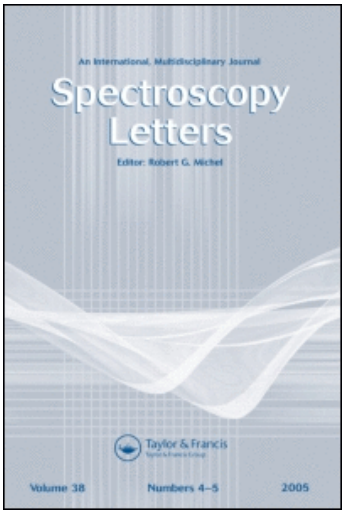

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SIMULTANEOUS SPECTROPHOTOMETRIC DETERMINATION OF FOLIC ACID, THIAMIN, RIBOFLAVIN, AND PYRIDOXAL USING PARTIAL LEAST-SQUARES REGRESSION METHOD

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ABSTRACT

A multivariate calibration method (Partial least squares regression, PLS-1), is applied to the simultaneous determination of folic acid (vitamin B_0), thiamin (vitamin B_1), riboflavin (vitamin B_2) and pyridoxal (vitamin B_6), in artificial mixtures by ultraviolet/visible absorption spectrophotometry. The concentration ranges used to construct the calibration matrix are 0.4–15.0, 0.7–30, 0.2–11 and 0.8–30 $\mu g\,mL^{-1}$ for vitamin B_0, B_1, B_2 and B_6 respectively. The absorption spectrum of the quaternary mixtures is used to perform the optimization of the calibration matrices by the PLS-1 method. For this purpose a cross-validation procedure for estimating the number of principal components in a matrix, is used. Satisfactory

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recovery values are obtained in most of the quaternary samples analyzed. Mean recovery values for these vitamins are 95–105%.

Key Words: Simultaneous analysis; Folic acid; Thiamin; Riboflavin; Pyridoxal; Partial least squares

INTRODUCTION

Multideterminations of analytes that exhibit absorbance signals in the UV-Vis region have become common place in most analytical laboratories such as clinical, food control, and pharmaceutical. UV-Vis spectroscopic methods have the advantage of simplicity, speed and low cost.

The greatest difficulties with UV-Vis multidetermination methods arise when the analytes to be determined give partly or fully overlapped spectra as is the case with the ingredients of most pharmaceutical preparations. It has been proven that multivariate calibration methods, especially partial least squares (PLS) has a high potential as a calibration-prediction methodology for processing absorbance signals of drugs. In spectrophotometric multicomponent analysis there is a large number of variables for each object, corresponding to the absorbance measured at the various wavelengths of the spectrum. Multidetermination methods are not highly influenced by the prevailing strong spectral overlap throughout the absorption wavelength range[1,2].

A mixture of vitamins, such as vitamin B complex and multivitamins in tablets and other pharmaceutical preparation, are used in treatment of several diseases. Therefore the simultaneous determination of mixture of vitamins is of great importance for the pharmaceutical industry. Several chemometric methods, such as Kalman filtering, principal component regression(PCR), partial least squares(PLS) and artificial neural network (ANN) were applied for simultaneous spectrophotometric and spectrofluorimetric determination of folic acid, thiamine, riboflavin and pyridoxal in combination with other pharmaceutical preparations[3–8]. To the best of our knowledge, no report has been published on the determination of quaternary mixtures of these vitamins.

In the present work, the partial least-squares method is applied for resolving both the matrix and the overlapped signals, with a view to carrying out the simultaneous spectrophotometric determination of these vitamins.

EXPERIMENTAL SECTION

Apparatus

Electronic absorption measurements were carried out on a CECIL 9000 spectrophotometer (slit width 0.2 nm and scan rate 350 nm/min) using 1.00 cm quartz cells, that was interfaced to a 386 PC computer. A Metrohm 692 pH-meter furnished with a combined glass-saturated calomel electrode was used for pH measurements. The meter was calibrated with at least two buffer solutions at pH 3.00 and pH 8.00.

Computer Hardware and Software

All absorbance spectra were digitized and recorded at wavelengths from 220 to 490 nm in steps of 1 nm and then transferred (in ASCII format) to a Pentium 200 MHz computer for subsequent manipulation by the PLS program.

The data treatment was done with MATLAB for windows (Math Works, version 4.2). The PLS program (for calibration-prediction and experimental design) was applied as an in-house program written in MATLAB according to the algorithm described in references[9,10].

Reagents and Chemicals

All experiments were performed with analytical-reagent grade chemicals. Doubly distilled water was used. Stock solutions (500 $\mu g\,mL^{-1}$) of folic acid (vitamin B_0[11]), thiaminum hydrochloride (vitamin B_1), riboflavin (vitamin B_2) and pyridoxal (vitamin B_6) were prepared by direct weighing of the required amount of commercially available reagent (all from Merck). Then by dissolving vitamin B_0 and vitamin B_2 in NaOH 0.01 M, and the two other vitamins in doubly distilled water. These solutions were spectrophotometrically stable for at least two weeks.

Working solutions containing 100 $\mu g\,mL^{-1}$ of each vitamin were prepared by the appropriate dilution of the stock solutions and were stored at room temperature.

To adjust the optimum pH for the simultaneous determination of these compounds, a 0.5 M HCl and a 0.5 M NaOH aqueous solutions were used. Buffer solutions were made from 1 M sodium acetate (Merck), the pH 3.5 to 6 was adjusted by adding of 1 M HCl or NaOH as required and diluting to 500 mL with bidistilled water.

Analytical Procedures

Individual Calibration

Known amounts of the standard solutions of each vitamin were placed (with a standard microsyringe) in a 10 mL volumetric flask and 1 mL of HAcO/NaAcO buffer solution added to it and then diluted to the final volume with deionized water (final pH: 5.8). The concentrations of vitamin B_0, vitamin B_1, vitamin B_2 and vitamin B_6 were between 0.2–24 $\mu g\,mL^{-1}$, 0.2–32 $\mu g\,mL^{-1}$, 0.2–16.6 $\mu g\,mL^{-1}$ and 0.2–36 $\mu g\,mL^{-}$, respectively and then their spectra were recorded between 220 nm and 490 nm against buffer solution as a blank. The absorbance signals were measured at λ_{max} of each vitamin spectra, that is, 284 nm, 235 nm, 265 nm and 325 nm, for vitamin B_0, B_1, B_2 and B_6, respectively.

Preparation of Calibration and Problem (Test) Sets

In order to design a series of mixtures, the maximum and minimum concentrations of each compound must first be estimated with the use of a linear calibration range of each compound, given by c_{maxk} and c_{mink}. By changing these values, an upper and lower limit to the summed absorbance are obtained as follows:

$$A_{maxj} = \sum_{k=1}^{K} c_{maxk}\varepsilon_{jk} \quad (1)$$

$$A_{minj} = \sum_{k=1}^{K} c_{mink}\varepsilon_{jk} \quad (2)$$

The concentration ranges were chosen so that the absorbance obtained for all standard samples was not greater than 1.0 (A_{max}) and was not smaller than 0.1 (A_{min}).

The composition of solutions used for the PLS-1 is presented in Tables 1A and 1B. Quaternary mixtures (calibration and problem/validation) of vitamin B_0, B_1, B_2 and B_6 were prepared as follows: A 25 mL volumetric flask was filled with 1 mL of HAcO/NaAcO buffer at pH 5.8, an appropriate volume of vitamin solution to obtain final concentrations of 0.4–15 $\mu g\,mL^{-1}$ of vitamin B_0, 0.7–30 $\mu g\,mL^{-1}$ of vitamin B_1, 0.2–11.0 $\mu g\,mL^{-1}$ of vitamin B_2 and 0.8–30 $\mu g\,mL^{-1}$ of vitamin B_6, with the

Table 1A. Composition of Vitamin B Solutions for the Synthetic Series; Concentration in μg mL^{-1} (Calibration Solutions)

Solution	B_0	B_1	B_2	B_6
1	2.35	2.55	1.45	3.50
2	0.75	4.35	0.95	1.35
3	1.00	2.50	2.35	2.25
4	4.15	1.45	2.90	0.80
5	4.70	2.35	1.30	1.20
6	0.45	0.95	0.25	2.15
7	1.60	1.80	4.90	1.55
8	2.15	0.70	1.65	1.05
9	1.00	3.00	2.00	3.00
10	3.30	9.60	8.30	3.50
11	1.00	8.80	6.40	4.70
12	4.00	2.60	8.90	8.30
13	0.90	2.70	6.10	4.30
14	1.40	4.50	3.90	4.00
15	5.70	4.20	9.70	4.30
16	0.00	3.10	4.40	3.10
17	0.00	4.00	6.00	0.00
18	8.20	9.60	1.20	26.40
19	1.40	4.50	1.10	12.90
20	11.60	1.30	1.90	1.60
21	4.20	18.10	1.70	19.60
22	4.80	2.20	4.00	9.30
23	1.60	1.80	5.00	3.60
24	1.40	12.60	5.10	1.20
25	9.40	7.70	0.50	28.90
26	9.50	5.10	2.00	17.10
27	7.30	21.60	1.00	8.40

addition of deionized water to the end volume. The compositions of the samples were randomly designed in order to obtain maximum information on each vitamin from the calibration procedure.

Because of highly overlapping spectra at the wavelength region below 226 nm, mixed spectra were registered over the wavelength range from 226 to 490 nm against buffer solution as a blank. An integration time of 1 sec was used throughout.

Table 1B. Composition of Vitamin B Solutions for the Synthetic Series; Concentration in μg mL^{-1} (Problem Solutions)

Solution	B_0	B_1	B_2	B_6
1	4.00	1.00	6.00	1.00
2	4.00	6..00	4.00	4.00
3	5.10	9.00	0.20	2.60
4	4.20	2.00	2.70	2.70
5	2.00	1.60	2.00	1.00
6	1.80	5.90	1.30	6.50
7	3.30	5.10	5.40	2.00
8	1.10	22.50	1.90	12.10
9	3.80	4.80	5.00	9.00
10	3.20	4.00	1.60	9.10
11	0.60	5.30	7.80	1.30
12	7.90	5.60	2.70	10.70
13	14.00	8.20	1.40	1.30

RESULTS AND DISCUSSION

Conventional Univariate Calibration

Figure 1 shows the absorption spectra for aqueous solutions of vitamin B_0, B_1, B_2 and B_6 and their quaternary mixture at pH 5.8. As can be seen, vitamin B_0 gave a single absorption maximum (at 284 nm), vitamin B_1 gave two absorption maxima (a stronger one at 235 nm and a weaker one at 265 nm), vitamin B_2 gave three absorption maxima (a strong band at 265 nm and two other at visible region) and vitamin B_6 two maxima (a stronger one at 325 nm and a weaker one at 253 nm). The spectra of vitamin B_2 and B_1 overlap for wavelengths greater than 247 nm and also with the B_0 at wavelengths lower than 272 nm. One of the characteristics of this multideterminationis the specific absorbance of single compound at the visible region (vitamin B_2).

In order to establish the optimal measurement conditions for the joint determination, we used the univariate method to investigate the effect of experimental variables on the absorption spectra of the vitamins.

At first the stability of the four vitamins in the aqueous solution was checked. For this purpose, the UV-Vis absorption spectra for solutions of the four compounds were recorded as a function of time and it was found that the spectra for all vitamins did not vary appreciably for at least two weeks, provided that the solutions were kept at room temperature and in the dark.

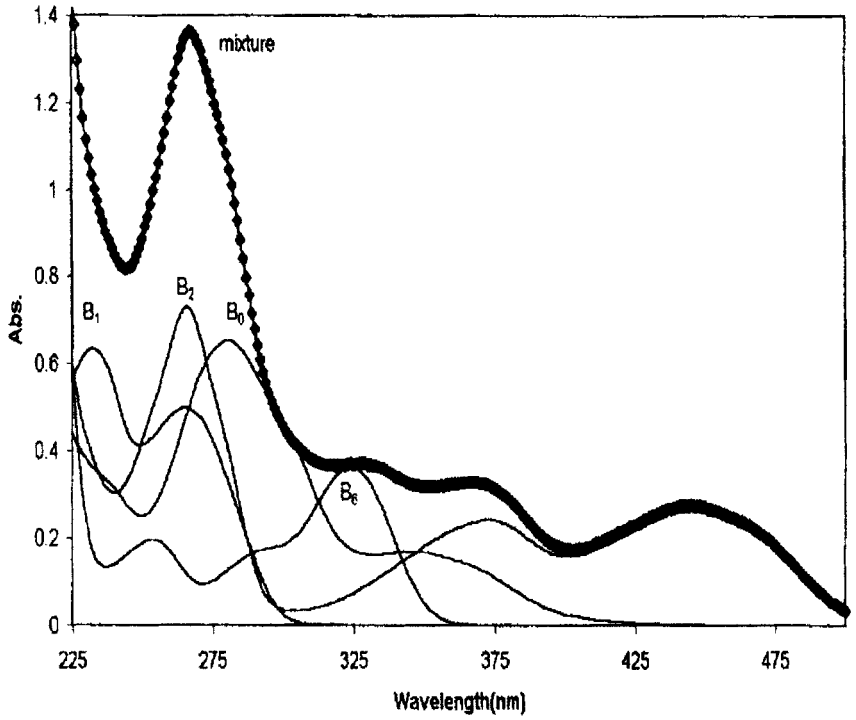

Figure 1. Spectra of vitamin B_0, B_1, B_2, B_6, and their mixture at pH 5.8. The concentration of vitamins are: B_0 (10.91 ppm), B_1 (19.25 ppm), B_2 (8.39 ppm), B_6 (11.74) and in mixture are: 7.27, 9.62, 8.39, and 5.87-ppm respectively.

The influence of the acidity and basicity of the medium on the absorption spectra of vitamins was studied (over the pH range 1.0–12.0), in order to select the optimum pH value at which the minimum overlap occurs. pH values were adjusted by addition of small volumes of dilute solutions of HCl or NaOH. The spectrum of vitamin B_1 markedly affected by the pH of the medium, the absorbance signal increased with decreasing acidity and a new absorption maximum gradually appeared at 235 nm due to the hydrolysis product. The spectrum of vitamin B_2 exhibits a little significant change at whole pH range. In contrast, the signal for vitamin B_6 was decreased with decreasing acidity at 290 nm and increased at 325 nm until the pH 12.0, coinciding with a hypsochromic shift in absorption maximum. In the case of vitamin B_0 (folic acid), this compound precipitated at pH values below 4.0.

In order to minimize the spectral overlapping and also to avoid the potential hydrolysis of the vitamins by highly acidic or alkaline media, pH 5.8 was adopted as optimal for subsequent experiments.

Several buffer solutions were tested and the HAcO/NaAcO was selected according to its minimum effect of structural and spectral characteristics of their compounds on the vitamin spectral behavior and thus this solution was employed for subsequent work. In order to determine the optimal buffer concentration, a series of experiments was conducted in the range 0.01–0.1 M. This concentration range were found to have no considerable effect on the spectra of the compounds so an intermediate buffer concentration 0.04 M, was accepted. The temperature was found to have no appreciable effect on the spectra of the analytes, so 25°C was chosen for subsequent work.

The linearity of the maximal signals was examined to select an adequate concentration range (for each individual compound) suitable for spectrophotometric measurements. Individual calibration curves were constructed with several points (Fig. 2) as absorbance versus vitamin concentration in the range of 0.3–30 $\mu g\,mL^{-1}$ and evaluated by linear regression. (their equations and correlation coefficients are presented in Fig. 2).

PLS Calibration Results

Multivariate calibration methods are suitable for the analysis for a large number of samples. However, they are not advisable for the determination of large numbers of analytes due to the complexity of the calibration matrix. Moreover, the preparation and analysis of the standards belonging to the calibration set are the most expensive step in any multivariate calibration procedure.

Multivariate calibration methods such as PLS require a suitable experimental design of the standards belonging to the calibration set in order to provide good predictions. A synthetic set of 40 solutions of mixtures of vitamin B_0, B_1, B_2 and B_6 were prepared (Tables 1A and 1B). From the series, 27 solutions were chosen for the calibration set and the other 13 were used as problem solutions. This selection was performed on the basis of their distributions on the first and second principal components as are shown in Fig. 3. Problem solutions were chosen according to their plotted position on the graph, inside the range of calibration solutions. (i.e., lies in the calibration range).

The spectral region between 226 and 490 nm, which implies working with 264 experimental points (λ) per spectrum (as the spectra are digitized at

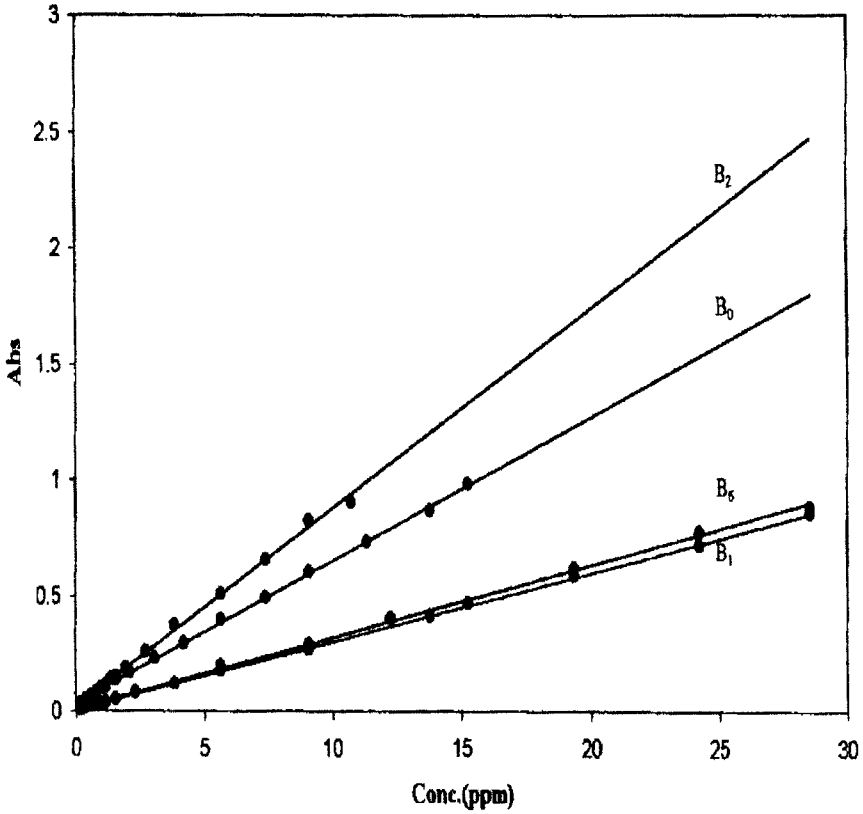

Figure 2. Analytical curves for univariate determination of synthetic vitamin B mixtures. Vitamin B_0: $Abs = 0.0618 \times Conc. + 0.0328$, $R = 0.99987$, $\lambda = 284$ nm; Vitamin B_1: $Abs = 0.0311 \times Conc. + 0.0064$, $R = 0.99971$, $\lambda = 235$; Vitamin B_2: $Abs = 0.8555 \times Conc. + 0.0188$, $R = 0.99945$, $\lambda = 265$ nm; Vitamin B_6: $Abs = 0.0317 \times Conc. + 0.0050$, $R = 0.99742$, $\lambda = 325$.

every 1 nm interval) was selected for analysis. In this spectral region, the maximum spectral information is shown.

PLS Program Output

The spectral data as ASCII format are fed into the MATLAB program and the processing started by mean centering and scaling of the data and

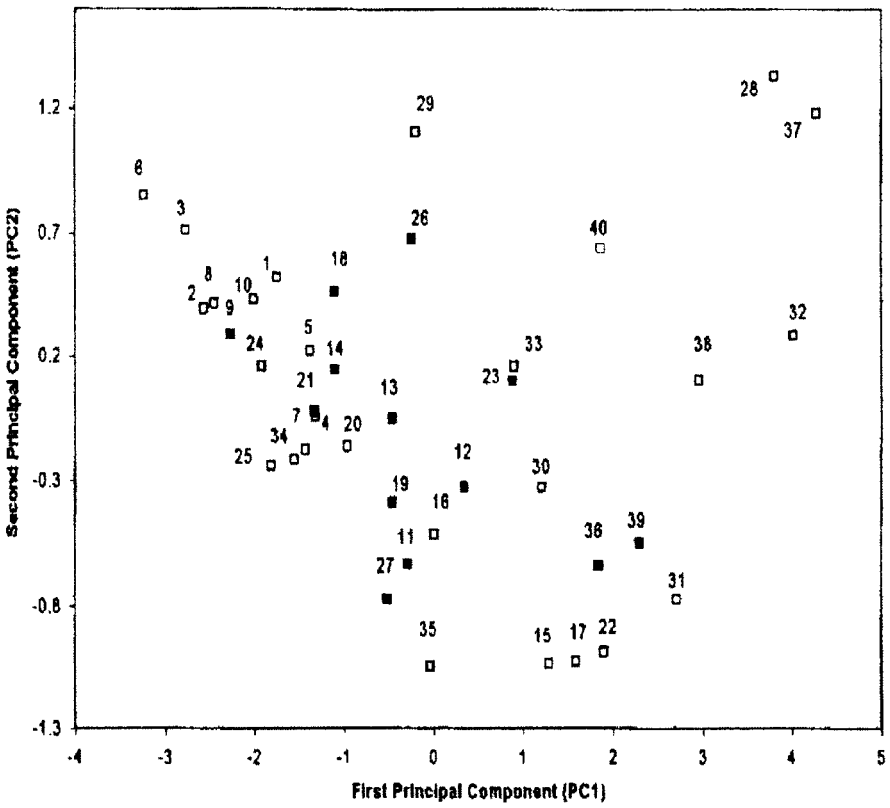

Figure 3. Distribution of vitamin B spectra for synthetis series on the first and second principal component. □, Calibration; ■, prediction.

spectral data are separated into either training or prediction sets. In the case of the training set, the various parameters are calculated as scores, loadings and corresponding weights of X (absorbance data matrix) and Y (concentration data matrix).

Figure 4 shows the model loadings, each of which is related to one or more of the analytes in solution. The relationship is reflected in the spectral shape, which may coincide with that of one of the analytes or a combination of two or more analytes. In our case, the first loading accounts for the variance of vitamin B_0, the second for that of B_1, the third for that of B_2 and the fourth for that of B_6. Finally, inducing a fifth loading lacks chemical significance.

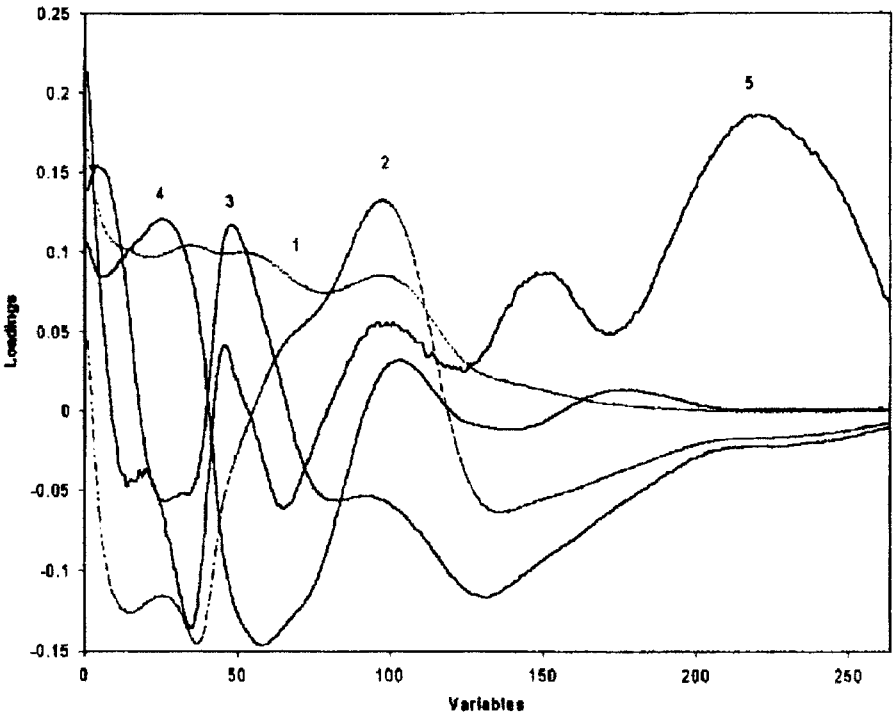

Figure 4. Loadings plot as a function of wavelength (variables); (1) first loading (related to vitamin B_0); (2) second loading (related to vitamin B_1); (3) third loading (related to vitamin B_2); (4) fourth loading (related to vitamin B_6); (5) fifth loading (lacks chemical significance).

Selection of the Optimum Number of Factors

To select the number of factors (latent variables) in the PLS algorithm, in order to model the system without overfitting the concentration data, a cross-validation method, leaving out one sample at a time, was used[12].

Given the set of 27 calibration spectra, the PLS calibrations on 26 calibration spectra were performed, and with using this calibration, the concentrations of the compounds in the sample left out during calibration was predicted. This process was repeated 27 times until each calibration sample had been left out once. The predicted concentrations of the compounds in each sample were compared with the known concentrations of the compounds in this reference sample and the prediction error sum of squares (PRESS) was calculated as follows:

$$\text{PRESS} = \sum_{i=1}^{n} (\hat{C}i - Ci)^2 \tag{3}$$

where, $\hat{C}i$ represents the estimated concentration and C_i is the reference concentration. The PRESS was calculated in the same manner, each a new factor was added to PLS model. In Fig. 5, the PRESS obtained by optimizing the calibration matrix of the absorption spectra are shown. The maximum number of factors used to calculate the optimum PRESS was selected as 14 (half the number of standards plus one).

One reasonable choice for the optimum number of factors would be the number h* which yielded the minimum PRESS. However using the number of factors (h*) that yields a minimum PRESS usually leads to some overfitting.

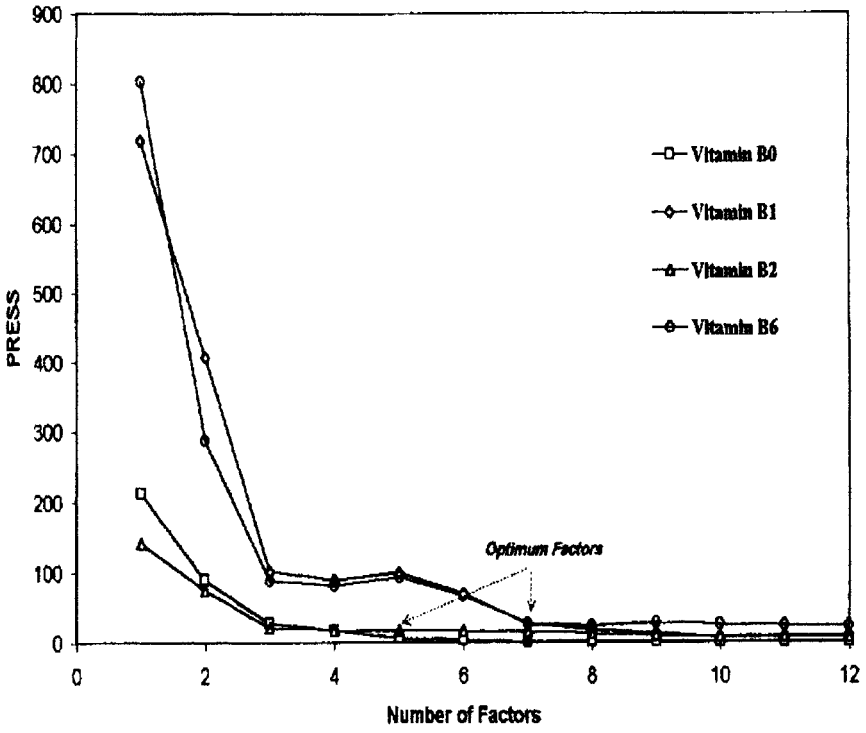

Figure 5. Representation of PRESS values generated from the prediction of various vitamin B by PLS-1 method as a function of number of factors used in the calibration.

A better criterion for selecting the optimum number of factors involves the comparison of PRESS from models with fewer than h* factors. The model selected is that with the fewest number of factors such that PRESS for that model is not significantly greater than PRESS from the model with h* factors. The F statistic was used to make the significance determination. Haaland and Thomas[13] empirically determined that an F-ratio probability of 0.75 is a good choice. We selected as the optimum number of factors for the first PRESS value the F-ratio probability of which drop below 0.75.

Notice that individual components are independently modeled by PLS-1, using an optimum h* value for each of them[13]. In the present case, factors were used in predicting the concentration of vitamin B_0, 7; for vitamin B_1, 7; for B_2, 5 and for B_6, 7. The F-ratio values in their h* factor are: 1.42, 1.43, 1.01 and 1.20, respectively.

The results obtained by applying PLS-1 algorithm to the 13 problem samples are listed in Table 2. As can be seen, the errors were also quite acceptable, as they never exceeded 5%.

In Fig. 6 the recovery results obtained by the application of the PLS-1 method are represented. Satisfactory recovery values are obtained in most of the quaternary samples analyzed. Recovery values for vitamin B_0, B_1, B_2 and B_6 are: 98.5–103.0%, 96.9–104.0%, 97.4–103.6% and 95.4–104.0%,

Table 2. Simultaneous Determination of B_0, B_1, B_2, and B_6 in Different Synthetic Mixture Using PLS-1 Prediction Method

	Amount Added ($\mu g\,mL^{-1}$)				Amount Found ($\mu g\,mL^{-1}$)				Error (%)			
Mixture	B_0	B_1	B_2	B_6	B_0	B_1	B_2	B_6	B_0	B_1	B_2	B_6
1	2.00	1.60	2.00	1.00	2.06	1.55	2.03	0.96	3.0	−3.1	1.5	−4.0
2	4.00	1.00	6.00	1.00	3.96	1.03	6.04	0.96	−1.0	3.0	0.6	−4.0
3	4.00	6.00	4.00	4.00	4.08	6.02	4.04	4.19	2.0	0.3	1.0	4.7
4	5.10	9.00	0.20	2.60	5.10	9.08	0.20	2.48	0.0	0.8	0.0	−4.6
5	4.20	2.00	2.70	2.70	4.17	2.08	2.86	2.63	−0.7	4.0	2.6	−2.5
6	1.80	5.90	1.30	6.50	1.80	5.91	1.34	6.57	0.0	0.1	3.1	1.1
7	3.30	5.10	5.40	2.00	3.35	6.06	5.46	2.08	1.5	−0.7	1.1	4.0
8	1.40	4.50	3.90	4.00	1.41	4.55	3.99	3.86	0.7	1.1	2.3	−3.5
9	3.80	4.80	5.00	9.00	3.86	4.93	5.08	8.86	1.6	2.7	1.6	−1.5
10	3.20	4.00	1.60	9.10	3.15	3.97	1.62	9.16	−1.5	−0.7	1.25	0.6
11	14.0	8.20	1.40	1.30	13.98	8.38	1.45	1.31	−0.1	2.2	3.6	0.7
12	7.90	5.60	2.70	10.7	7.89	5.81	2.72	10.21	−0.1	3.7	0.7	−4.6
13	1.10	22.5	1.90	12.1	1.13	23.14	1.85	12.01	2.7	2.8	−2.6	−0.7

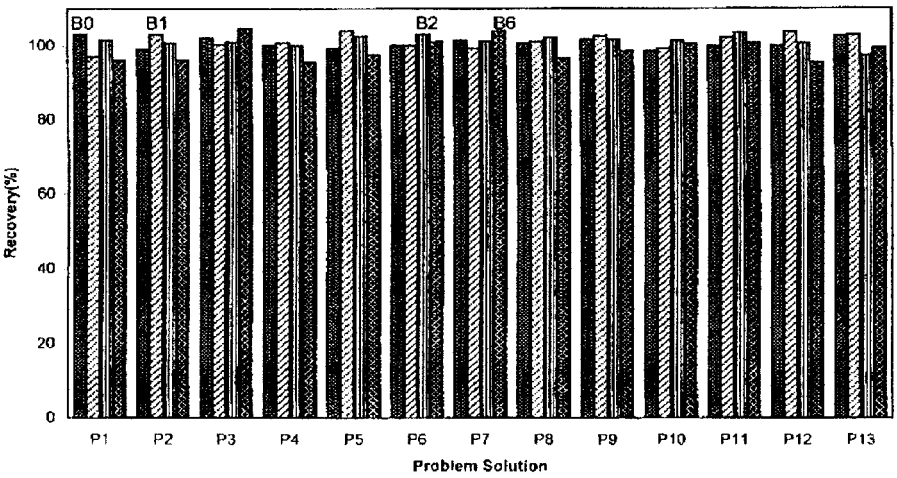

Figure 6. Diagramatic representation of percentage recovery found in the analysis of mixtures of vitamin B_0, B_1, B_2, and B_6 by PLS-1 method.

respectively. Figure 7 show the results of the determination of these vitamins in the problem samples compared with their actual values. It can be seen in all graphs, especially for vitamins B_0 and B_2, there was good agreement between the real and calculated values.

Statistical Parameters

The root mean squares difference (RMSD) is an indication of the *average error* in the analysis for each component:

$$\mathrm{RMSD} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(\hat{C}i - Ci)^2} \tag{4}$$

where n is the total number of calibration samples, $\hat{C}i$ represents the estimated concentration, and C_i is the reference concentration.

The squares of the correlation coefficient (R^2), which is an indication of the *quality fit* of all the data to a straight line is presented by:

$$R^2 = \frac{\sum_{i=1}^{N}(\hat{C}i - \bar{C})^2}{\sum_{i=1}^{N}(Ci - \bar{C})^2} \tag{5}$$

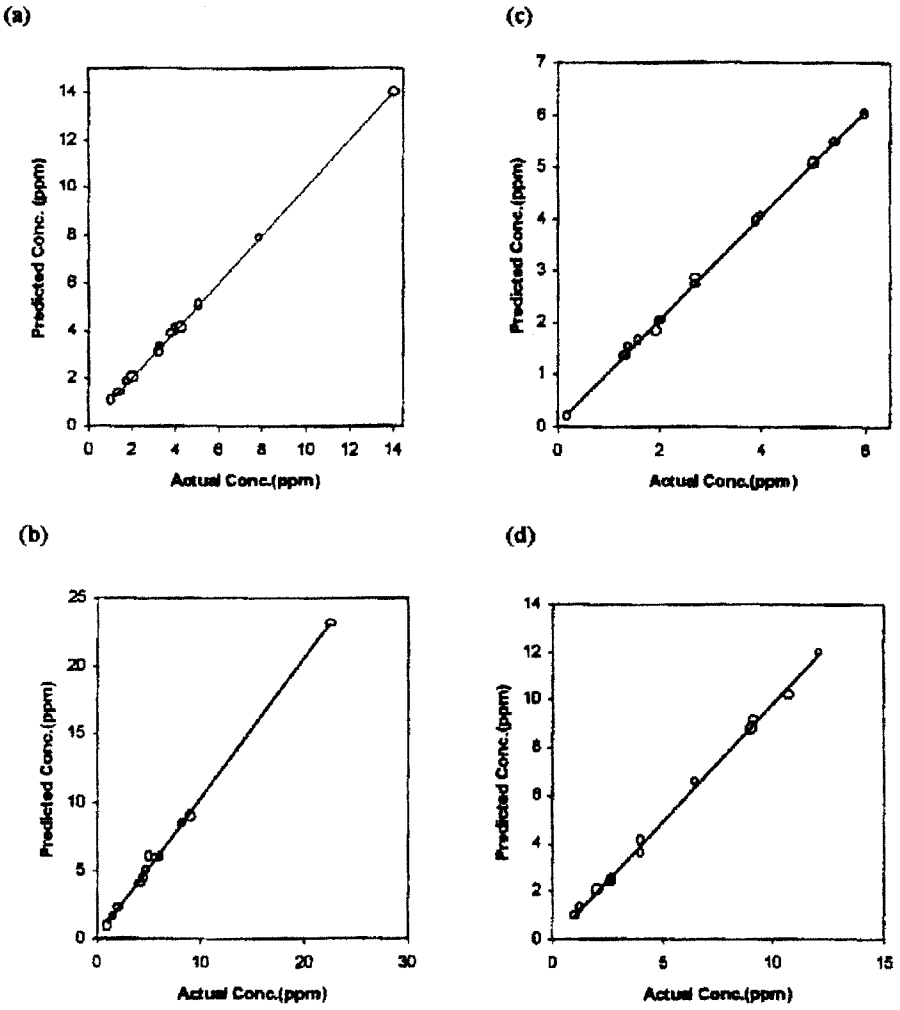

Figure 7. The plot of predicted concentration vs. actual concentration obtained with PLS-1 method. (a) vitamin B_0, (b) vitamin B_1, (c) vitamin B_2, and (d) vitamin B_6.

where $\bar{C}$ represents the mean of the true concentrations in the prediction set[14].

The RMSD values are an estimate of the *absolute error* of prediction for each component. The prediction ability of each method and for each component can also be described in terms of the relative error of prediction (REP)[15] as follow:

Table 3. Statistical Parameters of the PLS-1 Method with Use of the Absorption Spectral Data

Component	NPC*	RMSD	R^2	REP (%)
Vitamin B_0	7	0.8386	0.9999	0.92
Vitamin B_1	7	3.8694	0.9962	3.24
Vitamin B_2	5	3.1554	0.9988	3.15
Vitamin B_6	7	4.0845	0.9978	1.66
Mean	—	2.9869	0.9981	2.24

*Number of principal components (Factors).

$$\mathrm{REP} = \frac{100}{\bar{\mathrm{C}}} \sqrt{\frac{1}{\mathrm{n}} \sum_{\mathrm{i}=1}^{\mathrm{n}} (\hat{\mathrm{C}}\mathrm{i} - \mathrm{Ci})^2} \tag{6}$$

RMSD, R^2 and REP values for each component (correspond to its optimum factor) have been summarized in Table 3.

CONCLUSION

The proposed method for the determination of mixtures of vitamins, with respect to obtained results and statistical parameters, is simple and versatile in comparisons to the chromatographic and electrochemical methods.

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