

Optimization of Verapamil Drug Analysis by Excitation-Emission Fluorescence in Combination with Second-order Multivariate Calibration

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Abstract Excitation emission fluorescence matrices (EEMs) of Verapamil drug were obtained by direct and by derivatization fluorescence spectroscopy. The fluorescence excitation and emission wavelengths were displaced to longer wavelengths and the fluorescence intensity was enhanced upon derivatization with respect to the native fluorescence of the drug. The complete EEM of the native fluorescence of the drug and of the derivatization product were rapidly acquired by using a charged-coupled device detector (CCD), which is advantageous in terms of speed in the analysis, with respect to the use of a conventional photomultiplier detector. The EEMs were analyzed by several second-order multivariate calibration methods exploiting the second order advantage. The three-dimensional decomposition methods used, based in different assumptions about the trilinearity of the three

way data structure under analysis, were parallel factor analysis (PARAFAC), bilinear least squares (BLLS), parallel factor analysis 2 (PARAFAC2) and multivariate curve resolution—alternating least squares (MCR-ALS). The determination was performed by using the standard addition approach. The figures of merit of the PARAFAC and BLLS methods were calculated, obtaining a lower limit of detection with the derivatization procedure, when compared with the direct measurement of the fluorescence of the drug. In Verapamil drug the best estimations were found with the BLLS and the MCR-ALS models. In the quantification of Verapamil in a pharmaceutical formulation the best estimation, when compared with the result obtained by the US Pharmacopeia high performance liquid chromatography approach, was obtained by direct fluorescence spectroscopy with MCR-ALS and by derivatization fluorescence spectroscopy with the PARAFAC2 model.

Keywords Verapamil drug · Pharmaceutical formulation · Direct and derivatization fluorescence EEM · PARAFAC · BLLS · PARAFAC2 · MCR-ALS

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Introduction

Spectrofluorimetry is a well known analytical technique used as a quantitative tool in several areas, such as chemistry, medicine, environmental and food science, owing to its intrinsic sensitivity, easiness of use and availability of instruments [1, 2]. Nevertheless, traditional fluorescence spectra are broad and featureless and analyte spectra are often irresolvable from interferent spectra or background fluorescence. In consequence, the analysis of multi-component

or complex samples can be hindered when the measurements are carried out in only one excitation or emission wavelength, and, in these cases, high order data are particularly suitable.

Nowadays, the availability of high-order instrumental data, coupled to newly chemometric algorithms for data processing, is increasing the regular use of multi-way analysis for complex analytical problems [3, 4]. Second order, i.e., matrix data for a given sample, can be produced in a variety of ways, among which one of the simpler is an excitation-emission matrix (EEM). It is well known that molecular fluorescence generates multidimensional excitation-emission spectral data per sample and that the recording of this type of data conveys the advantage of been carried out in a single instrument. EEMs present simultaneously all the excitation and emission spectra of one sample, and can be defined as a three dimensional plot with the excitation and emission wavelengths and corresponding intensities as *xyz* axes. Alternatively, EEMs can be represented as a numerical data matrix of the fluorescence intensities as function of the excitation and emission wavelengths. Because EEMs contain a lot of information about the sample under analysis they are particularly useful for the analysis of complex mixtures [2]. Examples on the use of second-order spectrofluorimetric data to analyze complex samples can be found in recent reports [4, 5].

A drawback for the use of the full potential of the information content in the EEMs is the time needed to obtain one data matrix, if a conventional photomultiplier detection system is used. Nowadays, a solution to this problem is the use of a spectrofluorimeter equipped with a charged-coupled device detector (CCD), that allows fast acquisition of full EEMs [1, 2]. In consequence, the outlined advances in molecular fluorescence instrumentation and chemometrics three-dimensional decomposition methods make feasible the use of multi-parametric determinations to achieve selective measurements [3, 4]. The rapid acquisition, coupled to the use of three-dimensional decomposition methods, gives these analytical methodologies a great potential for their application on rotinary quality analytical control [5].

However, fluorescence based analytical measurements may have some limitations due to: chemical interferences; Rayleigh or Raman scattering; overlapped fluorescence bands; fluorescence background or instrumental noise [1, 2, 6]. Nevertheless, fluorescence is inherently linear in all dimensions [7]. An excitation-emission matrix consists of a trilinear part plus some random (noise) and minor deviations from the trilinearity due to scattering and other chemical and/or instrumental interferences [8]. The latter are also called three-way data, and are characterized by following the trilinear or PARAFAC model. Interestingly, the decomposition of a three-dimensional cube of data is

often unique. This is due to the fact that three-way data are not merely a collection of two-way data sets, but there is actually an internal relationship between each of the matrices. As a consequence, the decomposition of a three-way data array built with response matrices measured for a number of samples, allows emission and excitation spectral profiles, as well as relative concentrations of individual sample components to be extracted directly. If the model is correctly specified with the right number of components, and the trilinear structure is approximately valid, then the PARAFAC solution will provide estimates of the underlying parameters. This property has been named the second-order advantage [9]. Hence, the combination of EEMs with multi-way calibration methods, achieving the second-order advantage, makes possible the correct modeling of the data in the presence of possible chemical and/ or instrumental interferences, and can minimize or eliminate traditionally time-consuming sample pretreatments.

Verapamil is a calcium channel blocker widely used as an antihypertensive, antianginal and antiarrhythmic drug [10]. When excited with ultraviolet light exhibits fluorescence in different solvents. In a previous work, the adequate quantification of Verapamil drug, by measuring the direct native fluorescence of the drug, using a spectrofluorimeter with a photomultiplier detector, was demonstrated to be feasible by PARAFAC calibration [11].

It has been demonstrated that, by catalysis of the tertiary amino group present in his molecule, by means of the reaction of malonic acid with acetic anhydride, a fluorescence derivate is obtained at higher wavelengths than the intrinsic fluorescence of Verapamil. In this work, several basic objectives were pretended, a substantial increment of the speed of the analysis, better chemical conditions to perform the Verapamil determination and the use of several second-order multivariate calibration algorithms, allowing an enhancement of the prediction results. In this context, EEMs obtained by direct and by derivatization fluorescence, using a spectrofluorimeter with a CCD detector, will be analyzed with three-dimensional decomposition methods, namely: parallel factor analysis (PARAFAC); bilinear least squares (BLLS); parallel factor analysis 2 (PARAFAC2) and multivariate curve resolution-alternating least squares (MCR-ALS).

The PARAFAC [12, 13] and BLLS [14, 15] models deal with trilinear three-way data, PARAFAC2 model [16, 17] handle with small deviations from trilinearity in one of the dimensions and with different rows (or columns) dimensions of the slices in the three-way data and MCR-ALS model [18] deals with non trilinear three-way data. The recently introduced BLLS algorithm, is an alternative to PARAFAC, also exploiting the second-order advantage. These chemometric methods are being used in analytical methodologies coupled to EEM measurements, namely:

environmental studies [19–28]; clinical analysis [11, 29–32]; and food research [33, 34].

The analytical performance of PARAFAC, BLLS, PARAFAC2 and MCR-ALS, to quantify Verapamil drug, from EEMs obtained by direct and by derivatization fluorescence spectroscopy, will be compared using standard solutions and one pharmaceutical formulation. Also, the results obtained will be compared with those obtained by a reference US Pharmacopeia high performance liquid chromatography with ultraviolet detection method (USP HPLC-UV).

Experimental

Reagents and solutions

Verapamil reference standard and Malonic acid (both $\geq 99\%$ purity) were obtained from Sigma-Aldrich Química S.A. (Spain). Anhydride acetic, methanol and ethanol were purchased from Merck, Darmstadt (Germany). Deionised water with resistivity higher than $4 \text{ M}\Omega/\text{cm}$ was used. The pharmaceutical formulation under analysis was Isoptin[®] injectable solution (5 mg/2 ml) from Knoll.

The Verapamil standard stock solutions were prepared in methanol (direct fluorescence spectroscopy) or in ethanol (derivatization fluorescence spectroscopy) by rigorous weighting. For the preparation of the pharmaceutical formulation stock solution the volume of a vial was rigorously diluted to obtain the required concentration.

Procedures

For Verapamil direct fluorescence spectroscopy the standard concentrations were obtained using successive three 10 μl additions of a 750 ppm standard solution to 3 ml of a 5 ppm standard or sample solution contained in a 1 cm quartz cell - to obtain concentrations increments of 2.5, 5 and 7.5 ppm.

For Verapamil derivatization fluorescence spectroscopy the dilution of the 1,000 ppm Verapamil standard or sample solution to an intermediate 10 ppm solution was done in a way as to obtain the fluorescent derivatization product. For a final volume of 10 ml the dilution was done after solvent evaporation, addition of 2 ml malonic acid reagent 0.5%, promotion of the reaction at 100°C during 5 min followed by dilution to 10 ml. The concentration of the diluted standard or sample solutions of the fluorescent derivatization product in 5.00 ml glass volumetric flasks was 0.3 ppm. As for the Verapamil direct fluorescence spectroscopy three additions of the 10 ppm standard solution were done in order to obtain standard concentrations of 0.1, 0.3, and 0.5 ppm.

For the chromatographic quantification the mobile phase, standard and sample solutions were prepared following the USP recommendations [10]. Standards were prepared in the mobile phase by rigorous weighting to a final concentration of 2,000 ppm. Diluted standards and sample solutions were obtained by dilution with methanol to the required concentrations. Standard solutions in the range from 50 to 500 ppm and an expected concentration of 250 ppm for the pharmaceutical formulation sample solution were used.

Instrumentation

A Spex 3D Spectrofluorimeter with a 75 W xenon lamp with a CCD detector was used. EEM were acquired, in an excitation wavelength range from 250 to 550 nm, and in an emission wavelength range from 250 to 710 nm, with a resolution of 5 nm and one accumulation. For the direct fluorescence measurements, an integration time of 18 s (slits with 0.05 mm) was used and, for the derivatization fluorescent measurements, an integration time of 0.4 s (slits with 0.1 mm) were used.

The HPLC determinations were made with a system constituted by a HP1100 isocratic pump, a manual injection valve Rheodyne, model 7752i, a Rheodyne 20 μL loop, a pre-column Agilent (20 \times 4 mm) and a Supelco column (100 \times 4.6 mm) Hypersil[®] ODS of 3 μm particle diameter and a diode array detector (DAD) Ati Unicam Crystal 250 model. The DAD spectra were acquired in a wavelength range from 200 to 452 nm with a resolution of 2 nm. The wavelength used for the quantification was 280 nm.

Chemometric analysis

The matrices of experimental data points [excitation (nm) \times emission (nm)] obtained at different standard concentrations were analyzed using the PARAFAC, BLLS, PARAFAC2 and MCR-ALS and implemented in MATLAB code. The PARAFAC, PARAFAC2 and MCR-ALS estimations are found by an iterative alternating least squares procedure. As convergence criteria a value of 1×10^{-6} , 1×10^{-7} and 1×10^{-3} and a maximum number of iterations 2,500, 2,000 and 50 were used for PARAFAC, PARAFAC2 and MCR-ALS, respectively. The initial estimates used for the PARAFAC, PARAFAC2 and the MCR-ALS were the estimates of a model without constraints. The BLLS estimations are found by a direct least-squares procedure. No initialization or constraint procedures are required.

For the analysis with PARAFAC and BLLS the data sets are structured as [excitation (nm) \times emission (nm) \times concentration], for the analysis with PARAFAC2 the data sets are structured as [emission (nm) \times excitation (nm) \times concentration] and for the analysis with MCR-ALS as

[(concentration × emission (nm)) × excitation (nm)]. Except for PARAFAC2 on the second dimension, non-negativity constraints are applied in all the dimensions of the Verapamil drug matrices analyzed.

Raw EEMs were reduced for chemometric analysis (Fig. 1). EEMs obtained by direct spectroscopy were reduced for an excitation wavelength range from 251.70 to 301.83 nm (10 wavelengths) and an emission wavelength range from 252.27 to 548.65 nm (58 wavelengths). EEMs obtained by derivatization spectroscopy were reduced for an excitation wavelength range from 251.70 to 452.23 nm (37 wavelengths) and an emission wavelength range from 397.86 to 600.64 nm (40 wavelengths).

All routines employed to carry out the calculations described in this paper were written in MATLAB [35]. Those for applying PARAFAC and PARAFAC2 models are available from <http://www.models.kvl.dk/source/>

[nwaytoolbox/](http://www.nwaytoolbox/) and the MCR-ALS program is available from <http://www.ub.es/gesq/mcr/>. For BLLS and for the calculation of the figures of merit with both PARAFAC and BLLS models, the MVC-2 routine, available from the authors on request, was used, including a useful graphical interface for data input and parameter settings, of the type already described for first order calibration [36].

Multi-way model fit analysis

The results obtained from the different PARAFAC, BLLS, PARAFAC2 and MCR-ALS models were compared using the model fit [Fit (%)]. The model fit (%) for PARAFAC and PARAFAC2 models is defined by Eq. 1.

$$\text{Fit}(\%) = 100 \times \left(1 - \frac{\sum_{i=1}^I \sum_{j=1}^J \sum_{k=1}^K (x_{ijk} - \hat{x}_{ijk})^2}{\sum_{i=1}^I \sum_{j=1}^J \sum_{k=1}^K (x_{ijk})^2} \right) \quad (1)$$

In this equation \hat{x}_{ijk} is the ijk element of the estimated three-dimensional matrix and x_{ijk} is the ijk element of the experimental three-dimensional matrix. The model fit (%) for MCR-ALS models is defined by Eq. 2.

$$\text{Fit}(\%) = 100 \times \left(1 - \frac{\sum_{l=1}^L \sum_{j=1}^J (x_{lj} - \hat{x}_{lj})^2}{\sum_{l=1}^L \sum_{j=1}^J (x_{lj})^2} \right) \quad (2)$$

In this equation \hat{x}_{lj} is the lj element of the estimated bidimensional column-wise augmented data matrix and x_{lj} is the lj element of the experimental bidimensional column-wise augmented data matrix with $l=k \times i$.

Also, the results obtained with PARAFAC models were assessed using the CORCONDIA or core consistency test [Corcondia(%)] defined by Eq. 3 [8, 12].

$$\text{Corcondia}(\%) = 100 \times \left(1 - \frac{\sum_{d=1}^N \sum_{e=1}^N \sum_{f=1}^N (g_{def} - t_{def})^2}{\sum_{d=1}^N \sum_{e=1}^N \sum_{f=1}^N (t_{def})^2} \right) \quad (3)$$

In this equation g_{efg} and t_{efg} denote the elements of the calculated core and of the intrinsic super-diagonal core, respectively, and N the number of components of the model. If they are equal, the core consistency is perfect and has a value of unity (100%). The appropriate number of components is accessed with the model with the highest number of components and a valid value of core consistency diagnostic test. Usually, the number of components is

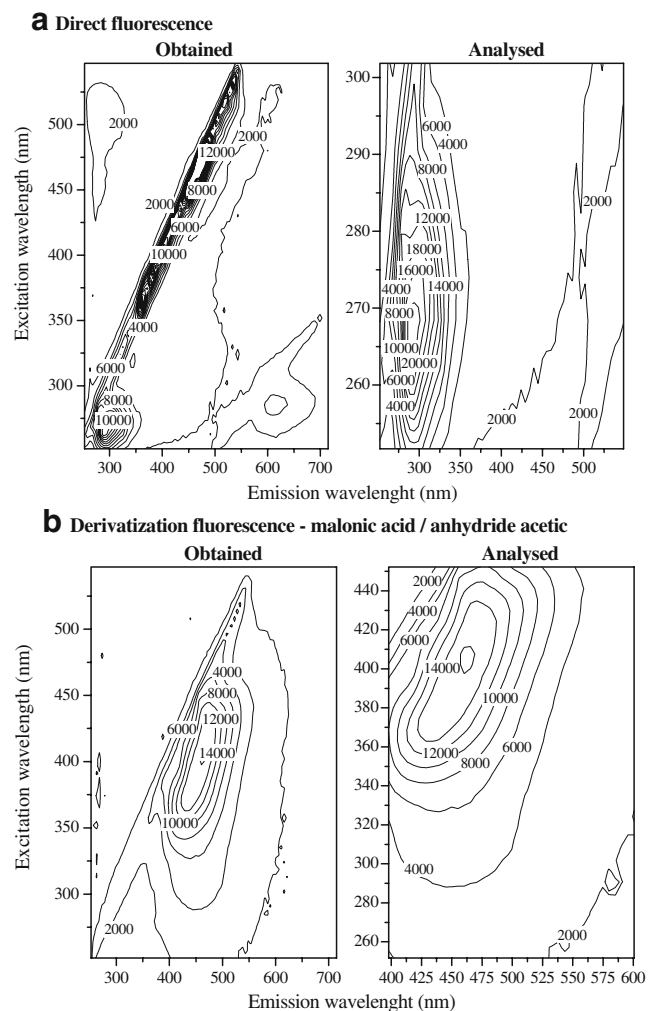


Fig. 1 Raw and analysed Verapamil drug EEM. Obtained in **a** by direct fluorescence (4.98 ppm) and in **b** by derivatization fluorescence spectroscopy (0.30 ppm)

taken as the largest number for which the latter parameter is larger than around 50.

The results obtained with MCR-ALS models were assessed using the lack of fit [LOF(%)] of the MCR-ALS model defined by Eq. 4 [18].

$$LOF(\%) = 100 \times \left(\sqrt{\frac{\sum_{l=1}^L \sum_{j=1}^J (x_{lj} - \hat{x}_{lj})^2}{\sum_{l=1}^L \sum_{j=1}^J (x_{lj})^2}} \right) \quad (4)$$

In this equation \hat{x}_{lj} is the lj element of the estimated bidimensional column-wise augmented data matrix by the MCR-ALS model and x_{lj} is the lj element of the experimental column-wise augmented data matrix in the $LOF_{MCR \text{ vs } Exp}$ calculus and of the estimated with principal component analysis in the $LOF_{MCR \text{ vs } PCA}$ calculus.

Figures of merit

For both linear models, PARAFAC and BLLS, the figures of merit were also evaluated. Two important sensitivity equations have been discussed in the literature, both based on extensions of Lorber’s net analyte signal (NAS) theory [37] to the second-order domain. More useful equations are obtained by considering that the various high-order chemometric methods provide estimated component profiles in the different dimensions. In the present context, a useful sensitivity parameter is the so-called MKL (after Messick et al. [38]) sensitivity for constituent n , whose mathematical expression as a function of the component profiles, is [39]:

$$SEN_{n, MKL} = k_n \left\{ [(\mathbf{B}^T \mathbf{B}) * (\mathbf{C}^T \mathbf{C})]^{-1} \right\}_{nn}^{-1/2} \quad (5)$$

where \mathbf{B} and \mathbf{C} are matrices containing, as columns, the profiles for all components in the first and second dimension respectively (all normalized to unit length), ‘ nn ’ designates the (n,n) element of a matrix, k_n is the total signal for component n at unit concentration, and the symbol ‘ $*$ ’ is the element-wise Hadamard product matrix. This MKL approach to NAS has already been rigorously

proved to be connected to cases where the second-order advantage is not exploited, for example, when employing PARAFAC [8], or BLLS with the so-called least-squares predictor [40].

In Eq. 5, the profiles are provided with three-way decomposition using the appropriate algorithm, while the value of k_n is also computed by the algorithm, using information on the nominal calibration concentrations for component n .

The selectivity is obtained by dividing the sensitivity by the total signal for the pure component, and therefore a suitable expression is [14]:

$$SEL_{n, MKL} = \left\{ [(\mathbf{B}^T \mathbf{B}) * (\mathbf{C}^T \mathbf{C})]^{-1} \right\}_{nn}^{-1/2} \quad (6)$$

More useful than the sensitivity appears to be the analytical sensitivity γ_n , defined, in analogy with univariate calibration, as the quotient between the sensitivity and the instrumental noise level. Its inverse establishes the minimum concentration difference which can be appreciated across the linear range, and is independent on instrument or scale [41].

The limit of detection (LOD_n) should also be considered. An approximation to the LOD_n can be gathered from the expression:

$$LOD_n = 3s_X / SEN_n \quad (7)$$

where s_X is an estimate of the instrumental noise level.

Results and discussion

Figure 1 shows typical raw and reduced EEMs obtained with direct and derivatization fluorescence spectroscopy of Verapamil. The analysis of this figure shows that both EEMs contain first order scattering which interferes with the main fluorescence band. In the EEM obtained by direct fluorescence it is also possible to verify the existence of the minor second order scattering bands. In the EEM obtained by derivatization fluorescence spectroscopy there is a shift towards higher wavelengths of the excitation and emission

Table 1 Explained variance of the first five components obtained by SVD

Principal component	Direct fluorescence		Derivatization fluorescence	
	Verapamil, 5 ppm	Four standards [exc. × (conc. × emi.)]	Verapamil, 0.3 ppm	Four standards [exc. × (conc. × emi.)]
1	97.87	98.55	93.63	90.54
2	1.30	0.87	5.34	8.09
3	0.50	0.43	0.88	1.20
4	0.18	0.07	0.07	0.11
5	0.08	0.04	0.04	0.04

[exc. × (conc. × emi.)] Matrix row augmented in which the rows are the excitation and the columns are the emission spectra obtain in the four concentrations levels

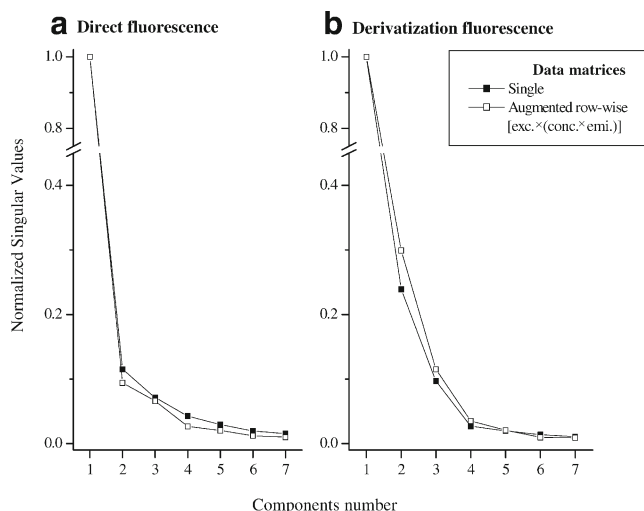


Fig. 2 Singular value decomposition of single and augmented row-wise data matrices of Verapamil drug obtain in **a** by direct fluorescence (4.98 ppm) and in **b** by derivatization fluorescence spectroscopy (0.30 ppm)

bands. Indeed, derivatization with malonic acid/anhydride acetic provokes a displacement of the excitation maximum from 375 to 425 nm and a displacement of the emission maximum from 425 to 475 nm.

The strategy used in this work to reduce the effect of the scatter bands was to eliminate the section of the EEMs that contain them. However, it was not possible to separate completely the scatter bands from the main fluorescence bands—this is particular critical in the direct fluorescence EEMs. This information must be accounted for when the number of linear independent factors present in the EEMs are estimated.

Interestingly, it is clear from Fig. 1 that the derivatization approach is advantageous over the direct approach, as the excitation and emission maxima of the derivatized product are more separated from the scatter bands than in the direct procedure.

Singular value decomposition (SVD)

Table 1 and Fig. 2 show the results of the SVD of the single and augmented row-wise [excitation × (concentration × emission)] data matrices. In Fig. 2, for an easier comparison, normalized singular values for the single and augmented row-wise matrix are used.

From the analysis of the explained variance of the singular values of the augmented EEM (Table 1) it is shown

Table 2 Comparison of the results obtained by the PARAFAC, BLS, PARAFAC2 and MCR-ALS models in the analysis of a Verapamil drug EEM (4.98 ppm) obtained by direct fluorescence spectroscopy

Model evaluation	Number of components				
	Two	Three	Four	Five	Six
Fluorescence at maximum fluorescence intensity (excitation 263 nm and emission 289 nm)					
Estimated concentration (ppm)=5.71 (0.06), Recovery=114.7%					
PARAFAC					
Estimated concentration (ppm)	4.83 (0.21)	4.94 (0.20)	5.40 (0.10)	5.46 (0.13)	5.44 (0.11)
Recovery (%)	97.0	99.2	108.4	109.6	109.2
Fit (%)	91.7	94.8	95.8	93.6	94.5
Number of Iterations	38	120	686	2500	2500
Corcondia (%)	98.1	-0.3	4.6	11.4	40.6
BLS					
Estimated concentration (ppm)	4.97 (0.11)	4.97 (0.068)	5.21 (0.064)	8.31 (0.95)	5.00 (0.072)
Recovery (%)	99.8	99.8	104.6	166.9	100.4
Number of Iterations	89	96	384	6614	5374
PARAFAC2					
Estimated concentration (ppm)	6.02 (0.05)	4.76 (0.25)	8.74 (1.45)	9.11 (1.46)	5.33 (0.21)
Recovery (%)	120.9	95.5	175.5	182.9	107.0
Fit (%)	91.7	94.9	96.1	96.9	97.7
Number of Iterations	2000	755	2000	2000	2000
MCR-ALS					
Estimated concentration (ppm)	4.99 (0.22)	4.82 (0.15)	4.82 (0.14)	5.18 (0.21)	4.84 (0.21)
Recovery (%)	100.2	96.8	96.8	104.0	97.2
Fit (%)	92.4	96.2	97.2	98.0	98.5
Number of Iterations	50	50	50	50	50
LOF _{MCRvs.PCA} (%)	0.02	0.03	0.18	0.55	0.55
LOF _{MCRvsExp.} (%)	7.59	3.81	2.77	1.96	1.55

For the estimated concentration the values in brackets are the standard deviation of the extrapolated concentration

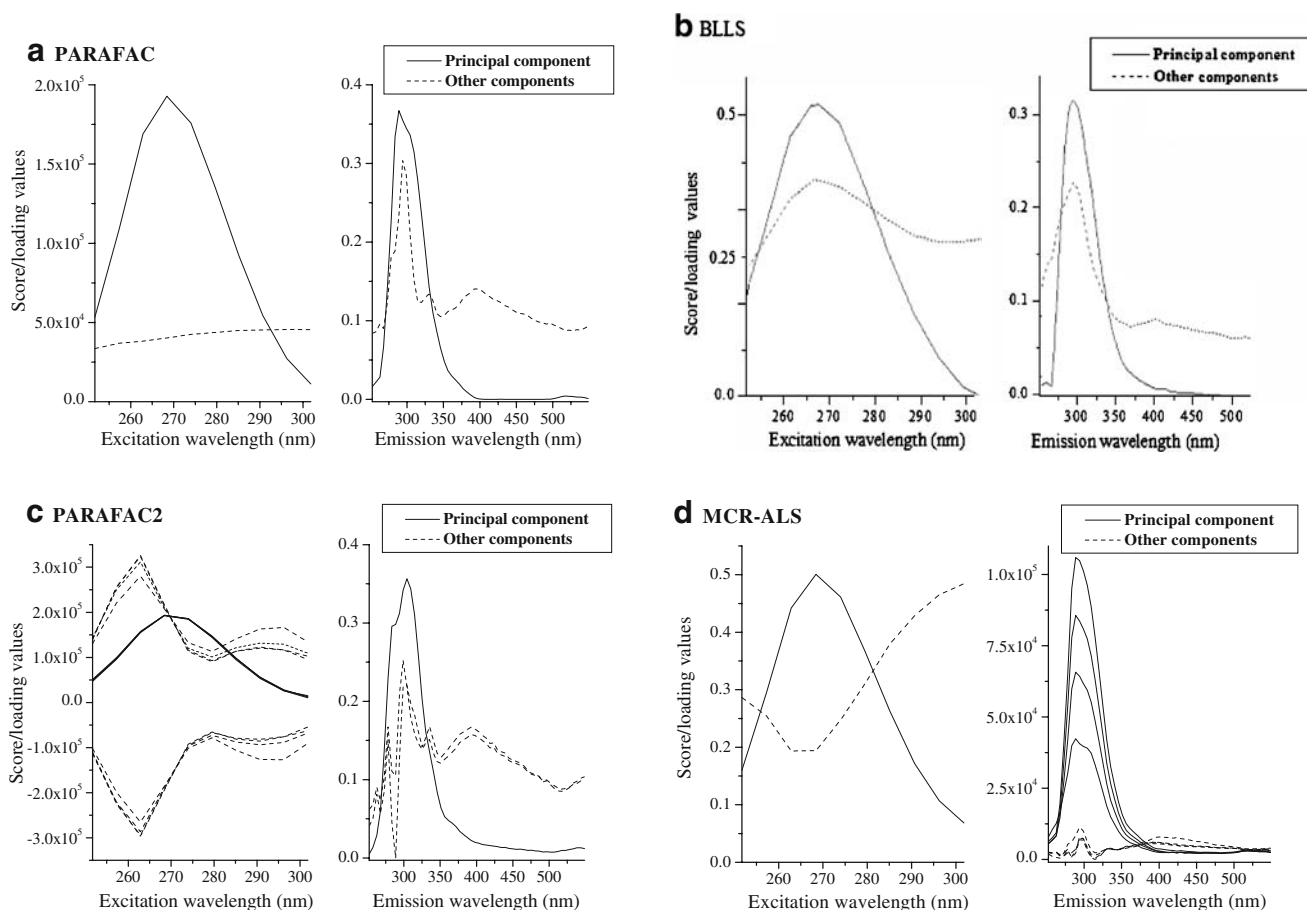


Fig. 3 Excitation and emission spectra estimated from the analysis of the Verapamil drug EEM obtained by direct fluorescence: **a** two components non-negativity constraint PARAFAC model, **b** one component and one interferent BLS model, **c** three components

non-negativity constraint in the first and third dimensions PARAFAC2 model and **d** two components non-negativity constraint MCR-ALS model

that two components explain almost 99% of variance. From the analysis of the normalized singular values of the single and augmented row-wise matrices (Fig. 2), a minimum of two and a maximum of five components may be expected for the single and augmented data matrices of the direct and derivatization EEM. It is known that, when there is scattering superimposed with the fluorescence signal, several minor components appear to explain the spectral variance. Taking into consideration this information, these numbers of components will be considered in the subsequent analysis by PARAFAC, BLS, PARAFAC2 and MCR-ALS.

Three-dimensional decomposition analysis

Direct fluorescence spectroscopy

Table 2 presents the results obtained using the fluorescence intensities at the maximum emission (excitation at 263 nm and emission at 289 nm) and with PARAFAC, BLS,

PARAFAC2 and MCR-ALS modelling of the direct EEM. The analysis of Table 2 shows that readings at maximum fluorescence intensity overestimates the concentration of Verapamil (recovery=115%) and only multivariate models allow good concentrations estimations.

The fit achieved with MCR-ALS are always higher than the fit obtained with PARAFAC and PARAFAC2. Similar

Table 3 Comparison of the figures of merit calculated for the PARAFAC and BLS models at the number of components indicated obtained by direct fluorescence spectroscopy

	PARAFAC	BLS
Number of components	2	2
Sensitivity (SEN) AFU (ml μg^{-1})	8,650	4,937
Analytical sensitivity (γ) (ml μg^{-1})	10.0	8.6
γ^{-1} ($\mu\text{g ml}^{-1}$)	0.10	0.11
Selectivity (SEL)	0.42	0.23
Limit of detection (LOD) ($\mu\text{g ml}^{-1}$)	0.30	0.38

AFU Arbitrary fluorescence units

Table 4 Comparison of the results obtained by the PARAFAC, BLS, PARAFAC2 and MCR-ALS models in the analysis of a Verapamil drug EEM (0.30 ppm) obtained by derivatization fluorescence spectroscopy

Model evaluation	Number of components				
	Two	Three	Four	Five	Six
Fluorescence at maximum fluorescence intensity (excitation 408 nm and emission 466 nm)					
Estimated concentration (ppm)=0.33 (0.06), recovery=110.0%					
PARAFAC					
Estimated concentration (ppm)	0.37 (0.04)	0.28 (0.04)	0.27 (0.05)	0.24 (0.05)	0.31 (0.09)
Recovery (%)	123.3	93.3	90.0	80.0	103.3
Fit (%)	87.6	93.9	96.5	97.2	97.9
Number of Iterations	188	246	364	360	1592
Corcondia (%)	87.6	32.9	26.9	92.6	2.5
BLS					
Estimated concentration (ppm)	0.29 (0.08)	0.30 (0.02)	0.32 (0.02)	0.33 (0.03)	0.42 (0.26)
Recovery (%)	96.7	100.0	106.7	110.0	140.0
Number of Iterations	44	40	192	819	4385
PARAFAC 2					
Estimated concentration (ppm)	0.288 (0.05)	0.284 (0.05)	0.259 (0.03)	0.327 (0.06)	0.298 (0.04)
Recovery (%)	96.0	94.7	86.3	109.0	99.3
Fit (%)	88.5	96.1	97.7	98.3	98.7
Number of Iterations	758	2,000	2,000	2,000	2,000
MCR-ALS					
Estimated concentration (ppm)	0.35 (0.03)	0.39 (0.04)	0.30 (0.03)	0.30 (0.03)	0.29 (0.03)
Recovery (%)	116.7	130.0	100.0	100.0	96.7
Fit (%)	88.1	95.5	97.4	98.2	98.3
Number of Iterations	21	50	50	50	21
Lo _f _{MCRvs.PCA} (%)	1.8	1.6	0.6	0.7	1.1
Lo _f _{MCRvsExp.} (%)	11.8	4.5	2.6	1.7	1.7

See footnote of Table 2

model fits were found for the PARAFAC, and PARAFAC2 from two to four components. However, the analysis of the PARAFAC CORCONDIA test shows that a two component model is the most appropriate, i.e. it corresponds to the highest value (98.1%), before the Core consistency value is lower than 50%. The analysis of the lack of fit of the MCR-ALS shows that a two and three component models originates relatively low values for Lo_f_{MCRvs.PCA} (0.02% and 0.03%, respectively).

With a two component model, as suggested by the CORCONDIA test, PARAFAC, and MCR-ALS are adequate to model the Verapamil drug EEM obtained by direct fluorescence. With these models, the predicted concentration and described recoveries of the expected Verapamil concentration (4.98 ppm) were: 4.83 and 97.0% with PARAFAC and 4.99 ppm and 100.2% with MCR-ALS. A slightly lower Verapamil concentration estimation of 4.82 ppm and a recovery of 96.8% was found with a three component MCR-ALS model. For two components PARAFAC2 model the predicted concentration was not good and only a three component model allows to obtain recovery of 95.6%. When BLS was applied, taken into account one com-

ponent and one interferent in the system, an estimation of 4.97 ppm and a recovery of 99.8% were found, on the same order of magnitude than MCR-ALS. A similar result was found with one component and two interferents in the system. Nevertheless, the best estimation was found with MCR-ALS.

The Fig. 3 presents the excitation and emission spectra estimated with the models that allows the best Verapamil concentration estimation: (a) with a two components non negativity constraint PARAFAC model; (b) with a one component and one interferent system BLS model; (c) with a three components non negativity constraint in the first and third dimensions PARAFAC2 model; (d) with a two components non negativity constraint MCR-ALS model. For all the models two components correspond to the Verapamil intrinsic fluorescent and to a background signal. For the PARAFAC2 model the other component correspond to scatter bands and background signal.

The figures of merit calculated for the PARAFAC and BLS models are shown in Table 3. By the analysis of this table it is possible to observe that similar results were found for the figures of merit by the two models. Nevertheless a

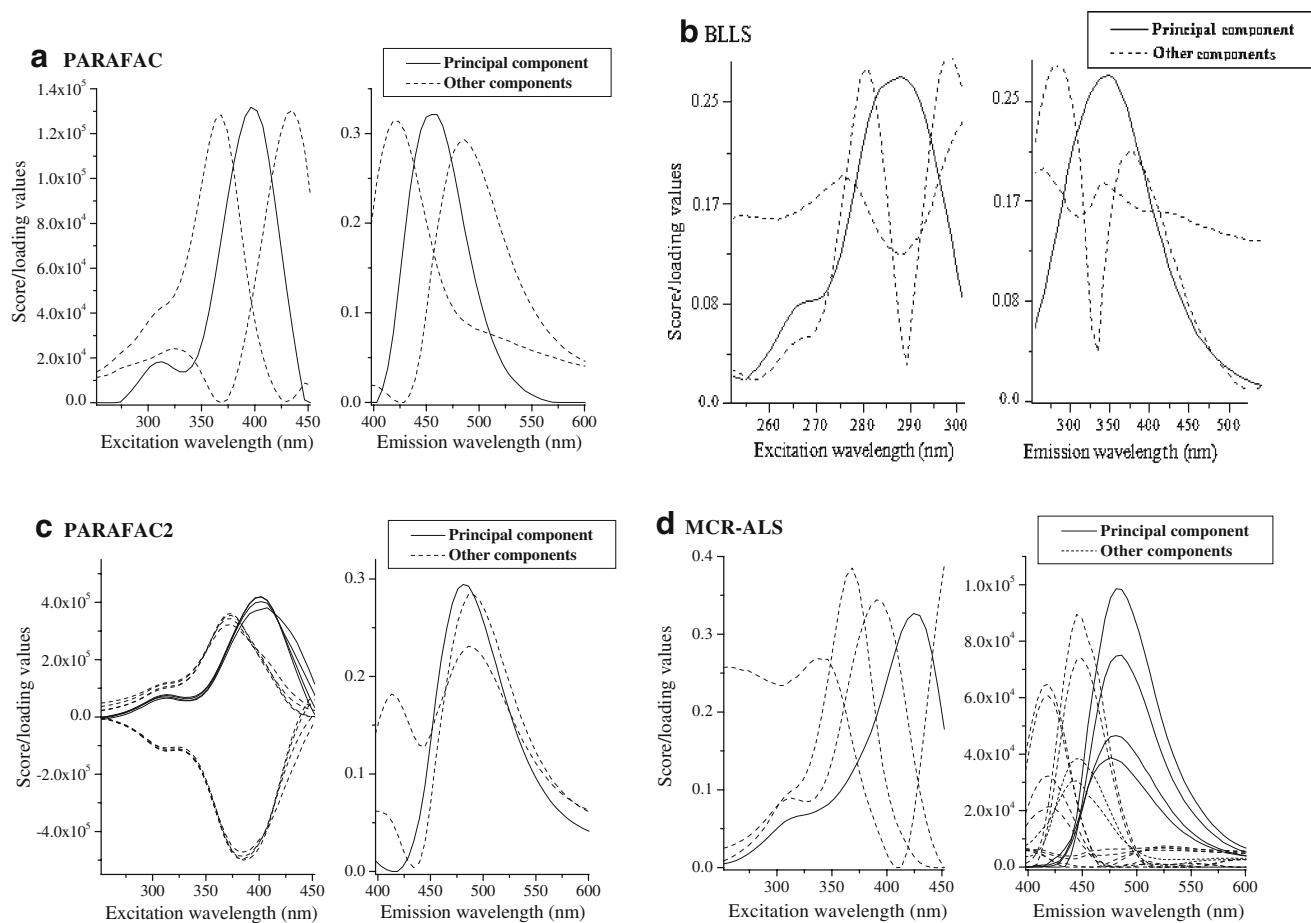


Fig. 4 Excitation and emission spectra estimated from the analysis of the Verapamil drug EEM obtained by derivatization fluorescence: **a** three components non-negativity constraint PARAFAC model, **b** one component and two interferents BLLS model, **c** three components

non-negativity constraint in the first and third dimensions PARAFAC2 model and **d** four components non-negativity constraint MCR-ALS model

greater sensitivity and selectivity with a slight lower LOD were found with the PARAFAC model.

Derivatization fluorescence spectroscopy

Table 4 presents the results obtained using the fluorescence intensities at the maximum emission (excitation 408 nm and emission 466 nm) and with PARAFAC, BLLS, PARAFAC2 and MCR-ALS modelling of the Verapamil drug EEM obtained by derivatization fluorescence spectroscopy. The analysis of Table 2 shows that readings at maximum fluorescence intensity overestimates the concentration of Verapamil (recovery=110%) and only multi-variate models allow good concentrations estimations.

Similar models fits are achieved with the four methods, although PARAFAC originates a slightly lower fit. If the analysis of the PARAFAC CORCONDIA test is strictly applied, a two component model seems the most appropriate, i.e. it corresponds to the highest value (87.6%) before the core consistency drops to a value lower than 50. However,

the core consistency values of the three component model, 32.9, and five component model of 92.6, are indicating that more than two components may also be appropriated. The analysis of the lack of fit of the MCR-ALS models shows that with a four component model, relatively low values are obtained for $LoF_{MCRvs.PCA}=0.6\%$.

Table 5 Comparison of the merit figures calculated for the PARAFAC and BLLS models at the number of components indicated obtained by derivatization fluorescence spectroscopy

	PARAFAC	BLLS
Number of components	3	3
Sensitivity (SEN) AFU (ml μg^{-1})	140,000	198,839
Analytical sensitivity (γ) (ml μg^{-1})	183	652
γ^{-1} ($\mu\text{g ml}^{-1}$)	0.005	0.002
Selectivity (SEL)	0.37	0.34
Limit of detection (LOD) $\mu\text{g ml}^{-1}$	0.02	0.02

AFU Arbitrary fluorescence units

Table 6 Comparison of the results obtained by the PARAFAC, BLLS, PARAFAC2 and MCR-ALS models in the analysis of a Verapamil pharmaceutical formulation EEM, obtained by direct fluorescence (4.98 ppm) and by derivatization fluorescence (0.30 ppm) spectroscopy

Model evaluation	PARAFAC	BLLS	PARAFAC 2	MCR-ALS
Direct fluorescence				
Estimated concentration (ppm)	4.35 (0.41)	4.94 (0.16)	4.51 (0.44)	4.76 (0.18)
Recovery (%)	87.4	99.2	90.6	95.6
Components number	4	3 (1+2 interferents)	4	5
Derivatization fluorescence				
Estimated concentration (ppm)	0.29 (0.11)	0.31 (0.0012)	0.28 (0.10)	0.29 (0.11)
Recovery (%)	96.7	103.3	93.3	96.7
Components number	5	3 (1+2interferents)	4	4
Standard USP HPLC-UV ($n=3$)				
Estimated concentration (ppm)=234.0 (3.3), recovery=93.6%				

See footnote of Table 2

A three component model was used to model the Verapamil drug EEM obtained by derivatization fluorescence with PARAFAC and PARAFAC2. With these models, the predicted concentration and described recoveries of the expected Verapamil concentration (0.30 ppm) were 0.28 ppm and 93.3% with PARAFAC; 0.284 ppm and 94.7% with PARAFAC2. With MCR-ALS four component model a predicted concentration of 0.30 ppm and a recovery of 100.0%. When BLLS was applied, taken into account one component and two interferents in the system, an estimation of 0.30 ppm and a recovery of 100.0% were found. With the derivatization procedure, similarly to that observed in the direct analysis, the best estimation of the four methods was also found with MCR-ALS and BLLS methods.

The Fig. 4 presents the excitation and emission spectra estimated with the models that allows the best Verapamil concentration estimation: (a) with a three components non negativity constraint PARAFAC model; (b) with a one component and two interferents system BLLS model; (c) with a three components non negativity constraint in the first and third dimensions PARAFAC2 model; (d) with a four components non negativity constraint MCR-ALS model. For all the models three of the components correspond to the fluorescence of the derivatization products, and the four component corresponds to a background signal.

The figures of merit calculated are shown in Table 5. By the analysis of this table it is possible to see that similar results were found for the figures of merit by the two models. Even so a greater sensitivity was found by the BLLS model and greater selectivity by the PARAFAC model. Similar LODs were found with the two models.

Pharmaceutical formulation quantification

Table 6 shows the results of the analysis of Verapamil in a pharmaceutical formulation using the four decomposition methods. A greater number of components it is generally needed in order to obtain good concentrations estimations

which is due to the existence of a more complex background. In the direct fluorescence approach, four components have been used for PARAFAC and PARAFAC2, three components for BLLS (one component and two interferents), and five components for MCR-ALS. In the derivatized procedure, five components have been used for PARAFAC, three components for BLLS (one component and two interferents) and four components for PARAFAC2 and MCR-ALS. A global comparison of the results shows that, taking as reference the recovery obtained by the USP HPLC-UV method (93.6%), the best quantification result is found by direct fluorescence spectroscopy with the MCR-ALS model (95.6%) and by derivatization fluorescence spectroscopy with the PARAFAC2 model (93.3%).

Conclusions

The results obtained by the different three-dimensional decomposition methods allow to conclude that the EEM of Verapamil drug obtained by direct fluorescence has marked deviations from the trilinear structure, and the EEM obtained by derivatization fluorescence spectroscopy has an approximately trilinear structure. The deviations of the EEM to the trilinear model are essentially due to the presence of scatter bands.

For the two EEM structures under investigation the highest model fit is observed with MCR-ALS. In the analysis of the EEM obtained by direct fluorescence spectroscopy at least a two components PARAFAC, BLLS and MCR-ALS models and a three components PARAFAC2 model are needed. In the analysis of the EEM structures obtained by derivatization fluorescence spectroscopy at least a three components PARAFAC, BLLS and PARAFAC2 and a four components MCR-ALS models are needed. The limit of detection found in the derivatized procedure is lower than in the direct procedure. The best model estimations in the Verapamil drug are found by the MCR-ALS and BLLS models. Worse model estimations are

obtained for the analysis of the EEM obtained by direct fluorescence spectroscopy when compared with the derivatization procedure.

The best estimations for the Verapamil concentration in the pharmaceutical formulation were obtained by direct fluorescence spectroscopy with the MCR-ALS model and by derivatization fluorescence spectroscopy with the PARAFAC2 model. Also, these concentration estimations agree with those obtained with the USP HPLC-UV reference method.

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