Simultaneous Spectrophotometric Determination of Xanthine, Hypoxanthine and Uric Acid in Real Matrix by Orthogonal Signal Correction-Partial Least Squares

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The simultaneous determination of xanthine (XA), hypoxanthine (HXA) and uric acid (UA) mixture using spectrophotometric methods is a difficult task in analytical chemistry because of the spectral interferences. Partial least squares (PLS) modeling, as a powerful multivariate statistical tool, were applied to the spectrophotometric simultaneous determination of these substances. The concentration ranges for XA, HXA and UA were 3.0-24.3, 2.7-19.0 and 3.4-25.2 μg ml^{-1}, respectively. The experimental calibration set consisted of 21 sample solutions with a mixture design for three-component mixtures. The absorption spectra were recorded from 220 to 320 nm. After orthogonal signal correction (OSC), the unrelated information was removed and the results were proved. The root mean square error of prediction (RMSEP) for xanthine, hypoxanthine and uric acid with OSC were 0.5161, 0.2997 and 0.5739 and without OSC were 1.6087, 0.8580 and 1.4009, respectively. This process afforded the simultaneous determination of XA, HXA and UA in human urine and human serum.

INTRODUCTION

Xanthine (XA), hypoxanthine (HXA) and uric acid (UA) are formed during urine metabolism and are found in tissues and bodies such as blood and urine. Extreme abnormal levels of XA, HXA and UA in blood, plasma and urine may provide sensitive indicators of certain pathologic states, including xanthinuria, gout, renal failure, toxaemia during pregnancy, and other diseases [1]. Hence, the measurement of these compounds is of considerable significance in biochemical and clinical diagnosis. These compounds have similar chemical structures and UV-Vis spectra, which has prompted the use of analytical separation techniques such as chromatographic methods [2-4] electrochemical methods [5], the voltammetric

method [6] and the HPLC technique [7]. Multivariate calibration techniques, such as partial least squares (PLS), have been devised for the analysis of mixtures with overlapping spectra [8,9]. The advantage of multicomponent analysis using partial least squares is the speed of the interest in a mixture, as a separation step can be avoided. The theory and application of partial least squares (PLS) in spectrometry have been discussed by several researchers [8,10]. Some multicomponent determinations, based on the application of these methods to spectrophotometric data, have been reported in the literature [11,12].

The orthogonal signal correction (OSC) was introduced by Wold and others to remove the systematic variation from the *X* response matrix that is either unrelated or orthogonal to the *Y* property matrix [13]. Therefore, it is certain that the important information on the analyte is retained. Since then, several

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groups [14,15] have published various OSC algorithms in an effort to lessen model complexity by removing the orthogonal components from the signal.

This paper describes an analytical method for the simultaneous determination of xanthine, hypoxanthine and uric acid using the spectrophotometric method and a multivariate calibration technique (partial least squares) with pre-processing by orthogonal signal correction. The aim of this work is to propose the orthogonal signal correction-partial least squares (OSC-PLS) method to resolve ternary mixtures of xanthine, hypoxanthine and uric acid in real matrixes. Generally, for the evaluation of the predictive ability of a multivariate calibration model, the root mean square error of prediction (RMSEP) with the relative standard error of prediction (RSEP) can be used [16].

EXPERIMENTAL

Materials

Xanthine, hypoxanthine and tricholoroacetic acid were purchased from Fluka, while uric acid was supplied by Merck. The stock solutions of XA, HXA and UA were prepared by dissolving them in KOH (0.1 M). A buffer solution (pH = 7) was prepared by KH_2PO_4 and NaOH (Merck). The serum and urine samples were prepared by spiking human serums and human urine, with appropriate amounts from the stock solutions of XA, HXA and UA. All the chemicals used were of analytical reagent grade. Throughout the experiments, distilled water was used.

Apparatus and Software

The electronic absorption measurements were carried out on a Lambda EZ210 Spectrophotometer (slit width: 2 nm, scan rate: 800 nm min^{-1}) with 1.00 cm quartz cells. The pH measurements were made with a Metrohm 692 pH meter, illustrating a combined electrode. All absorption spectra were digitized and stored at wavelengths from 220 to 320 nm in steps of 1 nm and, then, transferred in txt format to a Pentium 4, 2.4 GHz computer using MATLAB software, version 7 (The Math Works). The PLS and OSC calculus was conducted with the PLS_Toolbox (Eigenvectors Company, Version 2.5). Also, the calculations of the mixture design were made with a program written in MATLAB by the authors.

Procedure

Standard calibration set. A mixture design for three-component mixtures was used for the calibration set [17,18] to provide good prediction in the PLS method. A training set of 21 samples was taken. Figure 1 shows the ternary mixtures of xanthine, hypoxanthine and uric acid in the calibration set. The concentration of XA, HXA and UA was varied between 3.0-24.3, 2.7-19.0 and 3.4-25.2 μg ml^{-1}, respectively. The mixed standard solutions were placed in a 10.0 ml volumetric flask and completed to final volume with buffer solution (pH = 7.0). Finally, the absorption spectra of all the prepared solutions were recorded between 220 and 320 nm against a blank of universal buffer. The spectral region between 220 and 320 nm was selected for the analysis, implying that 101 experimental points per spectra were involved as the spectra were digitized every 1.0 nm. This region was selected because it offered the maximum spectral information from the mixture components of interest. All absorption data were pre-processed by standard mean centering and scaling.

Prediction set. For the prediction set, seven mixtures were prepared randomly (Table 1). The concentration ranges were added to be 3.0-24.3, 2.7-19.0 and 3.4-25.2 μg ml^{-1} for XA, HXA and UA, respectively.

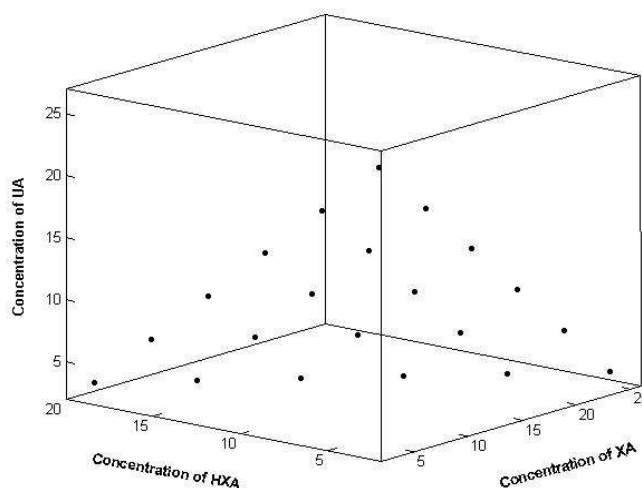**Fig. 1.** Ternary mixtures of xanthine, hypoxanthine and uric acid in the calibration set (μg ml^{-1}).

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Table 1. Added and Found Results of the XA, HXA, UA Synthetic Mixtures

Added (µg ml^{-1})			Found (µg ml^{-1})			Recovery (%)		
XA	HXA	UA	XA	HXA	UA	XA	HXA	UA
PLS method								
15.60	6.75	7.14	15.08	5.95	5.92	96.7	88.2	82.9
11.10	14.04	8.84	10.70	14.00	8.62	96.4	99.7	97.5
7.50	12.96	21.76	6.54	14.78	21.24	87.2	114.1	97.6
7.20	5.67	6.80	6.80	5.41	5.54	94.4	95.4	81.5
19.20	9.45	14.96	15.60	8.68	12.89	81.3	91.8	86.2
14.40	5.40	19.04	13.13	5.97	16.95	91.2	110.5	89.0
15.00	8.37	10.54	13.58	7.93	9.23	90.5	94.7	87.6
OSC-PLS method								
15.60	6.75	7.14	15.83	6.31	7.60	101.5	93.5	106.4
11.10	14.04	8.84	10.50	14.67	8.75	94.6	104.5	99.0
7.50	12.96	21.76	7.71	12.98	23.01	102.8	100.2	105.7
7.20	5.67	6.80	6.68	5.53	7.10	92.8	97.5	104.4
19.20	9.45	14.96	18.43	9.51	14.93	96.0	100.6	99.8
14.40	5.40	19.04	14.80	5.31	19.50	102.8	98.3	102.4
15.00	8.37	10.54	15.62	8.45	10.07	104.1	101.0	95.5

Sample Preparation

Serum sample. The serum samples were homogenized. For the deproteinization, 1 ml of 24% w/v trichloroacetic acid was added to 1.0 ml of serum. After 15 min, the resulting mixture was centrifuged for 15 min at 3000 rpm [19]. Then, a certain amount of NaOH solution was added to the supernatant solution to reach the final pH value of 7.0. Afterwards, an appropriate amount from the stock solution of XA, HXA and UA was added to 0.5 ml of the lastly prepared serum and completed to the final volume (10.0 ml volumetric flask) with buffer solution to obtain the desired concentration. The electronic absorption spectrum was recorded in the range of 220-320 nm against a blank solution of universal serum.

Urine sample. The urine sample was diluted 1:3 with distilled water. Then, the cell debris and the particulate matter were removed from the urine using low-speed centrifugation (for 5 min at 1500 rpm) [20]. Afterwards, a certain amount of

NaOH solution was added to the supernatant solution until its final pH value was equal to 7.0. Also, appropriate amounts from the stock solutions of XA, HXA and UA were added to 0.5 ml of the lastly prepared urine and completed to the final volume (10.0 ml volumetric flask) with buffer solution to get the desired concentration. The electronic absorption spectrum was recorded in the range of 220-320 nm against a blank solution of universal serum.

RESULTS AND DISCUSSION

Selection of Optimum Chemical Conditions

Figure 2 depicts the absorption spectra in the aqueous solutions of xanthine, hypoxanthine and uric acid at pH 7.0. A universal buffer solution of pH 7.0 was selected. To select the optimum pH value at which the minimum overlap occurred, the pH influences on the absorption spectra of XA, HXA and
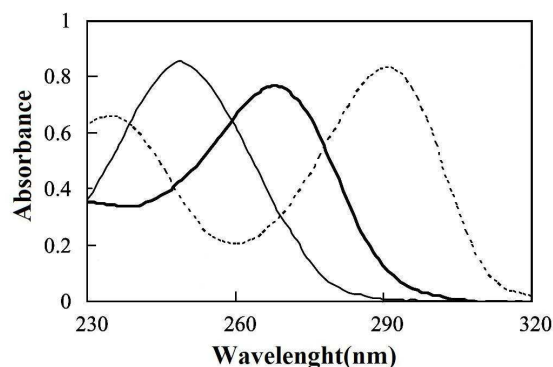
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Fig. 2. Pure spectra of xanthine (12 μg ml^{-1}), hypoxanthine (11 μg ml^{-1}) and uric acid (13 μg ml^{-1}): (━) XA, (—) HXA, (----) UA.

UA were studied over the pH range of 5.0-10.0. The individual calibration curves were constructed with several points, with the absorbance versus the XA, HXA and UA concentrations in the ranges of 3.0-24.3, 2.7-19.0 and 3.4-25.2 μg ml^{-1}, respectively. The wavelengths, used to produce the calibration curve, were 268, 249 and 291 nm for XA, HXA and UA, respectively.

Pre-processing by Orthogonal Signal Correction

For the calibration set, three OSC components were used for filtering. To evaluate the prediction errors for the validation set, it was revealed that the OSC-treated data provided substantially lower RMSEP values than those of the original data. Also, the OSC-filtered data gave much simpler calibration models with fewer components than the ones based

on the original data. The results revealed that the OSC method removed the information from the UV-Vis data that was not indeed necessary for the *Y*-variables fitting. Occasionally, the OSC method also removed the nonlinear relationships between *X* and *Y*.

Selection of the Optimum Number of Factors

The optimum number of factors (latent variables) to be included in the calibration model was determined by computing the prediction error sum of squares (PRESS) for the cross-validated models, using a high number of factors (half the number of total standard + 1), which is defined as follows in Eq. (1):

$$PRESS = \sum (y_i - \hat{y}_i)^2 \qquad (1)$$

where y_i is the reference concentration for the ith sample and $\hat{y}_i$ represents the estimated concentration. The employed cross-validation method was to eliminate only one sample at a time and, then, the remaining standard spectra would be calibrated by PLS. With this calibration, the sample concentration, being left out, was predicted. This process was repeated until each standard had been left out once. To select the optimum number of factors, the criteria proposed by Haaland and Thomas [20] were applied. The optimum number of factors, obtained by the application of the PLS and OSC-PLS models, are summarized in Table 2. In all cases, the value of the F-ratio probability dropped below 0.75, which was selected as the optimum one. As can be seen from Table 2, the OSC-PLS method has used fewer number of factors than the PLS method.

Table 2. Statistical Parameters of the Optimized Matrix Using the OSC-PLS and PLS

Component	NF[a]	PRESS	RMSEP	RSEP (%)
Xanthine[b]	5	0.3329	0.5161	3.8233
Hypoxanthine[b]	5	0.4209	0.2997	3.1555
Uric acid[b]	5	0.1486	0.5739	4.1383
Xanthine[c]	6	0.5013	1.6087	11.9167
Hypoxanthine[c]	6	1.3876	0.8580	9.035
Uric acid[c]	6	0.2273	1.4009	10.1018

[a]Number of factors. [b]Using OSC-PLS. [c]Using PLS.

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Determination of Xanthine, Hypoxanthine and Uric Acid in Synthetic Mixtures

The predictive ability of the method was determined with the use of seven three-component mixtures (their compositions are given in Table 1). The deriving data after the application of the PLS and OSC-PLS algorithm to seven synthetic samples are listed in Table 1. Table 1 also depicts the recovery for the prediction series of XA, HXA and UA mixtures. As it can be
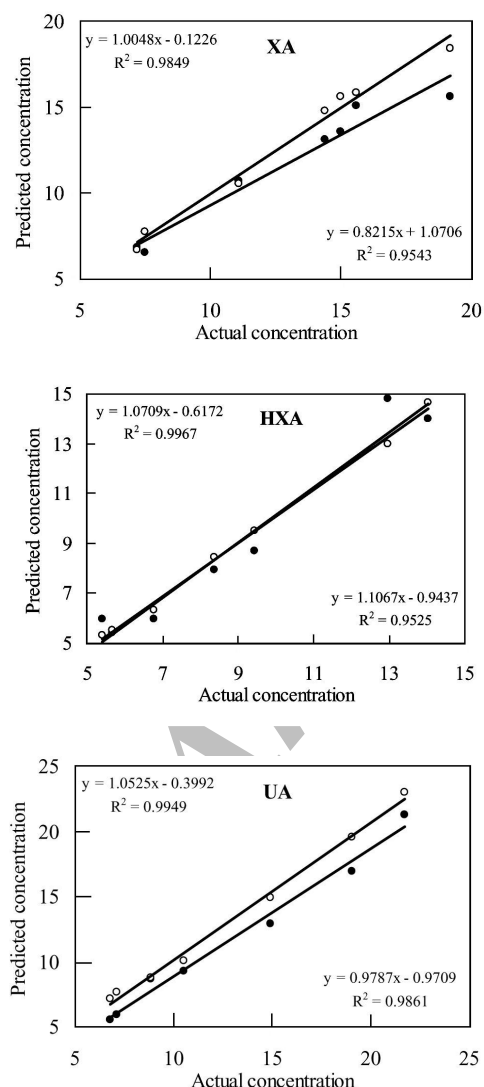
seen, the recovery was also acceptable. The root mean square error of prediction and the relative standard error of prediction values are summarized in Table 2. The plots of the predicted concentration actual values for XA, HXA and UA are illustrated in Fig. 3 (line equations and R^2 values are displayed, as well).

Determination of Xanthine, Hypoxanthine and Uric Acid in Real Matrix

To test the applicability and matrix interferences of the recommended method to the analysis of real samples, the OSC-PLS method was applied for the determination of XA, HXA and UA in human urine and human serum. For the serum and urine samples, the spectral region was selected between 230-320 nm and 250-320 nm, respectively, since this was the zone with the maximum spectral information from the mixture components of interest (Figs. 4 and 5). Table 3
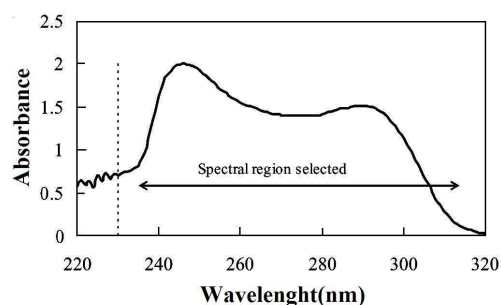

Fig. 3. Plots of the predicted concentration *vs.* the actual concentration of xanthine, hypoxanthine and uric acid by PLS (●) and OSC-PLS (○).

Fig. 4. Absorption spectra of serum sample, spiked with XA (10 μg ml^{-1}), HXA (14 μg ml^{-1}) and UA (20 μg ml^{-1}).

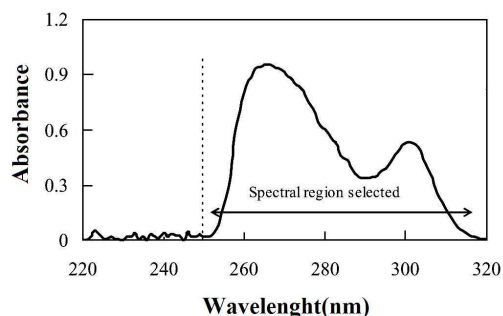**Fig. 5.** Absorption spectra of urine sample, spiked with XA (10 μg ml^{-1}), HXA (14 μg ml^{-1}) and UA (20 μg ml^{-1}).

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Table 3. OSC-PLS Results Applied on the Real Matrix Samples

Real matrix	Set	Added (µg ml^{-1})			Found (µg ml^{-1})		
		XA	HXA	UA	XA	HXA	UA
Serum	I	20.0	5.0	7.0	19.41	5.20	7.36
	II	6.5	15	6.0	6.84	15.12	5.81
	III	10.0	7.2	14.2	10.22	6.86	14.73
Urine	I	20.0	5.0	7.0	19.14	5.10	7.13
	II	6.5	15	6.0	6.20	14.52	5.83
	III	10.0	7.2	14.2	9.82	7.11	14.02

demonstrates the results for real matrix samples. Accordingly, the OSC-PLS model was able to predict the concentrations of XA, HXA and UA in the human serum and human urine samples.

CONCLUSIONS

The XA, HXA and UA mixture is a difficult complex system because of the high spectral overlapping observed between the absorption spectra for their components. To overcome the drawback of the spectral interferences, PLS multivariate calibration approaches were applied. Furthermore, the present study revealed that the OSC could be a good method to remove the systematic variation from the response matrix *X,* which was either unrelated or orthogonal to the *Y* property matrix. Consequently, one can be certain that the important information on the analyte was retained. The good agreement clearly displayed the utility of this procedure for the simultaneous determination of xanthine, hypoxanthine and uric acid, without tedious pretreatment with complex samples in synthetic and real matrix.

ACKNOWLEDGEMENTS

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