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Simultaneous determination of three fluoroquinolones by linear sweep stripping voltammetry with the aid of chemometrics

Yongnian Ni [a,*], Yuerong Wang [a], Serge Kokot [b]

[a] *Department of Chemistry, Nanchang University, Nanchang, Jiangxi 330047, China*
[b] *Inorganic Materials Research Program, School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Qld 4001, Australia*

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Abstract

A linear sweep stripping voltammetric (LSSV) method has been researched and developed for simultaneous quantitative determination of mixtures of three antibiotic drugs, ofloxacin, norfloxacin and ciprofloxacin. It relies on reductive reaction of the antibiotics at a mercury electrode in a Britton–Robinson buffer (pH 3.78). The voltammograms of these three compounds overlap strongly, and show non-linear character. Thus, it is difficult to analyse the compounds individually in their mixtures. In this work, chemometrics methods such as classical least squares (CLS), principal component regression (PCR), partial least squares (PLS) and radial basis function-artificial neural networks (RBF-ANN) were applied for the simultaneous determination of these compounds. The prediction performance of the calibration models constructed on the basis of these methods was compared. It was shown that satisfactory quantitative results were obtained with the use of the RBF-ANN calibration model relative prediction error (RPE_T) of 8.1% and an average recovery of 101%. This method is able to accommodate non-linear data quite well. The proposed analytical method based on LSSV was applied for the analysis of ofloxacin, norfloxacin and ciprofloxacin antibiotics in bird feedstuffs and their spiked samples, as well as in eye drops with satisfactory results.

Keywords: Fluoroquinolones; Voltammetry; Chemometrics; Radial basic function-artificial neural network

1. Introduction

Fluoroquinolones are important antibacterial agents developed in the 1980s, and have many applications in veterinary and human medicine. The pharmaceuticals have a broad spectrum of activity and good oral absorption [1]. These drugs are bactericidal over a wide range of therapeutically achievable concentrations, and act via selective inhibition of bacterial DNA synthesis. They have a broad range of action against both Gram-negative and Gram-positive bacteria [2,3]. In this work, three important fluoroquinolones (Fig. 1), ofloxacin [9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid], ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid] and norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid] were investigated and quantitatively analysed by voltammetric methods.

Many methods have been developed for the determination of ofloxacin, ciprofloxacin and norfloxacin including spectrophotometric methods [4–6], high-performance liquid chromatography (HPLC) [7–9], fluorescence methods [10–12] and capillary electrophoresis [13–15]. Of these methods, HPLC has been widely applied because of its high sensitivity and selectivity and the ability to minimize interferences. Generally, linear calibration may be obtained over the range of 0.1–0.5 $\mu g\,ml^{-1}$ with mean recovery of $97 \pm 6\%$ [8].

Electrochemical methods, such as polarography and voltammetry have high sensitivity and are widely used in many areas of analytical chemistry. Over the last 15 years, direct determination of ofloxacin, ciprofloxacin and norfloxacin as individual analytes, has been investigated by these two techniques with polarographic methods being favoured in the early 1990s. Thus, in 1990, a differential pulse polarographic (DPP) investiga-

* Corresponding author.
E-mail address: ynni@ncu.edu.cn (Y. Ni).

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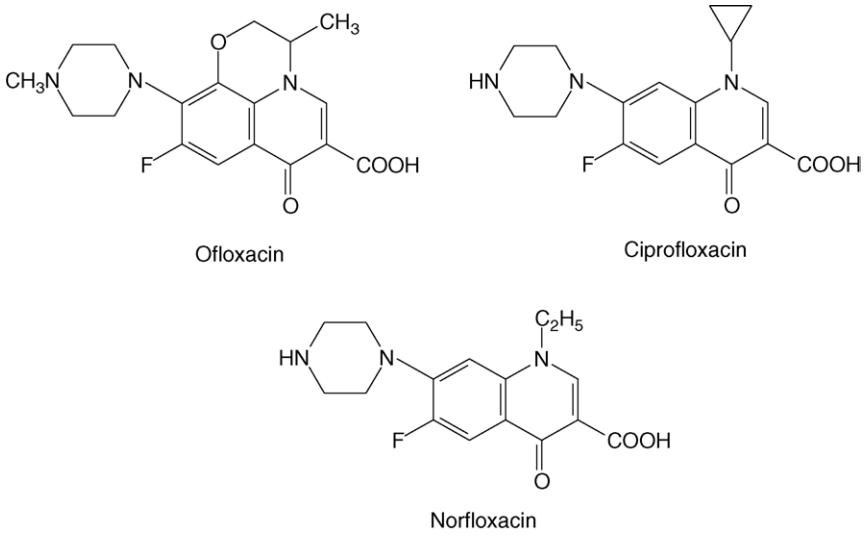

Fig. 1. Chemical structures of the antibiotics, ofloxacin, ciprofloxacin and norfloxacin.

tion of ciprofloxacin dissolved in Britton–Robinson buffer of pH 8.5 showed two reduction waves at −1.44 and −1.64 V. The former wave was shown to be useful for the determination of the concentration of ciprofloxacin between 5×10^{-7} and 3×10^{-5} mol l^{-1}; the method was also applied for the determination of ciprofloxacin in formulated tablets, and the results indicated a relative standard deviation of less than 0.4% [16]. In the same year, an adsorption stripping voltammetry (ASV) study of the determination of ofloxacin was reported [17]. The compound was also dissolved in a Britton–Robinson buffer (pH 6.00), and a well-defined stripping peak was observed with a peak potential of −1.675 V (versus Ag/AgCl). A pre-concentration step of 60 s at −1.0 V, led to the development of a linear calibration model in the concentration range of 0.079–197.5 μg ml^{-1}. This method was applied for the determination of ofloxacin tablets with satisfactory results. In the mid-1990s, Jaber and Lounici [18] investigated the fluoroquinolone, norfloxacin, with the use of direct current polarography (DCP) and differential pulse polarography. A reduction peak for this compound was obtained from solutions in different electrolytes over a pH range of less than 1–10 as well as in highly alkaline solutions of 0.1 mol l^{-1} NaOH. The same authors [19] applied cyclic voltammetry (CV) to study the adsorptive processes of norfloxacin at a hanging mercury drop electrode (HMDE) in several electrolytes with pH <9. Three differential pulse cathodic stripping voltammetry (DPCSV) waves were observed from solutions of various electrolytes over a potential range of −1.02 to −1.54 V with the one at −1.02 V being the most sensitive. Satisfactory linear calibration models were obtained over two concentration ranges of 0.32–900 ng l^{-1} and 0.32–65 μg l^{-1}. In subsequent years, Zhou and Pan [20] reported that a linear sweep voltammetric (LSV) peak of ofloxacin was obtained at −1.343 V (versus SCE) in Britton–Robinson buffer (pH 4.00). The characteristics of the peak and the mechanism of the reduction process were studied with the results showing that the electrochemical process of ofloxacin was irreversible, and displayed adsorption characteristics at the electrode. The peak current was proportional to the concentration over the range 8×10^{-4} to 2×10^{-5} mol l^{-1} with a limit of detection of 4×10^{-6} mol l^{-1}. Rizk et al. [21] applied DPP technique for the determination of ofloxacin in its pure form, medicinal preparations and biological fluids. The relationship between current and concentration was found to be linear over the range 5×10^{-5} to 5×10^{-4} mol l^{-1} and 1×10^{-5} to 5×10^{-4} mol l^{-1} using the DC_t and DPP modes, respectively. Ghoneim et al. [22] reported that in acetate buffer of pH 5.0, the adsorptive and electrochemical behaviour of norfloxacin on a glassy carbon electrode were studied using CV and square wave voltammetry (SWV). The peak current of norfloxacin was linear with the concentration in the range of 5–50 μg ml^{-1}, and the detection limit was 1.1 μg ml^{-1}. In general, it can be shown that voltammetric and polarographic methods will give similar or higher sensitivity as compared to the HPLC method [8].

As described above each of the three antibiotic drugs, ofloxacin, norfloxacin and ciprofloxacin, will give well-defined voltammetric waves in a suitable medium. However, these drugs are sometimes used in mixtures [8], and several studies have indicated the presence of their residues in municipal wastewater and surface waters [23], live animals and animal products [24]. In such samples, measured voltammograms from the three antibiotic analytes would overlap significantly. Thus, it is difficult or indeed impossible to determine the analytes individually from their mixtures by conventional means without a pre-separation.

In general, multivariate calibration methods have the ability to resolve overlapping peaks, and electrochemical responses in particular, leading to satisfactory prediction results. Thus, Guiberteau et al. [25] resolved the overlapping differential pulse voltammetric (DPV) signals from a mixture of two carbamate pesticides carbaryl and carbofuran with the aid of chemometrics. Partial least squares (PLS) and classical least squares (CLS) methods were examined for the resolution of the overlapped peaks of both compounds in mixtures, and it was found that the PLS-1 method gave the best results with a relative error of prediction (%RPE) of 8.2% and 5.1% for the prepared binary mixtures of carbaryl and carbofuran, respectively. Navalon et

al. [26] reported the application of principal component regression (PCR) to resolve the ASV voltammograms of enrofloxacin in the presence of its metabolically derived, ciprofloxacin. The linear concentration ranges of application for enrofloxacin were 4–25 ng ml^{-1} and 18–55 ng ml^{-1} by using a pre-concentration potential of −0.3 V and 180 s or 60 s accumulation time, respectively. Ni et al. [27] applied multivariate calibration methods for the determination of nitrobenzene and nitro-substituted phenols by differential pulse voltammetry, and determination of chlorpromazine hydrochloride and promethazine hydrochloride by differential pulse stripping voltammetry (DPSV) [28] in both cases with satisfactory prediction results. In the case of the group of industrially important chemicals—the nitro-phenols PLS and PCR modeling gave %RPE$_T$ values of approximately 10 with LODs in the order of 3 μg l^{-1}. Similarly, with the pharmaceuticals, chlorpromazine and promethazine hydrochlorides, applying the same chemometrics modeling methods, the %RPE$_T$ were about 5 with LODs in the order of 40 μg l^{-1}. Guiberteau Cabanillas et al. [29] used PLS and artificial neural networks (ANN) to determine binary mixtures of atrazine–simazine and terbutryn–prometryn by DPP techniques in the concentration ranges of 5×10^{-7} to 5×10^{-6} mol l^{-1} with LODs of 9.4, 5.2, 7.2 and 4.4 mol l^{-1} for atrazine, simazine, terbutryn and prometryn, respectively. In comparison to the PLS modeling, the ANN calibration method notably improved the results for terbutryn and prometryn, and yielded a significant decrease in the R.S.D. For atrazine and simazine an insignificant slight decrease in the R.S.D. was found. Simons et al. [30] applied singular value decomposition (SVD) and ANN techniques to resolve the overlapping voltammetric signals of solution mixtures consisting of two nitrophenols with two disk electrodes (gold and rhodium). In their experiments, measurements were made on 117 solutions in order to obtain stable ANN model. Carvalho et al. [31] used ANN to model overlapped DPV peaks with modified carbon fiber electrode of binary mixtures of catechol and hydroquinone, in the concentration range of 1.0×10^{-4} to 6.0×10^{-4} mol l^{-1} with root mean square errors of predictions (%RMSEP) of 7.42 and 8.02, respectively. Gutes et al. [32] applied ANN to determine phenolic compounds with biosensor measurements with good prediction results. Sarabia et al. [33] developed a new methodology for complex matrices, based on a neural network, to determine the detection capability of an analytical procedure. The procedure was applied to the polarographic determination of Tl(I)/Pb(II) mixtures, and indomethacin/tenoxicam mixtures. Ni et al. [34] reported an application of chemometrics for the simultaneous determination of three organophosphorus pesticides by DPSV including methods such as PCR, PLS, Kalman filter (KF), and ANN in concentration range of 15–60 μg l^{-1}. The best performing method was ANN (%RPE$_T$—approximately 9; %recovery—approximately 100 and LODs in the order of 5 μg l^{-1}) with quite uniform figures of merit for the prediction of individual analytes. On the other hand, PLS performed much more erratically on the same basis resulting in significantly higher figures of merit, and the other three methods performed unsatisfactorily. This work illustrated the power of ANN to model non-linear responses, and this issue was explored in this paper.

In this paper, we describe the research and development of an analytical method for simultaneous determination of ofloxacin, ciprofloxacin and norfloxacin antibiotics with the use of linear sweep stripping voltammetry (LSSV) at an HMDE, with the aid of different chemometrics methods for prediction of the analytes, including CLS, PLS, PCR, and ANN.

2. Theory of the chemometrics methods

In electrochemical techniques such as LSSV, the measured current i_j at a given potential is proportional to the analyte concentration over a given concentration range. Thus, in the absence of interactions between the different components of a mixture of n electroactive species,

$$i_j = k_{0j} + \sum_{i=1}^{n} k_{ij} c_i + e_j, \quad (j = 1, 2, \ldots, s) \tag{1}$$

where k_{ij} is the proportional coefficient for component i at potential point j (total s points are selected) and k_{0j} is the corresponding background. Let $c_0 = 1$ and merge k_{0j} into the main term; Eq. (1) can further be simply written as:

$$i_j = \sum_{i=0}^{n} k_{ij} c_i + e_j \qquad (j = 1, 2, \ldots, s) \tag{2}$$

If m standard samples are prepared, Eq. (2) can be extended and expressed in matrix form:

$$\boldsymbol{I}_{m\times s} = \boldsymbol{C}_{m\times(n+1)} \boldsymbol{K}_{(n+1)\times s} + \boldsymbol{E}_{m\times s} \tag{3}$$

where the first row in matrix $\boldsymbol{K}$ represents the background vector. According to this equation, it is possible to determine the components individually by suitable chemometrics methods. In this work, the electrochemical data were treated by multivariate calibration methods, such as CLS, PLS, and PCR. The theory and applications of all these methods are well documented in the literature [35–38].

Classical least squares, which has often been labeled as the $\boldsymbol{K}$ matrix method, is a common multivariate calibration method that uses multiple linear regression (MLR) techniques and has been used for quantitative spectral analysis. This method has generally presumed that there is a linear relationship between the response signal and component concentrations. In addition, this method has a calibration step where the relationship between the measured data and component concentrations is estimated from a set of reference samples. This step is followed by prediction in which the results of the calibration are used to predict or estimate the component concentrations from the 'unknown' sample data. A major disadvantage of CLS is that all interfering chemical components in the spectral region of interest need to be known and included in the calibration.

Another two important multivariate calibration methods, partial least squares and principal component regression, were also applied for analysis of the overlapping voltammetric signals in this work. PLS and PCR are factor analysis multivariate statistical tools, which have many of the full-spectrum advantages of the CLS method, and have been successfully applied to anal-

ysis of multicomponent mixtures. Early PCR modeling harks back to the work of Gunst and Mason [39], Mandel [40] and Joliffe [41], while for PLS, Wold's pioneering work in chemometrics [42,43] was quickly followed by that of others such as Lindberg et al. [44], Geladi and Kowalski [38] and Lorber et al. [45]. Thomas and Haaland [37] have compared different multivariate calibration methods for the resolution of overlapping signals and quantitative analysis. Like CLS, PLS and PCR need a calibration step where the models for the measured data and the component concentrations are deduced from a set of standards, followed by a prediction step in which the concentrations of the unknown are estimated from the sample measured data. Both of these methods involve spectral matrices decomposition. The PCR decomposition is based entirely on response variations without regard for the component concentrations.

$$\boldsymbol{I}_{m\times s} = \boldsymbol{T}_{m\times d}\boldsymbol{P}^{\mathrm{T}}_{s\times d} = \sum_{i=1}^{d} \boldsymbol{t}_i \boldsymbol{p}_i^{\mathrm{T}} + \boldsymbol{E} \tag{4}$$

where $\boldsymbol{T}$ and $\boldsymbol{P}^{\mathrm{T}}$ are the score and loading matrices of $\boldsymbol{I}$, respectively. PCR decomposition is significantly influenced by variations, which have no relevance to the analyte concentrations. In this method, the following relationship between score $\boldsymbol{T}$ and the concentration matrix $\boldsymbol{C}$ can be established:

$$\boldsymbol{C}_{m\times n} = \boldsymbol{T}_{m\times d}\boldsymbol{G}_{d\times n} \tag{5}$$

where $\boldsymbol{G}$ is the regression coefficient matrix, and it can be calculated by least squares method: $\boldsymbol{G} = (\boldsymbol{T}^{\mathrm{T}}\boldsymbol{T})^{-1}\boldsymbol{T}^{\mathrm{T}}\boldsymbol{C}$. It is noted that, as compared with CLS, for PCR and PLS of factor analysis based methods, the last column in $\boldsymbol{C}$ is omitted. In PLS, the response signal decomposition is weighted to the concentration. Corresponding to Eq. (4), matrix $\boldsymbol{C}$ can be decomposed to:

$$\boldsymbol{C}_{m\times n} = \boldsymbol{U}_{m\times d}\boldsymbol{Q}^{\mathrm{T}}_{n\times d} = \sum_{i=1}^{d} \boldsymbol{u}_i \boldsymbol{q}_i^{\mathrm{T}} + \boldsymbol{F} \tag{6}$$

where $\boldsymbol{U}$ and $\boldsymbol{Q}^{\mathrm{T}}$ are the score and loading matrices for $\boldsymbol{C}$, respectively. The two matrices, $\boldsymbol{I}$ and $\boldsymbol{C}$, are correlated by their scores $\boldsymbol{T}$ and $\boldsymbol{U}$, for each latent variable, as follows:

$$\boldsymbol{u}_i = b_i \boldsymbol{t}_i \tag{7}$$

where b_i is the regression coefficient for the i latent variable.

The major difference in the predictive abilities of these two methods is that PLS seems to predict better than PCR when there are random linear baselines or independently varying major spectral components, which overlap with the spectral features of the analysis.

This is not surprising when one considers that the PCR decomposition of the spectral matrix is based entirely on *I*-variable variation, whereas PLS decomposition considers both *i*- and *c*-criteria. It can also describe non-linear systems to some extent. This can be carried out either by incorporating a larger number of latent variables than would be required for a linear system or with the use of non-linear or quadratic versions of the algorithms [46].

Radial basis function (RBF) network is an ANN model, which is a variant of the three-layer feed forward network. It contains a pass-through input layer, a hidden layer and an output layer. A different approach for modeling the data is used as compared to that with the back propagation (BP) algorithm, a detailed description of which can be found elsewhere [47,48]. The transfer function in the hidden layer of RBF networks is called a kernel or basis function, and thus, each node in the hidden unit contains a kernel function. The main difference between the transfer function in BP network and the kernel function in the RBF network is that the latter (usually a Gaussian function) defines an ellipsoid in the input space. RBF networks divide the input space into hyperspheres by means of the kernel function with specified widths and centres. The output of a hidden unit for a Gaussian kernel function is defined as:

$$\text{output}_j = o_j(\boldsymbol{x}) = \exp\left[-\left(\frac{|\boldsymbol{x} - c_j|}{b_j}\right)^2\right] \tag{8}$$

where $|\boldsymbol{x} - c_j|$ is the Euclidean distance between the input vector, $\boldsymbol{x}$, and c_j, the centroid of the Gaussian kernel function. The parameter b_j represents the width of the Gaussian function. The centroids, c_j, and the width, b_j of all the hidden units together define the so-called activation space to perform the non-linear transformation, where the Gaussian function has a value larger than a given threshold value. The output of these hidden nodes, o_i, is then forwarded to all output nodes through weighted connections. The output y_i of these nodes consists of a linear combination of the kernel function:

$$y_j = \sum_{i=1}^{n} w_{ji} o_i(\boldsymbol{x}) \tag{9}$$

where w_{ji} represents the weights of the connections between the hidden layer i and output layer j.

3. Experimental

3.1. Reagents

Stock solutions of ofloxacin, ciprofloxacin and norfloxacin (500 mg l^{-1}) were prepared by dissolving the appropriate amount of each compound in a small amount of 0.1 mol l^{-1} hydrochloric acid, and diluted to 100 ml with distilled water. Britton–Robinson buffers were prepared by adding different amounts (22.5 ml for pH 3.78) of sodium hydroxide solution (0.2 mol l^{-1}) into 100 ml of mixed acid, containing 0.04 mol l^{-1} of each of boric acid, *ortho*-phosphoric acid and acetic acid. All chemicals were analytical grade reagents and all the solutions were prepared with doubly distilled water.

3.2. Apparatus

The linear sweep stripping voltammetry was carried out with a BAS 100B/W electrochemical analyzer (BAS) equipped with a PARC 303A cell stand (EG & G). A three-electrode cell, containing a hanging mercury drop electrode as working electrode, an Ag/AgCl electrode as reference electrode and a platinum wire as auxiliary electrode was employed for the electrochemical measurements. The pH measurements were performed on an Orion

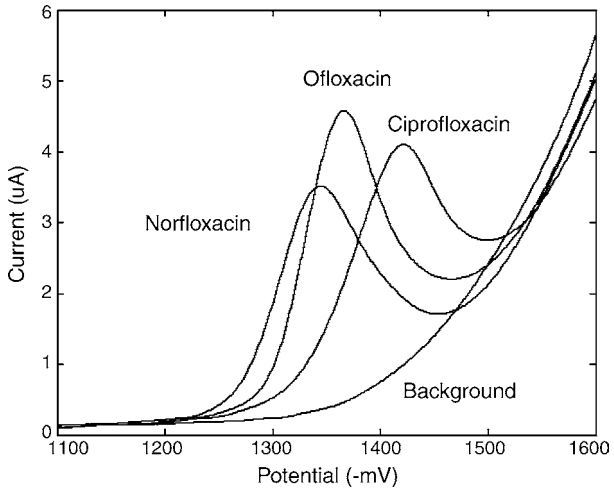

Fig. 2. Linear sweep stripping voltammograms of ofloxacin (0.03 μg ml^{-1}), ciprofloxacin (0.096 μg ml^{-1}) and norfloxacin (0.10 μg ml^{-1}) in Britton–Robinson buffer solution (pH 3.78); deposition potential: −1000 mV; deposition time: 30 s; scan rate: 140 mV s^{-1} and solution's rest time: 10 s.

SA 720 pH meter equipped with an Ag/AgCl glass combination pH electrode. All experiments were carried out at 25 °C. Data analysis was performed on a Pentium IV computer. Software of RBF-ANN was employed with the use of the Neural Network Toolbox, Matlab 6.5 (Mathworks) and other chemometrics programs were written in-house.

3.3. Procedure

Each sample solution contained an aliquot of either one of the antibiotics, ofloxacin, ciprofloxacin or norfloxacin or a mixture of these compounds, together with a Britton–Robinson buffer (2.0 ml, pH 3.78). Such a sample was transferred to an electrochemical cell, and diluted to 10.0 ml with distilled water. This solution was deoxygenated by purging with purified nitrogen gas for 200 s, and then submitted to a pre-concentration step at an HMDE set at a deposition potential of −1000 mV with stirring for 30 s. The solution was then allowed to rest for 10 s followed by a linear sweep stripping voltammetric scan (−1100 to −1600 mV) at the HMDE with a scan rate of 140 mV s^{-1}. The resulting voltammograms were electronically sampled at 151 potential points in the range of −1200 to −1500 mV at 2 mV interval. Overlays of examples of the voltammograms (Fig. 2) of the ofloxacin, ciprofloxacin and norfloxacin, show that they overlap strongly, and thus, if measured in mixtures, these antibiotics could interfere with each other at the electrode giving a complex combined signal from the mixture.

4. Results and discussion

Several electrochemical techniques were investigated on the basis of peak intensity and peak shape of the electrochemical responses from the three analytes, ofloxacin, ciprofloxacin and norfloxacin. These included: differential pulse voltammetry, differential pulse stripping voltammetry, square wave voltammetry, square wave stripping voltammetry (SWSV), linear sweep voltammetry and linear sweep stripping voltammetry. The experimental results showed that maximum peak current and well-defined peak shapes were obtained when the LSSV technique was employed. Therefore, LSSV was selected in this work.

4.1. Selection of pH and supporting electrolyte

The effect of pH was examined on the reduction peak current of ofloxacin, ciprofloxacin and norfloxacin voltammograms in different Britton–Robinson buffers (pH 1.98–6.37). The results showed that no obvious LSSV signals from these three antibiotics were observed when pH <2. Above pH 6 the peak shape of the voltammograms was poor, and it was difficult to obtain any quality results. The peak shapes were better defined in the pH range 2–6, but the peak current behaviour was erratic, and maximum peak current for the three compounds was obtained at pH 3.78. The relationship of peak potential and pH was roughly linear for all three compounds with peak potential shifting towards negative values with increase in pH.

The effect of supporting electrolyte was examined with the use of different solutions, including Britton–Robinson, acetic acid–sodium acetate, sodium citrate–hydrochloric acid and sodium tartrate–tartaric acid buffers. It was found that for the three pharmaceuticals, voltammograms of relatively high sensitivity could be obtained in both Britton–Robinson and sodium citrate–hydrochloric acid buffers. However, relatively better defined peak shapes were recorded with the Britton–Robinson buffer electrolyte. Variable results were observed with the other buffers, e.g. with the sodium tartrate–tartaric acid buffer, peak intensities were satisfactory for ofloxacin and nofloxacin but were relatively poor for ciprofloxacin, and with the acetic acid–sodium acetate buffer, the results were acceptable only for ciprofloxacin. Thus, Britton–Robinson buffer at pH 3.78 was selected as the electrolyte of choice.

4.2. Optimization of experimental parameters

The influence of deposition potential on the stripping reduction current was examined over the potential range of −500 to −1200 mV. With a shift to more negative deposition potentials, peak currents of the fluoroquinolone antibiotics did not change significantly. Thus, a deposition potential of −1000 mV was selected for all the experimental work.

The relationship between peak current of each compound and deposition time was also investigated. It was found that the peak current increased with increasing deposition time. Also, the quality of the peak shape of ciprofloxacin deteriorated when the deposition time exceeded 30 s, although the peaks of the other two compounds remained well-defined. Therefore, a deposition time of 30 s was selected for this work.

An investigation of the influence of scan rate on the peak current found that the peak potential of each compound became more negative with increasing scan rate from 40 to 200 mV s^{-1}, and the peak current of each analyte increased with the increase in the scan rate. The quality of the reduction peak of ciprofloxacin became poor when the scan rate exceeded 140 mV s^{-1}, and thus, this value was chosen as the scan rate for this work.

The net i_p behaviour as measured by LSV has been described [49] as the sum of diffusion ($i_{p,diff}$) and adsorption ($i_{p,ads}$) currents at the electrode:

$$i_p = i_{p,diff} + i_{p,ads} = 0.446\frac{n^{3/2}F^{3/2}}{R^{1/2}T^{1/2}}AD^{1/2}v^{1/2}c + \frac{n^2F^2}{4RT}vA\Gamma \quad (10)$$

where A is the area of the electrode; D, the diffusion coefficient; v, the scan rate; c, the concentration of the analyte, Γ, the amount of the adsorbed analyte, and n, R, F and T have their common meanings.

The first term in Eq. (10) refers to the well-known Randle–Sevcik relationship, which describes the diffusion-controlled process at the working electrode, while the second term relates to the adsorption process and shows that in this case, i_p is directly proportional to the scan rate v. In the present work, the plot of reduction peak current of each compound versus scan rate was linear:

$i_p(\text{ofloxacin}) = 0.017v + 2.301 \quad (r = 0.989)$,

$i_p(\text{norfloxacin}) = 0.007v + 1.666 \quad (r = 0.998)$ and

$i_p(\text{ciprofloxacin}) = 0.011v + 3.851 \quad (r = 0.989)$

Thus, this result indicated that the electrochemical reduction process of these three compounds at the HMDE was mainly adsorption-controlled [49].

4.2.1. Investigation of ofloxacin, ciprofloxacin and norfloxacin by cyclic voltammetry

The reduction process of ofloxacin, ciprofloxacin and norfloxacin was studied by cyclic voltammetry. Each of the voltammograms of the three fluoroquinolone displayed only one reduction peak and no obvious oxidation peak was observed. This indicates that the electrochemical reaction of each of the antibiotics is irreversible.

4.2.2. Calibration curves and the limits of detection

Quantitative analysis of individual fluoroquinolones was carried out according to the previously described experimental procedure. The relationship of peak current and concentration of each fluoroquinolone was fitted to a linear regression model (Table 1). The linear concentration ranges were 0.024–0.240 μg ml^{-1}, 0.032–0.288 μg ml^{-1} and 0.020–0.180 μg ml^{-1} for ofloxacin, ciprofloxacin and norfloxacin, respectively. The detection limit values were 0.006, 0.011 and 0.008 μg ml^{-1} for ofloxacin, ciprofloxacin and norfloxacin, respectively, which compare well with the detection limit obtained for the HPLC method with a UV detector (ca. 0.02 μg ml^{-1}) [8]. Thus, these results clearly indicate that the proposed electrochemical method of analysis is appropriate for the determination of individual fluoroquinolones.

4.3. Non-linearity of the voltammograms

It is important to note that the linear calibration relationships were observed only at the actual peak potential, but as can be observed in Fig. 3a–c, the peak potential of ofloxacin and ciprofloxacin strongly shifted towards the negative applied potentials with increasing analyte concentration; however, the peak potential of norfloxacin shifted towards the positive applied potentials in the concentration range of 0.02–0.10 μg ml^{-1} but then turned in the negative direction with concentrations above 0.10 μg ml^{-1}.

Guiberteau Cabanillas et al. [51,52] reported similar observations of non-linear behaviour for binary and ternary mixtures of nitrofurantoin, furazolidone and furaltadone, analysed by DPP. The polarograms were generated by the irreversible electrochemical reductions of the three compounds, and the peak potentials shifted to more negative values with increase in concentration for each component.

Table 1
Parameters of linear calibration models for ofloxacin, ciprofloxacin and norfloxacin

Parameter	Ofloxacin	Ciprofloxacin	Norfloxacin
Number of samples (n)	10	9	9
Linear range (μg ml^{-1})	0.024–0.240	0.032–0.288	0.020–0.180
Slope (μA ml μg^{-1})	33.3	18.6	23.8
Intercept (μA)	3.21	2.06	1.22
Standard deviation of slope	0.319	0.281	0.416
Standard deviation of intercept	0.048	0.051	0.047
Correlation coefficient	0.999	0.999	0.999
Detection limit (μg ml^{-1})[a]	0.006	0.011	0.008

[a] Detection limit for all compounds were calculated according to the method described in Miller and Miller [50].

4.3.1. Prediction of ofloxacin, ciprofloxacin and norfloxacin in synthetic mixtures

For quantitative analysis of mixtures of ofloxacin, ciprofloxacin and norfloxacin, a calibration set was prepared according to the orthogonal array design method, in order to extract maximum quantitative information efficiently. A four-level orthogonal array design, denoted by $OA_{16}(4^3)$ was selected [53], and Table 2 shows the composition of the calibration

Table 2
Composition of calibration samples (ng ml^{-1})

Samples	Ofloxacin	Ciprofloxacin	Norfloxacin
1	30	40	24
2	30	88	56
3	30	144	88
4	30	200	120
5	60	40	56
6	60	88	24
7	60	144	200
8	60	200	88
9	90	40	88
10	90	88	200
11	90	144	24
12	90	200	56
13	120	40	120
14	120	88	88
15	120	144	56
16	120	200	24

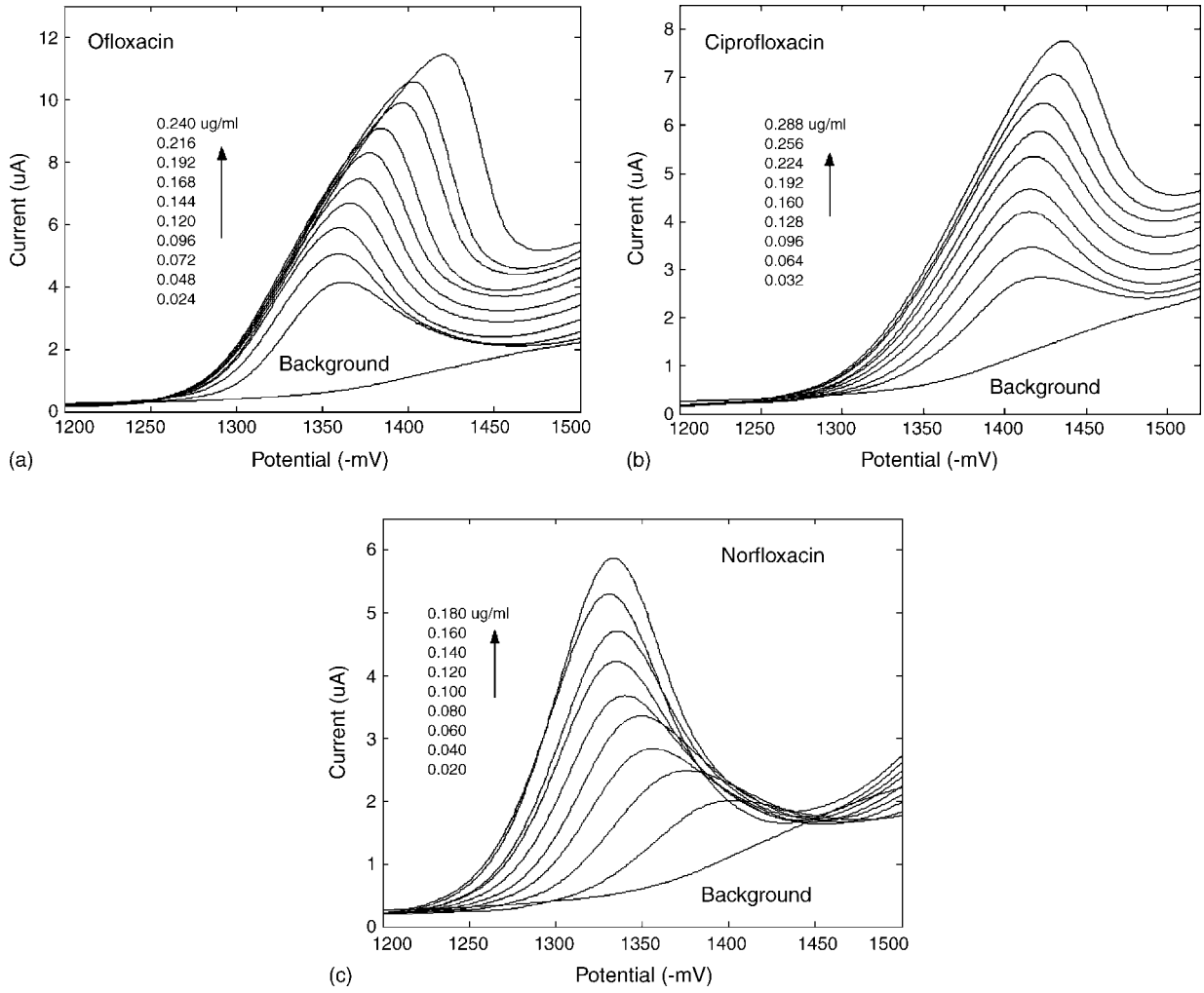

Fig. 3. Voltammograms of fluoroquinolone compounds as a function of concentration ($\mu g\,ml^{-1}$). Experimental conditions are as in Fig. 2.

samples. The voltammograms of these samples containing mixtures of the antibiotics show serious overlapping of individual responses, and there are only small differences between these curves (Fig. 4). Such voltammograms were analysed by LSSV according to the experimental procedure previously described, and different calibration models, based on CLS, PCR, PLS and RBF-ANN methods, were established. To test and evaluate the prediction ability of these chemometrics models, a set of ten synthetic verification mixtures containing the three compounds was prepared (Table 3), and submitted for prediction by each of the calibration models. The prediction ability was expressed in terms of the relative prediction errors—RPE_S for individual

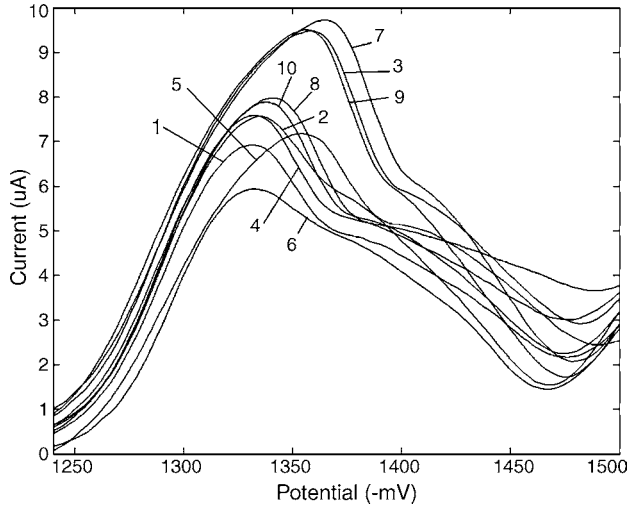

Fig. 4. Voltammograms of the prepared antibiotic mixtures as in Table 3 (numbers correspond to the sample numbers in Table 3).

Table 3
Composition of verification samples ($ng\,ml^{-1}$)

Samples	Ofloxacin	Ciprofloxacin	Norfloxacin
1	66	96	52
2	66	56	112
3	108	184	80
4	108	136	28
5	42	184	52
6	42	56	80
7	84	184	112
8	84	96	80
9	108	96	112
10	108	56	52

Table 4
Figures of merit for the different calibration models applied to the verification set of the three antibiotics

Compounds	RPE_S (%)			
	PLS[a]	PCR[b]	CLS	RBF-ANN[c]
Ofloxacin	10.8 (103)[d]	9.26 (104)	12.8 (105)	8.86 (105)
Ciprofloxacin	20.5 (107)	21.6 (113)	23.1 (91.6)	8.35 (101)
Norfloxacin	19.8 (116)	23.8 (113)	27.8 (103)	6.18 (97.8)
RPE_T (%)	18.4	19.9	22.3	8.06

[a] Five significant factors were extracted by cross-validation for PLS and PCR modelling.
[b] Five significant factors were extracted by cross-validation for PLS and PCR modelling.
[c] Parameters for spread coefficient and the number of neurons in the hidden layer are 30 and 25, respectively.
[d] The value in the parentheses is the mean %recovery for each compound, and it was calculated as: $\%\text{recovery} = 100 \times \Sigma(c_{ij,\text{pred}} - c_{ij,\text{added}})/n$, where n is the number of samples, c_{ij} is the concentration of the jth component in the ith sample.

compounds, and RPE_T for total compounds (Table 4).

$$RPE_s = \left[\frac{\sum_{i=1}^{n}(c_{ij,\text{pred}} - c_{ij,\text{added}})^2}{\sum_{i=1}^{n}(c_{ij,\text{added}})^2}\right]^{0.5} \quad (11)$$

and

$$RPE_T = \left[\frac{\sum_{i=1}^{n}\sum_{i=1}^{m}(c_{ij,\text{pred}} - c_{ij,\text{added}})^2}{\sum_{i=1}^{n}\sum_{i=1}^{m}(c_{ij,\text{added}})^2}\right]^{0.5} \quad (12)$$

where $c_{ij,\text{added}}$ indicates the concentration of ith component in jth mixture and $c_{ij,\text{pred}}$ is its estimation [35].

For CLS, PCR and PLS calibrations, the $\%RPE_T$ values were around 20 (Table 4), and thus, unsatisfactory. Even the PLS method, which sometimes can account for some non-linearity in the measured responses [46], was apparently unable to cope adequately with the non-linearities of the voltammograms from the three antibiotic analytes.

It was found that the performance of the RBF-ANN calibration was acceptable for the simultaneous prediction of the fluoroquinolones with a $\%RPE_T$ of 8.1% and an average recovery of 101%. These figures of merit compare well with our other recent work, on the voltammetric determination of three organophosphorus pesticides with the aid of PLS and RBF-ANN [54].

Over the last few years, we have been particularly interested in the research, and development of relatively uncomplicated analytical methods for the simultaneous determination of analytes of industrial, environmental, pharmaceutical and pesticides importance in real mixtures. Chemometrics methods for prediction have played a central role in facilitating the simultaneous analysis, and we have compared the performance of many chemometrics methods for prediction abilities of analytes from responses obtained by spectrophotometric or voltammetric means. In this context, we have found that sometimes PLS, PCR and several ANN methods perform about equally [54–58], but we noted that the RBF-ANN method was performing consistently better or at least as well as others [54,56–58]. The underlying reasons for this efficient performance were not obvious. However, in this work, similar to our recent voltammetric research on the organophosphorus pesticides [54], we were able to demonstrate quite clearly the significant non-linear behaviour of the analytes' responses, which could be responsible for the poor performance of the PLS calibration models.

4.4. Determination of the fluoroquinolones in eye drops and feedstuffs

4.4.1. Analysis of feedstuffs

The preparation of the feedstuff samples for analysis was carried out according to the methods described in the literature [59]. The neat feedstuff sample (2.0 g) or that spiked with the antibiotics was placed into a 100 ml beaker; 5.0 ml of hydrochloric acid (0.1 mol l^{-1}) were added, and the sample was leached for an hour. The sample was then filtered into a 25 ml flask; the pH of this solution was adjusted to 4, and then diluted to mark with distilled water. A suitable aliquot of this solution was transferred into the electrochemical cell and analysed according to the method described in Section 3. The resulting voltammetric data were submitted for prediction with the use of the RBF-ANN calibration. The results (Table 5; Analyses 1 and 2) show that values of each fluoroquinolone antibiotic found in the feedstuff samples were in the range 0.71–1.45 mg g^{-1}, and the procedure

Table 5
Determination of ofloxacin (Floxin), ciprofloxacin (Cipro) and norfloxacin (Noroxin) in feedstuffs and eye drops by linear sweep stripping voltammetry and RBF-ANN

Samples[a]	Found by RBF-ANN (mg g^{-1})			Added (mg g^{-1})			Recovery (%)		
Feedstuffs	Floxin	Cipro	Noroxin	Floxin	Cipro	Noroxin	Floxin	Cipro	Noroxin
1	1.16	0.71	1.23	1.35	0.80	1.40	85.9	88.8	87.9
2	0.95	1.45	1.03	1.10	1.60	1.10	86.3	90.6	93.6
Eye drops	Found by RBF-ANN (mg ml^{-1})			Labeled amount (mg ml^{-1})			Found (%)		
3	2.99	ND	ND	3.0	–	–	100	–	–
4	ND[b]	2.73	ND	–	3.0	–	–	91.0	–
5	ND	ND	2.89	–	–	3.0	–	–	96.3

[a] Samples: (1) feedstuff for ducks and (2) feedstuff for quails, Nanchang Hongxing Feedstuffs Co. Ltd.; (3) ofloxacin ophthalmic solution, Henan Zhufeng Pharmaceutical Production Co. Ltd.; (4) ciprofloxacin hydrochloride, Wuhan Wujin Pharmaceutical Co. Ltd. and (5) Norfloxacin eye drops, Wuhu Sanyi Pharmaceutical Co. Ltd.
[b] Not detected.

was further verified by standard addition of the three fluoroquinolones. The results showed good %recovery values in the range of 85.9–93.6.

4.5. Analysis of eye drops

Eye drops (0.70 ml) were transferred into a 100 ml volumetric flask and diluted to the mark with distilled water. A suitable aliquot of this solution was transferred directly into the electrolytic cell, and analysed with the procedure described in experimental section. The analytical results obtained by RBF-ANN (Table 5, Analyses 3–5) agreed with the amounts of the antibiotics shown on the commercial labels of the eye drop packages with recovery values between 91% and 100%.

5. Conclusion

The behaviour of three fluoroquinolone antibiotics, ofloxacin, ciprofloxacin and norfloxacin, at an HMDE was studied with the use of LSSV. It was found that each of the analytes reacted irreversibly at the working electrode, and produced non-linear voltammetric responses as a function of concentration. The plot of peak current with scan rate was linear, which strongly suggested that the process at the electrode was adsorption-controlled. Important method parameters were investigated, and a quantitative analytical procedure was developed to facilitate the individual estimation of the three antibiotics. Chemometrics calibration models were constructed for CLS, PCR, PLS and RBF-ANN methods with the use of a statistically designed calibration set of samples containing the three fluoroquinolones in mixtures. The calibration models were verified for prediction with a separate set of similar synthetic samples, which indicated that the RBF-ANN model produced the most satisfactory figures of merit—$\%RPE_T$ of 8.1% and an average recovery of 101%. This RBF-ANN model was then used for prediction of the antibiotics of bird feedstuff and eye drops. Satisfactory %recoveries from these samples supported the conclusion that the proposed method is appropriate for the determination of mixtures of the three antibiotics.

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