH, low recovery in the extraction of polar analytes, and the presence of residual silanol groups[162]. SPE is a well-established alternative to conventional solvent extractions. Such a technique imparts sample preparation with reduced sample manipulation and solvent economy; also, SPE-based technologies eliminate the use of specific, high-cost, and advanced instruments or accessories [163].

Recently, significant efforts have been made for enhancement and characterization of modern formats and advanced sorbent materials to increase selectivity or specificity towards target analytes, higher capacity and enhanced physicochemical or mechanical stability [164].

Waters has designed Oasis® HLB sample extraction products which is a universal polymeric reversed-phase sorbent that was developed for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices using a simple, generic protocol. This cartridge can overcome the limitations of reversed-phase SPE and to streamline the sample preparation process. Another type of HLB called Oasis® PRiME HLB is the first-of-its-kind SPE sorbent that sets the new performance standard for routine analyses. The unique, Oasis PRiME HLB Sorbent provides cleaner samples in less time and with less effort [165]. In addition, this sorbent allows avoiding the tedious steps such as conditioning, equilibrating and washing. Thus, the sample throughput is higher than when using the conventional HLB technology.

I.4.3.1 Molecularly Imprinted Polymers

Another approach for selective SPE was the development of molecularly imprinted polymers (MIPs) applied in molecular recognition. In order to enhance the selectivity of the extraction, MIPs based on specific recognition of the template molecule, allowing a selective extraction of a target molecule or its structural analogues, were developed [166,167,168].MIPs are defined as synthetic materials with artificially generated recognition sites able to rebind specifically the target molecule in preference to other highly related compounds, in analogy to antibody-antigen recognition [169,170]. These materials may create by polymerizing functional and cross-linking monomers around a template molecule, which leading a highly cross-linked three-dimensional network polymer. In addition, it is notable that the synthesis of MIP is also consider relatively cheap and easy comparing with other selective materials such as immunosorbent (IS), thus making MIP a clear alternative to the use of natural receptors [171]. Fig.I.7 shows the MIPs interaction.

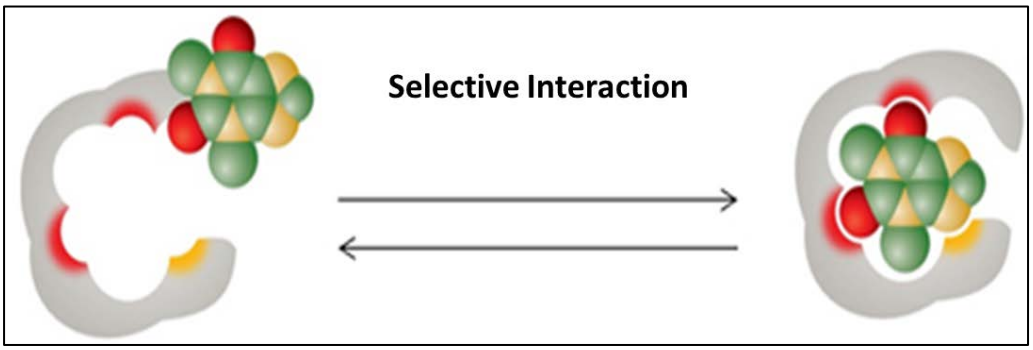

Figure I.7. The MIPs interaction. Reproduced from [172].

In this sense, one of the most exciting utilization of MIPs is as sorbent for solid-phase extraction (MISPE). Fig.I.8 shows the principal of MISPE.

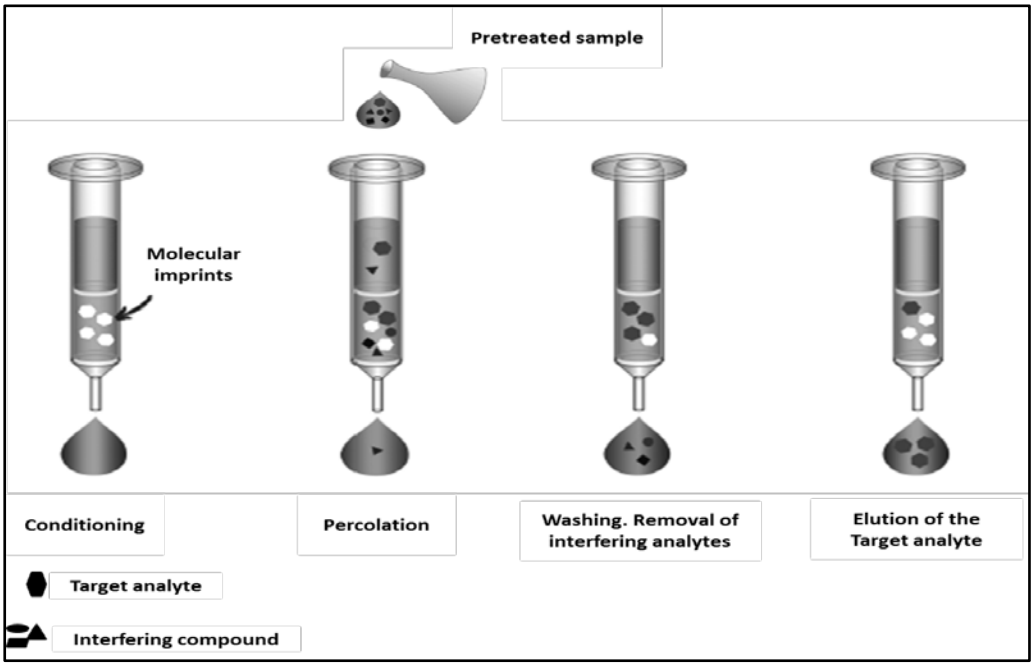

Figure.I.8. The principle of MISPE. Reproduced from [173].

This procedure has several advantages: particularly it is less time consuming than liquid–liquid extraction (LLE) procedure; it decreases the utilization of toxic solvents, provides the possibility of automation, shows a high mechanical and thermal stability and, the most important aspect, provides a higher selectivity [174,167]. In recent years, the food contaminant analysis field has devoted considerable interest to MISPE. For instance, there are some reports describing the use of the MISPE protocols for the selective extraction of antibiotics such as TCs and QNs [175,176]. Moreover, this technique has been applied successfully to overcome several challenging issues especially in very complex samples where analyte selectivity is required [177,178,179].

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CHAPTER 1

Determination of aflatoxins in yogurt by dispersive liquid–liquid microextraction and HPLC with photoinduced fluorescence detection

1

Abstract

An HPLC method with fluorescence detection for the determination of aflatoxins B1, B2, G1, G2 and M1 in yogurt using dispersive liquid–liquid microextraction as alternative sample treatment has been developed. To enhance the fluorescence of aflatoxins B1 and G1 a post-column photochemical derivatization has been proposed, avoiding the use of derivatization reagents. The method was validated using natural yogurt as representative matrix, showing a good linearity in the studied range (25-500 ng kg^{-1}) and limits of quantification below the maximum level established by European Union in milk for the manufacture of milk-based products. Satisfactory recoveries ranging from 69.4 to 99.7%, with relative standard deviations lower than 11.2% were obtained for all the compounds. The proposed method is simple, rapid, with low solvent consumption, inexpensive, and environmentally friendly.

1.1.Introduction

Mycotoxins are secondary metabolites produced by a wide range of fungi which can contaminate crops and other foods and feed [1]. Mycotoxins show mutagenic, teratogenic, carcinogenic and immunosuppressive effects in animals and humans [2]. Thus, their presence in food, even at very low concentrations, is a matter of global concern. Among mycotoxins, five aflatoxins (AFs) namely, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1), produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi are considered the most toxic group [3]. They have been classified by International Agency for Research on Cancer (IARC) as potential carcinogens for humans [4,5]. AFB1 is the most common and toxic AF, and AFM1 is the hydroxylated metabolite of AFB1, excreted in milk in the mammary glands of lactating animals fed with feedstuff contaminated with AFB1. It has been reported that approximately 0.3% to 6.2% of AFB1 initially present in animal feed appears as AFM1 in milk, with a linear relationship between the intake of AFB1 in feed and the AFM1 content of cow milk. Moreover, as AFs show resistance to heat treatment and mild acidic conditions used in the production of dairy products, if milk is contaminated there is a great possibility of AFM1 appearance in the final product [6,7,8]. Considering the above-mentioned, European Union (EU) has set maximum permitted levels for AFs in different foodstuff and AFM1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products (50 ng kg^{-1}), and in infant formulae and follow-on formulae (25 ng kg^{-1}), including infant milk and follow-on milk [9,10]. However, maximum levels have not been established yet for other milk-based products. As a consequence, accurate analytical methods for the determination of AFs in dairy products are required.

Different methods have been proposed for the determination of AFs in food, including milk [11,12]. For screening purposes, enzyme-linked immunosorbent assay (ELISA) and other immunochemical tools are frequently reported [6,13,14,15], while high performance liquid chromatography (HPLC) with mass spectrometry (MS) [7,16,17] or, more frequently, fluorescence detection (FLD) [18,19,20,21,22,23] are preferred when an accurate quantification is required [24]. However, FLD of AFB1 and AFG1 requires their conversion into higher fluorescent derivatives. Different alternatives have been proposed, such as pre-column derivatization with trifluoroacetic acid (TFA), or post-column derivatization with iodine, pyridinium hydrobromide perbromide or electrochemically generated bromine. Other option that avoids the use of reagents is photoinduced (PI) fluorescence using an on-line post-column photoreactor [11,25].

Concerning sample treatment, milk and related products are very complex matrices. In order to extract AFs from milk and dairy products, different sample treatments have been proposed, as immunoaffinity columns (IAC) [12,19,22,23,26], solid phase extraction (SPE) [27] or liquid–liquid extraction [28,29]. Recently, liquid-phase microextraction techniques are becoming more popular, as they require a lower consumption of reagents (being more environmentally friendly), allowing a high preconcentration factor. Among them, dispersive liquid–liquid microextraction (DLLME) is being increasingly used. Some 82 recent reviews describe the main principles and advantages of this sample treatment based on an 83 ternary component solvent system [30,31]. The applications of DLLME in food analysis have increased during the last years [32], including some methods for determination of AFs in different matrices such as edible oils [33] or cereal products [34]. Regarding dairy

products, only few papers about the determination of AFM1 in milk by DLLME [35] or DLLME combined with vortex-assisted hydrophobic magnetic nanoparticles-based SPE [36], and AFB1 and AFB2 in powdered milk by DLLME followed by IAC [37] have been proposed. However, to the best of our knowledge, there are no applications of DLLME for the extraction of the five main AFs in dairy products different than milk.

Thus, the aim of this work was the evaluation of a method for the sensitive, simple and rapid determination of the five AFs regulated by European legislation (AFB1, AFB2, AFG1, AFG2 and AFM1) in yogurt based on the simultaneous precipitation of protein and extraction by DLLME followed by HPLC-PI-FLD. The performance characteristics of the whole analytical procedure were established for different yogurts (natural, liquid and skimmed yogurt).

1.2.Materials and Methods

1.2.1 Chemicals and solvents

All reagents were of analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (MeCN), and chloroform ($CHCl_3$) were purchased from VWR BDH Prolabo (West Chester, Pennsylvania, USA) and NaCl was purchased from Panreac Química (Barcelona, Spain). Analytical standards of each AF were supplied by Sigma-Aldrich (St Louis, MO, USA). Individual stock standard solutions containing 1 µg mL^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeCN. These solutions were stable for at least 6 months. From these stock solutions, intermediate working solutions in MeCN

were prepared. All these solutions were stored at -20 °C. Working standard solutions containing all the AFs were freshly prepared by proper dilution of the stock standard solutions with MeOH: water (1:1, v/v).

Syringe filters (13 mm, 0.22 µm nylon membranes, Agela Technologies, DE, USA) were used for filtration of extracts before the injection into the chromatographic system.

1.2.2 Instrumentation and software

All experiments were carried out using a modular HPLC system including a quaternary high pressure pump (Model PU–2089, Jasco, Tokyo, Japan), an autosampler with a 100 µL loop (Model AS-2055, Jasco) and a fluorescence detector (Model FP 2020, Jasco). A post-column UV derivatization module (LCTech, Dorfen, Germany) was used to enhance the fluorescence. Separations were performed in a Kinetex C18 column (150 mm×4.6 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA). Data were collected using the software Jasco Chrom NAV (Version 1.09.03).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), and a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany) were used for sample treatment.

1.2.3 Sample treatment

Yogurt samples (natural, liquid and skimmed yogurt) were purchased in local stores (Granada, Spain) and stored at 4°C. Their compositions were as follows: (1) natural yogurt: 2.9% fat, 4% carbohydrates, 3.2% proteins, 0.1% salts and 0.1% calcium; (2) liquid yogurt: 0.4 % fat, 3.6% carbohydrates, 3.0% proteins, 0.1% salts, 0.8% vitamin D and 0.2% vitamin B6; (3) skimmed yogurt: 0.1% fat, 4.5% carbohydrates, 4.3% proteins, 0.1% salts and 0.1% calcium. Sample

treatment was based on a previous study for determination of AFM1 in milk [35]. Natural and liquid yogurt samples were defatted by centrifugation at 6,000 rpm (4 °C, 5 min) to eliminate the fat in the upper phase, whereas skimmed yogurt samples were directly processed. A portion of 5 g of sample was placed in a 15-mL falcon tube. Then, 1.5 g of NaCl and 6 mL of MeCN were added to the sample. The mixture was shaken for 30 s by vortex and centrifuged for 5 min at 6,000 rpm. Subsequently, the upper phase was quantitatively transferred (5.1±0.2 mL) into a 10-mL vial. A mixture of this organic phase containing the extracted analytes (disperser solvent) and 1,500 µL of $CHCl_3$ (extractant) was injected in 5 mL of deionized water for DLLME. Then, the ternary system was vigorously shaken by hand for 10 s and a stable cloudy solution was formed. The mixture was centrifuged for 5 min at 6,000 rpm for phase separation. The organic phase was collected and dried under a gentle nitrogen stream. Finally, the residue was reconstituted with 500 µL of MeOH: water (1:1, v/v) and filtered before injection into the chromatographic system.The DLLME procedure is summarized in Fig.1.1.

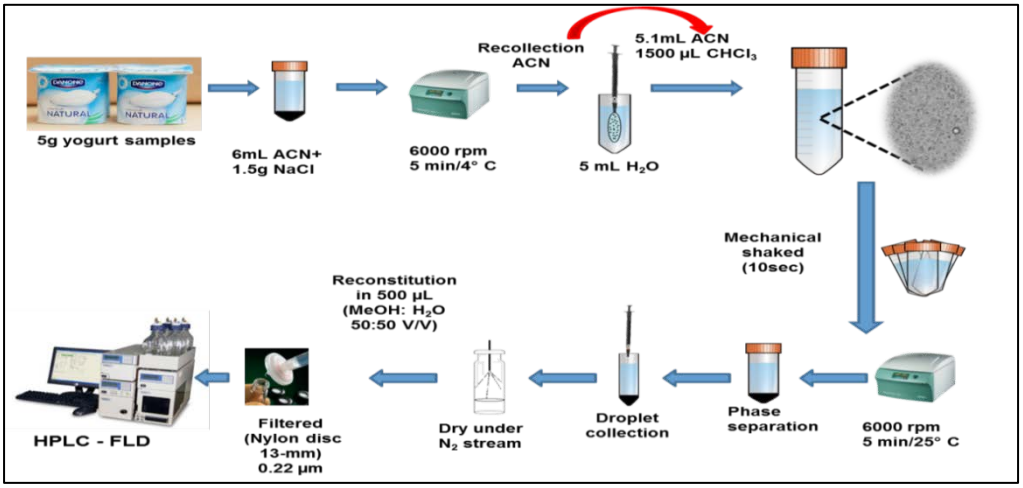

Figure.1.1. Diagram of DLLME for the determination of aflatoxins in yogurt samples.

1.2.4 Chromatographic conditions

The chromatographic separation was performed in a C_{18} Kinetex separation column (150 mm×4.6 mm, 2.6 µm). The mobile phase consisted on a gradient of MeCN, MeOH and water, as follows: 0-18 min: MeCN:MeOH:water (15:12:73); 21-26 min: MeCN:MeOH:water (40:10:50); 27 min: MeCN:MeOH:water (10:80:10); finally, return to the initial conditions in 1 min and maintained for 7 min for column equilibration. The flow rate was set at 0.8 mL min^{-1}, the column temperature was 35 °C, and an injection volume of 70 µL was selected. A photochemical post-column reactor was used to enhance AFB1 and AFG1 fluorescence. The excitation and emission wavelengths were 360 and 430 nm, respectively. The fluorescence detector operated at gain ×100.

1.3.Results and discussion

1.3.1.Optimization of chromatographic condition

Peak area, efficiency and analysis time were taken into account to select the adequate determination procedure. Different natures of mobile phase (water with acetic acid as solvent A, water as solvent B, MeOH as solvent C and MeCN as solvent D) were tested. The best results were obtained with water, MeOH and MeCN. Subsequently, the percentages of solvent B,C and D were studied in order to get the best peak shape and sensitivity in the shortest time; the following conditions were selected: MeCN, MeOH and water, as follows: 0-18 min: MeCN:MeOH:water (15:12:73); 21-26 min: MeCN:MeOH:water (40:10:50); 27 min: MeCN:MeOH:water (10:80:10); finally, return to the initial conditions in 1 min and maintained for 7 min for column equilibration (total run time of 35 min). Flow rate was set at 0.8 mL/min, with oven temperature at 35° C, and injection volume was 70 µL. The excitation and emission

wavelengths for the determination of the AFs derivatives were 360 and 430 nm, respectively. The fluorescence detector operated at gain ×100. Then, the percentage of acetic acid in solvent A was optimized between 0 and 5%. The best results were obtained with 0% acetic acid (Fig.1.2).

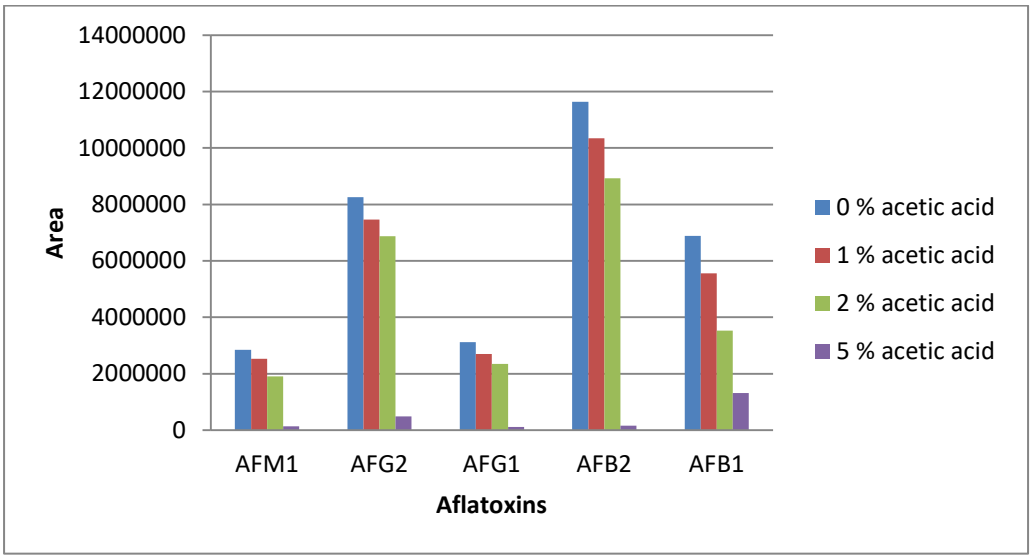

Figure 1.2. Influence of the percentage of acetic acid in the mobile phase on peak area.

The effect of column temperature was examined in the range of 25-45°C; an optimum value of 35 °C was chosen in terms of the peak area, reproducibility and peak shape, as a compromise between analysis time and column life (Fig.1.3).

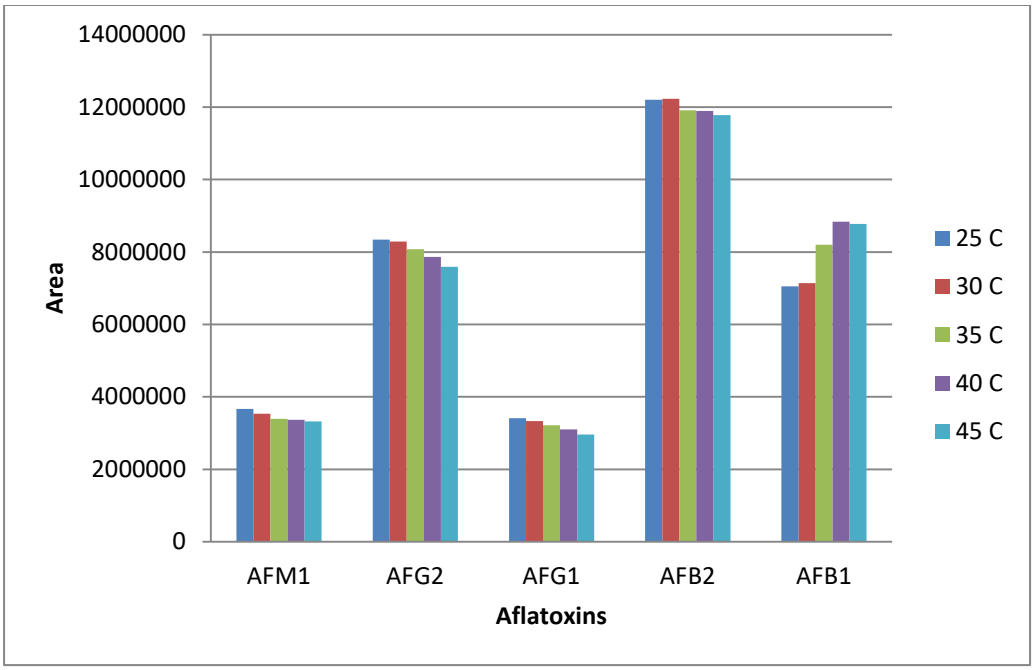

Figure 1.3. Influence of column temperature on peak area.

The flow rate was also tested between 0.6 -1.0 mL min^{-1}, selecting a final optimum value of 0.8 mL min^{-1}, as higher flow rates involved higher pressure without a significant improvement in the analysis. Finally, the injection volume was increased from 60 -90 µL, selecting 70µL as optimum depending on the peak shape, peak areas, high sensitivity and reproducibility, as higher volumes involved peak splitting.

Table 1.1. Summary of the optimum values for the chromatographic procedure.

Parameter	Specification
Column	C18 (2.6µ, 150 x 4.6mm)
Mobile phase	MeCN, MeOH and water, as follows: 0-18 min: MeCN: MeOH : water (15:12:73); 21-26 min: MeCN:MeOH:water (40:10:50); 27 min: MeCN:MeOH:water (10:80:10); finally, return to the initial conditions in 1 min and maintained for 7 min for column equilibration.
Flow rate	0.8 mL min^{-1}
Injection volume	70 µL
Column Temperature	35 °C

1.3.2 Optimization of sample treatment

The optimization of the sample treatment was carried out using 5 g of AF-free natural yogurt (as representative matrix), spiked with 1 µg kg^{-1} of each AF. The recovery percentage was used to evaluate the extraction efficiency. The extraction of AFs is not an easy task due to the high complexity of the yogurt sample (proteins and fat components). Samples were defatted prior to the extraction procedure by centrifugation at 6,000 rpm at 4 °C for 5 min to eliminate the fat in the upper phase (this step was not necessary for skimmed yogurt). Then, the protein precipitation was carried out using MeCN, which acts

also as extraction solvent and subsequently, as disperser solvent in the following DLLME process. Different volumes of MeCN between 2-6 mL were tested, adding 1 g of NaCl. The best recoveries for all AFs were obtained using 6 mL of MeCN (see Fig.1.4).

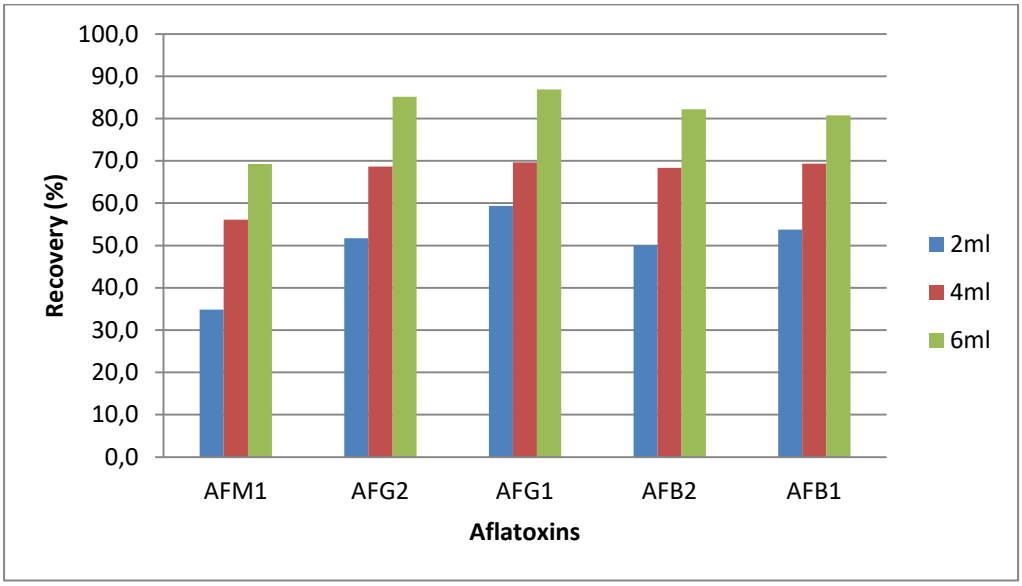

Figure 1.4. Influence of different the volume of MeCN on DLLME efficiency.

As the addition of salt to the system could significantly improve the extraction efficiency by salting-out effect, the influence of the ionic strength was investigated by adding different amounts of NaCl (0.5, 1 and 1.5 g). With 0.5 g the phase separation was not complete, whereas with 1 g and 1.5 g the phase separation was well-defined, being the highest recoveries obtained with 1.5 g of NaCl, which was selected as optimum (see Fig.1.5). The final volume of collected organic phase was approx. 5.1 mL.

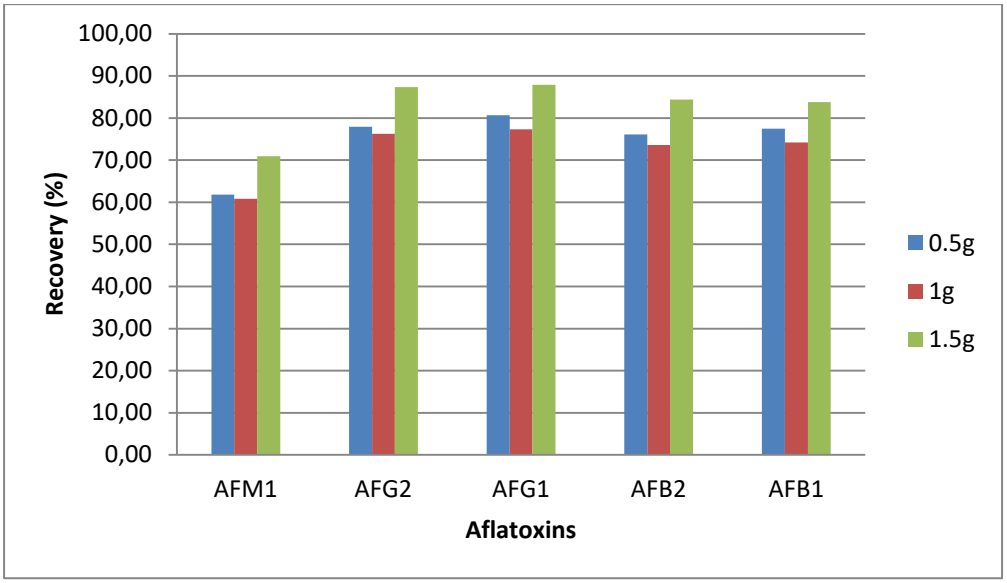

Figure 1.5. Influence of different the volume of NaCl on DLLME efficiency.

Subsequently, main factors affecting the extraction efficiency of DLLME were tested, such as: volume of extractant ($CHCl_3$), volume of water and final reconstitution volume. Extraction solvent plays a key role in the DLLME process and must fulfil certain criteria, namely: a higher density than water; be immiscible with water; and provide the best extraction efficiency for all the analytes. It has been previously reported that $CHCl_3$ is the best extractant to perform DLLME in the case of AFM1 [35], so different volumes of this solvent (1, 1.5 and 2 mL) were added to the MeCN, and injected into 5 mL of water. The recovery percentage increased with volumes of $CHCl_3$ up to 1.5 mL, which was selected as optimum value.

Then, different volumes of water (3-7 mL) were tested. The recovery of analytes increased up to 5 mL of water, remaining constant thereafter. Therefore, 5 mL was selected as the optimum volume.Finally, the effect of the volume of reconstitution solvent was studied in the range of 500 to 1000 µL. A

volume of 500 µL of MeOH:water (1:1 v/v) was selected to obtain the best enrichment factors for AFs.

1.3.3 Characterization of the method

In order to assay the suitability of the method for the determination of AFs in yogurt samples, it was characterized in terms of linear dynamic ranges, limits of detection (LODs) and quantification (LOQs), precision and trueness.

1.3.3.1 Calibration curve and performance characteristics

Calibration curves were established using natural yogurt samples as representative matrix, spiked with different concentrations of each AFs (25, 50, 100, 250 and 500 ng kg^{-1}). Each sample was analyzed following the DLLME method and injected in triplicate. Peak areas were considered as a function of analyte concentration in the sample. A blank sample was also checked, and no AFs were detected. Statistics and performance characteristics of the proposed method in natural yogurt are shown in Table 1.2.

Table1.2. Statistical and performance characteristics of the method for natural yogurt

Analyte	Linear range (ng kg^{-1})	Slope	Intercept	R^2	LOD (ng kg^{-1})	LOQ (ng kg^{-1})
AFM1	18-500	621.3	921.7	0.9992	5.5	18
AFG2	5.0-500	3192.2	-3877.9	0.9998	1.5	5.0
AFG1	18-500	1011.3	1282.0	0.9994	5.5	18
AFB2	11-500	5469.9	2349.1	0.9996	3.2	11
AFB1	10-500	2664.9	848.3	0.9995	2.9	10

The responses were linear in the studied range with determination coefficients higher than 0.9992 for all AFs. LODs and LOQs were assigned taking into

account signal-to-noise (S/N) ratio criterion (3 and 10 for LOD and LOQ, respectively). As could be observed in Table 1, LOQs lower than 18 ng kg^{-1} were obtained in all cases. It has to be highlighted that the LOQ for AFM1 was lower than the established maximum level in milk and infant milk [38,10]. The proposed method shows LODs similar or even better than other HPLC-FLD methods for determination of AFs in yogurt, based on the use of IAC [19,22,23]. Moreover, not only AFM1 but all main AFs can be determined using this method. In addition, the use of DLLME as green, simple and efficient methodology makes the method a real alternative for the control of AFs in dairy products.

1.3.3.2 Precision study

The precision of the method was evaluated in terms of repeatability and intermediate precision. Experiments were carried out with natural yogurt samples spiked at two different concentration levels of AFs (25 and 50 ng kg^{-1}). To check the repeatability, three samples were analyzed and injected in triplicate on the same day, under the same conditions (that is, intra-day precision). Similar procedure was carried out in the case of intermediate precision. Thus, during five consecutive days, one sample per day was prepared and injected in triplicate (intra-day precision). The results, expressed as %RSD of peak areas, are shown in Table 1.3. RSD values lower than 10% were obtained in all cases, fulfilling current regulation laying down the methods of analysis for the official control of the limits of mycotoxins in food [9].

Table 1.3. Precision study (%RSD of peak areas) for the determination of AFs in natural yogurt.

	AFM1	AFG2	AFG1	AFB2	AFB1
Repeatability (n = 9)					
Level 1 (25 ng kg^{-1})	4.4	8.5	8.3	2.1	5.7
Level 2 (50 ng kg^{-1})	3.7	3.1	5.9	1.8	2.3
Reproducibility (n = 15)					
Level 1 (25 ng kg^{-1})	5.2	8.5	6.0	5.1	8.3
Level 2 (50 ng kg^{-1})	9.8	3.9	4.8	4.4	2.4

1.3.3.3 Turness studies

In order to analyze the trueness of the proposed methodology, recovery experiments were carried out in different yogurt samples (natural, liquid and skimmed) spiked at two different concentration levels of AFs (see Table 1.4).

Always, a blank sample was analyzed to test the presence of AFs, and none of them gave a positive result. Table 3 shows the recoveries obtained with the proposed method (ranging between 69.4 to 99.7%), which can be considered in agreement with the current demand [9]. However, as could be observed in Fig. 1.6, AFG2 could not be detected in skimmed yogurt, due to a co-eluting peak. This fact could be due to the high percentage of proteins of this type of yogurt. To overcome this problem, several gradient profile modifications were carried out. Unfortunately, it was not possible to improve the resolution between AFG2 and the interference. An alternative solution could be the use of other stationary phase, which could allow a higher resolution (i.e. UHPLC).

Table 1.4. Recovery study (n = 9) for the determination of AFs in different yogurts.

Sample	Analyte	Level 1 (25 ng kg^{-1})		Level 2 (50 ng kg^{-1})	
		%Recovery	%RSD	%Recovery	%RSD
Natural yogurt	AFM1	74.0	4.4	76.5	3.7
	AFG2	82.7	8.5	90.0	3.1
	AFG1	92.0	8.3	99.7	5.9
	AFB2	87.7	2.1	91.1	1.8
	AFB1	89.0	5.7	91.1	2.3
Liquid yogurt	AFM1	70.1	7.8	79.7	2.3
	AFG2	90.2	2.7	89.2	3.2
	AFG1	72.5	6.4	92.0	2.5
	AFB2	78.1	3.4	90.9	1.5
	AFB1	77.0	6.2	91.8	1.8
Skimmed yogurt	AFM1	69.4	8.9	79.4	7.4
	AFG2	----	----	----	----
	AFG1	92.7	6.7	83.1	1.6
	AFB2	99.3	11.2	82.0	2.7
	AFB1	98.3	10.9	82.5	3.0

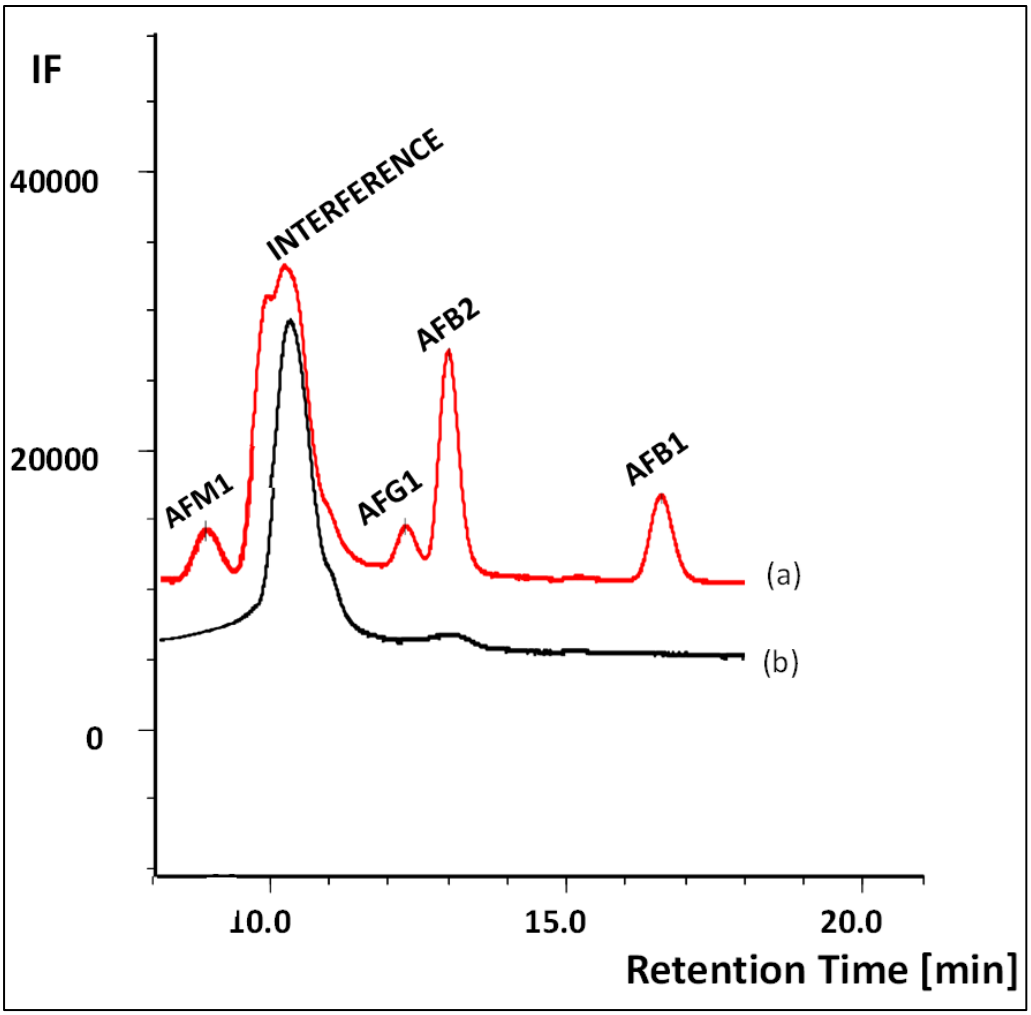

Figure.1.6. HPLC-PI-FLD chromatograms of (a) a spiked skimmed yogurt sample (50 ng kg^{-1} for each AFs); and (b) a blank sample.

Typical chromatograms corresponding to natural yogurt samples analyzed by the proposed DLLME-HPLC-PI-FLD method under optimum conditions are shown in Fig.1.7.

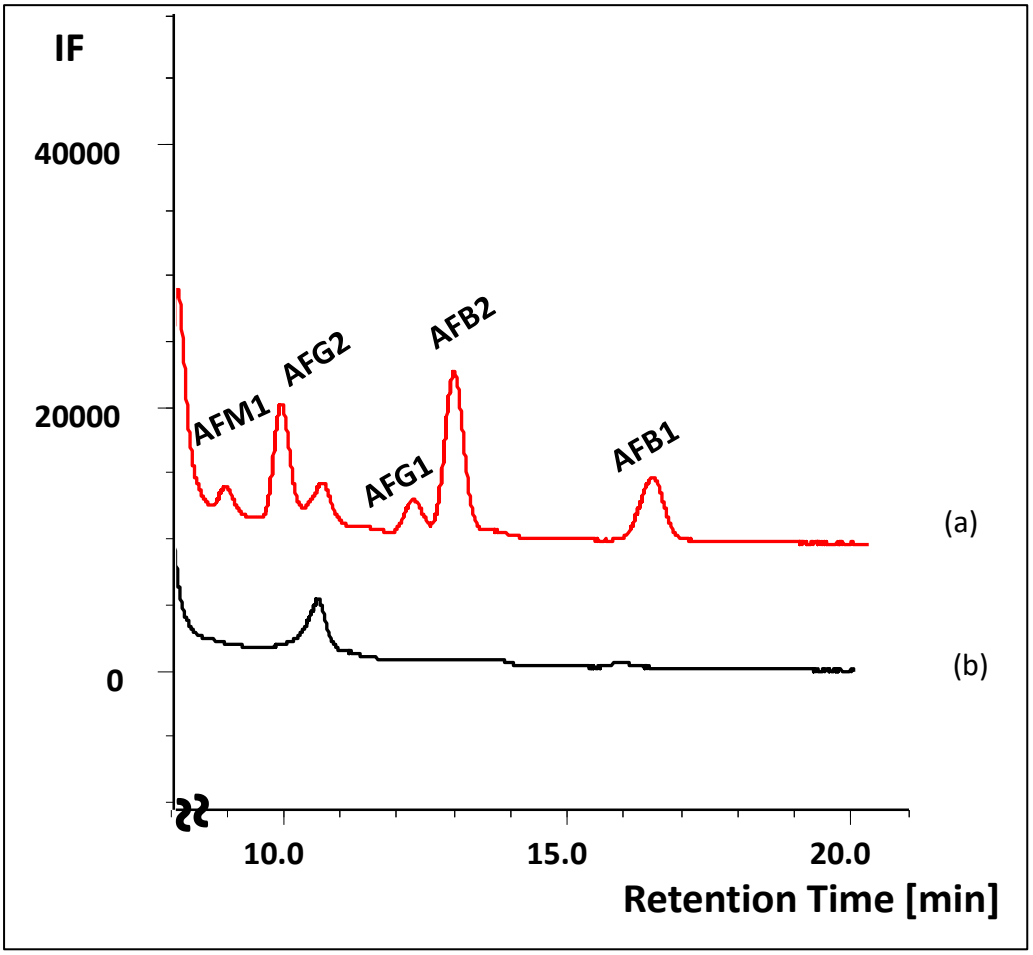

Figure 1.7. HPLC-PI-FLD chromatograms of (a) a spiked natural yogurt sample (50 ng kg^{-1} for each AFs); and (b) a blank sample.

1.4. Conclusions

DLLME has been successfully evaluated for the determination of five AFs (AFM1, AFB1, AFB2, AFG1 and AFG2) in different kinds of yogurt. The procedure based on the sequential application of DLLME before analysis by HPLC-PI-FLD provided very good results in terms of recovery and extract cleanliness. The extract was analyzed by HPLC-PI-FLD to significantly increase the fluorescence of AFB1 and AFG1, avoiding the use of derivatization reagents.

The method has been fully validated for natural yogurt. Calibration curves were established in the presence of matrix and the low LOQs obtained allowed determining the five AFs at concentrations lower than the limits established by current legislation for AFM1 in milk, with satisfactory precisions. In addition, trueness has been successfully evaluated for natural, liquid and skimmed yogurt, obtaining good recoveries for all AFs, except AFG2 in skimmed yogurt due to a co-eluting peak. The main advantages of the proposed method are its simplicity and low solvent consumption, allowing the simultaneous determination of five mycotoxins, being an alternative to other traditional sample treatments such as IACs. Moreover, to the best of our knowledge, this is the first time that DLLME is applied for extraction of the five main AFs in dairy products.

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CHAPTER 2

Determination of Fusarium toxins in functional vegetable milks applying salting out assisted liquid-liquid extraction combined with ultra-high performance liquid chromatography tandem mass spectrometry

2

Abstract

Most common vegetable milks, such as those derived from soybean, rice, oat or seeds are considered as functional foods due to their physiological benefits. However, they may include some mycotoxins resulting from the use of contaminated raw materials. Although the offer and consumption of these products have significantly increased, these relatively new matrices have received little attention in legislation, as regard to mycotoxin control. In this work, ultra-high performance liquid chromatography tandem mass spectrometry has been proposed for the determination of most relevant *Fusarium* toxins (fumonisin B_1 and B_2, HT-2 and T-2 toxins, zearalenone, deoxynivalenoland fusarenon-X) in different functional beverages based on cereals, legumes and seeds. Sample treatment consisted of a simple salting-out assisted liquid-liquid extraction with no further clean-up. The proposed method was optimized and characterized obtaining limits of quantification between 3.2-57.7 μg L^{-1}, recoveries above 80% and precision with RSD lower than 12%. The method was also applied for studying the occurrence of these mycotoxins in market samples of vegetable functional beverages and deoxynivalenol was found in three oat-based commercial drinks.

2.1.Introduction

Mycotoxins are highly toxic natural secondary metabolites produced by filamentous fungi belonging mainly to *Aspergillus*, *Penicillium*, and *Fusarium* genera that grow in a wide range of agricultural goods before, during and after the harvest process. Specifically, *Fusarium* species can produce several mycotoxin groups, being the most important: trichothecenes, such as T-2 and HT-2 toxins, deoxynivalenol (DON)and fusarenon-X (F-X); fumonisins as fumonisin B1 (FB_1) and fumonisin B2 (FB_2); and zearalenone (ZEA). They are commonly found world-wide on cereals such as wheat, rye, barley, oat and maize, and subsequently in derived products. Moreover, the co-existence of different *Fusarium* spp. In the same crop is frequent, making possible the co-ocurrence of several mycotoxins in the same commodity [1,2].

Due to the high occurrence of *Fusarium* mycotoxins and their toxic effects in animals and humans, maximum levels (MLs) for these contaminants in foodstuffs have been established worldwide. Particularly, the European legislation has established MLs for ZEA, DON and fumonisins, and a recommendation for monitoring the presence of T-2 and HT-2 toxins, including also indicative levels in cereals and cereal-based products [3,4,5] . However, by the moment no legislation exists in relation to other products such as leguminous plants or seeds, such as soyabean or bird seeds, although certain risk of contamination by fungi and mycotoxins should be considered [6,7].

Different analytical approaches have been proposed for determination of *Fusarium* toxins in cereal and cereal-based products, including immunological methods such as enzyme linked immunosorbent assay (ELISA) [8,9], or gas chromatography coupled with mass spectrometry (GC-MS) [10,11,12].

However, for multiple mycotoxin determination, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is considered as the method of choice [13,14,15] due to its several advantages such as selectivity, sensitivity and ability to cover a wide range of mycotoxins. For extraction and clean-up, liquid extraction followed by a solid phase extraction (SPE) using immunoaffinity columns (IACs) has been traditionally proposed for mycotoxin determination. However, IACs present some disadvantages such as rather high cost, cross reactivity, limited lifetime and, the most important, they are limited to a reduced number of compounds, not allowing a multi-class mycotoxin determination [16,17]. Therefore, alternative sample treatments have been proposed for mycotoxin determination in cereal and cereal-based products. An extensive revision of analytical methodologies as well as sample treatments on this topic has been published [18]. Among them, QuEChERS procedure is being increasingly adopted as a simple methodology based on extraction/partitioning in the presence of salts followed by dispersive-SPE (d-SPE) for clean-up. It has been widely reported for mycotoxin determination in food samples including cereals and cereal-based products due to its low solvent consumption, little cost, together with its good versatility and flexibility [11,12,19,20,21,22,23,24,25]. When there is no need for sample clean up, salting-out assisted liquid–liquid extraction (SALLE) is a simple and efficient strategy for sample treatment, by adding an appropriate amount of salt to the mixture of aqueous sample and water–miscible organic solvent which induces the separation of the solvent from the mixture, containing the target analytes [26]. This strategy has been previously used for the mycotoxin determination in pig urine [27].

However, despite the considerable number of publications devoted to determination of mycotoxins in cereal and cereal-based products, including studies of occurrence in different commodities [126], scarce attention has been paid to beverage based on cereals, legumes or seeds, commonly named as vegetable milks. Most common vegetable milks are based on soybean, rice or oat and they may be contaminated by mycotoxins resulting from the use of contaminated raw materials. Moreover, during the last decade, both offer and consumption of vegetable milks have significantly increased due to lactose intolerance, the decision to avoid consumption of animal products, or the healthy claims attributed to these products, considered as functional foods. Anyway, they should be considered as a potential source for ingestion of mycotoxins, as the rest of cereal-based products, and new analytical methodologies are required for these matrices. To the best of our knowledge, very few articles have been published on this topic [28] and none of them has been focused on the determination of *Fusarium* mycotoxins (the most common in cereals) in vegetable milks.

The goal of this study was therefore to develop a sensitive method based on the application of SALLE combined with UHPLC-MS/MS for the simultaneous determination of most relevant *Fusarium* toxins (FB_1, FB_2, T-2, HT-2, ZEA, DON and F-X) in different types of functional beverages produced from oat, soyabean, rice and bird seeds. From our point of view, the proposed method for *Fusarium* toxins determination in these types of matrices could contribute to food safety and the study of the relationship between mycotoxin-contaminated raw material (cereals, leguminous plants or seeds) and their derived products. The method was characterized in terms of matrix effect (ME), linear dynamic ranges, limits of detection (LODs) and quantification

(LOQs), precision and trueness. Finally, it was applied to control of mycotoxins in different commercial functional beverages.

2.2.Materials and methods

2.2.1.Chemicals, reagents and standard solutions

Ultrapure water (18.2 MΩ/cm, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout all the work. All reagents were of analytical reagent grade and solvents were LC-MS grade. Methanol (MeOH), acetonitrile (MeCN), ammonium formate, formic acid (analysis grade) and chloroform ($CHCl_3$) were supplied by VWR International Eurolab, S.L. (Barcelona, Spain). Magnesium sulphate ($MgSO_4$), tri-sodium citrate ($Na_3C_6H_5O_7.5\ 1/2H_2O$) and sodium chloride (NaCl) were purchased from Panreac Química (Barcelona, Spain). Potassium dihydrogen phosphate and disodium hydrogen citrate sesquihydrate were supplied by Merck (Darmstadt, Germany). Formic acid eluent additive for LC–MS was obtained from Sigma Aldrich (St. Louis, MO, USA).

Nylon syringe filters, 0.22 µm x 25 mm (Agela Technologies, New York, USA) were used for filtration of samples prior to the injection into the chromatographic system. Individual standard solutions (10 µg mL^{-1} in MeCN) of FB_1, FB_2, HT-2, T-2, ZEA, DON and F-X were obtained from Techno Spec (Barcelona, Spain) and stored in a glass vial at -20 °C.

2.2.2.Instrumentation

Separation was performed on an Agilent 1290 Infinity LC using a C18 Zorbax Eclipse Plus Rapid Resolution High Definition (RRHD) (50 x 2.1 mm, 1.8 µm) as

chromatographic column. The measurements were performed on a triple quadrupole (QqQ) mass spectrometer API 3200 (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI). The instrumental data were collected using the Analysts Software version 1.5 with Schedule MRM^{TM} Algorithm (AB Sciex).

In addition, a centrifuge (Universal 320 model from Hettich, Leipzig, Germany), a vortex (Genie 2 model from Scientific Industries, Bohemia, NY, USA), a bench mixer multi-tube vortex agitator (model BV1010, Edison, NJ 08818, USA), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany), and a pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain) were used during the sample preparation procedure.

2.2.3.Sample treatment procedure

Oat milk (14% oat), soybean milk (15% soya), rice milk (15% rice) and bird seed milk (15% bird seeds) were purchased in local markets from Granada (Spain) and stored at 4 $^{\circ}$C. An aliquot of 5 mL of sample and 5 mL of 50 mM potassium dihydrogen phosphate at pH 7.0 were placed into a 50-mL screw cap test tube with conical bottom and shaken by vortex for 10 s. Subsequently, 10 mL of MeCN with 5% formic acid was added, and the mixture was shaken again using the bench mixer for 2 min. Then, 4 g $MgSO_4$, 1 g NaCl, 1 g tri-sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate were added and the tube was shaken for 1 min using the bench mixer and centrifuged at 4500 rpm for 5 min. After that, 2 mL of the upper MeCN layer was transferred to a glass vial, evaporated to dryness under a gentle stream of nitrogen and reconstituted with 500 µL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 µm filter prior their injection in the UHPLC–MS/MS system.

2.2.4.UHPLC–MS/MS analysis

Separation was performed in a C18 column (50x2.1 mm, 1.8 µm), using a mobile phase consisting of 0.3% aqueous formic acid solution with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}. The eluent gradient profile was as follows: 0 min: 5% B; 0.5 min: 50% B; 1.5 min: 72% B; 2.5 min: 80% B and 4.5 min: 90% B. Finally, it was back to 5% B in 0.2 min and maintained for 2 min for column equilibration. The temperature of the column was set at 35 °C and the injection volume was 5 µL. The UHPLC system was coupled to a mass-spectrometer with ESI operating in positive ion mode, under the multiple-reaction monitoring (MRM) conditions shown in Table 2.1, previously reported in references [21,22].

Table 2.1. Monitored ions of the target analytes and MS/MS parameters

Analyte	Precursor ion(m/z)	Rt	Molecular ion	DP	EP	CEP	Product ion*	CE	CXP
DON	297.1	1.0	[M+H]+	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	355.1	1.1	[M+H]+	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
HT-2	442.0	1.9	[M+NH4] +	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB1	722.2	2.0	[M+H]+	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	484.0	2.1	[M+NH4] +	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEN	319.0	2.3	[M+H]+	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
FB2	706.2	2.5	[M+H]+	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0

*(Q) Transition used for quantification, (I) Transition employed to complete the identification.
Rt: Retention Time. DP: Declustering Potential. EP: Entrance Potential. CEP: Collision Cell Entrance potential. CE: Collision Energy. CXP: Collision Exit Potential.

The ionization source parameters were: dry gas temperature, 500°C; curtain gas (nitrogen), 30 psi; ion spray voltage, 5000 V; collision gas, 5 and dry gas pressure (GS 1 and GS 2, both of them nitrogen) 50 psi.

2.3.Results and discussion

2.3.1.Optimization of UHPLC-MS/MS

For the determination of *Fusarium* toxins, the UHPLC-MS/MS was adapted from a previous method developed in our laboratory for multiclass mycotoxin determination in cereals and cereal-based products [21,22]. However, in order to reduce analysis time, the gradient was modified as follows: 0 min: 5% B; 0.5 min: 50% B; 1.5 min: 72% B; 2.5 min: 80% B and 4.5 min: 90% B. Finally, it was back to 5% B in 0.2 min and maintained for 2 min for column equilibration. It allows the separation of the seven *Fusarium* toxins in less than three minutes.

2.3.2.Optimization of 137imple preparation

A very simple methodology based on SALLE was explored as sample treatment for the determination of *Fusarium* toxins in the selected vegetable milks. During the optimization, oat milk sample was used as representative matrix and different extraction solvents and mixtures of salts were tested. As initial conditions, the extraction was carried out using 5 mL of sample plus 5 mL of 50 mM potassium dihydrogen phosphate, pH 7.0. In addition, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate were added for partitioning. Acid media can help the extraction process by breaking interactions between the toxins and sample constituents such as proteins [29]. Moreover, the addition of formic acid to the extraction solvent could help the

simultaneous extraction of fumonisins from cereals [30]. Thus, the extraction of *Fusarium* mycotoxins from vegetable milks was carried out using acidic conditions. On the other hand, MeCN is the preferred extraction solvent as it extracts the widest range of mycotoxins and least amount of matrix components [15]. Therefore, MeCN with different percentages (between 0 and 10%) of formic acid was tested as extraction solvent. The best results in terms of recoveries and sensitivity were obtained with 5% formic acid; thereby it was selected for further experiments.

Subsequently, different combinations of solvents and salts were tested for extraction /partitioning: (a) 5 mL of H_2O, 4 g $MgSO_4$ and 1 g NaCl; (b) 5 mL of H_2O, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate; (c) 5 mL of 50 mM phosphate buffer at pH 7.0, 4 g $MgSO_4$ and 1 g NaCl; and (d) 5 mL of 50 mM phosphate buffer at pH 7.0, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate. In all the cases a volume of 10 mL of MeCN with 5% formic acid was added as extractant. The best results in terms of recovery were obtained using the buffered conditions (5 mL of 50 mM phosphate buffer at pH 7.0) and the salt mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate. After that, different concentrations of phosphate buffer (between 50 and 150 mM) were tested. The best results in terms of recovery were obtained using 50 mM phosphate buffer and it was therefore selected for the rest of the work. Finally, the effect of the volume of reconstitution solvent was studied in the range of 500 to 1000 µL. A volume of 500 µL of MeOH:H_2O (1:1 v/v) was selected to obtain the best enrichment factor for the studied mycotoxins. With this SALLE, no further clean-up was necessary, as extract was clean enough for quantification and identification purposes.

A typical chromatogram corresponding to a spiked oat-based beverage (75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA) submitted to the proposed method is shown in Figure 2.1.

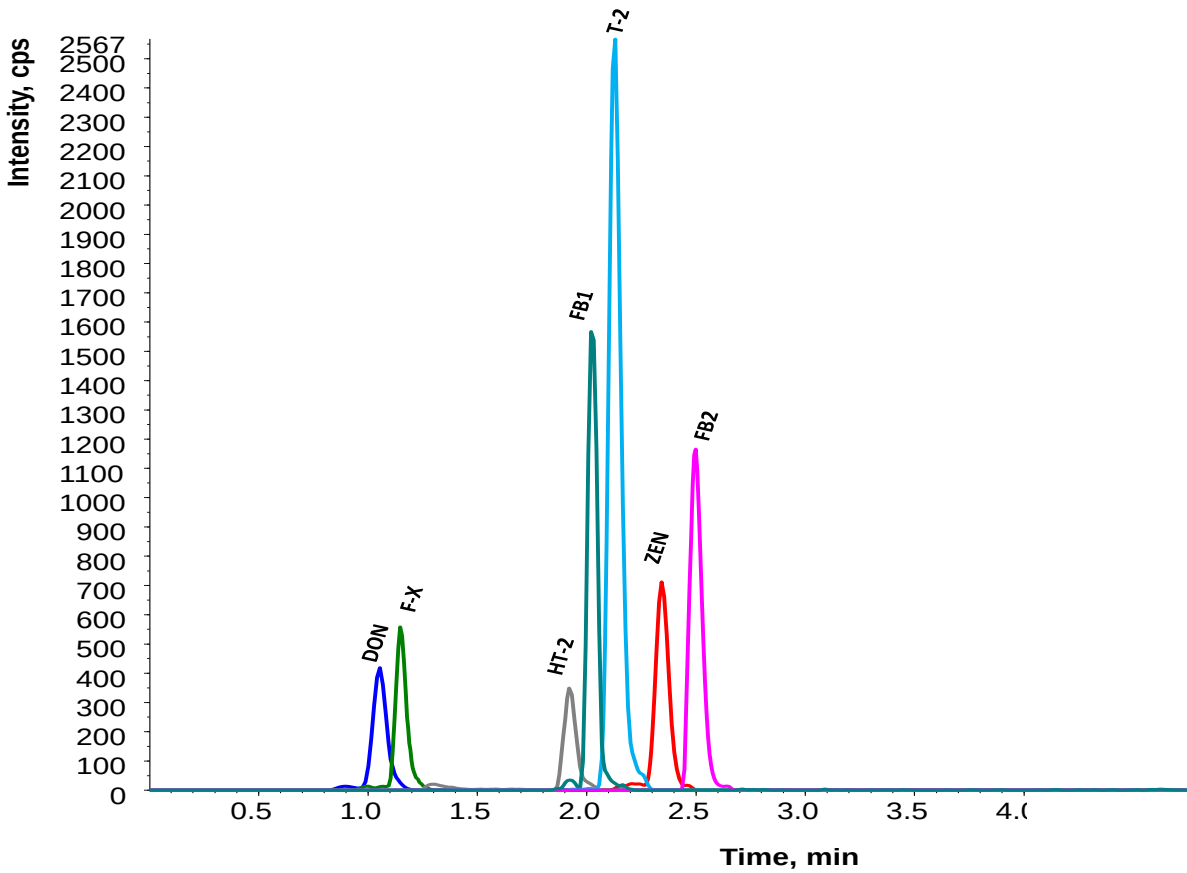

Figure 1. Chromatogram of a spiked oat milk sample applying the proposed method (75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA).

2.3.3.Method characterization

According to EU regulation requirements, the suitability of the proposed method for *Fusarium* mycotoxin determination in different cereal and legume-based milks (oat, soybean, rice and birdseed) was evaluated in terms of matrix effect (ME), linear dynamic ranges, limits of detection (LODs) and quantification (LOQs), precision and trueness.

2.3.3.1.Matrix effect

ME is an analytical signal interference caused by co-eluting sample components that causes an increase or suppression of the analyte signal in presence of the matrix components, compared with the signal of the same analyte when injected in solvent. As it can cause systematic errors in the quantification process, ME was evaluated in this work at two different concentration levels (75 and 300 μg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 μg L^{-1} for T-2, HT-2 and ZEA) in the different samples. Peak areas of the most abundant product ions were considered as analytical signals and ME was calculated as 100 × [(signal of spiked extract – signal of standard solution)/signal of standard solution]. Table 2.2 shows the results and, as can be seen, the Mes ranged between -7 to -39, depending on the mycotoxin. Thus, a moderate ME (<20%) was obtained for FB_1, FB_2, T2 and ZEA in all matrices. However, higher Mes were obtained for HT2, F-X and DON. As a consequence, in order to compensate ME, procedural calibration curves were established.

Table 2.2. ME % for all samples studied.

Analyte	OAT MILK		SOYA MILK		RICE MILK		BIRDSEED MILK	
	level 1	level 2	level 1	level 2	level 1	level 2	level 1	level 2
FB_1	-11	-8	-11	-12	-9	-14	-11	-8
FB_2	-9	-7	-9	-7	-8	-13	-8	-13
T-2	-14	-16	-12	-16	-11	-14	-18	-19
HT-2	-21	-25	-13	-26	-21	-20	-13	-13
F-X	-26	-29	-28	-33	-23	-34	-23	-23
DON	-37	-29	-36	-28	-37	-31	-30	-39
ZEA	-18	-18	-10	-12	-8	-8	-13	-13

Level 1: 75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA.
Level 2: 300 µg L^{-1} for FB_1, FB_2, DON and F-X and 50 µg L^{-1} for T-2, HT-2 and ZEA.

2.3.3.2.Calibration curves and analytical performance characteristics

Procedural calibration curves were used for quantification purposes, established by spiking blank samples at five concentration levels (ranging from 75 to 750 µg L^{-1} for FB_1, FB_2, DON and F-X; and from 10 to 100 µg L^{-1} for T-2, HT-2 and ZEA) before the extraction process. Each level was prepared in duplicate following the proposed QuEChERS-based extraction procedure and injected in triplicate. Performance characteristics of the method are shown in Table 2.3. The statistical parameters were calculated by least-square regression, and LODs and LOQs were considered as 3xS/N ratio and 10xS/N ratio, respectively. For all analytes, the response was linear with a coefficient of determination (R^2) higher than 0.99. As can be seen, low LOQs were obtained, showing the suitability of the proposed method for the determination of very low concentrations of these toxins in the selected matrices. Although there is no specific MLs for mycotoxin content in this kind of products, EU legislation stablishes a maximum contents for these mycotoxins in cereals (1250–1750 µg kg^{-1} for DON and 100-350 µg kg^{-1} for ZEA in unprocessed cereals, and 4000 µg

kg^{-1} sum of FB_1 and FB_2 in un processed maize) [4], and a recommendation for T-2 and HT-2 content (100-1000 μg kg^{-1} for unprocessed cereals) [5]. Taking into account the cereal, legume or seed content in the studied vegetable milk samples (14-15%), the proposed method could also provide an estimation of the mycotoxin contamination of the raw materials used for the processing of these beverages.

Table 2.3. Statistics and performance characteristics of the QuEChERS-UHPLC-MS/MS method for the determination of *Fusarium* mycotoxins in vegetable milk.

ANALYTE	OAT MILK			SOYA MILK			RICE MILK			BIRDSEED MILK		
	LOD*	LOQ	R^2	LOD	LOQ	R^2	LOD	LOQ	R^2	LOD	LOQ	R^2
FB_1	17.3	57.7	0.998	12.4	41.2	0.999	10.7	35.7	0.997	9.0	30.0	0.999
FB_2	11.8	39.5	0.999	17.2	57.3	0.998	9.0	30.1	0.998	10.0	33.3	0.998
T-2	1.8	5.9	0.996	1.3	4.2	0.997	1.6	5.3	0.995	1.0	3.2	0.998
HT-2	2.7	9.1	0.998	2.2	7.4	0.997	2.3	7.7	0.998	1.5	5.1	0.993
F-X	8.7	28.8	0.997	12.2	40.5	0.997	9.8	32.6	0.998	14.1	46.9	0.996
DON	16.1	53.6	0.996	12.9	42.9	0.997	11.3	37.5	0.997	14.2	47.5	0.996
ZEA	1.4	4.5	0.998	2.4	8.0	0.997	2.4	8.0	0.998	2.3	7.6	0.999

*LOD and LOQ are expressed as $\mu g\ L^{-1}$

2.3.3.3. Precision study

The precision of the whole method was evaluated in terms of intraday precision (repeatability) and interday precision (intermediate precision). Intraday precision was assessed by application of the proposed SALLE-UHPLC-MS/MS method in samples spiked at two different concentration levels (75 and 300 μg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 μg L^{-1} for T-2, HT-2 and ZEA). Each sample was prepared in triplicate (experimental replicates) and injected in triplicate (instrumental replicates) on the same day. Similar procedure was carried out during three consecutive days in order to evaluate intermediate precision. The results, expressed as %RSD of peak areas, are shown in Table 2.4. Good precision (RSD lower than 12%) was obtained in all cases, being in agreement with current legislation [31].

Table 2.4. Precision expressed as %RSD of peak areas for spiked vegetable milk samples (n=9).

Analyte	OAT MILK				SOYA MILK				RICE MILK				BIRDSEED MILK			
	Intraday		Interday		Intraday		Interday		Intraday		Interday		Intraday		Interday	
	Level		Level		Level		Level		Level		Level		Level		Level	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
FB_1	7	4	6	8	6	9	7	5	8	3	7	6	5	6	8	4
FB_2	4	5	7	5	6	5	6	5	5	5	7	8	3	4	9	6
T-2	7	7	11	7	8	9	9	9	5	7	11	8	7	6	11	8
HT-2	9	7	10	8	8	7	10	8	5	9	10	9	9	8	11	7
F-x	7	8	8	9	9	5	6	8	9	8	10	10	7	7	8	9
DON	9	9	9	5	6	9	10	9	9	7	12	9	5	4	10	7
ZEA	6	5	12	5	4	6	11	7	6	7	10	9	4	9	9	9

Level 1: 75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg $L^{-1}$for T-2, HT-2 and ZEA.

Level 2: 300 µg L^{-1} for FB_1, FB_2, DON and F-X and 50 µg L^{-1} for T-2, HT-2 and ZEA.

2.3.3.4.Trueness assessment

In order to check the trueness of the proposed method, recovery experiments were carried out in the selected commercial beverages previously analysed in order to detect the presence of mycotoxins. None of them gave a positive result above the LODs of the method. These samples were spiked at two different concentration levels (75 and 300 μg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 μg L^{-1} for T-2, HT-2 and ZEA), processed, and injected in triplicate into the UHPLC–MS/MS system. The results are shown in Table 2.5, and as can be seen, very good recoveries were obtained, ranging between 80 and 99%.

Table 2.5. Recoveries % and (RSD %) for different spiked samples (n=9).

Analyte	OAT MILK				SOYA MILK				RICE MILK				BIRDSEED MILK			
	Level 1		Level 2		Level 1		Level 2		Level 1		Level 2		Level 1		Level 2	
	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%
FB_1	83	7	98	4	85	6	91	9	89	8	98	3	84	5	98	6
FB_2	80	4	80	5	80	6	95	5	81	5	96	5	83	3	97	4
T-2	97	7	80	7	95	8	89	9	97	5	98	7	92	7	83	6
HT-2	83	9	96	7	86	8	91	7	96	5	85	9	87	9	99	8
F-x	90	7	82	8	90	9	81	5	92	9	93	8	90	7	91	7
DON	87	9	84	9	82	6	87	9	82	9	90	7	83	5	89	4
ZEA	81	6	81	5	80	4	81	6	82	6	82	7	80	4	85	9

Level 1: 75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA.

Level 2: 300 µg L^{-1} for FB_1, FB_2, DON and F-X and 50 µg L^{-1} for T-2, HT-2 and ZEA.

2.3.4.Application to the analysis of commercial samples

Different samples of commercial vegetable milks, including 8 oat milk, 8 soybean milk (7 natural and 1 light), 5 rice milk and 1 bird seed milk, purchased in local markets from the south of Spain (Granada, Córdoba and Jaén) and the north of Morocco (Tangier) were analysed in order to monitor the presence of *Fusarium* toxins. All of them were supplied as 1 L packs and were stored at 4 °C.

Three oat milk samples (all of them from the same brand but purchased in different cities) gave a positive result for DON at concentrations of 191, 221 and 270 μg L^{-1}. These results were confirmed by standard addition calibration, obtaining concentrations of 192 μg L^{-1} ($y = 14.537x + 3163$; $R^2 = 0.997$), 218 μg L^{-1} ($y = 14.523x + 3163$; $R^2 = 0.998$) and 263 μg L^{-1} ($y = 14.532x + 3163$; $R^2 = 0.996$). Considering the percentage of cereals in these milk samples (14%), these concentrations would correspond to DON concentration in raw cereals of approximately 1364, 1577 and 1929 μg kg^{-1}, respectively. That would indicate that the raw oat used for milk processing was contaminated at concentrations close or even above the MLs allowed by European legislation for unprocessed oat (1750 μg kg^{-1}) [4]. The rest of samples analysed did not show any contamination by Fusarium toxins above the LODs.

Figure 2.2 shows the extracted ion chromatogram for DON of a contaminated oat milk sample applying the proposed method.

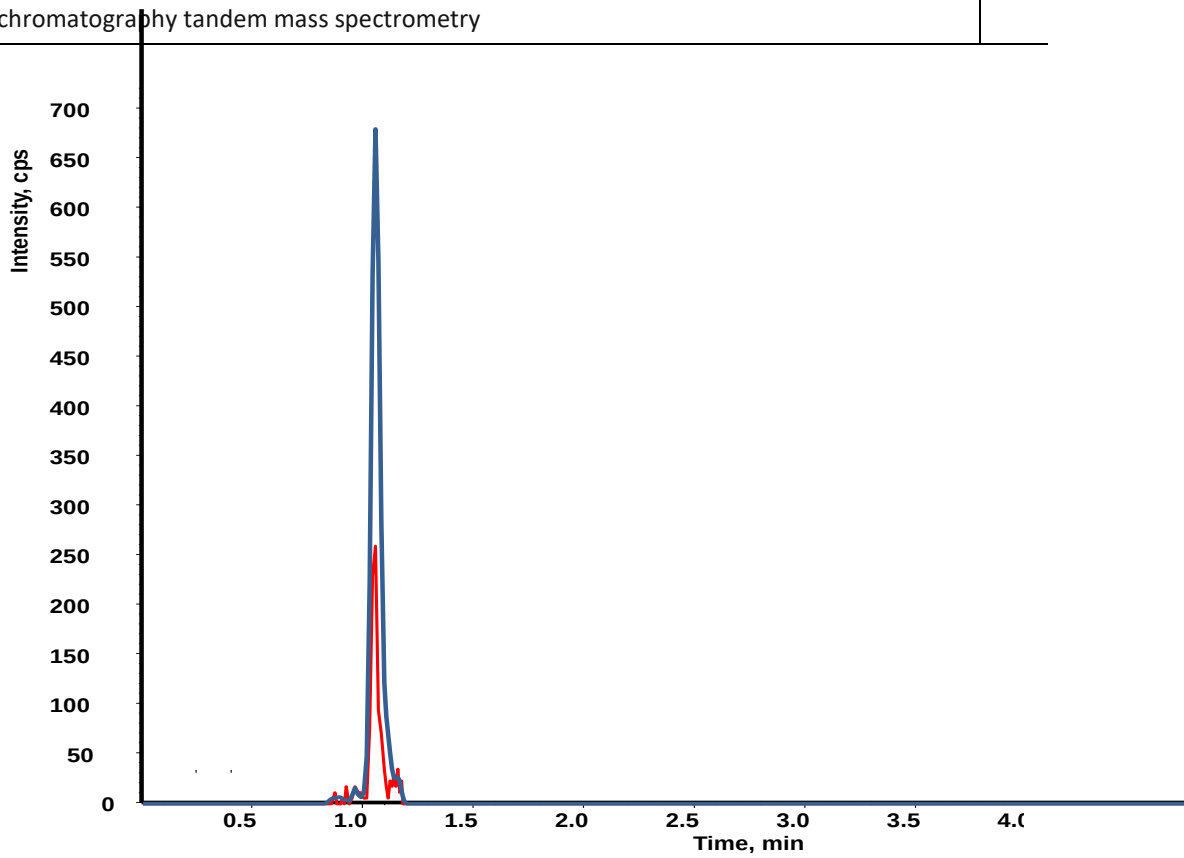

Figure 2. Extracted ion chromatogram for DON of a contaminated oat milk sample applying the proposed method.

2.4.Conclusions

A SALLE-based procedure followed by an UHPLC–MS/MS method have been proposed and successfully applied to the determination of most relevant *Fusarium* toxins in functional beverages based on cereals, leguminous plant and seeds, such as oat, soybean, rice and bird seed milk samples, being a relevant analytical proposal for the control of these important toxins in this kind of relatively new products, not included in the present legislation. The proposed method showed low LODs and LOQs and both, recovery and precision studies, meet the performance criteria required for mycotoxin

analytical methods in foodstuffs. By applying the method on commercial samples, DON with concentrations between 191 and 270 µg L^{-1} was found in three oat milk samples from Spain, indicating that the raw oat used for milk processing was contaminated at concentration close or above the maximum limits allowed by European legislation. This data reveal a matter of concern, indicating that more control should be applied on these increasingly consumed products belonging to functional foods.

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2

CHAPTER 3

Evaluation of a new modified QuEChERS method for the monitoring of carbamate residues in high-fat cheeses by using UHPLC–MS/MS

3

Abstract

A simple and efficient method for the determination of 28 carbamates in high-fat cheeses is here proposed. The methodology is based on a modified quick, easy, cheap, effective, rugged, and safe procedure as sample treatment using a new sorbent (Z-Sep$^+$) followed by ultra–high performance liquid chromatography-tandem mass spectrometry determination. The method has been validated in different kind of cheeses (Gorgonzola, Roquefort and Camembert), achieving recoveries between 70-115%, relative standard deviations lower than 13% and limits of quantification lower than 5.4 μg kg^{-1}, below the maximum residue levels tolerated for these compounds by the European legislation. Matrix effect was lower than ±30% for all the studied pesticides. The combination of ultra–high performance liquid chromatography-tandem mass spectrometry with this modified quick, easy, cheap, effective, rugged, and safe procedure using Z-Sep$^+$ allowed a high sample throughput and an efficient cleaning extracts for the control of these residues in cheeses with high-fat content.

3.1.Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), more than 6 billion people worldwide consume milk and milk products. Moreover, dairy products play a key role in human nutrition, especially for vulnerable groups, such as infants, school-age children and elderly people [1,2]. The presence of pesticide residues in food derived from animals, mainly due to the contamination of feedstuff, is a matter of concern because their possible adverse effects in humans. In fact, the Rapid Alert System for Food and Feed (RASFF) database shows that it is possible to found some alerts of contamination of this kind of food with high levels of pesticides[3]. A potential risk for pesticide contamination must be taken into account. To ensure the safety of consumer, European Union (EU) by Regulation No. 396/2005 and subsequent amendments, has established groups of different products of animal origin, including milk and some milk-based products, for which maximum residue level (MRL) for some pesticides are established. In the case of milk-based products with a 2% fat content of or more, the MRL is 25-fold that set for raw milk [4].

The use of carbamates (CRBs) as insecticides, fungicides or herbicides is a common practice in agriculture. They are acetylcholine esterase inhibitors in the nervous system and its toxicity in human is related to some symptoms such as miosis, fasciculation, and even long term neurological and teratogenic effects [5]. In relation to food quality, the European Union also sets MRLs for these residues in dairy products [6]. In fact, CRB residues have been found in milk samples [7]. Henceforth, it is critical the development of highly sensitive methods for the determination of these pesticides in scarcely explored samples with high fat content, such as some dairy products. LC or GC coupled with MS,

or MS/MS detection, has been generally used for the determination of pesticides in foods [8]. Several methods for the determination of pesticide residues in milk and dairy products can be found in literature [9], using GC-MS [10,11,12,13] and, more frequently LC-MS and LC–MS/MS [6,14,15,16]. Additionally, the advantages of ultra-high performance LC (UHPLC) make it a good alternative to conventional LC, and it has also been proposed for pesticide determination in dairy products [17,18,19]. The last trends in pesticide determination by LC-MS have been compiled in a review article [20].

Dairy products are generally highly complex matrices, having proteins, fats and carbohydrates as main components, which could often interfere in the analytical determination. For this reason, a sample treatment to remove the high molecular-mass fat from the sample is required. However, sample treatment can be tedious, involving several steps to remove the co-extracted material from the matrix. With this purpose, different methodologies have been proposed as sample clean-up step for determination of pesticides in different dairy products, being most of them based on SPE [15,16,17,21]. Concerning CRBs, a review article summarized the most commonly employed methodologies for sample preparation [22].

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a generic extraction procedure which involves microscale extraction using acetonitrile, ethyl acetate or other organic solvents, and partitioning with magnesium sulphate alone or in combination with other salts, generally NaCl. This extract could be either directly injected in the chromatographic system or cleaned-up by dispersive solid phase extraction (dSPE) [23]. This sample treatment has been widely used for multiresidue pesticide determination in vegetables, fruits, and many other matrices, including high-fat foods [24]. This approach has been

successfully used in the determination of pesticides in milk samples [17,25]. In these works, different dSPE sorbents such as primary secondary amine (PSA) and C18 were tested. However, some lipophilic pesticides were removed by the dSPE sorbent together with the other fatty compounds. To overcome these problems, a new dSPE sorbent, named Supel™ QuE Z-Sep+ consisting of both, C18 and zirconia bound to the same silica particles, has been developed. The C18 binds fats through hydrophobic interaction, while the zirconia acts as a Lewis acid, attracting compounds with electron donating groups. It has been recently applied for highly fatty vegetable commodities such as avocado, almond and edible oils [26,27]. This sorbent could remove more fats and pigments from sample extracts than the traditional dSPE sorbents for QuEChERS, eliminating matrix interferences [28]. Nevertheless, as far as we know, it has not been employed in the determination of pesticides in dairy products.

The goal of this study was to evaluate this new sorbent for the UHPLC-MS/MS determination of 28 CRBs in a dairy product of high-fat content, as cheese. For this purpose, three different cheeses such as Gorgonzola, Roquefort and Camembert, with fat contents of 50, 32 and 45%, respectively, were selected as representative high-fat dairy products. The obtained results showed that the sample treatment shows enough clean-up efficiency to remove fatty components which may cause matrix effect (ME). The proposed method has been validated according to the recently approved EU guidance document [29]. The results show the suitability of this procedure for monitoring CRBs in these products in a single run.

3.2.Materials and Methods

3.2.1.Chemicals and solvents

LC-MS "purity grade" methanol (MeOH) and acetonitrile (MeCN) were supplied by VWR (Radnor, PA, USA). Formic acid and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ.cm, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout all the work.

Pestanal grade analytical standards of propamocarb (PRM), asulam (ASL), aldicarbsulfoxide (ALDSFX), oxamyl (OX), methomyl (MTY), carbendazim (CBZ), pirimicarb desmethyl (PIRDES), methiocarb sulfoxide (MTHSFX), 3-hidroxycarbofuran (3-CF), methiocarb sulfone (MTHSFN), cymoxanil (CY), aldicarb (ALD), pirimicarb (PIR), propoxur (PX), carbofuran (CF), carbaryl (CAR), ethiofencarb (ETH), thiodicarb (TH), isoprocarb (ISO), fenobucarb (FEN), diethofencarb (DETH), methiocarb (MTH), promecarb (PR), napropamid (NP), fenoxycarb (FNX), pyraclostrobin (PY), benthiocarb (BTH), and furathiocarb (FURA) were supplied by Fluka Analytical (Steinheim, Germany). Individual stock standard solutions of each compound were prepared by dissolving accurately weighed amounts in MeOH and were stored in the dark at 4° C. Working standard solutions containing all the CRBs (1 mg L^{-1} and 10 mg L^{-1}) were freshly prepared by proper dilution of the stock standard solutions with MeOH. QuEChERS extraction tubes were prepared in the lab. They consisted of a 50-mL tube with 4 g MgSO4 and 1 g NaCl (Panreac Química, Barcelona, Spain) for extraction, and a 15-mL dSPE tube with different quantities of bulk C18, PSA, (Agilent Technologies, Waldbron, Germany) or Supel™ QuE Z-Sep+ (Supelco, Bellafonte, PA, DE, USA). Nylon syringe filters, 0.2 mm, 13 mm (Bonna-Agela Technologies Inc., Wilmington, USA) were used for filtration of sample extracts prior to the injection into the UHPLC system.

3.2.2 Instrumentation and software

Separation was performed on an Agilent 1290 Infinity LC using a C_{18} column (Zorbax Eclipse plus RRHD 50 mm×2.1 mm, 1.8 µm) supplied by Agilent Technologies. The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB Sciex, Darmstadt, Germany) with ESI. Analysts Software version 1.5 with Schedule MRM^{TM} Algorithm (AB Sciex) was used for data collection and treatment.

A vortex (Genie 2 model from Scientific Industries, Bohemia, NY, USA), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA), and a centrifuge (Universal 320 model from Hettich, Leipzig, Germany) were used also during the sample preparation procedure.

3.2.3 Sample treatment

Cheese samples (Gorgonzola, Roquefort and Camembert) were purchased from local market (Granada, Spain) and stored at 4°C until analysis. The QuEChERS procedure was as follows: 3 g of sample was placed in a 50-mL falcon tube. Then, 7 mL of water and 10 mL of MeCN (1% acetic acid) were added to the tube, and it was mechanically shaken for 10 min. QuEChERS extraction salts (4 g $MgSO_4$, 1 g NaCl) were added to the tube and it was shaken again for another 10 min. Then, the sample was centrifuged at 5000 rpm for 5 min. After that, 3 mL of the supernatant was transferred to the dSPE tube containing 100 mg of Z-Sep$^+$, stirred in vortex for 2 min and centrifuged (5000 rpm, 2 min). An aliquot of 2 mL of the MeCN extract was transferred to a vial, dried under N_2 stream and the final residue was re-dissolved first with 300 µL of MeOH, shaken by vortex for 2 min. Subsequently 700 µL of H_2O were added, shaken 2 min more, filtered through syringe filters and injected into the UHPLC–MS/MS system.

The QuEChERS method for determination of carbamates in cheese is summarized in Fig.3.1.

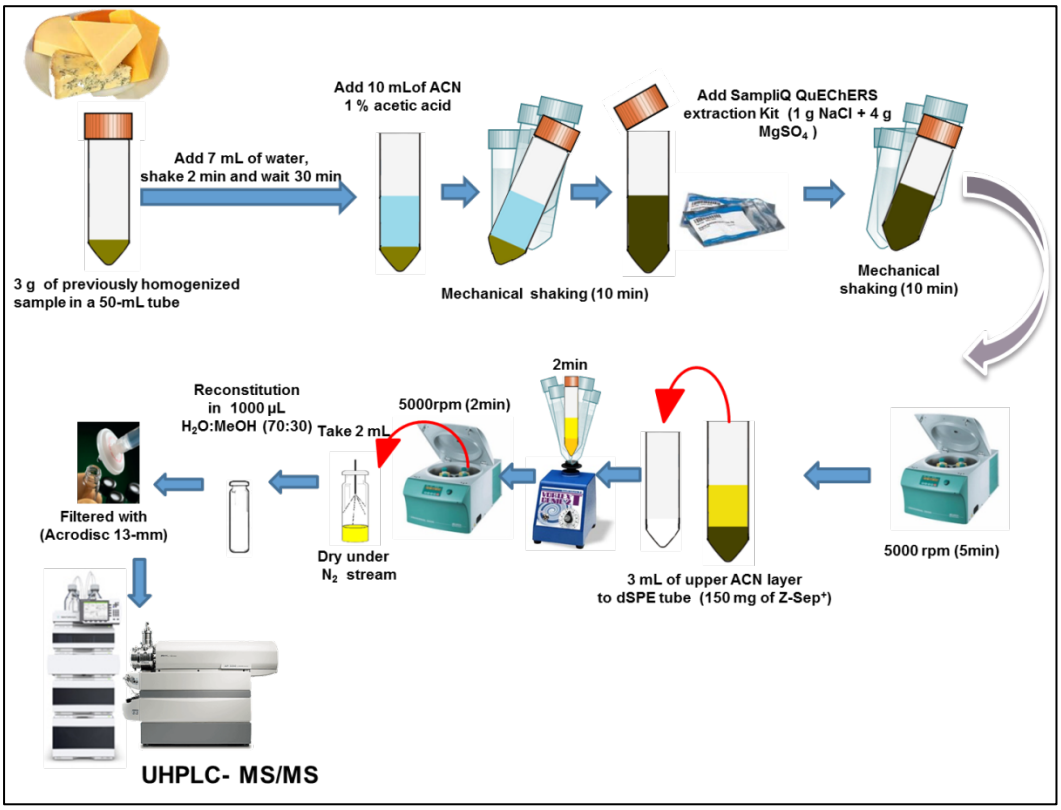


Figure 3.1. Sample treatment for the determination of carbamates in cheese.

3.2.4 UHPLC–MS/MS analysis

The chromatographic method for the determination of CRBs was previously developed in our laboratory [30]. UHPLC separations were performed on a C18 column using a mobile phase consisting of 0.01% aqueous formic acid (solvent A) and MeOH with the same percentage of formic acid (solvent B) at a flow rate of 0.5 mL min^{-1}. The eluent gradient profile was as follow: 5% B at the beginning; 20% B from 0.7 to 1.2 min; 50% B from 2.5 to 3.0 min; 100% B from 6.5 to 8.5 min; 5% B from 8.5 to 9.0 and finally 5% B for 1.5 min. Under optimum conditions, all the analytes were eluted in 6 min, while the run time

for each injection was 10.5 min. The temperature of the column was 25° C and the injection volume was 10 µL. The mass spectrometer was working with ESI in positive mode under the multiple reaction monitoring (MRM) conditions shown in supplementary data (Table 3.1). The ionization source parameters were: source temperature 400° C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

Table 3.1. Optimized MS/MS parameters.

	Precursor ion	Rt	DP	EP	CEP	Product ion	CEN	CXP
PRM	189.2	1.0	41	6.5	22	102.1 (Q)	25	4
						73.8 (I)	37	4
ASL	231.1	1.2	36	8	12	155.9 (Q)	15	4
						92.1 (I)	33	4
ALDSFX	207.2	1.3	21	8.5	16	132.1 (Q)	11	4
						89.1 (I)	17	2
OX	237.2	1.5	16	6.5	12	71.9 (Q)	27	4
						90.2 (I)	13	2
MTY	163.2	1.7	26	7.5	10	88.2 (Q)	13	2
						106.0 (I)	15	2
CBZ+BY	192.2	1.9	36	9	18	160 (Q)	41	4
						132.1 (I)	25	4
PIRDES	225.1	2.3	26	9	14	72.1 (Q)	27	4
						168.1 (I)	19	6
MTHSFX	242.1	2.5	26	6.5	14	185.2 (Q)	19	6
						170.0 (I)	29	4
MTHSFN	258.1	2.7	41	6	12	122.0 (Q)	33	6
						201.0 (I)	13	6
CY	199.2	2.7	21	7	15	128.0 (Q)	13	4
						111.2 (I)	25	4
ALD	116.1	3.0	31	9.5	12	89.1 (Q)	13	0
						70.3 (I)	13	0
PIR	239.2	3.2	36	7.5	16	72.1 (Q)	29	4
						182.2 (I)	21	4
PX	210.3	3.3	21	7.5	15	111.1 (Q)	21	4
						168.2 (I)	13	4
CF	222.2	3.4	41	6.5	15	165.1 (Q)	17	4
						123.1 (I)	29	4
CAR	202.2	3.6	26	9	15	145.0 (Q)	17	4
						127.2 (I)	39	4
ETH	226.1	3.8	26	8	15	107.1 (Q)	23	4
						164.4 (I)	13	2
TH	355.2	3.9	21	7	19	88.1 (Q)	33	4
						108.1 (I)	19	2
ISO	194.2	4.0	26	8.5	15	95.1 (Q)	21	2
						137.2 (I)	13	4
FEN	208.2	4.5	36	7	15	95.2 (Q)	23	2
						151.9 (I)	13	4
DETH	268.3	4.6	31	8.5	16	226.0 (Q)	15	6
						124.2 (I)	41	4
MTH	226.1	4.6	21	8.5	15	169.1 (Q)	15	4
						121.1 (I)	25	4
PR	208.3	4.7	26	8.5	15	151.1 (Q)	13	4
						109.0 (I)	25	4

NP	272.2	5.0	36	11	17	129.2 (Q) 171.3 (I)	23 25	4 6
FNX	302.1	5.2	36	5.5	17	87.9 (Q) 116.1 (I)	33 17	4 4
PY	388.2	5.4	26	7	20	163.1 (Q) 194.1 (I)	21 19	4 4
BTH	258.2	5.5	46	7.5	16	125 (Q) 89.2 (I)	33 71	4 4
FURA	383.1	5.8	56	8	8	195.2 (Q) 252 (I)	23 19	4 6

Rt, Retention Time; DP, Declustering Potential; EP, Entrance Potential; CEP, Collision Cell; CEN, Collision Energy; CXP, Collision Exit; Q, Transition used for quantification; I, Transition employed to complete the identification.

3.3.Results and discussion

3.3.1 Optimization of sample preparation

The complexity of the matrix combined with potential interferences of food components can affect significantly the analysis of pesticide residues, producing ME [31]. Thus, the analyte signal may be enhanced or suppressed compared to the signal of the same analyte when injected in solvent due to the presence of co-eluting sample components, mainly in fatty matrixes [32]. Thus, to avoid these problems the use of a proper sample treatment is mandatory. As stated before, QuEChERS methodology allows quick and effective extractions, so it was the method chosen in this work.

The optimization of QuEChERS was carried out with 3 g of gorgonzola cheese (as representative high-fat cheese) spiked at 10 μg kg^{-1} of each CRB. The recovery of each analyte was employed to evaluate the extraction efficiency. The first extraction step of CRBs was based on the application of non-buffered QuEChERS method with MeCN extraction. However, the recoveries for some analytes (PRM, PIRDES, CY, ALD and ASL) were lower than 60%. This fact may be due to these CRBs could exhibit pH-dependent stability problems and suffer a possible degradation [33]. To overcome this inconvenience in the extraction

process, the addition of acetic acid in MeCN was considered [34]. The use of 1% of acetic acid allowed recoveries higher than 70% for all studied CRBs, so MeCN with 1% acetic acid was selected as extraction solvent.

Subsequently, different sorbents were tested for dSPE. This optimization was carried out using 100 mg of PSA, Z-Sep$^+$ or C18. The average recovery was 75%, 86% and 84% for C18, Z-Sep$^+$ and PSA, respectively. Moreover, Fig.3.2 shows the ME with the different sorbents, calculated as [(signal of spiked extract-signal of standard solution)/signal of standard solution] x100. As it can be observed, the average ME in the case of Z-Sep$^+$ was -13%, while with the other sorbents this effect was higher than -25 %. Thus, Z-Sep$^+$ allows to remove the fat components of the matrix more efficiently than the traditional dSPE sorbents with the best recoveries.

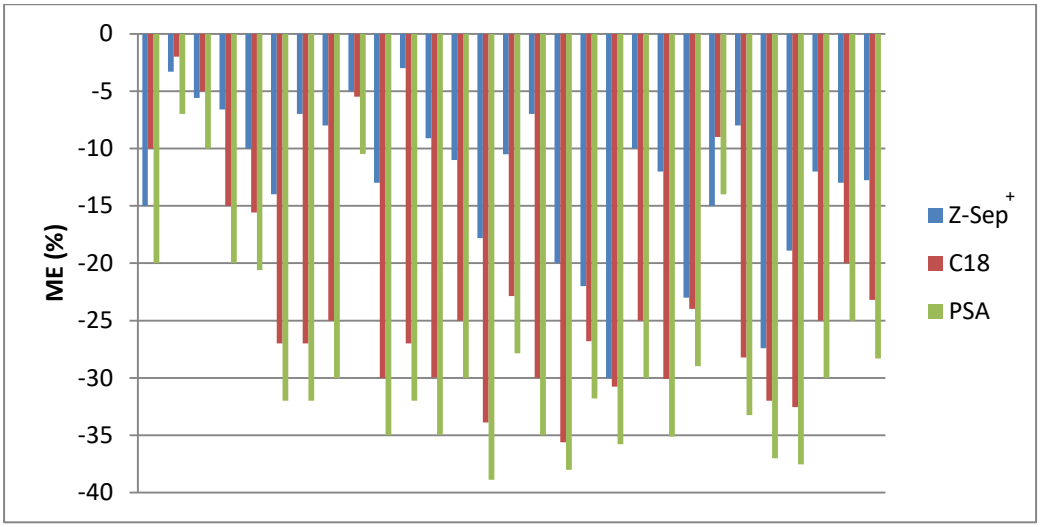
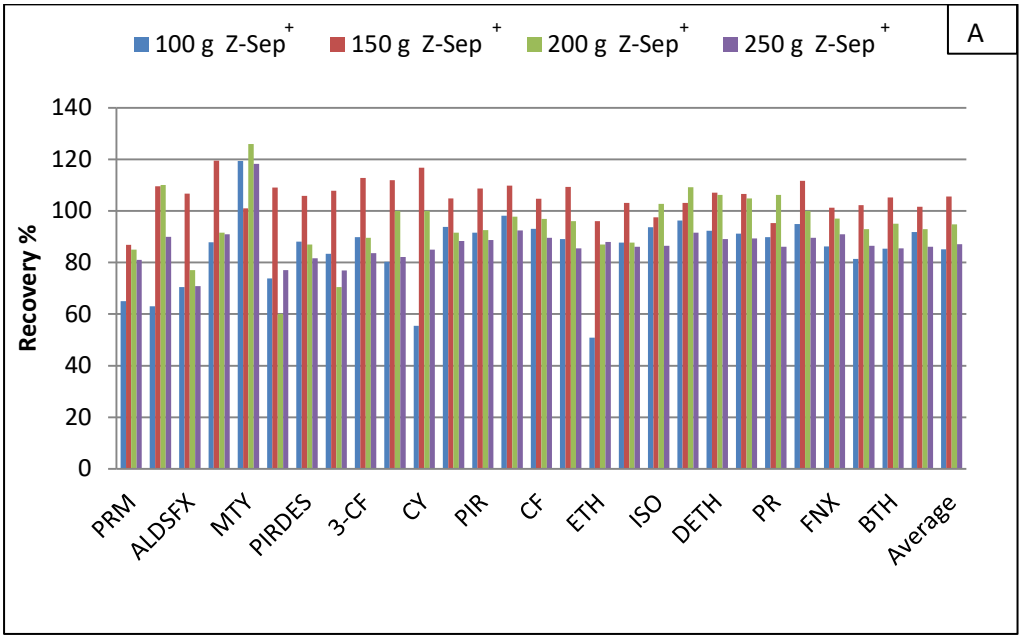


Figure 3.2. Matrix effect (%) using Z-Sep $^+$, C18 and PSA as dSPE sorbents (3 g of gorgonzola cheese spiked at 10 µg kg^{-1} of each CRB).

Finally, different amounts of Z-Sep$^+$ were tested (from 100 to 250 mg, with increments of 50 mg). As it can be observed in Figure 3.3 (a and b), using 100 mg of Z-Sep$^+$ the recoveries for the studied CRBs were higher than 90% in all cases, and the average ME were -13%. Higher amounts did not reduce the ME and the extraction efficiency was similar. Thus, the use of 100 mg of Z-Sep$^+$ provided good results in terms of recoveries and ME, so it was selected as optimum.



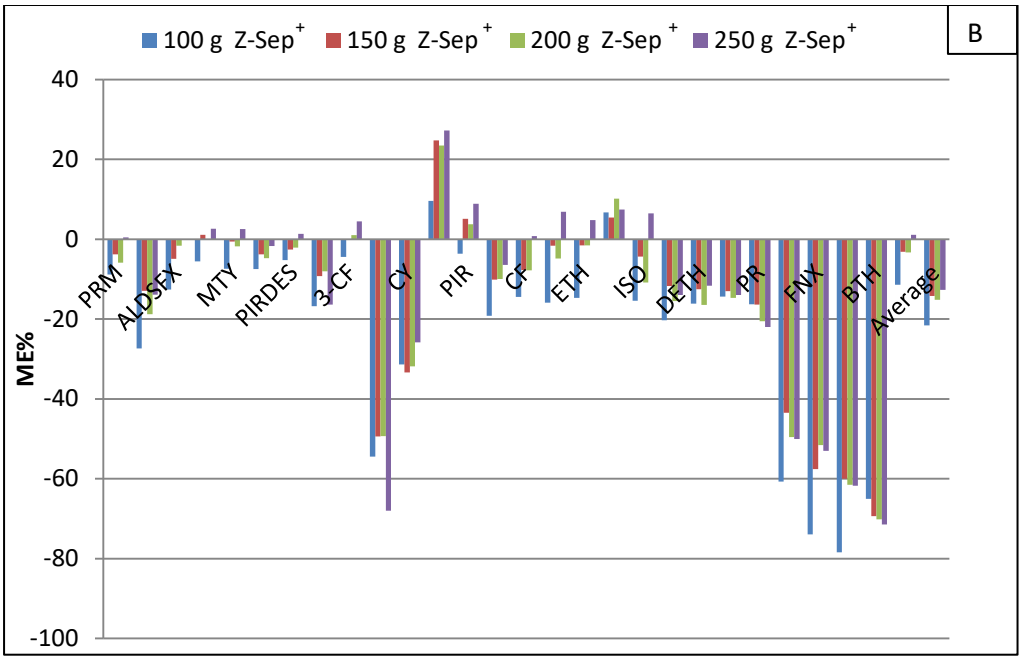


Figure 3.3. Effect of different amounts of Z-Sep$^+$ (from 100 to 250 mg, with increments of 50 mg) on the recoveries (a) and matrix effect (b) of the studied CRBs.

Another key point is the composition of the solvent used for reconstituting the dried sample extract after the application of dSPE. It was observed that, when a mixture of MeOH/H_2O (20:80) was used as reconstitution solvent, very low recoveries for some non-polar CRBs (BTH, BFU and FURA) were obtained. However, for the rest of pesticides the recoveries were adequate. This fact could be due to some matrix compounds that are not removed with dSPE, which are poorly soluble in water and could trap non-polar pesticides, remaining in the undissolved layer on the bottom of the vial. To avoid this problem, the percentage of MeOH was increased in order to dissolve non-polar CRBs, observing that a mixture of MeOH/H_2O (30:70) was enough to obtain satisfactory recoveries for these analytes. However, it should be noted that MeOH was added firstly (as explained in "Extraction procedure" section) to

facilitate the recovery of the non-polar compounds. Finally, the volume of this reconstitution solvent was studied. The results showed that a volume of 1000 µL was sufficient to obtain the best enrichment of CRBs with satisfactory MEs. Therefore, 1000 µL of the mixture MeOH/H_2O (30:70 v/v) was selected as reconstitution solvent.

3.3.2 Characterization of the method

The proposed method for the determination of CRBs in cheeses was characterized in terms of linear dynamic ranges, LODs and LOQs, ME, precision, trueness and selectivity. Gorgonzola cheese was selected as representative matrix.

3.3.2.1 Calibration curve and performance characteristics

Calibration curves were assessed by spiking blank samples of Gorgonzola cheese (5, 10, 25, 50, 100 and 150 µg kg^{-1}) before the extraction process. Each sample was prepared following the proposed QuEChERS method and injected in triplicate. According to SANTE guidance document [29], two product ions were selected, the most intense one was used as quantification ion (Q) and the following was considered as confirmation ion (I). These product ions together with the retention times were employed to ensure adequate analyte identification according the current legislation (see Table 3.1) [30].

Performance characteristics of the method are shown in Table 1. LODs and LOQs have been calculated based on the response observed at the lowest detected point of the calibration curve for each carbamate. Calculations were carried out considering an extrapolation at S/N=3 for LODs and S/N=10 for LOQs. As can be seen in Table 3.2, LOQs were always lower than the permitted MRLs. Therefore, the proposed method could be used for the determination of these compounds in the selected matrices at very low concentration levels.

Table 3.2. Statistics and performance characteristics of the QuEChERS-UHPLC-MS/MS method for the determination of CRBs in cheese.

Analyte	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Linear range (μg kg^{-1})	R^2	MRL in cheese (μg kg^{-1})	ME %
PRM	0.6	2.0	2.0 - 150	0.9938	250	-15
ASL	0.5	1.5	1.5 – 150	0.9918	500	-3
ALDSFX	1.4	4.5	4.5 – 150	0.9936	250	-6
OX	1.2	4.0	4.0 – 150	0.9954	250	-7
MTY	0.9	3.2	3.2 - 150	0.9983	Not established	-10
CBZ	1.1	3.7	3.7 – 150	0.9964	1250	-14
PIRDES	0.1	0.5	0.5 – 150	0.9957	1250	-7
MTHSFX	0.2	0.7	0.7 - 150	0.9989	1250	-8
3-CF	1.0	3.2	3.2 – 150	0.9987	250	-5
MTHSFN	1.0	3.4	3.4 – 150	0.9994	1250	-13
CY	1.4	4.7	4.7 – 150	0.9972	1250	-3
ALD	1.4	4.6	4.6 – 150	0.9969	250	-9
PIR	0.2	0.8	0.8 – 150	0.9973	1250	-11
PX	1.1	3.8	3.8 – 150	0.9984	250	-18
CF	0.4	1.3	1.3 – 150	0.9919	1250	-11
CAR	0.7	2.4	2.4 – 150	0.9988	Not established	-7
ETH	0.6	2.0	2.0 – 150	0.9952	500	-20
TH	0.5	1.8	1.8 – 150	0.9934	Not established	-22
ISO	1.2	3.9	3.9 – 150	0.9949	Not established	-30
FEN	1.1	3.8	3.8 – 150	0.9931	1250	-10
DETH	0.6	2.1	2.1 – 150	0.9948	1250	-12
MTH	0.2	0.8	0.8 – 150	0.9936	Not established	-23
PR	0.6	1.9	1.9 – 150	0.9916	250	-15
NP	1.3	4.4	4.4 – 150	0.9912	1250	-8
FNX	1.3	4.4	4.4 – 150	0.9935	250	-27
PY	0.9	2.9	2.9 – 150	0.9942	250	-19
BTH	1.2	3.8	3.8 – 150	0.9912	250	-12
FURA	0.2	0.8	0.8 – 150	0.9944	1250	-13

R^2, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification; MRL, maximum residue limit; ME, matrix effect

3.3.2.2 Matrix effect

In order to check any possible difference among the three selected cheese, ME was estimated for each CRB in the different matrices, by comparing the slopes of matrix-matched calibration curves (obtained by adding CRB standards to an extract of a blank sample) with the slopes of external standard calibration curves, both of them were obtained with the same final concentrations levels.

ME was estimated for each CRB in Gorgonzola cheese samples by application of the following equation [35].

$$ME\ \% = \left(\left(\frac{slope\ of\ matrix\ matched\ calibration}{slope\ of\ standard\ solution\ calibration}\right) - 1\right) \times 100$$

Table 3.1 show the results of ME values for each CRB and, as can be observed, all MEs were lower than $|30\ \%|$.

3.3.2.3 Precision study

Both repeatability and intermediate precision were tested by application of the proposed QuEChERS-UHPLC-MS/MS method in Gorgonzola samples spiked at two different concentration levels of CRBs (5 and 10 μg kg^{-1}). To check the repeatability (intraday precision), three samples were prepared and injected in triplicate on the same day, under the same conditions. Similar procedure was carried out in the evaluation of intermediate precision (interday precision). Thus, during three consecutive days, one sample per day was prepared and injected in triplicate. The results, expressed as %RSD of peak areas, are shown in Table 3.3. Good precision (RSD lower than 13%) was obtained in all cases. So, it could be concluded that the obtained results are in agreement with the current demand [29,36].

Table 3.3. Intraday (n=9) and interday precision (n=9) expressed as %RSD of peak areas for spiked cheese samples.

	Intraday		Interday	
	level 1	**level 2**	**level 1**	**level 2**
PRM	4	3	8	6
ASL	8	8	10	9
ALDSFX	8	7	10	7
OX	7	5	8	6
MTY	9	8	12	9
CBZ	5	4	7	6
PIRDES	5	6	9	9
MTHSFX	7	5	10	9
3-CF	9	5	11	7
MTHSFN	8	6	10	9
CY	10	8	11	9
ALD	9	7	10	11
PIR	10	8	12	10
PX	6	5	8	4
CF	10	6	11	8
CAR	9	8	11	9
ETH	8	6	11	7
TH	8	7	10	10
ISO	5	4	9	8
FEN	8	6	9	7
DETH	9	8	12	9
MTH	8	9	9	10
PR	9	5	10	8
NP	9	6	10	8
FNX	9	7	11	10
PY	9	8	10	12
BTH	9	7	13	9
FURA	7	6	10	8

Level 1: 5 μg kg^{-1}, level2: 10 μg kg^{-1}

3.3.2.4 Trueness and assessment

The trueness of the proposed method was assessed by recovery studies in different types of cheese samples (Gorgonzola, Roquefort and Camembert) spiked at two different concentration levels of each CRB (5 and 10 μg kg^{-1}). Each sample was analysed in triplicate and injected three times. Blank samples were previously analysed to check the presence of CRBs; none of them gave a result above the LODs of the method. Recoveries between 70% and 115% were obtained, with satisfactory precisions (see Table 3.4), fulfilling current legislation requirements [29, 36]. A typical extracted ion chromatogram corresponding to a gorgonzola cheese sample spiked with 10 μg kg^{-1} for each CRB, and analysed by the proposed QuEChERS–UHPLC–MS/MS method is shown in Fig. 3.4.

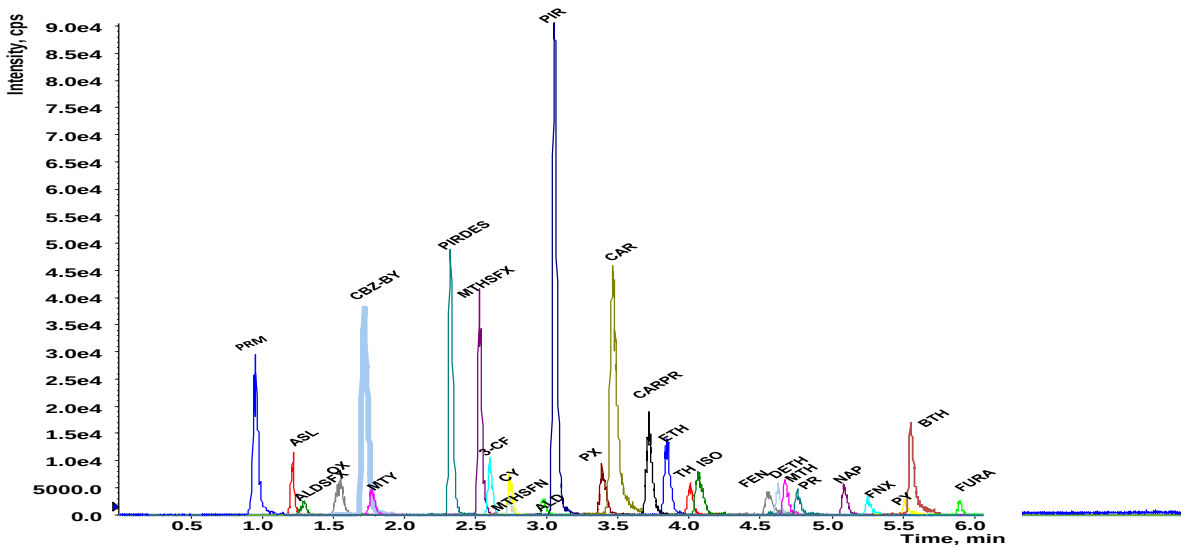


Figure 3.4. Extracted ion chromatogram of a spiked cheese sample applying the proposed method (10 μg kg^{-1} for each CRB).

Table 3.4. Recoveries and RSD for different spiked cheese samples (n=9).

Analyte	Gorgonzola cheese				Roquefort cheese				Camembert cheese			
	Level 1 (5 µg kg^{-1})		Level 2 (10 µg kg^{-1})		Level 1 (5 µg kg^{-1})		Level 2 (10 µg kg^{-1})		Level 1 (5 µg kg^{-1})		Level 2 (10 µg kg^{-1})	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R(%)	RSD (%)	R (%)	RSD (%)
PRM	72	4	70	3	71	5	72	3	73	4	72	3
ASL	72	8	77	8	93	10	83	7	75	6	82	9
ALDSFX	74	8	77	7	77	10	71	10	78	6	82	7
OX	112	7	106	5	105	9	112	7	78	9	82	9
MTY	103	9	89	8	103	8	98	7	87	6	114	9
CBZ	83	5	83	4	86	10	80	10	89	4	77	6
PIRDES	88	5	90	6	97	9	82	10	85	4	83	2
MTHSFX	86	7	97	5	93	10	82	10	80	7	85	2
3-CF	103	9	109	5	109	9	88	9	90	8	96	4
MTHSFN	97	8	103	6	113	9	89	11	92	6	92	9
CY	91	10	98	8	114	11	93	10	88	10	86	5
ALD	104	9	110	7	102	10	91	7	85	6	91	6
PIR	103	10	100	8	98	6	95	6	90	3	92	3
PX	104	6	105	5	101	6	95	4	103	3	101	3
CF	104	10	98	6	98	7	98	6	94	3	94	3
CAR	105	9	100	8	97	7	96	4	94	8	97	7
ETH	94	8	80	6	79	7	70	6	97	4	77	6
TH	111	8	97	7	100	7	95	4	73	6	100	5
ISO	95	5	97	4	102	10	87	8	89	6	92	5
FEN	110	8	95	6	98	6	91	5	97	5	86	6
DETH	98	9	86	8	94	5	85	8	97	6	80	6
MTH	89	8	91	9	100	6	86	4	97	5	85	6
PR	82	9	85	5	94	7	83	6	98	8	76	6
NP	88	9	90	6	108	10	83	10	106	8	81	10
FNX	92	9	97	7	109	10	108	9	115	8	105	7
PY	97	9	94	8	86	10	83	8	104	7	81	6
BTH	99	9	100	7	96	7	89	7	108	10	87	7
FURA	100	7	109	6	105	9	92	9	88	3	97	2

R, recovery; RSD, relative standard deviation

3.3.2.5. Selectivity

According to SANTE guidance document [29], to confirm positive results, the selectivity of the method must be checked. The relative intensity or ratios of selective ions (Q and I MRM transitions) in real samples has to fulfil a certain tolerance level, obtained from Q/I ratio from a standard solution measured under the same conditions. The ion ratio Q/I from real samples should not deviate more than 30%. As could be observed in Table 3.5, the Q/I ratios obtained from the different spiked cheese samples (5 μg kg^{-1} for each CRB) compared to that from a standard solution (3 μg L^{-1} for each CRB) fulfilled this criterion. Thus, the proposed method provided enough selectivity for the 28 selected CRBs in this type of samples.

Table 3.5 Ratio between the quantifier (Q) and qualifier (I) ions in standard solutions and spiked cheese samples**.**

	Standard solution (3 µg L^{-1})		Cheese samples (5 µg kg^{-1})		
	Q/I	**Range (30%)**	**Q/I (Gorgonzola)**	**Q/I (Roquefort)**	**Q/I (Camembert)**
PRM	3.1	2.19-4.07	3.5	3.9	3.4
ASL	2.3	1.63-3.02	2.8	1.6	2.2
ALDSFX	1.0	0.69-1.28	1.1	1.0	1.1
OX	2.3	1.58-2.93	2.3	2.1	1.2
MTY	1.3	0.91-1.68	1.5	1.3	1.5
CBZ	13.9	9.76-18.12	13.3	17.2	12.9
PIRDES	1.4	1.00-1.86	1.7	1.9	1.6
MTHSFX	4.0	2.78-5.16	2.6	2.8	3.6
3-CF	1.5	1.04-1.94	0.2	0.1	1.7
MTHSFN	1.4	1.00-1.86	1.5	1.3	1.6
CY	1.5	1.06-1.96	1.5	1.4	1.4
ALD	0.9	0.65-1.20	0.8	0.7	1.1
PX	1.1	0.77-1.43	1.2	0.8	1.2
CF	1.3	0.94-1.75	1.4	1.3	1.3
CAR	2.6	1.85-3.43	3.0	3.2	2.8
ETH	5.3	3.72-6.91	5.9	5.7	6.4
TH	3.1	2.15-4.00	2.3	2.5	2.8
ISO	1.5	1.08-2.00	1.3	1.9	1.5
FEN	2.7	1.91-3.55	3.2	3.2	3.0
DETH	1.6	1.09-2.02	1.3	1.3	1.3
MTH	0.9	0.64-1.20	1.0	1.1	0.9
PR	0.9	0.60-1.12	0.8	0.8	0.9
NP	1.9	1.31-2.43	1.8	1.9	1.8
FNX	1.0	0.68-1.26	0.9	0.9	1.0
PY	0.4	0.28-0.51	0.4	0.5	0.4
BTH	8.0	5.57-10.35	8.3	8.1	6.1
FURA	1.6	1.10-2.04	1.5	1.7	1.6
PIR	2.1	1.46 -2.72	2.1	2.1	2.2

3.4. Conclusions

A rapid and sensitive analytical method for the simultaneous determination of 28 CRBs in different high-fat cheese samples (Gorgonzola, Roquefort and Camembert) was developed and validated. The results showed that Z-Sep$^+$ sorbent allowed removing co-extractive interferences such as fats and proteins. Good recoveries (70-115 %) with RSD (%) lower than 13% were obtained. The ME was moderate, showing the suitability of the clean-up procedure for matrices with a high percent of fatty components. The developed method is simple, rapid, low solvent consumption and inexpensive, providing good sensitivity and selectivity and high sample throughput. Thus, these results showed the suitability of this QuEChERS-UHPLC-MS/MS procedure for the monitoring of CRB residues in less explored samples, such as cheese.

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3

CHAPTER 4

Evaluation of hydrophilic interaction liquid chromatography–tandem mass spectrometry and extraction with molecularly imprinted polymers for determination of aminoglycosides in milk and milk-based functional foods

Abstract

An analytical method for the determination of eleven aminoglycosides in different types of milk and milk-based functional products has been optimized and validated. A hydrophilic interaction chromatography (HILIC) column was proposed for the separation of analytes by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). A commercially molecularly imprinted polymer has been used for the solid phase extraction of the analytes, in order to achieve high selectivity in the sample treatment. The proposed method was characterized for different types of milk (whole cow milk, skimmed cow milk, whole goat milk) and functional dairy products, such as follow-on milk, Omega 3-enriched milk and isoflavones-enriched milk. Matrix effect was studied in the different matrices, being lower than |15|% in all cases, showing that the proposed procedure provided very clean extracts. Limits of quantification in the range 4.2-49 μg kg^{-1} were estimated, well below the maximum residue limits established by the European Union. Recoveries ranged from 70-106% with RSD lower than 13%, in compliance with the current legislation. The combination of HILIC to solve the difficulties of the separation of these very polar compounds in reverse phase with the use of MISPE for sample treatment and MS/MS detection provided a very sensitive, highly selective, robust and useful method for identification and quantification of AG residues in different types of milk and milk-based products.

4.1.Introduction

It is well known that aminoglycosides (AGs) are classes of antibiotics widely used in veterinary practice to treat bacterial infections of animals in livestock farming and bovine milk production [1]. However, the misuse of antibiotics in husbandry practices can generate the presence of residues of these compounds in foods of animal origin, such as milk. In general, nephrotoxicity, ototoxicity, and neuromuscular blockade are the main toxic effects in the human being [2]. More specifically, streptomycin (STP) and gentamicin (GNT) are primarily vestibulotoxic, causing dizziness, ataxia, and/or nystagmus [3]. On the other hand, amikacin (AMK), neomycin (NEO), dihydrostreptomycin (DHS) and kanamycin (KNY) are primarily cochleotoxic, causing permanent hearing loss [4]. As a consequence, several organizations have invested resources to address these negative effects of the presence of AGs residues in food through of awareness campaigns [5,6]. Moreover, bearing in mind this matter of concern and in order to protect consumer health, the European Union (EU) has controlled their use in veterinary medicine thought the council regulation 470/2009/EC [7]. In addition, the EU has set maximum residue limits (MRLs) in milk between 100 and 200 μg kg^{-1} for these compounds [8]. Thus, the development of very sensitive methods to determine AGs in foodstuffs of animal origin at trace levels is mandatory.

Several methods have been proposed for the determination of these antibiotics using spectrophotometric, immunochemical, microbiological and chromatographic techniques [1,9]. Among them, liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) has been selected as the better choice to obtain an unambiguous identification and quantification of AGs in a wide range of samples [10,11,12,13]. However, these

compounds have a high polarity due to the presence of some amino and hydroxyl groups in their structure [14]. So, this polarity is a drawback for their analysis by LC, as they are scarcely retained in reverse-phase columns. This poor retention could be overcome by the use of ion-pair reagent (such as trifluoroacetic acid, heptafluorobutyric acid or pentafluoropropionic acid) in the mobile phase [11,13,15]. Nevertheless, these mobile phase additives can affect the performance of MS such as suppression of analytes and contamination of the ion source. Another encouraging recent development has been the use of hydrophilic interaction chromatography (HILIC) for the analysis of these compounds coupled to MS [10,12,14,16,17]. The main advantage of this approach is that polar compounds show good solubility in the aqueous mobile phase used in HILIC, which overcomes the drawbacks of the poor solubility often encountered in reverse-phase chromatography [18]. Other key point is the use of high organic content mobile phase, which offer readily compatibility with MS. Taking into account these characteristics, HILIC methodology could be an attractive alternative to the widely used reverse-phase chromatographic separations for the analysis of AGs and other antibiotics [19].

Another critical challenge in the monitoring of trace-level AGs in highly complicated matrices, such as milk, is the extraction and clean-up procedure. Different strategies, such as liquid extraction [11], solid phase extraction (SPE) [10,13,17,20,21,22], *online* SPE [23,24], disposable pipette extraction [25] and matrix solid phase dispersion [26] have been employed to AG extraction and clean-up. Generally, in this purification step particular attention is paid on the recovery efficiency. However, another relevant issue, when ESI-MS is used, is the matrix effect (ME). The presence of co-eluting interfering species may

cause signal enhancement or suppression of the analytes of interest [27,28,29]. These MEs hamper the accuracy of the results, reducing laboratory throughput [30]. To overcome, minimize or compensate ME during quantitative analytical LC–MS measurements several strategies could be carried out [31], such as the application of specific clean-up protocols. In this sense, molecularly imprinted polymers (MIPs) used as sorbent in solid phase extraction (MISPE) can provide cleaner extracts because the strong and selective interaction between MIPs and target molecules, being of special interest for complex matrices. MIPs are synthetic materials with artificially produced recognition sites capable of specifically catch target molecules [32]. So, several matrix components are removed from the final extract, reducing the ME. This methodology has been successfully applied for the determination of AGs in honey samples, achieving excellent results in terms of recovery and ME [33,34].

The aim of this study was to develop a fast multi-residue method for routine analysis of 11 AG residues in different types of milk (whole cow pasteurized milk, skimmed cow pasteurized milk, whole goat pasteurized milk) and milk-based functional foods, such as follow-on-milk (for children 6 to 12 months), omega 3-enriched milk and isoflavones-enriched milk, using a recently commercially available MISPE. To the best of our knowledge, this is the first report about the use of MIPs combined with HILIC–based UHPLC-MS/MS, demonstrating the potential of both methodologies for the determination of these antibiotics in milk and milk-based products.

4.2.Materials and methods

4.2.1. Reagents and materials

Due to the high absorption affinity of the AGs to polar surfaces and their high photosensitivity, polypropylene amber vessels (flasks, glass and vials) were used during sample preparation, storage, and injection.

Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (MeCN) and n-hexane (LC-MS HiPerSolv grade) were supplied by VWR (Radnor, PA, USA). Formic acid (LC-MS grade, 99%) and heptafluorobutyric acid (HFBA, > 99.5%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium hydroxide (30%) and dichloromethane (stabilized with 20 ppm of amylene) were obtained from Panreac-Química (Barcelona, Spain). Potassium dihydrogen phosphate (99%) was purchased from Alfa Aesar (Haverhill, USA). Ammonium acetate (98%) and trichloroacetic acid (TCA) (99.5 %) were supplied by Merck (Darmstadt, Germany). Vetranal grade analytical standards of Gentamicin sulfate (GENT, 63.9%), that was a mixture of GENT C1, GENT C1a and GENT C2C2a, Apramycin sulfate salt (APM, 95%), Paromomycin sulfate salt (PRM, 74%), Dihydrostreptomycin sesquisulfate (DHS, 98%), Spectinomycin dihydrochloride pentahydrate (SPC, 60.3%), Kanamycin acid salt (KAM, 68.9%), Amikacin sulfate salt (AM, 74%), Tobramycin sulfate salt (TOM, >99.9%) and Streptomycin sulfate salt (STP, >99.9%) were supplied by Fluka Analytical (Steinheim, Germany). Individual stock standard solutions of 3 g L^{-1} were prepared by dissolving accurately weighed amounts in water and stored in the dark at 4 ˚C. They were stable for at least 2 months. Standard solutions containing all the

AGs were freshly prepared by proper dilution of the stock standard solutions with MeCN:H_2O (25/75; v/v). These solutions were stored in plastic tubes at 2-4 °C and remained stable for up to 1 week.

MISPE cartridges (SupelMIP AGs SPE Column, 50 mg, 3 mL) supplied by Supelco (Bellefonte, PA, USA) were used for extraction and clean-up process. Nylon syringe filters, 0.22 mm x 13 mm (Agela Technologies, New York, USA) were used for filtration of the sample extracts before injection into the UHPLC-MS/MS system.

4.2.2. Instrumentation

Separation was performed on an Agilent 1290 Infinity LC using a Kinetex HILIC column (100 × 2.1 mm, 1.7 µm) supplied by Phenomenex (Torrance, CA, USA). The mass-spectrometer measurements were performed on a triple quadrupole (QqQ) mass spectrometer API 3200 (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI). The instrumental data were collected using the Analysts Software version 1.5 with Schedule MRMTM Algorithm (AB Sciex).

MISPE was carried out on a Visiprep^{TM} DL vacuum manifold (Supelco) for 12 cartridges. A centrifuge (Universal 320 model from Hettich, Leipzig, Germany), a vortex (Genie 2 model from Scientific Industries, Bohemia, NY, USA) and a pH-meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ±0.01 pH unit were used also during the sample preparation procedure.

4.2.3 UHPLC–MS/MS analysis

Separation was performed in a HILIC column using a mobile phase consisting of 150 mM ammonium acetate containing 1% formic acid (solvent A), and MeCN (solvent B) at a flow rate of 0.5 mL min^{-1}. The eluent gradient profile was as follow: 80% B at the beginning; 30% B at 2 min (held for 2 min); 5% B at 5 min (held for 5 min) and finally go back to the initial conditions at 12 min (held for 6

min). The temperature of the column was 35 °C and the injection volume was 20 µL.

The mass-spectrometer was working with ESI in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table 4.1. The ionization source parameters were: dry gas temperature, 700 °C; curtain gas

Table 4.1. Optimized MS/MS parameters.

	Precursor ion	Rt	DP	EP	CEP	Product ion*	CE	CXP
AM	586.2	1.7	66	4.5	24	163.0 (Q)	45	6
						424.9 (I)	27	8
APM	540.2	2.1	66	6.0	22	217.0 (Q)	35	6
						377.9 (I)	25	6
DHS	584.2	1.6	76	9.0	20	263.0 (Q)	39	8
						246.0 (I)	49	4
GENT C2C2a	464.3	2.5	51	4.5	16	322.0 (Q)	21	6
						160.0 (I)	29	4
GENT C1	478.3	2.6	66	5.0	20	322.0 (Q)	21	6
						157.0 (I)	29	4
GENT C1a	450.3	2.5	41	4.5	18	322.2(Q)	21	6
						160 (I)	29	4
KAM	485.2	1.9	41	4.5	16	163.1 (Q)	35	6
						323.9 (I)	23	6
PRM	616.3	2.0	66	8.5	20	163.1 (Q)	47	6
						293.0 (I)	33	6
SPC	351.2	1.3	41	4.5	14	333.0 (Q)	23	6
						98.0 (I)	45	4
STP	600.2	1.5	121	10.0	22	263.0 (Q)	41	8
						246.0 (I)	53	6
TOM	468.5	2.0	41	6.5	16	163.1 (Q)	33	4
						324.1 (I)	21	6

*(Q) Transition used for quantification, (I) Transition employed to complete the identification. Rt: Retention Time. DP: Declustering Potential. EP: Entrance Potential. CEP: Collision Cell Entrance potential. CE: Collision Energy. CXP: Collision Exit Potential.

(nitrogen), 30 psi; ion spray voltage, 4000 V; collision gas, 5 and dry gas pressure (GS 1 and GS 2, both of them N_2) 50 psi.

4.2.4 Sample treatment procedure

A portion of 2 g of milk (obtained from a local store), free of AGs was spiked at different concentration levels using the working standard solutions of AGs. After spiking and homogenizing in vortex, 250 µL of TCA (15%, w/v) were added for protein precipitation. Then the mixture was homogenized by vortex and centrifuged at 9000 rpm for 5 min. The aqueous phase was collected and transferred to a falcon tube. Subsequently, 1 mL of n-hexane was added to remove the fatty components of the sample. The mixture was shaken during 1 min and centrifuged at 9000 rpm for 5 min and the upper layer, containing fatty components, was withdrawn. The aqueous phase was diluted with 3.5 mL of 50 mM potassium phosphate buffer at pH 7.0, and shaken manually for 10 s. The pH of the final solution was checked to be 7.0 and adjusted with ammonium hydroxide, if necessary. Then, the final volume was adjusted to 5 mL with 50 mM potassium phosphate buffer pH 7.0. A 3 mL aliquot of this solution was loaded onto a SupelMIP AG SPE column (previously conditioned with 1 mL of MeOH and 1 mL of 50 mM potassium phosphate buffer at pH 7.0) at a flow rate of approximately 0.2 mL min^{-1}. After sample loading, the cartridge was washed with 3 mL of water at a flow rate lower than 0.5 mL min^{-1}. Subsequently, strong vacuum was applied for 5 min. Then, the MISPE cartridge was washed again with 1 mL of a mixture of dichloromethane: MeOH (50:50, v/v). After this washing step, a slight vacuum was applied for 10 s. Finally, the elution of the analytes was achieved using 1 mL of 1% formic acid in MeCN:H_2O (20:80, v/v) with 5 mM HFBA. Finally, 2 mL of MeCN:H_2O (20:80,

v/v) were added to this final extract in order to make it compatible with the HILIC conditions. This extract was filtered and injected in the UHPLC-MS/MS system. The MISPE procedure is summarized in Fig.4.1.

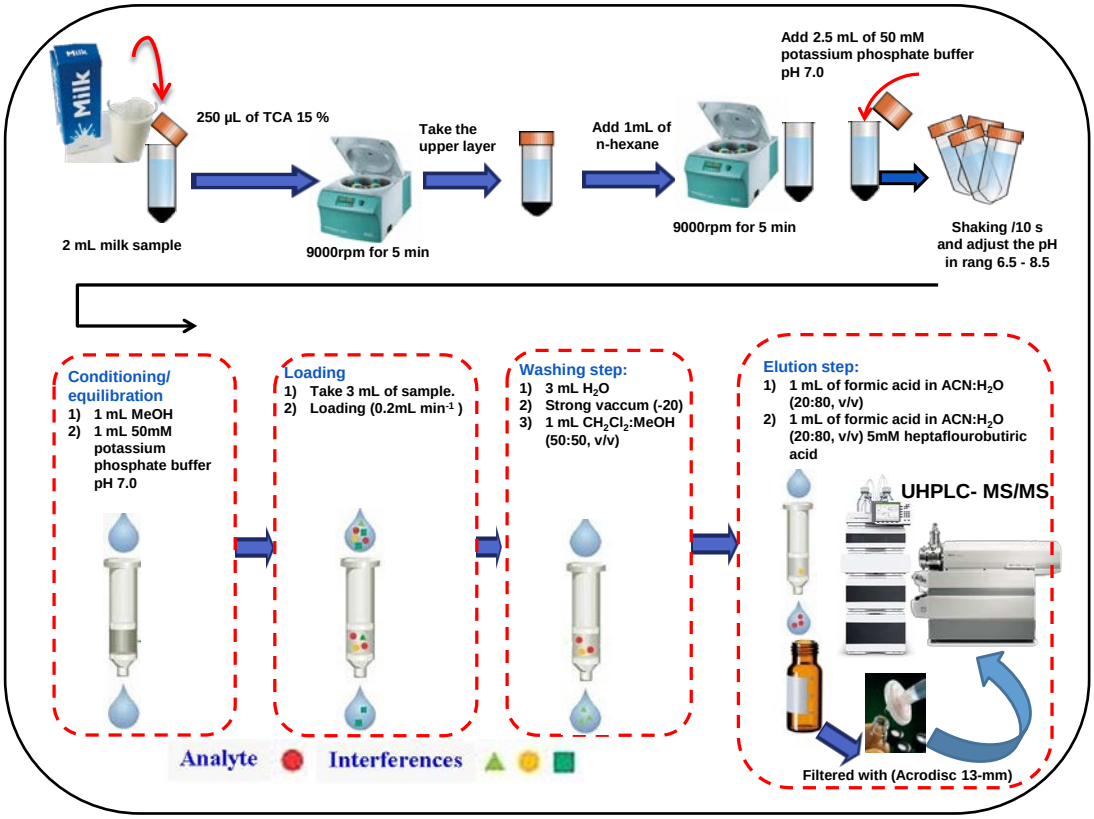

Figure.4.1. Diagram of MISPE for the determination of AGs in milk samples.

4.3.Results and discussion

3.1. Optimization of chromatographic separation and MS/MS detection

For the analysis of the target antibiotics by UHPLC-MS/MS, an MRM method was developed. Individual optimization for each AG (0.5 mg L^{-1} in 0.1% aqueous formic acid solution: MeCN (50:50, v/v)) was conducted using an

external syringe pump connected to the mass spectrometer. During analyte infusions, Declustering Potential (DP), Entrance Potential (EP), Collision Cell Entrance Potential (CEP), Collision Gas (CAD), Collision Energy (CE) and Collision Exit Potential (CXP) of the two most abundant transitions were also optimized. The detailed optimized parameters and MRM transitions are shown in Table 1. Protonated molecular ions $[M+H]^+$ were found for most of the studied compounds in ESI positive mode and were selected as precursor ions [35]. Only in the case of SPC and STP, the highest peak corresponded to the water adduct $[M+H+H_2O]^+$, probably due to the unusual structural feature of these compounds, in which the carbonyl group is hydrated in an aqueous solution [36]. Two product ion transitions were set up (Table 4.1), the most intense one was used as quantification ion (Q) and the following was considered as confirmation ion (I). The MS/MS experiments were performed in scheduled MRM mode, with a target scan time for each MRM transition of 0.2 s, which provided 15 data points per peak.

As stated in the introduction, the use of an HILIC column is mandatory in order to increase chromatographic retention of polar antibiotics and to achieve higher MS sensitivity. Kinetex HILIC column based on an un-bonded silica phase was selected, as it can provide satisfactory results in terms of relative retention factor, selectivity and peak shape, according to Kumar *et al* [20]. First of all, the mobile phase composition was evaluated. This step plays an important role in LC–ESI-MS/MS because it influences in the ionization efficiency and the separation quality [36]. Buffer salts such as ammonium acetate and ammonium formate are commonly used to improve peak shape and the ionization of the compounds. Thus, these buffer salts were evaluated using a concentration of 100 mM in solvent A. No strong differences were observed between both

buffers in terms of peak shape. However, the sensitivity was slightly better when ammonium acetate was used, in agreement with previous studies [37,38]. Thus, the concentration of ammonium acetate was checked from 50 to 200 mM and the peak shapes improved up to 150 mM. Above this value, a higher ionic strength had a modest effect in the peak quality, so 150 mM of ammonium acetate (solvent A) was selected. The effect of the formic acid concentration was also studied using different percentages of formic acid (0-2%, v/v) in solvent A. A 1.5% formic acid concentration (v/v) provided sharp and symmetrical peaks due to minimized silanol interactions. So, the final composition of the mobile phase was 150 mM ammonium acetate containing 1% formic acid (v/v) (solvent A) and MeCN (solvent B). The gradient was optimized to get the best separation and peak shape in the shortest time. In order to delay the elution of the most polar AGs, it was necessary to start using 20% of solvent A. The rest of gradient program was as follow: 70% A at 2 min (held for 2 min); 95% A at 5 min (held for 5 min) and finally go back to the initial conditions at 12 min (held for 6 min). The flow rate was studied from 300 to 600 µL min^{-1} and finally 500 µL min^{-1} was selected as a compromise between signal, peak shape and run time. The column temperature was studied between 25 °C and 55 °C, selecting 35 °C as optimum.

Sample solvent nature was investigated and optimized in terms of sensitivity and peak shape. The sample solvents tested were MeCN; MeCN:H_2O (80/20); MeCN:H_2O (60/40); MeCN:H_2O (40/60); MeCN:H_2O (20/80) and H_2O. It was observed that the higher the percentage of MeCN, the lower the sensitivity. This fact may be due to the poor solubility of AGs in this organic solvent. However, the use of 20% MeCN allowed an improvement in the peak shape for most of the AGs. Injection volume was evaluated from 5 to 20 µL (full loop).

The maximum injection volume was used, obtaining the best sensitivity without losing peak efficiency.

Finally, to obtain the maximum response the ionization source parameters were evaluated. Curtain gas (nitrogen) was tested between 20 and 35 psi and finally 30 psi was selected as optimum. Turbo V ion source temperature was evaluated between 300 and 750 ºC, achieving a satisfactory solvent evaporation at 700 ºC. The response of the ion spray voltage was checked from 5000 to 5500 V. However, the response was not improved when this voltage was increased and finally 5000 V was selected as optimum. Nitrogen nebulizer gas (Gas 1) and nitrogen heater gas (Gas 2) pressure were optimized at the same time, obtaining the best signals when both parameters were set to 50 psi.

4.3.2 Optimization of MISPE

Milk and milk based products are complicated samples that present a high content in polyunsaturated fatty acids, minerals, vitamins, proteins and salts. So, in order to remove the main matrix components, the sample treatment is mandatory. The use of MISPE simplifies the extraction of AGs from these complicated matrices, providing a higher selectivity and reducing sample manipulation. Initially, the protocol proposed by Supelco for the SupelMIP AGs SPE Columns for the determination of Neomycin, GENT C1, DHS, STP, Geneticin (G418-2), AM, TOM, KAM, APM, Hygromycin, Puromycin and SPC in honey samples was followed with some modifications [39], using 2 mL of whole cow milk as representative sample. First of all, the use of TCA for precipitation of proteins and inhibition of protein binding of the analytes was checked [40,41]. Thus, 1 mL of different concentrations of TCA solutions (15%, 25% and 50%, w/v) was added to the sample, and 15% of this agent showed to be enough to precipitate all proteins. Then, a study of the TCA volume was carried out (from

100 to 1000 µL). Volumes lower than 250 µL were insufficient to obtain a complete precipitation. On the other hand, the precipitation efficiency was similar from 250 µL, so 250 µL of 15% TCA was selected as optimum value. The original protocol proposed by Supelco included four consecutive washing steps: (1) 3 mL of water; (2) 1 mL of 0.1%, ammonium hydroxide (v/v); (3) 1 mL of a mixture of MeCN:H_2O (40/60, v/v); and (4) 1 mL of a mixture of dichloromethane:MeOH (50/50, v/v). However, in the case of milk samples satisfactory recoveries (average recovery, 84 %) and very low MEs (average, |5|%) were obtained using only steps (1) and (4), achieving an important simplification of the sample treatment. Finally, the concentration of HFBA in the elution step was studied between 0 and 10 mM. A concentration of 5 mM was enough to obtain satisfactory extraction efficiency for all AGs. The final MISPE procedure is described in detail in section 2.4.

4.3.3 Method characterization

The method was characterized in a wide range of milk samples and milk-based functional foods. Parameters such as linear dynamic range, limits of detection (LODs) and limits of quantification (LOQs), ME, precision (both inter- and intraday precision) and trueness were taking into account.

4.3.3.1 Calibration curves, LODs and LOQs

Procedural standard calibration curves were established at five different concentration levels (10, 25, 50, 100 and 150 µg kg^{-1} for SPC and DHS; and 50, 100, 150, 200 and 250 µg kg^{-1} for the rest) by spiking blank whole milk cow samples (as representative matrix) before the extraction process. Each level was prepared following the proposed MISPE procedure and injected in triplicate. Two product ions were selected, the most intense one was used as

quantification ion (Q) and the following was considered as confirmation ion (I). These transitions together with the retention times were employed to ensure adequate analytes identification. Performance characteristics of the method are shown in Table 4.2. LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. As can be seen, LOQs lower than the MRLs were obtained for all AGs. Therefore, the proposed method is adequate for the determination of very low levels of these residues in the selected matrix.

Table 4.2. Statistics and performance characteristics of the MISPE-UHPLC-MS/MS method for the analysis of AGs in whole cow milk.

	Linear dynamic range (µg kg^{-1})	Slope (SD)	Intercept (SD)	R^2 (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	MRL[a] (µg kg^{-1})
AM	31.3 – 250	8202 (126)	-9116 (21005)	0.995	9.4	31.3	NA
APM	38.5-250	1955 (29)	19166 (4948)	0.990	11.5	38.5	NA
DHS	7.7 -150	96120 (164)	-121633 (2738)	0.988	2.3	7.7	200
GENT C2C2a	45.5 -250	2039 (32)	8766 (5330)	0.996	13.6	45.5	100
GENT C1	30.9 -250	2567 (39)	- 16666 (6535)	0.998	9.3	30.9	100
GENT C1a	49.0 -250	5544 (96)	56900 (15948)	0.989	14.7	49.0	100
KAM	45.5 -250	9208 (113)	- 51566 (18761)	0.993	13.6	45.5	150
PRM	47.6 -250	3390 (27)	- 3966 (4544)	0.994	14.3	47.6	NA
SPC	4.2 -150	137772 (3217)	-438164 (301408)	0.993	1.3	4.2	200
STP	45.9 -250	1588 (18)	- 2606 (3051)	0.993	13.8	45.9	150
TOM	45.5 -250	2159 (16)	3833 (2681)	0.990	13.6	45.5	NA

[a] MRL: Maximum residue limit in milk [8]; NA: Non-authorized in milk

4.3.3.2 Precision study

Both repeatability (interday precision) and intermediate precision (intraday precision) were tested by application of the proposed MISPE-UHPLC-MS/MS method in whole cow milk samples spiked at two different concentration levels of AGs: 10 and 25 μg kg^{-1} for DHS and SPC; and 50 and 100 μg kg^{-1} for the rest. To check the repeatability, three samples were prepared and injected in triplicate on the same day, under the same conditions. Similar procedure was carried out in the evaluation of intermediate precision. Thus, during three consecutive days, one sample per day was prepared and injected in triplicate. The results, expressed as %RSD of peak areas, are shown in Table 4.3. Good precision (RSD lower than 12%) was obtained in all cases. So, it could be concluded that the obtained results are in agreement with the current demand [42].

Table 4.3. Precision expressed as %RSD of peak areas for spiked whole cow milk samples (three samples, injected in triplicate, n=9)

	AM	APM	DHS	GENT C2C2A	GENT C1	GENT C1a	KAM	PRM	SPC	STP	TOM
Repeatability											
Level 1[a]	9	4	2	5	9	4	4	5	5	7	6
Level 2[b]	6	9	6	5	3	3	4	7	5	5	7
Intermediate precision											
Level 1[a]	11	7	9	8	10	12	7	10	9	10	11
Level 2[b]	8	9	10	6	8	10	7	10	5	9	9

[a] **Level 1 :** 10 μg kg^{-1} for DHS and SPC, 50 μg kg^{-1} for the rest.

[b] **Level 2:** 25 μg kg^{-1} for DHS and SPC, 100 μg kg^{-1} for the rest.

4.3.3.3 Matrix effect

ME was estimated for each AGs in the different milk samples and milk-based functional foods (whole cow milk, skimmed cow milk, whole goat milk, follow-on milk, Omega 3-enriched milk and isoflavones-enriched milk). To evaluate this factor, the slope of matrix-matched calibration curves and the slope of external standard calibration curves were compared according to the following equation: [(calibration curve slope in matrix/ calibration curve slope in solvent)-1] x 100 [43]. The obtained data are shown in Table 4.4. ME was always lower than |15|% which involves that the proposed sample treatment is enabled to remove co-extractants in all the studied matrices. Thus, MEs were negligible in all studied cases, so that the use of matrix-matched calibration would not be mandatory.

Table 4. ME% for all samples studied.

	Whole cow milk	Skimmed cow milk	Whole goat milk	Follow-on milk	Isoflavones-enriched milk	Omega 3-enriched milk
AM	-6	4	-8	-7	-9	-8
APM	1	-5	-4	-5	-11	-6
DHS	-9	-8	-7	-9	-8	-10
GENT C2C2a	-7	-1	-6	-7	-8	-4
GENT C1	-8	-8	-7	-8	-6	-6
GENT C1a	-1	-2	-3	-5	-8	-5
KAM	-4	7	-5	-6	-10	-4
PRM	-1	-1	-2	-3	-4	-5
SPC	1	1	2	2	-1	3
STP	-3	-3	-5	-6	-7	-8
TOM	-12	-10	-8	-4	-15	-14

4.3.3.4 Trueness assessment

The trueness of the proposed method was assessed by recovery studies in the different types of milk samples spiked at two different concentration levels of each AG: 10 and 25 μg kg^{-1} for DHS and SPC; and 50 and 100 μg kg^{-1} for the rest. The absolute recoveries have been calculated by comparing the concentration of AGs in milk samples spiked before the MISPE procedure with the concentration in extracts of milk samples spiked after the MISPE procedure. Each sample was analysed in triplicate and injected three times. Blank samples were previously analysed to check the presence of AGs; none of them gave a result above the LOQs of the method. The recoveries were between 70% and 106% for all analytes except for TOM (45 to 65%) in all samples tested and also for APM, GENT C1a, KAM, PRM and TOM in Omega 3-enriched milk (recoveries lower than 70%). Regarding precision, satisfactory RSD% were obtained for all analytes in all samples (see Table 4.5), fulfilling current legislation [42].

A typical extracted ion chromatogram corresponding to a whole cow milk sample spiked with 25 μg kg^{-1} for DHS and SPC and 100 μg kg^{-1} for the rest and analysed by the proposed MISPE-UHPLC–MS/MS method is shown in Fig.4.2.

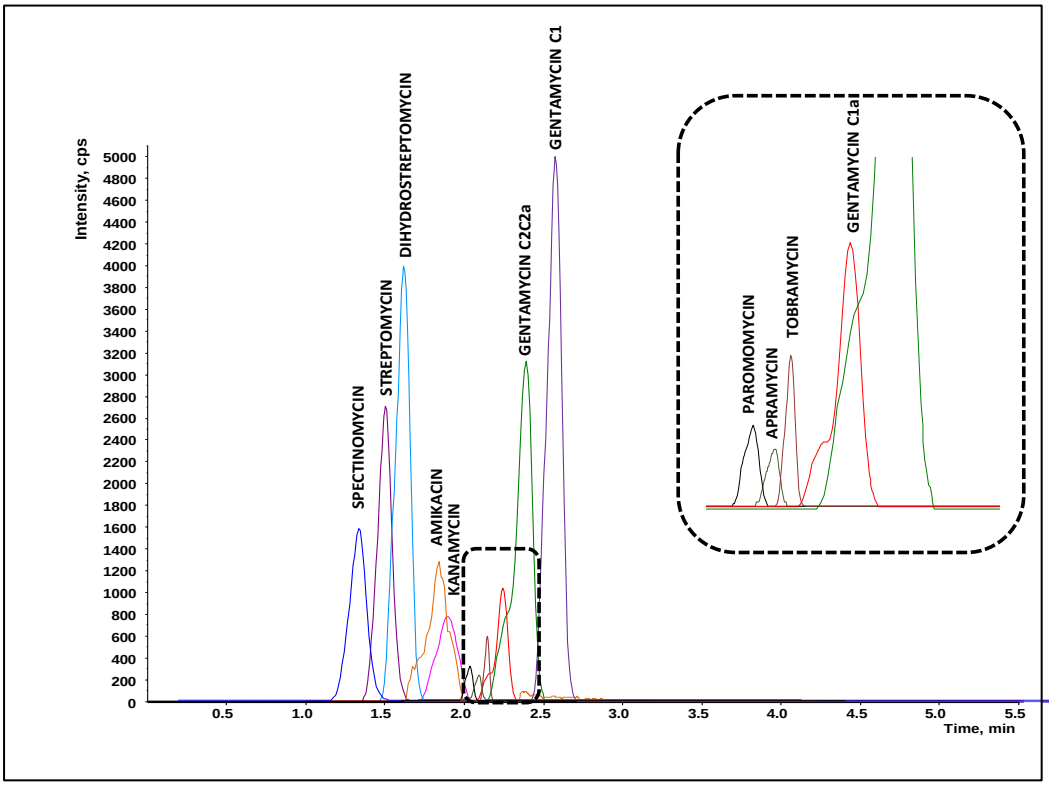

Figure 4.2. Extracted ion chromatogram of a spiked whole cow milk sample applying the proposed method (25 µg kg^{-1} for DHS and SPC, 100 µg kg^{-1} for the rest).

Table 4. 5. Recoveries % and (RSD %) for different spiked samples (three samples, injected in triplicate, n=9).

	AM	APM	DHS	GENT C2C2a	GENT C1	GENT C1a	KAM	PRM	SPC	STP	TOM
Whole cow milk											
Level 1[a]	83(9)	75(4)	74(2)	87(5)	103(9)	76(4)	82(4)	81(5)	78(5)	85(7)	63(6)
Level 2[b]	92(6)	76(9)	96(6)	105(5)	85(3)	101(3)	76(4)	76(7)	75(5)	89(5)	53(7)
Skimmed cow milk											
Level 1[a]	74(6)	77(4)	86(7)	80(3)	96(10)	82(10)	78(5)	74(8)	85(9)	77(7)	54(13)
Level 2[b]	75(5)	73(7)	74(9)	94(6)	96(9)	74(10)	81(9)	73(9)	81(5)	81(7)	49(11)
Whole goat milk											
Level 1[a]	84(10)	79(9)	75(6)	100(9)	103(9)	91(10)	80(8)	82(10)	89(10)	76(6)	57(8)
Level 2[b]	73(5)	73(9)	81(9)	98(9)	91(8)	74(9)	79(10)	76(10)	80(6)	75(10)	45(9)
Follow-on milk											
Level 1[a]	75(9)	74(10)	73(9)	78(7)	86(8)	76(10)	80(9)	79(6)	77(6)	79(7)	59(12)
Level 2[b]	72(4)	74(10)	75(7)	73(9)	80(10)	72(8)	76(9)	73(10)	77(6)	77(10)	56(8)
Isoflavones-enriched milk											
Level 1[a]	83(10)	85(9)	70(9)	94(10)	103(8)	106(10)	84(6)	82(7)	89(10)	78(10)	65(11)
Level 2[b]	80(10)	74(8)	70(6)	92(6)	104(7)	84(10)	80(3)	81(5)	95(8)	81(8)	57(8)
Omega 3-enriched milk											
Level 1[a]	70(10)	53(8)	73(10)	74(8)	82(10)	55(11)	49(10)	28(10)	82(9)	74(8)	37(12)
Level 2[b]	74(7)	51(8)	70(7)	72(6)	80(9)	50(9)	43(8)	29(7)	82(7)	71(9)	33(11)

[a] **Level 1:** 10 μg kg^{-1} for DHS and SPC, 50 μg kg^{-1} for the rest.
[b] **Level 2:** 25 μg kg^{-1} for DHS and SPC, 100 μg kg^{-1} for the rest.

4.3.3.5 Comparison with other methods

A comparative overview of the main analytical performance characteristics of the proposed method with other published methods for the determination of AGs in milk samples are shown in Table 4.6. The results in terms of LOQs, recoveries and number of AGs simultaneously studied were similar or even better than those obtained by the other methods. Moreover, the amount of required sample in the proposed MISPE procedure (2 g) was usually lower. However, the most relevant issue was the lower MEs: as could be observed, the proposed method provided significantly lower ME results than the other methods. In fact, some of these methods needed two consecutive SPE to obtain satisfactory MEs. So, it can be concluded that MISPE provides greater cleanup than traditional SPE methods.

Table 4.6. Comparison of the proposed method with other reported methods for the determination of AGs in milk samples.

Method	Sample treatment	Number of AGs	LOQ (μg kg^{-1})	Recovery (%)	Amount of sample	ME (%)	Ref.
LC-MS/MS	SPE (C18)	10	25-125	87-95	1.0 mL	-	[11]
LC-MS/MS	Consecutive SPE (C18-Weak cation exchange)	4	7.7-19.4	73.9-86.2	2.0 g	(-19.2) to (34.9)	[12]
Ion pair chromatography-MS/MS	Consecutive SPE (HLB-HLB)	13	-	64-108	5.0 mL	-	[13]
HILIC-MS/MS	SPE (Weak cation exchange)	14	2-152	91-114	6.0 g	(-81) to (-16)	[22]
HILIC -MS/MS	MISPE	11	4.2-49	70-106	2.0 g	(-15) to (1)	This work

4.4.Conclusions

In the present study, the use of a HILIC column in UHPLC-MS/MS combined with MISPE has been presented as a reliable, selective and highly sensitive methodology for the simultaneous quantification and confirmation of 11 aminoglycosides in different types of milk (whole cow milk, skimmed cow milk, whole goat milk) and milk-based functional foods (follow-on milk, Omega 3-enriched milk and isoflavones-enriched milk). The results showed that MISPE is a robust tool for extraction of AGs and sample clean-up, achieving ME lower than 15% in all cases. Calibration curves were established in the presence of matrix and the low LOQs obtained allowed determining the 11 AGs at concentrations lower than the limits established by current legislation for AGs in milk, with satisfactory precisions. In addition, trueness has been successfully evaluated, achieving good recoveries for all AGs, except for TOM in Omega 3-enriched milk. The developed method is rapid, low solvent consumption and inexpensive providing good sensitivity. Thus, these results showed the suitability of this MISPE-UHPLC-MS/MS procedure for the monitoring of AGs residues in milk and milk-based products.

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CHAPTER 5

Evaluation of multiresidue capillary electrophoresis- quadrupole-time-of-flight mass spectrometry method for the determination of antibiotics in milk samples

5

Abstract

A selective and rapid method has been developed to determine 15 antibiotic residues (eight tetracyclines and seven quinolones) in milk samples by capillary zone electrophoresis coupled with quadrupole time-of-flight mass spectrometry (CZE-Q-TOF-MS). The use of this hybrid mass spectrometer allowed obtaining full scan and full MS/MS spectra for quantification/confirmation purposes in a single run. In addition, solid phase extraction (SPE) using the new Oasis PRiME HLB cartridge was proposed for the extraction, achieving excellent results in terms of sample throughput. The proposed method was validated using whole cow milk as representative matrix. Good linearity was obtained ($R^2 > 0.99$) for all the studied compounds. The precision, expressed as relative standard deviation (%, RSD), at two concentration levels (50 and 100 μg kg^{-1}) was below 13%. Recoveries obtained from goat milk, whole cow milk and semi-skimmed cow milk, at two concentration levels, ranged from 76 to 106%, while limits of quantification ranged from 1.5 to 9.6 μg kg^{-1}, being lower than the established maximum residue limits in the European legislation. Matrix effect was negligible in all cases, showing that with this new SPE sorbent cleanest extracts were obtained with a minimum number of steps in the sample treatment. Thus, the proposed SPE-CZE-Q-TOF-MS method is suitable for multiclass multiresidue monitoring in different types of milk samples.

5.1.Introduction

Milk and related products play an unquestionable role in a healthy and balanced diet [1]. Several benefits of dairy products are related with nutrition, strengthening of the immune system and prevention of some illnesses such as hypertension, respiratory problems, osteoporosis and even some forms of cancer [2]. It comes as no surprise that the world production of milk reached 816 million tons/y in 2016 [3]. In spite of its benefits, some residues of veterinary drugs can be found in milk and dairy products, mainly antibiotics, being necessary their monitoring. Extensive use of antibiotics in veterinary medicine and medicated feed is a common practice in intensive production of animals intended for human consumption, leading to a significant increase in antibiotic resistance and allergic reactions, having therefore important consequences for public health [4]. To minimize these risks, the European Union (EU) has set maximum residue limits (MRLs) for some antibiotics in foodstuffs of animal origin, including milk, by means of Commission Regulation No 37/2010 [5]. These limits range between 30 and 100 μg kg^{-1}. Bearing in mind these MRLs, to detect low levels of these compounds in milk samples sensitive and selective analytical methods are highly advisable.

Among the different families of antibiotics, quinolones (QNs) and tetracyclines (TCs) are widely used in veterinary medicine in prophylaxis or therapy of bacterial infections. The determination of these veterinary drugs in milk and related products have been usually carried out by liquid chromatography (LC) coupled with UV/Vis [6,7] or fluorescence detection [8,9,10]. However, these methods tend to lack selectivity. To overcome this problem, the use of tandem mass spectrometry detection (MS/MS) offers the selectivity and sensitivity

required by the EU regulation [11]. Thus, LC-MS/MS is often the selected option, especially for multiresidue analysis purposes [12,13,14,15,16]. In this scenario, capillary electrophoresis (CE) coupled with MS has been proposed as an interesting alternative to LC for the analysis of veterinary drugs in food samples due to its advantages such as high separation efficiency, short analysis time and low reagent consumption [17,18,19,20,21]. Whatever the separation technique, methods based on MS/MS usually work in the multiple reaction monitoring (MRM) mode, which provides excellent quantitative performance using a thorough optimized acquisition method. Nevertheless, one of the main drawbacks of this approach is the previous optimization of the MS/MS transition conditions and precise knowledge of the retention time window of each studied analyte. Another problem is the possibility of false positives due to the presence of ionized isobaric compounds that could be fragmented in the collision cell together with the studied compounds [22,23]. This fact, together with the low resolution power (RP) of the most used triple quadrupole mass spectrometers, increase the number of false positives. Thereof, these spectrometers could not distinguish between a target compound and an interfering compound with a decimal difference. These problems can be overcome by the use of high-resolution mass spectrometers (HRMS), which allows improving selectivity with a higher RP, under full scan conditions [24]. Quadrupole Time-of-Flight (Q-TOF) mass spectrometry is a hybrid technique that combines the advantages of the TOF accurate mass and the MS/MS technique. Thus, the unequivocal confirmation according to the most stringent EU criteria is possible [11]. The main advantage of Q-TOF MS is the availability of full MS/MS spectra after a single injection for identification and confirmation purposes. In addition, the data obtained from a Q-TOF can be analysed

retrospectively when new substances of interest emerge [25]. Nevertheless, the coupling CE-Q-TOF has been restricted so far to bioanalytical applications [26] and it has not been used for the monitoring of small molecules such as antibiotics or pesticides.

Milk is composed of a mixture of carbohydrates, proteins, fat components, phospholipids, vitamins, minerals and enzymes, so the sample treatment is a previous critical step for the determination of antibiotic residues [27]. In order to isolate the analytes from the matrix, different sample treatments or clean-up procedures have been proposed including liquid-liquid extraction [15,16], QuEChERS [8,28,29], dispersive liquid-liquid microextraction [30] or solid phase extraction (SPE) using HLB cartridges [10,15,31,32]. Recently, Waters Corporation has released a new HLB sorbent called PRiME (process, robustness, improvements, matrix effects, ease of use), with specific adsorption for lipids carrying fatty acid chains, among other features [33]. In addition, this sorbent allows avoiding the tedious steps such as conditioning, equilibrating and washing the cartridge. Thus, the sample throughput is higher than when using the conventional HLB technology [34,35].

With this background, the main objective of this work was to develop a new analytical method based on CZE-Q-TOF-MS/MS as an alternative to LC-MS/MS for the identification and simultaneous quantification of fifteen antibiotics (7 QNs and 8 TCs) in milk samples. To the best of our knowledge, this is the first attempt that this technique in combination to Oasis HLB PRiME has been proposed for the determination of antibiotic residues in milk samples at very low concentrations levels.

5.2.Expremental

5.2.1. Chemicals and reagents

Methanol (MeOH), 2-propanol (IPA) and acetonitrile (MeCN) HPLC grade, sodium acetate (reagent grade) and formic acid (FA) were supplied by Sigma-Aldrich (Madrid, Spain). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was employed throughout the study to obtain the LC-grade water used during the analyses. Ammonium hydroxide (30%) was obtained from Panreac-Química (Madrid, Spain). Vetranal grade analytical standards of QNs (oxolinic acid, flumequine, ciprofloxacin, danofloxacin, enrofloxacin, marbofloxacin, sarafloxacin and difloxacin) and TCs (methacycline, doxycycline, tetracycline, 4-epitetracycline, minocycline, demeclocycline, and chlortetracycline) were purchased from Sigma-Aldrich and Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions (500 mg L^{-1}) of each antibiotic were prepared by dissolving the appropriate amount of each analyte in H_2O/MeCN (80/20, v/v) (difloxacin, marbofloxacin, danofloxacin, enrofloxacin and sarafloxacin) or 100% MeCN (oxolinic acid, flumequine, methacycline, doxycycline, tetracycline, 4-epitetracycline, minocycline, demeclocycline and chlortetracycline) and were stored in the dark at 4 °C. A mixture of 0.1 mg L^{-1} of each antibiotic was prepared in ammonium hydroxide (1 M) and stored at 4 °C. The working solutions were prepared by proper dilution with ammonium hydroxide (1 M).

Ammonium carbonate, ammonium acetate and sodium hydroxide were provided by VWR (West Chester, PA, USA). To obtain EDTA-McIlvaine buffer solution, ethylendiaminetetraacetic acid sodium salt (Na_2EDTA), citric acid and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) were purchased from Merck. This buffer was prepared as described below: 18.6 g of Na_2EDTA was dissolved

in a mixture of 125 mL of 0.1 M citric acid solution and 70 mL of 0.2 M $Na_2HPO_4 \cdot 2H_2O$; the solution was diluted to 500 mL with water and pH was tested to be 4.

The reference compound solutions for internal Q-TOF recalibration (i.e. 5 mM of purine, $C_5H_4N_4$, in 90:10 MeCN/water, v/v, and 2.5 mM HP-0921, hexakis(1H,1H,3H-tetrafluoropropoxy) phosphazine, a polyfluorinated compound, $C_{18}H_{18}O_6N_3P_3F_{24}$, in MeCN) were obtained from Agilent Technologies (Waldbronn, Germany).

Oasis PRiME HLB was supplied by Waters (Milford, MA, USA). All sample extracts were filtered using nylon syringe filters, 0.2 µm x 13 mm (Bonna-Agela Technologies Inc, Wilmington, USA).

5.2.2. Instrumentation

CZE-MS experiments were performed using a 7100 CE System (Agilent Technologies) coupled to a 6530 Accurate-Mass Q-TOF (Agilent Technologies) with a dual-nebulizer ESI source. The sheath-liquid was delivered by an Agilent 1260 series isocratic pump equipped with a 1:100 flow splitter. The MassHunter workstation software (Version B.05.01, Agilent Technologies) was employed for the control, data acquisition and analysis of CE-Q-TOF system.

SPE was carried out on a Visiprep™ DL vacuum manifold (Supelco) for 12 cartridges. A pH-meter (Crison model GLP21; Barcelona, Spain), a nitrogen evaporator (Turbo Vap LV from Zymark, Hopkinton, USA), a centrifuge (Sigma 2-16P model from Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and a vortex (Genie 2 model from Scientific Industries; Bohemia, NY, USA) were also employed for sample treatment steps.

5.2.3 CE condition

Separation was carried out in a bare fused-silica capillary (90 cm total length, 50 µm i.d., 375 µm o.d.) from Polymicro Technologies (Phoenix, AZ, USA). The electrophoretic separation was achieved using a voltage of 25 kV. The background electrolyte (BGE) was an aqueous solution of 75 mM ammonium acetate and 2.5 mM EDTA (pH=9.0). The temperature of the capillary was kept constant at 25°C. The sample was hydrodynamically injected for 100 s at 50 mbar. The sample solvent was 1 M ammonium hydroxide. Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min, then with water for 10 min, and finally with the BGE for 20 min. At the beginning of each session, the capillary was prewashed with water (3 min), 1 M ammonium hydroxide (3 min), water again (3 min) and BGE (20 min). In order to obtain a satisfactory repeatability of run-to-run injections, before each run the capillary was pre-washed with 1 M ammonium hydroxide for 2.5 min, water for 1 min and finally with the running buffer for 5 min. At the end of the analysis, the capillary was washed with water during 5 min, and dried with air for 5 min. All these steps were carried out applying a N_2 pressure of 1 bar.

5.2.4 MS conditions

The mass spectrometer was operating in the positive ion mode and scanned from 50-600 m/z. The analytes were detected in ESI^+. To obtain the precursor ion and product ions for each compound, all-ion mode MS/MS (also known as MS^E) was selected. In this acquisition mode two different experiments are conducted alternatively, full-scan acquisition and collision-induced dissociation (CID), where the collision cell switches rapidly and continuously between low and elevated collision energy states. At low energy state, no fragmentation

occurs and precursor ion spectra are recorded. At high energy state, ramped collision energy is used to generate fragment ions. In this way, both precursor ions (obtained in full scan mode) and product ions spectra of all precursors are recorded [36,37]. All-ion mode full-scan acquisition was used at two different collision energy conditions (0 V (full-scan with no fragmentation) and 20 V), using 400 ms for each experiment (1.25 spectra/acquisition points per second). The detailed optimized parameters are shown in Table 5.1.

Table 5.1. List of analysed antibiotics with retention time, RSD of migration time (n=5), theoretical and experimental mass of extracted precursor and product ions and their mass error in parts per million (ppm), relative abundance (%) of fragments for each compound using all ion mode fragmentation (0, 10, 20 and 30 V).

	t_R (min)	[a] RSD (%) t_R	Elemental composition	Ion detected	Theoretical (m/z)	Experimental (m/z)	Error (ppm)	All ion mode MS/MS fragmentation Relative Abundance (%)			
								0V (Full Scan MS)	10V	20V	30V
Oxolinic acid	19.6	0.14	$C_{12}H_{11}NO$	$[M+H]^+$	262.0710	262.0714	1.5	100	45	4	2
Oxolinic acid F1				$C_{13}H_{10}NO_4$	244.0604	244.0607	1.2	1	100	100	100
Oxolinic acid F2				$C_{11}H_6NO_4$	216.0291	216.0290	-0.5	0	5	80	30
Flumequine	20.2	0.11	$C_{14}H_{12}FNO_3$	$[M+H]^+$	262.0874	262.0876	0.8	100	100	7	3
Flumequine F1				$C_{14}H_{11}FNO_2$	244.0768	244.0768	0.0	0	67	100	100
Flumequine F2				$C_{11}H_5FNO_2$	202.0299	202.0300	0.5	0	25	30	45
Ciprofloxacin	13.1	0.08	$C_{17}H_{18}FN_3O_3$	$[M+H]^+$	332.1405	332.1406	0.3	100	85	2	18
Ciprofloxacin F1				$C_{16}H_{19}FN_3O_2$	314.1299	314.1301	0.6	0	0	58	52
Ciprofloxacin F2				$C_{16}H_{19}FN_3O$	288.1507	288.1509	0.7	2	2	3	3
Danofloxacin	12.4	0.10	$C_{19}H_{20}FN_3O_3$	$[M+H]^+$	358.1561	358.1567	1.7	100	100	52	12
Danofloxacin F1				$C_{17}H_{15}FN_2O$	283.1241	283.1243	0.7	0	42	4	4
Danofloxacin F2				C_6H_9N	96.0808	96.0810	2.1	1	3	1	1
Enrofloxacin	12.7	0.08	$C_{19}H_{22}FN_3O_3$	$[M+H]^+$	360.1718	360.1718	0.0	100	100	52	14
Enrofloxacin F1				$C_{19}H_{21}FN_3O_2$	342.1612	342.1614	0.6	0	6	15	3
Enrofloxacin F2				$C_{18}H_{23}FN_3O$	316.1820	316.1823	0.9	0	15	10	52
Marbofloxacin	13.3	0.12	$C_{17}H_{19}FN_4O_4$	$[M+H]^+$	363.1463	363.1468	1.4	100	100	83	49
Marbofloxacin F1				$C_{15}H_{15}FN_3O_4$	320.1041	320.1043	0.6	0	52	15	4
Marbofloxacin F2				$C_{14}H_{15}FN_3O_2$	276.1143	276.1142	-0.4	0	15	3	0
Sarafloxacin	13.8	0.12	$C_{20}H_{17}F_2N_3O_3$	$[M+H]^+$	386.1311	386.1315	1.0	100	100	47	27
Sarafloxacin F1				$C_{20}H_{16}F_2N_3O_2$	368.1205	368.1210	1.4	0	52	64	73
Sarafloxacin F2				$C_{19}H_{18}F_2N_3O$	342.1412	342.1416	1.2	0	13	25	29
Difloxacin	17.3	0.08	$C_{21}H_{19}F2N_3O_3$	$[M+H]^+$	400.1467	400.1466	-0.2	100	100	61	25
Difloxacin F1				$C_{21}H_{18}F_2N_3O_2$	382.1362	382.1365	0.8	0	40	93	100
Difloxacin F2				$C_{20}H_{20}F_2N_3O$	356.1569	356.1571	0.6	0	25	30	32
Methacycline	15.4	0.08	$C_{22}H_{22}N_2O_8$	$[M+H]^+$	443.1449	443.1449	0.0	100	73	25	10
Methacycline F1				$C_{22}H_{19}NO_8$	426.1183	426.1185	0.5	0	80	45	25
Methacycline F2				$C_{22}H_{18}NO_7$	408.1078	408.1080	0.5	0	100	70	23

	t_R (min)	[a] RSD (%) t_R	Elemental composition	Ion detected	Theoretical (m/z)	Experimental (m/z)	Error (ppm)	All ion mode MS/MS fragmentation			
								Relative Abundance (%)			
								0V (Full Scan MS)	10V	20V	30V
Difloxacin	17.3	0.08	$C_{21}H_{19}F2N_3O_3$	$[M+H]^+$	400.1467	400.1466	-0.2	100	100	61	25
Difloxacin F1				$C_{21}H_{18}F_2N_3O_2$	382.1362	382.1365	0.8	0	40	93	100
Difloxacin F2				$C_{20}H_{20}F_2N_3O$	356.1569	356.1571	0.6	0	25	30	32
Methacycline	15.4	0.08	$C_{22}H_{22}N_2O_8$	$[M+H]^+$	443.1449	443.1449	0.0	100	73	25	10
Methacycline F1				$C_{22}H_{19}NO_8$	426.1183	426.1185	0.5	0	80	45	25
Methacycline F2				$C_{22}H_{17}NO_7$	408.1078	408.1080	0.5	0	100	70	23
Doxycycline	14.6	0.12	$C_{22}H_{24}N_2O_8$	$[M+H]^+$	445.1605	445.1608	0.7	100	100	14	0
Doxycycline F1				$C_{22}H_{22}NO_8$	428.1340	428.1342	0.5	0	45	100	100
Doxycycline F2				$C_{22}H_{19}NO_7$	410.1332	410.1333	0.2	0	0	4	25
Tetracycline	15.7	0.12	$C_{22}H_{24}N_2O_8$	$[M+H]^+$	445.1605	445.1605	0.0	100	0	0	0
Tetracycline F1				$C_{22}H_{22}NO_8$	428.1340	428.1342	0.5	0	100	100	0
Tetracycline F2				$C_{22}H_{19}NO_7$	410.1332	410.1335	0.7	0	25	63	24
4-epitetracycline	16.2	0.14	$C_{22}H_{24}N_2O_8$	$[M+H]^+$	445.1605	445.1607	0.4	100	100	20	0
4-epitetracycline F1				$C_{22}H_{22}NO_8$	428.1340	428.1343	0.7	0	40	100	22
4-epitetracycline F2				$C_{22}H_{19}NO_7$	410.1332	410.1333	0.2	0	0	5	32
Minocycline	14.7	0.13	$C_{23}H_{27}N3O_7$	$[M+H]^+$	458.1922	458.1930	1.7	100	65	10	8
Minocycline F1				$C_{19}H_8N$	250.0651	250.0653	0.8	0	25	100	0
Minocycline F2				$C_{23}H_{26}N_2O_7$	221.0865	221.0869	1.8	15	42	62	40
Demeclocycline	16.6	0.14	$C_{21}H_{21}ClN_2O_8$	$[M+H]^+$	465.1059	465.1058	-0.2	100	100	10	0
Demeclocycline F1				$C_{21}H_{19}ClNO_8$	448.0794	448.0797	0.7	0	80	60	5
Demeclocycline F2				$C_{21}H_{17}ClNO_7$	430.0688	430.0686	-0.5	0	10	81	5
Chlortetracycline	18.8	0.12	$C_{22}H_{23}ClN2O_8$	$[M+H]^+$	479.1216	479.1217	0.2	100	75	0	0
Chlortetracycline F1				$C_{22}H_{21}ClNO_8$	462.0950	462.0953	0.6	0	25	100	100
Chlortetracycline F2				$C_{22}H_{19}ClNO_7$	444.0845	444.0847	0.5	26	21	14	5

[a] RSD calculated from 5 replicates (different days) in solvent at 100 µg L^{-1}.

A coaxial sheath-liquid sprayer was used for CE-MS coupling. The sheath-liquid consisted of purine (5×10^{-3} mM) and HP-0921 (1.25×10^{-3} mM) dissolved in MeOH: water: FA (70:29.9:0.1, v/v/v) and was delivered at a flow rate of 2 μL min^{-1}. The rest of optimized parameters were as follows: capillary voltage, 5500 V; nebulizer pressure, 5 psi; dry gas flow rate, 3 L min^{-1}; and dry gas temperature, 250 °C; fragmentor voltage, 50 V; skimmer voltage, 50 V; OCT 1 RF voltage, 800 V.

5.2.5. Sample treatment procedure

Milk samples were purchased in local markets from Granada and Jaen (south-eastern Spain) and stored at 4°C. Samples of 1 g of milk, 4 mL of 0.2% FA in MeCN and 1 mL McIlvaine's buffer solution were mixed and adjusted to pH 4 with 1 M NaOH. This mixture was placed into a 15 mL screw cap test tube and shaken by vortex for 10s. Finally, it was centrifuged for 6 min at 7500 rpm. Then the supernatant was passed through the SPE cartridge at a flow rate of 1 mL min^{-1} (previously conditioned with a mixture of 3 mL 0.2% FA in MeCN and 1 mL McIlvaine's buffer solution at the same flow rate) and collected. Then, this extract was evaporated to dryness under a gentle nitrogen stream and reconstituted in 10 mL of 1 M ammonium hydroxide. The final extract was filtered and transferred to a vial for CZE-Q-TOF-MS/MS analysis.

5.3. Results and discussion

5.3.1. Optimization of electrophoretic separation

To achieve a satisfactory electrophoretic separation, the main CE parameters were carefully studied. However, in order to maintain the performance of MS, there are two requirements, which must be taken into consideration when a CE-MS method is developed: firstly, the BGE must be volatile and, secondly,

this buffer must present a low conductivity (i.e., electric current below 50 µA). This second point is of critical importance to avoid plugging of the dielectric capillary between the spray chamber and the MS [17]. According with the pK values of TCs and QNs previously reported, a satisfactory separation between these compounds can be achieved with basic buffers [38,39]. Thus, ammonium acetate and ammonium carbonate buffers at pH 9.0 were checked as BGE. Better peak shapes were obtained with ammonium acetate, so it was chosen for subsequent experiments. Using this buffer, pH variations in the BGE were studied between pH 8 and 10 and finally a pH of 9.0 was selected, as it provided the best resolutions among the studied analytes. Thus, ammonium acetate concentration was modified between 50 and 150 mM, keeping the pH at 9.0. The best results in terms of analysis time and peak shape were obtained using a concentration of 75 mM, achieving also a very low electric current (≈28 µA). The effect of the separation voltage was tested between 20 and 30 kV. The resolution between compounds did not change significantly with varying the voltage, but as expected, the analysis time decreased with increasing voltage. However, the separation voltage was limited to 25 kV in order to avoid excessive Joule heating. Capillary temperature was studied from 20 to 30°C. It was observed that this parameter hardly affected the separation, probably due to the portion of the capillary that remains outside the CE system at room temperature. So, a capillary temperature of 25°C was selected. As it can be observed in Table 1, the precision in terms of retention time was satisfactory, obtaining RSD (%) lower than 0.15% for all the compounds.

Finally, to reach the maximum sensitivity, on-line preconcentration by field amplified sample stacking (FASS) mode was checked [40]. To obtain a pH higher than the pKs of the studied compounds, a 1 M ammonium hydroxide

solution was selected as sample solvent. Thus, QNs and TCs were as zwitterionic and anionic forms, respectively.At this conditions, the injection time was studied from 5 to 120 s and finally, 100 s at 50 mbar was used as optimum, equivalent to a volume of approximately 76.7 nL (≈4% of the capillary volume). Finally, sensitivity enhancement factors based on peak heights (SEF_{height}) were estimated from this equation:

$$SEF_{height} = \frac{\text{Peak height under FASS injection / Analyte concentration in FASS injection}}{\text{Peak height under hydrodynamic injection/Analyte concentration in hydrodynamic injection}}$$

SEF_{height} values ranging from 95 to 450 were obtained, considering FASS in relation to a conventional hydrodynamic injection (50 mbar for 10 s, sample solvent: BGE), employing optimum separation and detection conditions. Bearing in mind these results, the use of this on-line preconcentration strategy significantly improved the sensitivity in CE.

5.3.2. CE-ESI-MS optimization

The composition of the sheath-liquid and its flow rate and other nebulizer parameters including nebulizer pressure, dry gas flow rate and temperature are key issues in CE-ESI-MS, as these parameters can strongly influence the spray stability and sensitivity of the method. Thus, they were optimized using a test solution of 0.1 mg L^{-1} of each antibiotic, selecting the signal-to-noise ratio (S/N) for each compound as response variable.

In order to obtain an accurate mass calibration correction during the CE-Q-TOF runs, an internal mass calibration solution should be infused continuously during each analysis. This procedure could be carried out by the second spray needle in the dual nebulizer ESI-source. Unfortunately, this common practice in

LC-TOF is not adequate for CE systems, because the nebulization of this second sprayer interferes with the CE-MS analysis [41]. This problem can be overcome by adding the internal mass calibration solution into the sheath-liquid [41,42,43], so the concentration of purine and HP-0921 were optimized. The best results were obtained using a sheath-liquid with 5×10^{-3} mM of purine and 1.25×10^{-3} mM of HP-0921. These concentrations allowed obtaining an accurate mass calibration correction without excessive ion suppression. Then, the influence of the nature of the organic solvent (MeOH or IPA) in the sheath-liquid was investigated. The best results in terms of S/N were obtained with MeOH. Moreover, it was observed that an increase of the percentage of MeOH up to 70% showed a steady increase of the S/N for all tested compounds, while higher percentages decreased the S/Ns; thus, 70% MeOH was selected. The percentage of FA was evaluated between 0.05 to 1.0%. An increase in the S/Ns was observed up to 0.1%, while higher values caused instability of the spray. So, 0.1% FA was used for the rest of studies. In conclusion, the optimum sheath-liquid composition consisted of a solution of purine (5×10^{-3} mM) and HP-0921 (1.25×10^{-3} mM) dissolved in MeOH: water: FA (70:29.9:0.1, v/v/v).

Subsequently, the flow rate of the sheath-liquid was studied in the range of 1.0-5.0 µL min^{-1}. An increase on the flow rate caused a reduction of S/N ratios due to the dilution produced at the nebulizer. However, an unstable spray was observed with flow rates lower than 2.0 µL min^{-1}, so this value was selected as optimum. The influence of the nebulizer pressure on the S/N ratios was studied from 3 to 7 psi. The increase of the nebulizer pressure up to 5 psi had a positive effect on the S/N ratio of the studied compounds, as higher nebulizer pressures produce smaller spray droplets, enhancing analyte desolvation, and thus promoting ESI. However, at a higher pressure, spray stability decreased, so an

optimum value of 5 psi was selected. The dry gas flow rate and its temperature were varied in the range of 3-10 L min^{-1} and 150-300 ˚C, respectively. The magnitude of these parameters were less critical and slightly better results were found at 5 L min^{-1} and 300 ˚C.

The next step was to set the Q-TOF-MS/MS parameters. Capillary voltage was studied from 4000 to 5500 V, achieving the best intensity signal with 5500 V. Fragmentor voltage was varied from 50 to 150 V, observing that the increase of the voltage caused a reduction of the precursor ion signal. This fact is principally related to in-source CID (i.e. fragmentation) of the analytes [44], so in order to reduce this effect, the minimum fragmentor voltage (50 V) was selected. Then, several skimmer voltage values (from 40 to 100 V) and OCT 1 RF voltage values (from 150 to 800 V) were also checked. It was observed that none of these parameters had a significant effect on the analytical response of the studied compounds, so 50 and 800 V were selected, respectively. According to the current European legislation, to achieve unambiguous identification of the studied antibiotics in the case of HRMS, it is necessary to obtain the precursor ion and one product ion with mass accuracy <5 ppm [11]. As stated in section 2.4, all-ion mode (full scan combined with CID MS/MS fragmentation without precursor ion isolation) was chosen. So, different collision energies (10, 20 and 30 V) were tested in CID and 20 V was selected to obtain at least one product ion of each antibiotic (Table 1). The mass accuracy was lower than 2 ppm in all studied compounds, being in compliance with the current legislation [11].

5.3.3. Optimization of sample preparation

In this work a HLB PRiME SPE sorbent recently available has been evaluated for the determination of different families of antibiotics in a complex matrix such as milk. The use of this new sorbent simplifies the extraction, providing an excellent capacity for removing lipids and fat components. The protocol proposed by Waters for the determination of multi-residue veterinary drugs in milk was initially followed [34], and was subsequently adapted for the compounds of interest. Thus, the optimization of the sample treatment was carried out with 1 g of whole cow milk (as representative matrix) spiked at 200 μg kg^{-1} of each antibiotic. The recovery of each analyte was used to evaluate the extraction efficiency. According to the protocol, in order to precipitate proteins while extracting antibiotics from the matrix, 4 mL of 0.2% FA in MeCN must be used. Although the extraction efficiency for QNs was satisfactory, the recoveries were nul for TCs. It is well known that TCs rapidly form strong complexes with different divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) [45]. In order to release TCs from their metallic complexes, the addition of a chelating agent as EDTA or citric acid to the matrix was mandatory. Thus, EDTA-McIlvaine buffer solution, which the preparation has been indicated in section 2.1, was also added to the sample before applying the SPE procedure. Different volumes of this buffer were tested (0.5, 1.0 and 2.0 mL), achieving recoveries higher than 75% for all compounds when 1.0 mL was used. So, this value was selected as optimum. Finally, in order to obtain lower conductivity in the sample than in the BGE, the eluate was diluted 10-fold with 1 M ammonium hydroxide.

Figure 5.1 shows an electropherogram corresponding to a whole cow milk sample (spiked at 100 μg kg^{-1} for each TC and 50 μg kg^{-1} for each QN). It should be noted that, the proposed method allowed enough resolution between tetracycline and its epimer 4-epitetracycline (Rs >1.5).

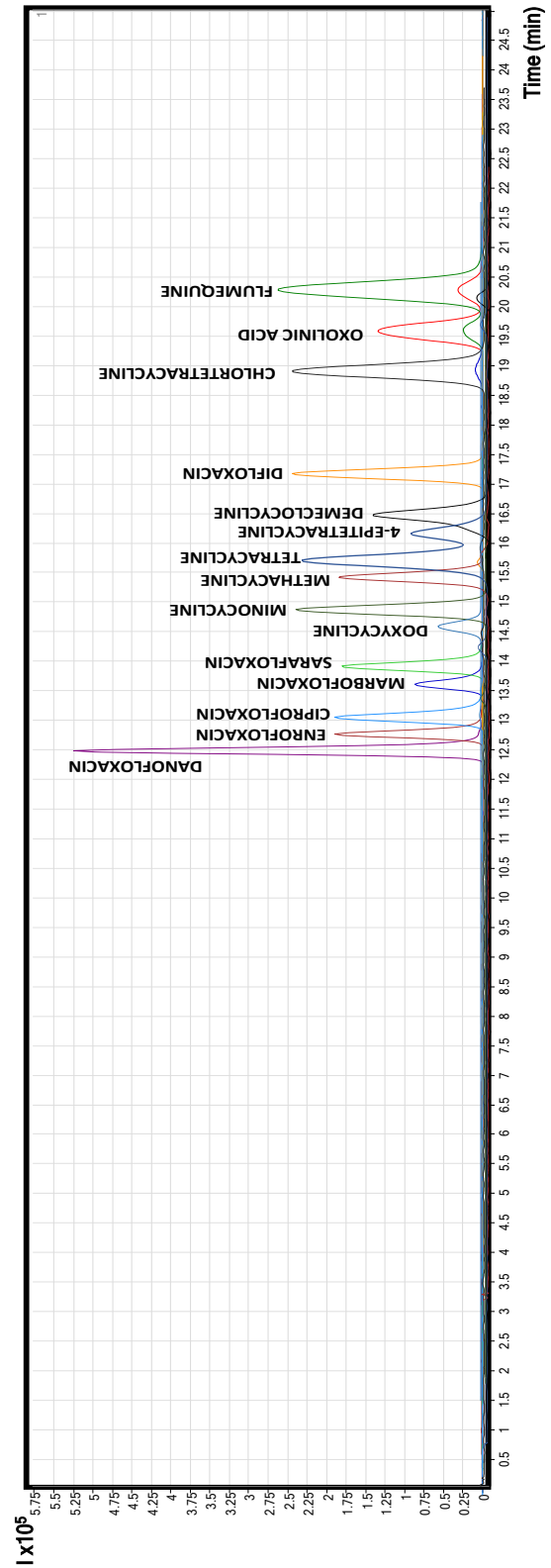

Figure 1. Electropherogram of a spiked whole cow milk sample (100 µg kg^{-1} for each TC and 50 µg kg^{-1} for each QN) applying the proposed SPE-CZE-Q-TOF-MS/MS method.

5.3.4. Method characterization

To check the suitability of the proposed method for the determination of TCs and QNs in milk samples, linearity, limits of detection (LODs) and quantification (LOQs), matrix effect (ME), precision and trueness were evaluated.

5.3.4.1. Calibration curves and analytical performance characteristics

Procedural calibration curves were established by spiking whole cow milk samples at six concentration levels (5, 10, 50, 100, 150 and 200 μg kg^{-1} for each QN and 10, 20, 50, 100, 150 and 200 μg kg^{-1} for each TC) by spiking blank samples before the extraction process. Two experimental replicates at each level were processed following the SPE-CZE-Q-TOF-MS/MS method, and each one was injected in duplicate. The peak area of each precursor ion (see Table 5.1) was considered as analytical signal. Previously, a blank sample was also processed, and none of the selected antibiotics were detected. Performance characteristics of the method are available in Table 5.2. As it can be observed, coefficients of determination (R^2) were higher than 0.99 in all cases. LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N ratio equal to three and ten, respectively. Excellent LOQs, lower than 10 μg kg^{-1}, were obtained in all cases.

Table 5.2. Statistics and performance characteristics of the PRiME HLB-CZE-Q-TOF-MS/MS method for the analysis of QNs and TCs in whole cow milk

Analytes	R^2	Slope	Intercept	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	MRL (µg kg^{-1})	ME (%)
Oxolinic acid	0.993	70415	-38542	0.7	2.5	NA	3.1
Flumequine	0.995	85283	148083	0.5	1.6	50	3.4
Ciprofloxacin	0.995	14851	12722	0.7	2.2	100	-1.0
Danofloxacin	0.997	128398	54636	0.7	2.3	30	-1.8
Enrofloxacin	0.997	29695	2207	0.9	3.1	100	-6.2
Marbofloxacin	0.995	43745	-18127	0.7	2.2	75	-5.0
Sarafloxacin	0.998	52517	-50269	0.8	2.6	NA	-1.8
Difloxacin	0.996	69923	-1649	0.6	2.1	NA	-1.3
Methacycline	0.995	20080	2581	2.1	7.0	NA	6.7
Doxycycline	0.993	15980	5190	1.5	5.1	100	-8.7
Tetracycline	0.999	31840	-8375	2.3	7.8	NA	1.1
4-epitetracycline	0.995	48370	-8056	1.7	5.8	100	-1.7
Minocycline	0.996	42790	-9348	2.6	8.7	NA	-2.8
Demeclocycline	0.995	14520	7279	2.9	9.7	NA	-7.8
Chlortetracycline	0.998	28760	-565	2.5	8.4	100	8.4

5.3.4.2. Matrix effect

ME is a key issue when MS methods are developed. It affects the analyte ionization, having as a consequence ion suppression or signal enhancement [46]. So, it is necessary to stablish sample treatments which could minimize or overcome this effect. In this sense, HLB PRiME could be useful to retain the majority of phospholipids and fats in milk, thus reducing ME. To evaluate this factor, the slope of matrix-matched calibration curves and the slope of external standard calibration curves were compared according to the following equation: [(calibration curve slope in matrix/ calibration curve slope in solvent)-1] x 100 [47]. As could be observed in Table 2, ME was negligible in all cases, obtaining ME values lower than |9|%. Thus, it can be concluded that this new sorbent recovered the most part of the compounds studied and simultaneously removed co-extractants, increasing the overall performance of the method by means of decreasing ME without any significant analyte losses.

5.3.4.3. Precision study

The precision of the whole method was evaluated by means of application of the proposed procedure to whole cow milk samples spiked at two concentration levels (50 and 100 μg kg^{-1}), processed during five consecutive days and injected in duplicate. The results expressed as relative standard deviation (RSD %) of peak areas, are given in Table 5.3. As can be observed, good precision (RSD lower than 14%) was obtained in all cases, being in agreement with the current demand [11].

Table 5.3. Precision of the method for spiked whole cow milk samples (five samples analysed in five different days and injected in duplicate)

Analyte	RSD% (n=10)	
	50 μg kg^{-1}	100 μg kg^{-1}
Oxolinic acid	10.1	9.2
Flumequine	10.0	9.8
Ciprofloxacin	11.4	12.4
Danofloxacin	13.4	11.2
Enrofloxacin	12.1	11.0
Marbofloxacin	9.3	8.2
Sarafloxacin	10.8	10.5
Difloxacin	12.3	10.5
Methacycline	12.3	9.8
Doxycycline	12.2	11.1
Tetracycline	13.2	12.8
4-epitetracycline	8.4	11.2
Minocycline	11.0	10.0
Demeclocycline	13.3	12.0
Chlortetracycline	14.2	12.0

5.3.4.4. Trueness assessment

The trueness of the proposed method was assessed by recovery studies in different types of milk samples (whole and semi-skimmed cow milk and whole goat milk) spiked at two different concentration levels of each compound (50 and 100 μg kg^{-1}). Two samples of each level were prepared and injected in duplicate. Recoveries were calculated for each TC by the ratio of concentrations (that estimated from the matrix-matched calibration curves and the added concentration). The results are shown in Table 5.4. Recoveries between 72 and 106% were achieved in all studied matrices. The repeatability was also satisfactory (RSD ≤ 10.5%), thus fulfilling the current legislation requirements [11].

Table 5.4. Recoveries (R) obtained for each antibiotic at different spiked levels in whole cow milk, goat milk and semi-skimmed cow milk samples (n=4)

Analytes	Whole cow milk				Goat milk				Semi-skimmed cow milk			
	50 µg kg^{-1}		100 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
Oxolinic acid	87.1	8.0	90.4	7.6	79.0	6.4	82.0	5.8	92.1	8.3	87.1	6.9
Flumequine	76.3	9.1	81.1	6.5	79.8	5.8	97.9	7.0	86.2	9.5	83.2	8.1
Ciprofloxacin	86.1	10.0	97.4	6.4	80.2	3.1	87.5	4.9	93.7	8.7	91.9	7.5
Danofloxacin	90.7	6.4	90.6	10.2	97.5	5.7	77.2	10.1	90.6	8.8	98.9	9.9
Enrofloxacin	80.7	9.7	90.8	10.3	78.4	7.6	75.7	9.3	85.1	7.7	96.6	9.5
Marbofloxacin	77.6	5.6	103.8	7.4	79.8	2.9	81.8	5.8	97.2	10.0	101.6	3.1
Sarafloxacin	88.9	7.2	105.8	9.2	102.4	4.1	103.3	9.2	81.6	4.6	97.1	10.5
Difloxacin	87.5	7.5	91.0	9.9	96.2	9.8	78.7	6.8	105.5	7.3	99.2	9.1
Methacycline	81.2	5.8	78.0	8.4	78.8	7.3	81.1	9.7	87.2	5.6	84.6	7.6
Doxycycline	101.5	6.2	99.7	9.7	98.5	7.5	99.3	9.8	86.1	9.6	98.3	5.3
Tetracycline	97.3	9.2	98.9	8.0	95.1	5.1	89.6	8.5	84.1	9.0	75.3	4.9
4-epitetracycline	75.4	9.6	87.9	9.3	73.6	9.6	75.8	8.2	73.6	7.4	87.6	6.8
Minocycline	86.2	6.6	104.6	4.4	85.7	6.3	91.9	2.1	89.0	8.2	76.8	5.4
Demeclocycline	76.5	8.3	89.3	10.4	95.9	7.1	84.1	8.6	75.7	7.6	90.1	8.3
Chlortetracycline	78.3	8.4	78.4	6.8	72.6	7,1	77.4	6.3	82.3	6.4	76.4	4.6

5.3.4.5. Comparison with other methods

A comparative overview of the main analytical performance characteristics of the proposed method with other published methods for the determination of these veterinary drugs in milk samples are shown in Table 5.5**.** The results in terms of LOQs and recoveries were similar or even better than those obtained by the other methods. Moreover, the amount of required sample in the proposed SPE procedure (1 g) was usually lower. However, the most relevant issue was the low MEs. This new HLB PRiME sorbent allowed obtaining a negligible ME for all studied compounds. So, it can be concluded that this new SPE sorbent provides greater clean-up than traditional SPE methods and can be an effective sample treatment combined with a green technique such as CE, with excellent quantification and identification power.

Table 5.5. Comparison of the proposed method with other reported methods for the determination of TCs and QNs in milk samples.

Method	Sample treatment	LOQ (µg kg^{-1})	Recovery (%)	Amount of sample	ME (%)	Reference
UHPLC-QQQ-MS/MS	Liquid–liquid extraction with partition at low temperature	0.3-35.5	67.4-112.5	2.0 mL	Medium: Between 20 and 50%	[12]
UHPLC-QQQ-MS/MS	SPE (HLB)	0.2-1.7	87-117	2.0 mL	-	[14]
HPLC-QQQ-MS/MS	SPE (HLB)	0.1-0.5	39-101	2.0 g	-	[15]
UHPLC-QQQ-MS/MS	QuEChERS	3.0-10.0	73.1-108.6	10.0 g	Strong: Higher than 50% for some compounds	[29]
CE-Q-TOF-MS/MS	SPE (HLB PRiME)	1.6-9.7	72.6-105.8	1.0 g	Negligible: Lower than 9%	This work

5.3.5. Real sample analysis

The developed method was applied for the determination of TC and QN residues in twenty four milk samples obtained from local supermarkets: five samples of whole cow milk, five samples of semi-skimmed cow milk, five samples of skimmed cow milk, three samples of goat milk, three samples of milk with omega 3, one sample of milk enriched with calcium, one sample of milk with isoflavons and one samples of milk without lactose. All milk samples were stored under the recommended conditions in their original packaging prior to use. None of the studied antibiotics were detected at a concentration higher than the LOD.

5.4. Conclusions

A simple, sensitive and high-throughput method for determination of 7 QNs and 8 TCs in milk samples has been developed and validated. The results in terms of ME showed that HLB PRiME sorbent allowed removing co-extractive interferences such as fats and phospholipids. Thus, CE-Q-TOF-MS/MS seems a good alternative for the determination of veterinary compounds, with excellent capability of quantification and unequivocal confirmation, achieving good results in terms of sensitivity and mass accuracy (lower than 2 ppm). LOQs lower than 10 μg kg^{-1} were obtained in all studied compounds, being lower than the MRLs established by current legislation for these antibiotics in milk, with satisfactory precisions. To the best of our knowledge, this is the first report about the use CZE-Q-TOF-MS/MS for determination of antibiotics in food commodities. Furthermore, the proposed method could be used in routine analysis for the simultaneous detection and quantification of the

studied antibiotics from different types of milk samples, offering the possibility to control also non-targeted compounds by using HRMS.

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FINAL CONCLUSIONS

The main goal of this thesis was the development of analytical methods to control contaminants (mycotoxins, pesticides and antibiotics) in milk, dairy products and vegetable milks, trying to achieve a high sensitivity, selectivity, throughput, and accuracy in the identification and confirmation of the selected analytes. Moreover, proper validations according to the criteria of "fitted for purpose" and current recommendations have been carried out.

Regarding to the different separation techniques coupled with several detection systems used during the development of this Thesis, they have their advantages and their drawbacks, achieving the following conclusions:

- The use of HPLC-FLD with photoinduced derivatization for the determination of aflatoxins in different types of yogurt provided very good results in terms of selectivity and sensitivity (especially for AFB1 and AFG1), avoiding the use of derivatization reagents.
- UHPLC-MS/MS has shown to be a good alternative to conventional LC for determination of different families of contaminants (such as *Fusarium* mycotoxins, carbamates and aminoglycosides) in different matrices, as vegetable milks, cheeses and different types of milks. It provided good results in terms of resolution and sensitivity, very short analysis time, and a number of identification points fulfilling with current demands of European regulation for the control of food contaminants.
- Moreover, HILIC was successfully used for the separation of aminoglycosides by UHPLC–MS/MS as an alternative to ion pair chromatography, solving the difficulties of the separation of these very polar compounds.

- CZE-Q-TOF has proved to be an advantageous alternative to LC for the identification and simultaneous quantification of different families of antibiotics (QNS and TCs) in milk samples. This coupling takes advantage of CE as a miniaturised green analytical technique, while Q-TOF/MS shows the availability of full MS/MS spectra after a single injection for identification and confirmation purposes, with an enhanced accuracy. LOQs of the method were comparable or better than those reported for other methods.

Another aim of this Thesis has been the study of alternative sample treatments for the target analytes (mycotoxins, pesticides and antibiotics), in order to take advantage of their simplicity, selectivity, recovery, as well as their impact on the environment. Thus, different sample treatments (DLLME, QuEChERS, SPE and MISPE) have been applied for determination of selected contaminants. The main conclusions about this aspect are summarized as follows:

- DLLME has been successfully applied and evaluated for the determination of five AFs (AFM1, AFB1, AFB2, AFG1 and AFG2) in yogurt, and the low LOQs obtained allowed their determination at concentrations lower than the limits established by current legislation, being an alternative to other traditional sample treatments such as IACs. Trueness has been successfully evaluated for natural, liquid and skimmed yogurt, obtaining good recoveries for all AFs, except AFG2 in skimmed yogurt due to a co-eluting peak.
- QuEChERS using a new sorbent (Z-Sep^{+}) for d-SPE has been evaluated as sample treatment for the determination of CRBs in different kind of

high-fat cheeses (Gorgonzola, Roquefort and Camembert), achieving satisfactory recoveries and low limits of quantification, below the MRLs tolerated for these compounds by the European legislation. Moreover, matrix effect was moderate for all studied CRBs.

- Also, a QuEChERS-based extraction (without further clean-up) has been successfully applied in the determination of *Fusarium* toxins in vegetable milks, achieving LOQs below the maximum limits established or recommended for these compounds by the European legislation. This simple methodology was applied in the analysis of a significant number of commercial samples.
- MISPE has proved to be a highly selective and efficient sample treatment for determination of AGs in milk and related products, providing cleaner extracts thanks to the strong and selective interaction between MIPs and target molecules. This relatively new sorbents allowed achieving low ME and LOQs below the limits established by current legislation.
- Also, the new sorbent Oasis HLB PRiME proved to be a valuable alternative for other SPE sorbents, avoiding tedious steps such as rinsing and washing the cartridge, thus increasing the throughput. It was successfully validated for multiclass determination of antibiotics (QNs and TCs) in milk samples and derived products.

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

Table.C1. Summary of the analytical characteristics of the methods developed in this Thesis.

Analyte	Matrix	Sample treatment	Technique	Recovery %	LOQs
AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1	Yogurt	DLLME	HPLC- PI-FLD Kinetex C18 column (150 ×4.6 mm, 2.6 µm) Mobil phase: A) MeCN - B) MeOH - C) H2O	69.4-99.7	5-18 ng kg^{-1}
FB_1, FB_2, HT-2, T-2, ZEN, DON, NIV and F-X.	Vegetable milk	QuEChERS-based extraction	UHPLC-MS/MS C18 column (Zorbax Eclipse plus 50×2.1 mm, 1.8 µm) Mobil phase: A) 0.3% formic acid (5 mM ammonium formate) B) MeOH 0.3% formic acid (5 mM ammonium formate)	80-99	3.2-57.7 µg L^{-1}
PRM, ASL, ALDSFX, OX, MTY, CBZ, PIRDES, MTHSFX, 3-CF, MTHSFN, CY, ALD, PIR, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP, FNX, PY, BTH, and FURA	High-fat cheeses	QuEChERS (Z-Sep+)	UHPLC-MS/MS C18 column (Zorbax Eclipse plus 50 ×2.1 mm, 1.8 µm) Mobil phase: A) 0.01% formic acid B) MeOH 0.01% formic acid	70-115	0.5 – 4.7µg kg^{-1}
AM, APM, DHS, GENT C2C2a, GENT C1, GENT C1a, KAM, PRM, SPC, STP, TOM	Milk and enriched milks	MISPE	UHPLC-MS/MS Kinetex HILIC column (100 × 2.1 mm, 1.7 µm) Mobil phase: A)150 mM ammonium acetate containing 1% formic acid B) MeCN	70-106	4.2-49 µg kg^{-1}
OXA, FLU, CIPRO, DNFX, ENRO, MAR, SAR, DIF , METH, DOXY, DOXY, TC, 4-epiTC, MIN, DMC, CTC	Milk and enriched milks	SPE (Oasis HLB PRiME)	CZE-Q-TOF-MS/MS Silica capillary: 90 cm x 50 µm i.d. BGE: 75 mM ammonium acetate and 2.5 mM EDTA (pH=9.0)	76 -106	1.5 - 9.6 µg kg^{-1}

Finally, in relation to the occurrence of the studied chemical contaminants (mycotoxins, pesticides and antibiotics) in milk and milk products in different regions in Spain, this study indicated that there were no contaminated samples by carbamates or antibiotics at concentrations higher than the LOQ of the developed methods. Thus, it can be stated than the analysed samples fulfilled with current food safety legislation.

However, regarding the determination of *Fusarium* toxins in vegetables milks, DON was found in three oat milk, at concentrations that could indicate that the raw material was contaminated at concentrations close or even above the maximum limits allowed by European legislation in these matrices. Thus, awareness regarding occurrence of mycotoxins in food commodities derived from cereals and scarcely explored, is necessary. In addition, more control in the processing manufacturing of these products must be applied.

CONCLUSIONES FINALES

El principal objetivo de esta Tesis Doctoral ha sido el desarrollo de metodologías analíticas para el control de contaminantes (micotoxinas, plaguicidas y antibióticos) en leche, productos lácteos y leches vegetales, intentando alcanzar una alta sensibilidad, selectividad, rendimiento y exactitud para la identificación confirmación de los analitos seleccionados. Además, se ha llevado a cabo la validación de los métodos propuestos de acuerdo con el criterio de "adecuado para el propósito", teniendo en cuenta las actuales recomendaciones en cuanto a criterios analíticos se refiere.

Respecto a las diferentes técnicas de separación acopladas a diversos sistemas de detección que se han empleado durante el desarrollo de la Tesis, todas presentan múltiples ventajas y algún inconveniente, y se ha podido llegar a las siguientes conclusiones:

- En empleo de HPLC-FLD con derivatización fotoinducida para la determinación de aflatoxinas en diferentes tipos de yogur ha proporcionado muy buenos resultados en términos de selectividad y sensibilidad, (especialmente para AFB1 y AFG1), sin requerir el empleo de agentes derivatizantes.
- UHPLC-MS/MS ha demostrado ser una excelente alternativa a la LC convencional para la determinación de diferentes familias de contaminantes (como toxinas de *Fusarium*, carbamatos y aminoglicósidos) en diferentes matrices, como leches vegetales, quesos y diferentes tipos de leche y productos relacionados. Esta técnica proporciona buenos resultados en términos de resolución y sensibilidad, tiempos de análisis cortos, y un número adecuado de puntos de identificación, necesarios para cumplir con las demandas actuales de la

regulación europea en lo que respecta a determinación de residuos de contaminantes en alimentos.

- Asimismo, la cromatografía HILIC se ha empleado satisfactoriamente para la separación de aminoglicósidos mediante UHPLC–MS/MS, como alternativa ventajosa a la cromatografía de pares iónicos, tradicionalmente empleada para la determinación cromatográfica de estos compuestos altamente polares.
- CZE-Q-TOF ha demostrado ser una alternativa a los métodos de LC convencionales para la determinación simultánea de diversas familias de antibióticos (QNS y TCs) en muestras de leche y preparados lácteos. Este acoplamiento saca ventaja de la CE como técnica miniaturizada y respetuosa con el medioambiente, y de la alta resolución de Q-TOF, que permite obtener un espectro MS/MS complete en una sola inyección. Los LOQs del método fueron comparables o incluso mejores que los obtenidos por otros métodos.

Otro objetivo importante de esta Tesis fue la propuesta de tratamientos de muestra alternativos para la determinación de los analitos objeto de estudio (micotoxinas, plaguicidas y antibióticos), con objeto de proponer métodos más sencillos, selectivos, con elevadas recuperaciones, así como con menor impacto medioambiental. Así, se han empleado diferentes tratamientos de muestra (DLLME, QuEChERS, SPE y MISPE) para la determinación de los contaminantes seleccionados en muestras de diversa naturaleza. Las principales obtenidas en este sentido han sido:

- La DLLME se ha evaluado satisfactoriamente para la determinación de cinco AFs (AFM1, AFB1, AFB2, AFG1 y AFG2) en yogur, y los bajos LOQs

obtenidos permiten su determinación a concentraciones inferiores a los límites establecidos por la legislación actual, siendo una alternativa a los métodos tradicionales de tratamientos de muestra, como las IACs. La veracidad fue evaluada en muestras de yogur natural, líquido y desnatado, obteniendo buenos valores de recuperación para todas las AFs, excepto AFG2 en yogures desnatados debido a un pico interferente.

- El método QuEChERS empleando un nuevo sorbente (Z-Sep$^+$) para la d-SPE ha sido evaluado para el tratamiento de muestra en la determinación de CRBs en diversas muestras de quesos de alto contenido graso (Gorgonzola, Roquefort y Camembert), obteniéndose recuperaciones muy satisfactorias y bajos LOQs, por debajo de los MRLs tolerados para estos compuestos por la legislación europea. Más aún, el efecto matriz fue moderado para los compuestos estudiados, confirmando la alta eficacia de este nuevo sorbente en matrices complejas.
- Asimismo, se ha probado una extracción basada en QuEChERS, sin necesidad de limpieza, para la determinación de toxinas de *Fusarium* en leches vegetales, alcanzándose LOQs por debajo de los valores legislados o recomendados para estos compuestos. Esta simple metodología se aplicó al estudio de la presencia de estos compuestos en leches vegetales comerciales.
- La extracción basada en MISPE ha demostrado ser una alternativa altamente selectiva y eficaz para la determinación de AGs en leche y preparados lácteos, proporcionando extractos muy limpios gracias a la fuerte y selectiva interacción entre los analitos y el polímero. Este

sorbente comercializado recientemente, proporciona bajos ME y LOQs, de acuerdo a los requisitos exigidos para estas determinaciones.

- Además, una nueva fase (Oasis HLB PRiME) se ha ensayado como alternativa a otros sorbentes tradicionales de SPE para la extracción multiclase de diversos antibióticos (QNs and TCs) en muestras de leche y preparados lácteos. Con esta nueva fase se consiguen evitar pasos como el lavado del cartucho, incrementándose el rendimiento y acortándose el tratamiento de muestra.

Como resumen, las características analíticas más relevantes de los métodos propuestos en la Tesis se muestran en la Tabla C1.

Tabla.C1. Resumen de las características analíticas de los métodos desarrollados en la Tesis.

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental	Recuperación %	LOQs
AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1	Yogur	DLLME	HPLC- PI-FLD Columna: Kinetex C18 (150 ×4.6 mm, 2.6 µm) Fase móvil: A) MeCN - B) MeOH - C) H2O	69.4-99.7	5-18 ng kg^{-1}
FB_1, FB_2, HT-2, T-2, ZEN, DON, NIV and F-X.	Leches vegetales	QuEChERS-extracccioón basada	UHPLC-MS/MS Columna:C18 (Zorbax Eclipse plus 50×2.1 mm, 1.8 µm) Fase móvil: A) 0.3% ácido fórmico (5 mM Formato de amonio) B) MeOH 0.3% ácido fórmico (5 mM Formato de amonio)	80-99	3.2-57.7 µg L^{-1}
PRM, ASL, ALDSFX, OX, MTY, CBZ, PIRDES, MTHSFX, 3-CF, MTHSFN, CY, ALD, PIR, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP, FNX, PY, BTH, and FURA	Queso de alto contenido graso	QuEChERS (Z-Sep+)	UHPLC-MS/MS Columna: C18 (Zorbax Eclipse plus 50 ×2.1 mm, 1.8 µm) Fase móvil: A) 0.01% ácido fórmico B) MeOH 0.01% ácido fórmico	70-115	0.5 – 4.7µg kg^{-1}
AM, APM, DHS, GENT C2C2a, GENT C1, GENT C1a, KAM, PRM, SPC, STP, TOM	Leche y leches enriquecida	MISPE	UHPLC-MS/MS Columna: Kinetex HILIC (100 × 2.1 mm, 1.7 µm) Fase móvil: A)150 mM Acetato de amonio contiene ácido fórmico al 1% B) MeCN	70-106	4.2-49 µg kg^{-1}
OXA, FLU, CIPRO, DNFX, ENRO, MAR, SAR, DIF , METH, DOXY, DOXY, TC, 4-epiTC, MIN, DMC, CTC	Leche y leches enriquecida	SPE (Oasis HLB PRiME)	CZE-Q-TOF-MS/MS Capilar de sílice: 90 cm x 50 µm i.d. BGE: 75 mM ammonium acetate and 2.5 mM EDTA (pH=9.0)	76 -106	1.5 - 9.6 µg kg^{-1}

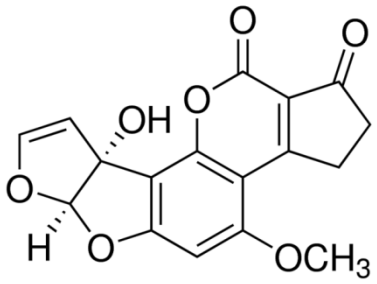
Finalmente, en lo relativo a la presencia de los contaminantes estudiados (micotoxinas, plaguicidas y antibióticos) en leche y productos lácteos, el estudio indica que no encontraron restos de CRBs o antibióticos en ninguna de las muestras estudiadas por encima de los LODs de los diversos métodos. Por tanto, se puede concluir que las muestras analizadas cumplen con la legislación vigente en cuanto a residuos de estos contaminantes se refiere.

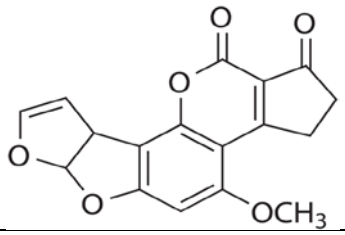
Sin embargo, respecto a la determinación de toxinas de *Fusarium* en leches vegetales, DON se encontró en tres muestras diferentes de avena, a concentraciones que hacen sospechar que el cereal empleado para su fabricación estaba contaminado a concentraciones próximas o incluso superiores a los límites permitidos por la legislación para estas matrices. Por tanto, se debería prestar mayor atención a la posible contaminación de estos productos derivados de cereales, cada vez más consumidos, como posible fuente de ingesta de micotoxinas, estableciéndose un mayor control en los procesos de manufactura.

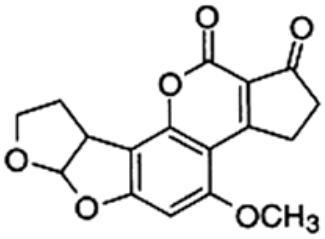
APPENDIX

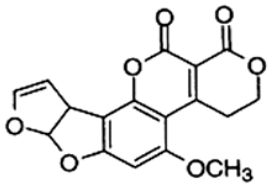
Chemicals and physicals properties in the analytes included in the study:

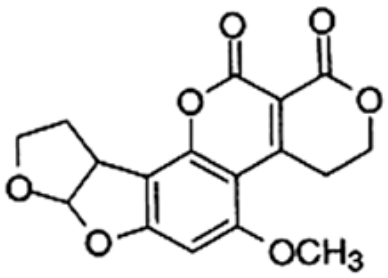
1. Physical and chemical properties of mycotoxins

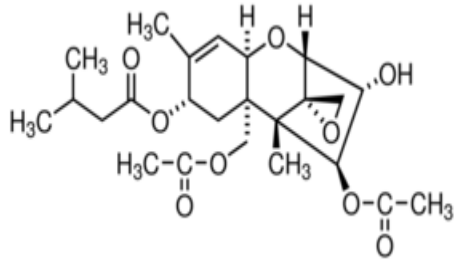
Aflatoxin M1 (AFM1)

IUPAC name: (6aR,9aR)-9a-Hydroxy-4-methoxy-2,3,6a,9a-tetrahydrocyclopenta[c]furo[3',2':4,5]furo[2,3-h]chromene-1,11-dione
Formula: $C_{17}H_{12}O_7$
Molecular Weight: 328.058289 Da
pKa: 11.42
Solubility: 0.994 g/L
Melting point: 299°C

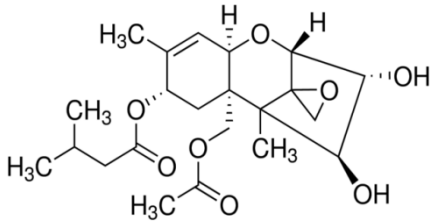
Aflatoxin B1 (AFB1)

IUPAC name: (6aR,9aS) -4-methoxy- 2,3,6a, 9a cyclopentane tetrahydro [c] furo [3',2': 4,5] furo [2,3-h] chromene-1,11-dione
Formula: $C_{17}H_{12}O_6$
Molecular Weight: 312.0634 Da
pKa: ---
Solubility: 0.087 g/L
Melting point: 528.151 °C a 760 mmHg

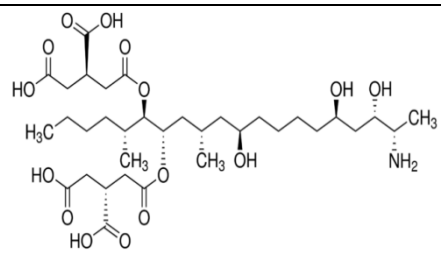
Aflatoxin B2 (AFB2)

IUPAC name: 4-methoxy-2,3,6a, 8,9,9-hexahydro-cyclopenta [c] furo [3',2': 4,5] furo [2,3-h] chromene-1,11-dione
Formula: $C_{17}H_{14}O_6$
Molecular Weight: 314.0790 Da
pKa: ---
Solubility: 0.012 g/L
Melting point: 520.999 °C a 760 mmHg

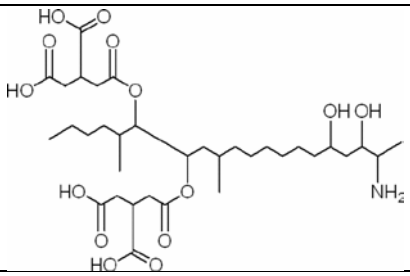
Aflatoxin G1 (AFG1)

IUPAC name: 5-methoxy-3,4,7a, 10a tetrahydro- 1H, 12H-furo [3',2': 4,5] furo [2,3- h] pyrano [3,4-c] chromene-1,12-dione
Formula: $C_{17}H_{12}O_7$
Molecular Weight: 328.0583 Da
pKa: ---
Solubility: 0.28 g/L
Melting point: 602.505 °C a 760 mmHg

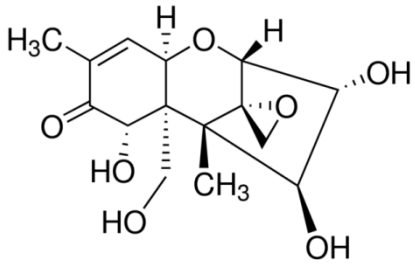
Aflatoxin G2 (AFG2)

IUPAC name: 5-Methoxy- 3,4,7a, 9,10,10a-hexahydro-1H, 12Hfuro [3',2': 4,5] furo [2,3-h] pyrano [3,4-c] cromeno- 1,12-dione
Formula: $C_{17}H_{14}O_7$
Molecular Weight: 330.0739 Da
pKa: ---
Solubility: 0.28 g/L
Melting point: 602.505 °C a 760 mmHg

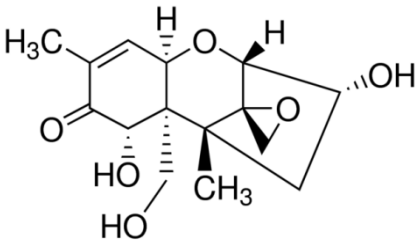
Toxin T-2 (T-2)

IUPAC name: (3β,4α,8α)-4,15-Diacetoxi-3-hidroxi-12,13-epoxitricotec-9-en-8-il 3-methylbutanoate
Formula: $C_{24}H_{34}O_9$
Molecular Weight: 466.220276 Da
pKa: 13.24
Solubility: 0.38 g/L
Melting point: 544.877 °C a 760 mmHg

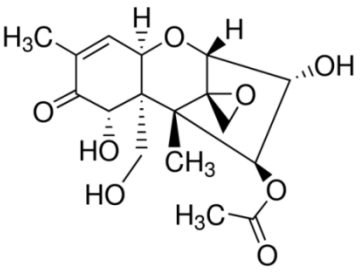
Toxin HT-2 (HT-2)

IUPAC name: (3β,4α,8α,12R)-15- Acetoxy-3,4-dihydroxy-12,13-epoxytrichothec-9-en -8-yl 3-methylbutanoate
Formula: $C_{22}H_{32}O_8$
Molecular Weight: 424.209717 Da
pKa: 13.56
Solubility: 1.1 g/L
Melting point: 537.081 °C at 760 mmHg

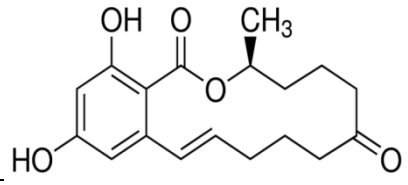
Fumonisin B1 (FB1)

IUPAC name: 2,2'-{(19-Amino- 11,16,18 -trihidroxi-5,9-dimetil-6,7-icosanediyl)bis[oxi(2-oxo-2,1- ethanediyl)]} disuccinic acid
Formula: $C_{34}H_{59}NO_{15}$
Molecular Weight: 721.388489 Da
pKa: 3.24 and 9.24
Solubility: 1000 g/L at pH 10
Melting point: 924.914 °C a 760 mmHg

Fumonisin B2 (FB2)

IUPAC name: (2R, 2'R) -2.2'- {[(5R, 6R, 7S, 9S, 16 R, 18S, 19S) -19-Amino-16,18- dihydroxy-5,9-dimethyl-6,7-icosanediyl] bis [oxy (2-oxo-2,1-ethanediyl)]} disuccinic acid
Formula: $C_{34}H_{59}NO_{14}$
Molecular Weight: 705.393555 Da
pKa: 3.64 and 9.25
Solubility: 1000 g/L at pH 10
Melting point: 864.399 °C a 760 mmHg

Nivalenol (NIV)

IUPAC name: **(3β,4α,7α,12ξ)-3,4,7,15-Tetrahydroxy-12,13-epoxytrichothec-9-en-8-one**
Formula: C22H32O8
Molecular Weight: 424.209717 Da
pKa: 13.56
Solubility: 1.1 g/L
Melting point: 537.081 °C a 760 mmHg

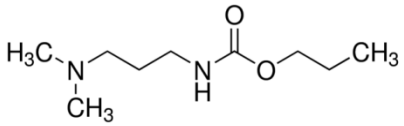
Deoxinivalenol (DON)

IUPAC name: **(3β,7α)-3,7,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one**
Formula: C15H20O6
Molecular Weight: 296.125977 Da
pKa: 11.91
Solubility: 19 g/L
Melting point: 543.875 °C a 760 mmHg

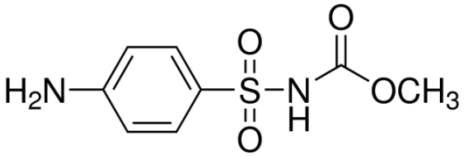
Fusarenon X (F-X)

IUPAC name: **(3β,4α,6β,11ξ)-3,7,15-Trihydroxy-8-oxo-12,13-epoxytrichothec-9-en-4-yl acetate**
Formula: C17H22O8
Molecular Weight: 354.13147 Da
pKa: 11.70
Solubility: 8.9 g/L
Melting point: 569.219 °C at 760 mmHg

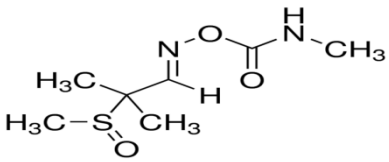
Zearalenon (ZEN)

IUPAC name: **(3S,11E)-14,16-Dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione**
Formula: C18H22O5
Molecular Weight: 318.146729 Da
pKa: 7.58
Solubility: 1000 g/L at pH 10
Melting point: 600.396 °C a 760 mmHg

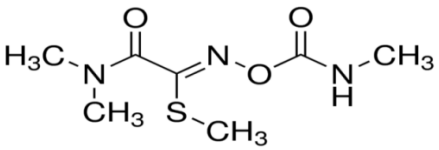
2. The physical and chemical properties of carbamates

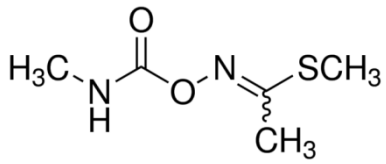
The physico-chemical properties of CRBs have been obtained from various sources such as Scifinder, chemispider or Chemfinder as follow:

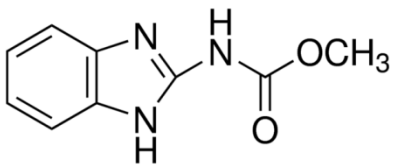
Propamocarb (PRM)

IUPAC name: propyl N-[3-(dimethylamino)propyl]carbamate
Formula: C9H20N2O
Molecular Weight: 188.2673 g/mol
pKa: 9.5 and 12.7
Melting point: 45-55°C

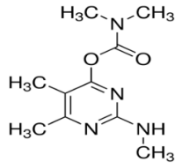
Asulam (ASL)

IUPAC name: methyl-N- (4-aminophenyl sulfonyl) carbamate
Formula: $C_8H_{10}N_2O_4S$
Molecular Weight: 230.24
pKa: 1.4 and 4.6
Melting point: 142-144°C

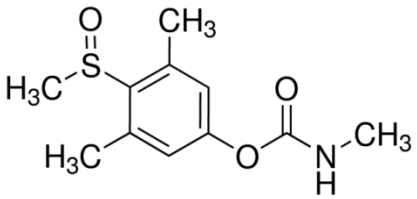
Aldicarb sulfoxide (ALDSFX)

IUPAC name: 2-methyl-2-(methylthio) propanal O-[methylamino) carbonyl]oxime
Formula: $C_7H_14N_2O_3S$
Molecular Weight: 206.26
pKa: -1.2 and 13.5
Melting point: 99-100°C

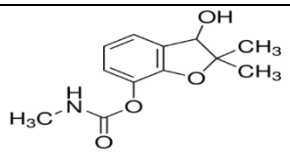
Oxamyl (OX)

IUPAC name: N, N-dimethyl-2-imino-2- methyl carbamoyl(Methylthio) acetamide
Formula: $C_7H_{13}N_3O_3S$
Molecular Weight: 219.26
pKa: -1.7 and 10.4
Melting point: 108-110 °C

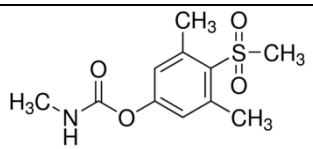
Methomyl (MTY)

IUPAC name: **Methyl (1E)-N-[(methylcarbamoyl)oxy]ethanimidothioate**
Formula: $C_5H_{10}N_2O_2S$
Molecular Weight: 162.21
pKa: -1.3 and 13.3
Melting point: 78 to 79 °C

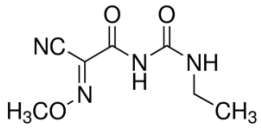
Carbendazim (CBZ)

IUPAC name: Methyl 1H-benzimidazol-2-ylcarbamate
Formula: $C_9H_9N_3O_2$
Molecular Weight: 191.19
pKa: 5.6 and 11.6
Melting point: 302 to 307 °C

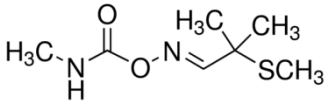
Pirimicarb desmethyl (PIRDES)

IUPAC name: (2-Dimethylamino-5,6-dimethylpyrimidin-4-yl)*N*,*N*-dimethylcarbamate
Formula: $C_{10}H_{16}N_4O_2$
Molecular Weight: 224.26
pKa: 4.4 and 8.7
Melting point: ---

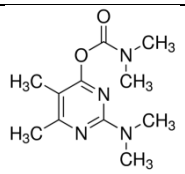
Methiocarb sulfoxide (MTHSFX)

IUPAC name: 3,5-dimethyl-4-(methylsulfinyl)-phenomethylcarbamate
Formula: $C_{11}H_{15}NO_3S$
Molecular Weight: 241.31
pKa: -1.6 and 12.0
Melting point: 118.5 °C

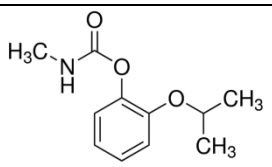
3- hidroxycarbofuran (3-CF)

IUPAC name: 3,7-Benzofurandiol, 2,3-dihydro-2,2-dimethyl-, 7-(methylcarbamate)
Formula: $C_{12}H_{15}NO_4$
Molecular Weight: 237.25
pKa: -1.5 and 12.3
Melting point: 140-148 °C

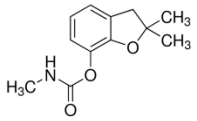
Methiocarb sulfone (MTHSFN)

IUPAC name: (3,5-dimethyl-4-methylsulfonylphenyl) N-methylcarbamate
Formula: $C_{11}H_{15}NO_4S$
Molecular Weight: 257.31
pKa: -1.5 and 11.9
Melting point: ----

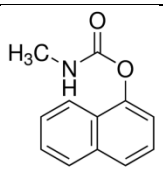
Cymoxanil (CY)

IUPAC name: **1-(2-Cyano-2-methoxyiminoacetyl)-3-ethylurea**
Formula: $C_7H_{10}N_4O_3$
Molecular Weight: 198.18
pKa: -1.7 and 7.4
Melting point: 162°C

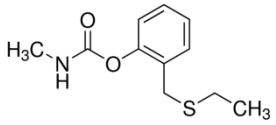
Aldicarb (ALD)

IUPAC name: 2-Methyl-2-(methylthio)propanal *O*-(*N*-methylcarbamoyl)oxime
Formula: $C_7H_{14}N_2O_2S$
Molecular Weight: 190.26
pKa: -1.1 and 13.8
Melting point: 100 °C

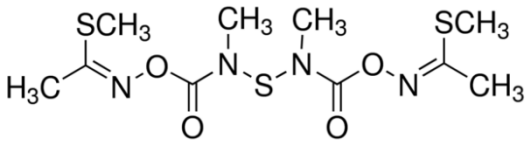
Pirimicarb (PIR)

IUPAC name: (2-Dimethylamino-5,6-dimethylpyrimidin-4-yl)*N*,*N*-dimethylcarbamate
Formula: $C_{11}H_{18}N_4O_2$
Molecular Weight: 238.29
pKa: 5.6 and 9.0
Melting point: 90.5 C

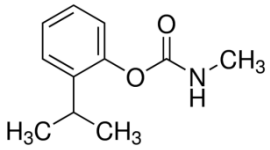
Propoxur (PX)

IUPAC name: 2-Isopropoxyphenyl *N*-methylcarbamate
Formula: $C_{11}H_{15}NO_3$
Molecular Weight: 209.25
pKa: -1.5 and 12.3
Melting point: 86 to 92 °C

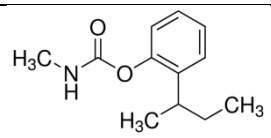
Carbofuran (CF)

IUPAC name: 2,2-Dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate
Formula: $C_{12}H_{15}NO_3$
Molecular Weight: 221.26
pKa: -1.5 and 12.3
Melting point: 151 °C

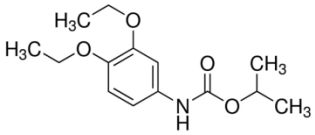
Carbaryl (CAR)

IUPAC name: 1-naphthyl methylcarbamate
Formula: $C_{12}H_{11}NO_2$
Molecular Weight: 201.23
pKa: -1.5 and 12
Melting point: 142 °C

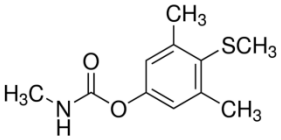
Ethiofencarb (ETH)

IUPAC name: α-Ethylthio-*o*-tolyl methylcarbamate
Formula: $C_{11}H_{15}NO_2S$
Molecular Weight: 225.31
pKa: -1.5 and 12.1
Melting point: 33.4 °C

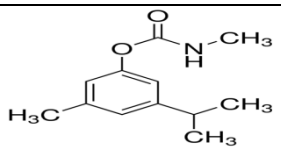
Thiodicarb (TH)

IUPAC name: Dimethyl-N,N'-{thio bis[(methylamino)carbonyl oxy]}bis(ethanimidothioat)
Formula: $C_{10}H_{18}N_4O_4S_3$
Molecular Weight: 354,47
pKa: -1.8
Melting point: 173–174°C

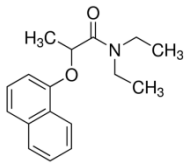
Isoprocarb (ISO)

IUPAC name: **2-Isopropylphenyl N-methylcarbamate**
Formula: $C_{11}H_{15}NO_2$
Molecular Weight: 193.24
pKa: 12.22
Melting point: 88-93° C

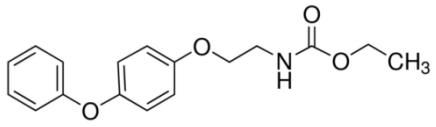
Fenobucarb (FEN)

IUPAC name: (2-Butan-2-ylphenyl) *N*-methylcarbamate
Formula: $C_{12}H_{17}NO_2$
Molecular Weight: 207.27
pKa: -1.5 and 12.2
Melting point: 32°C

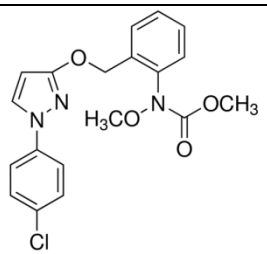
Diethofencarb (DETH)

IUPAC name: **Isopropyl (3,4-diethoxyphenyl)carbamate**
Formula: $C_{14}H_{21}NO_4$
Molecular Weight: 267.32
pKa: 0.9 and 12.8
Melting point: 100 °C

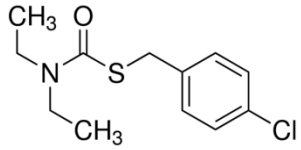
Methiocarb (MTH)

IUPAC name: 3,5-Dimethyl-4-(methylsulfanyl)phenyl *N*-methylcarbamate
Formula: $C_{11}H_{15}NO_2S$
Molecular Weight: 225.31
pKa: -1.6 and 12.3
Melting point: 118.5 °C

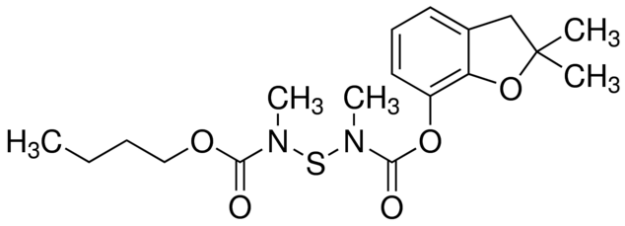
Promecarb (PR)

IUPAC name: 3-Isopropyl-5-methylphenyl methylcarbamate
Formula: $C_{12}H_{17}NO_2$
Molecular Weight: 207.27
pKa: -1.5 and 12.4
Melting point:87.0

Napropamid (NP)

IUPAC name: N, N-diethyl-2- (1-naphthyloxy) propionamide
Formula: $C_{17}H_{21}NO_2$
Molecular Weight: 271.35
pKa: -0.8
Melting point: 75 °C

Fenoxycarb (FNX)

IUPAC name: Ethyl 2-(4-phenoxyphenoxy)ethylcarbamate
Formula: $C_{17}H_{19}NO_4$
Molecular Weight: 301.34
pKa: -1.5 and 12.5
Melting point: 53.5 °C

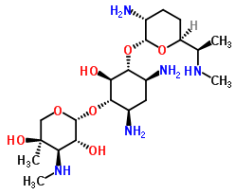
Pyraclostrobin (PY)

IUPAC name: **Methyl {2-[1-(4-chlorophenyl)-1*H*-pyrazol-3-yloxymethyl]phenyl}methoxycarbamate**
Formula: $C_{19}H_{18}ClN_3O_4$
Molecular Weight: 387.82
pKa: -0.3
Melting point: 59-62 °C

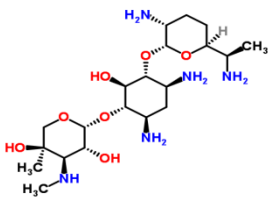
Benthiocarb (BTH)

IUPAC name: *S*-(4-Chlorobenzyl) diethylcarbamothioate
Formula: $C_{12}H_{16}ClNOS$
Molecular Weight: 257.780
pKa: -1.3
Melting point: 3.3 °C

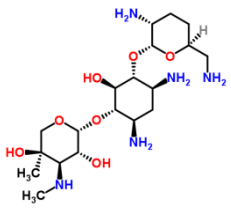
Furathiocarb (FURA)

IUPAC name: (2,2-dimethyl-3H-1-benzofuran-7-yl) N-[butoxycarbonyl(methyl)amino]sulfanyl-N-methylcarbamate
Formula: $C_{18}H_{26}N_2O_5S$
Molecular Weight: 382.48
pKa: -2.1
Melting point: 160 °C

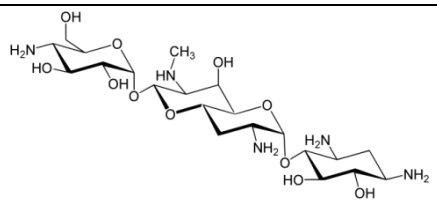
3. The physical and chemical properties of Antibiotics

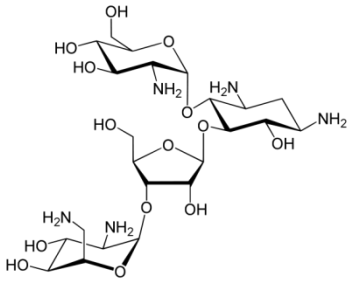
3.1. Aminoglycosides

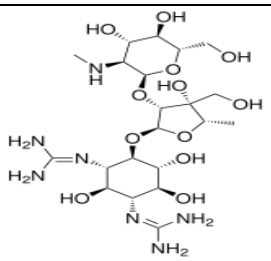
Gentamicin C1 (GENT C1)

IUPAC name: (1S,2S,3R,4S,6R)-4,6-Diamino-3-({(2R,3R,6S)-3-amino-6-[(1R)-1-(methylamino)ethyl]tetrahydro-2H-pyran-2-yl}oxy)-2-hydroxycyclohexyl 3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranoside
Formula: $C_{21}H_{43}N_5O_7$
Molecular Weight: 477.6 Da
pKa: 8.6
Melting point: 218-237 °C

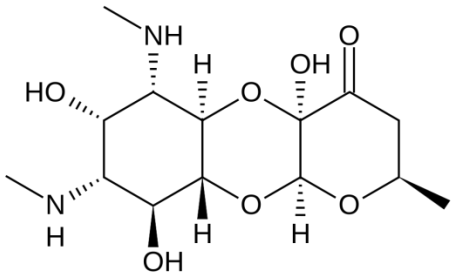
Gentamicin C2 (GENT C2)

IUPAC name: (2R,3R,4R,5R)-2-[(1S,2S,3R,4S,6R)-4,6-diamino-3-[(2R,3R,6S)-3-amino-6-[(1R)-1-aminoethyl]oxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-5-methyl-4-(methylamino)oxane-3,5-diol
Formula: $C_{20}H_{41}N_5O_7$
Molecular Weight: 463.569 Da
pKa: ----

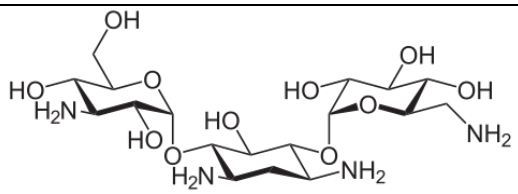
Melting point: 218-237 °C
Gentamicin C1a (GENT C1a)

IUPAC name: (1R,2S,3S,4R,6S)-4,6-Diamino-3-{[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]oxy}-2-hydroxycyclohexyl 2,6-diamino-2,3,4,6-tetradeoxy-α-D-erythro-hexopyranoside
Formula: $C_{19}H_{39}N_5O_7$
Molecular Weight: 449.5
pKa: ----
Melting point: 218-237 °C

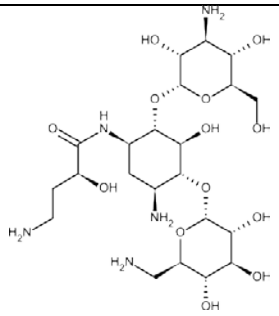
Apramycin (APM)

IUPAC name: (2R,3R,4S,5S,6S)-2-[[(2R,3S,4R,4aR,6S,7R,8aS)-7-amino-6-[(1R,2R,3S,4R,6S)-4,6-diamino-2,3-dihydroxycyclohexyl]oxy-4-hydroxy-3-(methylamino)-2,3,4,4a,6,7,8,8a-octahydropyrano[3,2-b]pyran-2-yl]oxy]-5-amino-6-(hydroxymethyl)oxane-3,4-diol
Formula: $C_{21}H_{41}N_5O_{11}$
Molecular Weight: 539.58
pKa:8.5
Melting point: 245-247°C

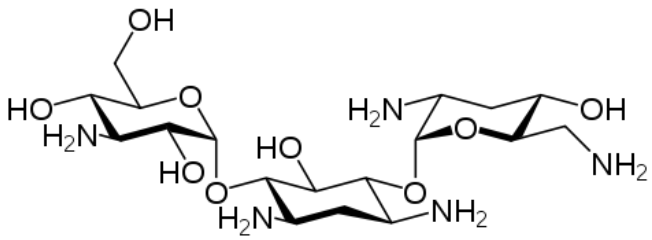
Paromomycin (PRM)

IUPAC name: (2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[(1*R*,2*S*,3*S*,4*R*,6*S*)- 4,6-diamino-2-[(2*S*,3*R*,4*R*,5*R*)-4-[(2*R*,3*R*,4*R*,5*R*,6*S*)- 3-amino-6-(aminomethyl)-4,5-dihydroxy-oxan-2-yl] oxy-3-hydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy- 3-hydroxy-cyclohexyl]oxy-2-(hydroxymethyl)oxane-3,4-diol
Formula: $C_{23}H_{47}N_5O_{18}S$
Molecular Weight: 615.629
pKa: 12.23 and 9.94
Melting point: -----

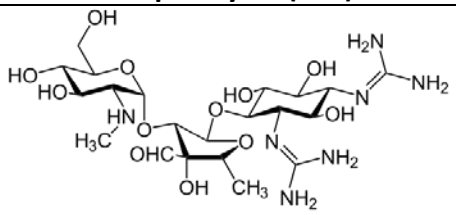
Dihydrostreptomycin (DHS)

IUPAC name: 2-[(1*S*,2*R*,3*R*,4*S*,5*R*,6*R*)-5-(Diaminomethylideneamino)-2-[(2*R*,3*R*,4*R*,5*S*)-3-[(2*S*,3*S*,4*S*,5*R*,6*S*)- 4,5-dihydroxy-6-(hydroxymethyl)-3-methylaminooxan-2-yl]oxy-4-hydroxy-4-(hydroxymethyl)- 5-methyloxolan-2-yl]oxy-3,4,6-trihydroxycyclohexyl]guanidine
Formula:$C_{21}H_{41}N_7O_{12}$
Molecular Weight: 583.59
pKa: 11.25 and 8.08
Melting point: ----

Spectinomycin (SPC)

IUPAC name: (1*R*,3*S*,5*R*,8*R*,10*R*,11*S*,12*S*,13*R*,14*S*)-8,12,14-trihydroxy-5-methyl-11,13-*bis*(methylamino)-2,4,9-trioxatricyclo[8.4.0.0^{3,8}]tetradecan-7-one
Formula: $C_{14}H_{24}N_2O_7$
Molecular Weight: 332.35
pKa: 6.95
Melting point: 205-207°

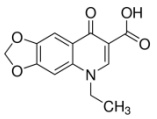
Kanamycin (KAM)

IUPAC name: 2-(aminomethyl)- 6-[4,6-diamino-3- [4-amino-3,5-dihydroxy-6-(hydroxymethyl) tetrahydropyran-2-yl]oxy- 2-hydroxy- cyclohexoxy]- tetrahydropyran- 3,4,5-triol
Formula: $C_{18}H_{36}N_4O_{11}$
Molecular Weight: 484.499
pKa: 12.11 and 9.75
Melting point: 275° C

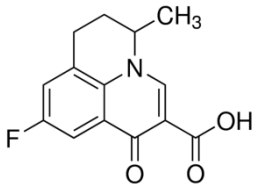
Amikacin (AM)

IUPAC name: (2*S*)-4-Amino-*N*-[(2*S*,3*S*,4*R*,5*S*)-5-amino-2-[(2*S*,3*R*,4*S*,5*S*,6*R*)-4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4-[(2*R*,3*R*,4*S*,5*R*,6*R*)-6-(aminomethyl)-3,4,5-trihydroxy-oxan-2-yl]oxy-3-hydroxy-cyclohexyl]-2-hydroxybutanamide
Formula: $C_{22}H_{43}N_5O_{13}$
Molecular Weight: 585.603
pKa: 12.1 and 9.7
Melting point: 203-204 °C

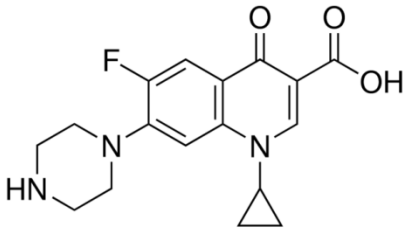
Tobramycin (TOM)

IUPAC name: (2*S*,3*R*,4*S*,5*S*,6*R*)-4-amino-2-{[(1*S*,2*S*,3*R*,4*S*,6*R*)-4,6-diamino-3-{[(2*R*,3*R*,5*S*,6*R*)-3-amino-6-(aminomethyl)-5-hydroxyoxan-2-yl]oxy}-2-hydroxycyclohexyl]oxy}-6-(hydroxymethyl)oxane-3,5-diol
Formula: $C_{18}H_{37}N_5O_9$
Molecular Weight: 467.515
pKa: 12.54 and 9.83
Melting point:

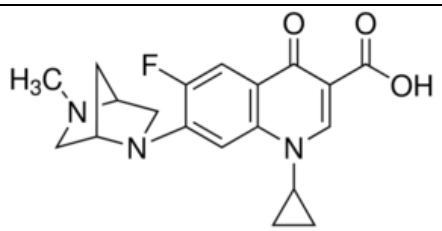
Streptomycin (STP)

IUPAC name: 5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)- 4-[4,5-dihydroxy-6-(hydroxymethyl) -3-methylamino-tetrahydropyran-2-yl] oxy-3-hydroxy-2-methyl -tetrahydrofuran-3-carbaldehyde
Formula: $C_{21}H_{39}N_7O_{12}$
Molecular Weight: 581.574
pKa: 10.88 and 11.9
Melting point: 12 °C

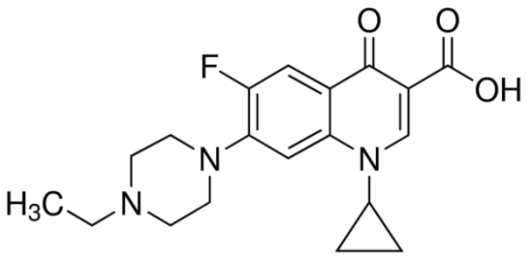
3.2. Quinolones

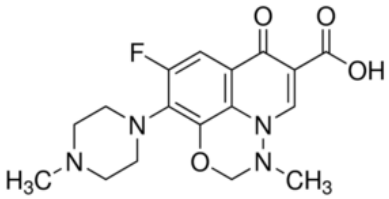
Oxolinic acid

IUPAC name: 5-Ethyl-8-oxo-5,8-dihydro[1,3]dioxolo[4,5-*g*]quinoline- 7-carboxylic acid
Formula: $C_{13}H_{11}NO_5$
Molecular Weight: 261.23
pKa: 5.94
Melting point: 314-316° C

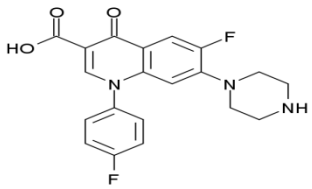
Flumequine

IUPAC name: 7-fluoro-12-methyl-4-oxo-1-azatricyclo[7.3.1.0^{5,13}]trideca-2,5,7,9(13)-tetraene-3-carboxylic acid
Formula: $C_{14}H_{12}FNO_3$
Molecular Weight: 261.25
pKa: 6 and –4.3
Melting point: 253 to 255 °C

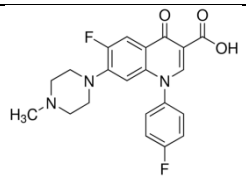
Ciprofloxacin

IUPAC name: 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxylic acid
Formula: $C_{17}H_{18}FN_3O_3$
Molecular Weight: 331.346
pKa: 6.09
Melting point: 255-257 °C

Danofloxacin

IUPAC name: 1-Cyclopropyl-6-fluoro-7-[(1*S*,4*S*)-3-methyl-3,6-diazabicyclo[2.2.1]heptan-6-yl]-4-oxoquinoline-3-carboxylic acid
Formula: $C_{19}H_{20}FN_3O_3$
Molecular Weight: 357.37
pKa: 6.2 and 9.4
Melting point: 328°C

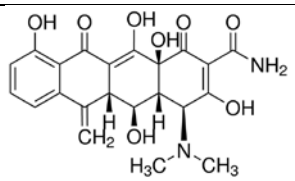
Enrofloxacin

IUPAC name: 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid
Formula: $C_{19}H_{22}FN_3O_3$
Molecular Weight: 359.4
pKa: 7.7
Melting point: 225 °C

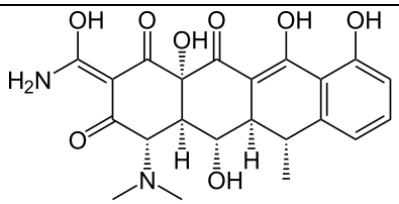
Marbofloxacin

IUPAC name: 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyridol(3,2,1-ij)(4,2,1)benzoxadiazin-6 carboxylic acid
Formula: $C_{17}H_{19}FN_4O_4$
Molecular Weight: 362.356
pKa: 6.02 and 7.34
Melting point: 269 °C

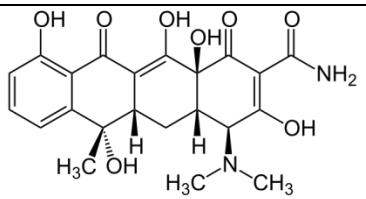
Sarafloxacin

IUPAC name: 6-fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid
Formula: $C_{20}H_{17}F_2N_3O_3$
Molecular Weight: 385.36
pKa: **----**
Melting point: > 300°C

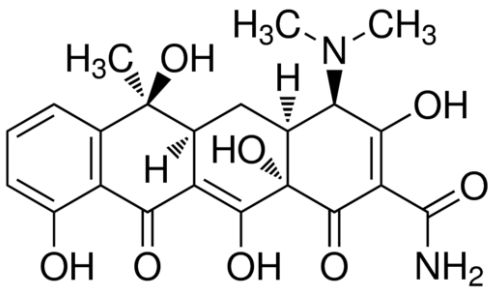
Difloxacin

IUPAC name: 6-fluoro-1-(4-fluorophenyl)-7-(4-methylpiperazin-1-yl)-4-oxoquinoline-3-carboxylic acid
Formula: $C_{21}H_{19}F_2N_3O_3$
Molecular Weight: 399.39
pKa: ------
Melting point: ------

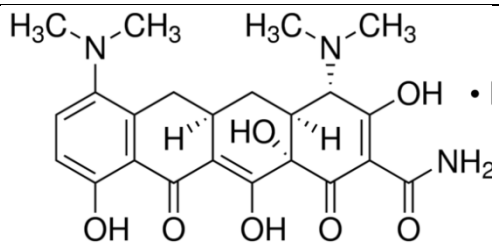
3.3. Tetracycline

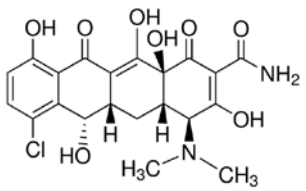
Metahcycline

IUPAC name: (2*Z*,4*S*,4a*R*,5*S*,5a*R*,12a*S*)-2-[amino(hydroxy)methylene]-4-(dimethylamino)-5,10,11,12a-tetrahydroxy-6-methylene-4a,5a,6,12a-tetrahydrotetracene-1,3,12(2*H*,4*H*,5*H*)-trione
Formula: $C_{22}H_{22}N_2O_8$
Molecular Weight: 442.419
pKa: 3.5 and 9.5
Melting point:-----

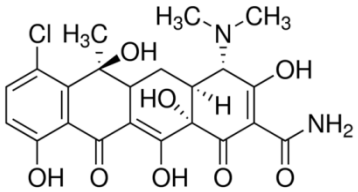
Doxycycline

IUPAC name: (4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy- 6-methyl- 1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene- 2-carboxamide
Formula: $C_{22}H_{24}N_2O_8$
Molecular Weight: 444.43
pKa: -2.2 and 7.75
Melting point: 201 °C

Tetracycline

IUPAC name: (4S,4AS,5as,12as)-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide
Formula: $C_{22}H_{24}N_2O_8$
Molecular Weight: 444.435
pKa: -2.2 and 8.24
Melting point: 170-173°C

4-Epitetracycline

IUPAC name: (4R,4aS,5aS,6S,12aS)-4-(DiMethylaMino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-Methyl-1,11-dioxo-2-naphthacenecarboxaMide
Formula: $C_{22}H_{24}N_2O_8$
Molecular Weight: 444.44
pKa: -----
Melting point: 217 °C

Minocycline

IUPAC name: (2*E*,4*S*,4a*R*,5aS,12a*R*)- 2-(amino-hydroxy-methylidene)-4,7-bis(dimethylamino)- 10,11,12a-trihydroxy-4a,5,5a,6-tetrahydro-4H-tetracene- 1,3,12-trione
Formula: $C_{23}H_{27}N_3O_7$
Molecular Weight: 457.477
pKa: -2.3 and 8.25
Melting point: ----

Demeclocycline

IUPAC name: (2*E*,4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-[amino(hydroxy)methylidene]-7-chloro-4-(dimethylamino)-6,10,11,12a-tetrahydroxy-1,2,3,4,4a,5,5a,6,12,12a-decahydrotetracene-1,3,12-trione
Formula: $C_{21}H_{21}ClN_2O_8$
Molecular Weight: 464.853
pKa: -2.6 and 8.23
Melting point: 220-223 °C

Chlortetracycline

IUPAC name: (4*S*,4a*S*,5a*S*,6*S*,12a*S*,*Z*)-2-[amino(hydroxy)methylene]-7-chloro-4-(dimethylamino)-6,10,11,12a-tetrahydroxy-6-methyl-4a,5,5a,6-tetrahydrotetracene-1,3,12(2*H*,4*H*,12a*H*)-trione
Formula: $C_{22}H_{23}ClN_2O_8$
Molecular Weight: 478.88
pKa: 3.3 and 9.27
Melting point: 168.50 °C

ABBREVIATIONS

List of abbreviations and acronyms:

ACO:	Acetone
ADI	Acceptable daily intake
AFB_1:	Aflatoxin B1
AFB_2:	Aflatoxin B2
AFG_1:	Aflatoxin G1
AFG_2:	Aflatoxin G2
AFM_1:	Aflatoxin M1
AFs:	Aflatoxins
AGs:	Aminoglycosides
ALD:	Aldicarb
ALDSFX:	Aldicarb sulfoxide
AMK:	Amikacin
APM:	Apramycin
ASL:	Asulam
BGE	background electrolyte
BTH:	Benthiocarb
CAR:	Carbaryl
CBZ:	Carbendazim
CCα :	Decision limit
CCβ:	Detection capability

CDC	Disease Control and prevention
CE:	Capillary electrophoresis
CEC:	Capillary electrochromatography
CEN:	Collision energy
CEP:	Collision cell entrance potential
CF:	Carbofuran
CGE:	Capillary gel electrophoresis
CIEF:	Capillary isoelectrofocusing
CITP:	Capillary isotacophoresis
CRBs:	Carbamates
CSF:	Carbosulfan
CUR:	Curtain gas
CXP:	Collision exit potential
CY:	Cymoxanil
CZE:	Capillary zone electrophoresis
DAD:	Diode array detection
DETH:	Diethofencarb
DHS:	Dihydrostreptomycin
DLLME:	Dispersive liquid-liquid microextraction
DP:	Declustering potential
dSPE:	Dispersive solid phase extraction

EFSA:	European Food Safety Authority
ELISA:	Enzyme linked immunosorbent assays
EP:	Entrance potential
EPA:	Environmental protection agency
ESI (+):	Electrospray ionization positive mode
ESI:	Electrospray ionization
ETH:	Ethiofencarb
EtOH:	Ethanol
EU:	European Union
FA:	Formic acid
FAO:	Food and Agriculture Organization
FASS	Field amplified sample stacking
FB1:	Fumonisin B1
FB2:	Fumonisin B2
FDA	Food and Drug Administration
FEN:	Fenobucarb
FLD:	Fluorescent detection
FNX:	Fenoxycarb
FURA:	Furathiocarb
F-X:	Fusarenon-X
GC:	Gas chromatopraphy

GNT:	Gentamicin
GS1/GS2:	Dry gas
HILIC:	Hydrophilic interaction liquid chromatography
HPLC:	High performance liquid chromatography
HPLC-PI-FLD:	High performance liquid chromatography-photoinduced-fluorescence detection
HRMS:	High resolution mass spectrometry
HT-2:	HT-2 toxin
I:	Identification ions.
IAC:	Immunoaffinity column
IARC:	Agency for Research on Cancer
IL-DLLME:	Dispersive liquid-liquid microextraction with ionic liquid
I.D	Inner diameter
IPA:	Isopropanol
IPLC:	Ion-pair liquid chromatography
IS:	Ion spray voltage
ISO:	Isoprocarb
IT:	Ion trap
KNM:	Kanamycin
LC:	Liquid chromatography

LIF:	Laser induced fluorescence
LLE:	Liquid-liquid extraction
LLME:	Liquid-liquid microextraction
LOD:	Limit of detection
LOQ:	Limit of quantification
ME:	Matrix effect
MeCN:	Acetonitrile
MEKC:	Micellar electrokinetic chromatography
MeOH:	Methanol
METOL:	Metolcarb
MIPs:	Molecularly imprinted polymers
MISPE:	Molecularly imprinted solid phase extraction
MRL:	Maximum residue limit
MRM:	Multiple reaction monitoring
MS/MS:	Tandem mass spectrometry
MS:	Mass spectrometry
MTH:	Methiocarb
MTHSFN:	Methiocarb sulfone
MTHSFX:	Methiocarb sulfoxide
MTY:	Methomyl
NEO:	Neomycin

NIV:	Nivalenol
NP:	Napropamid
OCP:	Organochlorine pesticide
OPA:	O-phtalaldehide
OTA:	Ochratoxin A
OX:	Oxamyl
PIR:	Pirimicarb
PIRDES:	Pirimicarb desmethyl
PMTDI:	Provisional maximum tolerable daily intake
PR:	Promecarb
PRM:	Paromomycin
PRM:	Propamocarb
PSA:	Primary secondary amine
PX:	Propoxur
PY:	Pyraclostrobin
Q:	Quantification ions
QNs	Quinolones
QqQ:	Triple quadrupole
QuEChERS:	Quick, Easy, Cheap, Effective, Rugged and Safe
Q-ToF:	Quadrupole time-of-flight
R (%):	Percentage of recovery

RASFF:	Rapid alert system for food and feed
RPLC	Reversed phase liquid chrpmatography
rpm:	Revolution per minute
RSD:	Relative standard deviation
Rt:	Retention time
S/N:	Signal to noise ratio
SEF_{height}	Sensitivity enhancement factors based on peak heights
SPC:	Spectinomycin
SPE:	Solid phase extraction
SPME:	Solid phase microextraction
STP:	Streptomycin
T-2:	T-2 toxin
TC:	Tetracycline
TCA:	Trichloroacetic acid
TFA:	Trifluoroacetic acid
TH:	Thiodicarb
TLC:	Thin layer chromatography
TOF:	Time of flight
TOM:	Tobramycin
UASEME:	Ultrasound-assisted surfactant-enhanced emulsification microextraction

UHPLC:	Ultra-high performance liquid chromatography
UV-Vis:	Ultraviolet-visible
WHO:	World health organization
ZEN:	Zearalenone
ZIC:	Zwitter ionic