Application of principal component regression to luminescence data for the screening of ciprofloxacin and enrofloxacin in animal tissues

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A new screening method for the analysis of enrofloxacin and ciprofloxacin in edible animal tissues is described. The method is based on the application of principal component regression to luminescence measurements after reaction of quinolones with terbium(III) in a micellar medium. The method was used, first, to discriminate between quinolone-containing or quinolone-not-containing samples (concentration below the detection limit) and, then, to quantify the sum of both analytes. Standards in a pure-water matrix, using the first three principal components, were used for the determination. RRMSE range from 4 to 10% depending on the analyte. Calibration was successfully applied to the analysis of spiked chicken and trout muscle at concentrations between 10 and 50 μg kg⁻¹.

Introduction

Enrofloxacin [1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxilic acid] is a veterinary drug widely used in the treatment of diseases in cattle, poultry and swine. Enrofloxacin (ENR) is extensively metabolised to the de-ethylated compound ciprofloxacin (CIP) which also has antibacterial activity. The presence of residues of these compounds in edible tissues of animals treated could lead to episodes of allergy or bacterial resistance. To ensure consumers’ health, regulations have been developed regarding the maximum permissible residue limit (MRL) for every veterinary drug used in food-producing animals. The MRL for the sum of ENR and CIP has been established at 30 μg Kg⁻¹ in several edible animal tissues.¹ Therefore, development or improvement of analytical methods for monitoring their levels in farm animals and their primary products is necessary.

Current methods of analysis of quinolones are based on liquid chromatography (LC), mainly with fluorimetric detection.2–5 However, luminescence spectroscopy offers other possibilities for sensitive and selective detection, such as the use of lanthanide-sensitised luminescence.⁶,⁷ The main advantages of this technique include long wavelength emission and long luminescence lifetimes, thus resulting in the elimination of light scattering interference and a significant decrease in background fluorescence.

Recently, terbium-sensitised luminescence spectroscopy has been applied to the joint determination of CIP and ENR.⁸ In this case, calibration was carried out univariately by means of matrix-matched standards, which made the calibration step cumbersome. The application of a multivariate calibration method, such as principal component regression (PCR) could be a useful alternative. PCR is a two-step process. The first step consists of the principal component analysis (PCA) of the original data, i.e. absorption spectra, to obtain a reduced number of variables, the factor scores. Then, multiple linear regression is used to relate these scores to the concentration values.⁹,¹⁰

In this work, we explore the advantages that PCR offers for the screening of ENR and CIP. The principal components obtained after the PCA of the excitation spectra are used for two purposes. Firstly, the study of the representation of the principal components allows the differentiation between quinolone-containing and non-quinolone-containing samples. The second step consists of the application of PCR to the quantification of both quinolones in the samples that were previously classified as quinolone-containing.

Experimental

Materials and reagents

Ciprofloxacin hydrochloride and enrofloxacin (Fig. 1) standards were kindly supplied by CENAVISA (Reus, Spain). Stock standard solutions (100 mg l⁻¹) of the analytes in 0.01 M aqueous nitric acid were stored in dark glass bottles at 4 °C. These solutions were stable over three months. Working solutions were prepared daily by dilution in 0.01M nitric acid. A 1 g l⁻¹ Tb(III) solution was prepared by dissolving the appropriate amount of TbCl₃·6H₂O (Alfa, Karlsruhe, Germany) in 0.01M aqueous nitric acid. The solution was stored in polyethylene bottles. Buffer solutions of 0.25 M acetic acid, sodium acetate and 0.1 M diethylmalonic acid–NaOH were used. A 0.2 M stock solution of sodium laurylsulfate (SLS) (Merek, Darmstadt, Germany) was prepared. Doubly-deionised water (Milli-Q, Millipore, Molsheim, France) with a resistivity of 18.2 MΩ cm⁻¹ was used throughout. All other reagents and solvents were of analytical reagent grade. All glassware used for

![Fig. 1 Molecular structure of enrofloxacin and its metabolite ciprofloxacin.](image)
experiments was soaked in 10% nitric acid for 24 h and rinsed with doubly-deionised water.

Apparatus

Luminescence was measured using a Perkin-Elmer (Beaconsfield, Buckinghamshire, UK) LS-50 fluorescence spectrophotometer equipped with a pulsed xenon lamp (60 Hz) and a 10 nm quartz cell. Excitation and emission slits were set to 5 and 20 nm, respectively. The instrument was set in the phosphorescence mode and a delay time of 0.03 ms and a gate time of 5 ms were used. A Radiometer (Copenhagen, Denmark) PHM 84 pH-meter equipped with an Orion (Boston, MA, USA) 81-02 Ross combination electrode, was used for pH measurements. A Breda Scientific rotary shaker (Breda, Netherlands) and a Heraeus Christ centrifuge (Osterode am Harz, Germany) were used to carry out the extractions. A rotary Resona Technics LABO ROTA S300 evaporator (Gossau, Switzerland) was used to remove the extracting solvent.

Samples

Drug-free tissues of trout and chicken were obtained from Navarra Food (Yusa, Spain) and from the Public Health Laboratory of the Generalitat de Catalunya (Barcelona, Spain), respectively. Skin and bones were removed before gridding the muscle. Minced muscle was stored at -20°C and each sample was thawed before analysis. Samples were spiked by addition of 10 ml of an aqueous standard solution and leaving it to stand for 30 min before the extraction procedure.

Procedures

Trout and chicken samples. Weigh 5.00 g of thawed sample in a 30 ml centrifuge tube. Add 1.5 ml 0.1 M diethylmalonic acid buffer at pH 7.4 and 20 ml CH2Cl2. Shake for 10 min. Centrifuge for 10 min at 3500 rpm. Transfer the organic phase to a 50 ml heart-shaped flask. Rinse sample with another 10 ml CH2Cl2. Centrifuge again for 10 min at 3500 rpm. Combine both organic extracts and add 1 ml of aqueous 0.5 M NaCl in 0.01 M HNO3. Evaporate under vacuum in the rotary evaporator at room temperature until only aqueous phase remains (about 7 min). Defat by rinsing with 10 ml n-hexane. Transfer 0.5 ml of aqueous phase into a 10 ml volumetric flask. Add 1.5 ml of Tb(III) solution, 0.5 ml of SLS solution, 5 ml of buffer solution (0.25 M acetic acid–sodium acetate at pH 6.0) and dilute with water. Record the excitation spectrum from 250 to 400 nm every 0.5 nm, setting the emission wavelength at 549 nm.

Standard solutions. Four series of standard solutions consisting of fifteen solutions each series were prepared: (i) ENR in pure water, (ii) CIP in pure water, (iii) ENR as matrix-matched standard, (iv) CIP as matrix-matched standard. The concentration of these solutions range from 10 to 200 μg kg⁻¹. Ten standard solutions of each series were used for calibration graphs, and the remaining five solutions of each series were used as validation sets. To prepare standards in pure water, transfer up to 2.0 ml of diluted CIP or ENR stock solutions into a 10 ml volumetric flask. Add Tb(III) sol, SLS and buffer solution and record the spectrum as described. For matrix-matched standard solutions, proceed as described for standard solutions in pure water but adding 0.5 ml of the extract from a drug-free tissue.

Data processing

Data were processed on a 133 MHz Pentium PC-compatible computer. For the principal component analysis (PCA) the algorithms from the PLS_ToolBox, written in MATLAB language (MathWorks, Inc.), were used. The parameter chosen to compare the different models was the relative root mean squared error (RRMSE):

\[
\text{RRMSE}(\%) = 100 \times \sqrt{\frac{\sum_{i=1}^{I} (c_i - \hat{c}_i)^2}{\sum_{i=1}^{I} c_i^2}}
\]

where \(c_i\) and \(\hat{c}_i\) are the predicted and the real concentrations, respectively, for the compound in the standard or sample \(i\). \(I\) is the number of standards or samples.

Results and discussion

Addition of terbium ions to a quinolone solution leads to the formation of complexes that absorb energy at the characteristic wavelength of the organic ligand and emit radiation at the characteristic wavelength of terbium, 549 nm (Fig. 2). Consequently, excitation spectra can be performed at the same emission wavelength for both enrofloxacin and ciprofloxacin. In a previous paper, sensitised luminescence of Tb ion by ENR and CIP was investigated, and chemical and instrumental parameters affecting the fluorescence intensity were optimised. The excitation spectra, which are quite similar for both quinolones, exhibit two bands centred at 276 and 325 nm (Fig. 3). Although both bands are characteristic of the quinolone, the first one (between 250 and 300 nm) includes some contribution from the blank. Given this spectral profile, the principal component analysis was performed on three different data matrices: the whole spectrum, the spectral range corresponding to the first band (between 250 and 300 nm), and the range between 300 and 350 nm, corresponding to the second band. Fig. 4a to 4c show the representation of the first principal component vs. the second principal component for each one of the three different data sets tested (whole spectrum, first band and second band).

![Fig. 2: Scheme of the terbium-sensitised luminescence process. * indicates an excited state.](Image 307x246 to 542x294)

![Fig. 3: Excitation spectrum of the Tb-quinolone complex.](Image 309x71 to 539x219)
As can be observed, the best results—lowest dispersion and highest linearity for samples or blanks—are obtained when only the second band is used, since the effect of the blank is minimised. For this reason, further work was carried out in the spectral range 300–350 nm. As can be deduced from Fig. 5, the decomposition in factors is complete with no more than three factors.

The study of the representation of the first principal component vs. the second principal component for several solutions (including reagents and matrix blanks, standards in pure water, matrix-matched standards and spiked samples) analysed over several days shows that a screening between negative and positive samples is possible. As is shown in Fig. 6, the blanks obtained over several days (which includes blanks of reagents, chicken and trout, indistinctly) fall in a well-defined line (PC#2 = −0.209PC#1 − 7.19; r = 0.984). From the standard deviation limits of the correlation line of the blanks, a zero level concentration zone is defined. Those samples whose principal component representation is located within these limits will be considered as quinolone-not-containing samples, i.e. with a quinolone concentration below the detection limit (2 μg l⁻¹ in the measurement cell). In practice, this means that, once the zero-level concentration zone has been defined, the screening between positive and negative samples is very easy. It is only necessary to perform the excitation spectrum, to obtain its PC and test whether they are located within this zone.

For samples with concentrations above the zero-level, a further procedure based on a quantitative determination using a PCR calibration model with three factors was applied. For this purpose, calibration graphs from standard solutions in pure water and from matrix-matched standards were obtained. The parameters of each calibration graph are shown in Table 1. In this table, ENR refers to calibration graphs obtained from just standard solutions of enrofloxacin [(i) or (iii)], CIP refers to those obtained from just solutions of ciprofloxacin [(ii) or (iv)], and QUI refers to calibration graphs obtained with both CIP and ENR standard solutions [(i) and (ii) or (iii) and (iv)].

Table 2 lists RRMSE values obtained from the validation sets. As can be seen, errors obtained in pure water solutions were quite similar (RRMSE about 5%), regardless of the analyte (ENR or CIP) and the calibration graph used. Results from

![Fig. 4](image1.png) Representation of the PC#1 vs. PC#2 obtained after PCA of different zones of the excitation spectrum. (a) Whole spectrum; (b) first band; (c) second band.

![Fig. 5](image2.png) Plots of CUMPRESS versus the number of principal components for the standard solutions in several matrices.

![Fig. 6](image3.png) Calibration graph of the blanks (○) with zero-quinolone zone (between prediction interval lines) and plot of samples spiked at different concentration levels: □ 0 μg kg⁻¹, ▲ 25 μg kg⁻¹, ▼ 50 μg kg⁻¹, ■ 100 μg kg⁻¹, + 200 μg kg⁻¹.

**Table 1** Calibration parameters according to the function $C = v_0 + v_1PC#1 + v_2PC#2 + v_3PC#3$

<table>
<thead>
<tr>
<th></th>
<th>$v_0$</th>
<th>$v_1$</th>
<th>$v_2$</th>
<th>$v_3$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENR</td>
<td>9.395</td>
<td>2.53 × 10⁻²</td>
<td>4.88 × 10⁻²</td>
<td>−0.321</td>
<td>0.9975</td>
</tr>
<tr>
<td>CIP</td>
<td>7.454</td>
<td>2.70 × 10⁻²</td>
<td>4.67 × 10⁻²</td>
<td>−0.285</td>
<td>0.9986</td>
</tr>
<tr>
<td>QUI</td>
<td>9.430</td>
<td>2.54 × 10⁻²</td>
<td>5.12 × 10⁻²</td>
<td>−0.310</td>
<td>0.9979</td>
</tr>
</tbody>
</table>

From standards in pure water

<table>
<thead>
<tr>
<th></th>
<th>$v_0$</th>
<th>$v_1$</th>
<th>$v_2$</th>
<th>$v_3$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENR</td>
<td>9.437</td>
<td>3.52 × 10⁻²</td>
<td>6.34 × 10⁻²</td>
<td>−0.380</td>
<td>0.9971</td>
</tr>
<tr>
<td>CIP</td>
<td>7.454</td>
<td>4.02 × 10⁻²</td>
<td>4.94 × 10⁻²</td>
<td>−0.174</td>
<td>0.9921</td>
</tr>
<tr>
<td>QUI</td>
<td>4.730</td>
<td>3.68 × 10⁻²</td>
<td>5.47 × 10⁻²</td>
<td>−0.292</td>
<td>0.9894</td>
</tr>
</tbody>
</table>

From matrix-matched standards
matrix-matched solutions show a greater dispersion (RRMSE from about 5 to 10%), which may be due to differences between tissue extracts. Spectra of the different sets of standard solutions, as well as those of the validation sets, were obtained on different days over a three-month period. This points out that the proposed calibration model, based on three PCs, is independent of the day in which the measures are taken, assuming that the sensitivity of the fluorimeter does not undergo significant changes. In order to verify the instrumental response, a tetraphenylbutadiene standard in a polymethylmethacrylate matrix was used at the beginning of each session.

Since matrix-matched standards are more time-consuming to prepare than standards in pure water, the possibility of quantifying analytes in matrix by means of standards in pure water has been assessed. For this purpose, the concentrations of the validation set of matrix-matched standards were calculated without the need for a new set of standards each time a sample is processed. Moreover, once the correction factor between standards in a pure-water matrix and matrix-matched standards has been established, there is no need to use standards where matrix is present, which makes the method much easier to perform.

Finally, the method has also been validated using edible tissues of trout and chicken spiked with different amounts of CIP and ENR. Table 3 shows the mean concentrations predicted for three replicated determinations per sample from standards in a pure-water matrix. A calibration graph established from standard solutions of both analytes were used, but similar results were obtained with just CIP (or ENR) standards. For calculations, recoveries reported elsewhere were used. As can be seen, accuracy is good enough to screen samples at concentrations of ciprofloxacin and enrofloxacin near their MRLs.

Conclusions

The work carried out shows the effectiveness of the proposed method as a screening procedure for detection of positive samples (quinolone-containing edible animal tissues). A few days pre-calibration matrix allows for the overall amount of both quinolones (enrofloxacin and ciprofloxacin) to be determined. The obtained values are close to the real composition, without the need for a new set of standards each time a sample is processed. Moreover, once the correction factor between standards in a pure-water matrix and matrix-matched standards has been established, there is no need to use standards where matrix is present, which makes the method much easier to perform.

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