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Direct Determination of Danofloxacin and Flumequine in Milk by Use of Fluorescence Spectrometry in Combination with Partial Least-Squares Calibration

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ABSTRACT: A new method for the simultaneous determination of danofloxacin and flumequine in milk samples was developed by using the nonlinear variable-angle synchronous fluorescence technique to acquire data and a partial least-squares chemometric algorithm to process them. A calibration set of standard samples was designed by combination of a factorial design with two levels per factor and a central star design. Whey was used as the third component of the calibration matrix. In order to assess the goodness of the proposed method, a prediction set of 11 synthetic samples was analyzed, obtaining recovery percentages between 96.1% and 104.0%. Limits of detection, calculated by means of a new criterion, were 0.90 and 12.4 ng mL^{-1} for danofloxacin and flumequine, respectively. Finally, the simultaneous determination of both fluoroquinoles in milk samples containing the analytes was successfully carried out, obtaining an average recovery percentage of 99.3 ± 4.4 for danofloxacin and 100.7 ± 4.4.

KEYWORDS: *danofloxacin, flumequine, milk, partial least-squares multivariate calibration, fluorimetry*

INTRODUCTION

Fluoroquinolones have been the most important group of synthetic antibacterials used in medical and veterinary drugs for the treatment and prevention of various infectious diseases ever since they were developed about 40 years ago.

Despite their benefits, misuse of antibiotics has resulted in the development of nonpathogenic and pathogenic resistance by living organisms. Also, the illicit use of antibiotics can increase the risk of food-borne infections with antibiotic-resistant pathogenic bacteria contaminating food for human consumption.[1] Milk is one of the products from food-producing animals that may be easily contaminated with drug residues. The presence of antibiotic residues in milk can trigger allergic reactions in especially sensitive individuals. Also, there are concerns that the widespread use of antibiotics may be responsible for the development of resistant bacterial strains.

To ensure safety in human foodstuffs, the European Union (EU)[2] has taken steps such as setting tolerance levels for quinolones in products of animal origin. This has raised the need to develop sensitive methods for measuring residual amounts of theses drugs with a view to facilitating quality control analyses of food products and verifying correct application of withdrawal times.

A number of methods for the multiresidue analysis of quinolones in biological samples and animal tissues have been reported. Quinolones in milk are typically determined by capillary electrophoresis and chromatography (whether gas or liquid) in combination with UV, mass spectrometry, or fluorescence detection.[3−13] These methods usually include deproteination of the milk and subsequent cleanup and preconcentration by solid-phase extraction. This step is usually performed off-line before separation, which can reduce the effective sample throughput. Recently, danofloxacin[14] residue in bovine muscle was screened at 200 ng/g by terbium-sensitized luminescence (TSL) directly measured on C18 sorbent strips. The analyte was first adsorbed on the sorbent surface by immersion in defatted homogenates. After reagent application and desiccation, TSL was directly measured on sorbent surfaces at λ_{ex} = 273 nm and λ_{em} = 546 nm.

Spectrofluorimetry has been widely used to determine trace elements in clinical, biomedical, and environmental analysis on account of its high selectivity and sensitivity and relatively low cost. However, the analytes usually exhibit broad spectral bands that tend to overlap when the sample contains several components; this entails using a separation pretreatment or a highly specific method not only to improve selectivity and detection limits but also to expedite analyses. This challenge has been met by using synchronous fluorescence spectroscopy (SFS),[15] the whole fluorescence excitation−emission matrix spectrum,[16,17] or, very frequently, derivative fluorescence spectra of both the conventional and the synchronous type.[18]

SFS is a modified version of the conventional fluorescence technique affording increased selectivity thanks to the narrowing of spectral bands and simplification of spectra. SFS can be implemented in various modes including nonlinear variable-angle synchronous fluorimetry (NLVASF), which involves continuously changing the scan path via the excitation and emission matrix. Following a curved path through the excitation−emission matrix allows light-scattering peaks to be suppressed.[20] Although this technique affords selection of the paths yielding the strongest signals and usually provides very good results by virtue of its overcoming energy transfer processes by circumventing "conflictive" spectral areas, total resolution of multicomponent mixtures is not always possible owing to the presence of strong background fluorescence of unknown origin from the matrix. Milk is an especially complex fluid containing emulsion, colloid, and solution phases. The aqueous phase of

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milk, whey, contains a variety of organic substances, most of which are present at low concentrations or possess a low fluorescence efficiency. In fact, only a few such substances are fluorescent and exhibit different excitation and emission maxima that contribute significantly to the overall fluorescence spectrum for whey. This ultimately results in a high background fluorescence that interferes with the determination of danofloxacin and flumequine. The interference can be circumvented by using an effective mathematical algorithm to resolve the data matrix. Partial least-squares (PLS) is one of the most useful calibration methods for this purpose and has the ability to additionally solve multicollinearity problems.[19−21] However, it has rarely been used in combination with NLVASF.[22,23]

To our knowledge, the simultaneous quantitation of danofloxacin and flumequine without their chromatographic separation and extraction has never to date been reported. In this work, we developed a new method for the simultaneous determination of danofloxacin and flumequine in milk by use of NLVASF in combination with PLS calibration. With easy prior steps, it is only necessary to precipitate the protein fraction of milk.

MATERIALS AND METHODS

Apparatus. All fluorimetric measurements were made on an Aminco Bowman Series 2 luminescence spectrometer governed via the software AB2 for the OS2 operating system. The instrument was equipped with a continuous 150 W xenon lamp, Czerny-Turner monochromators of 200 mm focal distance and 1200 lines/mm holographic gratings, adjustable slits on both monochromators, and a gated photomultiplier. Quartz glass cuvettes of 1.0 cm × 1.0 cm light path were used throughout.

Thermostating equipment, a Crison model 2001 pH-meter furnished with a glass-saturated calomel combination electrode, and a Mixtaxel Selecta centrifuge were also used.

The software AB2 affords file management and setting of instrumental parameters for acquisition of excitation and emission spectra.

Software. The software Ftotal[24] was used for optimal characterization of each fluorescent compound from a total luminescence spectrum. Three-dimensional spectra can be obtained and presented as contour maps. The software affords autoscaling and suppression of light scatter, which allows the fluorescent properties of the target compound to be exploited in full.

For application of partial least-squares regression, synchronous spectra were exported as ASCII files and transferred to a PC running MatLab 7.0.1 and the routine MVC1, developed by Olivieri,[25] which is freely available on the Internet.

Reagents and Solutions. All experiments were performed with analytical reagent grade chemicals, pure solvents, and Milli-Q water. Danofloxacin and flumequine were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions containing a 50 mg L^{-1} concentration of either analyte were prepared in amber-colored flasks by dissolving appropriate amounts in ethanol. The stock solutions were used on a daily basis to prepare working-strength solutions. A 1 M buffer solution at pH 4.8 was prepared by mixing appropriate amounts of sodium acetate and hydrochloric acid. All stock standard solutions were stable at room temperature for at least 1 month, and the working samples of each compound for at least 2 h.

Whey. Whey was obtained by precipitating the casein protein fraction of milk at pH 4.6. To this end, a volume of 50 mL of whole milk was placed in a 100 mL calibration flask, to which 45 mL of Milli-Q water at 40 °C and 1 mL of acetic acid solution (10%, w/v) were added. The flask contents were smoothly mixed and allowed to settle for 10 min. Then, 1 mL of 1 M sodium acetate buffer was added, followed by mixing and making to volume with water, after which the flask was allowed to cool.

Once the casein protein fraction was precipitated, the soluble phase (1:1 whey) was separated by passage through filter paper and stored in a dry container at low temperature.

Procedure. *Construction of the Calibration and Validation Matrices.* The analyte concentrations in the calibration matrix ranged from 0 to 75.0 ng mL^{-1} for danofloxacin and 0 to 500.0 ng mL^{-1} for flumequine, and whey dilutions from 1:4 to 1:20. In order to accurately represent these entire concentration ranges, a factor design involving two levels per factor in addition to a central composite design was used to obtain a matrix of 15 standards plus 1 analyte blank. The predictive ability of the ensuing model was assessed by using a validation matrix consisting of 10 samples containing both analytes at different concentrations spanning the previous ranges that were chosen with the aid of custom-made random number generation software.

Calibration and Validation Samples. Calibration and validation samples were prepared by adding appropriate amounts of each analyte from its stock solution, dilute whey, and 5.0 mL of buffer to a 25 mL volumetric flask, which was then made to volume with ethanol and distilled water in a 20:80 (v/v) ratio.

A total of 61 emission spectra were acquired at 0.5 nm intervals over the wavelength range 300−540 nm by using excitation wavelengths of 200−440 nm in steps of 4.0 nm. The total luminescence spectrum was obtained by using Ftotal,[24] which was also used to select appropriate paths and synchronous spectra. The spectra were exported as ASCII files for application of the MVC1 routine[25] under Matlab and processed with the PLS-1 algorithm.

Procedure for Milk Samples. The calibration model was validated by using 10 mL volumes of commercial whole milk spiked with appropriate amounts of danofloxacin and flumequine in a 100 mL volumetric flask. All samples were allowed to stand at room temperature in the dark for 30 min prior to processing and analysis in order to facilitate interaction between the antibiotics and the matrix. These samples were prepared in triplicate. Whey was prepared and processed by following the above-described procedure for calibration and validation. Finally, the proposed model was used to predict the concentration of each fluoroquinolone in the milk samples.

RESULTS AND DISCUSSION

Influence of Experimental Variables. Chemical variables were optimized in order to ensure the optimum measurement conditions, maximum fluorescence intensity, and adequate selectivity by examining the influence of the ethanol content of the medium, pH, temperature, and analyte concentrations. An organic solvent was required since flumequine is not water-soluble. On the basis of its physical and chemical properties, ethanol was a suitable choice. The influence of its concentration was studied by using it to prepare a sample containing danofloxacin, flumequine, and whey. The fluorescence intensity of danofloxacin peaked at 10% ethanol, remained constant up to 50%, and then decreased to 0 at 100%. The fluorescence intensity of flumequine increased slightly with increasing ethanol content up to 20% and then decreased similarly to danofloxacin. Whey also exhibited an increase in fluorescence intensity with an increase in ethanol content; some whey components precipitated above 60%, however. A proportion of ethanol of 20% was thus selected in order to maintain flumequine in solution and avoid too high a background signal from whey.

The influence of pH was examined by adding HCl or NaOH to a solution containing 1.0 mg L^{-1} flumequine and 0.5 mg L^{-1} danofloxacin in 20% ethanol plus whey. Both fluoroquinolones responded virtually identically to a change in pH. Thus, the fluorescence intensity increased slightly up to pH 4, decreased sharply to pH 7, and then remained fairly constant at pH 8−12. Also, the characteristic peaks for the two analytes retained their positions in the spectrum. The intensity of the whey signal decreased with increasing pH. Therefore, pH 4.8, adjusted with 0.2 M sodium acetate/acetic acid buffer to ensure a high buffering capacity, was selected as optimal.

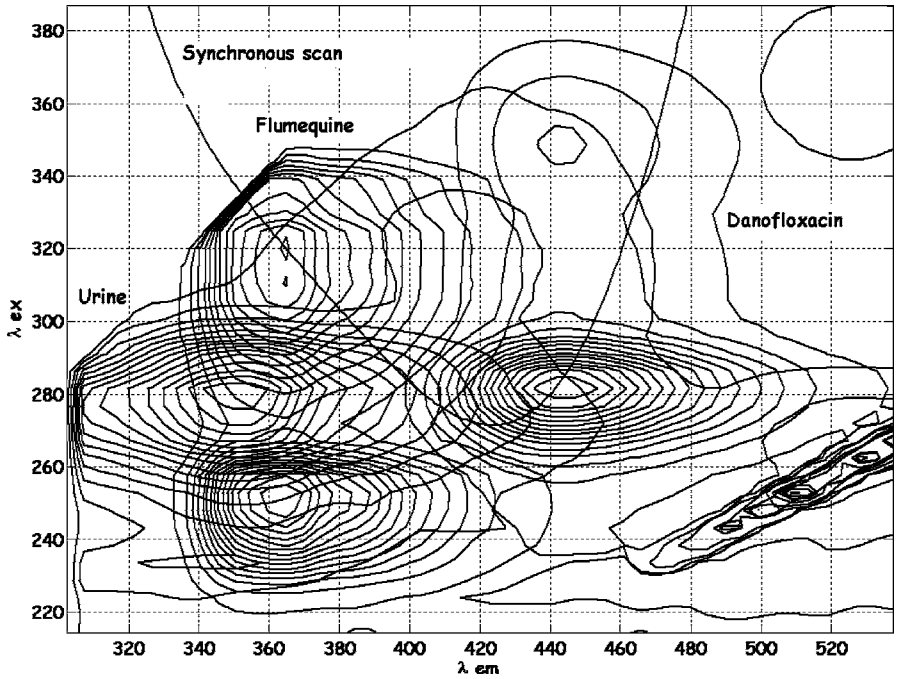

Figure 1. Overlapped contour maps of 800.0 ng mL^{-1} flumequine, 50.0 ng mL^{-1} danofloxacin, whey (1:12.5), and selected trajectory.

Temperature was another factor influencing the fluorescence intensity. The fluorescence intensity of both fluoroquinolones decreased with increasing temperature from 4 to 35 °C. A measurement temperature of 20 °C, which was close to the ambient level and hence easy to maintain with thermostating equipment, was therefore selected.

The influence of the fluoroquinolone concentrations on the fluorescence intensity was studied under the above-described optimum conditions, where the fluorescence emission of danofloxacin and flumequine was linearly related to their concentration up to 500 and 1000 ng mL^{-1}, respectively.

Spectral Features. After optimizing the variables influencing the fluorescence intensity of the analytes and establishing their linear concentration ranges, we recorded three-dimensional spectra for solutions containing 50 ng mL^{-1} danofloxacin and 800 ng mL^{-1} flumequine plus whey at a 1:12.5 dilution

Figure 1 shows the total fluorescence spectra for danofloxacin, flumequine, and whey (three-dimensional spectra are shown as contour lines). The spectra for flumequine and whey were completely overlapped, and both interfered with the determination of danofloxacin. Spectral overlap therefore precluded direct determination of the analytes by conventional spectrofluorimetry in the presence of whey and required using an effective alternative such as NLVASF.

The greatest difficulty in using the synchronous scan technique is that it entails indentifying the best NLVASF scan path beforehand in order to obtain optimum results. In our case, this required careful inspection of the contour maps for a standard solution of the two compounds at concentration levels yielding an identical fluorescence intensity in the presence of the matrix (Figure 1). The path was optimized to minimize spectral interferences from the analytes and matrix in the mixture with no appreciable loss of sensitivity.

Based on the contour plots, the best scan path was that defined by a function passing through the maximum for flumequine at λ_{ex} = 324.0 nm, λ_{em} = 363.0 nm, where the signal due to the matrix was lower, the maximum for danofloxacin at λ_{ex} = 284.0 nm, λ_{em} = 440.0 nm, and two additional points (λ_{ex} = 350.0 nm, λ_{em} = 340.0 nm and λ_{ex} = 286.0 nm, λ_{em} = 450.0 nm). The chosen path was represented by the following function: $\lambda_{ex} = 165188.2522 - 2034.426904\lambda_{em} + 9.99579792(\lambda_{em})^2 - 0.02439658385(\lambda_{em})^3 + 2.951907092 \times 10^{-5}(\lambda_{em})^4 - 1.413756613 \times 10^{-8}(\lambda_{em})^5$ (see Figure 1).

Figure 2 shows the NLVASF spectra for danofloxacin, flumequine, and whey. Although the synchronous mode efficiently resolved the bands for the two fluoroquinolones, the results were still poor owing to the high background signal of the matrix. Figure 3 shows the first-derivative NLVASF spectra for the three components. These spectra afforded the determination of danofloxacin in the presence of flumequine and whey, but not

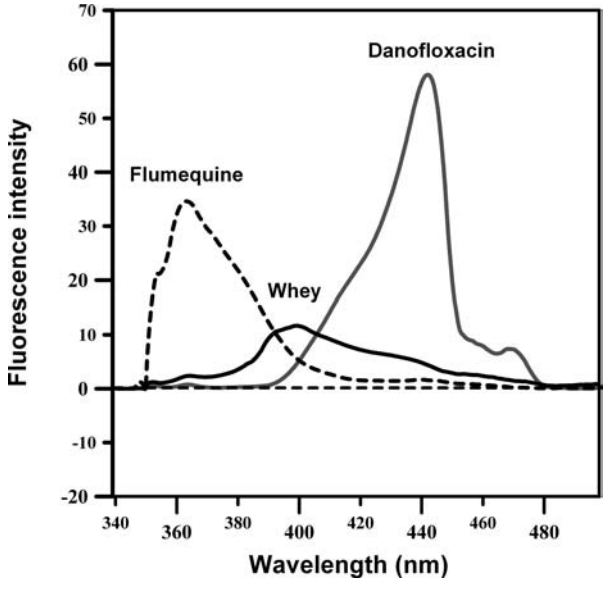

Figure 2. Nonlinear variable-angle synchronous spectra of flumequine, danofloxacin, and whey.

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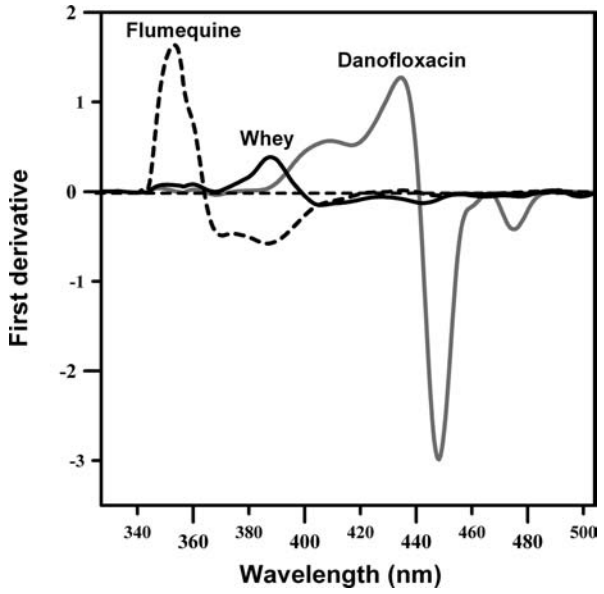

Figure 3. First derivative of NLVAS spectra of flumequine, danofloxacin, and whey.

that of flumequine, because its signal was interfered with by whey. This required using the PLS-1 multivariate algorithm, which has the added advantage of addressing multicollinearity.[26]

Calibration Matrix and Selection of Spectral Zones for Analysis with PLS-1. In order to resolve mixtures of the fluoroquinolones in milk, we considered whey to be the third component of the samples and performed additional tests with variable proportions of matrix in the calibration mixtures.

Devos et al.[27] recently found the accuracy of PLS models to depend on the way the calibration set is constructed and provided recommendations to minimize collinearity ratings and improve accuracy.

A combination of a factor design with two levels per factor, a central star design, and a whey blank was used to statistically maximize the information contained in the spectra. The combined design included individual standards; however, because the determination was conducted in whey, the standard in the matrix was considered and a whey blank used as calibration matrix. A set consisting of 16 samples was thus used. The concentrations of danofloxacin and flumequine in the calibration matrix ranged from 16.0 to 75.0 and 105.0 to 500.0 ng mL^{-1}, respectively, and whey dilution from 1:4 to 1:20. Table 1 shows the composition of the calibration matrix.

Although all multivariate calibration methodologies are considered full-spectrum procedures and a whole spectrum obviously provides a more accurate description of a sample than does a single measurement at a specific wavelength, using the full spectrum for the purpose of calibration is unwarranted since part of the information gathered is redundant and the measured signal at some wavelengths may be noisy or nonlinear, or contain useless information for the intended purpose. Accordingly, choosing an appropriate wavelength region is essential toward constructing an accurate model.

We optimized the spectral region by cross-validation at variable wavelengths, using the coefficient of determination, R^2, and the predicted error sum of squares (PRESS) as optimization criteria. In this way, we selected the region between points 103 and 150 for danofloxacin and that between points 225 and 248 for flumequine.

Table 1. Concentration Data for the Different Mixtures Used in the Calibration Set for the Determination of Flumequine and Danofloxacin in Whey

sample	flumequine (ng mL^{-1})	danofloxacin (ng mL^{-1})	whey dilution
1	105.0	16.0	1:4
2	105.0	16.0	1:16
3	105.0	60.0	1:4
4	105.0	60.0	1:16
5	394.0	16.0	1:4
6	394.0	16.0	1:16
7	394.0	60.0	1:4
8	394.0	60.0	1:16
9	0	37.5	1:10
10	500.0	37.5	1:10
11	250.0	0	1:10
12	250.0	80.0	1:10
13	250.0	37.5	0
14	250.0	37.5	1:10
15	250.0	37.5	1:10
16	0	0	1:10

Selection of the Optimum Number of Factors. An appropriate choice of the number of principal components, or factors, is necessary for PLS to perform properly. The selected factors should account as much as possible for the experimental data without overfitting. Various criteria have been developed to select the optimum number. We used cross-validation and the leave-one-out method in combination with the PLS-1 algorithm for this purpose. The predicted concentrations were compared with the known concentrations of the compounds in each calibration sample in order to calculate PRESS,[28] which was recalculated upon addition of each new factor to the PLS-1 model.

The optimum number of factors was selected according to Haaland and Thomas.[28] The selected model was that with the smallest number of factors resulting in a PRESS value not significantly greater than that for the model leading to the lowest PRESS. The significance level was determined via an F-test (α = 0.25). The optimum number of factors was thus found to be 2 for danofloxacin and 3 for flumequine.

Table 2 shows the specific factors selected for each analyte together with the standard error of calibration or prediction

Table 2. Statistical Parameters of the PLS-1 Method

component	factors	PRESS	SEP	REP %
flumequine	3	852.7	7.8	3.5
danofloxacin	2	207.6	4.0	9.4

(SEP), relative error of prediction (REP) for the sample series (n = 16), and PRESS.

External Validation of the Method. The predictive ability of the proposed method was assessed by applying PLS-1 to a series of 11 unknown mixtures. The concentrations of danofloxacin and flumequine, and whey dilution, were chosen from a distribution of random numbers,[29] falling within the ranges of the calibration matrix. Table 3 lists the added and found concentrations of both fluoroquinolones. The series was evaluated by statistical analysis of the experimental data, which were fitted to the equation $y = a + bx$ by using least median of squares regression (LMS) and least-squares (LS) regression. LMS[30] is a robust regression method that affords outlier

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Table 3. Data of Prediction Set of Flumequine and Danofloxacin

flumequine concentration (ng mL^{-1})			danofloxacin concentration (ng mL^{-1})		
added	found	recovery (%)	added	found	recovery (%)
380.0	386.5	101.7	75.0	78.0	104.0
400.0	404.0	101.0	60.0	57.7	96.2
480.0	473.8	98.7	66.0	68.6	104.0
580.0	574.2	99.0	32.0	30.8	96.2
320.0	331.2	103.5	70.0	71.3	101.8
340.0	333.5	98.1	26.0	26.7	102.7
220.0	221.9	100.9	54.0	57.2	106.0
464.0	466.3	100.5	18.0	18.7	104.0
372.0	368.7	99.1	48.0	49.4	103.0
180.0	175.5	97.5	52.0	49.8	95.8
260.0	257.9	99.2	68.0	65.3	96.1

detection. Outliers cause a rotation in the true line when experimental data are fitted by LS regression. Consequently, LS regression should be applied after outliers have been discarded by using a robust regression method such as LMS. Table 4

Table 4. Regression Parameters of LMS and LS Regression of the Prediction Set

	flumequine		danofloxacin	
regression parameter	LMS	LS	LMS	LS
A	0.3361	0.3561	0.1851	0.2032
B	1.0285	1.0171	1.0051	1.0135
R^2	0.999	0.998	0.995	0.990

summarizes the results of the statistical analysis. As can be seen, the slopes of the LMS and LS regression plots were similar to each other and near unity. On the basis of these results, the regression model (PLS-1) allows the fluoroquinolone mixture studied to be accurately resolved.

Precision and Selectivity. The repeatability of the proposed method was assessed by analyzing a series of 10 standard samples containing 40.0 ng mL^{-1} danofloxacin, 300.0 ng mL^{-1} flumequine, and 1:20 diluted whey. The samples were processed as described above to obtain their spectral data and construct the calibration model. As can be seen from the results (Table 5), PLS affords resolving this complex mixture.

Table 5. Precision Study

	flumequine	danofloxacin
$\bar{x} \pm$ sd	300.9 ± 1.9	39.8 ± 0.90
RMS	6.5	1.9
REP	2.8	5.0
SEP	8.1	3.2
r^2	0.995	0.977

Selectivity was assessed by determining the analytes in the presence of other fluoroquinolones. A compound was assumed to interfere when the recovery of either fluoroquinolone in its presence fell outside the range 95−105%.

Marbofloxacin, ofloxacin, and levofloxacin in proportions of 1:1 exhibited no interference.

On the other hand, lomefloxacin, pefloxacin, and norfloxacin interfered with the determination of danofloxacin, and ciprofloxacin and enrofloxacin in proportions of 1:0.25 exhibited no interference with this analyte. Also, oxolinic acid and nalidixic acid interfered with flumequine.

That is, although the method is characterized by an acceptable sensitivity and near 100% recoveries of fortified samples, selectivity should improve. Circumventing this problem would require using a different scan path to record the synchronous spectra, but lose sensitivity.

Detection Limit. The use of no regression lines in multivariate calibration methods makes calculating detection limits difficult. Murillo et al.[31] developed a procedure based on the use of parameters obtained from plots of added versus found concentrations (with an optimized calibration model), which are then substituted into the IUPAC equation for the limit of detection (LOD):[32]

$$\mathrm{LOD} = \frac{3s_{\mathrm{blank}}}{b}$$

where s_{blank} is the standard deviation of the blank and b the slope of the curve. The LODs thus obtained were 0.90 ng mL^{-1} for danofloxacin and 12.4 ng mL^{-1} for flumequine. Since the milk was diluted to 1:4, the lowest concentration that can be detected with the proposed method is 3.6 ng mL^{-1} for danofloxacin and 49.6 ng mL^{-1} for flumequine.

Applications. *Milk Samples.* The proposed models were validated by application to the simultaneous determination of the two fluoroquinolones in random whole milk samples. Recovery was assessed by using three sets of three samples each spiked with the analytes at variable concentrations. The milk was diluted 1:9. Table 6 compares the results obtained with the proposed method

Table 6. Recoveries of Flumequine and Danofloxacin in Milk

		proposed method		HPLC method	
analyte	added	found	recovery (%)	found	recovery (%)
danofloxacin (ng mL^{-1})	16.0	15.4	96.3	15.1	94.5
	40.0	38.9	97.2	40.8	102.1
	64.0	66.6	104.4	66.0	103.2
flumequine (ng mL^{-1})	120.0	114.5	104.8	117.8	98.2
	220.0	223.1	101.4	227.0	103.2
	320.0	307.2	96.0	324.2	101.3

and a previously reported HPLC method.[13] Recoveries ranged from 96% to 104% and were thus acceptable for quantitative analysis.

The use of NLAVFS spectra as analytical signals in multivariate calibration therefore provides an effective tool for resolving complex mixtures of compounds with overlapping fluorescence spectra, as illustrated here with the simultaneous determination of a mixture of fluoroquinolones in milk by using PLS-1 in combination with a calibration matrix and a central composite design.

The PLS model used affords good correlation between the synchronous fluorescence spectra for the fluoroquinolones and their concentrations in milk. Therefore, synchronous fluorescence measurements in combination with appropriate calibration allow the quantitative determination of fluoroquinolones with only some simple preliminary steps.

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Notes

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