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Two different strategies for the fluorimetric determination of piroxicam in serum

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Abstract

Two different spectrofluorimetric methods for the determination of piroxicam (PX) in serum are presented and discussed. One of them is based on the use of three-way fluorescence data and multivariate calibration performed with parallel factor analysis (PARAFAC) and self-weighted alternating trilinear decomposition (SWATLD). This methodology exploits the so-called second-order advantage of the three-way data, allowing to obtain the concentration of the studied analyte in the presence of any number of uncalibrated (serum) components. The method was developed following two different procedures: internal standard addition and external calibration with standard solutions, which were compared and discussed. The second approach investigated is based on the combination of solid-phase extraction (SPE) and room temperature fluorimetry. Both methods here presented yield satisfactory results. The concentration range in which PX could be determined in serum was 1–10 μg ml^{-1}. The limits of quantification for the experimental solutions using the chemometric approach were 0.09 μg ml^{-1} for the standard addition mode and 0.12 μg ml^{-1} using external calibration (both for PARAFAC and SWATLD algorithms). In the solid-surface fluorimetric method, the calibration graph was linear up to 0.22 μg ml^{-1} and the limit of quantification was 0.02 μg ml^{-1}.

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1. Introduction

Piroxicam (PX), 4-hydroxy-2-methyl-*N*-(2-pyridyl)-2H-1,2-benzothiazine-3-carboamide-1,1-dioxide, is a compound with both anti-inflammatory and analgesic properties [1]. Several methods have been proposed involving either spectroscopic or chromatographic techniques for the determination of PX in pharmaceuticals and biological fluids [2–6]. Damiani et al. [7] quantified PX in pharmaceutical preparations employing fluorescence signals, and subsequently the method was notably improved using β-cyclodextrin as fluorescence enhancer [8].

It is noteworthy that spectrofluorimetric methods have not been reported for the analysis of PX in serum. An explication to this fact lies in the strong overlapping between the fluorescence emis-

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sion for human serum and PX, which in principle precludes the direct spectrofluorimetric quantification of the studied compound. Due to the importance of luminescence methods as alternative to chromatographic ones, we have focussed our interest in the challenges posed by the above determination. Among the several different approaches investigated, only two yielded successful results. One of them corresponds to a three-way data analysis performed on excitation–emission fluorescence matrices (EEFMs). The wealth of information offered by this kind of data can be enhanced by the potentiality of three-way chemometric tools [9]. Especially useful are parallel factor analysis (PARAFAC) [10] and self-weighted alternating trilinear decomposition (SWATLD) [11], particularly when the data follow the so-called trilinear model [10]. The decomposition of a three-dimensional cube of data built with response matrices measured for a number of samples is often unique, allowing spectral profiles as well as relative concentrations of individual sample components to be extracted directly. This property has been named the 'second-order advantage' [12,13], and permits correction for uncalibrated sample constituents. It is fully exploited by the PARAFAC and SWATLD models, which operate in a three-step mode: (1) trilinear decomposition of the spectral data cube formed by stacking the calibration matrices together with that of the unknown sample, (2) calibration of the concentration scale by linear regression using external calibration or standard addition, and (3) prediction by inter- or extrapolation of the unknown in the pseudo univariate graph. Although these chemometric tools are chemically attractive and very useful for the analysis of complex samples, only few reports are devoted to its use in biological materials [14,15]. Besides solving a specific problem, the present report represents a contribution to the recognition and application of trilinear fluorescence data.

On the other hand, a simple method based on the combination of solid-phase extraction (SPE) and fluorescence measurements was developed. Recently, we have applied a similar methodology for the determination of PX and pyridoxine in pharmaceutical formulations [16]. This method is rapid and highly sensitivity, and the present paper demonstrates that a time consuming serum pretreatment serum is not necessary.

The advantages and limitations of each method are herein discussed, both from the experimental point of view and from the quality of the analytical results.

With the purpose of validating the methods developed, PX was also quantified by HPLC.

2. Experimental

2.1. Reagents

Analytical-reagent grade chemicals were employed in all experiments. PX was obtained from Sigma. Benzene, NH_3, HCl and sodium acetate were purchased from Merck (Darmstadt, Germany). For the preparation of an aqueous stock solution of PX (1000 μg ml^{-1}) the compound was dissolved in 0.2 M NH_3. The benzene stock solution (1000 μg ml^{-1}) was prepared by dissolving PX in benzene. From these solutions, more diluted PX solutions were obtained in aqueous 0.2 M NH_3 or benzene. An acetate/acetic acid buffer (pH 3.5) was prepared from sodium acetate and HCl.

2.2. Apparatus

Measurement of fluorescence signals were carried out with an Aminco Bowman (Urbana, IL, USA) Series 2 spectrofluorimeter fitted with a 150 W continuous xenon lamp and interfaced to a 486 personal computer. Data acquisition and data analysis were performed with AB2 software, running under OS/2 version 4.0. The measurements were done using 1.00 cm quartz cells and slit widths of 4 nm.

2.3. Three-way trilinear data

When the data structure is trilinear, a cube can be written as the sum of tensor products of three vectors for each component. In such a case, if $\mathbf{A}_n$, $\mathbf{B}_n$ and $\mathbf{C}_n$ collect the relative concentrations ($I \times 1$, I = number of samples), emission profile ($J \times 1$,

$J =$ number of data points) and excitation profile $(K \times 1)$ for component, n, respectively, the data cube $\underline{\mathbf{R}}$ can be written as [17]:

$$\underline{\mathbf{R}} = \sum_{n=1}^{N} \mathbf{A}_n \otimes \mathbf{B}_n \otimes \mathbf{C}_n + \underline{\mathbf{E}} \quad (1)$$

where, $\otimes$ indicates tensor product, N is the total number of responsive components and $\underline{\mathbf{E}}$ is a residual error term of the same dimensions as $\underline{\mathbf{R}}$. The column vectors $\mathbf{A}_n$, $\mathbf{B}_n$ and $\mathbf{C}_n$ are usually collected into the three loading matrices **A**, **B** and **C**. A characteristic property of $\underline{\mathbf{R}}$ is that it can be uniquely decomposed, providing access to the profiles **B** and **C** and to the relative concentrations (**A**) of individual components in the I mixtures, whether they are chemically known or not. This constitutes the basis of the so-called second-order advantage (‘second-order’ refers to the tensor order of a single sample data matrix, in contrast to ‘third-order’, as referred to the cube formed by the matrices of I samples). Theoretically, this property should allow the analyst to obtain the concentration values of calibrated constituents in the presence of any number of uncalibrated components.

In general, this type of data can be analysed by different procedures: those based on solving an eigenvalue problem, such as the generalised rank annihilation method (GRAM) [18] and those employing a alternating least-squares minimisation, as implemented in both PARAFAC [19] and SWATLD [20]. The theory of these methods has already been described [18,19]. In the present report, we use the latter type of methods because, according to our experience [14], they offer better results than other algorithms.

When multi-way calibration models are applied, the relevant subjects which should be taken into account are: (a) how model uniqueness can be assessed, (b) how to establish the number of responsive spectral components, (c) how to identify specific components from the information provided by the model and (d) how to calibrate the model in order to obtain absolute concentrations for a particular analyte in an unknown sample.

Uniqueness is usually checked through the residuals of the least-squares fit, i.e. the size of the error term $\underline{\mathbf{E}}$ in Eq. (1). In all the presently studied cases, the residuals were lower than the estimated instrumental noise, indicating a satisfactory fit.

Bro suggested obtaining the number of responsive components [N in Eq. (1)] by consideration of the internal parameter known as core consistency, which is a measure of how well a given model is able to reproduce the so-called Tucker core of a cube of data [17]. The core consistency is calculated as a function of a trial number of components. It remains near a value of 100 when the number is less than or equal to the optimum; for higher component numbers it drops below 50%.

Identification of the chemical constituent under investigation is usually done with the aid of the **B** and **C** profiles, as extracted by the employed algorithms, and comparing them with those for a standard solution of the analyte of interest.

Absolute analyte concentrations are obtained after proper calibration, since only relative values (**A**) are provided by decomposing the cube of data. Two different modes were employed: internal standard additions and external calibration set (see below). Both modes exploit the second-order advantage, which takes into account the presence of constituents not modelled by the calibration set and which can be present in the evaluated sample.

The experimental procedure for the standard addition mode was developed as follows: a test set of ten samples was prepared by spiking different human sera with PX, obtaining concentration levels between 1 and 10 μg ml^{-1}, which were in the order of those found in blood after multiple 20 mg doses [1]. A volume of 0.600 ml of a given serum sample was placed in a capped flask and the corresponding volume of aqueous PX stock solution, 1 ml of acetate/acetic acid buffer (pH 3.5) and 7.00 ml of benzene were added. The tube was then shaken for 5 min and briefly centrifuged (5 min at $2000 \times g$). On the other hand, different volumes of PX bencenic stock solution were added to three 2.00 ml flasks and evaporated by use of nitrogen. Finally, the organic phase separated after centrifugation was added to each flask to the mark, in order to obtain final concentrations for PX which were 0, 10 and 20 μg ml^{-1} higher than the original ones. For the external calibration method, a calibration set was constructed with six bencenic samples containing PX in the concentration range 0.1–1 μg ml^{-1}. The EEFMs for these standard solutions were then read. In this case, the determination of the PX concentration in the serum samples was performed using the EEFMs previously measured for the standard addition mode, selecting those which corresponded to the bencenic extract without any addition of PX, i.e. those containing each of the original PX concentrations. Finally, the EEFMs for the standards and for each unknown sample were combined into a cube and subjected to three-way data analysis.

In principle, exploratory excitation and emission wavelength ranges were 270–380 nm (excitation) and 370–550 nm (emission), but in order to avoid the presence of Rayleigh scattering, the EEFMs were then recorded in restricted excitation and emission wavelength ranges. Depending on the specific sample, two or three factors were selected, as suggested by core consistency analysis [17].

2.4. SPE-solid surface fluorescence

The fluorimetric determination of PX in disks was carried out following a procedure previously described [16]. Briefly, solid-phase extraction concentrator (SPEC®) octadecyl (C18AR) membranes were dissected into 13 mm disks, loaded into a 13 mm stainless steel filter syringe kit (Alltech, Deerfield, IL, USA) and placed into a 5 ml syringe (Hamilton, Reno, NV, USA). The disk was first conditioned with 0.5 ml of methanol and then a positive pressure was used to force all sample solution (5 ml) through the membrane in approximately 30 s per sample. Note that this flow rate is in the optimum range for maximum breakthrough volume (10–30 ml min^{-1}) [21]. After the sample was dried by forcing air through the membrane using a 25 ml syringe (Hamilton, Reno, NV, USA), the disk was placed in a holder and the fluorescence spectrum was collected at 90°, irradiating at 320 nm and obtaining the emission at 440 nm.

For the construction of the calibration curve, convenient dilutions of the PX aqueous standard solution with acetate/acetic acid buffer were performed in order to obtain concentrations in the range 0–0.22 μg ml^{-1} and pH 3.5. Then, solid-phase-extraction and fluorescence measurements were carried out by the procedure described above. For the serum analysis, the same spiked samples described for trilinear fluorescence determination were used. Thus, 100 μl of serum (without any pretreatment) were transferred into a 5.00 ml flask and diluted with acetate/acetic acid buffer (pH 3.5). SPE and fluorescence measurements were subsequently performed as already indicated.

2.5. HPLC

The proposed methods were validated by HPLC, following the procedure suggested by Velpandian et al. [5].

3. Results and discussion

As can be appreciated in Fig. 1, the fluorescence spectral overlapping of a normal serum and PX is very important and, even after organic solvent extraction, it hinders its direct spectrofluorimetric

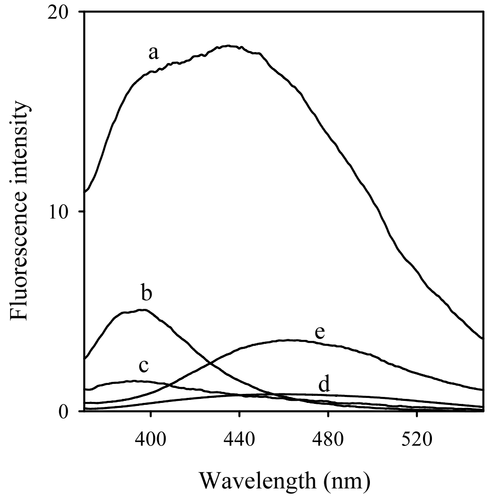

Fig. 1. Emission spectra for: (a) a diluted (1/10) typical serum, free of PX, (b) and (c) two different sera after solvent extraction with benzene, (d) 1.0 μg ml^{-1} PX in aqueous solution at pH 3.5, and (e) 1.0 μg ml^{-1} PX in benzene; $\lambda_{ex} = 320$ nm, PMT sensitivity = 540 V.

determination in this type of matrix. It can also be observed that although the PX emission in benzene is higher than in acid aqueous solution, a serious overlapping remains.

This problem was overcome by the use of three-way multivariate calibration and by isolation of PX in an adequate surface, where the undesired constituents are either not retained or do not fluoresce.

3.1. Chemometric analysis

The main advantage of three-way multivariate calibration is that it allows concentration information of an individual component to be extracted in the presence of any number of uncalibrated constituents. Therefore, it is highly useful for solving analytical problems involving a complex matrix like serum, where numerous compounds (both exogenous and endogenous ones) can be present.

Fig. 2 shows examples of typical EEFMs for a normal serum after solvent extraction and for a PX bencenic solution, in wide spectral ranges (top figure) and in appropriately selected wavelength ranges which avoid Rayleigh and Raman scatterings (bottom figure).

Table 1 shows the nominal and predicted PX concentrations, and the corresponding recoveries using PARAFAC and SWATLD, applying both standard addition and external standard calibration. The standard addition procedure allows one to take into account possible spectral variations of the studied compound due to chemical interactions with the matrix. Although in principle multiple additions of standard solutions could be carried out over each sample, from the experimental point of view this is rather difficult to implement, especially when several samples have to be analysed. In fact, an experiment involving more than three additions is undesirably time-consuming, and involves the use of a significant amount of serum. On the other hand, the external standard calibration is simpler to implement because a single standard set is used for all unknown samples, and the possibility of working with several standard solutions allows a better modelling of the spectral variability. However, this latter procedure does not consider possible interactions between the matrix and the analyte, which can modify its spectral signals. If such interactions do not occur, this mode can be successfully applied, as in the presently studied system. An inspection of the values quoted in Table 1 using either calibration mode seems to indicate reasonably good figures with all methods employed.

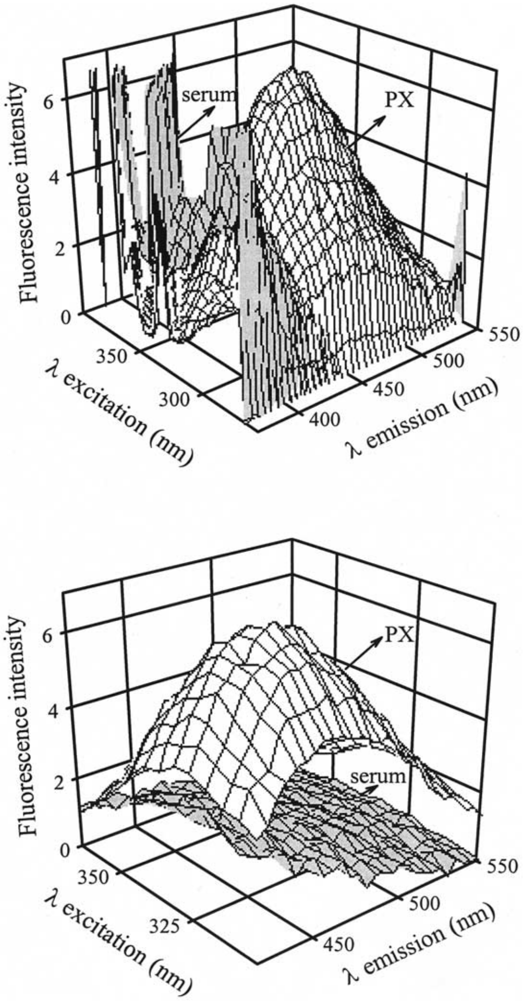

Fig. 2. Three-dimensional plots of the EEFMs for the extracts of a typical serum sample and PX bencenic solution (1 μg ml^{-1}) in their global wavelength ranges (top) and in selected wavelength ranges (bottom).

A summary of validation statistics is shown in Table 2, which demonstrates that, in general, the procedure where the external calibration is used

Table 1
Determination of PX in human serum

Nominal value (μg ml^{-1})	Found (μg ml^{-1})[a]					
	Three-way data				SPE	HPLC
	Standard addition		External calibration			
	PARAFAC	SWATLD	PARAFAC	SWATLD		
1.10	1.2(1) [109]	1.1(1) [100]	0.9(1) [82]	1.1(1) [100]	1.05(6) [95]	1.07
2.48	2.3(1) [93]	2.6(3) [105]	2.0(1) [81]	2.5(1) [101]	2.5(2) [101]	2.52
2.91	2.9(1) [100]	2.9(1) [100]	2.9(1) [100]	3.2(1) [110]	2.97(2) [102]	2.84
4.00	4.0(2) [100]	3.9(3) [98]	3.9(3) [98]	4.0(3) [100]	4.2(2) [105]	4.00
5.51	5.8(2) [105]	5.6(1) [102]	5.3(2) [96]	5.4(2) [98]	5.61(8) [102]	5.22
5.96	6.0(1) [101]	6.0(2) [101]	6.7(3) [112]	5.8(3) [97]	5.6(2) [94]	5.72
6.98	6.7(1) [96]	7.2(3) [103]	7.5(1) [107]	7.0(1) [100]	7.0(2) [100]	7.05
7.69	6.4(1) [83]	7.2(4) [94]	7.5(4) [98]	7.8(4) [101]	7.59(1) [99]	8.01
9.09	8.9(3) [98]	9.1(9) [100]	9.5(2) [105]	8.6(2) [95]	8.9(4) [98]	8.85
10.4	11.7(1) [112]	11.5(1) [110]	10.4(4) [100]	10.4(4) [100]	10.3(1) [99]	9.95
Real sample	3.0(1) [114]	2.7(1) [103]	2.7(1) [103]	2.6(1) [99]	2.5(2) [95]	2.62

[a] Standard deviation (S.D.) in parentheses. Average of three determinations. The recoveries (%) are indicated in square brackets.

shows a better analytical performance. It should be noted that these values correspond to the PX concentration in the experimental solutions (after the serum pre-treatment indicated in Section 2). In order to gain further insight into the accuracy of these methods, linear regression analysis of nominal versus found concentration values by using each procedure was applied. The estimated intercept and slope ($\hat{a}$ and $\hat{b}$, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test [22]. Fig. 3 (split in two plots for clarity) shows the EJCRs for the two employed modes when either PARAFAC or SWATLD are applied. As can be

Table 2
Validation statistical results for the determination of PX by three-way data

	Standard addition		External calibration	
	PARAFAC	SWATLD	PARAFAC	SWATLD
RMSEP (μg ml^{-1})	0.05	0.04	0.03	0.02
REP (%)	11	7	7	4
R^2	0.956	0.981	0.984	0.995
γ (μg^{-1} ml)	209	216	100	192
LOD (μg ml^{-1})	0.03	0.03	0.04	0.04
LOQ (μg ml^{-1})	0.09	0.09	0.12	0.12

RMSEP, root mean square error of prediction $= [\frac{1}{I-1}\Sigma_1^I(c_{act}-c_{pred})^2]^{1/2}$ where, I is the number of samples, c_{act} and c_{pred} are the actual and predicted concentrations, respectively, REP (relative error of prediction) $= \frac{100}{\bar{c}} \times$ RMSEP where $\bar{c}$ is the average component concentration, R^2 (square correlation coefficient) $= 1 - \frac{\Sigma_1^I(c_{act}-c_{pred})^2}{\Sigma_1^I(c_{act}-\bar{c})^2}$, γ (analytical sensitivity) $=$ SEN/$[V(\mathbf{R})]^{1/2}$ where SEN is the sensitivity (estimated as the net analyte signal at unit concentration) and $V(\mathbf{R})$ is the variance of the instrumental signal [26], LOD (limit of detection) = 3.3 $s(0)$ where $s(0)$ is the S.D. in the predicted concentration of PX in a blank sample [27], LOQ (limit of quantification) = 10 $s(0)$.

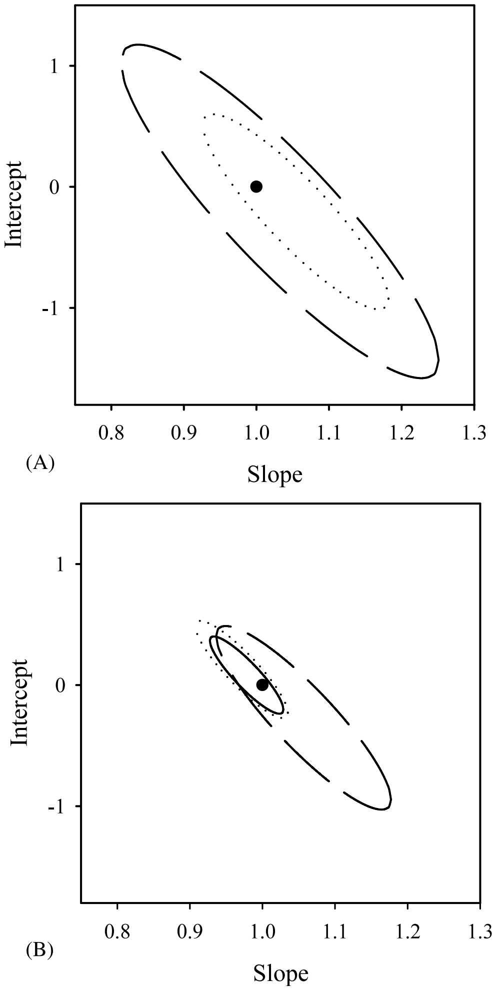

Fig. 3. EJCRs for the slope and intercept corresponding to regressions of predicted vs. actual concentrations of PX applying PARAFAC (dashed line) and SWATLD (dotted line): (A) standard addition; (B) external calibration. The solid line corresponds to the SPE-fluorescence method. The black circles mark the theoretical ($a = 0$, $b = 1$) points.

seen, all EJCR contain the theoretical ($a = 0$, $b = 1$) point, but the elliptic sizes corresponding to the standard addition mode are significantly larger. The conclusion is that although this procedure is statistically accurate, it is however, less precise. From the inspection of Fig. 3 it can be also concluded that the SWATLD algorithm yields better results.

HPLC was used as a reference method (see Table 1) and the obtained values suggest that the method here described and the reference one produce statistically comparable results concerning the content of PX in serum.

3.2. PX fluorescence in solid surface

The retention of PX in a C18AR disk as a function of its ionisation state was previously studied [16]. From this investigation, it could be concluded that in the pH range 3–4, PX is present as the uncharged species (with the zwitterion form predominant over the neutral one) and is completely retained in the membrane. It is important to point out that the sample with the highest concentration of PX (fixed by the upper linear limit) contains about 1 μg of this compound, and does not exceed the extraction capacity of the used membrane (about 1–10% w/w) [23].

Fig. 4 shows the fluorescence spectrum of PX in aqueous solution (pH 3.5) and that obtained from the extraction membrane after 5 ml of diluted serum containing PX were forced to pass through the disk. The comparison of these spectra indicates that: 1) the fluorescence properties of PX do not change when it is retained in a solid surface, 2) the PX emission does not overlap with the background membrane signal and 3) the serum components do not promote significant changes in the fluorescence of PX. The intense fluorescence signal produced from a very diluted PX solution suggests that a very sensitive method can be developed. The analytical figures of merit calculated for PX on the extraction disks were: linear range = 0.02–0.22 μg ml^{-1}, correlation coefficient = 0.999, intercept = 1.64 (0.05), slope = 36.3 (0.4), analytical sensitivity (γ) = 279 μg^{-1} ml, limit of detection (calculated according to Clayton et al. [24] using 0.05 as assurance probabilities) = 0.008 μg ml^{-1}, limit of quantification = 0.02 μg ml^{-1}. The number of data for the calibration curve corresponded to eight different concentration levels, with three replicates for each level ($n = 24$). The linearity for the curve was tested by an ANOVA method [25]. As can be appreciated, the analytical sensitivity (γ) obtained is significantly higher than that for PX in solution determinations [8].

PX was satisfactorily determined in serum using the SPE method (Table 1) and the absence of interference from the matrix was confirmed by the

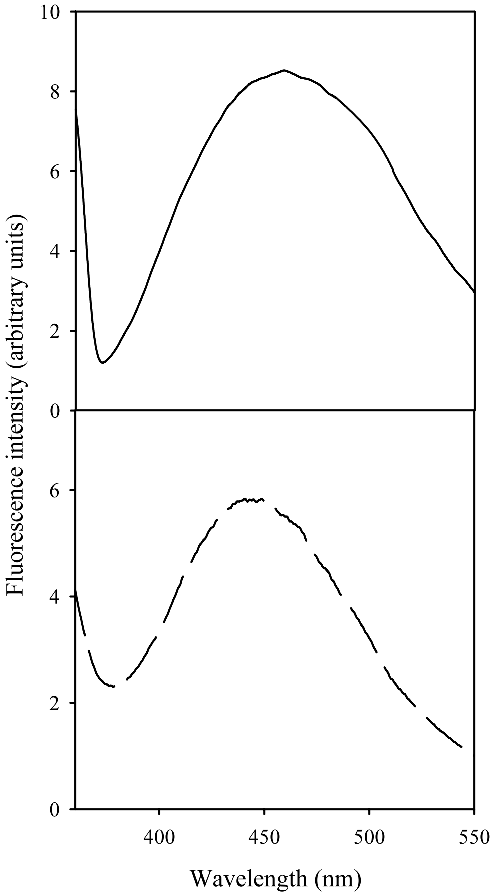

Fig. 4. Fluorescence spectra for an aqueous solution of 1.0 μg ml^{-1} PX at pH 3.5 (solid line, PMT sensitivity = 1000 V) and for 0.12 μg ml^{-1} of PX contained in a diluted serum sample and extracted on a C18AR membrane (dashed line, PMT sensitivity = 300 V), $\lambda_{ex} = 320$ nm.

very good recoveries. These values are also statistically comparable to those obtained by HPLC.

In addition, as can be seen in Fig. 3, the EJCR corresponding to these results contains the theoretical ($a = 0$, $b = 1$) point and the ellipse has a small size. A fact which should be remarked is that excellent results are obtained without the need of extracting PX with organic solvents or performing serum deproteinisation.

3.3. Comparison of methods and real sample evaluation

In comparing the accuracies of the investigated methods for determining PX in serum from the EJCR test, one may note that the chemometric results are as satisfactory as those furnished by SPE-fluorescence analysis. In general, however, better figures of merit are obtained when SPE-fluorescence data are applied. Further, from the experimental point of view, the use of the latter method is significantly simpler to implement than the three-way calibration. A point that should be taken into account by the analyst is the possible presence in serum of analytes, which could be retained by the disk at the working pH. If they emit fluorescence signal in the solid surface, then it may be possible that this emission interferes with the PX signal. Although these facts can be considered as a potential limitation in the applicability of the method, it is unlikely that they will simultaneously occur.

Concerning the three-way method, it was demonstrated, as in previous works, that it is an excellent tool for the determination of analytes in complex matrices. However, it cannot be applied when the data loose the trilinear property, because in this case the cube decomposition is no longer unique [14].

Finally, with the purpose of evaluating the proposed methods in a real sample, the serum of a volunteer administered with PX was investigated. The values obtained (average of three replicates) by both methods (Table 1) are statistically comparable to that obtained with the HPLC reference one. These results suggest that the presence of the PX metabolites in serum cause no serious interference when applying the proposed methods.

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