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Simultaneous determination of pteridines in multicomponent mixtures using derivative spectrophotometry and partial least-squares calibration

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Abstract

Simple binary mixtures composed of very similar pteridines (neopterin and pterin) have been resolved by derivative spectrophotometry. Detection limits of 0.30 μg ml^{-1} and 0.12 μg ml^{-1} for pterin and neopterin, respectively, have been calculated. Also, different mixtures of pteridines considered as disease markers, such as pterin, neopterin, xanthopterin and isoxanthopterin, have been determined by using a partial least-squares (PLS-2) model. Calibration set containing 0–7 μg ml^{-1} for each component was used. The resolution of several mixtures and single determination was tested in artificial samples.

Keywords: Pteridines; Multicomponent analysis; Derivative-spectrophotometry; Partial least-squares method

1. Introduction

Pteridin derivatives are a family of organic compound with very similar chemical structures which play an important biochemistry role. Pteridines metabolism and its regulation in normal and pathological conditions have not been extensively investigated due to the difficulty of their quantification. In the last decade, their relationship to patients with various disorders has been described [1]. Increased concentration of neopterin in body fluids has been reported in patients suffering from three groups of disorders [2], hyperphenylalaninaemia, malignancy and several inflammatory disease states. Results about the imbalance in pterins and possibly folate metabolism, are associated with the presence of malignant diseases. A significant increase in the urinary excretion of xanthopterin, neopterin and pterin and a significant decrease of isoxanthopterin were determined in cancer patients [3]. In addition, a disproportion was noted in the relative excretion levels of neopterin and biopterin in cancer patients. An increased excretion in the neopterin/biopterin ratio was reported. The mentioned studies suggest that monitoring of pteridin levels may be of potential value for controlling the course of malignant diseases.

Several difficulties were described for the simultaneous determination of pteridin compounds.

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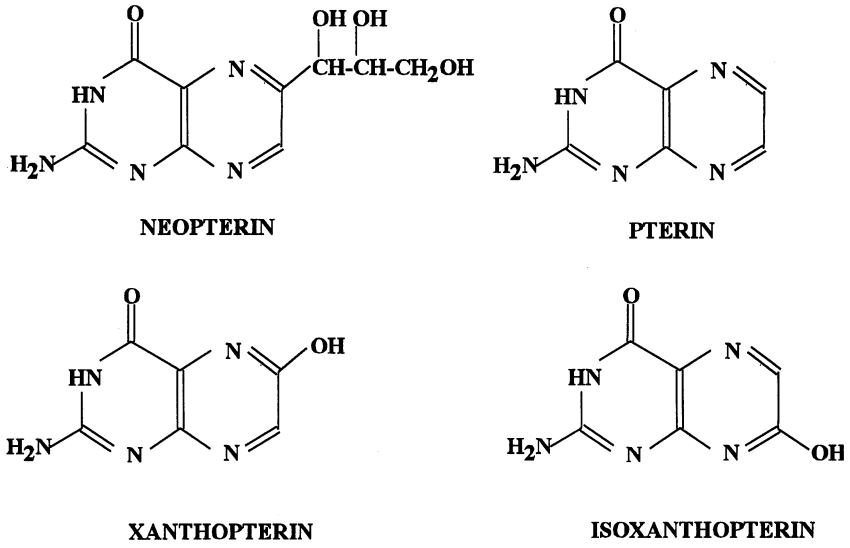

Fig. 1. Formulations of different pteridines.

Photolability (exposure to direct sunlight has to be avoided), similar chemical structures and small concentrations in biological fluids are some of them. Separation processes have often been used for determining pteridines in multicomponent mixtures. HPLC (high performance liquid chromatography) is the most widely applied. Pteridines have been essentially determined in body fluids [4] and in urine [5]. Woolf et al. [4] developed a chromatographic method for resolving mixtures of six pteridines, considered as diseases markers (biopterin and other pterins). This method have been applied in tissues and in urine. Trehan et al. [5] determined eight pteridines in urine by HPLC, at a very time-consuming 20 min per chromatogram; however, this method does not provide separation between isoxanthopterin and pterin.

No data about the application of chemometrical methods has been found in the literature. However, in the recent years, several multicomponent analysis methods based on spectral properties have [6] been demonstrated as powerful tools in diverse analysis fields. Derivative spectrophotometry has been widely applied in pharmaceutical analysis to analyse simple mixtures of drugs [7,8]. Partial least-squares methods (PLS) [9,10] have been applied to resolve complex mixtures of organic compounds [11] and metals [12] by using conventional, and also, kinetic signals [13].

The aim of this paper was to investigate, for the first time, the possibility of using derivative spectrophotometry and PLS methods for quantifying pteridines, such as neopterin, pterin, xanthopterin and isoxanthopterin (Fig. 1), in mixtures. The advantage of multicomponent analysis using multivariate calibration is the speed of these methods, as a separation step is avoided.

2. Experimental

2.1. Apparatus

A Milton Roy Spectronic 3000 diode array spectrophotometer, provided with the Rapid Scan Milton Roy software package V2.2, was used for all acquisition of spectrophotometric data. The Beckman Data Leader software, version 3.0 was used for manipulation of the spectral data. Differentiation was performed according to the simplified least squares procedure of Savitzky and Golay [14,15]. The GRAMS-386 Version 3.0 software package with the PLS plus/IQ application software [16], was used for the statistical treatment of the data and the application of the PLS methods.

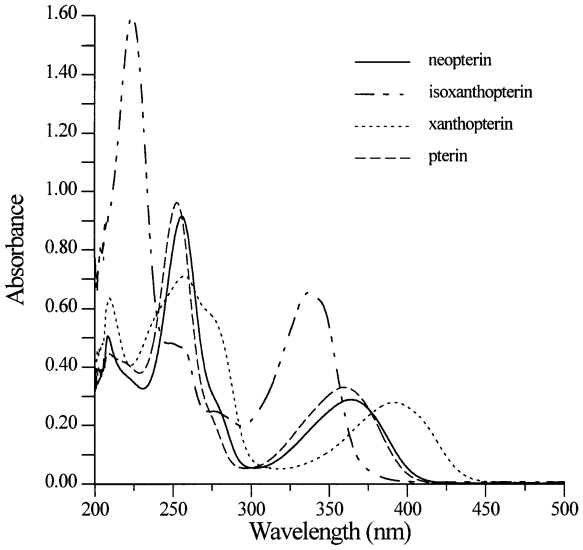

Fig. 2. Absorption spectra of neopterin, pterin, xanthopterin, and isoxanthopterin at pH 10.2. Concentration of each pteridin is 10 μg ml^{-1}.

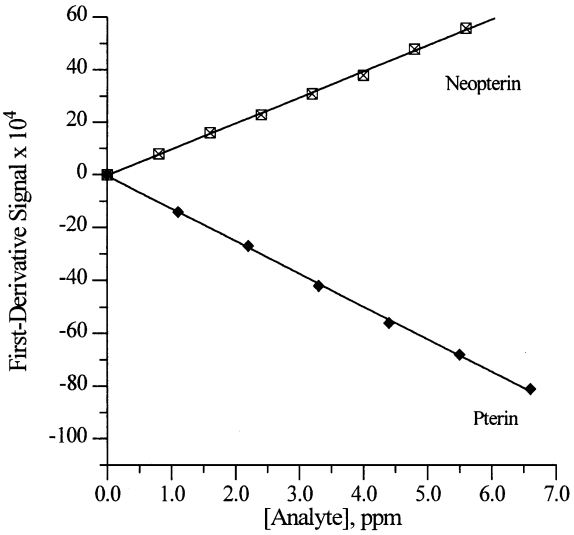

Fig. 4. Calibration graphs for the determination of (A) neopterin and (B) pterin.

2.2. Reagents

All solvents used were of analytical reagent quality. Pterin (97%), xanthopterin (97%), isoxanthopterin (97%) and neopterin (97%) were purchased from Sigma and standard solutions were prepared by dilution in water (avoid the exposure to direct sunlight). Buffer solution (pH 10.2) was prepared from ammonium chloride/ammonia (0.5 M).

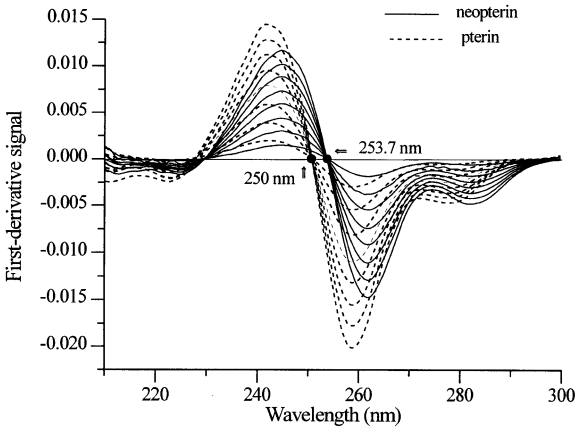

Fig. 3. First-derivative spectra of neopterin and pterin ($\Delta\lambda = 11$ nm) at different concentrations. The wavelengths for the simultaneous determination of both compounds, zero-crossing points, are marked.

2.3. Procedures for analysing binary mixtures of neopterin and pterin

In a 10-ml calibrated flask, place an aliquot of the sample containing up to 100 μg of neopterin and/or pterin, 1 ml of pH 10.2 buffer ammonium chloride/ammonia solution and then dilute to the mark with deionized water. The absorption spectrum between 200 and 500 nm was recorded. The spectra of all solutions, were measured against a blank of buffer solution. The first-derivative spectra ($\Delta\lambda = 11$ nm) were obtained. Determine pterin content from first-derivative spectrum by measuring first-derivative signal at zero-crossing point for neopterin, at 253.7 nm, and comparing with appropriate calibration graph. Determine neopterin content by measuring first-derivative signal at zero-crossing point for pterin, at 250.0 nm and comparing the values with the appropriate calibration graphs.

Table 1
Statistical parameters for calibration graphs for the simultaneous determination of pterin and neopterin

Component	Slope	σ_{slope}	Intercept	$\sigma_{intercept}$	R	LD[a]	LD[b]
Pterin	-1.17×10^{-3}	2.11×10^{-5}	-2.43×10^{-4}	1.17×10^{-4}	0.9990	0.30	0.48
Neopterin	9.22×10^{-4}	9.32×10^{-6}	1.03×10^{-4}	3.77×10^{-5}	0.9997	0.12	0.22

σ, standard deviation.
LD, limit of detection in μg ml^{-1}.
[a] Winefordner criterion with $k=3$ [17].
[b] Clayton criterion ($\alpha=\beta=0.05$) [18].

2.4. Procedure for analysing mixtures of pterin, neopterin, xanthopterin and isoxanthopterin

In a 10-ml calibrated flask, place an aliquot of the sample containing up to 70 μg of each pterin compound, 1 ml of pH 10.2 buffer solution and dilute with deionized water to the mark. The absorption spectra of all solutions are measured against a blank of buffer solution. The optimized calibration matrix calculated by application of the PLS-2 method is applied to analyse the spectra of the samples and calculate the concentration of pterin, neopterin, xanthopterin and isoxanthopterin in the mixture.

3. Results and discussion

The four pteridines show a strong overlap between their absorption spectra. In Fig. 2, the absorption spectra (at pH 10.2) for the four components are shown. Pterin ($\lambda_{max}=359$ nm and 253 nm), neopterin ($\lambda_{max}=368$ nm and 256 nm) and xanthopterin ($\lambda_{max}=393$ nm, 275 nm and 260 nm) show very similar absorption spectra. Isoxanthopterin ($\lambda_{max}=338$ nm and 224 nm) exhibits the most different spectral characteristics. No better results are obtained at other pH values. At pH <10, a hypochromic effect is observed for neopterin and pterin, and a higher spectral overlapping with isoxanthopterin, in the range between 300 and 375 nm, is observed. Then, separation process prior to the application of univariate spectrophotometric methods is necessary.

3.1. Simultaneous determination of pterin and neopterin

In Fig. 3, the significant spectral overlapping between pterin and neopterin can be observed. Using pH 10.2, the ability of the derivative spectrophotometry to resolve binary mixtures of both pteridines was tested. The optimization of the derivative spectra was based on the influence of the $\Delta\lambda$ used in the differentiation of the absorption spectra with the object of optimized the relation signal to noise ratio. In the first derivative

Table 2
Results obtained in the resolution of binary mixtures of pterin and neopterin by derivative spectrophotometry

Problem	Pterin			Neopterin		
	Actual (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)	Actual (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)
M1	1.00	1.05	105	5.00	5.09	102
M2	5.00	4.99	99.8	1.00	1.00	100
M3	3.00	3.36	112	1.00	0.91	91
M4	2.00	1.90	96	4.00	3.91	98
M5	0.00	0.00	—	1.00	1.00	100
M6	1.00	1.05	105	2.00	2.00	100

Table 3
Calibration set composition and concentration predicted by the model

Standard	[Neopterin] ($\mu g\ ml^{-1}$)		[Xanthopterin] ($\mu g\ ml^{-1}$)		[Pterin] ($\mu g\ ml^{-1}$)		[Isoxanthopterin] ($\mu g\ ml^{-1}$)	
	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]
1	6.00	6.17	6.00	6.23	2.00	2.30	4.00	4.02
2	4.00	4.10	1.00	1.34	7.00	7.46	5.00	4.90
3	2.00	2.16	2.00	1.74	1.00	1.03	1.50	1.46
4	7.00	7.22	3.00	2.93	5.00	4.78	2.00	1.96
5	6.50	5.55	5.00	4.74	3.00	2.89	2.50	2.50
6	7.00	7.23	1.50	1.46	7.00	6.91	1.00	1.09
7	5.00	4.84	3.50	3.55	2.50	2.69	6.00	5.90
8	2.00	1.91	4.00	3.99	6.00	5.97	4.00	4.12
9	0.00	−0.36	3.50	3.45	1.00	0.72	7.00	7.07
10	2.50	2.67	0.00	−0.069	5.50	5.28	0.80	0.79
11	1.00	1.80	5.50	5.35	0.00	−0.11	6.00	5.93
12	1.00	1.22	4.50	4.42	6.50	6.01	0.00	−0.005
13	1.50	1.44	0.00	−0.08	0.00	0.019	1.00	1.00
14	0.00	−0.17	2.00	2.07	3.50	3.58	0.00	−0.01
15	6.00	5.88	0.00	−0.05	0.00	−0.09	6.50	6.56
16	0.00	−0.16	5.50	5.48	6.00	6.07	0.00	−0.02
17	0.00	−0.28	0.00	−0.03	0.00	0.094	0.50	0.50
18	0.00	−0.04	0.50	0.41	0.00	−0.011	0.00	−0.03
19	2.00	2.06	2.00	2.35	2.50	2.74	2.00	2.06
20	0.80	0.89	0.00	−0.39	0.00	−0.46	0.00	−0.08
21	3.00	3.22	3.00	3.25	0.00	0.12	1.00	1.03

[a] By using seven factors and PLS2 algorithm.

mode, when the $\Delta\lambda$ value increased from 3 to 25 nm, the signal amplitudes decreased slightly, and a $\Delta\lambda = 11$ nm was selected for the calculation by the Savitzky-Golay method as this gives the best signal to noise ratio. In Fig. 3, the first derivative spectra ($\Delta\lambda = 11$ nm) for different concentrations of pterin and neopterin are shown. Two zero-crossing points were located at 250 nm and 253.7 nm for pterin and neopterin, respectively. Ranges of concentrations up to 8 $\mu g\ ml^{-1}$ for pterin and neopterin, were tested to obtain calibration graphs for both compounds. Adequate linearity is observed in both cases, as we can be shown in Fig. 4. In Table 1, the statistical parameters calculated from the calibration graphs are summarized. Pterin and neopterin were determined simultaneously in synthetic mixtures by measuring the signals at the selected wavelengths in the first derivative spectra and the results of the determinations of the different binary mixtures are summarized in Table 2. Recovery values of 96–112% for pterin and 91–102% for neopterin were obtained.

3.2. Simultaneous determination of pterin, neopterin, xanthopterin and isoxanthopterin

The high overlap observed between the absorption spectra of pterin, neopterin, xanthopterin and isoxanthopterin indicates that univariate and/or derivative methods cannot be applied. PLS is a factor analysis-based method that was recently demonstrated to have a high capacity to resolve complex mixtures of components with similar spectral characteristics. PLS performs the spectral factoring by trying to account for the spectral variation while assuming that the new basis vectors relate to the calibration concentrations. When the PLS method is used to calibrate a single chemical constituent at a time it is termed PLS-1, for the calibration of several constituents simultaneously it is called PLS-2 regression. PLS-1 may

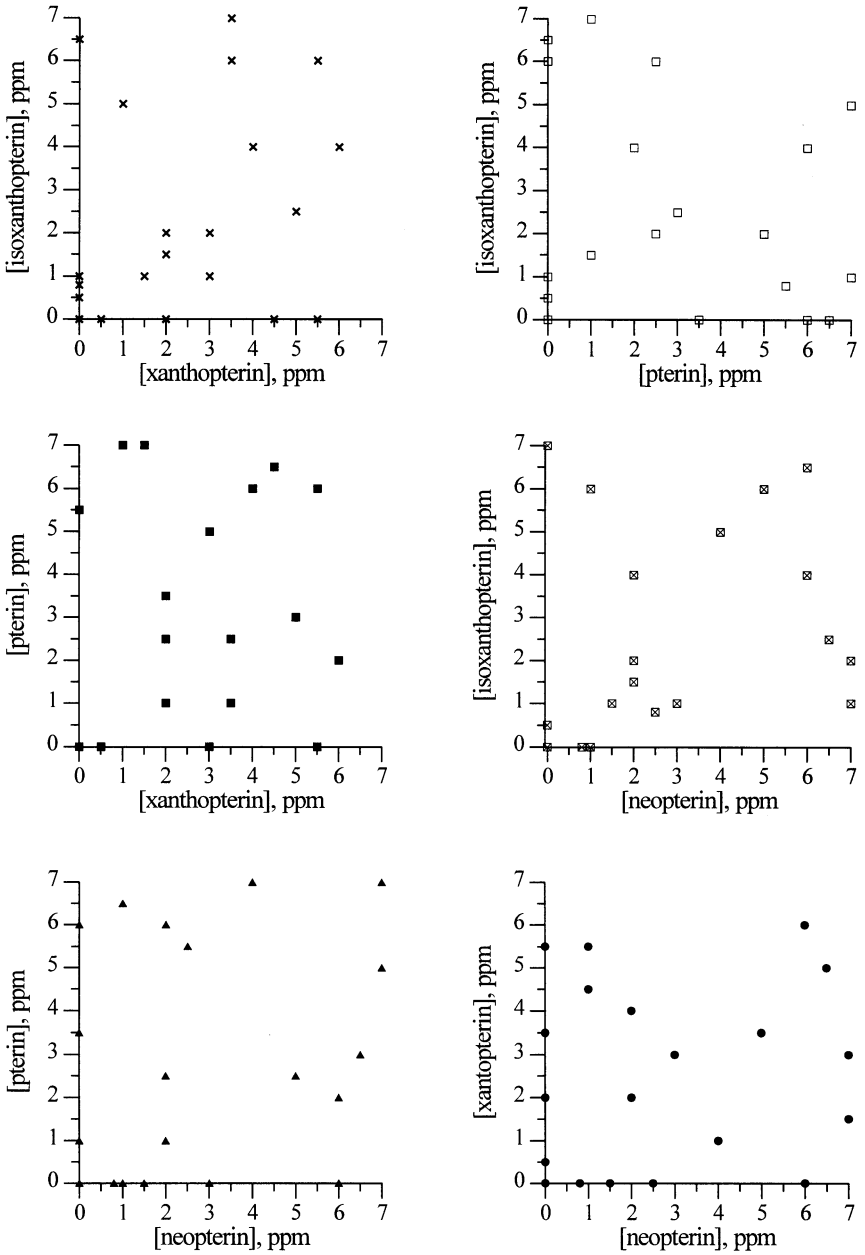

Fig. 5. Actual vs. actual concentration plots for the standard samples used in the calibration set for the evaluation of the predictive ability of the method.

have the greatest advantage when analyzing systems that have constituent concentrations that are widely varied. If the concentration ranges of the constituents are approximately the same, PLS-1 may have less of an advantage over PLS-2.

To choose the calibration samples, the components to be determined have been selected in order to span all dimensions. A training set of 21 standard samples (randomly selected) was taken from different mixtures of pteridines (Table 3). Each

Table 4
Statistical parameters of the PLS-2 model

Component	R^2	RMSD	PRESS[a]
Neopterin	0.98331	0.3311	4.0701
Xanthopterin	0.99161	0.1509	
Pterin	0.99245	0.1762	
Isoxanthopterin	0.99939	0.0524	

[a] By using seven factors.

concentration was varied between 0 and 7.0 μg ml^{-1} through the calibration matrix. The correlation between the different calibration samples has to be avoided because collinear components in the training set data will tend to cause underfitting in the PLS models. In Fig. 5, actual versus actual concentration plots for the different calibration samples for training set are represented. A no co-linear distribution of the component concentrations can be observed. The spectral region between 205 nm and 475 nm, which implies working with 743 experimental points per spectrum, was selected for analysis. In this spectral region, the maximum spectral information from the mixture is present.

To select the number of factors in the PLS algorithm, a cross-validation method leaving out one sample at a time, was employed. PLS-2 calibration on 20 calibration spectra was performed and, using this calibration, the concentration of the sample left out during the calibration process was calculated. These processes were repeated a total of 21 times until each sample had been left out once. The concentration of each sample was then predicted and compared with the known concentration of the reference sample. The prediction error sum of squares (PRESS) [19] was calculated in the same manner each time a new factor

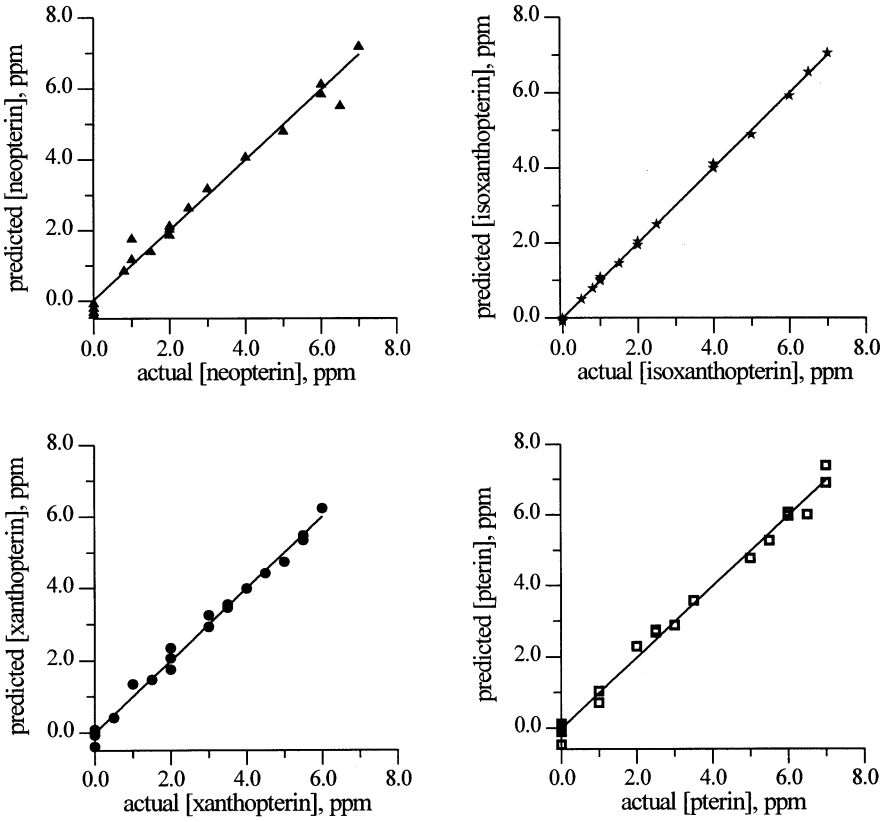

Fig. 6. Actual vs. predicted concentration plots obtained for the calibration set using PLS-2 calibration.

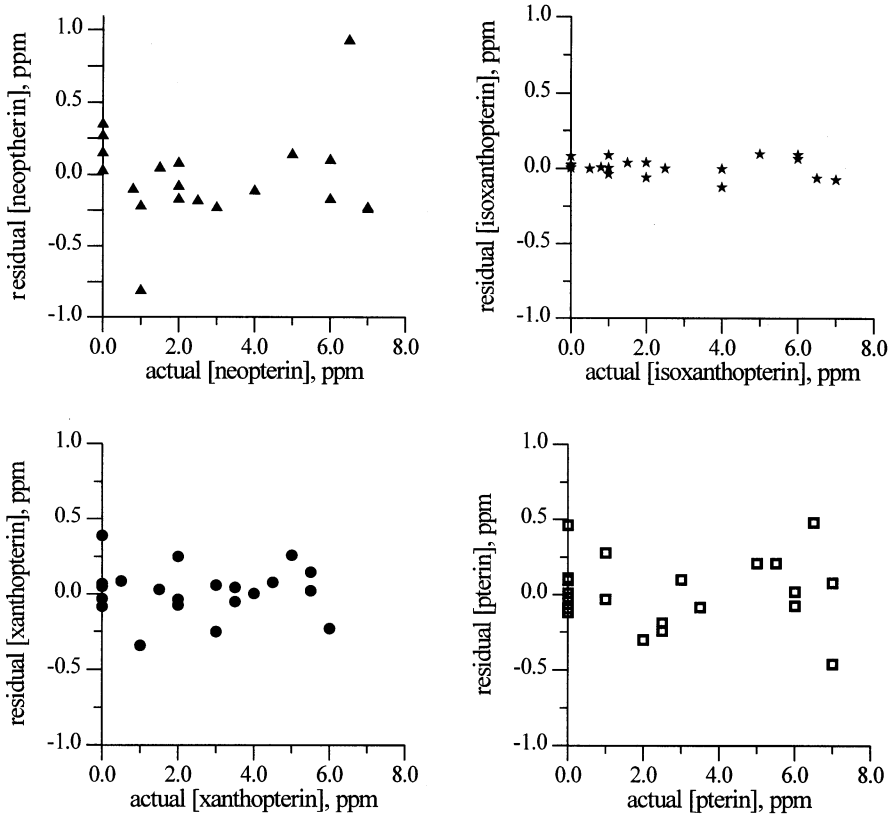

Fig. 7. Residual concentration plots obtained for calibration set by application of the PLS-2 calibration.

was added to the PLS model. The estimated time necessary for the total process was 3 min. Similar results were obtained by application of PLS-1 method. The maximum number of factors used to calculate the optimum PRESS was 13 and a *F*-statistic test was used to carry out the significance determination [19]. The optimum number of factors was seven for the optimized model and with the PLS-2 method.

The PRESS values obtained for each component with the optimum number of factors are summarized in Table 4. Statistical parameters about the quality of the optimized model were calculated. The values of the root mean square difference (RMSD) [19], which is an indication of the average error in the analysis for each component and the square of the correlation coefficient (R^2), which is an indication of the quality of fit of all the data to a straight line were calculated and the results are summarized in Table 4. The best R^2 value was obtained for isoxanthopterin determination and the lowest value for neopterin. The actual versus predicted concentration for each component has been calculated for this model (Fig. 6) and the predicted concentrations have been also included in Table 3. In addition, the residuals analysis [20] permits us to determine an amount that is not explained by the model for each data point,

$$\epsilon_i = y_i - \hat{y}i \text{ being } \hat{y}i = b_i x_i$$

where y_i is the data value and $\hat{y}i$ is the predicted value by the model; b_i is a regression parameter and x_i is the independent variable. In Fig. 7, the residual concentration values for all components are represented. The smallest residual values are obtained for neopterin and isoxanthopterin determinations. Higher values are obtained for pterin

Table 5
Resolution of a test set composed by neopterin, pterin, xanthopterin and isoxanthopterin in different mixtures by multivariate analysis

Test set	[Neopterin] ($\mu g\ ml^{-1}$)			[Xanthopterin] ($\mu g\ ml^{-1}$)			[Pterin] ($\mu g\ ml^{-1}$)			[Isoxanthopterin] ($\mu g\ ml^{-1}$)		
	Actual	Found	Recovery (%)	Actual	Found	Recovery (%)	Actual	Found	Recovery (%)	Actual	Found	Recovery (%)
1	2.00	2.13	106	1.00	0.82	82	0.00	−0.09	—	0.00	0.00	—
2	4.00	4.18	104	3.00	3.50	116	6.00	6.33	105	0.00	−0.06	—
3	0.00	−0.13	—	2.00	2.33	116	4.00	4.43	110	2.00	1.92	96
4	1.00	0.88	88	1.00	0.92	92	1.00	1.07	107	1.00	0.95	95
5	2.00	2.06	103	2.00	2.11	105	2.00	2.30	115	2.00	2.03	101
6	0.00	−0.30	—	0.00	0.00	—	1.00	1.13	113	2.00	1.94	97
7	0.00	−0.06	—	0.00	−0.26	—	0.00	−0.26	—	4.00	3.88	97
8	1.00	1.10	110	5.00	5.37	107	3.00	3.17	106	0.00	0.01	—
9	3.00	3.16	105	3.00	3.18	106	0.00	0.083	—	1.00	1.02	102
10	1.00	0.99	99	0.00	−0.30	—	0.00	−0.048	—	0.00	−0.033	—

determination. The model resolves satisfactorily all components assayed.

One set of ten unknown artificial samples were predicted by applying the model. The composition of the test set is summarized in Table 5. The recovery values obtained using the model are also included in Table 5. Satisfactory recovery values are obtained in most of the samples analyzed. The recovery values were 88–110% for neopterin, 82–116% for xanthopterin, 105–115% for pterin, and 95–102% for isoxanthopterin.

4. Conclusion

The proposed derivative method can be used to determine simple binary mixtures of pterin and neopterin. PLS-2 model can be used to make single or simultaneous determination of pterin, neopterin, xanthopterin and isoxanthopterin in complex mixtures. The PLS-2 model can be applied to resolve up to quaternary mixtures in dispensing with the chromatographic method. The chromatographic procedures are rather time-consuming for routine assays and require an expensive apparatus. For the first time, chemometrics have been applied to simultaneous resolution of pteridines and permit us to obtain an adequate resolution between the four pteridines assayed.

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