Anal. Chem. **1999,** *71,* 4361−4368

Enhanced Synchronous Spectrofluorometric Determination of Tetracycline in Blood Serum by Chemometric Analysis. Comparison of Partial Least-Squares and Hybrid Linear Analysis Calibrations

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Tetracycline has been determined in human serum samples by a combination of: (1) synchronous fluorescence spectra of whole sera treated with Mg^{2+}, and (2) the multivariate calibration methods of partial least-squares (PLS-1) and a variant of the recently introduced hybrid linear analysis (HLA), which does not require the knowledge of pure-component spectra. The calibration set was designed with 50 sera spiked with concentrations of tetracycline in the range 0.0−4.0 μg mL^{-1}. Studies concerning validation, precision, accuracy and figures of merit (selectivity, sensitivity and limit of determination) were also carried out. A novel wavelength-selection procedure was applied to minimize the effect of nonmodeled interferents present in serum samples containing bilirubin, triglycerides, and salicylate. Overall, the performance of the newly developed HLA approach seems to be better than that of PLS-1.

Tetracyclines possess a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria. They bind specifically to 30 S ribosomes and appear to inhibit the protein synthesis by preventing access of aminoacyl tRNA to the acceptor site on the mRNA−ribosome complex.[1] In addition to the use of tetracyclines in humans, there is an ever-increasing use of them for therapeutic veterinary purposes, to maintain the health of farm animals and to enhance the productivity of the farming industry.[2] Today, nearly 1000 tetracycline derivatives exist, but only seven have been in extensive clinical and veterinary use.[3]

To decrease the resistance in new strains, unnecessary usage of tetracyclines should be minimized. One way of achieving this is to decrease their consumption, and this requires monitoring methods for tetracycline residues in samples such as blood serum, urine, milk, egg, and animal tissues.[4] Tetracycline has been previously determined in blood serum by using liquid chromatography,[5,6] high-performance liquid chromatography,[7−9] capillary electrophoresis,[10] stopped-flow mixing,[11] and differential-pulse polarography.[12] Very recently, methods based on titrimetry,[13] recombinant *Escherichia coli* sensors,[14] and electrospray ionization mass spectrometry[15] have also been described for the determination of tetracyclines. In general, conventional microbial methods show limits of detection on the order of 0.2 μg mL^{-1}, whereas HPLC or MS techniques display limits of detection which are about 10 times smaller. For these reasons, the latter methods are normally used to confirm the results of screening assays, although they involve rather complex extraction protocols. On the other hand, several spectrofluorometric methods have been used in the past, but they involve deproteinization, a tedious prior extraction step and show rather poor recoveries.[16−18] The combination of spectrofluorimetry with multivariate calibration may allow the development of methods for determining tetracycline in human blood which are simple, fast, specific, and sensitive.

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10.1021/ac990374e CCC: $18.00
Published on Web 08/28/1999

Table 1. Composition and Purpose of the Studied Sets of Samples

set no.	purpose	type of sera	total no. of samples	tetracycline added (μg mL^{-1})
C1	calibration	10 different pools of sera	50	0.0, 1.0, 2.0, 3.0, and 4.0 to each pool
V1	validation of the PLS-1 and HLA/XS calibration	18 different sera	36	0.0−4.0
V2	study of precision and accuracy	pool of normal sera	4	2.0
V3	calculation of figures of merit	4 different sera	16	0.0, 0.1, 0.2, and 0.4 to each serum
V4	interference study of aspirin	pool of normal sera with added sodium salicylate (90 μg mL^{-1})	1	1.0
V5	interference study of triglycerides and bilirubin	lipemic and icteric	2	1.0 to each sample

Multivariate calibration methods[19−21] applied to both absorptive and emissive spectral data as well as to electrochemical signals are increasingly being used for the analysis of complex biological mixtures.[22] They have the advantage of using full spectral information and allow for a rapid determination of mixture components, often with no need of prior separation or sample pretreatment. Pertinent examples in the field of biomedical analysis are the determination of urinary metabolites of aspirin by spectrofluorometry,[23,24] glucose in blood by near-infrared spectroscopy,[25] and calcium and magnesium in plasma by UV−vis spectrophotometry.[26] As part of a program devoted to the application of multivariate calibration techniques to biomedical analysis, we have recently reported a simple determination of teophylline in human serum by PLS calibration of electronic absorption data.[27]

In the present report we discuss the possibility of quantitating tetracycline and its derivatives in blood-serum samples, by applying synchronous spectrofluorometric measurements together with calibrations based on partial least-squares (PLS-1) and a variant of the so-called hybrid linear analysis (HLA). As will be shown below, an important degree of spectral overlap exists in the spectral region of interest among the fluorescence spectra of tetracycline (as the Mg^{2+} complex) and normal serum components (which may substantially vary among individuals). The problem thus consists of a single analyte embedded in a highly complex matrix which is difficult to reproduce. We have explored the use of the well-known PLS-1 formalism and a modification of the recently introduced HLA[28,29] for accomplishing this goal, together with a calibration design prepared to take into account the natural variability of the serum samples. Furthermore, we have performed net analyte signal (NAS) calculations with both of these multivariate methods in order to obtain several figures of merit. Finally, the presence of nonmodeled interferences in certain serum samples has been minimized through a novel variable selection procedure based on NAS regression plots.[30−32]

EXPERIMENTAL SECTION

Apparatus. All fluorescence measurements were carried out on a Shimadzu RF5300 PC spectrofluorophotometer equipped with a 150 W xenon lamp, using 1.00-cm quartz cells. The spectra were saved in ASCII format and transferred to a PC Pentium 166 microcomputer for subsequent manipulation.

Reagents. All experiments were performed with analytical-reagent-grade chemicals. Tetracycline was obtained from Richet Laboratories, and its purity was checked according to Pharmacopeia recommendations. Stock solutions of tetracycline and sodium salicylate containing 1.000 g L^{-1} and 3.000 g L^{-1}, respectively, were prepared by dissolving the compounds in doubly distilled water. Serum samples were prepared by spiking blank sera with appropriate amounts of the stock solution of tetracycline.

Calibration Set. It was estimated that the analyte of interest is present within a complex mixture of an unknown, but rather large, number of components. Therefore, to mimic the possible variation in the latter components, serum pools were used for calibration, each of them consisting of sera from 15 different patients. The calibration set (C1) was designed with 10 serum pools with added tetracycline in the range 0.0−4.0 μg mL^{-1} (Table 1). The selected concentrations cover the therapeutic range of tetracycline, and were previously verified to lie in the known linear fluorescence concentration range. The spectra were measured in random order with respect to the analyte concentration.

Validation Sets. Several validation sets of samples were prepared. Both the composition and the purpose of each set are shown in Table 1.

Technique. The appropriate amount of the stock solution of tetracycline was added to 1.00 mL of serum in order to obtain the desired concentration (in all cases the change in the volume of the serum sample was negligible). The sample was then homogenized, and 2.00 mL of magnesium acetate, 5×10^{-3} mol L^{-1}, in ammonium hydroxide, 3 mol L^{-1}, was added.[17] After homogenization, and within a period of 30 min from the sample preparation (in order to avoid signal-intensity changes), the synchronous fluorescence spectrum was recorded in the region

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λ_{em} = 400−600 nm, digitized every 1 nm, with a constant difference between both monochromators ($\Delta\lambda$) of 120 nm.

Theory. Notation. The following matrixes and vectors will be used throughout the present work: an $I \times J$ data matrix $\mathbf{R}$ composed of the calibration responses of I samples at J sensors, a $J \times 1$ vector $\mathbf{s}_k$ containing the pure spectrum of analyte k at unit concentration and an $I \times 1$ vector $\mathbf{c}_k$ of calibration concentrations of analyte k.

Net Analyte Signal. The NAS for analyte k ($\mathbf{r}_k$*) is defined as the part of its spectrum which is orthogonal to the space spanned by the spectra of all other analytes.[34] In general, for inverse calibration methods, it is given by the following equation

$$\mathbf{r}_k^* = [\mathbf{I} - \mathbf{R}_{-k}(\mathbf{R}_{-k})^{+}]\,\mathbf{r} = \mathbf{P}\mathbf{r} \tag{1}$$

where $\mathbf{r}$ is the spectrum of a given sample (when $\mathbf{r}$ is the spectrum $\mathbf{s}_k$ of pure k at unit concentration, eq 1 becomes $\mathbf{s}_k^* = \mathbf{P}\mathbf{s}_k$), $\mathbf{I}$ is a $J \times J$ unitary matrix, $\mathbf{R}_{-k}$ is a $J \times A$ column space spanned by the spectra of all other analytes except k ($\mathbf{R}_{-k}^{+}$ is the pseudoinverse of $\mathbf{R}_{-k}$ and A is the number of spectral factors used to build the model), and $\mathbf{P}$ is a $J \times J$ projection matrix which projects a given vector onto the NAS space. In factor-based methods such as PCR, PLS, and HLA, this latter statement is true only to the extent that the selected factors are representative of the spectra of all other analytes. Very recently, the calculation of $\mathbf{R}_{-k}$ has been discussed for PLS or PCR. It has been defined by the following rank annihilation expression[33]

$$\mathbf{R}_{-k} = \hat{\mathbf{R}} - \alpha\hat{\mathbf{c}}_k\hat{\mathbf{r}}^{\mathrm{T}} \tag{2}$$

where $\hat{\mathbf{R}}$ is the response matrix rebuilt using the first A significant factors of $\mathbf{R}$, $\hat{\mathbf{c}}_k$ is the projection of the calibration concentration vector $\mathbf{c}_k$ onto the A-dimensional space, $\hat{\mathbf{r}}$ is a linear combination of the rows of $\hat{\mathbf{R}}$ which contains a contribution from the spectrum of component k, and α is a scalar defined by[33]

$$\alpha = \frac{1}{\hat{\mathbf{r}}^{\mathrm{T}}\hat{\mathbf{R}}^{+}\hat{\mathbf{c}}_k} \tag{3}$$

which may be seen as the inverse of the estimated concentration of k in $\hat{\mathbf{r}}$. Any spectrum $\hat{\mathbf{r}}$ can be used to compute $\mathbf{R}_{-k}$ through eqs 2 and 3 and not necessarily the pure spectrum $\mathbf{s}_k$. However, it is advisable to use a spectrum which contains maximum information regarding the analyte of interest. A good candidate is the average of the calibration spectra containing nonzero contributions from k.

On the other hand, in both the HLA algorithms described below, the contribution from pure k is removed from the response data matrix before the calculation of factors. This causes the latter models to work with less factors than either PCR or PLS.[28,29]

HLA. Although the PLS algorithm is well-known, the recently introduced HLA method[28] deserves some comments. The latter involves constructing a blank data matrix $\mathbf{R}_{-k} = [\mathbf{R} - \mathbf{c}_k\,\mathbf{s}_k^{\mathrm{T}}]$ and using the first significant A eigenvectors of ($\mathbf{R}_{-k}^{\mathrm{T}}\mathbf{R}_{-k}$) to define the projection matrix $\mathbf{P}$.[28] The concentration of component k in an unknown sample is obtained from its spectrum $\mathbf{r}$ as

$$c_k = \frac{\mathbf{s}_k^{\mathrm{T}}\mathbf{P}\mathbf{r}}{\mathbf{s}_k^{\mathrm{T}}\mathbf{P}\mathbf{s}_k} = \frac{\mathbf{s}_k^{\mathrm{T}}\mathbf{P}\mathbf{P}\mathbf{r}}{\mathbf{s}_k^{\mathrm{T}}\mathbf{P}\mathbf{P}\mathbf{s}_k} = \frac{(\mathbf{s}_k^*)^{\mathrm{T}}\mathbf{r}_k^*}{||\mathbf{s}_k^*||^2} \tag{4}$$

which is the basis of the prediction step in NAS based methods.[34]

The optimum number of factors A can be obtained by the well-known cross-validation procedure.[19,35] It should be noticed that HLA can be applied, provided a very accurately measured spectrum of pure k is available.

Xu and Shechter have recently described an approach similar to HLA,[29] which does not require the knowledge of the pure spectrum $\mathbf{s}_k$. Instead, to obtain the matrix $\mathbf{R}_{-k}$, the mean calibration spectrum is first obtained

$$\bar{\mathbf{r}}_{\mathrm{cal}} = \frac{1}{I}\sum_{i=1}^{I}\mathbf{r}_{i,\mathrm{cal}} \tag{5}$$

where $\mathbf{r}_{i,\mathrm{cal}}$ is the spectrum for the ith calibration sample. Then the contribution of analyte k is subtracted from the data matrix $\mathbf{R}$ in the following way

$$\mathbf{R}_{-k} = \mathbf{R} - \frac{\mathbf{c}_k\bar{\mathbf{r}}_{\mathrm{cal}}^{\mathrm{T}}}{\bar{c}_{k,\mathrm{cal}}} \tag{6}$$

where $\bar{c}_{k,\mathrm{cal}}$ is the mean calibration concentration of analyte k. The least-squares approximation to $\mathbf{s}_k$ is subsequently used for the calculation of $\mathbf{s}_k$*:

$$\mathbf{s}_k^* = \mathbf{P}\,\mathbf{s}_{k,\mathrm{LS}} = \mathbf{P}\,\frac{\mathbf{R}^{\mathrm{T}}\mathbf{c}_k}{\mathbf{c}_k^{\mathrm{T}}\mathbf{c}_k} \tag{7}$$

Notice that eq 7 will approximate $\mathbf{s}_k$* even if the least-squares fit $\mathbf{s}_{k,\mathrm{LS}}$ contains contributions from the spectra of other analytes, provided the matrix $\mathbf{P}$ is able to cancel out these latter contributions. Although Xu and Shechter suggest using all the I factors of $\mathbf{R}_{-k}$ for prediction, to build a method free from optimum factor estimation,[29] we have selected the optimum number A by cross-validation. We shall call this method HLA/XS. It works in a manner similar to HLA (it is, in fact, a variant of the latter) and can be used even when the number of samples exceeds the recommended limit of one-third the number of sensors.[29] The presently discussed application of HLA/XS seems to be the first concerning samples of the complexity of human sera.

Incidentally, it should be noticed that the name HLA seems somewhat unfortunate. On one hand, the term "hybrid" has been proposed since HLA uses information on pure component spectra, unlike PLS and other multivariate methods. However, in the version of Xu and Shechter, this pure spectrum is no longer needed. On the other, "linear" refers to the underlying assumption in computing the matrix $\mathbf{R}_{-k}$ (see eq 6). Nevertheless, when small nonlinearities occur in the calibration set, only their linear part

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will be removed from **R** through eq 6, causing $\mathbf{R}_{-k}$ to retain any remaining nonlinearities. Extra factors will be needed in order to accommodate for such effects. Overall, the performance of HLA is therefore expected to be similar to PLS in handling nonlinearities.[19]

Wavelength Selection. In the present work, wavelength selection has been performed by calculating, for each test sample, an error indicator (EI) as a function of a moving window, using information from the NAS regression plot (NASRP).[30−32] The latter is a plot of the elements of the sample vector $\mathbf{r}_k$* vs those of $\mathbf{s}_k$*, and the moving window was obtained by varying both the position of the first sensor and the sensor range. The expression for EI used in the present context is[31]

$$\mathrm{EI} = \frac{\left[s_{\mathrm{fit}}^{\,2}\left(1 + \frac{N^2 s_{\mathrm{fit}}^{\,2}}{4||\mathbf{r}_k^*||^2}\right)\right]^{1/2}}{||\mathbf{r}_k^*||} \qquad (8)$$

where $||\mathbf{r}_k^*||$ is the norm of the net analyte spectrum for the test sample, s_{fit} is the standard deviation of the best-fitted straight line to the NASRP (in a given spectral region), and N is the number of points in the latter plot. In this work, the vectors $\mathbf{r}_k$* and $\mathbf{s}_k$* were calculated from either PLS or HLA/XS, but the latter method seems to be simpler to implement.

Figures of Merit. Selectivity, sensitivity, and limit of determination can be calculated and used for method comparison or to study the quality of a given analytical technique. Different approaches exist in the literature for obtaining the former figures of merit for multivariate calibration methods.[36,37] One method relies on NAS calculations, as suggested by Boqué and Rius[38] for classical least-squares models and is extended to inverse multivariate methods by Lorber.[33] In this formulation, a limit of decision in signal space can be set as

$$\mathrm{LOD} = \Delta(\alpha,\beta)\,\delta r \qquad (9)$$

where $\Delta(\alpha,\beta)$ is the noncentrality parameter of a noncentral t-distribution, which can be calculated numerically[38] or taken from statistical tables,[39] and δr is an estimate for the standard deviation of the measurement errors.

NAS calculations also allow estimation of other figures of merit such as the selectivity[33,37]

$$\mathrm{SEL} = ||\mathbf{s}_k^*||/||\mathbf{s}_k|| \qquad (10)$$

and the sensitivity[37]

$$\mathrm{SEN} = 1/||\mathbf{b}|| \qquad (11)$$

where **b** is the vector of final regression coefficients, which can be obtained by any multivariate method.

A different strategy has been discussed to assess whether a sample does contain the analyte of interest, which is based on the calculation of confidence limits for the predicted concentrations.[33,37] In this approach, the uncertainty region for the concentration in a particular sample is checked for inclusion of the value of zero or not. The confidence interval (CI) is given by:

$$(\mathrm{CI})^2 = t_{J-A}^{\,2}\sum_{j=1}^{J} b_j^2 \delta r_j^2 + t_{I-A}^{\,2} s_{\mathrm{cal}}^{\,2}\sum_{i=1}^{I} h_i^2 \qquad (12)$$

In the latter expression, t_{J-A} and t_{I-A} are appropriate t coefficients at a given confidence level, with $(J - A)$ and $(I - A)$ degrees of freedom, respectively; b_j are the elements of the **b** vector; δr_j^2 are estimates of the variance at each sensor; s_{cal}^2 is the calibration variance; and h_i are the elements of the sample leverage.[33,37]

It should be noted that the very definition of the limit of detection in multivariate calibration, which is

$$\mathrm{LOD} = 3||\epsilon||\,||\mathbf{b}|| \qquad (13)$$

where $||\epsilon||$ is a measure of the instrumental noise, has been criticized because the amount of analyte which can be detected depends on the concentration of each interference.[37] Hence, the approaches described above tend to focus on each particular sample in order to test whether the analyte is present or not, rather than on obtaining a given concentration as the limit of detection. However, in the present context, eq 13 appears to give results which are consistent with the above analysis.

Programs. Multivariate calibration and wavelength selection were applied with in-house programs written in Visual Basic 5.0 according to ref 19 and refs 28 and 29, respectively.

RESULTS AND DISCUSSION

Analytical Methodology. The first step in applying synchronous fluorescence spectra is the estimation of the optimum value of $\Delta\lambda$.[40] One method for selecting the latter parameter involves the construction of the excitation−emission contour plots for overlapping signals, in this case those from tetracycline (as the Mg^{2+} complex) and blank sera. However, for reasons which will be clear below, the spectrum obtained for pure tetracycline is not useful in this regard. Instead, a more useful contour plot (shown in Figure 2) was built with spectra for a basal serum sample and for the spectral difference between the latter and its mixture with tetracycline (in both cases after prior treatment with Mg^{2+}). The straight line in Figure 2 corresponds to the path with $\Delta\lambda = 120$ nm, which was selected as optimum since it passes near the maximum of the tetracycline peak. Other choices of $\Delta\lambda$ led to poorer results.

Figure 3 compares the synchronous fluorescence spectra of an aqueous solution of pure tetracycline and the spectral difference between a serum sample with and without tetracycline (in all cases after treatment with Mg^{2+}). It is apparent that the interaction with the serum significantly modifies the spectral properties of the analyte. This fact has a deep influence on the selection of the

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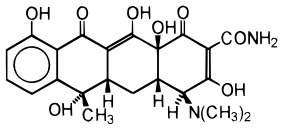

Figure 1. Structure of tetracycline.

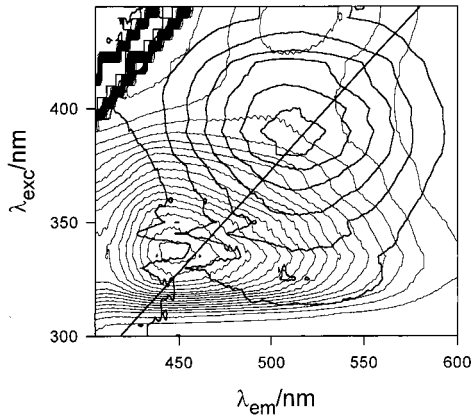

Figure 2. Excitation−emission contour plot for a representative serum sample (narrow solid lines) and for the spectral difference between a blank serum and a serum containing tetracycline 3.0 μg ml^{-1} (thick solid lines). Both samples were treated with Mg^{2+} prior to measurements. The straight line represents the path for $\Delta\lambda = 120$ nm.

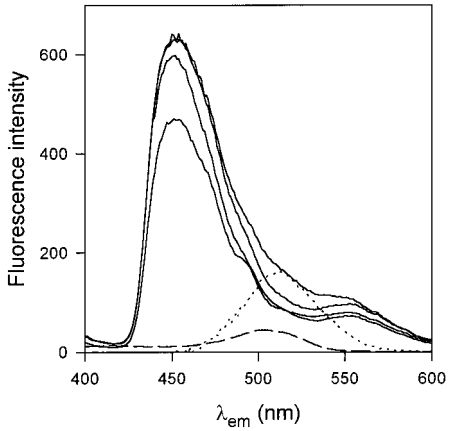

Figure 3. Synchronous fluorescence spectra of: (—), several human sera; (···), spectral difference between a blank serum and a serum containing tetracycline 3.0 μg mL^{-1}; and (− − −), pure tetracycline 3.0 μg mL^{-1}. In all cases the spectra were recorded with $\Delta\lambda = 120$ nm. Both samples were treated with Mg^{2+} prior to measurements.

multivariate calibration method. In particular, the original HLA formalism may not be useful in the present context, since it requires the knowledge of a very accurate pure spectrum of the analyte of interest. Since tetracycline/Mg^{2+} interacts with highly variable basal sera, it is apparent that the vector $\mathbf{s}_k$ required by HLA would be very difficult to obtain. Figure 3 also shows synchronous fluorescence spectra of several whole human sera, highlighting both their intrinsic variability and the high degree of overlapping of their signals with that of tetracycline. Maximum information concerning tetracycline can be apparently gathered from measurements in the region $\lambda_{em} = 450-550$ nm (Figure 3). Deproteinization of the serum samples did not significantly improve the resolution, and hence, this experimental procedure was not incorporated into the method.

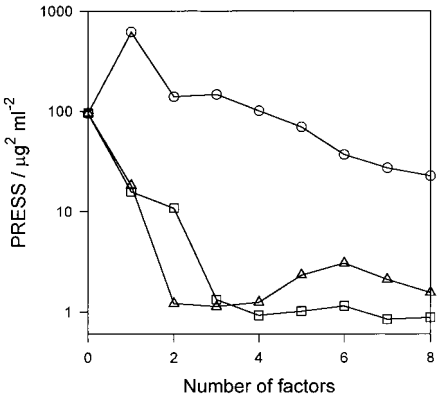

Figure 4. Values of the PRESS as a function of the number of factors using: PLS-1, squares; HLA, circles; and HLA/XS, triangles.

Table 2. Optimum Number of Factors and Calibration Statistical Parameters When Applying PLS-1 and HLA/XS Analyses

	PLS-1		HLA/XS	
statistical parameter[a]				
spectral range[b] (nm)	400−600	450−550	400−600	450−550
factors	4	4	5	2
RMSD (μg mL^{-1})	0.146	0.137	0.169	0.154
REP%	7.82	7.32	9.01	8.25
R^2	0.988	0.990	0.985	0.987
SEL	0.37		0.40	
SEN	103.2		109.7	
analytical SEN (γ)[c]/mL μg^{-1}	20.6		21.9	

[a] $R^2 = 1 - \sum_1^I (c_{act} - c_{pred})^2 / \sum_1^I (c_{act} - \bar{c})^2$, where $\bar{c}$ is the average component concentration in the I calibration mixtures; RMSD $= [(1/I)\sum_1^I (c_{act} - c_{pred})^2]^{1/2}$ and REP% $= (100/\bar{c})[(1/I)\sum_1^I (c_{act} - c_{pred})^2]^{1/2}$. [b] The range given is the emission spectral range corresponding to synchronous fluorescence spectra. [c] Calculated as (SEN/δr), with $\delta r \approx 5$ (see text and Table 4).

Since fluorescence spectra for Mg^{2+} (or Ca^{2+}) complexes of other tetracyclines are similar to that of the parent compound,[16−18] the technique could also be applicable to derivatives of the latter.

Calibration and Selection of the Optimum Number of Factors. The optimum number of factors to be used within the multivariate algorithms is an important parameter to achieve better performance in prediction. This allows modelling of the system with the optimum amount of information, avoiding overfitting. We have applied the cross-validation procedure in all cases, which consists of systematically removing one of the training samples, in turn, and using only the remaining ones for construction of the latent factors and/or regression coefficients.[19,35] The predicted concentrations were then compared with the actual ones for each of the calibration serum samples, and the predicted error sum of squares [PRESS $= \sum(c_{act} - c_{pred})^2$)] was calculated. The PRESS was computed in the same manner, each time a new factor was added to the PLS model. Figure 4 shows the changes in the PRESS as a function of the number of factors included in the model for the various multivariate methods employed. As can be seen, both PLS-1 and HLA/XS yield reasonable results, whereas the original HLA version is seen to be inappropriate. This may be traced to the observed differences in the spectrum of tetracycline/Mg^{2+} when it is interacting with serum components. To select the optimum number of factors, the criterion proposed by Haaland and Thomas was used.[19] In our case, the value of F

corresponding to a probability smaller than 0.75 yielded the optimum number of factors of 4 for PLS-1 and 2 for HLA/XS in the spectral region $\lambda_{em} = 450-550$ nm (Table 2). Notice that the performances of both methods improve when the latter region is selected from the full spectral range 400−600 nm (Table 2).

Usual statistical parameters giving an indication of the quality of fit of all the data are the root-mean-square difference (RMSD), square of the correlation coefficient (R^2), and relative error of prediction (REP%). The obtained values for the present calibration are summarized in Table 2. PLS-1 and HLA/XS are seen to yield comparable results, although the latter method works with less factors and is simpler to implement.

Selectivity and Sensitivity. Table 2 collects the selectivity and sensitivity for tetracycline using the presently discussed method, as calculated by both multivariate methods according to eqs 10 and 11, respectively. The reasonably good obtained values are in mutual agreement. Notice that $||\mathbf{s}_k||$ cannot be calculated for pure tetracycline, but should be estimated from the spectral difference shown in Figure 3. Also shown in Table 2 is the analytical sensitivity, γ, which may be more useful for method comparison, since fluorescence intensity units are arbitrary. The parameter γ may be defined, in analogy to univariate calibration, as the quotient (SEN/δr). It allows one to compare analytical methods regardless of the specific technique, equipment, and scale employed and establishes the minimum concentration difference (γ^{-1}) which is statistically discernible by the method.[41] In the present case, $\gamma^{-1} \approx 0.05\ \mu g\ mL^{-1}$. In comparison, HLA/XS yields better results than PLS-1 (Table 2).

Prediction. Samples of set number V1 (Table 1) were analyzed with the above procedure, with representative results shown in Table 3. Both the recoveries and values of REP% are acceptable. Notice that the latter parameter is calculated with the predicted values for the validation sets of samples and may differ from that quoted above for calibration.

With samples of set number V1 (those quoted in Table 3 and additional ones up to a total of 18), plots of c_{pred} vs c_{act} were constructed both for PLS-1 and HLA/XS. The results are: slope = 0.91(2), intercept = 0.01(5) (PLS-1), and slope = 1.00(2), intercept = −0.02(4) (HLA/XS). The joint confidence regions for the slope and intercept are shown in Figure 5. Both methods give regions containing the theoretically expected value of (1,0), but HLA/XS seems more successful than PLS-1 (Figure 5). This is so even when the calibration statistical indicators are slightly better for PLS-1 (see Table 2) and may be due to the higher selectivity and sensitivity of HLA/XS (Table 2).

Limit of Determination. To gather insight into the limit of determination of the presently described method, several samples were prepared with low concentrations of tetracycline (in the order of 0.00−0.40 μg mL^{-1}). For these samples, both approaches described in the Experimental Section were applied. The results concerning NAS calculations are summarized in Table 3. According to this Table, we may estimate $\delta r \approx 5$ (the standard deviation for several blank serum samples). Using the noncentrality parameter $\Delta(0.05,0.05) = 3.312$,[39] Table 3 and eq 9 predict that a given sample does contain the analyte provided the norm of its NAS is at ∼17 units from that of the blank. Table 3 indicates that samples containing 0.40 μg mL^{-1} yield NAS norm values well below the blank (notice that the concentrations are mean-centered, hence NAS norm values are actually lower for the sample than for the blank), while those with 0.20 μg mL^{-1} are close to the limit of decision based on eq 9. In those with 0.1 μg mL^{-1}, on the

Table 3. Predictions from PLS-1 and HLA/XS for a Serum Pool with Spiked Tetracycline in Concentrations from 0.0 to 4.0 μg mL^{-1} and NAS Norms and Confidence Itervals for Samples with Low Concentrations

tetracycline added (μg mL^{-1})	mean tetracycline predicted[a] (μg mL^{-1})	recovery (%)	confidence interval (μg mL^{-1})[a]	NAS norm[a]
		PLS-1		
0.00	0.00 (2)	-	0.15 (3)	195 (5)
0.10	0.06 (2)	60.0	0.20 (5)	194 (5)
0.20	0.20 (3)	100.0	0.13 (2)	175 (5)
0.40	0.39 (3)	96.9	0.12 (2)	155 (5)
0.60	0.67 (2)	111.3	0.11 (2)	
0.80	0.81 (1)	101.3	0.08 (1)	
1.00	1.02 (1)	101.5	0.06 (2)	
1.50	1.42 (3)	94.7	0.05 (2)	
2.00	1.98 (2)	99.0	0.04 (2)	
2.50	2.40 (2)	96.0	0.04 (2)	
3.00	2.91 (3)	97.0	0.07 (2)	
3.50	3.47 (2)	99.1	0.10 (2)	
4.00	3.86 (5)	96.5	0.11 (2)	
RMSD[b] (μg mL^{-1})/REP%[b]		0.060/4.3		
		HLA/XS		
0.00	−0.04 (2)	-	0.15 (2)	210 (5)
0.10	0.00 (2)	-	0.27 (5)	209 (5)
0.20	0.18 (3)	90.0	0.12 (2)	187 (6)
0.40	0.36 (2)	90.0	0.11 (2)	167 (5)
0.60	0.61 (2)	101.7	0.10 (2)	
0.80	0.77 (1)	96.3	0.09 (2)	
1.00	0.98 (1)	98.0	0.08 (1)	
1.50	1.49 (2)	99.3	0.06 (2)	
2.00	1.97 (2)	98.5	0.04 (1)	
2.50	2.51 (3)	100.4	0.02 (1)	
3.00	2.97 (2)	99.0	0.05 (2)	
3.50	3.57 (4)	102.0	0.07 (2)	
4.00	4.03 (5)	100.8	0.11 (3)	
RMSD[b] (μg mL^{-1})/REP%[b]		0.042/2.8		

[a] Four replicates. Values in parentheses are standard deviations (n = 4). [b] RMSD and REP% correspond to the validation set and may differ from calibration values.

Table 4. Predictions in μg mL^{-1} from PLS-1 and HLA/XS for a Serum Pool Spiked with 2.0 μg mL^{-1} Tetracycline during Three Weeks

	week 1		week 2		week 3	
replicate	PLS-1	HLA/XS	PLS-1	HLA/XS	PLS-1	HLA/XS
1	2.01	2.00	2.15	2.18	1.85	1.87
2	2.00	1.99	2.18	2.23	1.89	1.91
3	1.97	1.98	2.11	2.17	1.88	1.91
4	1.95	1.95	2.10	2.17	1.88	1.91
mean[a]	1.98(3)	1.98(2)	2.14(4)	2.19(3)	1.88(2)	1.90(2)
recovery (%)	99.0	99.0	107.0	109.5	94.0	95.0

	PLS-1	HLA/XS
RMSD (μg mL^{-1})/REP%[b]	0.110/5.48	0.125/6.25

[a] Values in parentheses are standard deviations (n = 4). [b] These values of RMSD and REP% correspond to the validation set and may be different than calibration values (see Table 3).

(41) Cuadros Rodríguez, L.; García Campaña, A. M.; Jimenez Linares, C.; Román Ceba, M. *Anal. Lett.* **1993**, *26*, 1243−1258.

Table 5. Results Obtained by Applying PLS-1 and HLA/XS Analyses to Sera with Potential Interferents and Calibration Statistics in Restricted Spectral Regions

serum	wavelength range (nm)	EI		tetracycline (μg ml^{-1})		
					found[a]	
		PLS-1	HLA/XS	actual	PLS-1	HLA/XS
lipemic serum	450−550	0.52	0.50	1.00	1.77 (4)[c]	0.97 (2)[c]
(triglycerides as interferent)	521−570[b]	0.03	0.02		0.78 (3)	1.00 (1)
icteric serum	450−550	0.38	0.50	1.00	1.14 (4)[c]	1.32 (2)[c]
(bilirubin as interferent)	491−550[b]	0.12	0.02		1.24 (4)[c]	1.04 (2)
pool of normal sera with	450−550	0.43	0.51	1.00	0.95 (4)[c]	1.58 (2)[c]
added sodium salicylate as interferent	491−552[b]	0.06	0.05		0.94 (4)	1.00 (2)

statistical parameter	PLS-1		HLA/XS	
spectral range (nm)	521−570	491−550	521−570	491−550
factors	3	2	2	1
RMSD (μg mL^{-1})	0.207	0.169	0.305	0.197
REP%	11.12	9.07	16.42	10.59
R^2	0.977	0.985	0.949	0.979

[a] The cross-validation optimum number of factors used for prediction in each spectral region is given in parentheses. [b] The minimum EI was found by varying both the spectral window and the number of calibration factors within each window. [c] Considered as outliers by PLS-1 and HLA/XS, on the basis of the calculation on the spectral $F(\mathbf{r})$ ratio, i.e., $F_{x,y}(\mathbf{r}) = I\sum_{j=1}^{J}(e_{r,j})^2/\sum_{i=1}^{I}\sum_{j=1}^{J}(e_{i,j})^2$, where $e_{r,j}$ are the spectral residues for the unknown sample $\mathbf{r}$ and $e_{i,j}$ are the corresponding residues for the I calibration samples, $x = (J - A)/2$ and $y = (J - A)(I - A - 1)/2$.[19]

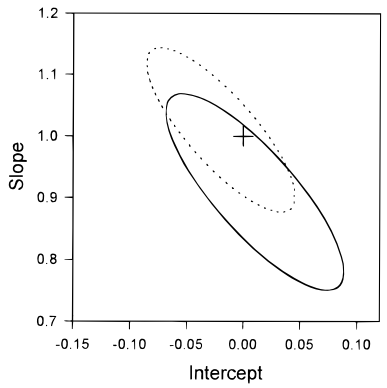

Figure 5. Joint confidence regions for slope and intercept as calculated from: (—), PLS-1; and (···), HLA/XS. The cross marks the point (1,0).

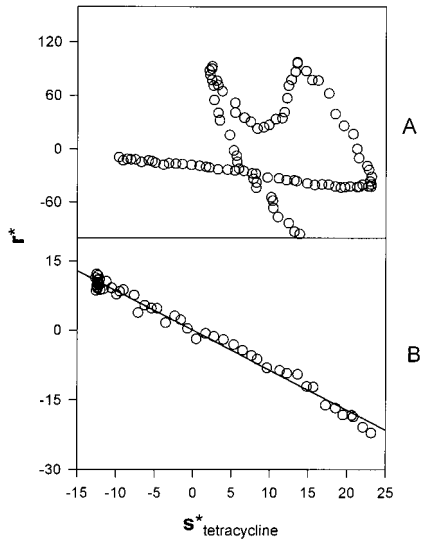

Figure 6. NASRPs for a serum containing triglycerides as interference: (A) in the region 450−550 nm and (B) in the region of minimum error indicator (521−570 nm).

other hand, the presence of the analyte cannot be statistically ascertained. Therefore, this study suggests that the limit of determination is near 0.20 μg mL^{-1}. Notice that PLS and HLA/XS give results concerning NAS norms which are in close mutual agreement.

A second approach relies on the estimation of confidence intervals for the predicted concentrations. Table 3 shows the results provided by PLS-1 and HLA/XS using eq 12. The confidence intervals increase for decreasing tetracycline concentrations (due to increasing leverages) and also suggest that the limit of determination is ~0.2 μg mL^{-1}.

Finally, eq 13 provides a simple estimation of the LOD as ~0.15 μg mL^{-1} ($||\epsilon|| \approx \delta r$ and $||\mathbf{b}|| \approx 100$), which is in agreement with the above discussion.

Precision. Four triplicates of set number V2 (Table 1) were analyzed by PLS-1 and HLA/XS during a period of three weeks, to study the intraassay and interassay variabilities. The results concerning predictions, standard deviations, and recoveries are acceptable for both methods (Table 4). The interassay standard deviation was 0.1 μg mL^{-1} in both cases.

Accuracy and Recovery. Tables 3 and 4 show the results concerning RMSD and recovery when PLS-1 and HLA/XS were applied to the validation sets V1 and V2 (Table 1). As can be seen, the statistical indicators are all reasonably good.

Interferences. Table 5 collects the results concerning the possible interference of triglycerides, bilirubin, and salicylate (the strongly fluorescent serum metabolite of aspirin, added in typical plasmatic concentrations) using sets number V4 and V5 (Table 1). To study the effect of nonmodeled interferences, a strategy based on HLA/XS/NAS calculations was applied to each of these test samples (see Experimental Section). This involves identifying the potential presence of an interference and alleviating its effects by selecting an appropriately restricted working region.[31,32] An example is provided by the NASRP for a lipemic serum shown in

Figure 6A in the spectral region $\lambda_{em} = 450-550$ nm. As can be seen, linearity is not fulfilled, strongly indicating the presence of nonmodeled interferences. A search for the minimum value of the EI as a function of all possible wavelength ranges indicates that the corresponding NASRP in the restricted region 521−570 nm (Figure 6B) is seemingly linear (see also the values of the EI in Table 5). It may be noticed that the latter spectral range avoids the region where the contribution from triglycerides is significant (i.e., 450−510 nm). Table 5 shows the predictions by HLA/XS and PLS-1 for all test samples containing potential interferences, before and after wavelength selection by the above procedure. It is apparent that, through the use of this NASRP method, regions are appropriately selected in which the interferences do not show significant contributions. Interestingly, HLA/XS considers the samples of sets number V4 and V5 as outliers in the spectral region 450−550 nm, but does not detect anomalies in the regions of minimum EI (Table 5). PLS-1, on the other hand, continues to treat the icteric serum as an outlier, even in the minimum EI region. Overall, the performance of HLA/XS with these samples is seen to be better than that of PLS-1.

It may be noticed that the calibration statistical indicators in the restricted regions shown in Table 5 are worse than those corresponding to the region 450−550 nm (Table 2), due to the fact that the former ranges exclude a portion containing spectral contributions from the analyte. The reward is a significantly improved prediction ability for samples containing nonmodeled interferents.

CONCLUSIONS

Quantification of tetracycline in human sera has been accomplished from synchronous fluorescence spectral data obtained after treatment with Mg^{2+}, in conjuntion with two multivariate calibrations: partial least-squares (PLS-1) and a modification of the recently introduced hybrid linear analysis (HLA). The calibration set was designed with 50 sera spiked with concentrations of tetracycline in the range 0.0−4.0 μg mL^{-1}. Validation, precision, accuracy, and figures of merit (selectivity, sensitivity, and limit of determination) were studied. A novel wavelength selection procedure was also applied, to alleviate the effect of nonmodeled interferents (bilirubin, triglycerides, and salicylate). Overall, the newly developed HLA/XS approach shows a better performance when compared with PLS-1.

ACKNOWLEDGMENT

Financial support from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), the University of Rosario, the Fundación Antorchas and the Agencia de Promoción Científica y Tecnológica (Project No. 06-00000-01765) is gratefully acknowledged. H. C. Goicoechea thanks FOMEC (Programa para el Mejoramiento de la Calidad de la Enseñanza Universitaria) for a fellowship. The authors thank Bioq. Darío Holman (Sanatorio 7 de Marzo, Santo Tomé, Santa Fe, Argentina) for providing the serum samples. We also thank Professor Arsenio Muñoz de la Peña (Departamento de Química Analítica, Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain), who pioneered the application of multivariate calibration to spectrofluorimetry, for his helpful advice and support.

Received for review April 8, 1999. Accepted July 7, 1999.

AC990374E