JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Ciprofloxacin, the Major Metabolite of Enrofloxacin, in Milk by Isopotential Fluorimetry

J. A. MURILLO PULGARÍN,* A. ALAÑÓN MOLINA, AND L. MUÑOZ FERNÁNDEZ

Department of Analytical Chemistry and Foods Technology, University Castilla-La Mancha, 13071 Ciudad Real, Spain

A new method for the determination of ciprofloxacin, the major metabolite of enrofloxacin, for concentrations between 20 and 200 ng/mL by means of matrix isopotential synchronous fluorescence spectrometry and derivative techniques is proposed. This new method is useful for the determination of compounds in samples with unknown background fluorescence, such as ciprofloxacin in whey, without the need of tedious preseparation. The determination was performed in an ethanol/water medium (20% v/v) at pH 4.8, provided by adding a sodium acetate/acetic acid buffer solution. Since enrofloxacin is widely used as an antibacterial agent in veterinary medicine, the method was successfully applied to the determination of its main metabolite in milk. An exhaustive statistical analysis has been developed to all calibration graphs. This treatment includes robust regression such as least median of squares, which also detects outliers and leverage points. The overall least-squares regression has been applied to find the more exact straight line that fits the experimental data. The error propagation has been considered to calculate the detection limit and the repeatability of the method.

KEYWORDS: Ciprofloxacin; enrofloxacin; milk; isopotential; fluorimetry

INTRODUCTION

Fluoroquinolones are the second-generation members of quinolone antibiotics fluorinated in position 6 and bearing a piperazinlyl moiety at position 7. These antimicrobial agents are highly active against a broad range of bacteria, and hence, their use is not restricted to human medicine but also finds wide application in the treatment and prevention of veterinary diseases in food-producing animals and even as growth-promoting agents. Because of the use in animals destined for human consumption, the risk of presence of unwanted residues in edible products exits. Milk is one of these products, where drug residues can be present especially if they are not used according to the label directions. The presence of antibiotic residues in milk may cause allergic reactions in sensitive individuals. There are concerns that the widespread usage of antibiotics may be responsible for resistant strains of bacteria.

Enrofloxacin, the first fluoroquinolone developed for veterinary application, is available in several oral and parental formulations for the treatment of some infectious diseases in pets and livestock. In different species, enrofloxacin is deethylated to ciprofloxacin, a fluoroquinolone used in human medicine. In fact, it is one of the most used clinical antibiotics in the world.

To protect consumers' health, the European Union set maximum residue limits for many drugs regarding milk, meat, and others foods. Ciprofloxacin is excreted with milk after enrofloxacin treatment (*1, 2*). Therefore, development or improvement of analytical methods for monitoring the levels of drugs in farm animals and their primary products is of increasing interest.

Only a few of them have focused on the determination of fluoroquinolones residues in milk. To determine ciprofloxacin, the most commonly employed technique is liquid chromatography by using fluorimetry (*3−8*) and photometry (*9*) as detection systems. Holtzapple et al. (*10*) determine four fluoroquinolones in milk by online immunoaffinity capture coupled with reserved-phase liquid chromatography. Liquid chromatography−tandem mass spectrometric method had been used for the quantification of quinolones in milk (*11, 12*). Electrochemical (*13*) and optical immunobiosensor (*14*) assays were developed for screening ciprofloxacin in bovine milk. In general, methods for the determination of this antibacterial agent involve tedious extraction and cleanup steps prior to determination. Therefore, our objective is to develop a method for the determination of ciprofloxacin in cow milk that involves minimal sample pretreatment in order to minimize times of analysis.

Fluorimetric methods are usually highly sensitive and selective. However, the selectivity can be poor in multicomponent analysis or determinations of analytes in the presence of substantial unknown background fluorescence, such as biological fluids. This problem is usually solved by using a

* To whom correspondence should be addressed. E-mail: joseantonio.murillo@uclm.es.

10.1021/jf801094g CCC: $40.75 © 2008 American Chemical Society
Published on Web 09/05/2008

prior separation step, which is rather time-consuming for routine analysis and occasionally calls for special expensive equipment.

The above-described problems can be circumvented by using a synchronous fluorimetric technique called matrix isopotential synchronous fluorescence spectrometry (*15*), which is especially useful for suppressing fluorescence matrix background effects and which affords the determination of individual compounds in complex samples. Provided the matrix to be used exhibits an almost invariable composition, a constant background signal can be maintained even if the fluorescence intensity changes. This is possible if a portion of the total fluorescence spectrum along a trajectory connecting points of identical intensity (isopotential trajectory) is selected from an initial point to final excitation and emission wavelengths. Such a trajectory can be obtained by using a software program developed by us in Basic (*16*). It is always possible to find the matrix trajectory that passes through the maximum fluorescence excitation and emission wavelengths of the component to be determined, and so, the same sensitivity is achieved as in direct determination in the absence of background fluorescence.

The performance of this technique can be improved by using derivative methods.

Milk is a complex fluid exhibiting simultaneously emulsion, colloidal, and solution phases. The aqueous phase of milk, whey, contains a variety of organic substances. Most are present at low concentration or possess low fluorescence efficiency. In fact, only a few are fluorescent and exhibit different excitation and emission maxima that contribute significantly to the overall fluorescence spectrum of whey. This ultimately results in a high background fluorescence that interferes with the direct determination of ciprofloxacin.

In previous works, we used the matrix isopotential synchronous fluorescence (MISF) technique to determine various drugs in biological fluids (*17*−*22*) with excellent results.

As shown in this paper, first derivative matrix isopotential synchronous fluorimetry is a simple, expeditious alternative to the determination of ciprofloxacin in whey without the need for a prior separation.

MATERIALS AND METHODS

Reagents. All experiments were performed with analytical reagent grade chemicals, pure solvents, and Milli-Q water. Acetic acid, sodium acetate, ethanol, and hydrochloric acid were supplied by Panreac (Barcelona, Spain).

The ciprofloxacin standard used was obtained from Fluka (Madrid, Spain). A stock solution of ciprofloxacin was prepared in a 500 mL volumetric flask by dissolving 25.0 mg of the product in ethanol. This stock solution was used to prepare working-strength solutions by appropriate dilution with water.

A 2.5 M buffer solution of pH 4.8 was prepared by mixing the required amounts of acetic acid and sodium hydroxide.

Milk was supplied by some commercial marks.

Apparatus. All fluorimetric measurements were performed on a Perkin-Elmer LS50 instrument equipped with a xenon lamp, connected to a computer running software (designed to handle fluorescence data on a personal computer) and an HP Deskjet 500C printer.

Thermostatic equipment and a Crison model 2001 pH meter turnished with a glass-saturated calomel combined electrode and Selecta centrifuge (model Mixtaxel) were also used.

Software. A program was developed to obtain λ_{ex} and λ_{em} at any constant fluorescence intensity value from a three-dimensional spectrum. Because the values obtained for specific curves were not equidistant, the Lagrange interpolation method was applied to all points, which were sequenced according to excitation wavelengths at 0.4 nm intervals. Once a trajectory was defined, the corresponding spectrum was obtained by using the Ftotal program (*16*), so that the spectra obtained display the same format as those performed directly from the Perkin-Elmer LS50 spectrofluorimeter.

Procedure. *Sample Whey Obtaining.* Whey is obtained by precipitation of the protein fraction of the milk at pH 4.6: 10 mL of milk were introduced into a 100 mL calibration flask; 75 mL of Milli-Q water at 40 °C and 1 mL of the acetic acid solution (10%, p/v) were added to the calibration flask. The content of the flask was smoothly mixed and left to settle for 10 min. Next, 1 mL of the 1 M sodium acetate was added and mixed again; the flask was then diluted to volume with water. The flask must be left to cool down.

Once protein fraction has been precipitated, soluble phase, whey 1:10, was separated using a filter paper. Whey must be stored in a dry container at low temperature.

Calibration Graph. An appropriate aliquot containing 200−2000 ng of ciprofloxacin was transferred into a 10 mL volumetric flask; 2.0 mL of acetate buffer at pH 4.8, 2.0 mL of ethanol, and 4.0 mL of whey were added and diluted to the mark with water. For the assay, a 1:25 dilution was adopted that ensures high enough sensitivity to determine the appropriate concentration of ciprofloxacin in therapeutic doses.

Various solutions prepared in the same way were also obtained, both from different whey and containing no whey.

Similarly, another calibration graph was obtained for ciprofloxacin in an aqueous solution.

An overall 61 emission spectra spanning a wavelength range of 240 nm were recorded at 4 nm intervals for each sample. The total luminescence spectrum was obtained by using Ftotal (*20*). Then, appropriate trajectory and matrix isopotential synchronous spectra for ciprofloxacin were obtained by using of the same software. The determination of ciprofloxacin is not possible by measuring the fluorescence intensity with regard to the value of the final extreme because the spectra do not display a constant value at the extreme; hence, the derivative values are essential in order to determine ciprofloxacin.

RESULTS AND DISCUSSION

Influence of Experimental Variables. Chemical variables were studied and optimized in order to ensure the best possible measurement conditions, maximum fluorescence sensitivity, and adequate selectivity. Specifically, the effects of ethanol content in the medium, pH, temperature, and the analyte concentration were investigated. Ciprofloxacin is not water-soluble, so organic solvent was required. On the basis of its physical and chemical properties, ethanol was a suitable solvent for this purpose. The influence of its content was investigated by using it to prepare a sample of ciprofloxacin and whey; its content ranged from 10 to 80% (v/v) in ciprofloxacin solutions and between 5 and 60% in whey solutions (above this level some whey components precipitated). The fluorescence intensity of ciprofloxacin increased slightly with the ethanol content to 50%, then decreased the similar way. Besides, the band suffers a bathochromic shift, of 276−280 nm and 430−448 nm for the excitation and emission wavelengths, respectively. Whey also exhibited an increase in fluorescence intensity with increase in ethanol content. Besides, the emission wavelength suffers little variation respecting to its normal position above 40% ethanol. A portion of 20% of ethanol was selected, so it is sufficient to maintain soluble ciprofloxacin and the background due to whey is not excessively high.

The influence of pH on the fluorescence intensity was studied by adding different amounts of HCl and NaOH to the ciprofloxacin and whey solutions. The fluorescence intensity of ciprofloxacin remained virtually constant at about pH 3−8 and then decreased with the pH. The intensity of whey decreased with increase in pH. pH 4.8 was selected as optimal. Such a

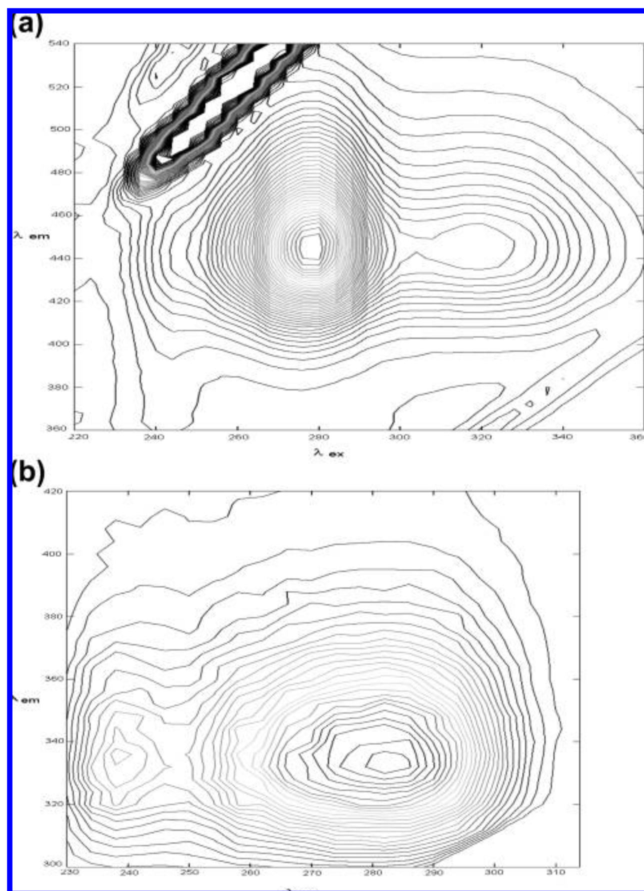

Figure 1. Total fluorescence spectra of 200 ng/mL ciprofloxacin (**a**) and whey 1:25 (**b**).

pH was adjusted with 0.5 M sodium acetate/acetic acid buffer to ensure the buffer capacity.

One other factor affecting fluorescence is temperature. The fluorescence intensity of ciprofloxacin and whey decreased, as the temperature was raised from 10 to 50 °C. Use of a thermostat was thus advisable in order to maintain a measurement temperature of 20 °C, which is usually close to room temperature.

The influence of the ciprofloxacin concentration on the fluorescence intensity was studied under these conditions. The optimum range for ciprofloxacin was found to span concentration up to 20 and 200 ng/mL.

Determination of Ciprofloxacin in Whey. The above-described operating conditions were used to develop a method for determining ciprofloxacin in whey, a very fluorescent matrix, using first derivative matrix isopotential synchronous fluorescence.

The fluorescence maxima characteristics of ciprofloxacin are located in the UV region. **Figure 1** shows the total fluorescence spectra for a standard solution of 200 ng/mL ciprofloxacin (**Figure 1a**) and for 1:25 whey (**Figure 1b**). Ciprofloxacin exhibits two bands at λ_{exc1}= 278 nm and λ_{exc2}= 314 nm, with emission at λ_{em}= 448 nm. Whey also exhibits two bands at λ_{exc1}= 238 nm and at λ_{exc2}= 282 nm, with emission at λ_{em}= 331 nm. The former band is weaker than the latter.

A previous work (*23*) demonstrated the constant shape of fluorescent spectra for the same wheys corresponding to different milk obtained from different mammals and subjected to several treatments and conditions (a necessary condition for application of this new synchronous technique). The different whey samples exhibited the same time of fluorescence, with a slight variation on the position of the former band depending on the nature of the milk. The relative intensity of the bands is also affected by the provenance of the milk. In the way, sheep milk shows higher fluorescence intensity than goat and cow milks, while human milk is characterized by its weak fluorescence.

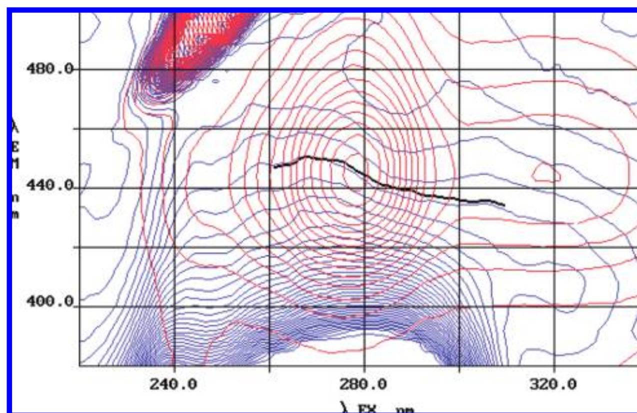

Figure 2. Total fluorescence spectra of 200 ng/mL ciprofloxacin (red line) and whey 1:25 (blue line), with isopotential trajectory selected.

As can be observed in **Figure 2**, in the UV region where ciprofloxacin exhibits fluorescence, whey interferes in the direct determination of ciprofloxacin with a high background fluorescence, so prior separation is necessary.

The spectrum corresponding to the arithmetical mean of three total fluorescence spectra of the different whey samples was obtained by means of the Ftotal program. In order to determine ciprofloxacin, we calculated the isopotential trajectory in the averaged whey spectrum, which passes through the excitation and emission maxima of the ciprofloxacin (278 and 448 nm), and so, the sensitivity achieved is the same as that achieved in a direct determination in the absence of whey fluorescence. Moreover, as a result of the selection of these wavelengths, the likelihood of interferences resulted low while the analyte intensity attractively high (**Figure 2**).

Whey sample solutions containing ciprofloxacin gave signals smaller than those obtained with aqueous standard solutions, owing to some type of binding with another component of the whey.

Total luminescence spectra of ciprofloxacin were obtained with different whey samples in order to construct calibration graphs and to carry out recovery experiments.

Figure 3a shows the effect of background fluorescence intensity (whey) on ciprofloxacin MISF. It can be seen that the spectra are identical in form, although their intensities are different by constant terms. These correspond to the values of the fluorescence intensity due to the three wheys in the isopotential trajectory applied. It was impossible to measure the fluorescence intensity with respect to the ends of the spectrum (i.e., 268 or 300 nm) because the fluorescence intensity of ciprofloxacin was not zero at both values. The problem arising from not knowing the matrix constant value can be avoided by using the first derivative spectrum. Because the trajectory is isopotential, the first derivative of the spectrum for whey will be zero and that the first derivative of the spectrum for ciprofloxacin in different wheys will coincide exactly (**Figure 3b**).

The first derivative was obtained using the Savitsky and Golay algorithm (*24, 25*) of the matrix isopotential synchronous spectra.

The number of points spanned by the derivative method was optimized; 25 points were found to provide derivative spectra with an adequate signal/noise ratio.

Figure 4 shows the spectra derived from the calibration of ciprofloxacin in whey. As can be seen, they have a maximum

Table 1. Statistical Data for the Determination of Ciprofloxacin in Whey by Means of First Derivative Matrix Isopotential Synchronous Fluorescence

fitting used[a]	sample	determination coefficient	intercept on ordinative (a)	SD of intercept	slope (b)	SD of slope	SD of estimation
1	whey I	1.0	9.1×10^{-3}		3.072×10^{-2}		2.4×10^{-3}
	whey II	1.0	1.57×10^{-1}		2.972×10^{-2}		1.2×10^{-2}
	whey III	1.0	2.12×10^{-1}		2.935×10^{-2}		2.0×10^{-2}
2	whey I	0.9991	9.12×10^{-2}	5.4×10^{-2}	2.983×10^{-2}	4.4×10^{-4}	5.4×10^{-2}
	whey II	0.9991	9.39×10^{-2}	5.6×10^{-2}	2.993×10^{-2}	4.6×10^{-4}	5.6×10^{-2}
	whey III	0.9960	9.85×10^{-2}	1.2×10^{-1}	3.054×10^{-2}	9.7×10^{-4}	1.2×10^{-1}
3	global	0.9998	9.45×10^{-2}	4.5×10^{-2}	3.016×10^{-2}	3.6×10^{-4}	9.2×10^{-2}

[a] 1 = least median of squares regression line according to $y = a + bx$; 2 = least-squares regression line according to $y = a + bx$; 3 = overall least-squares regression line according to $y = a + bx$.

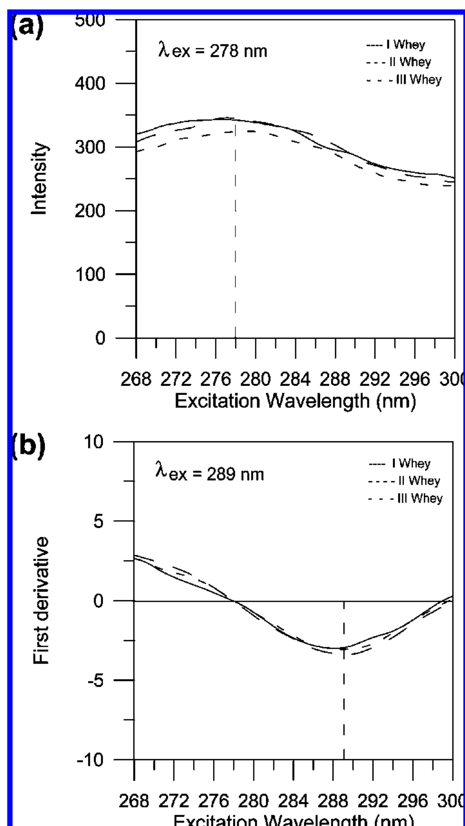

Figure 3. (**a**) Set of MISF spectra of 100 ng/mL ciprofloxacin in different whey samples. (**b**) Their first derivative spectra.

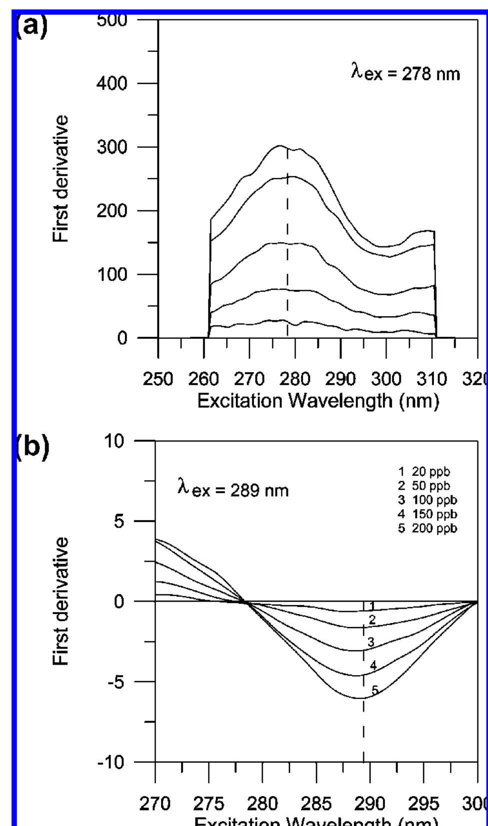

Figure 4. (**a**) Set of MISF spectra of ciprofloxacin in whey. Ciprofloxacin concentrations: (1) 20; (2) 50; (3) 100; (4) 150; (5) 200 ng/mL. (**b**) Corresponding first derivative spectra.

at 289 nm that coincides with the highest sensitivity and reproducibility. We thus adopted this wavelength as optimal to determine ciprofloxacin.

To test the independence of the analytical signal of ciprofloxacin (i.e., to show that the signal measured is independent of the whey), three calibration graphs were constructed from first-derivative signals from standards containing between 20 and 200 ng/mL of ciprofloxacin in different whey samples (1:25).

The proposed method was evaluated by a statistical analysis of experimental data by fitting the least-squares line according to $y = a + bx$, after discarding outliers with the help of the least median of squares regression (*26*) (LMS), since LMS is a robust regression method. **Table 1** shows the outstanding results of the statistical analysis.

In order to obtain the most representative calibration graph, an overall least-squares regression was developed. The regression line was homocedastic (*27*), so the residuals exhibited uniform variance (errors in measurements were independent of the ciprofloxacin concentration); it was therefore unnecessary to weigh the first derivative values relative to the mean standard deviation. The accuracy of the overall least-squares regression was checked via an ANOVA test (*27*). The variance of the residual error was therefore assumed not changed from sample to sample or among calibration graphs.

In order to perform an analysis of variance, the variance ratio (experimental F) must be calculated and compared to the theoretical value of F for an adequate number of degrees of freedom at the 95% of confidence level. The F_1 value compares total deviations from overall linear regression with the deviation within each set from the set line. The experimental value of F_1 was less than the theoretical $F_{4,13}$ value (**Table 2**); consequently, the departure of individual sets from the overall regression line was not significant. F_2 compares differences among the regression coefficients, slopes, with the deviation within each set from the set line. The experimental value of F_2 was less than the theoretical $F_{2,13}$ value (**Table 2**), so there were no significant differences among the regression coefficients and therefore their mean, overall slope can be adopted as the representative slope. In summary, the signal measured at 289 nm in the first derivative synchronous spectrum is only a function of the ciprofloxacin

Table 2. ANOVA Test

source of variation	sum of squares	degrees of freedom	mean squares
deviations within individual sets	8.510×10^{-2}	9	9.455×10^{-3}
differences between individual slopes	6.327×10^{-3}	2	3.164×10^{-3}
deviation between individual sets	2.535×10^{-2}	4	6.338×10^{-3}
total deviation over the global calibration	1.104×10^{-1}	13	8.496×10^{-3}

F statistic	experimental	theoretical ($\alpha = 0.05$)
F_1	6.60×10^{-1}	3.63
F_2	3.35×10^{-1}	4.26

concentration and hence independent of the whey, which is consistent with the theoretical predictions.

By using the first derivative matrix isopotential synchronous spectrofluorimetry technique in conjunction with overall least-squares regression ($y = a + bx$), a limit of detection of 3.9 ng/mL was obtained according to definition of the IUPAC (*28, 29*), which only considers the standard deviation of the blank. The error propagation approach provided a limit of detection consistent with the reliability of blank measurements and signal measurements of the standards (*30*). In this work, it is 5.9 ng/mL. The limit of detection determined according to Clayton et al. (*31*) considers the probability of false negatives and false positives values was 8.0 ng/mL. We proposed as limit of detection of our method of 8.0 ng/mL of ciprofloxacin.

In order to study the repeatability of the method, a series of 10 solutions was prepared containing 100 ng/mL of ciprofloxacin in whey. By applying the IUPAC definition, the standard deviation of replicates means was 3.5 ng/mL and the relative error 3.3%, while based on error propagation, the standard deviation obtained was 2.4 ng/mL and relative error 2.3% (95% confidence level).

The selectivity of the proposed method for ciprofloxacin was examined by using it in the presence of other quinolones. Compounds such as oxolinic acid, flumequine, nalidixic acid, enoxacin, ofloxacin, levofloxacin, and marbofloxacin exhibited no interference in a proportion of 1:1. A substance was assumed to interfere when the recovery obtained in the determination of ciprofloxacin in its presense was 95−105%.

The determination of ciprofloxacin was validated by least-squares regression (*27*). The performance of the proposed method in the determination of ciprofloxacin in milk was compared with that of a chromatographic method (*5*) by analyzing 10 samples containing the fluoroquinolone at levels within the application range. The concentrations, provided by the currently accepted method and the proposed method, were subjected to least-squared pair analysis. This procedure considers the effects of various types of error. The presence of random errors in the test method causes points to scatter around the least-squares line and the calculated slope and intercept to slightly depart from unity and zero, respectively. The random error can be estimated from the standard deviation in the *y*-direction (also called the standard deviation of the estimate of *y* on *x*). A proportional systematic error leads to a change in *b*, so the difference between *b* and unity provides an estimate of the proportional error. A constant systematic error shows up in a nonzero value for the intercept. If both methods provided identical concentrations, in the same samples, then the least-squares analysis would give a zero intercept and a unit slope. **Figure 5** shows the 95% confidence region for the true slope and estimated intercept. As can be seen, the point corresponding to the zero intercept and unity slope falls within the joint confidence region, which means that the accuracy of the proposed method and the currently accepted method is not significantly different.

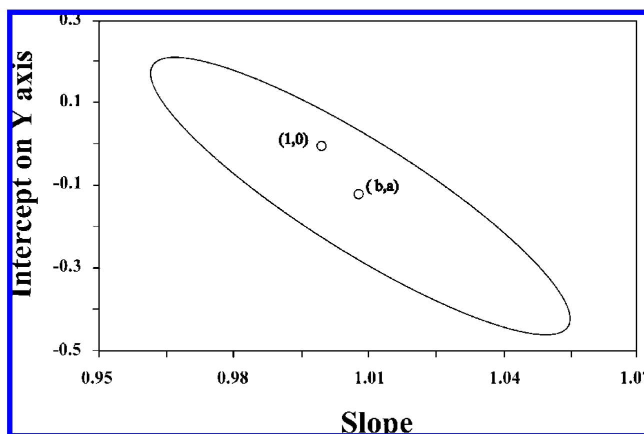

Figure 5. Comparison of the proposed method and the chromatographic method. The ellipse bounds the 95% confidence region for the true slope and intercept on *y*-axis as estimated the overall least-squares regression the concentration calculated in reverse through both. The point (b,a) is the center of the ellipse corresponding to the true intercept and estimated slope. The point (1,0) corresponds to a zero intercept and unity slope.

The experimental results obtained in this work testify to the ability of the first derivative matrix isopotential synchronous fluorescence technique to determine compounds in highly fluorescent matrices such as biological fluids (serum, urine, whey, etc.) without the need for a prior separation.

The method developed in this study has the advantage of simplicity and good precision and accuracy. Furthermore, the method uses simple reagents, with minimum sample preparation procedures, encouraging its application in routine analysis.

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Received for review April 8, 2008. Revised manuscript received July 1, 2008. Accepted July 1, 2008. We gratefully acknowledge financial support from the "Junta Castilla-La Mancha" (Project No. PCI08-0120).

JF801094G