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Fabric phase sorptive extraction of selected penicillin antibiotic residues from intact milk followed by high performance liquid chromatography with diode array detection

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ABSTRACT

Fabric phase sorptive extraction (FPSE), a novel sorbent-based microextraction method, was evaluated as a simple and rapid strategy for the extraction of four penicillin antibiotic residues (benzylpenicillin, cloxacillin, dicloxacillin and oxacillin) from cows' milk, without prior protein precipitation. Time-consuming solvent evaporation and reconstitution steps were eliminated successfully from the sample preparation workflow. FPSE utilizes a flexible fabric substrate, chemically coated with sol-gel derived, highly efficient, organic-inorganic hybrid sorbent as the extraction medium. Herein short-chain poly(ethylene glycol) provided optimum extraction sensitivity for the selected penicillins, which were analysed using an RP-HPLC method, validated according to the European Decision 657/2002/EC. The limit of quantitation was 10 μg/kg for benzylpenicillin, 20 μg/kg for cloxacillin, 25 μg/kg dicloxacillin and 30 μg/kg oxacillin. These are a similar order of magnitude with those reported in the literature and (with the exception of benzylpenicillin) are less than the maximum residue limits (MRL) set by European legislation.

1. Introduction

Milk is an important source of energy, protein and micronutrients for the human body (FAO & Rural Infrastructure, 2013). The presence of veterinary drug residues in milk, such as antibiotics, diminishes its nutritional value to a great extent. Penicillins belong to the β-lactam antibiotic family, which prevent the formation of the bacterial cell. This is achieved by inhibiting the final stage of peptidoglycan synthesis. Penicillins are classified into six groups, according to the time they were introduced into use. Herein, one member of the first group, benzylpenicillin or penicillin-G (PENG), as procaine (long-acting form), and three from the third group, cloxacillin (CLO), dicloxacillin (DICLO) and oxacillin (OXA), were studied as examples. Benzylpenicillin is active against gram-positive bacteria. Cloxacillin, dicloxacillin and oxacillin are active against penicillinase-producing *S. aureus* and *S. pseudintermedius*. Benzylpenicillin is administered to cattle for treating anthrax, clostridial and corynebacterial infections, mastitis, haemorrhagic septicaemia and listeriosis. Group 3 penicillins are acid stable are administered orally primarily for the treatment and even prevention of bovine staphylococcal mastitis (Giguère, Prescott, & Dowling, 2013). Chemical structures and other relevant physicochemical properties of penicillins studied are shown as **supplementary material in Table Suppl. 1.

Administration of all antibiotics to cattle must follow the regulations to avoid residues in the milk, which might pose a risk to public health and safety because these antibiotics are also used as therapeutic agents for humans and could, subsequently, increase antibiotic-resistance (Piñero, Bauza, Arce, & Valcárcel, 2014). Maximum residue limits (MRL), set by European Commission in the Regulation of 37/2010/EC, are 4 μg/kg for benzylpenicillin and 30 μg/kg for cloxacillin, oxacillin and dicloxacillin. Routine monitoring for the presence of these penicillin antibiotics in milk at such low concentrations can be achieved either by using sophisticated analytical instruments or applying suitable sample preparation strategies. The challenge is to extract and pre-concentrate these analytes from milk samples to a detectable range, appropriate for downstream analytical instruments.

Abbreviations: FPSE, fabric phase sorptive extraction; PENG, benzylpenicillin/penicillin-G; CLO, cloxacillin; OXA, oxacillin; DICLO, dicloxacillin; TFA, trifluoroacetic acid.

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The presence of lipids, proteins, amino acids, vitamins, minerals, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, and other bioactive peptides (Haug, Høstmark, & Harstad, 2007) in milk make it a very complex matrix from a sample preparation standpoint. For the isolation of penicillins in milk, various sample pre-treatment techniques have been employed. One of these techniques involves centrifugation of whole milk followed by liquid-liquid extraction (LLE) with hexane and clean-up by solid-phase extraction (SPE) in a C_{18} column (Hsieh, Huang, & Lee, 2009). In another study, milk was deproteinized and extracted *via* tandem SPE with an OASIS HLB cartridge, and the eluent passed through an Alumina N cartridge for sample clean-up and preconcentration of analytes (Bailón-Pérez, García-Campana, del Olmo-Iruela, Gámiz-Gracia, & Cruces-Blanco, 2009). Solid phase extraction has also been used for the isolation of penicillins from ewes' milk, following protein precipitation using acetonitrile (Cámara et al., 2013). A mixed micelle-cloud point extraction using Triton X-114 (TX-114) and cetyltrimethylammonium bromide as the mixed micellar extractant, has also been proposed. Prior to extraction, a fat and protein removal step was necessary, followed by evaporation and reconstitution with a buffer solution (Kukusamude et al., 2010). A modified matrix solid phase dispersion procedure was applied for the extraction and clean-up of antibiotics, using a mixture of QuEChERS and Strata-X sorbent (Karageorgou & Samanidou, 2011). A simple protein precipitation procedure was described by Jank, Hoff, Tarouco, Barreto, and Pizzolato (2012), using two parts of ACN vs. one part of milk, followed by the addition of sodium chloride, while Cepurnieks, Rjabova, Zacs, and Bartkevics (2015) used 5% trichloroacetic acid as deproteinizing agent. Ion-paired extraction, using tetrabutylammonium bromide as an ion-pairing agent and water-acetonitrile as a solvent, was proposed by Kukusamude, Burakham, Chailapakul, and Srijaranai (2012), while dispersive liquid-liquid microextraction was introduced by Junza et al. (2014) for the determination of quinolones and β-lactams in bovine milk.

The current trend in modern analytical chemistry is to follow the demands of green analytical chemistry (Armenta, Garriguez, & de la Guardia, 2008) using minimal amounts of organic solvent or not at all as well as the miniaturization of sample preparation techniques. Most of the analytical methods above use relatively large amounts of toxic, organic solvents and other consumables. In addition, most require time-consuming protein precipitation prior to the extraction of target analytes as well as time-consuming solvent evaporation. These steps often contribute to the substantial loss of analytes. There is, therefore, a strong need for a miniaturized sorptive microextraction approach that offer the advantages of being green, simple and cost-effective and effectively eliminate the necessity for solvent evaporation.

Fabric phase sorptive extraction (FPSE), a new generation sample preparation technology, developed by Kabir and Furton (2014), has overcome most of the drawbacks generally experienced in conventional sample preparation methodologies. FPSE requires no matrix modifications or clean-up steps. It can be used both in the equilibrium (as in solid phase microextraction) and the exhaustive extraction (as in solid phase extraction) mode. The highly efficient sol-gel sorbent is chemically bonded to the substrate that, in turn, provides high chemical and thermal stability, and allows the use of any solvent/solvent mixtures for extraction. The open geometry of the FPSE media, as well as the sponge-like porous architecture of sol-gel PEG sorbent, promote rapid sorption and desorption of the analytes. During sample preparation in the equilibrium extraction mode, the FPSE media are immersed directly in the sample matrix containing the target analytes. Diffusion of the analytes in the aqueous sample matrix can be accomplished using external stimuli, such as magnetic stirring, orbital shaking, and sonication. Once the extraction equilibrium is reached (ensuring maximum recovery of the target analytes), the FPSE medium with the adsorbed analytes is withdrawn from the sample, residual water from the FPSE medium is dried and the medium is submerged in a small volume of organic solvent/solvent mixture for analyte elution/back-extraction. Due to the strong capillary action exerted by the FPSE substrate, no external stimuli is needed for the rapid diffusion of the solvent for quantitative scavenging of the adsorbed analytes from the FPSE medium during this elution/back-extraction process. The small volume of eluate maintains the preconcentration factor achieved during sample preparation (higher volume of the sample and a significantly lower volume of solvent used in eluting the extracted analytes). As such, FPSE eliminates completely the necessity of solvent evaporation and sample reconstitution. The eluate containing the pre-concentrated analytes is then centrifuged to eliminate any particulate matter prior to injection into the analytical instrument. FPSE ensures the rapid extraction of target analytes directly from the raw sample, in presence of a high volume of matrix interference, and eliminates totally time-consuming and error-prone solvent evaporation and sample reconstitution prior to instrumental analysis, leading to high throughput for routine analysis, without sacrificing data quality under strict regulatory scrutiny.

All the target analytes were of high- to medium-polarity as evident by their $\log K_{ow}$ values i.e., 1.83 for benzylpenicillin, 2.38 for oxacillin, 2.44 for cloxacillin and 2.91 for dicloxacillin. (Hansch & Leo, 1987; Hansch, Leo, & Hoekman, 1995). Therefore, a relatively high polarity sorbent, as the extracting phase, was logical. In order to select the best sorbent for efficiently extracting residual penicillin antibiotics from milk samples, two high polarity sol-gel sorbents: sol-gel poly(ethylene glycol) (sol-gel PEG), sol-gel poly (ethylene)-block-poly(propylene glycol)-block poly(ethylene glycol) (sol-gel PEG-PPG-PEG) and a medium polarity sorbent sol-gel C_{18} were investigated. Preliminary analytical data favoured unambiguously sol-gel PEG coated FPSE medium as the optimum microextraction device. As such, this high polarity sol-gel PEG coated FPSE medium was selected as the optimum sorbent. Samanidou, Kabir, Galanopoulos, and Furton (2015) described the incorporation of short-chain polyethylene glycol (average molecular weight 300 Da) into the sol-gel matrix and anchoring the growing sol-gel PEG network on a flexible hydrophilic cellulose substrate. This high polarity FPSE sorbent has been used successfully for the extraction of amphenicols and sulphonamides from raw milk (Karageorgou, Manousi, Samanidou, Kabir, & Furton, 2016; Samanidou et al., 2015). Herein, the efficiency of sol-gel PEG coated FPSE medium in extraction of four target penicillin antibiotic residues from bovine milk was studied. The protocol developed was validated according to the European Directive 657/2002/EC.

2. Materials and methods

2.1. Instrumentation

The HPLC system consisted of an automatic Injector SIL-9A and an LC9AD pump from Shimadzu (Kyoto, Japan), with an FCV-9A solvent mixing system. Class-M10A software was used to control the photodiode array (PDA) detector (SPD-M6A), the wavelength was set at 240 nm. A DGU-2A unit was used to degas the solvent lines during analysis.

Chromatographic separation was performed on an Inertsil C_8 analytical column (250 mm × 4.0 mm, 5 μm particle size), purchased from MZ Analysentechnik (Mainz, Germany), operated at room temperature.

Aliquots of 100 μL standards and samples were injected, while peak monitoring and quantitation was performed at 240 nm for