The Analyst

Simultaneous fluorimetric determination of pyridoxal, pyridoxamine and pyridoxic acid by partial least squares using non-linear variable angle synchronous spectra

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The multivariate calibration method partial least squares regression type 1 (PLS-1) was applied to the simultaneous fluorimetric determination of pyridoxal, pyridoxamine and pyridoxic acid at low concentration levels (8.0–80, 30.0–300 and 2.4–24 μg l^{-1}, respectively). The non-linear variable-angle synchronous spectra were obtained, tracing a curved trajectory through the total luminescence spectroscopic data. Several calibration models were studied in order to select the best one. The statistical parameters obtained by internal and external validation of the optimum calibration model were in agreement, with satisfactory results.

Keywords: *Pyridoxal; pyridoxamine; pyridoxic acid; partial least squares; non-linear variable angle synchronous fluorescence; spectrofluorimetry*

Fluorescence spectroscopy is a powerful tool in quantitative analysis owing to its great sensitivity. However, satisfactory analysis of a fluorescent multicomponent system without any separation or derivatization step is not possible when there are interferences in the excitation and emission spectra. For this reason, the development of techniques which improve the selectivity of fluorimetric methods is desirable; among these techniques, synchronous and derivative fluorescence spectrometry are the most commonly used.

Conventionally, synchronous fluorescence spectroscopy involves the simultaneous scanning of the excitation and emission monochromators at a constant wavelength ($\Delta\lambda$) or energy ($\Delta\nu$) difference between them, obtaining two-dimensional spectra simpler, narrower and more distinctive than conventional spectra.[1,2] When the difference between the excitation and emission wavelengths is continuously varied, maintaining a linear trajectory throughout the scan, the technique is called linear variable-angle synchronous fluorescence and, if the trajectory is non-linear, the linear term is changed to non-linear.[3] However, it is necessary to define the wavelength or energy intervals before recording the spectra in order to obtain better results and some multicomponent systems cannot be resolved directly by these techniques.

The use of computer-controlled instrumentation with suitable software allowed the digitization, storage and processing of total luminescence spectroscopic data to obtain the complete characterization of fluorescent compounds in a reasonable time.[4–6] Thus, the combination of derivative or multivariate calibration techniques with emission, excitation and synchronous spectra was improved.

In published work, when non-linear variable-angle synchronous fluorimetry has been used the spectrum obtained is a combination of several linear variable-angle synchronous spectra. We use a program that we developed called FTOTAL.[6] This program generates a curved trajectory following a continuous and derivable mathematical function. This function can be an exponential, a polynomial, a hyperbolic function, *etc*.

Multivariate calibration methods,[7–9] such as principal component regression (PCR) and partial least squares (PLS), have also been widely used for the analysis of complex systems employing different detection techniques.[10–13] Since Lindberg *et al.*[14] performed the fluorimetric analysis of humic acid and lignin sulfonate by PLS, this statistical tool has been successfully applied to the resolution of multicomponent systems and enhanced the selectivity of fluorimetric methods.[15,16]

The determination of vitamin B_6 and related compounds is of great interest, although it is difficult owing to the existence of multiple forms with similar structures, such as pyridoxal, pyridoxamine and pyridoxic acid. Although several methods have been reported for the determination of these substances by distinctive techniques, fluorimetric methods are very sensitive and separation steps or derivatization reactions have been proposed for increasing their selectivity.[17–20] In contrast, some workers have suggested the use of chemometric techniques instead of the alternatives described above to reduce the interferences due to analytes and complex matrices. Thus, Berzas *et al.* proposed individual determinations of pyridoxal[21] and pyridoxamine[22] in biological fluids using matrix isopotential synchronous fluorescence spectrometry. In addition, derivative synchronous fluorescence spectrometry has been used for the resolution of vitamin B_6 and its derivatives in mixtures.[23–25]

The principal purpose of this paper is to demonstrate for the first time the possibilities that the combination of partial least squares and non-linear variable-angle synchronous fluorimetry offer. This procedure was applied to the resolution of a ternary mixture of compounds which have very similar chemical structures and whose fluorescence spectra overlap considerably so that they cannot be determined simultaneously by other fluorimetric techniques or by a combination of derivative techniques and fluorimetry.

In this work, the application of the partial least squares regression method type 1 (PLS-1) to the analysis of non-linear variable angle synchronous spectra is proposed for the simultaneous determination of pyridoxal, pyridoxamine and pyridoxic acid without separation or derivatization steps.

Experimental

Apparatus

An SLM Aminco Bowman Series 2 luminescence spectrometer (SLM, Rochester, NY, USA), equipped with a 150 W continuous xenon lamp and interfaced with a PC 386 Polac Tron computer, was used. The excitation and emission slit widths were set at 8 and 16 nm, respectively, and a scan rate of 30 nm s^{-1} was employed for the acquisition of three-dimensional excitation–emission spectra. A Selecta Frigiterm 6000382

thermostatic bath (J.P. Selecta, Barcelona, Spain) and a Crison Model 2002 pH meter (Crison Instruments, Barcelona, Spain) with a glass–saturated calomel combination electrode were also used.

Data acquisition, manipulation and statistical treatment were performed with AB2 software Version 1.40 running under 0S/2 2.0, the FTOTAL[6] program developed in TURBO BASIC language and PLSplus Version 2.1G for the GRAMS 386 Version 2.0 software package (Galactic Industries, Salem, NH, USA), respectively.

Reagents

Pyridoxamine dihydrochloride and 4-pyridoxic acid were obtained from Sigma (St. Louis, MO, USA) and pyridoxal hydrochloride from Merck (Darmstadt, Germany). All other reagents were of analytical-reagent grade and ultra-pure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Stock standard solutions of pyridoxal, pyridoxamine and pyridoxic acid containing 200 mg l^{-1} were prepared and stored at 0–4 °C, protected from light. Working standard solutions were prepared daily by appropriate dilution. A 2 M buffer solution of NH_3–NH_4Cl (pH 9.3) was also used.

Procedure

Appropriate volumes of the working standard solutions to give final concentrations of 8.0–80.0 μg l^{-1} of pyridoxal, 32.0–300.0 μg l^{-1} of pyridoxamine and 2.4–24.0 μg l^{-1} of pyridoxic acid were introduced into a 25 ml calibrated flask, 1 ml of 2 M NH_3–NH_4Cl buffer solution (pH 9.3) was added and the mixture was diluted to volume with water. The flask was protected from light. The total luminescence spectrum [excitation–emission matrix (EEM)] was recorded by scanning 61 emission spectra between 320 and 512 nm with increments of 3.2 nm of the excitation wavelength between 201.6 and 393.6 nm (2 min 54.5 s were necessary to obtain the 3D matrix when a scan speed of 100 nm s^{-1} was used). The three-dimensional spectrum was smoothed with the Savitzky–Golay algorithm using 15 experimental points.

Results and discussion

The best characterization of a fluorescent compound can be obtained from its total luminescence spectrum. However, the large amount of information implied requires the use of suitable software for the analysis and representation of data; in this case, the FTOTAL program was used.

The total fluorescence spectra of pyridoxal, pyridoxamine and pyridoxic acid are presented as contour maps in Fig. 1, where the *x*- and *y*-axes correspond to excitation and emission wavelengths and the fluorescence intensities are shown as a group of curves obtained by joining points of equal values. As can be seen, the spectra exhibit substantial overlapping and resolution of the mixture by means of conventional excitation or emission spectra is not possible when it is required to maintain the maximum sensitivities of the individual determinations. The conventional synchronous spectra, the constant energy synchronous spectra and the linear variable-angle synchronous spectra do not permit the simultaneous determination of these compounds. The use of excitation, emission, conventional synchronous, constant energy synchronous and linear variable-angle synchronous spectra would produce a decrease in the sensitivity of the determination because the spectra do not pass simultaneously through the fluorescence maxima of the three compounds. The simplest cut in the 3D matrix, which passes through the fluorescence maxima of the three compounds, is a quadratic curve, *viz*., the parabolic trajectory used by us. The combination of derivative techniques and excitation, emission and synchronous spectra does not permit the analysis of these components. For this reason, the two-dimensional spectra known as non-linear variable-angle synchronous spectra were obtained from the respective total fluorescence spectra following a parabolic trajectory (second-grade polynomial function). These spectra were used as data for the application of PLS-1, a mathematical tool for the analysis of multicomponent systems.

Optimization of experimental conditions

Several factors were studied in order to select the best conditions for the resolution of the ternary mixture. First, the influence of pH was evaluated by the addition of 2.5 ml of 0.5 M phosphoric acid solution with pH values between 4 and 12 adjusted with NaOH solution. The excitation and emission wavelengths corresponding to the highest fluorescence intensities for the three compounds *versus* pH are presented in Fig. 2; according to the plots, the maximum separation between these wavelengths occurs in the emission spectra at approximately pH 9; therefore, the recording of emission spectra at 61 excitation wavelengths and a buffer solution of NH_3–NH_4Cl (pH 9.3) were selected. Subsequently, the effect of buffer concentration on the fluorescence signals of pyridoxal, pyridoxamine and pyridoxic acid was studied. A slight decrease in the intensities occurred as a result of buffer concentration increments and a final concentration of 0.08 M was chosen as providing adequate buffering capacity.

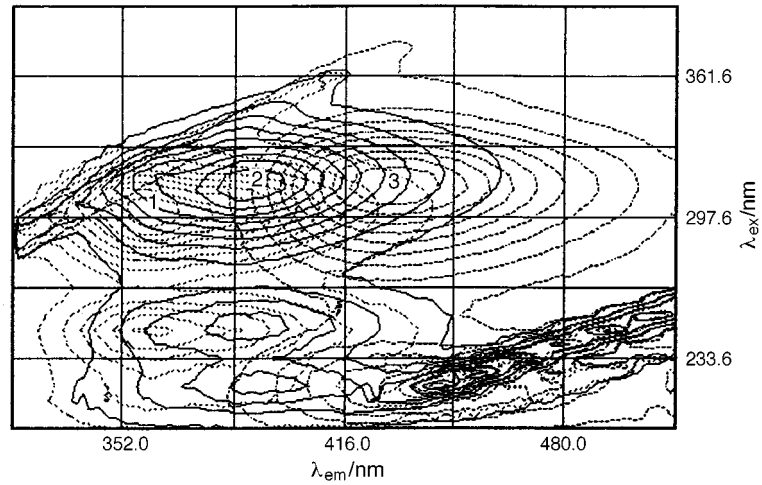

Fig. 1 Total luminescence spectra of pyridoxal (1), pyridoxamine (2) and pyridoxic acid (3).

Temperature also affects the fluorescence intensity. Therefore, a thermostatic bath was employed to maintain a temperature of 20 °C, close to room temperature. The pyridoxal, pyridoxamine and pyridoxic acid samples were stable for at least 2 h under these experimental conditions.

Construction of two-dimensional spectra

Since partial least squares regression methods (PLS, Types 1 and 2) require *x*–*y* data, it is not possible to introduce the total luminescence spectra for the statistical treatment. Therefore, the non-linear variable-angle synchronous spectra were obtained from their EEMs by the use of the FTOTAL program.[6]

Under the experimental conditions described under Procedure, the analytes exhibit their highest fluorescence intensities at the following wavelengths: pyridoxal, λ_{ex} = 301.2 and λ_{em} = 361.2 nm; pyridoxamine, λ_{ex} = 313.6 and λ_{em} = 388.8 nm; and pyridoxic acid, λ_{ex} = 312.0 and λ_{em} = 430.0 nm. For this reason, a parabolic trajectory through these wavelengths was projected with the aid of a second-degree polynomial fit, according to the algebraic form

$$\lambda_{ex} = a(\lambda_{em})^2 + b(\lambda_{em}) + c \quad (1)$$

and the mathematical function obtained was

$$\lambda_{ex} = -0.007\,094\,61\,(\lambda_{em})^2 + 5.770\,23\,(\lambda_{em}) - 857.406 \quad (2)$$

The trajectory is represented in the excitation–emission wavelengths plane in Fig. 3. Finally, each emission wavelength from 320 to 512 nm (in increments of 0.4 nm) was substituted in eqn. (2) in order to identify the series of emission–excitation wavelengths present in the EEM that were considered to generate the synchronous spectrum, a set of fluorescence intensities against their corresponding emission wavelengths. Fig. 4 shows the non-linear variable-angle synchronous spectra for the three components and their mixture.

Calibration set design

The most important part of the PLS regression method is the calibration. In order to establish the concentration ranges of interest for pyridoxal, pyridoxamine and pyridoxic acid, typical one-compound calibration experiments were carried out. As a result, linearity was assumed ($r > 0.9990$) for concentrations of

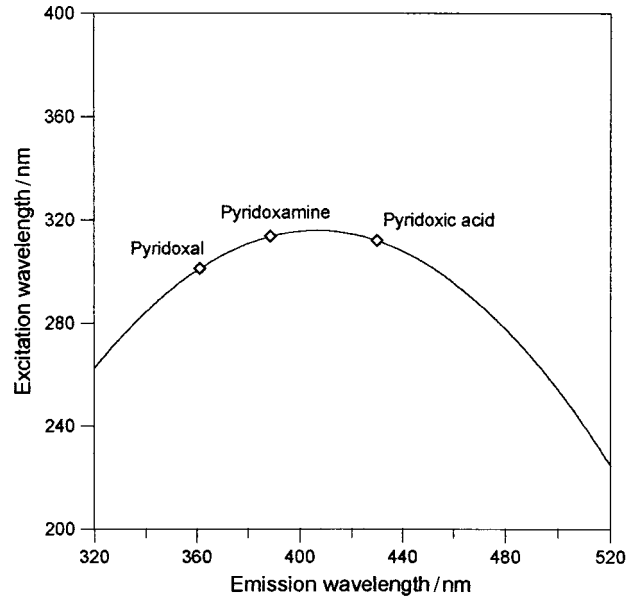

Fig. 3 Parabolic trajectory used to obtain the non-linear variable-angle synchronous spectra. The diamond symbols show the localization of the maximum fluorescence of pyridoxal, pyridoxamine and pyridoxic acid.

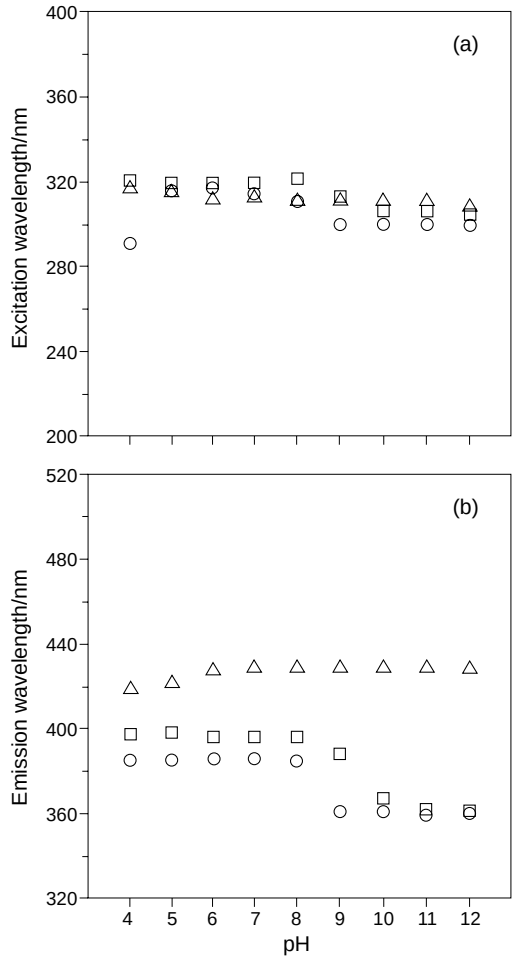

Fig. 2 Effect of pH on the localization of the maximum fluorescence intensity of pyridoxal (○), pyridoxamine (□) and pyridoxic acid (△): (a) excitation and (b) emission.

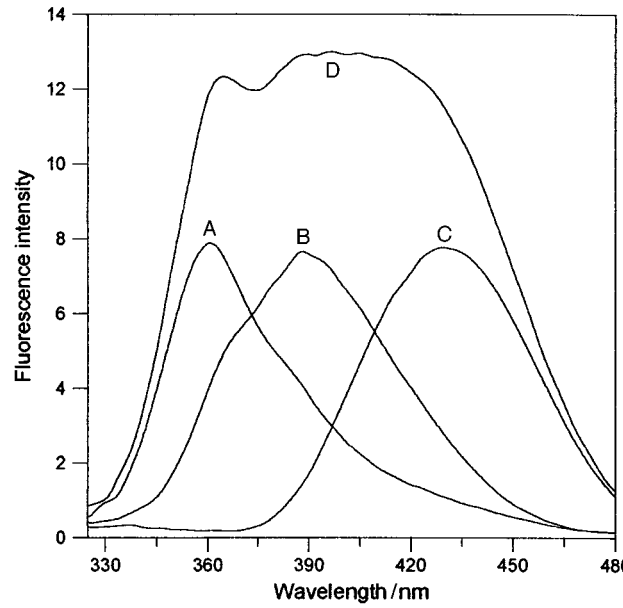

Fig. 4 Non-linear variable-angle synchronous spectra used as data for PLS-1: A, pyridoxal, 80 μg l^{-1}; B, pyridoxamine, 300 μg l^{-1}; C, pyridoxic acid, 24 μg l^{-1}; and D, their mixture.

pyridoxal between 8.0 and 80.0 μg l^{-1}, pyridoxamine between 32.0 and 300.0 μg l^{-1} and pyridoxic acid between 2.4 and 24.0 μg l^{-1}, without an inner filter effect in the mixture. A calibration set of 36 samples was then taken for the application of the multivariate method, and the compositions are summarized in Table 1. The training set design responds to some practical guidelines given by several workers,[9,12,26] *e.g.*, the use of mixtures and pure compounds for calibration, samples with several concentration and spectral ratios and a statistically significant number of samples considering the overlapping of synchronous spectra.

Calibration models and internal validation

In general, the PLS regression method is designed to use the full spectrum for calibration. However, there are some reasons for avoiding this. If there are spectral regions containing information not related to the analytes, including these wavelengths could contribute to an increase in the memory and time required to perform the statistical treatment, to the selection of a number of factors that lead to some overfitting and even to larger prediction errors.[26] In this case, the PLS-1 algorithm was used to construct four calibration models with different spectral regions: model A, from 320 to 484 nm (411 experimental points); model B, from 332 to 484 nm (381 experimental points); model C, from 348 to 464 nm (291 experimental points); and model D, from 352 to 464 nm (281 experimental points).

Table 1 Composition of training set samples. All concentrations in μg l^{-1}

Sample No.	Pyridoxal	Pyridoxamine	Pyridoxic acid
1	8.0	32.0	2.4
2	40.0	152.0	12.0
3	80.0	300.0	24.0
4	16.0	300.0	14.4
5	16.0	180.0	24.0
6	48.0	60.0	14.4
7	48.0	300.0	4.8
8	80.0	60.0	24.0
9	80.0	180.0	4.8
10	32.0	120.0	21.6
11	72.0	120.0	9.6
12	32.0	272.0	9.6
13	8.0	208.0	16.8
14	56.0	32.0	16.8
15	56.0	208.0	2.4
16	40.0	88.0	19.2
17	40.0	240.0	7.2
18	64.0	88.0	12.0
19	24.0	152.0	19.2
20	24.0	240.0	12.0
21	64.0	152.0	7.2
22	48.0	120.0	0
23	32.0	0	14.4
24	0	180.0	9.6
25	72.0	32.0	0
26	8.0	0	21.6
27	0	272.0	2.4
28	8.0	0	0
29	0	32	0
30	0	0	2.4
31	40.0	0	0
32	0	152.0	0
33	0	0	12.0
34	80.0	0	0
35	0	300.0	0
36	0	0	24.0

In order to establish the optimum number of factors to build the models without overfitting and to study their predictive ability in the calibration samples, the full cross-validation method was performed leaving out one sample at a time and the corresponding PRESS (prediction residual error sum of squares) were calculated. Then the *F*-test criterion recommended by Haaland and Thomas[8] for selecting the optimum number of factors was applied to the models, with a probability of 0.75. However, the number of factors was higher than expected (three) in some cases. Therefore, a smaller number of factors was selected according to another criterion for which the analysis of several tools given by the PLS software,[27] *e.g.*, view of PLS loading factors, calibration coefficients and statistical parameters, was carried out. In spite of both criteria being somewhat arbitrary, the purpose of the comparison was to identify the factors truly needed to build the calibration models.

Finally, the internal cross-validation of the proposed calibration models was performed by means of several statistical parameters; r^2 (square of the correlation coefficient), RMSD (root mean square deviation), SEC (standard error of calibration) and REP (%) (relative error of prediction) were calculated. The number of factors and statistical parameters for the models in the study are shown in Table 2.

According to the *F*-test and the proposed criteria, the optimum number of factors coincide in the calibration model D (three factors) for pyridoxamine and pyridoxic acid, and there are no significant differences between their internal validation parameters values and the corresponding parameters of models A, B and C. In contrast, four and three factors are suggested as the optimum according to both criteria in models C and D for the calibration of pyridoxal, without significant differences between the results obtained with three and four factors, even if these contrast with the parameters of models A and B chosen with the two criteria. Consequently, calibration model D was selected as the best one, constructed with three factors for all compounds. Probably the use of spectral regions with Raman scattering, noise or information not related to the compounds led to the overfitting observed in models A, B, C and D for pyridoxal, if the *F*-test criterion was considered.

Table 2 Internal validation: statistical parameters using the PLS-1 algorithm

Model	Component	Factors*	r^2	RMSD	SEC	REP (%)
A	Pyridoxal	6^F	0.9996	0.560	0.568	1.659
		4^O	0.9993	0.718	0.728	2.126
	Pyridoxamine	4^F	0.9996	2.162	2.193	1.702
		3^O	0.9986	3.814	3.869	3.004
	Pyridoxic acid	4^F	0.9999	0.098	0.100	0.972
		3^O	0.9998	0.126	0.128	1.241
B	Pyridoxal	5_F	0.9995	0.623	0.632	1.844
		4^O	0.9992	0.779	0.790	2.307
	Pyridoxamine	4^F	0.9995	2.303	2.336	1.813
		3^O	0.9987	3.767	3.821	2.966
	Pyridoxic acid	4^F	0.9999	0.099	0.101	0.989
		3^O	0.9998	0.126	0.128	1.241
C	Pyridoxal	4^F	0.9996	0.552	0.560	1.634
		3^O	0.9986	1.054	1.069	3.122
	Pyridoxamine	4^F	0.9996	1.971	1.999	1.552
		3^O	0.9993	2.751	2.790	2.166
	Pyridoxic acid	4^F	0.9999	0.096	0.097	0.944
		3^O	0.9998	0.111	0.112	1.092
D	Pyridoxal	4^F	0.9994	0.661	0.670	1.956
		3^O	0.9990	0.864	0.877	2.559
	Pyridoxamine	$3^{F,O}$	0.9995	2.363	2.396	1.860
	Pyridoxic acid	$3^{F,O}$	0.9998	0.105	0.107	1.038

* F = *F*-test criterion; O = proposed criterion.

However, the complete assessment of a calibration model requires prediction testing for a set of samples not present in the calibration.

External validation

In order to study the predictive ability of the calibration models proposed, a set of 15 synthetic mixtures selected in the same concentration ranges as those used in the calibration matrix was prepared; the composition is given in Table 3. Once the non-linear variable-angle synchronous spectrum had been obtained for each sample, it was used to provide data in the four models built by PLS-1.

To quantify the predictive performances for the three analytes, the recovery results expressed as percentages (mean ± standard deviation) were calculated for synthetic samples, in addition to RMSD, REP (%) and SEP (standard error of prediction). The results of the external validation performed are given in Table 4. As can be observed, the recoveries obtained for the three compounds were satisfactory in all cases. In fact, there are no significant differences between the statistical parameter values of models A, B, C and D.

Finally, if the internal and external validation results are compared, it can be shown that predictive errors are very similar and the calibration model D constructed with three factors for all components can be selected as the optimum, without overfitting or underfitting problems.

Table 3 Composition of synthetic mixtures. All concentrations in μg l^{-1}

Sample No.	Pyridoxal	Pyridoxamine	Pyridoxic acid
1	36.8	68.0	15.2
2	28.8	116.0	8.8
3	60.8	232.0	18.0
4	20.8	140.0	22.4
5	76.8	52.0	13.2
6	38.4	280.0	4.0
7	0	288.0	10.8
8	0	136.0	23.2
9	75.2	0	9.2
10	43.2	0	22.4
11	78.4	160.0	0
12	35.2	264.0	0
13	0	0	20.4
14	0	256.0	0
15	67.2	0	0

Table 4 External validation: statistical parameters using the PLS-1 algorithm

Model	Component	Factors*	*R* ± SD (%)†	RMSD	SEC	REP (%)
A	Pyridoxal	6^F	98.7 ± 1.6	0.894	0.926	2.388
		4^O	99.5 ± 1.2	0.591	0.612	1.579
	Pyridoxamine	4^F	99.9 ± 1.4	2.350	2.433	1.770
		3^O	100.4 ± 1.3	1.983	2.053	1.493
	Pyridoxic acid	4^F	99.6 ± 0.7	0.108	0.112	0.967
		3^O	99.4 ± 0.4	0.111	0.115	0.993
B	Pyridoxal	5_F	99.1 ± 1.4	0.657	0.680	1.755
		4^O	99.5 ± 1.2	0.628	0.650	1.677
	Pyridoxamine	4^F	99.9 ± 1.4	2.273	2.352	1.712
		3^O	100.4 ± 1.3	1.983	2.052	1.493
	Pyridoxic acid	4^F	99.6 ± 0.7	0.106	0.109	0.949
		3^O	99.4 ± 0.4	0.111	0.115	0.993
C	Pyridoxal	4^F	99.0 ± 1.2	0.656	0.679	1.752
		3^O	100.0 ± 1.6	0.704	0.729	1.880
	Pyridoxamine	4^F	100.4 ± 1.8	2.150	2.225	1.619
		3^O	100.2 ± 1.2	1.990	2.060	1.498
	Pyridoxic acid	4^F	99.6 ± 0.7	0.105	0.109	0.940
		3^O	99.5 ± 0.4	0.108	0.111	0.967
D	Pyridoxal	4^F	98.9 ± 1.5	0.795	0.823	2.123
		3^O	99.0 ± 1.4	0.649	0.672	1.733
	Pyridoxamine	$3^{F,O}$	100.2 ± 1.4	2.020	2.091	1.521
	Pyridoxic acid	$3^{F,O}$	99.5 ± 0.4	0.198	0.204	1.772

* F = *F*-test criterion; O = proposed criterion. † R ± SD = mean recovery ± standard deviation.

Conclusion

The development of instrumentation controlled by personal computers and appropriate software packages permits the digitization, storage and processing of total luminescence spectroscopic data, to obtain the complete characterization of fluorescent compounds in a reasonable time. Consequently, new possibilities in the chemometric area can be suggested; this is the case with non-linear variable-angle synchronous fluorescence spectra which are obtained by the projection of a curved trajectory throughout the excitation–emission matrices.

In addition, the use of techniques such as non-linear variable-angle synchronous fluorescence in conjuction with the partial least squares regression method, a powerful mathematical tool, can improve the selectivity of fluorimetric methods for the resolution of multicomponent systems without separation or derivatization steps, in cases where other techniques do not resolve the interference problems.

Finally, the application of PLS to the simultaneous determination of pyridoxal, pyridoxamine and pyridoxic acid using non-linear variable-angle synchronous spectra has been achieved with satisfactory results.

The authors gratefully acknowledge financial support from the Dirección General de Investigación Científica y Técnica (Project No. PB 94-0743).

References

1 Lloyd, J. B. F., *J. Forensic Sci. Soc.*, 1971, **11**, 83.
2 Inman, E. L., Jr., and Winefordner, J. D., *Anal. Chem.*, 1982. **54**, 2018.
3 Kubic, T. C., Kanabrocki, T., and Dwyer, J., Plenary Lectures at the Annual Congress of the American Academy of Forensic Science, 1980.
4 Muñoz de la Peña, A., Murillo, J. A., Vega, J. M., and Baringo, F., *Comput. Chem.*, 1988, **12**, 213.
5 Oms, M. T., Forteza, R., Cerdá, V., García Sánchez, F., and Ramos, A. L., *Anal. Chim. Acta*, 1990, **228**, 293.
6 Murillo, J. A., and Alañon, A., *Comput. Chem.*, 1993, **17**, 341.
7 Wold, H., and David, F., *Research Papers in Statistics*, Wiley, New York, 1966.
8 Haaland, D., and Thomas, E. V., *Anal. Chem.*, 1988, **60**, 1193.
9 Haaland, D., and Thomas, E. V., *Anal. Chem.*, 1988, **60**, 1202.
10 Brown, S. D., Blanck, T. B., Sum, S. T., and Weyer, L. G., *Anal. Chem.*, 1994, **66**, 315R.
11 Sánchez Peña, M., Muñoz de la Peña, A., Salinas, F., Mahedero, M. C., and Aaron, J. J., *Analyst*, 1994, **119**, 1177.
12 López-de-Alba, P. L., López-Martínez, L., Wróbel-Kaczmarzyc, K., Wróbel-Zasada, K., and Amador-Hernández, J., *Anal. Chim. Acta*, 1996, **330**, 19.
13 Berzas, J. J., Rodríguez, J., and Castañeda, G., *Anal. Chim. Acta*, 1997, **340**, 257.
14 Lindberg, W., Persson, J. A., and Wold, S., *Anal. Chem.*, 1983, **55**, 643.
15 Muñoz de la Peña, A., Durán-Merás, I., Moreno, M. D., Salinas, F., and Martínez-Galera, M., *Fresenius' J. Anal. Chem.*, 1995, **351**, 571.
16 Muñoz de la Peña, A., Durán-Merás, I., Moreno, M. D., Salinas, F., and Martínez-Galera, M., *Fresenius' J. Anal. Chem.*, 1995, **353**, 211.
17 Linares, P., Luque de Castro, M. D., and Varcárcel, M., *Anal. Chem.*, 1985, **57**, 2101.
18 Burton, D. E., Sepaniak, M. J., and Maskarinec, M. P., *J. Chromatogr. Sci.*, 1986, **24**, 347.

19 Tsuge, H., and Hirose, N., *Bitamin*, 1989, **63**, 349.
20 Shama, S. K., and Dakshinamurti, K., *J. Chromatogr.*, 1992, **578**, 45.
21 Berzas, J. J., Murillo, J. A., and Gómez, M. A., *J. Pharm. Biomed. Anal.*, 1996, **14**, 1487.
22 Berzas, J. J., Murillo, J. A., and Gómez, M. A., *Analyst*, 1995, **120**, 171.
23 Petidier, A., Rubio, S., Gómez-Hens, A., and Varcárcel, M., *Anal. Biochem.*, 1986, **157**, 212.
24 Chen, D., Luque de Castro, M. D., and Varcárcel, M., *Anal. Chim. Acta*, 1992, **261**, 269.
25 Berzas, J. J., Murillo, J. A., and Gómez, M. A., *Talanta*, 1995, **42**, 129.
26 Martens, H., and Naes, T., *Multivariate Calibration*, Wiley, New York, 1989.
27 *PLSplus for GRAMS/386 User's Guide*, Galactic Industries, Salem, NH, USA, 1993.

Paper 7/05548J
Received July 31, 1997
Accepted December 4, 1997