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Simultaneous quantification of antibiotics in wastewater from pig farms by capillary electrophoresis

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ABSTRACT

Pig farming is an important activity in the economic development of Mexico with millions of tons of meat produced annually. Antibiotics are used in therapeutic dose to prevent diseases, and sometimes as growth promoters. These compounds are not completely metabolized; they are carried into the environment in its active form at concentrations that could induce antibiotic resistance in bacteria, which could be transferred to human pathogens by horizontal gene transfer. The objective of this work was to develop methods of analysis for simultaneous quantification of the antibiotics Oxytetracycline (OXT), Chlortetracycline (CLT), Enrofloxacin (ENRO) and Ciprofloxacin (CIPRO) by field-amplified sampling injection in capillary zone electrophoresis (FASI-CZE). The method was validated by parameters of (1) linearity, obtaining a lineal range of 0.05 at 1 μg mL^{-1} for ENRO and CIPRO, and from 0.1 to 1 μg mL^{-1} for OXT and CLT; (2) precision, obtaining values < 5% of standard deviation for CIPRO and ENRO and < 10% of standard deviation for OXT and CLT; (3) accuracy, with recovery values from 93 to 115%; (4) selectivity, with values of resolution > 2 for the all antibiotics tested. To prove the method, a sample of wastewater from a local pig farm was analyzed, detecting a concentration of 0.140 ± 0.009 for OXT. This concentration was higher than the minimal selective concentration, indicating the point in which resistance to a determined antibiotic could develop. The methods were validated with precision and sensitivity comparable to chromatographic methods, which can be used to analyze wastewater from pig farms directly.

1. Introduction

Pig and aquaculture farming in Mexico are important economic activities that generate more than six thousand million tons of meat and aquaculture products [1]. To guarantee this productivity, antibiotics and hormones are frequently used as therapeutic agents and growth promoters. In this sense, Mexico should establish the official regulations on their use. These antibiotics, included in feed, are partially metabolized and discarded in the environment in its active form [2]. Generally, its concentrations are found in the nanomolar range, so they are considered micro-contaminants [3]. Antibiotics could induce resistance to bacterial communities while increasing the availability of resistant genetic determinants [4]. For example, some enterococcus that cause urinary infections have evolved in nosocomial pathogens, such as the case of *Enterococcus faecium*, which has mobilized from pig to human microbiota and resistant genes transferred of tetracyclines, macrolides, and fluoroquinolones to other pathogen bacteria as *Staphylococcus aureus* [5]. The relationship between resistance transfer and the use of antibiotics is of complex nature and data are insufficient. Thus, precise knowledge should exist of antibiotics present in the different environmental matrices: soil, water, and biota. The majority of the data comes from China, the United States, and some European countries [3]. In Mexico, not enough qualitative and quantitative information of these contaminants in water and sediments is available, although there is evidence of its use in some intensive livestock activities [6]. For these reasons, it is a priority to develop methods for their identification and quantification.

The most used analytic techniques in the environmental field are high-pressure liquid chromatography (HPLC) and gas chromatography (GC). On the other hand, the use of capillary electrophoresis (CE) has gained interest because of its high separation efficiency and low analysis cost [7, 8]. Different authors have quantified antibiotics using CE,

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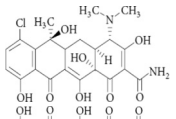
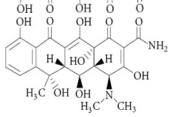
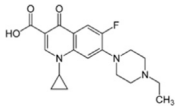
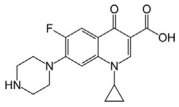
Table 1
Physico-chemical characteristics of oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin [16].

Compound	Formula	log K_{ow}	pK_a
CLT		−0.62	3.30, 7.44, 9.27
OXT		−0.92	3.27, 7.32, 9.11
ENRO		0.7	6.27, 8.30
CIPRO		0.28	5.90, 8.89

with detection limits comparable or higher than those reached with HPLC, as the case of amphenicols [9, 10], tetracyclines [11–13], and macrolides [14]. In these cases, the cost of reagents could be up to 76 times less than the HPLC analysis [15], an important aspect when the budget is limited and requires a laborious processing of samples. Another advantage of capillary electrophoresis is that it requires very low sample volumes, in the range of nanoliters, allowing direct injection in its aqueous phase, that is, it does not require evaporation or reconstitution of the sample in organic solvents.

In capillary electrophoresis, the analysis conditions are regulated by the characteristics of water charge and solubility of the analyte. The octanol/water partition coefficient (K_{ow}) indicates its hydrophobicity, and the ionizing potential establishes the ionizing state of a functional group. Table 1 shows the parameters K_{ow} and pK_a for some of the tetracyclines and fluroquinolones used in pig farming, inferring that tetracyclines are zwitterionic compounds with moderate solubility in water and mainly in the form of cations at pH < 4 [16]. Fluoroquinolones show greater solubility in water and are found totally in the form of cations at pH < 3.90 [17].

These antibiotics are commonly analyzed by chromatography or capillary electrophoresis. Nonetheless, few reports are available on methods for simultaneous quantification of this type of antibiotics. Kowalski [18] determined different antibiotics in meat tissues with a similar methodology; however, some integration peaks showed low resolution. On the other hand, many of the multi-residual techniques use high cost complex systems that usually require several stages of concentration and extraction with adsorbents or solvents [16, 19, 20]. The FASI-CZE technique is an operation mode in which the sample is pre-concentrated or stacked in the capillary as an effect of an amplified electric field between a sample with low conductivity and a running buffer of high conductivity.

If sample conductivity is higher, a modification must be made to reduce the conductivity of the sample. As stated by other authors, the addition of an adequate solvent, like acetonitrile or methanol reduce the conductivity of the sample [21, 22]. Additionally, the solvent and salts dissolved could enhance the sample stacking through a process called pseudo- isotachophoresis, where the salts and the solvent acts as leading and terminating ions, respectively [21].

Moreover, the electro-kinetic injection is the most convenient when method sensitivity is required. Because of the previous information, the objective of this work was to validate the FASI-CZE method for determining four antibiotics of veterinary interest simultaneously. The scope of the methods covers the determination of CIPRO, ENRO, OXT and CLOR in swine wastewater at concentrations of 0.1 to 1.0 μg mL^{-1}.

2. Materials and methods

2.1. Reagents

The reagents used for the preparation of samples and solutions were of reagent degree or higher. Methanol, boric acid, monosodium phosphate, phosphoric acid 85% w/w, and calibration standards were acquired from Sigma-Aldrich, USA.

2.2. Solution preparations

This work was carried out applying two methods, Method A for wastewater samples with low conductivity < 1.8 mS cm^{-1}, and Method B for wastewater samples with conductivities from 1.8 to 6.9 mS cm^{-1}.

Stock solutions of 1 mg mL^{-1} were prepared in methanol for each one of the standards, except for CIPRO due to its insolubility in methanol, so it was prepared at a concentration of 20 μg mL^{-1} in 5 mM phosphate buffer previous to its analysis. The solutions in methanol can be kept at -20 °C up to three months, maintaining their stability [21].

Working solutions were prepared from the stock solutions at concentrations of 100 μg mL^{-1} in 5 mM phosphate buffer (Test method A), and pH was adjusted to 3.5 with hydrochloric acid at 0.1 M. A solution was prepared with the mixture of antibiotics at concentration of 1 μg mL^{-1}, each one. The multi-residual calibration was prepared diluting the antibiotic solutions in 5 mM phosphate buffer to obtain concentrations in the range of 0.05 to 1.0 μg mL^{-1}. The multi-residual calibration solutions were stored at 4 °C up to seven days protected from light and filtered with 0.45 μm pore size nylon membrane before each analysis. An internal multi-residue calibration was prepared in sample wastewater diluted to 50% ACN v/v (Method B).

2.3. Experimental conditions

The running buffer was used at different monosodium phosphate and boric acid concentrations adjusted with phosphoric acid at 85% w/w. Table 2 shows the running conditions and the buffers used during the development of the method. The analyses were performed in a capillary electrophoresis system P/ACE MDQ (Beckman-Coulter, Denmark) equipped with a diode array detector (DAD) in a wavelength range from 190 to 400 nm. Separations were performed with a uncoated fused silica capillary of 75-μm internal diameter (Polymicro

Table 2
Running conditions assessed during the development of the method for simultaneous determination of oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin by capillary electrophoresis.

Variables	Levels			
	I)	II)	III)	IV)
A) Buffer composition	50 mM NaH_2PO_4	100 mM NaH_2PO_4	100 mM NaH_2PO_4 + 50 mM H_3BO_3	100 mM NaH_2PO_4 + 200 mM H_3BO_3
B) Buffer pH	2	3	5	7
C) Sample pH	2.5	3	3.5	4