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Molecularly imprinted polymer as in-line concentrator in capillary electrophoresis coupled with mass spectrometry for the determination of quinolones in bovine milk samples

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ABSTRACT

In this work molecularly imprinted polymers have been evaluated as sorbent for the construction of an in-line solid phase extraction analyte concentrator in capillary electrophoresis coupled with mass spectrometry for the determination of the eight regulated veterinary quinolones in bovine milk samples. Different parameters affecting the analyte concentrator performance, such as sample pH, volume and composition of the elution plug and injection time, were studied. Sample volumes of 22 μL (2 bar for 15 min) were loaded on the MISPE microcartridge and the retained analytes were eluted by injecting a plug of MeOH/H_2O/NH_3 (60/37/3 by volume) for 125 s at 50 mbar (60 nL). The proposed method is simple for the monitoring of these antibiotic residues in milk samples, allowing the direct injection of the samples with minimum sample pretreatment, achieving limits of detection between 3.8 and 4.7 μg kg^{-1} and unequivocal identification of the compounds working in tandem mass spectrometry. Recoveries ranging from 70.0 to 102.3% were obtained and satisfactory intra-day and inter-day RSDs were achieved (≤12% and 15% respectively). Reproducibility among different constructed analyte concentrators showed RSD ≤ 11%.

1. Introduction

Quinolones (Qns) are one of the classes of antibiotics most widely used in veterinary practice to treat bacterial infections of animals in livestock farming and bovine milk production, causing residues in foodstuffs that produce adverse reaction in humans, as allergic reactions or antibiotic resistance [1,2]. In this sense, European Governments are dedicating huge quantities of money in advertising campaigns and scientific projects to avoid the misuse of antibiotics in both animals and humans. Therefore, to ensure food safety, European Union (EU) has set maximum residue limits (MRLs) of antibiotics in foodstuffs of animal origin by means of the Commission Regulation 37/2010 [3]. 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 established for SAR while DIF and OXO are forbidden in animals from which milk is produced for human consumption. For the rest of Qns, in the case of bovine milk, these MRLs are 30 μg kg^{-1} for DAN, 50 μg kg^{-1} for FLU, 75 μg kg^{-1} for MAR and 100 μg kg^{-1} for the sum of ENR and its metabolite CIP. Consequently, very sensitive analytical methods to detect ultratrace levels of these compounds in milk are needed.

Several methods have been published for the determination of Qns in milk samples using mainly high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC) coupled with UV/vis detection [4], fluorescence [5,6], chemiluminescence [7] or mass spectrometry (MS) [8–10].

Capillary electrophoresis (CE) coupled with UV/vis [11,12], laser induced fluorescence [13] and MS [14] detections has also been proposed as an alternative to chromatographic techniques to determine Qns in milk samples. An interesting option to increase sensitive in CE was developed by Guzman et al. [15]. This option consists of in-line solid phase extraction (SPE) which is carried out using the so-called analyte concentrators (ACs) or preconcentrators. Several reviews have described theoretical aspects and different modes for coupling SPE with CE [16–18]. In in-line SPE–CE mode, the SPE column is integrated in the CE capillary, which is different from an on-line concentrator approach where the SPE column is not integrated in the CE capillary but is a part of a separate system that is coupled to the CE separation capillary *via* an interface. In the in-line CE approach, the complete analysis can be

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performed with a minimum of sample-handling steps, limited risk of sample losses, and automation [18]. This approach was applied in the monitoring of Qns in chicken muscle samples, employing MCX as sorbent in the AC [19]. After the sample treatment by pressurized liquid extraction the final extract was injected into the in-line-SPE–CE–MS/MS system. Limits of detection (LODs) lower than 0.47 $\mu g\,kg^{-1}$ were achieved with satisfactory precision.

On the other hand, molecularly imprinted polymers (MIPs) are becoming a relevant alternative in sample treatment for improving sensitivity. MIPs are synthetic materials able to selectively recognize a particular chemical in the presence of closely related interfering species, as they contain specific recognition sites with a shape and geometry of functional groups complementary to those present in the template molecule [20,21]. Thus, the strong retention between the MIP and its target molecules makes it ideal for the selective extraction of compounds at trace levels, being of special interest when the sample is complex. In the last years, several reviews show the characteristics of these materials and their applications in analytical chemistry [22–27]. Specifically, the use of MIPs as SPE sorbents (MISPE) for the selective extraction of Qns from food samples has grown significantly in the last few years, mainly using HPLC as separation technique [28–31]. In our research group we applied available commercial MISPE previous to CE analysis for the determination of these antibiotics in bovine milk samples with satisfactory results [13]. Moreover, the use of MIP as sorbent in an AC for CE analysis was first reported by Lara et al. who evaluated a commercial MIP sorbent for the determination of triazine herbicides in urine samples without any sample treatment [32]. Sensitivity was not as high as expected partially because of UV/vis detection was used in this study. Recently an open tubular approach has been proposed to use MIP sorbent as AC concentrator using light-emitting diode-induced polymerization technology for the on-line construction of an in-column MISPE concentrator for CE, becoming an useful alternative also for microchips [33]. Another interesting alternative for the improvement in the sensitivity obtained when applied in-line SPE in CE–MS could be the use of a sheathless interfacing. This interface avoids the use of an additional sheath liquid, allowing lower instrumental LOD, as no dilution of the analytes happens. Recently this approach has been successfully applied to build a fritless AC concentrator for the analysis of peptides by using a prototype porous-tip capillary [34].

In this work, we have proposed the construction of an AC using a commercially available MIP as sorbent for the in-line MISPE–CE determination of the eight Qns of veterinary use (MAR, CIP, ENR, DAN, DIF, SAR, OXO and FLU), including those for which MRLs are established in bovine milk. In-line MISPE–CE offers the possibilities of off-line MISPE in terms of selectivity and sensitivity in addition to the advantages of being carried out in an in-line mode, which involves miniaturization (lower consumption of sample and solvents and more environmentally friendly in term of waste generation) and automation (higher productivity and less prone to human errors). In this paper, we evaluate the obtained results when in-line MISPE is used in combination with tandem MS, offering selectivity, sensitivity and enough identification points for the confirmation of the studied residues in milk according to the EU regulation [35]. To the best of our knowledge, this is the first report about the use of MIPs as in-line concentrators in CE–MS/MS, demonstrating that both methodologies can be compatible and its potential for the determination of these antibiotics in bovine milk samples.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of analytical grade. n-Hexane, methanol (MeOH), ammonium hydroxide and trichloroacetic acid (TCA) were supplied by Panreac (Madrid, Spain); acetonitrile (ACN) was obtained from Fluka (Buchs, Switzerland), while isopropanol, formic acid, acetic acid, ammonium acetate and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). DAN, SAR, and DIF were obtained from Riedel-de-Haën (Seelze, Germany); FLU from Sigma (St. Louis, MO, USA); CIP, ENR, MAR and OXO from Fluka. Ultrapure water, purified with a Milli-Q Plus system (Millipore Bedford, MA, USA), was used.

Stock standard solutions (100 $mg\,L^{-1}$) of each Qn were prepared by dissolving the appropriate amount of each analyte in H_2O/ACN (80/20, v/v) (DIF, MAR, DAN, ENR and SAR,), H_2O/ACN (50/50, v/v) (CIP) or 100% of ACN (OXO and FLU) and were stored in the dark at 4 °C. Under such conditions, they were stable for at least 1 month. A mixture of 0.1 $mg\,L^{-1}$ of each Qn was prepared in 50 mM ammonium acetate at pH 5.0 and stored at 4 °C. The working solutions were prepared by dilution with ammonium acetate buffer.

MIP sorbent for the construction of the AC was obtained from commercial SupelMIP Qns SPE Columns (Supelco, Bellefonte, PA, USA), with an average particle size of 57 μm. Nylon syringe filters, 0.22 μm × 13 mm (Agela technologies, New York), were used for filtration of the sample extracts prior to injection into the in-line-MISPE–CE–MS/MS system.

2.2. Instrumentation

CE experiments were carried out with an HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with external pressure mode to apply pressure above 1 bar. The coaxial sheath-liquid sprayer was supplied by Agilent Technologies. MS was performed using an Agilent 1100 Series LC/MSD SL mass spectrometer equipped with an ion trap (IT) analyzer. MS spectrometer was controlled by a PC running the Esquire software 4.1 from Bruker Daltonics (Bremen, Germany).

A pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320R from Hettich Zentrifugen, Tuttlingen, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used.

2.3. Electrophoretic procedure

Separation was carried out in a bare fused-silica capillary of 130 cm total length × 50 μm id (360 μm od) from Polymicro Technologies (Phoenix, AZ, USA). Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min, then with water for 5 min, and finally with the running buffer solution for 20 min. Also, at the beginning of each working session, the capillary was prewashed with a N_2 pressure of 7 bar for 4 min with 1 M NH_3, 3 min with water, and 5 min with running buffer.

In order to activate the MIP sorbent, before each injection the capillary was conditioned as follows: 1 M NH_3 for 1 min at 7 bar, H_2O for 1 min at 7 bar, and 50 mM ammonium acetate (without pH adjustment) for 1 min at 7 bar. Sample injections were made at the anodic end using a pressure of 2 bar for 15 min (*ca.* 22 μL using the Hagen–Poiseuille equation). The sample solvent was 50 mM ammonium acetate at pH 5.0. After the injection, the sample solvent was displaced of the capillary with running buffer by applying 7 bar for 1 min. Subsequently, the analytes were eluted by injecting a plug of MeOH/H_2O/NH_3 (60/37/3 by volume), using a pressure of 50 mbar for 125 s (*ca.* 2.3% of the total capillary volume = 60 nL). Before applying the separation voltage, the elution plug was displaced from the AC with the running buffer by applying 1 bar for 1 min in order to avoid undesirable interactions of the analytes with the retention sorbent. The electrophoretic separation was then achieved with a voltage of 25 kV (normal mode) with an initial ramp of 1 min. The running buffer was an aqueous solution of 50 mM ammonium acetate adjusted to pH 9.1 with 5 M