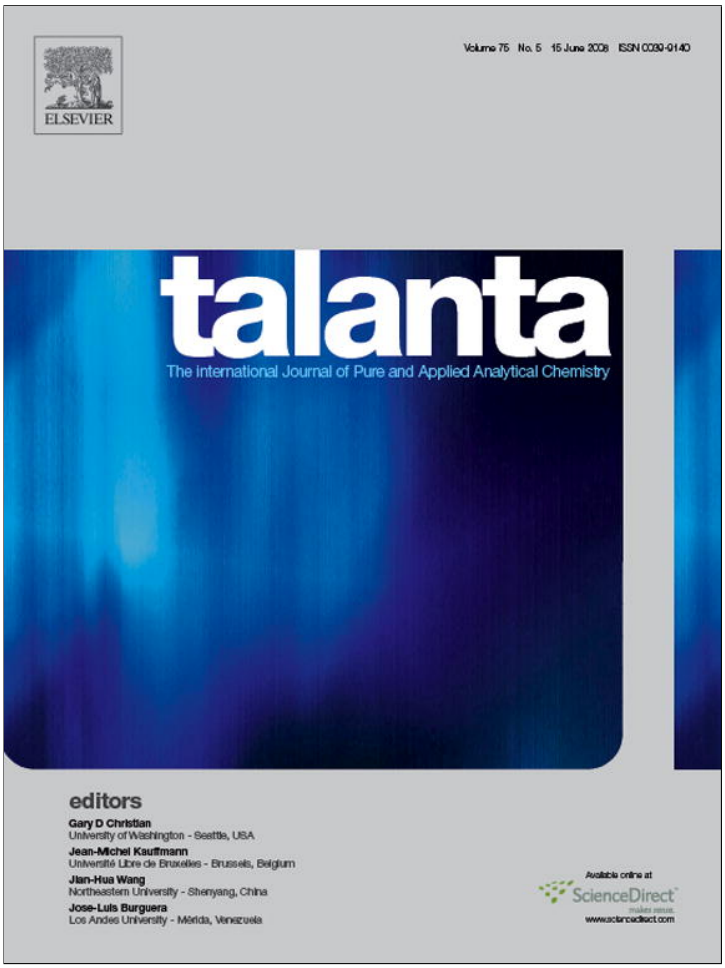

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Development and application of a capillary electrophoresis–electrochemiluminescent method for the analysis of enrofloxacin and its metabolite ciprofloxacin in milk

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Abstract

Enrofloxacin (ENR) is a fluoroquinolone developed exclusively for the use in veterinary practice for the treatment of respiratory and gastrointestinal infections, and ciprofloxacin (CIP) is its main active metabolite. Their contents are regulated by the EU Council Regulation no. 2377/90 in animal edible tissues. We developed a sensitive and rapid method for the determination of ENR and CIP by capillary electrophoresis (CE) with electrochemiluminescence (ECL) detection. The method is based on the detection of aliphatic tertiary or secondary amino moieties in ENR and CIP with end-column tris(2,2-bipyridyl)ruthenium(II) electrochemiluminescence. Parameters that affect separation and detection were optimized. Under the optimized conditions, the calibration functions were linear in the range of 0.03–1 $\mu g\,ml^{-1}$ for ENR and 0.05–1.2 $\mu g\,ml^{-1}$ for CIP. The detection limits of ENR and CIR were 10 $ng\,ml^{-1}$ and 15 $ng\,ml^{-1}$, respectively, based on the signal-to-noise ratio of 3. The relative standard derivations of the peak height and the migration time for ENR and CIP were less than 4.13%. The developed method was successfully applied to determine ENR and CIP in milk with a solid-phase extraction procedure.

Keywords: Electrochemiluminescence; Capillary electrophoresis; Enrofloxacin; Ciprofloxacin; Milk

1. Introduction

Quinolones are an important group of synthetic antibiotics with bactericidal action. They are derived from nalidixic acid, a naphthyridine derivative introduced for clinical applications in the livestock and farming industries [1]. Enrofloxacin (ENR) is a fluoroquinolone developed exclusively for the use in veterinary practice for the treatment of respiratory and gastrointestinal infections. It is an extended spectrum antimicrobial drug that has been employed successfully in the treatment of a variety of infections caused by susceptible bacterial pathogens in food animals [2]. The pharmacokinetic properties of ENR have been studied in a number of mammals and aquatic animals, such as cows [3], goats [4], sea bass [5], and juvenile Atlantic salmon [6]; and ENR's active ciprofloxacin (CIP), derived by ENR deethylation, was investigated in these animals. ENR and CIP are representatives of stimulants that belong to amine species. The molecular structures of ENR and CIP are shown in Fig. 1.

In the recent years, the utilization of antibiotics in food-producing animals has caused public concern due to the transfer of antibiotic-resistant bacteria to man [7]. This is an increasingly prominent problem because antibiotics are used in animals to treat infections and to act as growth promoters, this will leads to the antibiotic-resistant strains can emerge in both healthy and sick animals. This fact would make the antibiotics treatment useless in common human infections. European Community (EC) has fixed a maximum residue limit (MRL) in edible animal products for some quinolones, such as ENR and its metabolite CIP. The MRL values are in the range 100–300 $\mu g\,kg^{-1}$ for the sum of ENR and CIP in foodstuffs of animal origin [8]. Therefore, the development of rapid, simple, sensitive, and accurate methods for monitoring their levels in animal-producing foods is of increasing interest. Chromatographic methods such as high-performance liquid chromatography (HPLC) combined with either ultraviolet or fluorescence and mass spectrometry

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(MS) detection have been widely studied and were commonly used to analyze quinolone residues in biological samples [9–14]. However, these HPLC-based methods are sometimes limited by poor separation efficiencies, expensive columns, and the consumption of relatively high amounts of buffer solutions and organic solvents. Some non-chromatographic methods, such as luminescence spectroscopy [15,16], immunoassays [17,18], have also been reported for the determination of quinolones including ENR and CIP. Problems encountered by these methods include lack of required selectivity for complex mixtures, or only allow semi-quantitative analysis. Capillary electrophoresis (CE) seems to have potential for the analysis of quinolones. The advantages of CE for quinolones analysis include its speed and cost of analysis, reductions in solvent consumption and disposal, and the possibility of rapid method development. CE combined with ultraviolet (UV) detection [19,20], MS [7,21,22], or laser-induced fluorescence (LIF) [23], amperometric detection (AD) [24,25] for quinolones analysis has been reported. The UV detection is relatively non-specific and the achievable sensitivity is limited. LIF and MS can offer higher detection sensitivity, but the expensive instruments limit extensive application. AD shows high sensitivity and cheap facility. However, the detection of AD is easily affected by the high-voltage (HV) electric field and the adsorption of analytes on the working electrode [26,27]. Electrochemiluminescence (ECL) is a kind of chemiluminescence produced directly or indirectly as a result of electrochemical reactions. ECL detection, emerging as a very sensitive mode of detection, has many advantages including its simplicity, inexpensive instrumentation, low background noise, high sensitivity, good selectivity, and wide dynamic linear range [28–31]. The marriage of CE to ECL is a sensitive and efficient analytical technique and has got excellent performance for the analysis of amino acids [32,33], alkaloids [34,35], drugs [36–38], herbicide [39], and enzyme activities [40,41]. The purpose of this study is to develop a new simple and sensitive CE–ECL method for the analysis of ENR and CIP in milk samples. The method is based on the capillary electrophoretic separation and the detection of aliphatic tertiary or secondary amino moieties in ENR and CIP with end-column tris(2,2-bipyridyl)ruthenium(II) electrochemiluminescence. The pathway of this $Ru(bpy)_3^{2+}$/(ENR and CIP) system is similar to the reaction of $Ru(bpy)_3^{2+}$ with alkylamines which was firstly proposed by Noffsinger and Danielson [42]. It can be expressed as follows:

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+}$$

$$(\text{ENR or CIP}) - e^- \rightarrow (\text{ENR or CIP})^{\bullet +} \rightarrow (\text{ENR or CIP})^{\bullet} + H^+$$

$$Ru(bpy)_3^{3+} + (\text{ENR or CIP})^{\bullet} \rightarrow Ru(bpy)_3^{2+}* + \text{products}$$

$$Ru(bpy)_3^{2+}* \rightarrow Ru(bpy)_3^{2+} + h\upsilon \quad (\lambda = 620\,\text{nm})$$

In this study, samples cleanup and further preconcentration were executed by solid-phase extraction (SPE). Some parameters that affect separation and detection conditions were discussed in detail. The detection limits obtained in this work are low enough to determine concentrations below the permissible MRL in milk.

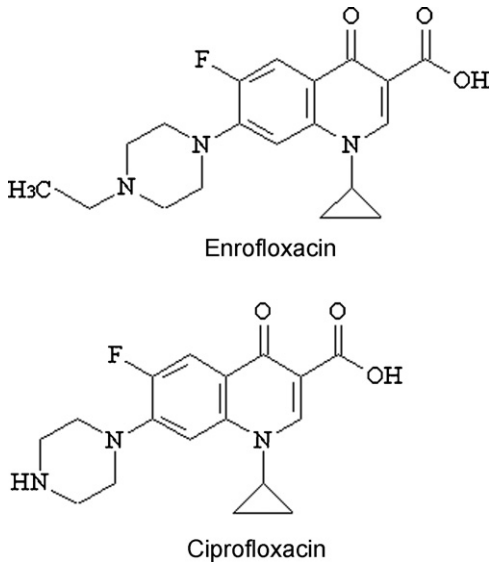

Fig. 1. Molecular structures of ENR and CIP.

2. Experimental

2.1. Chemicals and materials

Tris(2,2′-bipyridyl)ruthenium(II) chloride ($Ru(bpy)_3Cl_2$, 98%) was purchased from Aldrich (Milwaukee, WI, USA). Pure powder of ENR (≥98%) and CIP (≥98%) was obtained from Sigma (St. Louis, MO, USA). Water (≥18.2 MΩ) used throughout the experiments was generated by a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade. All solutions for CE were stored in the refrigerator at 4 °C prior to use. The solutions were made up to volume with appropriate buffer. The solutions used throughout the experiments were all passed through 0.22-μm filters before being injected into the CE system. Oasis HLB cartridges (60 mg, Waters, Milford, MA, USA) were used in the procedure of SPE.

2.2. Apparatus and equipments

A programmable high-voltage power supply (0–20 kV, Xi'an Remax Electronics Co. Ltd., Xi'an, China) was applied to perform the electrokinetic sample injection and electrophoretic separation. ECL detection was carried out with a MPI-A capillary electrophoresis ECL detector (Xi'an Remax Electronics Co. Ltd., Xi'an, China), using a three-electrode system consisting of a 300-μm diameter platinum wire as working electrode, Ag/AgCl (KCl saturated) as reference electrode, and a platinum wire as counter electrode. Before use, the working electrode surface was polished with 0.3 μm α-alumina powder and washed with water in an ultrasonic cleaner. A 300-μl aliquot of 100 mM pH 8.5 phosphate buffer containing 5 mM $Ru(bpy)_3^{2+}$ was added to the cell for CE–ECL detection and replaced every 3 h. The photons were captured by PMT which was located in the lower

layer of detection cell. The voltage of PMT was set at 800 V in the process of detection. An uncoated fused-silica capillary with 50 cm length, 50 μm i.d., and 360 μm o.d. was used for separation (Yong Nian Optical Fiber Factory, Hebei, China). The capillary-to-electrode distance was fixed at 120 μm according to the previous optimization [43].

2.3. Electrophoretic procedure

Running buffer solutions were prepared with NaH_2PO_4 and Na_2HPO_4 at different concentrations and pH was adjusted with NaOH and H_3PO_4. For preconditioning, the capillary was pretreated by rinsing at high pressure with 1 M NaOH for 10 min, followed by pure water for 10 min, and phosphate electrolyte for 15 min. In order to obtain better reproducibility, between runs, the capillary was rinsed at high pressure with 0.1 M NaOH for 1 min, pure water for 2 min, and buffer for 3 min. The injection was done electrokinetically and CE was executed in room temperature.

2.4. Standard solutions

A standard solution containing ENR and CIP was first dissolved in methanol as a stock solution at concentration of 1 mg ml^{-1} and stored in refrigerator at 4 °C. Working standard solutions were prepared daily with designated concentration by diluting the stocking standard solutions in 0.1% acetic acid.

2.5. Extraction procedure

The extraction procedure of ENR and CIP used here was a modification of the technique reported by Barbosa and coworkers [44]. A 5-g aliquot of milk purchased from a local supermarket was accurately weighed in a 50-ml test tube and spiked with ENR and CIP at different concentration levels. The sample was shaken on a vortex mixer for 30 s and then allowed to stand at 4 °C in the dark, for at least 20 min, to enable sufficient equilibration with the milk matrix. Then 1.5 ml of 0.1 M phosphate buffer (pH 7.4) and 20 ml of dichloromethane were added to the sample in order to extract the ENR and CIP. After agitating for 10 min the sample was centrifuged for 10 min at 3500 rpm. The organic phase was transferred into a 50 ml heart-shaped flask and the sample was re-extracted with another 10 ml portion of dichloromethane. The organic extracts were combined and 1 ml of 3 M H_3PO_4 was added. Dichloromethane was evaporated under vacuum in a rotary evaporator at room temperature until only aqueous phase remains. The above mixture was defatted with 10 ml of hexane. After centrifugation, the aqueous phase was passed through the HLB cartridge, which was previously conditioned with 2 ml of methanol and 2 ml of water. The cartridge was then washed with 2 ml of water and vacuum-dried. ENR and CIP were eluted from the cartridge with 3 ml of methanol. The eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 500 μl of 0.1% acetic acid.

3. Results and discussion

3.1. Optimization of CE–ECL conditions

3.1.1. Effects of detection potential

The ECL intensity is dependent on the rate of the light-emitting chemical reaction, and this reaction rate relies on the detection potential. Therefore, the potential applied to the working electrode directly affected the detection sensitivity. In order to obtain best detection results, optimum potential applied to the working electrode should be selected. In this study, the effect of detection potential from 1.0 V to 1.4 V on the signal intensities of both ENR and CIP was investigated. As can be seen from Fig. 2, the signal intensity of the two analytes exhibited same dependence on the detection potential. Increase in detection potential from 1.0 V to 1.15 V resulted in a strong increase in the signal intensity of ENR and CIP. The reason may be that the relative low oxidation rate of $Ru(bpy)_3^{2+}$ at the surface of electrode when applied potential was below 1.15 V and reached its maximum oxidation rate at 1.15 V. However, with the increasing detection potential from 1.15 V to 1.4 V, the signal intensity of both ENR and CIP decreased, possibly due to the oxidation of water which has negative effect on the ECL intensity. Thus, 1.15 V was chosen as the detection potential for further determination of the two analytes.

3.1.2. Effects of separation voltage

Both the electroosmotic and electrophoretic velocities are directly proportional to the field strength, so the microenvironment of the outlet of capillary aligned with the working electrode will be affected by the effluent from the capillary when separation voltage was changed. Based on this fact, the effect of separation voltage on and ECL intensity was investigated in the range of 8–20 kV. In these experiments it was found that ECL intensity increased and reached the maximum value at 14 kV for both ENR and CIP. This was also increased due to electroos-

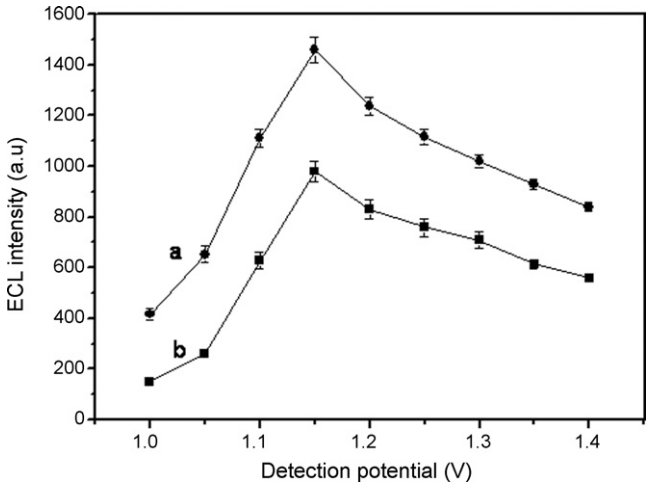

Fig. 2. Effect of detection potential on the ECL intensity (5 μg ml^{-1} standard solutions): (a) ECL intensity of ENR and (b) ECL intensity of CIP. Conditions—electrokinetic injection: 10 s at 10 kV and separation voltage: 14 kV.

mosis and more analytes in the effluent arrived in the diffusion layer of working electrode within a given time [37]. When the separation voltage exceeded 14 kV, the ECL intensity decreased, at the same time the noise of the background increased with the voltage, possibly due to the effect of high joule heat. On the other hand, the strong flow of effluent from the capillary decreased the concentration of $Ru(bpy)_3^{2+}$ at the working electrode surface, thereby reducing the efficiency of ECL reaction. For this reason, 14 kV was chosen as the optimum in the following experiments.

3.1.3. Effects of running buffer concentration and pH

An investigation of running buffer concentration was performed. Although buffer concentration has various influences on this CE–ECL method, for example, migration time, resolution, and ECL intensity, sensitivity is one of the most important parameters in the trace analysis. So, ECL intensity was carefully examined when the buffer concentration changed from 5 mM to 40 mM. Results have shown that the highest ECL intensity was obtained at a running buffer concentration of 15 mM. Higher buffer concentration had a negative effect on the ECL intensity, perhaps because other ions replace $Ru(bpy)_3^{2+}$ in the vicinity of the working electrode.

Besides the running buffer concentration, its pH value is also an important parameter because of its effect on the electroosmotic flow (EOF) as well as the net charge of the ENR and CIP. Because both ENR and CIP have two pK values, the pK_1 values of ENR and CIP are 5.86 and 5.88, respectively, and the pK_2 values of ENR and CIP are 8.24 and 7.74, respectively. In acidic running buffer the cationic species should be predominant while the basic pH of the running buffer should have shifted the equilibrium to the right, leaving the anionic species predominant for both the ENR and CIP. Previous reports have indicated that basic pHs provide better separation of quinolones than acidic pHs acidic buffer, possibly due to interactions on the interior capillary wall of the predominantly cationic form of quinolones and lack of an EOF [21]. Barbosa and coworkers have also established the model for predicting the optimum pH range for the separation of ENR and CIP, it is deduced that the best separation is around pH 8 [19]. On the other hand, the ECL response is also pH-dependent because the oxidation of alkylamines occurs only in their deprotonated form and at very low pH values the ENR and CIP radical cations are difficult to deprotonate to form high reducing free radical intermediate which is critical for ECL reaction. For these reasons, acidic buffer could not be assayed in this study. Therefore, the effect of the pH of running buffer on CE separation and ECL intensity was investigated in the pH range of 7.0–9.0 (7.0, 7.5, 8.0, 8.5, and 9.0). As shown in Fig. 3, it was found that the peak of ENR and CIP was completely overlapped at pH 7.0. With the increase of the pH of running buffer, the separation of ENR and CIP was improved gradually. Finally, baseline separation can be reached when background electrolyte was set at pH 8.5. But above this value there was no improvement in the resolution. As was expected, ECL intensity also showed the similar tendency. We noted that the ECL intensity decreased when the buffer pH exceeded 8.5. The reason of the decrease of the ECL intensity can be ascribed to the reduced availability of $Ru(bpy)_3^{3+}$ due to the competitive reaction with

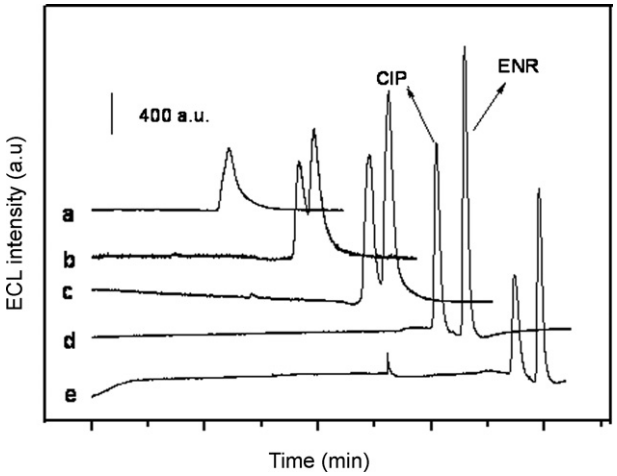

Fig. 3. Electropherograms showing the effect of the running buffer pH on the ECL intensity and separation (5 μg ml^{-1} standard solutions): (a) pH 7, (b) pH 7.5, (c) pH 8, (d) pH 8.5 and (e) pH 9. Conditions—detection potential: 1.15 V, 15 mM phosphate running buffer (pH 8.5), electrokinetic injection: 10 s at 10 kV and separation voltage: 14 kV. Note that the time axes for the electropherograms were shifted horizontally for clarity of presentation.

the OH^- ion which assumes considerable concentration levels at high pHs [45]. For a comprehensive thought, the buffer pH value of 8.5 was chosen to obtain sensitive ECL response and high separation efficiency.

3.1.4. Effect of injection time

In order to avoid excessive heat generation and bubble formation under these high electric field strength conditions, we chose 10 kV as the injection voltage. For injection time optimization, the studied interval was from 5 s to 30 s. The effect of the injection time on resolution was also investigated. The resolution (Rs) between ENR and CIP was calculated with the following equation: $Rs = 2(t_2 - t_1)/(W_{b1} + W_{b2})$, where t_1 and t_2 are migration times of CIP and ENR, respectively, W_{b1} and W_{b2} are the peak widths at half-height of CIP and ENR, respectively. Generally, at larger injection time, more analytes appeared at the working electrode, which produced a higher ECL signal. However, the sample zone may expand in the capillary during its running, which will lead the deterioration of resolution. In our experiments, the ECL intensity of both ENR and CIP increased with the increase of injection time in the range of 5–25 s. When the injection time exceeded 15 s, the resolution got deteriorated gradually (Fig. 4). We noted that the resolution was satisfactory even though the injection time extended as long as 20 s, and attended by a remarkable enhancement of sensitivity. Therefore, we selected 20 s as the injection time for subsequent experiments. The phenomena can be generally referred to as a stacking effect. A rational explanation is that sample injection was performed electrokinetically, and separation was performed using a basic background electrolyte (pH 8.5), 0.1% acetic acid used as samples solvent, which may prompt the protonation of analytes. When high voltage was applied, a discrete pH step was formed and literally stacked charged analytes at the interface of the sample and background electrolyte zones.

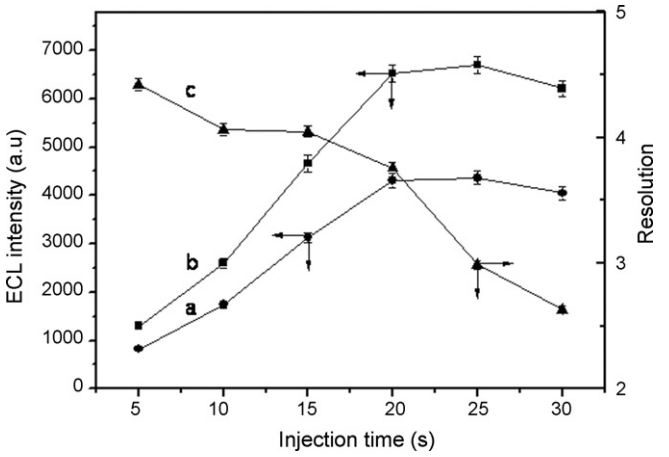

Fig. 4. Effect of injection time on ECL intensity and resolution (5 μg ml^{-1} standard solutions): (a) ECL intensity of CIP, (b) ECL intensity of ENR and (c) resolution between ENR and CIP. Conditions—detection potential: 1.15 V, 15 mM phosphate running buffer (pH 8.5) and separation voltage: 14 kV.

3.2. Linearity, repeatability, detection limit of ENR and CIP

Under optimized experimentation conditions: detection potential 1.15 V, separation voltage 14 kV, 15 mM phosphate running buffer (pH 8.5), injection voltage 10 kV and injection time 20 s, the different concentrations of ENR and CIP were measured from 0.01 μg ml^{-1} to 1.5 μg ml^{-1}. It was found that calibration was linear over concentration ranges of 0.03–1 μg ml^{-1} and 0.05–1.2 μg ml^{-1} for ENR and CIP, respectively. Regression analysis of the calibration data was performed by the use of OriginPro 7.5. The calibration equations and regression coefficients were $y = 1438(\pm 40)x + 32(\pm 13)$ and $R = 0.998$ for ENR, $y = 810(\pm 26)x + 45(\pm 18)$ and $R = 0.997$ for CIP. The calibration graphs of ENR and CIP are shown in Fig. 5. Limits of detection (LOD) were evaluated, by comparing signals from low concentrations of analytes with those from blank samples, and establishing the minimum concentrations at which the analytes could be detected reliably, on the basis of signal-to-noise ratio of 3. For ENR and CIP the LOD were 10 ng ml^{-1} and 15 ng ml^{-1}, respectively. The repeatability of the method was studied by six consecutive injections of standard solution of both ENR and CIP at 1 μg ml^{-1}. Relative standard derivations (R.S.D.s) of the ECL intensity and the migration time were 4.13% and 1.16% for ENR and 3.25% and 0.84% for CIP, respectively.

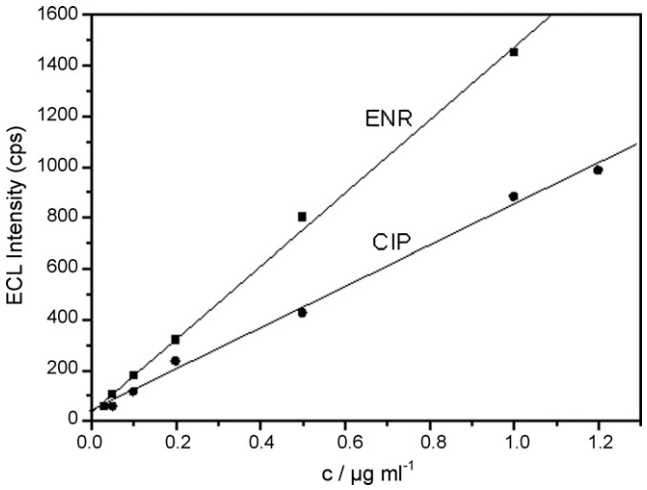

Fig. 5. The calibration graphs of ENR and CIP. Conditions—detection potential: 1.15 V, separation voltage: 14 kV, 15 mM phosphate running buffer (pH 8.5), injection voltage: 10 kV and injection time: 20 s.

3.3. Application to milk sample

The developed CE–ECL method in this report was applied to the separation and determination of ENR and CIP in milk samples. On account of the complexity of the milk samples which contain large concentrations of fat and proteins, thus samples need to be pretreated before to perform CE separation. In general, liquid–liquid extraction and SPE are the most frequently used methods to extract quinolones from complex samples. In this study, attempts were initially made to analyze ENR and CIP with a liquid–liquid extraction procedure. However, this proved unsuccessful as numerous interfering peaks appeared in the electropherogram. Therefore, additional sample preparation was required and involved off-line SPE to remove interfering compounds. For SPE procedure, there are different available solid-phase cartridges (for example, ion-exchange cartridge, polymeric phase cartridge, and reversed-phase exchange cartridge) which can be utilized for quinolones (include ENR and CIP) extraction from biological samples. Literature searches have revealed that the highest recoveries can be obtained for both ENR and CIP that was used a polymeric phase HLB cartridge [7,11,44]. Thus, HLB cartridge was chosen for this experiment. The detailed extraction procedure was described in Section 2. Chiefly, proteins and the fat were eliminated by solvent extraction using dichloromethane and hexane. Then the cleaner extracts obtained were used later for a second step using HLB cartridges to eliminate salts and a preconcentration factor 10 was applied. This method was validated using milk samples spiked with several levels of standard ENR and CIP mixture, and subjected to the entire extraction procedure. Recovery studies were determined by comparing the spiked sample peaks with an externally generated calibration curve at three concentration levels. The recoveries data are reported in Table 1. The mean recoveries were 77.4% for CIP and 80.6% for ENR, and R.S.D. was lower than 10% for both ENR and CIP. Calibration parameters in spiked milk samples were determined and were summarized in Table 2. The LOD and

Table 1
Recovery for ENR and CIP at different spiked levels in milk samples

Spiked concentration (μg kg^{-1})	Recovery (%)	R.S.D. (%) ($n = 3$)
CIP		
20	76.3	9.21
40	78.7	8.33
80	77.4	8.12
ENR		
20	79.6	8.48
40	80.8	7.36
80	81.4	7.71

Table 2
Calibration parameters in spiked milk samples

	ENR	CIP
Linearity ($\mu g\,kg^{-1}$)	5–400	8–400
Calibration equation[a]	$y = 10.1(\pm0.4)x + 22.4(\pm10.4)$, $R = 0.996$	$y = 6.11(\pm0.2)x + 25.4(\pm18.1)$, $R = 0.997$
LOQ ($\mu g\,kg^{-1}$)[b]	5	8
LOD ($\mu g\,kg^{-1}$)[c]	1.5	3

[a] y = ECL intensity; x = spiked concentration of the ENR and CIP in $\mu g\,kg^{-1}$.
[b] Calculated from the peak height based on a signal-to-noise ratio of 10.
[c] Calculated from the peak height based on a signal-to-noise ratio of 3.

Table 3
Comparison of the results obtained by the present method with other CE-based assay

	LOQ ($\mu g\,kg^{-1}$)		LOD ($\mu g\,kg^{-1}$)		Recovery (%)		Extraction procedure
	CIP	ENR	CIP	ENR	CIP	ENR	
CE–ECL	8	5	3	1.5	77.4	80.6	SPE
CE–UV [19]	50	25	25	10	54	74	SPE
CE–MS [7]	17	18	5	5	84	92	Two-step SPE
CE–LIF [23]	20	5	–	–	22	68	Liquid–liquid extraction

limit of quantitation (LOQ) of ENR and CIP were estimated on the basis of the results for two replicates of milk samples spiked at low concentration levels, considering a signal-to-noise ratio of 3 and 10, respectively. For ENR and CIP the LOQ was 5 $\mu g\,kg^{-1}$ and 8 $\mu g\,kg^{-1}$ and LOD was 1.5 $\mu g\,kg^{-1}$ and 3 $\mu g\,kg^{-1}$, respectively, considering that the MRL established for ENR plus CIP are 100 $\mu g\,kg^{-1}$, the proposed method is sensible enough for the analysis of ENR and CIP in milk or other biological samples, because the values of LOD and LOQ obtained were below the MRL established for ENR and CIP in the Council Regulation 2377/90 of European Union [8]. The linearity of the response was established from six calibration levels with a start point of the LOQ, covering the range from 5 $\mu g\,kg^{-1}$ to 400 $\mu g\,kg^{-1}$ for ENR (5 $\mu g\,kg^{-1}$, 10 $\mu g\,kg^{-1}$, 50 $\mu g\,kg^{-1}$, 100 $\mu g\,kg^{-1}$, 200 $\mu g\,kg^{-1}$, and 400 $\mu g\,kg^{-1}$) and from 8 $\mu g\,kg^{-1}$ to 400 $\mu g\,kg^{-1}$ for CIP (8 $\mu g\,kg^{-1}$, 10 $\mu g\,kg^{-1}$, 50 $\mu g\,kg^{-1}$, 100 $\mu g\,kg^{-1}$, 200 $\mu g\,kg^{-1}$, and 400 $\mu g\,kg^{-1}$). Injecting each level in triplicate and intending to establish the MRLs in the middle of the linear calibration range. The studied linearity ranges were considered valuable for analysis since real milk samples with higher content of ENR and CIP only occurs rarely. The calibration curves established for both ENR and CIP present correlation coefficients higher than 0.990. The data of comparing the results obtained by the present method with previously published CE-based assays are given in Table 3. As can be seen, the LOQ and LOD of CIP and ENR, obtained by our method, were lower than that of other CE-based assays. This indicates that the present method is one of the most sensitive methods for the analysis of ENR and CIP using CE to the present.

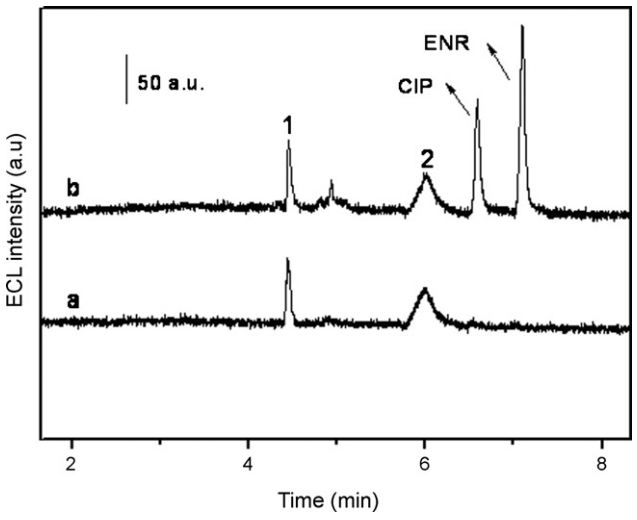

Fig. 6. Electropherograms of (a) blank milk sample and (b) the blank milk sample spiked 15 $\mu g\,kg^{-1}$ CIP and 15 $\mu g\,kg^{-1}$ ENR; (1) and (2) are the peaks of unknown compounds in extract. Conditions—detection potential: 1.15 V, 15 mM phosphate running buffer (pH 8.5), electrokinetic injection: 20 s at 10 kV and separation voltage: 14 kV.

Fig. 6 shows the typical electropherograms of a blank milk sample and milk sample spiked with 15 $\mu g\,kg^{-1}$ CIP and ENR, which was threefold lower than of the MRLs established by the European Union. No interferences were found co-migrating with ENR and CIP showing the proper specificity of the proposed method.

4. Conclusions

We have demonstrated that CE–ECL, combined with an effective sample cleanup, is the method of choice for the detection of ENR and its metabolite CIP in milk samples. Relative to other reported CE procedures for ENR and CIP, this approach offers improved detection limits. In addition, the whole method is simple, accurate, selective, and can detect the concentration of ENR and CIP residues in milk below MRLs. This work gives a demonstration of the feasibility of CE and ECL detection for trace analysis of ENR and CIP. However, there may be a much wider range of application of the area of food and other kinds of samples.

Acknowledgments

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