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Detection capability of tetracyclines analysed by a fluorescence technique: comparison between bilinear and trilinear partial least squares models

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Abstract

According to the committee decision of 12 August 2002 (2002/657/EC) the capability of detection, CCβ, must be set in all analytical methods not only at concentration levels close to zero but also at the maximum permitted limit (PL). In this work we describe a methodology which evaluates the capability of detection of a fluorescence technique with soft calibration models (bilinear and trilinear PLS) to determine tetracyclines (group B1 substances from annex 1 of Directive 96/23/EC). Its estimation is based on the generalisation of the procedure described in International Union of Pure and Applied Chemistry and in the ISO standard 11843 for univariate signals which evaluates the probabilities of false positive (α) and false negative (β). The capability of detection, CCβ, estimated from the second-order signal and the trilinear PLS model is 9.93 $\mu g\,l^{-1}$ of tetracycline, 17.75 $\mu g\,l^{-1}$ of oxytetracycline and 26.31 $\mu g\,l^{-1}$ of chlortetracycline, setting α and β at 0.05. The capability of detection, CCβ, determined around the PL (100 $\mu g\,kg^{-1}$ in milk and muscle) with the second-order signal is 109.4 $\mu g\,l^{-1}$ of tetracycline, 117.0 $\mu g\,l^{-1}$ of oxytetracycline and 124.9 $\mu g\,l^{-1}$ of chlortetracycline, setting α and β at 0.05. The results were compared with those obtained with zero and first-order signals. The effect of the interferences on the capability of detection was also analysed as well as the number of standards used to build the models and their calibration range.

When a tetracycline is quantified in presence of uncalibrated ones by means of the trilinear PLS model the errors oscillate between 14.70% for TC and 9.57% for OTC.

Keywords: Capability of detection; Fluorescence; Tetracyclines; ISO 11843; 2002/657/EC; Trilinear PLS

1. Introduction

Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are antibiotics of the tetracycline group frequently given to animals destined for human consumption not only to prevent and treat certain diseases but also to fraudulently accelerate growth. These compounds have been classified in group B1 (veterinary medicines and contaminants) of annex I of Directive 96/23/EC. Studies performed on their accumulation in the body conclude that, even if the added levels are low, they may cause allergic reactions, and daily intake would generate the evolution of micro-organisms provoking resistance to antibiotics. For this reason the European Union (Commission Regulation EC no. 281/96) has set the individual permitted limits (PL) of TC, CTC and OTC at 100 $\mu g\,kg^{-1}$ in milk and muscle, 200 $\mu g\,kg^{-1}$ in eggs, 300 $\mu g\,kg^{-1}$ in liver and 600 $\mu g\,kg^{-1}$ in kidney.

Most procedures for the determination of tetracyclines use high performance liquid chromatography (HPLC) with UV-Vis [1], or fluorescence [2–4] detectors. Also flow injection analysis with fluorescence detector is employed [5]. The fluorescent properties of tetracyclines [6] and above all of their chelates formed from metals such as Mg [2,7], Al [3], Fe [5], Zr [4], etc., have been studied for years. However, the derivatisation (mostly post-column) means a considerable increase in analysis time and complexity of the instrumentation.

The detection limit, or as it is named by the International Organization for Standardization in the guideline ISO 11843 [8], capability of detection, is a figure of merit which allows one to decide whether an analytical procedure is suitable for the determination of an analyte at low concentration levels. In accordance with the committee decision of 12 August 2002 (2002/657/EC) [9], the capability of detection, CCβ,

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must be established in all methods of screening, identification or identification plus quantification. In the validation of a procedure for analysis of permitted substances, one needs to check the validity of the method not only at concentration levels close to zero but also at higher amounts such as the maximum PL.

The detection limit with univariate calibrations, in which to each sample (analyte concentration) there corresponds a univariate or zero-order signal, is well established in the ISO [8] and the International Union of Pure and Applied Chemistry (IUPAC) [10], evaluating the probabilities of false positive and false negative. However, univariate calibration models do not distinguish the analyte signal from that contributed by interferences. To avoid the matrix effect it is absolutely necessary to have selective signals.

This problem can be solved by using two-way calibration models (e.g. bilinear partial least squares regression model, bilinear PLS) and first-order signals where each sample is characterised by a data vector. This solution requires the calibration of the interferences together with the analyte being analysed. If the interferences are not calibrated one can still detect their presence in a sample but it is not possible to quantify the analyte of interest.

The quantification of an analyte in presence of unknown or uncalibrated interferences can be achieved by means of three (and beyond) way models [11] (e.g. trilinear PLS) in which each sample is represented by a data matrix or a second-order signal. Fluorescence has the property of generating second-order signals, the excitation–emission matrix (EEM), which consist of emission spectra recorded at various excitation wavelengths. The information given by fluorescence in combination with chemometric techniques, such as three-way PLS, allows one to solve complex samples under fairly general conditions without having to calibrate or know the interferences present beforehand. Fluorescence is thus said to have the second-order advantage. The so-called hyphenated techniques also have this advantage, for example, HPLC [12] or FIA [13] with diode array detector, HPLC-fast-scanning fluorescence spectrometry [14], GC/MS [15]. Besides other techniques such us spectroelectrochemical techniques [16], spectrophotometry (pH/UV-Vis) [17], two-dimensional GC [18] and kinetic-spectrophotometric analysis [19] amongst others provide second-order signals.

The need to use three (and beyond) way calibration models is clear. However, there is not a general accepted definition such as given by the ISO and IUPAC to estimate the detection limit with multivariate signals [16,20,21]. In this work the methodology described by the ISO and IUPAC has been generalised to evaluate the capability of detection,

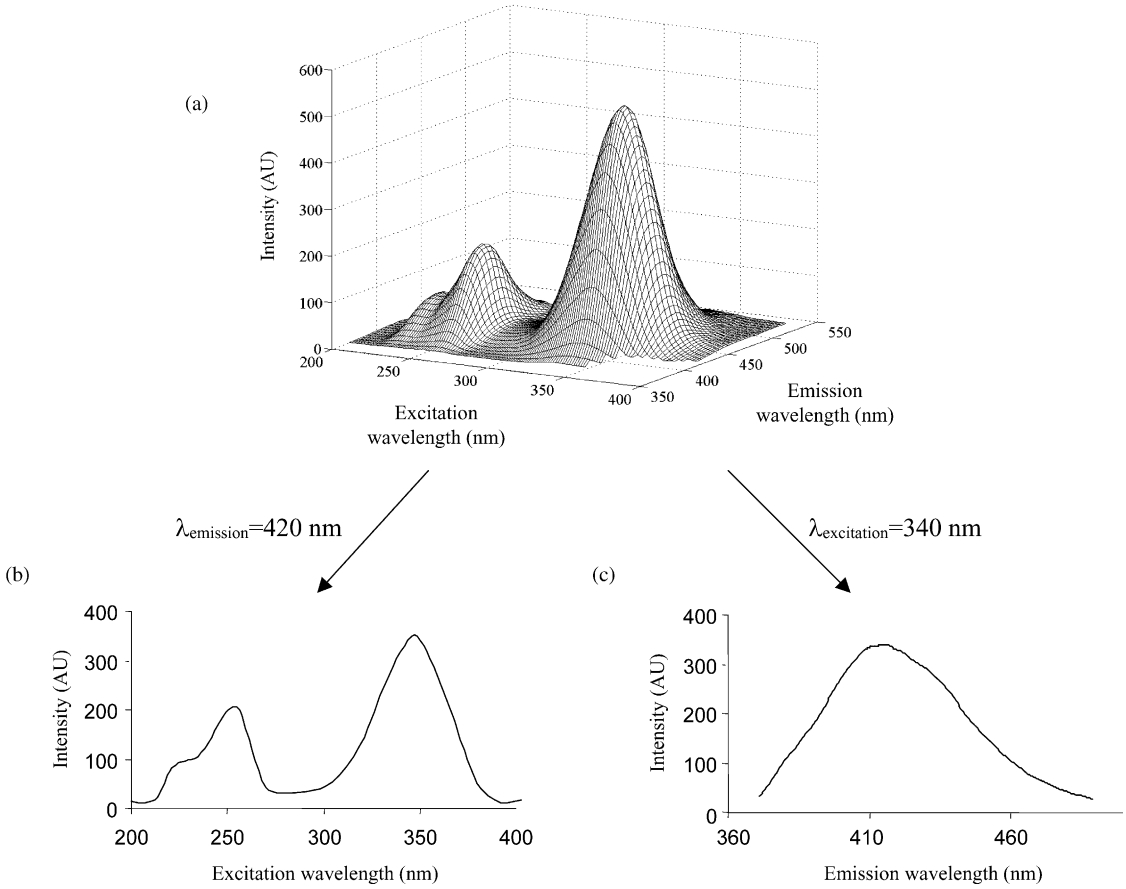

Fig. 1. (a) EEM (landscape) of 10 mg l^{-1} of pure CTC; (b) excitation spectrum at $\lambda_{emission} = 420$ nm; (c) emission spectrum at $\lambda_{excitation} = 340$ nm. The data which do not adjust to the trilinearity are not represented.

CCβ, with higher-order signals using the procedure described by Ortiz et al. [22,23]. The capability of detection, CCβ, was also determined at the maximum PL (100 μg kg^{-1} in milk and muscle).

The results were compared with those obtained with first-order signals (from the same EEM either the excitation spectrum, Fig. 1b, or the emission spectrum, Fig. 1c, was extracted) and with those of the zero-order signal (fluorescence intensity at the excitation–emission maximum).

2. Theory

2.1. Three-way PLS model

The trilinear PLS model is a generalisation of the two-way PLS model [24] to second-order data. The three-way PLS model is built by decomposing the data array, $\underline{X}$, in triads. A triad is a trilinear factor or rank-one model of the calibration array formed by a score vector in the sample direction, t, and two weight factors, w^J, in the excitation spectra direction and w^K, in the emission spectra direction. The model of $\underline{X}$ for each of its elements, x_{ijk}, is given by Eq. (1):

$$x_{ijk} = t_i w_j^J w_k^K + e_{ijk} \tag{1}$$

where e_{ijk} represents the error which affects each individual measure. The decomposition [25] is done such that the weight vectors w^J and w^K produce score vectors t_i with maximum covariance with the unexplained part of the dependent variable, y (Eq. (2)):

$$\max_{w^J w^K} \left[\mathrm{cov}(t, y) \left| \min \sum_{i=1}^{I} \sum_{j=1}^{J} \sum_{k=1}^{K} (x_{ijk} - t_i w_j^J w_k^K)^2 \right. \right] \tag{2}$$

A detailed description of the algorithm and the extension for higher-order signals can be found in Refs. [25,26].

Although the three-way decomposition is similar to that done by the PARAFAC model [27,28], the trilinear PLS has the advantage of seeking the maximum covariance between the independent and the dependent variables. The components in the two blocks are calculated simultaneously, thus improving the predictive capability of the model. However the trilinear PLS model does not estimate the true underlying profiles as PARAFAC does.

2.2. Trilinearity of the fluorescence technique

The model of Eq. (1) assumes that the data are trilinear. In PLS nomenclature, multilinear refers to the decomposition of the block of variables and not to the relation between dependent and independent variables, which implies that the response is linear and additive for all the analytes. The fluorescence emission intensity, I, in a sample which contains F fluorophores, at an emission wavelength λ_k, when excited at a wavelength λ_j, can be expressed as

$$I = \sum_{f=1}^{F} a_f b_{jf} c_{kf} \tag{3}$$

where the parameter b_{jf} includes the factors related to the excitation of the analyte f at excitation wavelength j. The parameter c_{kf} is related to the fluorescence intensity of the fluorophor f at an emission wavelength k and the concentration of the analyte f is represented by a_f. Eq. (3) is in agreement with the model of Eq. (1) for diluted solutions. It must not be forgotten that there are certain areas of the spectrum where this is not verified [29]. First, emission is not defined at wavelengths below the excitation wavelength. Secondly, fluorescence simultaneously occurs with other phenomena such as Rayleigh and Raman scatter which do not conform to the trilinear model.

2.3. Number of factors

The RMSEP (root mean square error of prediction) was determined, Eq. (4), using an independent validation test set which did not take part in the performance of the model. The number of factors to be included in the PLS models (bilinear and trilinear ones) is that which minimises RMSEP:

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_i (y_{i,\mathrm{cal}} - y_{i,\mathrm{true}})^2}{n}} \tag{4}$$

2.4. Capability of detection

The International Organization for Standardization (ISO) in the guideline 11843-1 [8] defines for a given probability of false positive, α, the capability of detection or minimum detectable net concentration, as the true net concentration of the analyte in the material to be analysed which will lead, with probability $1 - \beta$, to the correct conclusion that the concentration in the analysed material is different from that in the blank material. The detection limit is clearly established there with zero-order signals through the application of the unilateral Neyman–Pearson hypothesis test. Its statistical bases were published in 1987 by Clayton et al. [30] and implemented in the DETARCHI programme by Ortiz and Sarabia [31] in 1994. In order to apply this methodology it is necessary to: (i) have specific signals, (ii) build a calibration model (signal versus concentration) and (iii) apply the following Neyman–Pearson test:

- Null hypothesis: the concentration of the sample is x_0, $X = x_0$.
- Alternative hypothesis: the concentration of the sample is greater than x_0, $X > x_0$.
- α, probability of rejecting that the true sample concentration is x_0 when the null hypothesis is true.
- β, probability of accepting that the true sample concentration is x_0 when in fact the null hypothesis is false.

Since the decision to affirm that the concentration of the analyte is equal to or greater than x_0 is based on the signal, the critical region of the hypothesis test (CR) will be

$$\mathrm{CR} = \{\text{signals above the detection signal}\} = \{y > y_d\}$$

Taking into account the calibration curve to transform signals into concentration [31] the capability of detection, CCβ, can be estimated from Eq. (5):

$$\mathrm{CC}\beta = \frac{\Delta(\alpha, \beta) w_{x_0} \hat{\sigma}}{\hat{b}} \quad (5)$$

where $\Delta(\alpha, \beta)$ depends on the probabilities α and β. $\hat{\sigma}$ is the residual standard deviation of the regression and $\hat{b}$ the slope. w_{x_0} (Eq. (6)) is a function of the position of the standards, x_i, in the calibration curve and is inversely proportional to the number of replicates of the sample, K, and to the number of standards in the calibration model, I.

$$w_{x_0}^2 = \frac{1}{K} + \frac{1}{I} + \frac{(x_0 - \bar{x})^2}{\sum_{i=1}^{I}(x_i - \bar{x})^2} \quad (6)$$

The capability of detection at zero will be determined with $x_0 = 0$ and α and β will be the probabilities of false positive and false negative, respectively. The capability of detection at the maximum PL will be estimated with $x_0 = \mathrm{PL}$ and α and β are the probabilities of false non-compliance and false compliance, respectively.

This way of estimating the capability of detection is not only limited to specific zero-order signals but it is also possible to evaluate it with non-specific higher-order signals and soft calibration [22,23]. The extension is based on the idea that the capability of detection does not vary upon linear transformations in the response variable. Therefore the instrumental signal (first, second or higher-order signal) could be substituted by the standard concentration predicted by the calibration model. Thus the capability of detection with first and higher-order signals can be estimated in accordance with IUPAC and the ISO by means of a regression between the concentration calculated by the soft model (bilinear and trilinear PLS) and the true standard concentration. The methodology described is implemented in the NWAY-DET house programme which determines the capability of detection with evaluation of α and β.

The linear relationship, concentration calculated by the soft model versus true concentration of the standard, is established by means of the least squares regression, LS. Since this criterion is highly affected by data which deviate from linearity, either because the data are erroneous or for chemical reasons, the least median of squares regression was used (LMS) [32]. Those data whose standardised residual with respect to the LMS regression is in absolute value higher than 2.5, are removed from the data set and the LS regression is then performed. This procedure is known as reweighted least squares regression (RLS). The methodology described has already been applied with success to determine the detection limit with different analytical techniques [33–35].

3. Experimental

3.1. Apparatus

Measurements were performed at room temperature on a Perkin-Elmer LS50 B Luminescence Spectrometer equipped with a xenon discharge lamp and a gated photomultiplier. Excitation and emission slits were both set to 10 nm and the scan speed was 1500 nm min^{-1}. Neither the excitation nor the emission spectrum was corrected or smoothed.

3.2. Reagents

All chemicals were of analytical-reagent grade, and solvents were HPLC-grade. Tetracycline standards were obtained from Sigma (Steinheim, Germany) and stored in a freeze (below 0 °C) away from light. Stock solutions (1 g l^{-1}) of each tetracycline were prepared in methanol (Merck, Darmstadt, Germany) and stored at 4 °C in brown glass bottles for a maximum period of 1 month. Dilute standard solutions (10 mg l^{-1}) were prepared in methanol daily for each tetracycline. Working solutions were arranged immediately before use by dilution of the dilute solution to appropriate concentrations with EDTA-Britton Robinson buffer/methanol (30:70, v/v). All solutions were degassed by ultrasonification to avoid the oxygen quenching.

The buffer contained 0.04 M acetic acid (Panreac, Barcelona, Spain), 0.04 M phosphoric acid (Merck, Darmstadt, Germany) and 0.04 M boric acid (Merck, Darmstadt, Germany) adjusted to pH 9.0 using 0.2 M sodium hydroxide solution. The EDTA/Britton Robinson buffer was prepared by dissolving 3.72 g disodium ethylenediaminetetracetate dihydrate (Panreac, Barcelona, Spain) in 1 l of Britton Robinson buffer. The aim was to eliminate the interferences caused by several metals. Deionised water was obtained by the Milli-Q water purification system (Millipore).

3.3. Analytical method and data sets

In this work three standard sets were prepared for TC and OTC: the calibration set, the test set and the test set with uncalibrated interferences. For CTC the calibration set and the test set were only arranged. The concentrations are detailed in Table 1.

3.3.1. Calibration set and analytical signals

This set is constituted by those samples which will be used to perform the calibration models. Twelve standards (see first column in Table 1) were prepared at concentrations ranging between 10.10 and 282.80 μg l^{-1} of pure TC as described in Section 3.2. Independently, to study the effect of interferences on the capability of detection, 12 standards were arranged between 0 and 181.80 μg l^{-1} of TC in presence of 39.76 μg l^{-1} of CTC and 36.29 μg l^{-1} of OTC (fourth column in Table 1). In both cases, the emission spectra were recorded between 420 and 590 nm (intervals of 1.5 nm) at

Table 1
Concentration of the standards of the calibration set (Section 3.3.1) and the test set (Section 3.3.2) (for the standards where footnotes c and d were not given, one replicate was acquired)

Set	Section 4.1			Section 4.3	
	TC ($\mu g\,l^{-1}$)	OTC ($\mu g\,l^{-1}$)	CTC ($\mu g\,l^{-1}$)	TC[a] ($\mu g\,l^{-1}$)	OTC[b] ($\mu g\,l^{-1}$)
Calibration set	10.10[c]	10.08[c]	9.94[c]	0.00[c]	0.00[c]
	35.35[d]	40.32[d]	39.76[d]	15.15[d]	10.08[c]
	60.60	70.56	69.58	35.35	40.32[d]
	85.85	100.80	99.40	50.50	70.56
	106.05	131.04	129.22	60.60	100.80
	131.30	161.28	159.04	85.85	131.04
	156.55	191.52	188.86	95.95	161.28
	181.80	221.76	218.68	106.05	191.52
	207.05	252.00	248.50	131.30	221.00
	232.30	282.24	278.32	146.45	236.88
	257.55[d]	312.48[d]	308.14[d]	156.55[d]	252.00[d]
	282.80[c]	342.72[c]	337.96[c]	181.80[c]	282.24[c]
Test set	35.35	40.32	39.76	35.35	85.68
	85.85	100.80	99.40	85.85	100.80
	131.30	161.28	159.04	95.95	146.16
	156.55	221.76	278.32	106.05	176.40
	207.25	252.00	–	131.30	201.60
	232.30	282.24	–	146.45	221.00

[a] Fixed amounts of 39.76 $\mu g\,l^{-1}$ CTC and 36.29 $\mu g\,l^{-1}$ OTC as interference.
[b] Fixed amounts of 149.10 $\mu g\,l^{-1}$ CTC as interference.
[c] Three instrumental replicates.
[d] Two instrumental replicates.

excitation wavelengths between 325 and 420 nm (regular steps of 5 nm). Replicates were done at the extremes of the calibration curve thus obtaining data arrays ($18 \times 20 \times 114$). In all cases the first mode refers to the number of samples, the second to the number of excitation wavelengths and the third to the number of emission wavelengths.

The calibration of pure OTC was performed with 12 standards between 10.08 and 342.72 $\mu g\,l^{-1}$ (see second column in Table 1). The emission spectra were scanned between 410 and 590 nm at intervals of 1.5 nm at several excitation wavelengths between 310 and 405 nm. The dimensions of the array are ($18 \times 20 \times 121$). The calibration corresponding to OTC in presence of fixed amounts of interferences was built with 11 standards in concentrations between 0 and 282.24 $\mu g\,l^{-1}$ of OTC with 149.10 $\mu g\,l^{-1}$ of CTC as interference (last column in Table 1). The emission spectra were recorded between 465 and 600 nm and the excitation wavelengths between 300 and 405 nm (intervals of 5 nm) in such a way that the array dimensions are ($19 \times 22 \times 92$).

The model of pure CTC was built with 12 standards between 9.94 and 337.96 $\mu g\,l^{-1}$ of CTC (third column in Table 1), recording the emission spectra between 320 and 510 nm at various excitation wavelengths between 200 and 403 nm. As can be seen in Fig. 1a which displays the landscape (EEM) of 10 $mg\,l^{-1}$ of pure CTC, this has two excitation modes. In this work we only present the results relative to the second excitation mode (340 nm), because it leads to better values of the capability of detection than the first one (249 nm). We studied the emission spectra recorded between 368 and 510 nm (intervals of 0.5 nm) at excitation wavelengths registered between 298 and 382 nm (regular steps of 7 nm). Thus the dimensions of the final array are $18 \times 13 \times 286$.

3.3.2. Test set

This set of standards was used to choose the proper number of latent variables or factors to include in the PLS models (bilinear and trilinear). The number of factors included will be that which minimises RMSEP (Eq. (4)). In all cases, six pure standards were prepared with concentrations ranging between 35.35 and 232.30 $\mu g\,l^{-1}$ of TC, 40.32 and 282.24 $\mu g\,l^{-1}$ of OTC and 39.76 and 278.32 $\mu g\,l^{-1}$ of CTC for the validation of the calibration sets built with the pure standards (see last rows in Table 1). For the validation of those models built with fixed quantities of interference, the concentration of the test set of TC oscillates between 35.35 and 146.45 $\mu g\,l^{-1}$ (with 39.76 and 36.29 $\mu g\,l^{-1}$ of CTC and OTC, respectively, as interference) and the concentration of the test set for OTC varies between 85.65 and 221.00 $\mu g\,l^{-1}$ (with 149.10 $\mu g\,l^{-1}$ of CTC as interference).

3.3.3. Test set with uncalibrated interferents

The second-order property (predict the concentration of an analyte in a sample with uncalibrated interferences) was tested with the models performed with the pure standards and determining the concentration of TC in 10 samples which contain 39.76 and 36.29 $\mu g\,l^{-1}$ of CTC and OTC, respectively, as uncalibrated interferences. In the case of OTC there were nine samples with 99.40 $\mu g\,l^{-1}$ of CTC as

Table 2
True TC concentration of the test set with uncalibrated interferences[a] (Sections 3.3.3 and 4.2; predicted concentration by all four models built with the pure TC standards)

True concentration of TC ($\mu g\,l^{-1}$)	Predicted concentration ($\mu g\,l^{-1}$)			
	Univariate	Excitation	Emission	EEM
15.15	71.72	69.91	70.12	10.58
35.35	86.58	86.6	86.27	50.48
55.55	113.47	108.41	108.27	64.98
65.65	135.46	129.18	128.91	66.82
80.80	144.77	142.19	141.99	77.61
95.95	151.39	143.59	143.14	80.07
126.25	180.53	179.67	179.35	112.16
131.17	194.16	178.52	178.33	125.03
156.55	207.31	207.85	207.93	147.91
171.70	235.74	218.27	222.59	146.92
Mean absolute relative error (%)	102.66	96.23	96.35	14.70

[a] CTC: 39.76 $\mu g\,l^{-1}$; OTC: 36.29 $\mu g\,l^{-1}$.

uncalibrated interference. The true concentration of TC and OTC of this set is shown in Tables 2 and 3, respectively.

3.4. Data pre-processing

All data corresponding to the non-trilinear area (Section 2.2) were considered as missing so that the model will not be affected by these data [27]. This includes the Rayleigh and Raman scatter (first and second-order), and emission wavelengths below the excitation ones.

3.5. Software

Data collection was performed by a software package from FL WinLab version no. 3.00 and transformed into ASCII format through 3D Exported version no. 1.00. The multilinear PLS algorithm was obtained from the N-way Toolbox for Matlab [36]. The bilinear models were built with the PARVUS programme [37]. The univariate capability of detection was calculated using the DETARCHI programme [31] while the methodology applied to evaluate the multivariate capability of detection is implemented in the programme arranged by the authors, NWAYDET. The least squares regression was done with the PROGRESS programme [32].

Table 3
True OTC concentration of the test set with uncalibrated interferences[a] (Sections 3.3.3 and 4.2; predicted concentration of OTC by all four models carried out with pure OTC standards)

True concentration of OTC ($\mu g\,l^{-1}$)	Predicted concentration ($\mu g\,l^{-1}$)			
	Univariate	Excitation	Emission	EEM
40.32	70.16	66.08	9.58	27.68
75.60	118.10	116.23	61.37	88.49
110.88	134.80	129.05	87.68	103.22
146.16	176.82	170.18	120.43	136.77
181.44	208.27	200.83	150.85	166.84
216.72	260.82	249.17	196.93	207.99
252.00	284.90	260.37	224.87	239.79
287.28	325.43	301.73	268.66	284.96
322.56	353.30	329.94	283.64	301.19
Mean absolute relative error (%)	27.09	20.75	20.99	9.57

[a] CTC: 99.40 $\mu g\,l^{-1}$.

4. Results and discussion

In Fig. 1a the three-dimensional fluorescent signal of 10 $mg\,l^{-1}$ of pure CTC is displayed, and in Fig. 2a and b that corresponding to 1 $mg\,l^{-1}$ of pure TC and 10 $mg\,l^{-1}$ of pure OTC, respectively; the data which do not fit the trilinearity (Section 3.4) have been eliminated. The excitation–emission maximum ($\lambda_{excitation}$, $\lambda_{emission}$) for each analyte is (390, 510 nm) for TC, (370, 510 nm) for OTC and (249, 420 nm) for the first mode of CTC and (340, 420 nm) for the second mode. The landscapes will show a high degree of overlapping when the tetracyclines are mixed. In all three cases it was observed that there was a shift of the peaks towards smaller wavelengths when the concentration decreases as a result of the Rayleigh and Raman scatter.

4.1. Capability of detection, CCβ, of the models built with the standards in absence of interferences

Using the same EEM of pure standards, the following models were performed: (1) univariate calibration (intensity recorded at the excitation–emission maximum, zero-order signal), (2) bilinear PLS model with the excitation spectrum (recorded at the emission maximum, first-order signal), (3) bilinear PLS model with the emission spectrum (registered at the excitation maximum, first-order signal) and (4) trilinear PLS model (with the EEM second-order signal). Table 4 shows the results of all four models for pure TC, OTC and CTC.

In most cases, with a reduced number of latent variables, the explained variance in the dependent variable is around 99% which indicates that the models are suitable for describing the variability found in the data. Likewise, RMSEP (Eq. (4)) was determined as well as the relative mean error in absolute value when the concentration of the test set (Section 3.3.2) is predicted. The values are acceptable, only exceeding 9% in one case, which reflects the stability and good prediction capability of the models.

In the seventh column of Table 4 (CCβ, $x_0 = 0$) the capability of detection of the different models and analytes is listed. One replicate was fixed and the probabilities of false positive and false negative were set at 0.05. This was determined following the methodology described in Section 2.4, i.e. establishing a linear relationship between the concentration calculated by the soft model and the true concentration of the standard. The RLS models (detection of anomalous data with LMS and later fitting by LS) were applied. For

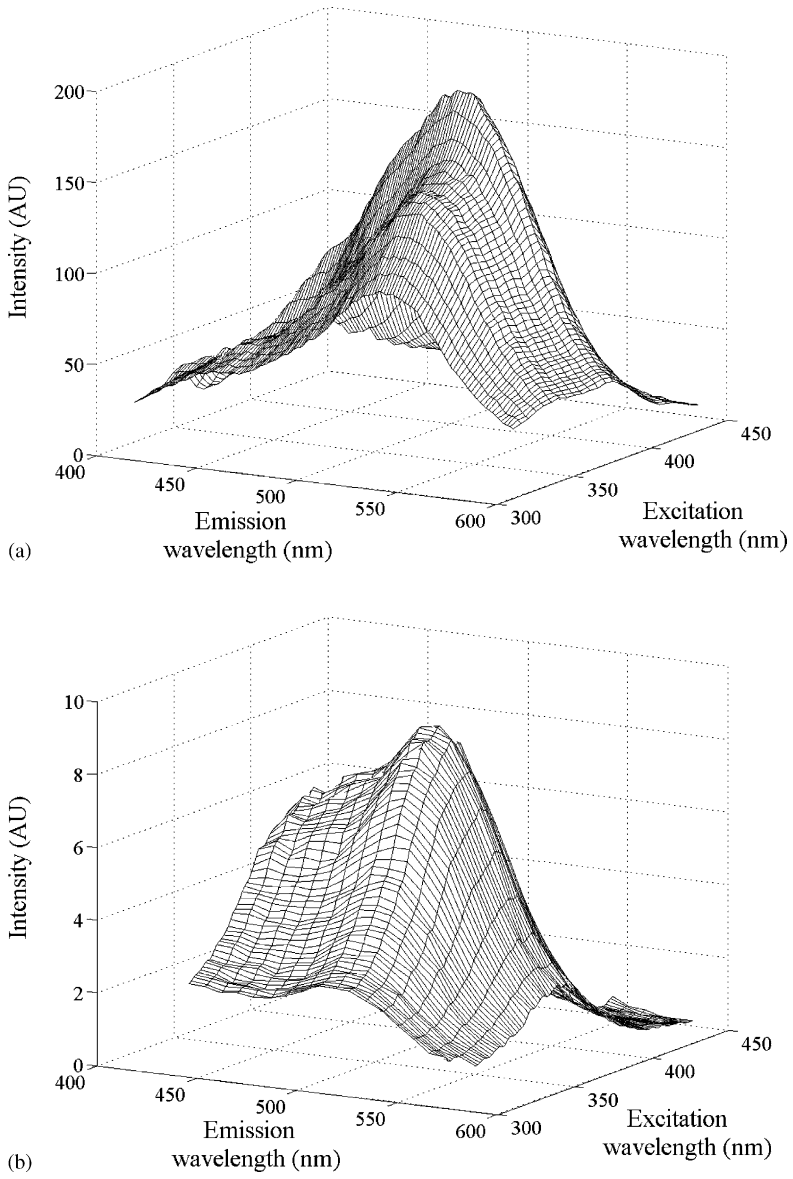

Fig. 2. EEM (landscape) of (a) 1 mg l^{-1} of pure TC and (b) 10 mg l^{-1} of pure OTC.

all three analytes, the best detection limit is obtained with the emission spectrum and the bilinear PLS calibration, in other words, the emission spectrum is the signal which presents least variability. This effect may be associated with the mode in which the signals have been recorded, that is fixing an excitation wavelength and registering the emission spectra. Therefore genuine emission spectra are registered whereas the excitation spectra are obtained by gathering the different excitation wavelengths of the experiment. In Ref. [38] excitation spectra were recorded at a solely emission wavelength, 510 nm, and the capability of detection was determined. The results obtained are comparable with those estimated in this paper with the emission spectra and bilinear PLS which supports the previous conclusion.

The capability of detection of the trilinear PLS model, which analyses the EEM, is worse than that determined by the emission spectrum. In other words, the capability of detection is conditioned by the addition of a new dimension with greater variability, the excitation spectra.

In view of the analytes, CTC is the compound for which the values of explained variance (Y, in Table 4) and the mean errors are the worst. The capability of detection of the different models for determining CTC are also the worst which may be related to its greater photosensitivity. Nevertheless, the values of the detection limit are below the maximum limits permitted by European regulations, such that it is possible to detect the presence of tetracyclines at these concentration levels.

The effect of the calibration range on the capability of detection (CCβ, $x_0 = 0$) together with that of the number of standards used for performing the trilinear PLS models was also checked. For doing that six standards with

Table 4
Parameters of the calibration models built with the pure standards (TC, OTC and CTC) and capability of detection, CCβ, at 0 and 100 μg l^{-1} (comparison between different kinds of calibration models with zero, first and second-order signals)[a]

Analyte	Signal (order)	L.V.	Y (%)	RMSEP (μg l^{-1})	Relative error (%)	CCβ	
						$x_0 = 0$[b] (μg l^{-1})	$x_0 = 100$[b] (μg l^{-1})
TC	Univ.(0)		–	9.83	8.11	20.39	119.5
	Exc.(1)	4	99.96	5.86	6.23	11.62	111.1
	Emis.(1)	5	99.99	6.51	4.43	5.93	105.7
	EEM(2)	4	99.93	6.65	4.79	9.93	109.4
OTC	Univ.(0)	–	–	5.98	6.19	21.58	120.7
	Exc.(1)	2	99.95	6.64	5.74	12.08	112.5
	Emis.(1)	3	99.99	3.65	2.39	7.72	107.4
	EEM(2)	4	99.96	4.62	3.16	17.75	117.0
CTC	Univ.(0)	–	–	13.44	8.71	22.91	121.9
	Exc.(1)	2	97.28	17.75	22.15	39.80	137.9
	Emis.(1)	5	99.94	6.99	8.71	15.72	115.1
	EEM(2)	6	99.73	9.30	4.49	26.31	124.9

[a] L.V.: the number of latent variables or components; Y: the percentage of explained variance in the response; RMSEP: the root mean square error of prediction; relative error: the mean relative error in absolute value when the concentration of the test set (Section 3.3.2) is determined.
[b] $\alpha = \beta = 0.05$.

the smallest concentration (see Table 1) were employed for building the models. Replicates were not taken into account. The capability of detection was evaluated fixing the number of replicates at 1 and probabilities of false positive and false negative at 0.05. CCβ was 3.07 μg l^{-1} of TC (versus 9.93 μg l^{-1} with the complete data set, Table 4), 17.65 μg l^{-1} of OTC (versus 17.75 μg l^{-1}) and 20.66 μg l^{-1} of CTC (versus 26.31 μg l^{-1}). From these results it can be deduced that the capability of detection does not depend neither on the number of standards nor on the calibration interval. Only in the case of TC its capability of detection has slightly decreased.

The behaviour of the analytical method at the maximum PL (100 μg kg^{-1}, in milk and muscle) must be also examined [9]. The values of the capability of detection (CCβ, $x_0 = 100$ μg l^{-1}) are given in the last column in Table 4. In all four cases the number of replicates was established at 1, the probability of false non-compliance, α, and the probability of false compliance, β, were fixed at 0.05. As can be seen the trend of the capability of detection at 100 μg l^{-1} is comparable to that observed around zero and the same conclusions can be drawn for the different models and analytes.

4.2. The second-order property

Once the models were built with pure standards and their validity for quantifying at low levels (CCβ, $x_0 = 0$) and at levels around the maximum PL (CCβ, $x_0 = 100$) was proved, the second-order advantage of the models was tested. In other words, the possibility of quantifying an analyte in samples which contain unknown interferences or simply have not taken part in the model. This property was proved with the models carried out with pure analytes and predicting the concentration of the test set with uncalibrated interferences (Section 3.3.3). The results are illustrated in Table 2 for TC and in Table 3 for OTC. Table 2 shows the true and the concentration of TC predicted by all four models performed with pure standards in 10 samples which contain 39.76 μg l^{-1} of CTC and 36.29 μg l^{-1} of OTC as uncalibrated interferences. The mean relative errors in absolute value are also displayed; Table 3 shows the true and predicted concentration of OTC in nine samples containing 99.40 μg l^{-1} of CTC as uncalibrated interference. As can be seen in Table 2 for TC, the errors with the univariate and bilinear models are about 100% which makes these calibration models unfeasible for the quantification of analytes in the presence of uncalibrated interferences. It is clear that the effect of the interferences is considerable in both situations. Only in the case of the bilinear PLS models these errors could be avoided, calibrating the interferences together with the analyte of interest, which was done in Ref. [38]. However, this solution implies the increase in the number of samples and additionally one has to know all the interferences and the ratio in which they occur in the samples. This difficulty frequently occurs in the analysis of biological samples. Despite the previous procedures of clean-up, extraction and chromatographic separation the instrumental signal is often not specific due to the coelution of several unidentified interferences.

According to Table 3 the effect of the interferences is less severe for OTC. The errors with the univariate model are about 30% and those with the bilinear PLS models around 20%. However errors with the trilinear PLS model have decreased (10%) which makes these kind of calibrations more appropriate for the analysis of OTC in presence of uncalibrated CTC.

The results presented in this section demonstrate that the problem of interferences can be solved using the second-order fluorescent signal in combination with the trilinear PLS model. Thus, for TC (Table 2), the relative errors were reduced from 100 to 14.70% with trilinear PLS and from 21 to 9.57% for OTC (Table 3).

4.3. Capability of detection, CCβ, of the models built with standards in presence of interferences

Eq. (3) which shows the fluorescence intensity of F fluorophores allows one to assume that the EEM complies with the requirements of the trilinear PLS model and consequently of the bilinear PLS and univariate models. However, the fluorescent additivity is only maintained when the contribution of each analyte remains independent of the rest. This therefore constitutes an important aspect to be verified since some fluorophores might cause data to fail the trilinearity assumptions either by concentration quenching (the excitation and emission spectra of the analyte and interference overlaps, favouring attenuation phenomena) or because the interference has a much more intense signal than the analyte.

The standard addition method is frequently carried out in analytical chemistry. This method consists of adding increasing amounts of the analyte of interest to aliquots of the sample to be analysed. That is why in this paper models were performed with the standards in presence of fixed amounts of interferences (Section 3.3.1 and Table 1). The results obtained with the different models are shown in Table 5. In the case of TC the standards contained 39.76 and 36.29 $\mu g\,l^{-1}$ of CTC and OTC, respectively, and the standards of OTC, 149.10 $\mu g\,l^{-1}$ of CTC. To compare the results, in all the models the capability of detection (CCβ, $x_0 = 0$ and $x_0 = 100\,\mu g\,l^{-1}$) was estimated fixing the number of replicates at 1 and α and β at 0.05.

As happened with the models built with pure standards, the best capability of detection is that estimated with the bilinear PLS and the emission spectrum.

However no difference was found between the univariate CCβ with pure standards and that with interferences. A constant variation in a univariate signal, e.g. due to the presence of interferences, does not affect the capability of detection when the Clayton method is used provided that the standard deviation, $\hat{\sigma}$ in Eq. (5), does not change. Naturally the standard deviation and hence, the capability of detection depends on the nature matrix. In this paper pure standards and standards with interferences were prepared in aqueous media so both the standard deviation and CCβ are not expected to get worse.

However, with first and second-order signals the residual standard deviation of the models together with the capability of detection worsen due to the effect of interferences on the multivariate signal. The influence of the interferences on the detection limit determined with the trilinear model can be graphically seen in Fig. 3a for TC and in Fig. 3b for OTC which show the curves of false negative, β, as a function of the capability of detection (CCβ, $x_0 = 0$) setting α at 0.05 and the number of replicates at 1. For any probability of false negative the capability of detection with the trilinear model worsens in presence of interferences. For any number of replicates, CCβ with interferences is always worse than that of the pure standards with one replicate which proves the effect of the interferences on the experimental variability of the second-order signal. It must be emphasised that the results obtained in this section for the different calibration models should not be extrapolated to all matrix interferences at any concentration. The capability of detection depends on the nature and the amount of the interferences.

As it was done in Section 4.1 with pure standards, the effect of the number of standards of the training set and the calibration range on the capability of detection was tested. Trilinear models were built with six standards (0–85.85 $\mu g\,l^{-1}$ of TC and 0–131.04 $\mu g\,l^{-1}$ of OTC, see Table 1). The capability of detection (CCβ, $x_0 = 0$) is 30.54 $\mu g\,l^{-1}$ for TC (versus 27.85 $\mu g\,l^{-1}$, with the complete data set, Table 5) and 13.48 $\mu g\,l^{-1}$ for OTC (versus 28.99 $\mu g\,l^{-1}$). Once again the capability of detection does not systematically depend neither on the number of standards of the calibration set nor on the calibration range.

Table 5
Parameters of the calibration models built with the standards (TC and OTC) in presence of interferences and capability of detection, CCβ, at 0 and 100 $\mu g\,l^{-1}$ (comparison between different types of calibration models with zero, first and second-order signals)[a]

Analyte	Signal (order)	L.V.	Y (%)	RMSEP ($\mu g\,l^{-1}$)	Relative error (%)	CCβ	
						$x_0 = 0$[b] ($\mu g\,l^{-1}$)	$x_0 = 100$[b] ($\mu g\,l^{-1}$)
TC[c]	Univ.(0)	–	–	8.57	8.88	23.73	122.7
	Exc.(1)	2	98.99	7.21	6.00	41.59	139.8
	Emis.(1)	3	99.76	7.73	8.24	15.11	114.4
	EEM(2)	3	99.67	6.56	8.11	27.84	125.9
OTC[d]	Univ.(0)	–	–	16.75	8.56	25.39	124.5
	Exc.(1)	3	99.09	14.91	6.42	45.79	144.7
	Emis.(1)	4	99.81	16.84	8.81	23.50	122.7
	EEM(2)	4	99.68	19.67	9.84	28.99	127.9

[a] L.V.: the number of latent variables; Y: the percentage of explained variance in the response; RMSEP: the root mean square error of prediction; relative error: the mean relative error in absolute value when the concentration of the test set (Section 3.3.2) is estimated.
[b] $\alpha = \beta = 0.05$.
[c] CTC: 39.76 $\mu g\,l^{-1}$; OTC: 36.29 $\mu g\,l^{-1}$.
[d] CTC: 149.10 $\mu g\,l^{-1}$.

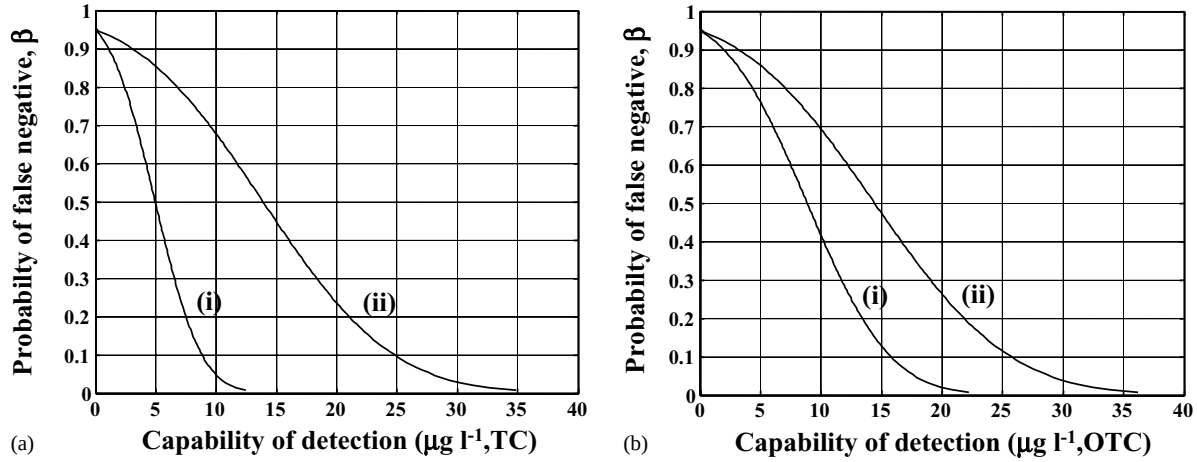

Fig. 3. Characteristic curves of the capability of detection, CCβ, of (a) TC: (i) pure TC and (ii) TC with 39.76 $\mu g\,l^{-1}$ of CTC and 36.29 $mg\,l^{-1}$ of OTC as interference. (b) OTC: (i) pure OTC and (ii) OTC with 149.10 $\mu g\,l^{-1}$ of CTC as interference. The probability of false positive, α, was set at 0.05 and the number of replicates at 1.

Values of the explained variance (around 99%, Table 5), the RMSEP and the mean errors in prediction of the test set are acceptable, which confirms that the presence of one or various tetracyclines as interferences in the determination of another tetracycline does not violate the assumptions made for the model expressed in Eq. (1).

Since tetracyclines are permitted substances their concentration will be often close to the PL. Therefore the calculation of CCβ around this concentration level is necessary to assure that the samples analysed fulfil the requirements established by the European Union. The capability of detection at 100 $\mu g\,l^{-1}$ in presence of interferences is listed in the last column of Table 5. The probabilities of false non-compliance and false compliance were set at 0.05.

5. Conclusions

The methodology described in this paper enabled us to determine the capability of detection, CCβ (unilateral hypothesis test either at zero, $x_0 = 0$ or the maximum PL, $x_0 = \text{PL}$) of the fluorescence technique to analyse tetracyclines using soft calibration models. This methodology can be applied to any analytical technique which provides first, second or even higher-order data.

The validation of the trilinear PLS models shows the possibility of quantifying tetracyclines in more or less complex samples with uncalibrated interferences, in comparison with the bilinear PLS models which require previous knowledge as to the potential interferences, their proportion in the samples and finally their inclusion in the calibration model.

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