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Analytical Methods

# Sample preparation combined with electroanalysis to improve simultaneous determination of antibiotics in animal derived food samples

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ABSTRACT

A procedure based on liquid–liquid extraction (LLE) and phase separation using magnetically stirred salt-induced high-temperature liquid-liquid extraction (PS-MSSI-HT-LLE) was developed to extract and pre-concentrate ciprofloxacin (CIPRO) and enrofloxacin (ENRO) from animal food samples before electroanalysis. Firstly, simple LLE was used to extract the fluoroquinolones (FQs) from animal food samples, in which dilution was performed to reduce interference effects to below a tolerable threshold. Then, adapted PS-MSSI-HT-LLE protocols allowed re-extraction and further pre-concentration of target analytes in the diluted acid samples for simultaneous electrochemical quantification at low concentration levels. To improve the peak separation, in simultaneous detection, a baseline-corrected second-order derivative approach was processed. These approaches allowed quantification of target FQs from animal food samples spiked at levels of 0.80 to 2.00 μmol $L^{-1}$ in chicken meat, with recovery values always higher than 80.5%, as well as in milk samples spiked at 4.00 μmol $L^{-1}$, with recovery values close to 70.0%.

## 1. Introduction

The growth of the pharmaceutical industry with consequent availability and use of large-scale veterinary antibiotics has become a global problem. Their use, to prevent or treat animal disease and to enhance production, combined with the discharge, has provided possible routes of entry of veterinary drug residues into the human food chain (Farré & Barceló, 2013; Kantiani, Llorca, Sanchís, Farré, & Barceló, 2010; Shao, Wang, Chen, & Wu, 2014) and the environment (Frade, Dias, Teixeira, & Palma, 2014). As a consequence, there has been the emergence of resistant “super-bacteria”. The contamination in the environment also results from activities of the pharmaceutical industry, which scientific studies have documented (Frade et al., 2014; Rutgersson et al., 2014). Studies developed in countries such as Brazil (Frade et al., 2014), India (Rutgersson et al., 2014), China (Li et al., 2013; Zou et al., 2011), Switzerland (Golet, Alder, & Giger, 2002), Japan (Adachi, Yamamoto, Takakura, & Kawahara, 2013), Estonia (Lillenberg et al., 2010) and the United States (Done & Halden, 2015; LaPara, Madson, Borchardt, Lang, & Johnson, 2015) show the presence of fluoroquinolones (FQ), or their metabolites, in considerable levels in several aquatic and agricultural samples, as well as in samples of food for animals that are part of the human food chain.

There are many concerns about the direct and indirect hazards of veterinary drug use and their potential impacts on public health as well as on the natural environment. As such, between 10 and 90% of the drug dosage remains in its unchanged or non-metabolized form in tissue, or is discarded into the environment. Moreover, summarizing the findings from literature studies (Brown, 1996; Ebert et al., 2011; Hernandez-Arteseros, Compañó, & Prat, 1998; Huang, Lin, Yu, & Feng, 2006; Murillo Pulgarín, Alañón Molina, & Muñoz Fernández, 2008), it can be concluded that ciprofloxacin (CIPRO) is the major metabolite of enrofloxacin (ENRO), which can increase the concentration of the target drug in the aforementioned samples. Considering the impact of these drugs on human health, sensitive and selective analytical methodologies, especially for the quantification of residual levels in complex samples, are very important and highly desirable.

From an analytical viewpoint, complex samples have several biological constituents, such as lipids, proteins, carbohydrates (Golet et al.,

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2002), and prior to analysis require several steps of preparation for the elimination of interference (Farré & Barceló, 2013). Even with separation techniques such as capillary electrophoresis (Lombardo-Agüí, García-Campaña, Gámiz-Gracia, & Cruces Blanco, 2010) and high-performance liquid chromatography (Garcés, Zerzaňová, Kučera, Barrón, & Barbosa, 2006; Moema, Nindi, & Dube, 2012; Turiel, Martín-Esteban, & Tadeo, 2006) the complexity of these samples has been challenging, and the sample preparation may be needed. In electroanalysis, the drawback regarding interference from complex samples is more pronounced (Bilibio, de Oliveira, Ferreira, & Trindade, 2014; de Oliveira & Trindade, 2016; Trindade, Bilibio, & Zanoni, 2014), due to the fact that the adsorption of concomitants at the electrode surface may cause the overlapping of target signals and/or a change in the ionic strength of the supporting electrolyte solution. Because of this, protein precipitation and subsequent dilution have been used to minimize this effect. However, in real samples, the concentration is very low and dilution is unfeasible.

New trends combining solid-phase and liquid-phase extraction have been documented as indispensable for sample preparation (Song et al., 2017) and pre-concentration (Sun, Qiao, Liu, & Liang, 2008). The phase separation based on magnetically stirred salt-induced liquid–liquid microextraction was used to determine five FQs in milk, chicken eggs and honey samples prior to HPLC analysis (Gao et al., 2015). Likewise, adapted methods that uses phase separation based on ultrasound-assisted salt-induced liquid–liquid microextraction were proposed (Wang, Gao, Wang, et al., 2015; Wang, Gao, Xu, et al., 2015) to process the samples: plasma, urine, fish, pork and beef muscle prior to determine some FQs using HPLC technique. The advantages noted when applying these methodologies were the use of less toxic solvents – in developing new applications for enhanced-solvent extractions – and the possibility to extract and preconcentrate target FQs from complex samples at very low concentration. Here, we explored the advantages highlighted in the previous reported methods to make some adaptions and study all the parameters variations using a compact device. The new device configuration allows the stirring in a hot plate and control the temperature without loses of the volatile solvent during the extraction process. The adapted method is named as phase separation based on magnetically stirred salt-induced high-temperature liquid–liquid extraction (PS-MSSI-HT-LLE).

Several electroanalytical methods have been developed for quantitation of FQs; however, the numbers are poorly reported when the application has been related to complex samples such as food samples of animal origin, especially those of solid foods. Therefore, we used the developed method, PS-MSSI-HT-LLE, to extract CIPRO and ENRO from milk and chicken meat prior to simultaneous electroanalytical determination. Target drugs are common FQ antibiotics (so called as emerging food contaminants) and were selected as model analytes. The challenge of simultaneous quantification of these two FQs was overcome by applying a correction factor to calculate the percentage of the ENRO signal overlapping the main CIPRO peak and so obtain an accurate quantification of both analytes. As for optimization of the extraction, where in some determinations the signals were almost indistinguishable, we also performed mathematical data processing using a baseline-corrected second-order derivative method.

## 2. Materials and methods

### 2.1. Chemicals and samples

The FQs: CIPRO and ENRO (Fig. S1, Supplementary material) were purchased from (Sigma-Aldrich®, São Paulo, Brazil) and the standard stock solutions were prepared at concentrations of 1.0 mmol $L^{-1}$ by dissolving in 0.05% acid acetic (Vetec®, Rio de Janeiro, Brazil) and further dilution with ultrapure water. Acetic acid, phosphoric acid, sodium acetate and boric acid were purchased from Sigma-Aldrich® (São Paulo, Brazil). The supporting electrolytes used in this work were: acetate buffer and Britton-Robinson (BR) buffer solutions, prepared as previously reported (de Oliveira & Trindade, 2016; Trindade et al., 2014). For both buffer solutions, the concentration was 0.04 mol $L^{-1}$ and the pH values were adjusted using hydrochloric acid or sodium hydroxide (both from Vetec®, Rio de Janeiro, Brazil) at concentrations of 0.10 mol $L^{-1}$.

The tested surfactants were: tetra-*n*-butylammonium bromide (TBAB), tetraethylammonium chloride (TEAC), Triton X-100 (TX-100), cetyltrimethylammonium bromide (CTAB), dioctyl sodium sulfosuccinate (DSS), sodium dodecyl sulfate (SDS); all purchased from Sigma-Aldrich® (São Paulo, Brazil) and prepared using ultrapure water ($R \geq 18.2\ M\Omega\ cm$). The chicken and meat samples were acquired in local supermarkets (Dourados, MS, Brazil) and stored at 4 °C during the months of January and March 2016. Prior to analysis, all samples were certified free of the target FQs.

### 2.2. Instrumentation

The electrochemical measurements were performed using a PGSTAT 204 potentiostat/galvanostat (Metrohm Autolab®, Utrecht, The Netherlands) and controlled by Nova 1.11 software. The electrochemical cell, with capacity of 5.0 mL, was composed of three conventional electrodes: Pt auxiliary electrode, $Ag/AgCl_{(KClsat.)}$ reference electrode, and a glassy carbon electrode (GCE) as the working electrode. Prior to use, the GCE was carefully polished with alumina slurry (1.0 and 0.30 μm) with a polishing pad and rinsed with ultrapure water. The procedure was carefully repeated before subsequent measurements. The ultrapure water ($R \geq 18.2\ M\Omega\ cm$) used for cleaning and for preparation of all chemical solutions was obtained in a reverse-osmosis water purifier OS 10 LTXE (Gehaka®, São Paulo, Brazil) and further ultra-purified in a Thermo Scientific® system (model Smart2Pure, San Jose, USA). All measurements and/or adjustments of pH were performed using a combined glass electrode (Hanna®, model HI 1131B, Texas, United States) connected to a digital pH meter (Hanna®, model HI 3221, Texas, United States). For stirring and homogenization, a magnetic stirrer (Fisatom®, model 701, São Paulo, Brazil) was used.

### 2.3. Mathematical processing

For improved peak resolution in simultaneous detection, a baseline-corrected second-order derivative method was used and processed by Originlab® software (version 8.5). The mathematical data processing was fundamental to simultaneous quantification, as previously published in works by de Oliveira and Trindade (2016) as well as by Trindade et al. (2014).

### 2.4. Sample preparation performed to extract FQs

#### 2.4.1. Extraction of FQs from animal food samples

Firstly, the chicken meat was cut into tiny cubes in order to present a larger contact area during the spiking process. After that, the spiked chicken meat and milk samples were prepared by adding the required amount of the working solutions (FQs), vortex shaking for approximately 5 min. and left overnight to complete the homogenization process. To evaluate the efficiency of the extraction of FQs from the samples, the procedure was developed as shown in Fig. S2 (Supplementary material), with further direct electroanalysis. As extractant solvents, HCl (0.70 mol $L^{-1}$) and Britton-Robinson buffer (0.04 mol $L^{-1}$, pH 3.00–9.00) were tested. Fig. S2 (step I, Supplementary material) shows the extraction performed, in which 0.40 g of chicken meat or milk sample containing between 0.80 and 4.00 μmol $L^{-1}$ of CIPRO and ENRO were placed into conical tubes. Then 10 mL of extraction solvent was added and shaken by vortex for 30 s (Fig. S2, step II, Supplementary material). Subsequently, 5 mL of the supernatant was removed (Fig. S2, step III, Supplementary material) and submitted to direct electroanalysis with no further treatment (Fig. S2, step IV,