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ORIGINAL PAPER

A simple method for electroanalytical determination of ceftiofur in UHT milk samples using square-wave voltammetry technique

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Abstract A simple electroanalytical method was developed to determine the antibiotic ceftiofur (CF) in milk. The method is based on the adsorptive accumulation of the drug on a hanging mercury-drop electrode (HMDE) and the accompanying initiation of a negative square wave, which yielded well-defined cathodic peaks at −0.60 V (1C) and −0.91 V (2C) vs. Ag/AgCl. Calibration graphs were constructed and statistical parameters were evaluated. At pH 2.5, the square-wave voltammetry method revealed linearity from 52.4 to 524 ng mL^{-1} ($r = 0.997$), which is in accordance with the tolerance level of 100 ng mL^{-1} for CF as a residue in bovine milk established by the Food and Drug Administration (FDA) and the European Union. The limits of detection and quantification were 1.86 and 6.20 ng mL^{-1}, respectively. The method was tested to determine CF in spiked milk samples using HPLC as reference method.

Keywords Ceftiofur · Lactams · Milk · Residue analysis · Voltammetry

1 Introduction

Antibiotics are widely used in dairy cattle management for the treatment of diseases and as dietary supplements. They may be administered orally as feed additives or directly by injection. The use of antibiotics may result in drug residues being present in milk, especially when they are not used according to label directions. The presence of antibiotic residues in milk may cause allergic reactions in sensitive individuals, interfere with starter cultures for cheese and other dairy products, or indicate that the milk was obtained from an animal with a serious infection [1].

Ceftiofur (CF) (Fig. 1) is a widely used broad-spectrum third-generation cephalosporin antibiotic approved for the treatment of infections in cattle, swine, sheep, goats, turkeys, and chickens [2]. No withdrawal period is required after treatment. An acceptable daily intake (ADI) for residues has been calculated at 20 g kg^{-1} of body weight. In the European Union, the antibiotic has been included in Annex I of Council Regulation 2377/90 and assigned a maximum residue limit (MRL) of 100 μg L^{-1} in milk. This MRL, along with those established for other edible tissues, corresponds to an intake of approximately 87% of the ADI for a standard diet [3]. CF metabolism has been described in rats [4], dairy cattle [5], and swine [6]. The thioester bond in this compound is rapidly cleaved to give desfuroylceftiofur, which is further metabolized to a disulfide dimer and various desfuroylceftiofur-protein and amino acid conjugates. Free desfuroylceftiofur is an active metabolite in which the intact cephalosporin portion of the molecule is responsible for biological activity [7].

Chromatographic procedures have been described for determination of a single cephalosporin [8] or simultaneous determination of cephalosporins [9] in milk. Methods have been reported for determination of CF [9, 10] or conjugated residues of CF [3] in milk. Electroanalytical techniques have been used to determine other cephalosporins, such as cefpodoxime proxetil [11], ceftazidime [12], cephalothin [13], cefminox [14], cefetamet–Na [15], cephalexin [16], cefaclor [17], ceftriaxone [18], cefixime [19], and cefepime [20], providing sensitivity equivalent to that of HPLC techniques. Most cephalosporins are electroactive by virtue

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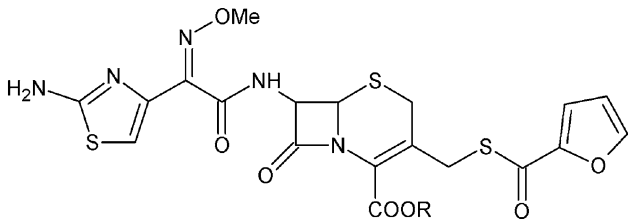

Fig. 1 Chemical structure of ceftiofur

of reduction of the (Δ^3) double bond of the cephem nucleus and possibly because of the presence of reducible substituents at the C_7 or C_3 positions in the molecule.

In bioanalytical methods, it is important to minimize sample preparation procedures, since solid phase extractions can sometimes compromise the stability and quantitativity of analytes. The simplest preparation procedure should therefore be the procedure of choice.

Some of the published methods suffer interference from milk components, while others are not sufficiently simple for use in routine analysis because they require sophisticated instruments and expensive reagents which are not available in many control laboratories. We therefore sought to develop a rapid, simple, and accurate procedure suitable for application in quality control laboratories.

In this study, a simple sample preparation method has been applied, consisting exclusively of protein precipitation and sample centrifugation techniques. The procedure did not influence the quantitative assay performed and yielded good recovery values.

We propose an electroanalytical methodology for determination of CF by square-wave adsorptive voltammetry on a hanging mercury-drop electrode (HMDE) in bovine milk. The resulting data were validated by comparing them against results obtained by a chromatographic method [9].

2 Experimental

2.1 Instrumentation

An μAutolab Type II device (Eco Chemie) controlled by General Purpose Electrochemical System (GPES) software (Eco Chemie BV) was used to perform the electrochemical measurements. A conventional three-electrode system was employed, which was composed of a Hanging Mercury-Drop Electrode (HMDE) (area: 0.52 mm^2), Ag/AgCl reference electrode and a glassy carbon auxiliary electrode.

A ProStar chromatographic system (Varian) equipped with a ProStar 210 ternary pump and a ProStar 325 UV–Vis detector (Varian, Melbourne, Australia) was employed. Sample injection was performed through a Rheodyne injector valve (Varian, Cotati, CA, USA) with a 20 μL sample loop. The isocratic chromatographic method utilized a Waters Nova-Pack phenyl column (4 μm, 150 mm, 3.9-mm i.d.) maintained at 25 °C. Before use, the mobile phase was degassed and vacuum-filtered through 0.45 μm nylon membranes (Alltech, Belgium), and then pumped at a flow rate of 1.0 mL min^{-1}. Detection of all analytes was performed at 270 nm. Data were acquired using ProStar Workstation software (Varian). Additional equipment included a Sigma D-37520 pH-meter, with appropriate standard buffer solutions, and an InoLab RS 232 centrifuge.

2.2 Reagents and solutions

Unless otherwise specified, all reagents were of analytical or higher quality. Ultrahigh-purity water was produced using an Elgastat system. HPLC-grade acetonitrile (Mallinckrodt, USA) and 85% orthophosphoric acid (Merck, Germany) were used. The mobile phase was prepared by mixing a solution of orthophosphoric acid and acetonitrile, which was filtered and degassed before use.

CF was obtained from Rhodia-Mérieux Ltda. Stock standard solutions (1.0×10^{-3} mol L^{-1}) of the compound were prepared by dissolving the solid substance in water. This stock solution was stored in the dark and under refrigeration in order to minimize decomposition. Standard solutions of this antibiotic at lower concentration were prepared daily by diluting the stock solution with distilled water. Britton–Robinson supporting buffer (pH 2.5, 0.04 mol L^{-1} each constituent) was prepared by dissolving 2.47 g of boric acid in 500 mL of distilled water containing 2.3 mL of glacial acetic acid, adding 2.7 mL of orthophosphoric acid, and diluting the mixture to 1.0 L with distilled water.

Milk samples were purchased from various markets located in the city of Campo Grande, Mato Grosso do Sul State, southwestern Brazil, stored at 4 °C after collection, tested by microbial assay, and used as blank samples in the application and validation of the new method.

2.3 Procedures

2.3.1 Milk sample preparation

Aliquots (500 μL) of milk were diluted with an equal volume of acetonitrile–water (50:50, v/v) as the milk-protein precipitating agent. After vortexing for 10–15 s, the precipitated protein was separated by centrifugation for approximately 30 min at 4000 rpm. The clear supernatant layer was used in a further procedure as protein-free milk. All samples were protected from light in amber vials covered with aluminum foil and stored at −20 °C until analysis.

2.3.2 Voltammetric determination

Square-wave voltammetry: 10 mL aliquots of Britton–Robinson (BR) buffer of different pH values were transferred to an electrochemical cell and deaerated for 10 min. A selected accumulation potential was then applied to a mercury drop for a uniform accumulation period while the solution was stirred at 1,500 rpm. After a 15-s rest period, adsorptive stripping square-wave voltammetry was applied in the negative direction over a voltage range of −0.20 to −1.20 V vs. Ag/AgCl. After recording the background voltammogram, 10 and 100 μL aliquots of the CF stock solutions were added to the cell and a square-wave voltammogram was recorded under the same conditions using a new drop. Quantification was performed by the standard addition method under optimized conditions. All measurements were made at room temperature.

Quality control samples were prepared at three CF concentrations (104.71, 209.43, and 418.86 ng mL^{-1}) of the supporting electrolyte and analyzed under the same conditions.

2.3.3 Chromatographic determination

Based on results published elsewhere [9], reversed-phase liquid chromatography was performed on a Waters Nova-Pack phenyl column (4 μm, 150 mm, 3.9-mm i.d.) and the mobile phase was prepared as follows: Eluent A, prepared by diluting acetonitrile (100 mL) to 1000 mL with octanesulphone acid solution (pH 2.52); Eluent B, prepared by diluting acetonitrile (300 mL) and methanol (150 mL) to 1000 mL with octanesulphonic acid solution (pH 2.52). The injection volume was 20 μL and the mobile phase flow rate was set at 1.0 mL min^{-1}. HPLC was run in isocratic mode with 90% eluent A and 10% eluent B for 8 min. The relative content of eluent B was then increased linearly to 45% over a 15-min period. The gradient was further increased linearly to 56% eluent B for 5 min and then to 100% eluent B for 17 min. The system was held constant for 3 min and returned to 90% eluent A for 2 min. The column temperature was kept at 20–24 °C. Ultraviolet detection was performed at 270 nm.

3 Results and discussion

For high-sensitivity monitoring of the accumulated drug, cathoding adsorptive stripping voltammograms of 5.0×10^{-7} mol L^{-1} CF were recorded in BR buffer (pH 2.5) at an accumulation potential of −0.25 V, followed by preconcentration for 50 s, using square-wave voltammetry (SWV) (Fig. 2, voltammogram a) and differential pulse voltammetry (DPV) (Fig. 2, voltammogram b). In the negative direction two well-defined peaks were observed at

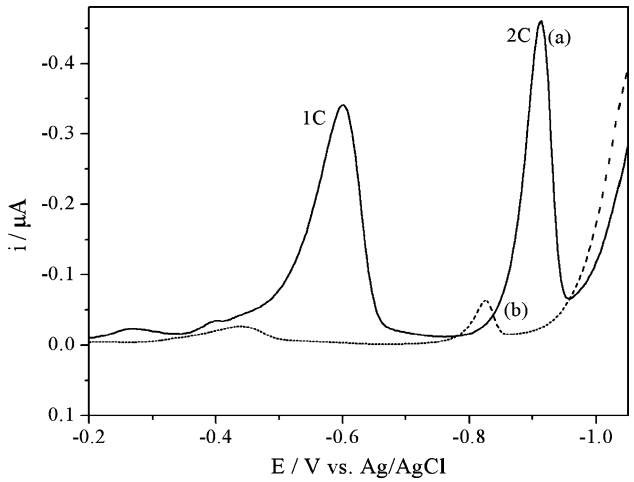

Fig. 2 Square-wave voltammogram (SWV) and differential pulse voltammogram (DPV) for a solution containing 5.0×10^{-7} mol L^{-1} of ceftiofur in 0.04 mol L^{-1} Britton–Robinson buffer at pH 2.5. Parameter: accumulation potential (E_{acc}) = −0.25 V, accumulation time (t_{acc}) = 50 s, frequency (f) = 60 Hz, scan increment (ΔE_s) = 25 mV for SWV and E_{acc} = −0.25 V, t_{acc} = 50 s, scan rate (v) = 2.5 mV s^{-1}, pulse amplitude (E_{sw}) = 50 mV for DPV

−0.60 and −0.91 V (SWV) and −0.44 and −0.82 V (DPV). Signal intensity of SWV was found to be 8.0 times higher than that of DPV. Since the analytical signal obtained using SWV was well-defined and much stronger than the signal obtained with differential pulse voltammetry after an accumulation step, the former was chosen as the preferred technique for further studies.

The influence of BR buffer (pH 2.0–8.0) on peak current was also analyzed for 5.0×10^{-7} mol L^{-1} CF after preconcentration for 50 s at −0.25 V. The nature and acidity of the supporting electrolyte are among the most important factors that strongly influence analyte stability and its cathodic and adsorption processes.

The dependence of the peak current (i_p) on pH for the buffer solutions utilized is shown in Fig. 3. A decrease in the peak current i_p (1C peak) can be observed for pH values from 2.5 to 6.0, whereas the peak virtually disappears at pH values greater than 6.0. This behavior confirms the presence of chemical reactions involving protons in the reduction process [19]. The value of i_p (2C peak) gradually decreases at pH values from 2.0 to 5.0 and remains almost constant up to pH 8.0, which indicates that the current is virtually unaffected by pH in this range. The pH dependence of i_p reveals that different ionic species, with different diffusion coefficients, are present in the solution. Since the pH of electrolysis media is a variable that tends to strongly influence the shape of voltammograms, the best results with respect to enhancement, shape, and reproducibility of the peak current were obtained with a 0.04 mol L^{-1} BR buffer at pH 2.5 thus constituting the most convenient pH value for further analytical determination.

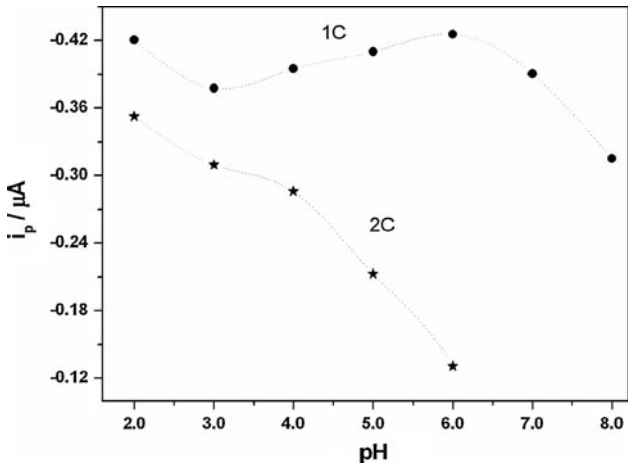

Fig. 3 Influence of pH on 1C (*a*) and 2C (*b*) peak current responses for 5.0×10^{-7} mol L^{-1} in Britton–Robinson buffer (pH 2.0–8.0) after a 50-s accumulation time; $E_{acc} = -0.25$ V, $f = 60$ s^{-1}, $\Delta E = 2.5$ mV, $E_{sw} = 25$ mV

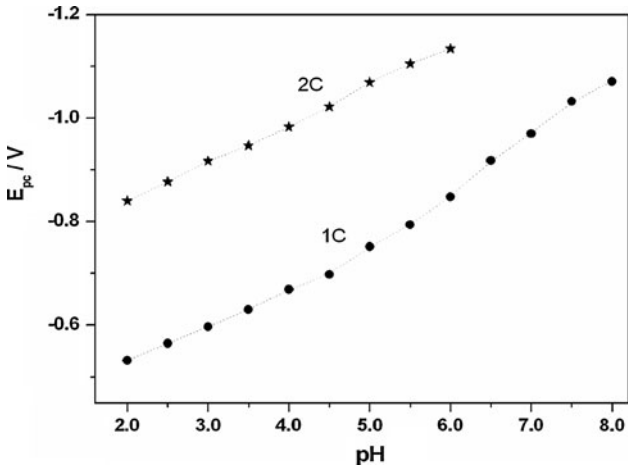

Fig. 4 Influence of pH on 1C (*a*) and 2C (*b*) peak potentials for 5.0×10^{-7} mol L^{-1} in Britton–Robinson buffer (pH 2.0–8.0) after 50-s accumulation time; $E_{acc} = -0.25$ V, $f = 60$ s^{-1}, $\Delta E = 2.5$ mV, $E_{sw} = 25$ mV

The influence of pH on peak potential (E_p) can be seen in Fig. 4. The peak potential (1C and 2C peaks) was found to shift to more negative values with increasing pH, which indicates the presence of a chemical reaction (proton-transfer reaction) preceding the electrode process. This behavior is consistent with that theoretically expected behavior for electrochemical reduction process consuming proton ions [21]. Also observed was the fact that E_p (2C peak) exhibited a linear relation with pH.

3.1 Influence of frequency on peak current

This and the subsequent parts of the study were performed with 5.0×10^{-7} mol L^{-1} CF in BR buffer at pH 2.5. At a constant scan increment (ΔE_s) of 2.5 mV and a pulse amplitude of 25 mV, the peak-current intensity increased linearly over the range of 30–210 Hz. Peak-current magnitude rose at increasing frequency values in this range. Although current values rose linearly in this frequency range, peak distortion was observed beyond 90 Hz, resulting in poor definition of the peak current shape.

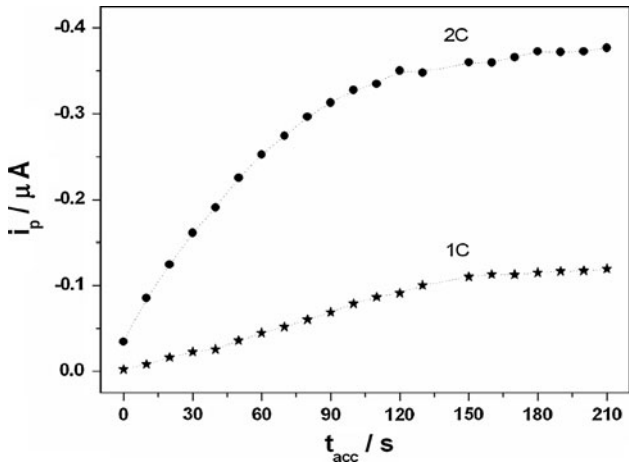

Fig. 5 Effect of accumulation time on 1C and 2C peak currents responses for a ceftiofur 5.0×10^{-7} mol L^{-1} solution in Britton–Robinson buffer (pH 2.5). Parameters: $E_{acc} = -0.25$ V, $f = 60$ s^{-1}, $\Delta E = 2.5$ mV, $E_{sw} = 25$ mV

3.2 Effect of accumulation time

The effect of accumulation time (from 0 to 210 s) on square-wave voltammetric peak-current intensities (Fig. 5) was investigated using 5.0×10^{-7} mol L^{-1} CF. As expected, the peak current rose with increasing accumulation time, although a plateau was reached after 120 s. For longer accumulation times, the peak current (1C and 2C) remained constant until 210 s, indicating that adsorptive saturation of the drug onto the mercury electrode surface was achieved [22–24]. Accumulation times shorter than 120 s—i.e., shorter than the time required for full saturation of the mercury electrode surface—were selected to evaluate the best working conditions for the proposed method.

3.3 Effect of accumulation potential

The dependence of the peak current on the accumulation potential was evaluated over the range from −0.10 to +0.80 V for 5.0×10^{-7} mol L^{-1} CF using 0.04 mol L^{-1} BR buffer at pH 2.5. The peak current was found to increase with increasing accumulating potential only from −0.20 to −0.45 V, reaching a maximum value at −0.30 V. $E_{acc} = -0.30$ V was therefore adopted as the optimum value for the subsequent steps.

3.4 Instrumental parameters

Drop size (0.25–0.52 mm^2), stirring rate (500–3000 rpm), scan increment (ΔE_s) (2.0–10 mV), and pulse amplitude (E_{sw}) (10–150 mV) were the experimental parameters examined in order to develop a suitable analytical procedure for CF determination. The working conditions selected were: drop area, 0.52 mm^2; stirring, 1,500 rpm; $\Delta E_s = 2.5$ mV; and $E_{sw} = 25$ mV. The current peak was not modified by changes to the rest period. The 15-s period was sufficient to allow a uniform concentration of the drug to be obtained on the mercury drop.

3.5 Analytical parameters

Based on the optimized operational parameters previously defined, an analytical calibration graph was constructed using CF concentrations from 52.4 to 524 ng mL^{-1} in 0.04 mol L^{-1} BR buffer at pH 2.5. Three calibration graphs were constructed. The peak current increased linearly over the entire concentration range investigated (data not shown), according to the equation $i_p2C\ (\mu A) = (-1.05 \times 10^{-7} \pm 1.70 \times 10^{-8}) + (-0.64 \pm 4.10 \times 10^{-3})\ C$ (C = ng mL^{-1}), with $r = 0.997$ and $n = 10$ (Table 1). The limits of detection (LOD) and quantification (LOQ) were calculated using the statistic treatment 3 × SD/b and 10 × SD/b, respectively, where SD is equivalent to the standard deviation of the average of the signals of 10 measurements of the blank at the 2C peak potential for CF and b is the slope of the analytical curve. LOD and LOQ were 1.86 and 6.20 ng mL^{-1}, respectively.

Comparison of the LOD and LOQ values obtained using the electroanalytical procedure against values available from the reference method (HPLC, Table 1) confirmed the higher sensitivity of the proposed method. The values obtained for the proposed method were also lower than those determined by HPLC [9] and liquid chromatography–electrospray mass spectrometry [10].

The satisfactory sensitivity of adsorptive voltammetry was accompanied by suitable repeatability. Evaluation of this analytical performance was based on 10 repeated measurements of the electrochemical signals of CF in a 5.0×10^{-7} mol L^{-1} pure solution and in the presence of milk. The precisions of the relative standard deviation were 0.55 and 1.74%, respectively.

Table 1 Analytical parameters for detection of the ceftiofur using SWV and HPLC

Parameter	SWV	HPLC
Concentration range (ng mL^{-1})	52.4–524	52.4–524
Correlation coefficient (r)	0.997	0.999
Intercept (μA)	-1.05×10^{-7}	16.3
Slope (μA mol L^{-1})	−0.64	−158
LOD (ng mL^{-1})	1.86	9.70
LOQ (ng mL^{-1})	6.20	30.6

3.6 Interference studies

Even at low concentrations, certain components of milk, such as casein, were found to decrease the height of 1C and 2C peaks, precluding direct determination of CF in milk samples using the experimental procedure described above. Separation of the drug is thus essential for its determination in milk. The best results were achieved by applying a simple and fast pretreatment (cleanup) procedure, which is in fact a slight modification on the sample preparation method developed for determination of CF in milk by HPLC-ES-MS [10]. By adding a small amount of 50% acetonitrile:water solution to the milk sample and centrifuging the mixture, most of the interfering substances (mainly proteins) were precipitated and easily removed. Using the proposed method, no sample pretreatment was required other than precipitation and dilution steps. Direct determination of the antibiotic in the milk sample was attempted, as was using pure acetonitrile as a deproteinizing agent, but the recovery results were extremely low.

3.7 Analysis of bovine milk sample using the electroanalytical method

The optimized procedure was successfully applied to the determination of CF in milk. Aliquots of milk spiked with CF were diluted with an equal volume of acetonitrile:water (50:50, v:v) as the milk-protein precipitating agent. After vortexing for 10–15 s, the precipitated protein was separated by centrifugation at 4000 rpm for approximately 30 min. Subsequently, an aliquot from the supernatant layer was diluted in the supporting electrolyte (BR buffer at pH 2.5) before measurement. Voltammograms of CF in BR buffer (pH 2.5) exhibited well-defined cathodic peaks. Current was proportional to the concentration along a convenient range. The good linearity of the calibration graph and the negligible scatter of the experimental points were clearly evident by the correlation coefficients (close to 1 in both cases). Representative voltammograms of a milk sample spiked with 209.42 ng mL^{-1} with successive additions of standard CF are shown in Fig. 6. For the milk samples, the peak potentials (E_p) shifted to more negative values in comparison to pure electrolyte. This was in fact the expected behavior, since those responses reflect interference of milk components, owing to the presence of these compounds at high concentration in the sample. This interference does not affect the analytical sensitivity, and the proposed procedure can be applied with success to

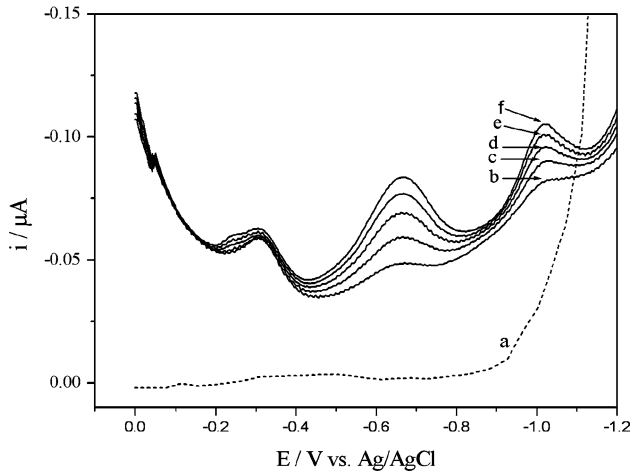

Fig. 6 Square-wave voltammograms obtained for the determination of ceftiofur using the optimized condition. (*a*) Blank UHT milk sample, (*b*) UHT milk sample (200 μL) spiked with 407.98 ng mL^{-1} of ceftiofur, (*c–f*) successive standard additions of ceftiofur; final concentrations in the cell: 52.4, 104.80, 157.20, and 209.60 ng mL^{-1}, respectively. Parameters: $t_{acc} = 60$ s, $E_{acc} = -0.30$ V; $f = 60$ s^{-1}, $\Delta E_s = 2.5$ mV, $E_{sw} = 25$ mV

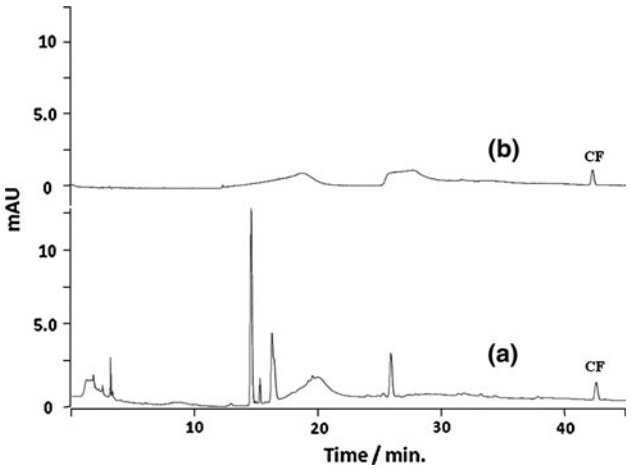

Fig. 7 Chromatograms obtained for (*a*) spiked milk sample containing ceftiofur (injected concentration = 209.42 ng mL^{-1}) and (*b*) standard solution of ceftiofur (injected concentration = 209.42 ng mL^{-1}). Conditions: mobile phase performed in gradient mode (Sect. 2.2.3); flow rate = 1.0 mL min^{-1}, wavelength = 270 nm

evaluating recovery levels using the standard addition method. The recovery rates of CF, based on the average of four replicate measurements on three levels of concentration (104.71, 209.42, and 418.85 ng mL^{-1}) were 101.03, 101.96, and 97.41% for SWV, respectively (Table 2). Therefore, the proposed procedure should be applicable to the quantification of CF in bovine milk samples and other similar samples containing this compound.

3.8 Analysis of milk samples using the HPLC method

For chromatographic evaluation, milk samples were spiked with CF antibiotic at 104.71, 209.42, and 418.85 ng mL^{-1}. Significant differences were found between the mean recoveries between HPLC and the proposed electroanalytical method. Analyte extraction in the chromatographic method may have contributed to the lower recovery values, when compared with the proposed method (Table 2). Mean recovery rates obtained with the proposed method (from 97.41 to 101.96%) revealed the satisfactory performance of the electroanalytical method for determining CF in milk samples. Figure 7 illustrates the chromatographic profiles of a spiked milk sample (chromatogram A) and a standard solution (chromatogram B) of the drug, both containing the same concentration of CF (209.42 ng mL^{-1}).

The results were also compared by applying the *F* test and Student's *t* test (Table 2). The experimental value (*t* calculated) exceeded the theoretical value, with significant differences between data obtained with each method. The pronounced differences revealed by the statistic test can be attributed to the low recovery rates achieved with chromatographic analysis, by reason of matrix pretreatment using solid phase extraction (adapted from reference 9). As a result, it is not possible to compare the proposed method with the reference method by applying the *F* test and Student's *t* test.

Table 2 Obtained results for the recovery of added ceftiofur in milk samples using the proposed method and the reference method

Methods	Added (ng mL^{-1})	Found[a] (ng mL^{-1})	Recovery (%)	RSD (%)	F^b	t^b
Electroanalytical (SWV)	104.71	105.79	101.03	3.75	6.33	7.36
	209.42	214.23	101.96	2.13	8.51	25.13
	418.85	407.98	97.41	1.51	2.28	15.79
HPLC	104.71	93.05	88.80	0.90	–	–
	209.42	170.31	81.30	1.84	–	–
	418.85	331.36	78.01	2.78	–	–

[a] Average of four determinations; *RSD* relative standard deviation, $F = F$ test confidence level of 95% ($F_{critical} = 9.28$), $t =$ Student's *t* test confidence level of 95% ($t_{critical} = 2.447$)

[b] Comparison between SWV and HPLC methods

4 Conclusions

The purpose of this study was to develop a square-wave adsorptive voltammetric method based on a new procedure for extracting CF from bovine milk samples. The voltammetric method proved to be as sensitive as methods utilized for the chromatographic determination of this antibiotic. Satisfactory selectivity was achieved, with quite suitable visibility of CF peaks. The procedure can be recommended as an alternative for routine quality control of CF in milk samples, having proven simpler and faster than the proposed and reference methods, while offering comparable precision and accuracy.

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