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Short communication

Fast-scanning fluorescence spectroscopy as a detection system in liquid chromatography for confirmatory analysis of flumequine and oxolinic acid

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Abstract

Oxolinic acid and flumequine were analysed by reversed-phase liquid chromatography after extraction from the sample matrix with dichloromethane and partitioning with NaOH. The detection system consisted of a fast-scanning fluorescence detector, which provides the full spectra of the eluting peaks and can thus be used to confirm the identity of analytes. Determination was performed by partial least squares (PLS) and three-way PLS over the three-dimensional data, i.e. fluorescence intensity versus retention time and excitation wavelength. In both cases, similar results, with prediction errors around 4%, were obtained. The method was successfully applied to the analysis of salmon, pork and chicken muscle spiked up to 300 ng g^{-1}.

Keywords: Fast-scanning fluorescence spectroscopy; Detection, LC; Edible animal tissue; Flumequine; Oxolinic acid

1. Introduction

Antibacterial agents, such as the quinolones flumequine (FLU) and oxolinic acid (OXO), are used as veterinary drugs in food-producing animals. Due to their misuse, competent organisms have fixed a maximum residue limit (MRL) for these substances in edible animal products. Effective control requires appropriate analytical methodology, which must provide enough sensitivity as well as the ability to confirm the identity of the analytes. Owing to its specificity, liquid chromatography–mass spectrometry (LC–MS) is a powerful confirmatory technique; however, it is expensive and thus not available to all laboratories. UV diode array (DAD) or fluorescence detectors able to provide full-spectra may be used as simpler alternatives.

In this paper, we report the use of fast-scanning fluorescence spectroscopy (FSFS) as a detection technique in LC for confirmatory analysis of FLU and OXO. Spectra of the effluent from the chromatographic column are recorded at a fixed excitation (or emission) wavelength by scanning emission (or excitation) wavelengths at a high rate, yielding three-dimensional chromatograms. Although it is as simple as DAD, FSFS shows higher selectivity and sensitivity. Few methods based on FSFS detection have been reported so far [1–4] and none of them deal with veterinary drug residues.

Here, FSFS was applied to the analysis of animal

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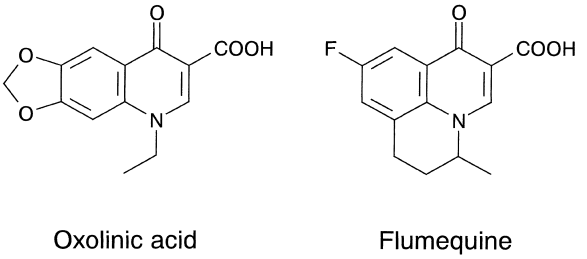
tissues following a method reported elsewhere [5], based on LC-fluorimetric detection at fixed wavelengths. This is the most usual approach for the determination of quinolones [6–8], whereas LC–MS [9,10] and GC–MS (after derivatization with $NaBH_4$) [11,12] are mainly applied for confirmatory purposes. Since FSFS provides three-dimensional data, the multivariate calibration models PLS-1 and three-way PLS [13,14] were applied for quantification.



Oxolinic acid Flumequine

Fig. 1. Structure of the analytes under study.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a double piston pump (Model 525, Bio-Tek Kontron Instruments, Milan, Italy) and an injection valve model 7125 (Rheodyne, Cotati, CA, USA) fitted with a 100-μl injection loop. The analytical column was a commercial Inertsil C_8 (250×4.6 mm), 5 μm (Alltech, Deerfield, IL, USA) equipped with a similar pre-column.

Detection was carried out by means of an Aminco Bowman Series 2 spectrofluorimeter (Rochester, NY, USA), equipped with a 25-μl flow cell (Hellma, Mülheim, Germany). pH was measured on a CRISON GLP 21 pH-meter (Alella, Barcelona, Spain), equipped with an Ag/AgCl combined glass electrode, CRISON 52-02.

A Heraeus Christ centrifuge (Osterode am Harz, Germany) was used.

2.2. Reagents and solutions

Flumequine (FLU) and oxolinic acid (OXO) (Fig. 1) were from Sigma (St Louis, MO, USA). Stock standard solutions of 100 mg l^{-1} of the quinolones were prepared by dissolving the compounds in 0.01 *M* NaOH and stored in dark glass bottles at 4°C. Working solutions were prepared daily by dilution with 0.01 *M* NaOH.

Acetonitrile of HPLC grade (Merck, Darmstadt, Germany) was used. Doubly de-ionised 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 experiments was soaked in 10% nitric acid for 24 h and rinsed with doubly de-ionised water.

2.3. Samples

Salmon, chicken and pork muscle samples were purchased from local shops. Skin, fat and bones were removed before grinding the muscle. Minced muscle was stored at −20°C and each sample was thawed before analysis.

2.4. Procedures

2.4.1. Sample treatment

Weigh 2.00 g of thawed sample in a 30-ml centrifuge tube. Add 10 ml CH_2Cl_2. Shake vigorously for about 20 s and centrifuge for 5 min at 4000 rpm. Transfer the organic phase to another tube. Rinse the sample with another 10-ml portion of CH_2Cl_2. Centrifuge again for 5 min at 4000 rpm. Combine both organic extracts. Add 2 ml 0.01 *M* NaOH and shake. Centrifuge for 10 min at 4000 rpm. Filter the supernatant through a 0.45-μm pore size nylon membrane and inject 100 μl into the chromatographic system.

Spiking was performed by adding a microvolume of FLU and OXO standard solution in water to each portion of the weighed sample. Spiked samples were left to stand at room temperature for 30 min in the dark before the analysis.

2.4.2. Chromatographic separation and detection

An isocratic elution procedure was used throughout. The mobile phase consisted of an AN–H_2O mixture (45:55, v/v) containing 10 m*M* oxalic acid. Both solvents were filtered separately through a 0.22-μm pore size nylon membrane and the mixture was degassed with a helium stream prior to use. Analyses were performed at room temperature. The mobile phase flow-rate was 1.5 ml min^{-1}.

For fast-scanning fluorescence detection, emission wavelength was set to 365 nm. Excitation spectra were recorded from 225 to 325 nm at 50 nm s^{-1} and readings were taken at 1-nm increments. One spectrum was recorded every 2.7 s (which includes the time required by the monochromator to return to the initial position).

2.5. Data treatment

The original data consisted of 148 fluorescence spectra (101 emission data per spectrum) for the whole chromatogram. Since the spectrofluorimeter takes an emission reading every 0.02 s, every spectral point corresponds to a different time and thus to a different portion of effluent from the chromatographic column. Therefore, the original data led to distorted chromatograms. A MATLAB algorithm that corrects the spectra and subtracts baseline was used to obtain valid data [3].

Most of the recorded spectra contained irrelevant information and so only the data obtained while analytes were eluting were considered. Selected data consisted of 21 spectra for each peak, one in the apex and 10 to each side thereof. As a result, data size was substantially reduced from 14 948 data points (101 wavelengths×148 spectra) for the whole chromatogram to 2121 data points (101 wavelengths×21 spectra) for the range selected for each peak.

For calibration and quantification, algorithms from the PLS_ToolBox, written in MATLAB language (MathWorks, Inc.), were used. Since MATLAB uses two-dimensional data, the three-dimensional chromatograms were unfolded before calculations. The parameter chosen to compare the models was the relative root mean squared error (RRMSE):

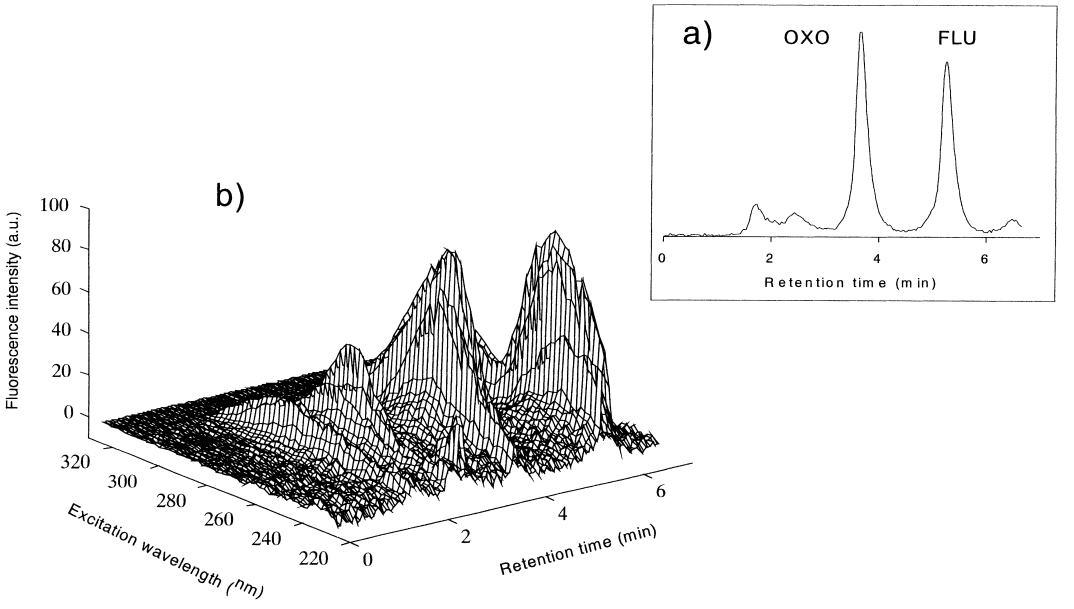


Fig. 2. Chromatograms of a salmon muscle spiked with OXO and FLU: (a) Chromatogram obtained at fixed wavelengths (328 nm for excitation and 365 nm for emission); spiking level: 50 ng g^{-1} for each analyte; (b) Chromatogram obtained by FSF after data correction; spiking level: 150 ng g^{-1} for OXO and 300 ng g^{-1} for FLU.

$$\text{RRMSE}\,(\%) = 100 \cdot \sqrt{\frac{\sum_{i=1}^{N}(c_i - \hat{c}_i)^2}{\sum_{i=1}^{N} c_i^2}}$$

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

A set of 52 standard solutions containing both OXO and FLU were prepared in 0.01 *M* NaOH. Concentration ranged from 0 to 300 ng ml^{-1} for OXO and from 0 to 400 ng ml^{-1} for FLU. The calibration set consisted of 35 standard solutions. The remaining 17 standards constituted the validation set. The number of factors in PLS was determined by leave-one-out cross-validation over the calibration set, leaving out one sample at a time. In the case of three-way PLS, the number of factors corresponds to that yielding the lowest RRMSE for the validation set.

3. Results and discussion

The optimal conditions for the chromatographic separation and sample treatment were taken from an earlier study [5]. The present study focused on the detection system to validate the method for determination and for confirmatory purposes.

Fluorescence spectra of OXO and FLU in acetonitrile–water (45:55) at pH 2 were recorded. Although both excitation and emission spectra showed maxima at similar wavelengths for both quinolones, the difference between emission maxima was smaller than that between excitation maxima. Thus, chromatographic detection was carried out by recording

Table 1
MRLs established by EU and JECFA for the quinolones under study

Pharmacologically active substance	Marker residue	Animal species	MRLs (ng g^{-1})	Target tissues	Other provisions
European Union					
Flumequine	Flumequine	Bovine, ovine, porcine	200	Muscle	Not for use in animals
			300	Fat or fat/skin	from which milk is
			500	Liver	produced for human
			1500	Kidney	consumption
		Bovine	50	Milk	
		Chicken, turkey	400	Muscle	Not for use in animals
			250	Skin + fat	from which milk is
			800	Liver	produced for human
			1000	Kidney	consumption
		Salmonidae	600	Muscle and skin in natural proportions	
Oxolinic acid	Oxolinic acid	Bovine, chicken, porcine	100	Muscle	Provisional MRLs
			50	Fat, fat/skin	expire on
			150	Liver, kidney	01.01.2001
			50	Eggs	
		Fin fish	300	Muscle and skin in natural proportions	
JECFA					
Flumequine	Flumequine	Bovine, ovine, porcine, chicken	500	Muscle	Provisional MRLs
			1000	Fat	
			1000	Liver	
			3000	Kidney	
		Trout	500	Muscle and skin in natural proportions	

excitation spectra with a fixed emission wavelength at 365 nm.

An increase in scan speed improves the acquisition of data, but also gives an increase in the instrumental noise, mainly due to the lower integration times. A scan speed of 50 nm s^{-1}, which provides one spectrum every 2.7 s, was selected as a compromise between both effects. Fig. 2 shows a two-dimensional chromatogram obtained at fixed wavelengths and a three-dimensional one, obtained by FSFS after correction of the data.

Several solutions containing both analytes were injected to determine the linear range, which was up to 300 ng ml^{-1} for OXO and to 400 ng ml^{-1} for FLU. As pointed out above, baseline is noisier in FSFS detection than when a single wavelength is used, and thus higher detection limits are obtained. Nevertheless, concentrations as low as 10 or 20 ng ml^{-1} for OXO and FLU, respectively, can be easily determined. These concentrations, which correspond to 10 and 20 ng g^{-1}, are far below the MRLs established by both the EU and the JECFA in several matrices (Table 1).

Since OXO and FLU peaks were completely resolved, data from each analyte were treated separately, and both PLS-1 and three-way PLS were used as calibration methods. Calibration requires five factors for FLU when either PLS or three-way PLS is used, and five or six factors for OXO when PLS or three-way PLS is applied. Although only one analyte is found in each peak, other factors, such as mobile phase and temperature, may affect signal, which increases the need for factors. For both OXO and FLU aqueous standards, RRMSE ranged between 3.8 and 4.5%. Individual errors were also low, irrespective of the calibration used.

The applicability of both calibration models to the analysis of OXO and FLU in edible animal tissues was tested. For this purpose, salmon, chicken and pork muscle samples spiked at various concentrations around their MRL were used. Fig. 3 shows the plots of the concentration values found for OXO and FLU versus the spiked ones, in salmon muscle. High linearity was obtained for both quinolones with both calibration models. A *t*-test at the 90% confidence level indicated that the recoveries obtained for the three matrices studied (Table 2) were not significantly different from those reported elsewhere [5].

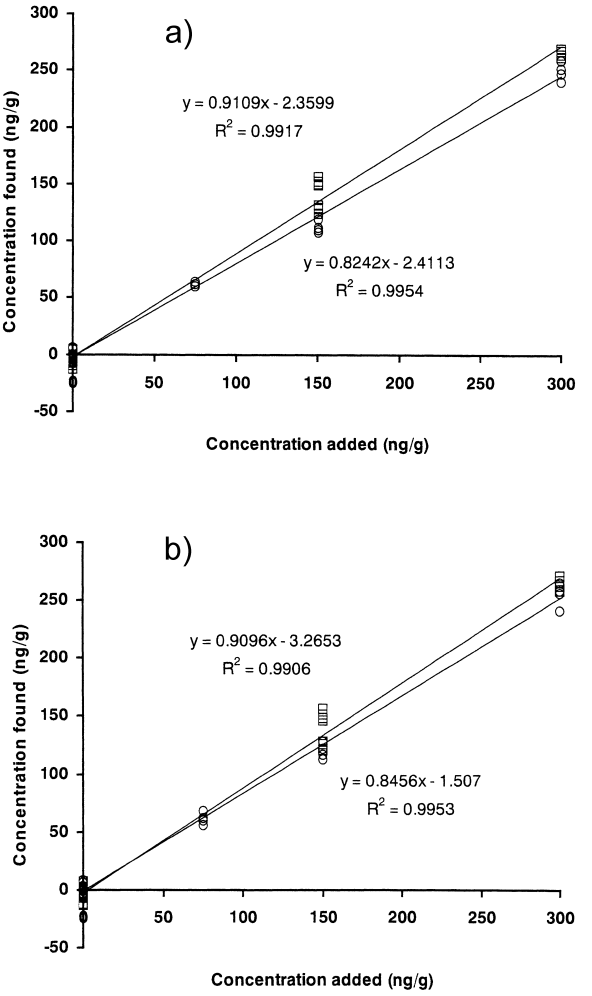


Fig. 3. Recovery lines for OXO and FLU in salmon muscle, according to both calibration models: (a) PLS; (b) three-way PLS. □, OXO; ○, FLU.

FSFS is useful mainly because it provides the excitation or emission spectrum along the chromatogram. This allows us to identify the analytes by

Table 2
Recoveries of FLU and OXO from several tissues spiked from 75 to 300 ng g^{-1}

Matrix	FLU		OXO	
	n	Recovery	*n*	Recovery
Salmon	20	85±7	20	91±8
Chicken	6	78±8	6	79±9
Pork	6	79±7	6	85±6

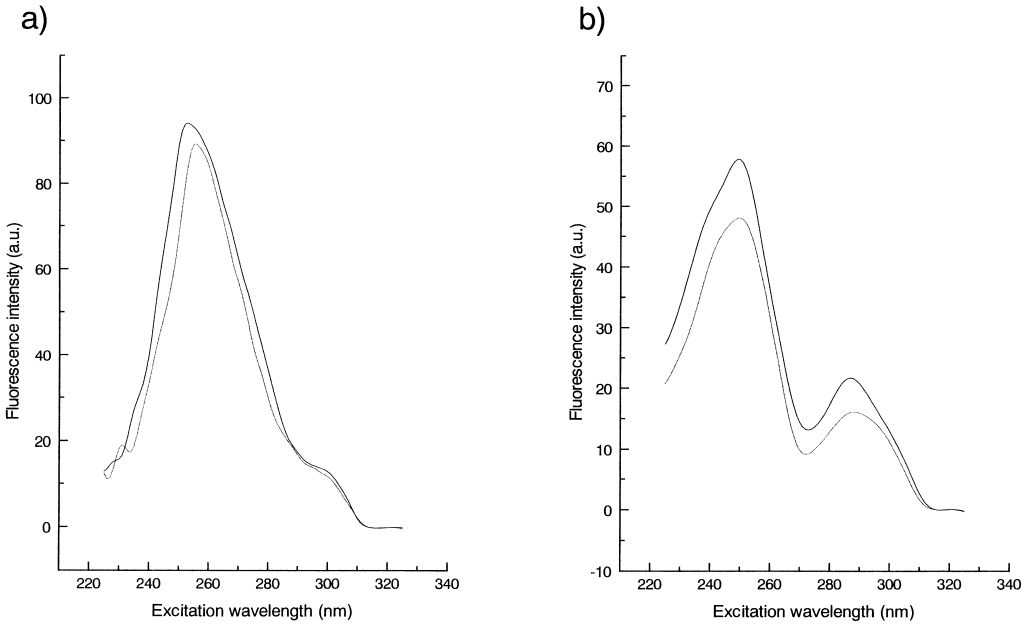


Fig. 4. Smoothed FSF excitation spectra at the apex of the chromatographic peak for OXO (a) and FLU (b) at 150 ng g^{-1}. Solid lines correspond to standard aqueous solutions and dotted lines to spiked salmon muscle samples.

comparing the spectrum of the suspected compound with that of a known standard. Fig. 4 shows the FSF excitation spectra of the apex of the chromatographic peak for both quinolones obtained from an aqueous standard solution and from a spiked salmon muscle. The spectra show good agreement for both compounds. In addition to its higher sensitivity, FSFS is more useful than DAD for confirmatory purposes since not only is the excitation spectrum recorded but the emission wavelength can also be selected.

4. Conclusions

LC-FSFS is a suitable technique for the analysis of quinolones as veterinary drug residues. Owing to its higher sensitivity and selectivity, it can be regarded as a reliable substitute for DAD for confirmatory purposes.

Both PLS and three-way PLS are useful to predict the concentration of analytes in salmon samples at levels corresponding to their MRL or lower. Thus, not only qualitative but also quantitative data are obtained in one chromatographic run.

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