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Multiclass method for the determination of quinolones and β-lactams, in raw cow milk using dispersive liquid–liquid microextraction and ultra high performance liquid chromatography–tandem mass spectrometry

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This article is dedicated to the memory of Professor Ramon Companyó of the University of Barcelona (Spain).

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ABSTRACT

An analytical method based on a sample treatment by dispersive liquid–liquid microextraction (DLLME) followed by ultra high performance liquid chromatography–tandem mass spectrometry analysis (UHPLC–MS/MS) for the determination of 17 quinolones and 14 β-lactams (penicillins and cephalosporins) in raw cow milk, was validated according to the European Commission guidelines as cited in the Decision 2002/657/EC. The extraction efficiency of the DLLME depends on several parameters such as the nature and volumes of extractant and dispersive solvents, pH, concentration of salt, shaking time and time of centrifugation. These variables were accurately optimized using multivariate optimization strategies. A Plackett–Burman design to select the most influential parameters and a Doehlert design to obtain the optimum conditions have been applied. Two different pH values were used for the extraction of compounds (pH 3 for acidic quinolones and β-lactams and pH 8 for amphoteric quinolones). The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. The limits of quantification found ranged from 0.3 ng g^{-1} for amoxicillin to 6.6 ng g^{-1} for ciprofloxacin, and the precision was lower than 15% in all cases as is required by the European Regulation. The decision limits (CC_α) ranged between 4.1 and 104.8 ng g^{-1}, while detection capabilities (CC_β) from 4.2 to 109.7 ng g^{-1}. These values were very close to the corresponding maximum residue limits (MLRs) for the studied antibiotics. Recoveries between 72 and 110% were also obtained. Finally, in order to evaluate the applicability of the method, 28 raw cow milk samples were analysed and it was observed that 28% of the samples were positive. However, only 11% were considered non-compliant with the current EU legislation (Commission Regulation 37/2010), due to some milk samples corresponded to treated cows with these antibiotics.

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1. Introduction

Antibiotics are widely used in human and veterinary medicine to treat bacterial infections. Moreover, they are also administered at sub-therapeutic doses in food-producing animals as prophylactics or growth promoters, although the European Union (EU) legislation has forbidden this last practice since 2006 [1]. The presence of antibiotics represents a risk to consumer health since it could cause problems of toxicity, allergy and bacterial resistance. In order to protect the consumer health, the EU Regulation 37/2010 [2] established the maximum residue limit (MRL) in animal products destined to human consumption. In this context, it is of great importance to have robust, accurate and sensitive analytical methods for the determination of these compounds in food of animal origin.

Due to their matrix complexity, the analysis of food of animal origin at trace levels, such as milk and dairy products, has nowadays main analytical problems. Thus, sample treatment is completely

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necessary in the determination of different kind of compounds in these matrices, to eliminate interferences and preconcentrate the analytes.

Traditionally, the sample treatment techniques used to determine antibiotics in animal products have been the liquid–liquid extraction (LLE) [3] and the solid-phase extraction (SPE) [4,5]. Despite the fact that LLE shows good analytical performance, several disadvantages are associated to this technique, including the formation of emulsions, the need of large sample volumes and toxic organic solvents, which make LLE a costly, time-consuming and environmentally unfriendly technique. Although SPE uses much less solvent amounts than LLE, it can still be considered significant and an extra step is usually needed to preconcentrate the analytes further into smaller volumes. SPE is also time-consuming and relatively expensive. The drive for "green" methods to overcome these inherent problems of conventional LLE and SPE has led to the development of miniaturized and solvent-minimized sample preparation techniques such as solid-phase microextraction (SPME). SPME has been used for the determination of tetracyclines in fish [6] and quinolones in eggs [7]; and other techniques like molecularly imprinted polymers (MIP) as sorbent in SPE (MISPE) have been applied for the determination of cephalosporins in milk [8] and tetracyclines in eggs [9]; automated techniques like pressurized liquid extraction (PLE) have been also used for analysis of tetracyclines in bovine, swine, poultry and lamb muscle tissues [10] and QuEChERS (quick, easy, cheap, effective, rugged and safe) for cephalosporins in beef muscle [11]. These new techniques have important improvements in comparison with classical techniques [12].

Despite the advantages provided by these techniques, they have some problems, for example, most commercial fibres used in SPME are relatively expensive, fragile and have limited lifetime. Moreover, sample carry-over is a possible problem. On the other hand, the initial investment to purchase PLE units is high, although the use of this technique will "repay" the costs because serial samples can be extracted in a fraction of the time, with a higher degree of automation.

Liquid-phase microextraction (LPME) with its various modes such as dispersive liquid–liquid microextraction (DLLME), single drop microextraction (SDME), hollow fibre–liquid phase microextraction (HF–LPME) and solvent-bar microextraction (SBME), among others, has emerged to overcome the problems of the other techniques. DLLME has gained increased prominence for its rapidity, simplicity of operation, cheapness, high enrichment factor, environmental friendliness, decreasing waste generation and ability to provide high extraction efficiencies. So, it is clear that the strong reduction of reagents and solvents is welcome from the environmental point of view. The heart of this method lies in the formation of a ternary solvent system composed of the aqueous solution containing the analytes, a water-immiscible extraction solvent and a water-miscible disperser solvent. Briefly, the extraction involves the rapid injection of a mixture of the extraction and disperser solvent in an aqueous sample. This injection causes the formation of small droplets which spread throughout the aqueous sample. The emulsified droplets have a great interstitial area and, consequently, the equilibrium is rapidly reached and the extraction is almost instantaneous. DLLME has been successfully applied for preconcentration of several trace analytes in water, environmental and biological samples, including food of animal origin [13–16]. In case of milk samples, although this technique has been used to analyse macrocyclic lactones [17], nonsteroidal anti-inflammatory drugs [18], triazole pesticides and aflatoxin M1 [19], there are few papers which have reported the determination of antibiotics in milk [20–23].

DLLME has attracted considerable attention and been widely accepted, as evidenced by its appearance in a continually increasing number of original articles and reviews since the technique's introduction. The number of publications on DLLME is growing exponentially, because it provides an extraordinary analytical potentiality using relatively ordinary instrumentation, being compatible with analytical techniques such as gas chromatography (GC) and high or ultra high performance liquid chromatography (HPLC or UHPLC).

Since quinolones and β-lactams (penicillins and cephalosporins) are among the most used families in veterinary medicine, the aim of the present work was the optimization and validation of a multiclass and multiresidue method for the determination of 17 quinolones and 14 β-lactams in raw cow milk using DLLME followed by ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis. The method showed important improvements in comparison with previously published works, since it determines by far a larger number of quinolone and β-lactam antibiotics. As well, the method allowed obtaining similar LODs, LOQs and recoveries in comparison with other methods proposed in the scientific literature for the determination of quinolones and β-lactams in cow milk that use SPE [4]. Moreover, this method based on DLLME offers better precision than other widely used techniques, such as LLE or SPE [3,4], which are also more tedious and require high solvent consumption. In addition, the proposed DLLME procedure was exhaustively optimized in order to disregard any further cleaning step or additional sample treatment, considering milk is a complex matrix, which reduce significantly costs and analysis time. The method has been validated according to European Directive 2002/657/EC [24] and FDA guideline [25] and has been applied to several samples from treated and untreated animals with antibiotics.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Analytical grade standards were purchased from different pharmaceutical firms. Moxifloxacin (MOX), marbofloxacin (MAR), ofloxacin (OFL), enrofloxacin (ENR), lomefloxacin (LOM), ciprofloxacin (CIP), enoxacin (ENO), norfloxacin (NOR), pipemidic acid (PIPE), difloxacin (DIF), sarafloxacin (SAR), danofloxacin (DAN), piromidic acid (PIRO), cinoxacin, (CIN), oxolinic acid (OXO), flumequine (FLU), nalidixic acid (NAL), cephoperazone (PER), cephalexin (LEX), amoxicillin (AMO), nafcillin (NAF), oxacillin (OXA), 2-phenyl-4-quinoline carboxylic acid (cincophen, CIC, used as surrogate) and caffeine (CAF, used as internal standard) were purchased from Sigma–Aldrich (St. Louis, MO, USA); ceftiofur (TIO), cephazolin (ZOL), cephapirin (PIR), cloxacillin (CLO) and piperacillin (PIP, used as surrogate) from Fluka (Buchs, Switzerland); cefquinome (QUI) from AK Scientific, Inc. (USA); cephalonium (LON) from Schering-Plough Animal Health Corporation (Ireland); and ampicillin (AMP), dicloxacillin (DIC) and penicillin G (PENG) from European Pharmacopoeia (Strasbourg Cedex, France). Individual standard solutions of quinolones (200 μg mL^{-1}) were prepared in a water–methanol mixture (1:4, v/v) and in water for β-lactams (200 μg mL^{-1}). These solutions were stored at −20 °C and prepared fresh monthly. Working standard mixtures were prepared by diluting the individual stock solution in water. They were stored at 4 °C and prepared fresh weekly. All solutions were stored in dark glass bottles to prevent degradation.

LC-grade methanol (MeOH), ethanol (EtOH), acetone (Ace) and acetonitrile (ACN) were purchased from Merck (Darmstadt,

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