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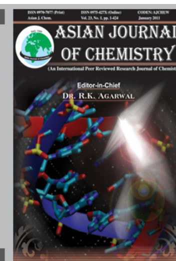

Simultaneous Spectrofluorometric Determination of Phenylalanine and Tryptophane Using Rank Annihilation Factor Analysis

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The application of rank annihilation factor analysis (RAFA) to the analysis of fluorescence excitation-emission matrices of mixtures of phenylalanine and tryptophan is described. The input of rank annihilation factor analysis consists of two bilinear data matrices, *i.e.*, one for unknown and one for the calibration sample. The excitation wavelength range was from 200-300 nm and the emission was recorded from 310-800 nm. Phenylalanine and tryptophan were determined in the concentration range (0.1-1.5) × 10^3 μg mL^{-1} and 0.5-10 μg mL^{-1}, respectively. To check the accuracy of the proposed method, several binary synthetic mixtures were analyzed successfully. No matrix effect was observed in mixture analysis, so a single external calibration sample was used for each analyte. The ability of rank annihilation factor analysis to quantify the studied compounds and the comparability of rank annihilation factor analysis results were evaluated by comparing them with those of partial least squares (PLS) regression as a standard first-order calibration.

Key Words: Rank annihilation factor analysis, Phenylalanine, Tryptophan, Excitation-emission matrices.

INTRODUCTION

The need for reliable quantitative analysis of multicomponent systems has prompted many workers to develop new instrumentation capable of quickly acquiring data from which the identities and concentrations of the components can be readily extracted. Recently, a video fluorometer was described[1] for accomplishing this with fluorescing components. This instrument acquires an excitation-emission matrix (EEM) which has been shown by Warner *et al*., to be useful for qualitative[2] and quantitative[3] analyses of fluorescent mixtures. The method of least squares used by Warner *et al*.[3] and by Sternberg *et al*.[4] is conceptually simple and easy to implement. For quantitative multicomponent analysis, however, the method of least squares yields predictably reliable results only if one has knowledge of all the major constituents present. Consequently other methods such as linear programming[3], non-negative least squares[5] and factor analysis[6] have been suggested as possible algorithms. In many analytical problems one is confronted with an analyte which contains a few known fluorescing species of interest mixed with other fluorescing unknowns. In such cases, it would be very convenient if one could obtain quantitative information for the known compounds without disturbing about the other species present. The method of rank annihilation proposed here offers a promising approach to this problem when combined with the video fluorometer[7].

Fluorescence spectroscopy is a versatile tool mainly used because of its selectivity and sensitivity. Fluorescence spectra can be recorded in difference modes such as emission, excitation, synchronous and excitation-emission matrix (EEM). The arrangement of fluorescence data in an array (*i.e.*, first-order data) can be analyzed with first-order calibration methods such as partial least squares (PLS) regression. This approach has been successfully applied to simultaneous mixture analysis[8,9]. However, first-order calibration methods require that both unknown and standard samples have the same chemical and physical characteristics, *i.e.*, all detectable species present in the unknown samples, including analytes and interferences, must also be present in the standard samples. The most expensive step in first-order multivariate calibration methods is the preparation and analysis of the large number of standards that have to be used for calibration. Alternatively, fluorimetric data can be arranged in data matrices (*i.e.*, second order data) for analysis with second-order calibration methods, which may take advantage of features of both excitation and emission spectra of the compound studied. The use of fluorescence excitation-emission matrices (EEMs) for the two orders of data is advantageous because (i) the measurements can be made on a single instrument with consistent channel registration, (ii) EEMs exhibit good sensitivity, selectivity and bilinearity and (iii) a range of options is available for the third order. Variation in the sample composition is the most common approach to introducing the third order in fluorescence EEMs[10,11].

In considering the simultanous analysis of multicomponent, multidimensional data sets, we have previously considered three analytical situations. In the first, all of the components are knowns, for which calibration data are available. Here, the method of maximum likelihood or least squares is appropriate for finding the quantity of each component[2,12]. In the past, this approach has required excellent reproducibility between calibration standards and the data obtained for the mixture. In the second analytical situation, none of the components are known and therefore only qualitative analysis is possible. Here, the method of factor analysis can be applied[13,14]. This method provides (a) a lower bound to the number of linearly independent components present in the mixture and (b) estimates for the spectral and retention vectors when low numbers of components are present. Factor analysis approaches have the advantage that no assumptions are made regarding the shapes of the spectral and retention vectors, other than that they all be positive.

In the final and most commonly encountered situation, one is attempting to quantitate a series of known in the presence of a variable background of unknown. For this purpose, the method of rank annihilation has been developed[15-18]. When there is one known component, the amount of that component can be found by iteratively subtracting it from the observed data until the rank of the remainder matrix is reduced by one. For multiple components, the rank of the remainder matrix is reduced by an amount equal to the number of components being subtracted[19]. Lorber[20] has developed a noniterative method for multicomponent rank annihilation and Sanchez and Kowalski[21] have further generalized the method.

One of the most serious problems that can occur in classical quantitative analysis is the presence of one or more spectral interferent-chemical species which affect the instrument response and which are unaccounted for in the calibration process. Advances in chemometric methods allow quantitative analysis in the presence of unidentified interferents if a three way experimental data matrix is available for each sample. This property is the so-called "second-order advantage"[22] and it is based on the earlier work done in the psychometrics field[23,24]. The use of this advantage in analytical chemistry was proposed by Ho *et al*.[7] (rank annihilation factor analysis, RAFA), for the multicomponent analysis of fluorescent mixtures using excitation-emission matrix (EEM). Later, Lorber[20] and Sanchez and Kowalski[21] proposed new and simple solutions for the method of Ho *et al*.[7] and now it is called the generalized rank annihilation method (GRAM). The method of rank annihilation qualitatively can be described as follows. For a multicomponent solution emission-excitation matrix, M, the rank, ideally, should equal thenumber of components. If we know one of the components with EEM, N present in the solution and if we subtract the correct amount of N from M, the original rank of M should be reduced by one. In such an instance, we should observe the eigenvalue of M corresponding to N becoming zero. Because of errors in actual experimental data, we cannot expect the eigenvalue to vanish completely. However, it will attain a minimum. The amount of N subtracted to achieve a minimum in the corresponding eigenvalue will correspond to the relative concentration of the known component in the mixture[7]. When both spectral and spatial vectors are known for the pure component, standard rank annihilation has demonstrated very accurate estimations for the concentrations. However, when only one of the vectors is known for the pure component, the technique is invalid. In this case, the method of rank annihilation with incomplete information is useful[25]. Some of rank annihilation factor analysis applications have came in below.

Rank annihilation factor analysis combined with the optimization of kinetic parameter is adopted to resolve the two-way kinetic-spectral data measured online from chemical reactions. To a multi-step reaction whose intermediate process is complicated and reaction order is fractional, the reaction order and rate constant of the first step can be determined without the knowledge of the kinetic model of the reaction[26].

To two-way kinetic-spectral data measured in chemical reactions, the pure spectrum of each reactant can be reached while that of the intermediate usually remains unknown and the concentration of each species in the reaction is not directly available, but they do change conforming to certain kinetics function. The resolving of such kind problem is similar to gray system with unknown background, where RAFA can be successfully adapted. The principles for the determination of the rate constants and absorption spectrum of each component are deduced through a two-step consecutive reaction model and it shows that this approach can be applicable to systems where all three components absorb and the intermediate or the final product does not absorb[27].

EXPERIMENTAL

All fluorescence measurements were done on a cary eclipse fluorescence spectrophotometer (Varian, Australia) equipped with a Xenon lamp pulsed at 80 Hz. The measurements were done using 1 cm quartz cell and slits widths of 5 nm for both excitation and emission monochromators. The scan rate of the monochromator was maintained at 120 nm/min in recording of emission and excitation-emission spectra. The pH measurements were carried out with a Metrohm 713 pH meter (Herisau, Switzerland) equipped with a glass electrode.

Data acquisition and data analysis were performed with Matlab 7.1 and the m-file of RAFA. Partial least square (PLS) analyses were carried out by using PLS-Toolbox software version 2.0.10

Analytical-reagent grade chemicals were employed in all experiments. Phenylalanine and tryptophane was obtained from Sigma (USA). KOH were purchased from Merck (Darmstadt, Germany). A stock solution of phenylalanine (5.0×10^3 μg mL^{-1}) was prepared by dissolving 500 mg of phenylalanine in 100 mL of distilled and organic free water in volumetric flask. An aqueous stock solution of tryptophane (100 μg mL^{-1}) was obtained by dissolving 10 mg of this amino acid in 100 mL of distilled and organic free water. 0.1 M of KOH solution was also employed as a buffer.

Procedure and acquisition of data sets: First order data (emission spectra) were recorded in the range of 278-800 nm with 1 nm intervals using an excitation wavelength of 258 nm for phenylalanine and 299-800 nm with 1 nm intervals using excitation wavelength of 279 nm for tryptophane. Excitation-emission matrices (second order data) were measured every 5

nm in the emission range 300-800 nm and every 5 nm in the excitation range 200-300 nm, making a total of 101 × 21 = 2121 data points.

RESULTS AND DISCUSSION

Individual calibration: Individual calibration curves were constructed with several points as fluorescence intensity *versus* amino acids concentration in the range (0.1-1.5) × 10^3 µg mL^{-1} for phenylalanine and (0.0100-0.0005) × 10^3 µg mL^{-1} for tryptophane and evaluated by linear regression. In order to obtain the calibration curves of amino acids, we measured the fluorescence intensity at 561 and 364 nm for phenylalanine and tryptophane, respectively, while the excitation wavelength was 258 and 279 nm for phenylalanine and tryptophane, respectively. The repeatability, expressed by relative standard deviation, the linear range, the sensitivity, the detection limits and the correlation coefficients were obtained for phenylalanine and tryptophane and they are presented in Table-1.

TABLE-1
ANALYTICAL FIGURE OF MERIT FOR BOTH ANALYTES

Phenylalanine	Tryptophane	Parameter
0.35-1.45	0.00075-0.0045	Dynamic linear (range/?g mL^{-1})
0.9932	0.9934	Correlation coefficient
11.6	5.4	RSD (%)
Y = 228.96x + 25.762	Y = 14407x + 430.66	Equation of calibration curve[a] (fluorescence intensity *versus* µg mL^{-1} of analyte)

a: In order to obtain the calibration curves of amino acids, we measured the fluorescence intensity at 561 and 364 nm for phenylalanine and tryptophane, respectively, while the excitation wavelength was 258 and 279 nm for phenylalanine and tryptophane, respectively.

Partial least squares (PLS): The arrangement of fluorescence data in an array (*i.e.*, first order data) was analyzed with partial least square regression as a first-order calibration method. Partial least squares was selected as a standard multivariate calibration method for comparing and evaluating the results of RAFA as a second-order method. However, first order calibration methods require that both unknown and standard samples have the same chemical and physical characteristics. The most expensive step in first-order multivariate calibration methods is the preparation and analysis of the large number of standards that have to be used for calibration. Fifteen binary mixtures were selected as the calibration set (Fig. 1). The composition of the samples was randomly designed in order to obtain non-correlated concen-tration profiles. The correlation between concentration vectors can be minimized if the correlation coefficient matrix is consi-dered as the criterion. The obtained model in the calibration step was validated with 5 synthetic mixture sets containing the amino acids in different ranges (Fig. 2). Fluorescence emission data from calibration set were taken between 278 and 800 nm. The concentration range of analytes in all synthetic samples was (0.1-1.5) × 10^3 µg mL^{-1} for phenylalanine and (0.5-100) µg mL^{-1} for tryptophane. The selection of the optimum number of factors was estimated by cross-validation, leaving out one sample at a time[28]. The predicted concentration of the analytes in each sample was

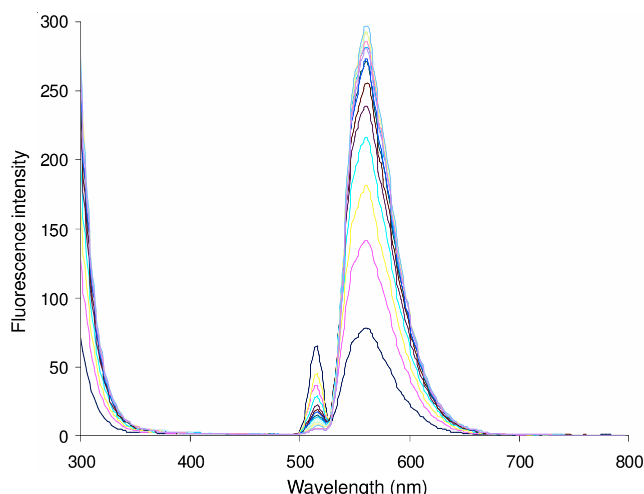

Fig. 1. Calibration of binary mixture

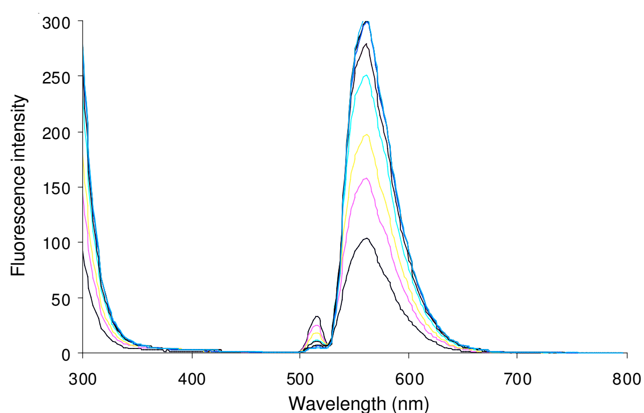

Fig. 2. Prediction of binary mixture

then compared with the known concentration of them in the respective sample and the prediction error sum-of-square (PRESS) was calculated. This parameter was calculated each time adding a new factor to the model and is shown in Figs. 3 and 4. A plot of the PRESS against the number of factors for each individual component indicates a minimum value for an optimal number of factors. For finding the smallest model (fewest numbers of factors) the F statistics was used to carry out the significance determination[29]. It was found that the optimum number of factors was nine for both phenylalanine and tryptophane (Figs.3 and 4). The results obtained from spectrofluorimetric simultaneous analysis of phenylalanine and tryptophane by PLS method are given in Table-2. The values of root mean square difference (RMSD), the square of the correlation coefficient obtained when plotting actual *versus* predicted concentration (R^2) and the relative error of prediction (REP) for each component in nine synthetic samples are included in order to give an indication both of the average error in analysis and the quality of fit of all data to a straight line.

$$RMSD = \left[\left(\frac{\Sigma(C_{real} - C_{found})^2}{\Sigma(C_{found})^2}\right)\right]^{1/2}$$

$$REP = 100 \times \left[\left(\frac{\Sigma(C_{real} - C_{found})^2}{n}\right)\right]^{1/2}$$

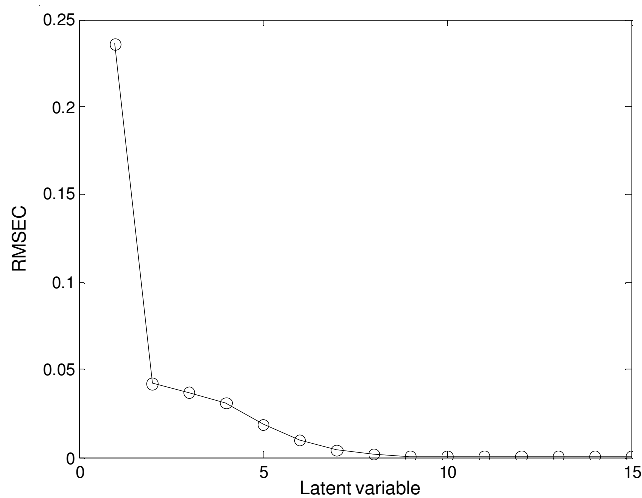

Fig. 3. Cross validation plots for phenylalanine

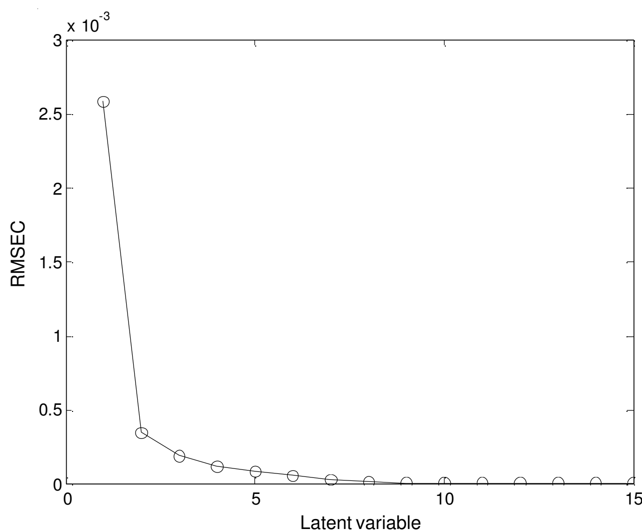

Fig. 4. Cross validation plots for tryptophane

TABLE-2 STATISTICAL PARAMETER FOR PHENYLALANINE AND TRYPTOPHANE IN THE VALIDATION SET OF PLS MODEL				
Statistical parameter	Phenylalanine		Tryptophane	
	Train	Pred.	Train	Pred.
RMSD[a]	0.0461	0.0641	0.6817	0.8571
REP[b]	4.1865	5.9864	0.0346	0.1817
(R^2)[c]	0.9906	0.9964	0.9888	0.9809

a: RMSD calculated according to: $RMSD = \left[\left(\frac{\Sigma(Y_{real} - Y_{found})^2}{\Sigma(Y_{found})^2}\right)\right]$.

b: REP calculated according to: $REP = 100\times\left[\left(\frac{\Sigma(Y_{real} - Y_{found})^2}{n}\right)\right]^{1/2}$.

c: Correlation coefficient for plotting the Y_{real} *versus* Y_{found}.

Rank annihilation factor analysis: The input of a RAFA calculation consists of J1 × J2 bilinear data matrices. Fig. 5 shows the EEMs for 800 and 10 µg mL^{-1} of phenylalanine and tryptophane, respectively, which were selected as standards for RAFA analysis. In order to test the performance of the proposed method, several synthetic binary mixtures of amino acids in various concentrations were analyzed by using the RAFA algorithm (Fig. 6). The results of estimating the concentration of both amino acids for 5 binary mixtures are

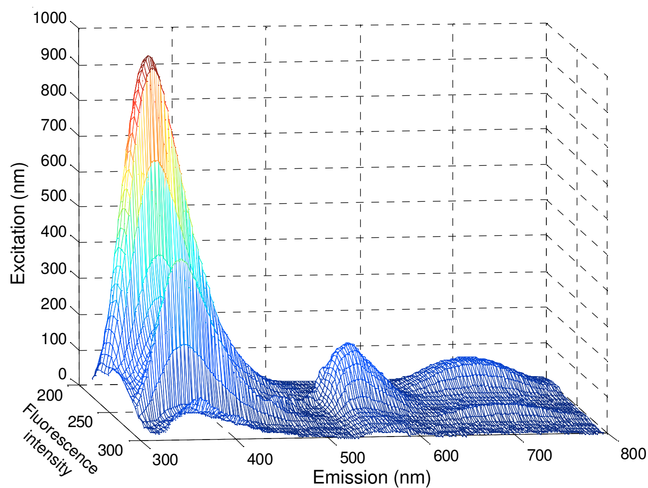

Fig. 5. EEM for 800 and 10 µg mL^{-1} of phenylalanine and tryptophane respectively, as standards for RAFA analysis

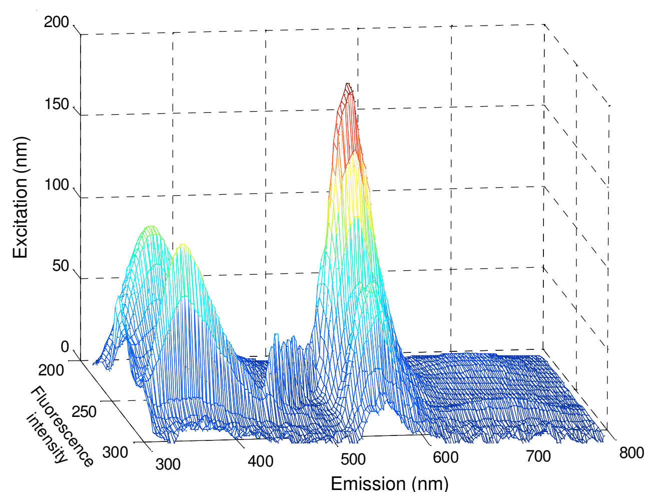

Fig. 6. EEM of various mixtures of phenylalanine and tryptophane as prediction set for RAFA analysis

presented in Table-3. As can be seen, the amounts added and found were consistent for the tested mixtures. In the presence of matrix effects which could be exist in very complex samples, RAFA can be combined with a standard addition method. In this work, the obtained results from standard addition method and using the external standards had not any significant difference. Therefore, the external standard calibration is proposed for simultaneous determination of phenylalanine and tryptophane. External standard calibration is simpler to implement because a single standard set is used for all unknown samples.

TABLE-3 RESULTS FOR ANALYSIS OF FIVE SYNTHETIC SAMPLES BY RAFA				
Sample	Phenylalanine (mg mL^{-1})		Tryptophane (mg mL^{-1})	
	Added	Found	Added	Found
1	0.30	0.35	0.00100	0.00130
2	1.50	1.57	0.00400	0.00480
3	1.00	0.96	0.00080	0.00086
4	0.70	0.72	0.00900	0.00880
5	1.30	1.17	0.00030	0.00027
RMSD (%)	6.9		8.7	

Conclusion

The potentials of two chemometric methods for determination of two important amino acids phenylalanine and tryptophane in binary mixtures of them have been demonstrated. Two types of fluorescence spectra data were used:emission spectra as first-order data and excitation-emission spectra as second-order data. PLS and RAFA were used for analyzing of first-order data and second-order data, respectively. The rank annihilation factor analysis requires only one standard sample for quantification, which make it easier than a first-order method such as PLS. The proposed method for simultaneous spectrofluorimetric determination of phenylalanine and tryptophane is a rapid one-step procedure which only requires the dissolution of the sample and the acquisition of excitation-emission spectra, so it is a simple, inexpensive and fast procedure which does not need a previous separation of the analytes or other previous sample treatments.

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