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Multivariate Curve Resolution Applied to Continuous-Flow Spectrophotometric Titrations. Reaction between Amino Acids and 1,2-Naphthoquinone-4-sulfonic Acid

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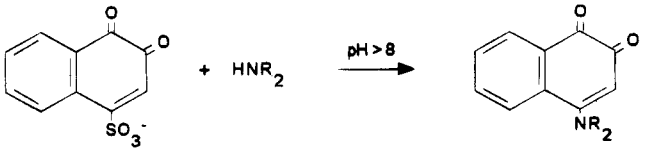
A continuous-flow acid–base titration system has been developed for the study and analytical application of chemical reactions between amino acids and 1,2-naphthoquinone-4-sulfonate. For each titration, a set of spectra are obtained at pH values from 6.5 to 12.5. Multivariate curve resolution is applied to provide an optimal estimation of concentration and spectra profiles of the species formed during the chemical reaction. Resolution of these chemical species is greatly improved when several runs of different spectrophotometric titrations of the same chemical reaction system (multiple correlated data matrices) are analyzed simultaneously, allowing the quantitative determination of the concentration of amino acid that has reacted in each case.

It has been pointed out that most traditional wet chemistry methods of analysis can be easily implemented using modern flow technologies. The term flow chemography[1] has been proposed for chemical techniques which exploit controlled flow and flow injection methodologies and translate the information obtained thereby into a graph or image. All these techniques, including chromatography, flow injection analysis, and flow injection synthesis, require control of the reaction conditions by the flow. Similarly to these methods, spectrophotometric titrations can be implemented using controlled flow methodologies. With any of these flow-based methods, a vector of ordered data (first-order tensor) or a matrix of ordered data (second-order tensor) is produced. The treatment of these types of data by computerized means, like multivariate calibration[2–5] and multivariate curve resolution methods,[6,7] permits the resolution and quantification of the components present in unknown mixtures.

The goal of multivariate curve resolution methods is the recovery of the true underlying concentration and spectra profiles of the individual species in the unresolved mixture. However, in general, the pure mathematical solutions obtained by multivariate curve resolution and other factor analysis-based methods are not the true solutions but a linear combination of them,[7,8] and a certain level of ambiguity persists (factor analysis ambiguity). Conditions to remove these ambiguities have been studied.[9] Detection of selectivity and zero concentration regions are important aspects to consider. Depending on the cases, additional constraints like non-negativity, unimodality, and closure considerably reduce the ambiguity of possible mathematical solutions.

The study of a single data matrix of an unresolved mixture by multivariate curve resolution is problematic, even applying the mentioned constraints. However, the simultaneous treatment of several sets of correlated data matrices having one or two orders in common by multivariate curve resolution[9–12] is a powerful way to improve the results with respect to those obtained by individual analysis of a single data matrix. This requires that the different data matrices simultaneously analyzed have at least one of the orders in common (i.e., the spectral order is easily maintained if the pure spectrum of common species is unique in the different titrations). Optimum resolution is achieved when the data present a trilinear structure,[13] which means that every species is defined by the same spectral and concentration vectors in the different experiments. Experimentally, this requires precise synchronization of the two orders of measurement for all data sets to be simultaneously analyzed.

In the present work, a recently developed multivariate curve resolution method is used for the analysis of reaction-based data obtained from a continuous-flow spectrophotometric titration. This iterative method is a general tool for the resolution and quantitation of the species present in different kinds of bilinear data systems, like multiequilibria and macromolecular spectrophotometric titrations,[10,11] process analysis,[12] coeluted chromatographic peaks,[14] and chemical sensors.[15] The reaction under study is the derivatization of amino acids with 1,2-naphthoquinone-4-sulfonate (NQS)[16] shown in the following scheme:



The reaction takes place at 70 °C in a three-channel continuous-flow system. The pH for the development of the reaction can be varied in a controlled manner. A previous continuous-flow titration

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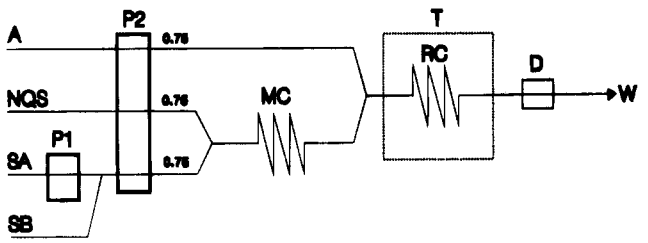

Figure 1. Continuous-flow manifold. P1, variable speed pump; P2, constant speed pump; MC, mixing coil of 2 m × 0.5 mm i.d.; RC, reaction coil of 10 m × 0.5 mm i.d.; T, thermostatic bath; D, spectrophotometric detector; A, amino acid channel; NQS, NQS channel; SA, stock solution A; SB, stock solution B; W waste. Flow rates of buffer, NQS, and amino acid solutions are 0.75 mL/min. Flow rates of stock solutions SA and SB are variable.

system[17] was designed to study the acid–base equilibria of the NQS reagent, demonstrating that flow systems allow titration of unstable analytes such as NQS. Although data produced were also spectra at different pH values, the data structures differ considerably in both cases. For a particular acid–base equilibrium, the relative concentrations of species depend only on the pH variation. This means that the simultaneous treatment of these data, apart from signal averaging, will not improve the resolution with respect to a single data matrix. However, in reaction-based systems, such as amino acids and NQS, different experiments can provide new and complementary information, reducing in this way the ambiguities of the mathematical solutions. Moreover, the present study is also focused on developing new constraints to be applied to the alternating least-squares optimization, profiting from the characteristics of the data structure in which the trilinearity is maintained only for some species.

EXPERIMENTAL SECTION

Reagents. All chemicals were of analytical-reagent grade. Amino acids were supplied by Merck. Sodium 1,2-naphthoquinone-4-sulfonate (NQS, Aldrich) was used to prepare a stock solution 1.1×10^{-3} M reagent in 0.1 M hydrochloric acid (Merck). Solution SA was 0.05 M NaH_2PO_4 + 0.05 M H_3BO_3 + 0.05 M $NaHCO_3$ + 0.05 M NaOH. Solution SB was 0.05 M NaH_2PO_4 + 0.05 M H_3BO_3 + 0.05 M $NaHCO_3$ + 0.35 M NaOH (all from Merck).

Apparatus. A Perkin-Elmer UV/vis/near-IR Lambda 19 spectrophotometer with a Hellma flow cell (10 mm path length and 18 μL volume) was used. Spectra were collected and translated to JCAMP ASCII files by means of Perkin-Elmer software. pH was measured with an Orion ROSS combined glass electrode connected to a Radiometer PHM84 pH meter.

Continuous-Flow System. The continuous-flow system developed in the present study is shown in Figure 1. All solutions were pumped through standard Tygon tubing by means of two low-pressure peristaltic pumps (Scharlau HP4). Connections, T-pieces, and reaction coils were made of PTFE. Buffer and reagent solutions merged in a mixing coil (MC) of 2 m × 0.5 mm i.d. The reaction between amino acids and NQS reagent took place in a reaction coil (RC) of 10 m × 0.5 mm i.d. immersed in a thermostatic bath (SBS TFB-3) at 70 °C.

The final buffer solution was generated on-line from two different stock solutions, SA and SB. Its composition was varied during the process by modifying the ratio of the flow rates of the two solutions. Therefore, the pH in the RC was varied in a controlled manner. Flow rates of amino acid, reagent, and buffer solutions were kept at 0.75 mL/min for all of them, while those of solutions SA and SB were varied during the process (from 0.65 to 0.1 mL/min for SA, and from 0.1 to 0.65 mL/min for SB).

At every titration point, the corresponding spectrum for each amino acid concentration was registered when the system achieved the steady state. The pH of each point was experimentally measured in the waste solution (W). The composition of solutions SA and SB was suitable to work in a buffered medium during the entire titration (from pH 6.5 to 12.5), increasing, therefore, the robustness of the method. The pH synchronization between different runs of titrations of the same chemical system was experimentally achieved by pumping sequentially through the system, at every pH, each solution with different amino acid concentration. When the corresponding spectra had been registered, the pH was modified and the whole procedure repeated for a new pH.

Chemical Reaction Systems under Study. Four amino acids were taken as models to study the reaction with NQS: proline (Pro) as a secondary amino acid, lysine (Lys) as a basic amino acid, aspartic acid (Asp) as an acidic amino acid, and phenylalanine (Phe) as a model for other amino acids. Every amino acid was assayed at three concentration levels: 5×10^{-5}, 1×10^{-4}, and 2.5×10^{-4} M (except for Asp, where the concentrations were 1×10^{-4}, 2.5×10^{-4}, and 5×10^{-4} M. In this case, the concentrations were higher because the sensitivity of the reaction for Asp is lower).

Validation. To validate the results obtained by multivariate curve resolution, the pure responses of the species present in the system were obtained by an independent method. Pure species spectra of the NQS were directly registered from a NQS solution at 70 °C at pH 6.5 (acidic NQS species, **I**) and 12.5 (NQS decomposition product, **II**). Pure spectra of amino acid derivatives were determined in the absence of the NQS reagent to avoid its interfering effect. This was done by chromatographic separation of the different amino acid derivatives after their corresponding precolumn derivatization. It was carried out in a vial (batch procedure) by mixing equal volumes of reagent, buffer, and amino acid solution containing Phe, Lys, Pro, and Asp. The reagent solution was 0.03 M NQS + 0.1 M HCl and buffer solution was 0.05 M H_3BO_3 + 0.24 M NaOH, providing a final pH of 10.0 in the reaction medium, and the concentration of every amino acid in the mixture solution was 0.002 M. The reaction was developed for 5 min in a water bath set at 65 °C. Under these conditions, the formation of derivatives was quantitative. Next, 50 μL of solution containing the amino acid derivatives was injected into a C_{18} column. Separation was achieved using an acetic acid/acetate mobile phase (pH 4.75). Every amino acid derivative was recovered by collecting the corresponding fraction of the eluate from the chromatographic system. For the characterization of

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the acidic (species III) and basic (species IV) amino acid derivative species, the pH of each fraction collected was adjusted to 4.8 or 13.0, respectively, setting the final volume to 5 mL in each case. These solutions were used to record the corresponding spectrum.

DATA TREATMENT

Data generated by the continuous-flow method consisted of independent sets of spectra at different pH values. Although spectra were initially registered from 290 to 590 nm in steps of 1 nm, data reduction was performed in steps of 6 nm; thus, 51 working wavelengths were finally used. The data were treated using MATLAB for Windows (version 4.0).

Although more detailed descriptions are given in other papers,[9-11,13-15] the steps of the multivariate curve resolution procedure are summarized as follows: (1) arrangement of spectral data in matrices; (2) determination of the number of species present in the system from the analysis of the singular values; (3) obtention of an initial estimation of pH profiles or pure spectra of these species either from evolving factor analysis[18] or by pure variable detection methods;[19] (4) detection of selective (rank one) windows using fixed-size moving window evolving factor analysis or local rank analysis;[20,21] and (5) alternating least-squares optimization of the pH or concentration profiles and of the pure spectra based on the compliance of Beer's law. Depending on the nature and structure of the data, different constraints are applied during the optimization.[9-11,13-15]

Points 1 and 5 deserve more attention, owing to the pecularities of the continuous-flow reaction system used in the present study.

Data Structure. Spectral data belonging to different titrations of the same amino acid were analyzed simultaneously by arranging the individual data matrices in a new augmented data matrix, one on top of the other, keeping common wavelengths in the same columns, assuming therefore that the spectra in different experiments were registered at the same wavelengths (spectral synchronization). This augmented data matrix has a trilinear structure[8] when every species is described by the same unique diad of vectors in the different experiments: a pure spectrum and a pH or concentration profile. Thus, trilinearity requires both pH and wavelength synchronization. Departures from strictly trilinear data structures are quite common in analytical chemistry, for instance, when different data matrices share only one of the two orders of measurement, or when one of these orders is not reproducible enough. In the present work, although there are synchronization and reproducibility in both orders of measure, concentration profiles have strictly equal shapes for only some species (amino acid derivative species III and IV), while for reagent species (species I and II), the shapes in their concentration profiles are slightly different in the different experiments.

Constraints Developed for Reaction-Based Systems. (A) Closure Constraint. Two closures must be simultaneously fulfilled at every point of the reaction process: (a) The sum of the concentrations of unreacted and derivative amino acid species must be equal to its total analytical concentration, which is kept constant during the experiment. However, only the amino acid derivative species are spectrophotometrically active, while the unreacted amino acid species do not absorb in the wavelength range under study. In practice, during the ALS optimization, if the sum of the concentrations of amino acid derivative absorbing species exceeds the total amino acid concentration, the pH or concentration profiles of the amino acid derivatives are suitably rescaled. (b) The sum of concentrations of all detected species containing the NQS reagent (including NQS species I and II and amino acid derivative species III and IV) must be equal to the analytical concentration of the NQS reagent.

(B) Equal Shape of pH Profiles Constraint. Equal shape contraints for concentration profiles have been previously developed.[9,13,17] In the present study, a new constraint has been implemented considering of some particularities of the reaction between amino acids and NQS. In this reaction, only the amino acid derivative species keeps the same shape of pH profiles in the different experiments, whereas the unreacted NQS species have pH profiles that depend on the amino acid concentration. This occurs because a certain amount of the reagent is consumed to produce the amino acid derivatives, which thus changes the pH profiles of the NQS reagent species. Therefore, the equal shape constraint is selectively applied to only those species whose pH profiles remain unchanged in the different experiments (amino acid derivative species).

Estimation of the Residual Degree of Ambiguity. The estimation of the residual degree of rotational ambiguity in the recovered solutions obtained by multivariate curve resolution has been calculated from the cosine of the angle between the two vectors representing the known spectrum vector (see Validation, above) and the estimated one by multivariate curve resolution. Cosine values equal to 1.0 indicate that the shapes of both vector profiles are equal, and the rotational ambiguity of that species is totally solved. Conversely, cosine values lower than 1 indicate that a certain degree of rotational ambiguity remains. Quantitative information is obtained by comparing the area of the pH profiles recovered, as is explained in Table 1.

RESULTS AND DISCUSSION

(A) Study of the NQS at 70 °C. Spectra of pure NQS reagent were obtained by pumping water instead of amino acid solution through channel A (see Figure 1). The spectra were ordered in a matrix, and they described the behavior of this reagent at 70 °C in the pH range from 6.5 to 12.5. Eigenanalysis of this data matrix reveals that two main factors are responsible for data variation, which is interpreted as the formation of two species during the titration. This analysis differs considerably from results obtained in previous studies performed at 20 °C, where three species were found.[17] This difference shows clearly that the process in both cases is different. At 70 °C, NQS quickly decomposes at pH values higher than 9, giving an orange-brown product. Conversely, at 20 °C, the decomposition of NQS is avoided, and the process observed was the acid–base equilibria of NQS. The initial purest spectra used in the alternating least-squares optimization were (a) spectrum at pH 6.5 for the NQS acidic species and (b) spectrum at pH 12.5 for the NQS decomposition species.

(B) Study of the Reaction between Phe and NQS. Figure 2 shows the spectral data set when a solution of 2.5×10^{-4} M Phe was pumped through channel A (see Figure 1) and a 1.1×10^{-3} M NQS solution was used as reagent (NQS channel). Eigenanalysis of the corresponding spectral data matrix indicates that four absorbing chemical species are present. According to

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Table 1. Effect of Constraints on the Recovery of Pure Species Spectra by Multivariate Curve Resolution in the Simultaneous Analysis of Four Continuous-Flow Acid–Base Spectrophotometric Titrations of the Phe–NQS Reaction System[a]

	cosine values[c]				% error[d]		
option[b]	I	II	III	IV	III	IV	total[e]
a	1	0.996	0.951	0.732	3.8	3.7	3.8
b	1	0.997	0.949	0.981	12.2	12.4	12.3
c	1	0.993	0.976	0.978	4.5	3.9	4.9
d	1	0.998	0.943	0.982	5.1	3.0	4.2
e	1	0.996	0.987	0.973	5.0	4.3	4.7

[a] Reaction conditions: NQS concentration, 1.1×10^{-3} M; Phe concentration, 0, 5×10^{-5}, 1×10^{-4}, and 2.5×10^{-4} M; pH range, 6.5–12.5 [b] Constraints applied in the ALS optimization: a, without applying any closure constraint or equal shape of pH profiles constraint; b, applying closure constraint with respect to the NQS reagent; c, applying closure constraint with respect to the NQS reagent and equal shape constraint of all the pH profiles; d, applying closure constraint with respect to the reagent and equal shape constraint to only the pH profiles of the amino acid derivative species; e, applying closure constraint with respect to both reagent and analyte and equal shape constraint to only the pH profiles of the amino acid derivative species. [c] Cosine values between experimental and calculated pure spectra. [d] Percentage of error in quantitative estimations of relative concentrations of amino acid derivative species **III** and **IV**: % error = $(\sum(C_{known} - C_{calcd})^2)^{1/2}/(\sum C_{known}^2)^{1/2}) \times 100$, where C_{known} is the relative concentration of an amino acid species known from the experimental concentration ratios of Phe solution used in the different experiments, and C_{calcd} is the calculated relative concentration of an amino acid species obtained from the area ratios of the recovered pH profiles of the species in the different experiments by multivariant curve resolution. [e] Total percentage of error considering both species **III** and **IV** simultaneously (using the same equation for % error described in *d*).

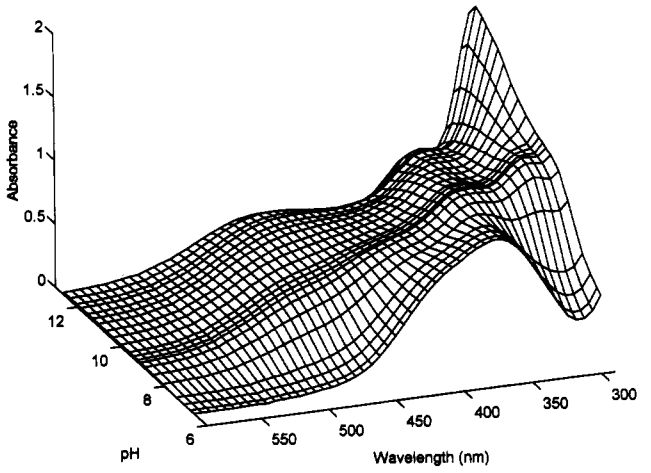

Figure 2. Experimental spectra obtained in a continuous-flow acid-base spectrophotometric titration of the Phe–NQS reaction system when a 2.5×10^{-4} M Phe solution is pumped through channel A. Conditions: NQS concentration, 1.1×10^{-3} M; pH range, 6.5–12.5.

the results of the previous study of the NQS decomposition, two of these species can be assigned to the NQS reagent, while the other two are assigned to the amino acid derivative species or reaction products. The spectra used as initial estimation of these two reaction products were those measured at pH 9.0 and 12.3, corrected (subtracted) for the NQS absorption spectra at these pH values.

In the resolution of this single data matrix by multivariate curve resolution, two closure constraints were simultaneously applied to the total analytical concentration of NQS and amino acid. The cosine values between the known pure spectra (obtained from independent experiments) and those recovered from the optimization procedure were 1 for species **I**, 0.971 for species **II**, 0.986 for species **III**, and 0.941 for species **IV**. From these results, species **I** is correctly recovered, while especially species **IV** still shows a certain degree of rotational ambiguity.

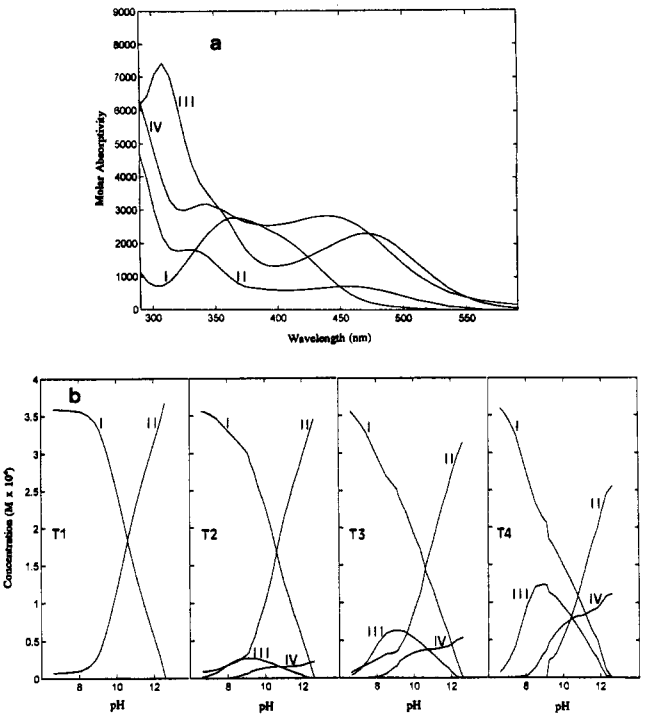

Figure 3. Results of the study of the reaction between Phe and NQS by simultaneous multivariate curve resolution analysis of four continuous-flow acid–base spectrophotometric titrations (T1, T2, T3, and T4). Titration conditions: NQS concentration, 1.1×10^{-3} M; pH range, 6.5–12.5; Phe concentrations, 0 in T1 (water instead Phe solution is pumped), 5×10^{-5} M in T2, 1×10^{-4} M in T3, and 2.5×10^{-4} M in T4. Constraints applied: (i) closure with respect to reagent and amino acid, (ii) equal shape for the pH profiles of the amino acid derivative species **III** and **IV**. (a) Recovered pure spectra of species **I**, **II**, **III**, and **IV**. (b) Recovered pH profiles of species **I**, **II**, **III**, and **IV** for the different experiments.

To improve the previous resolution of the system, a simultaneous analysis of several experiments is needed. Four different solutions pumped through channel A of Figure 1 (Phe solutions at 5×10^{-5}, 1×10^{-4}, and 2.5×10^{-4} M and water) were used to generate four acid–base spectrophotometric titrations. Eigenanalysis of the column-wise augmented data matrix (wavelength common) gives four major contributions, similarly to the eigenanalysis of the individual data matrices containing the amino acid and the NQS reagent. No rank augmentation is observed when more data matrices are included in the analysis, showing that a maximum of four independent absorbing species are detected in the system within the pH range under study (6.5–12.5). Eigenanalysis of the rowwise augmented data matrix indicates a contribution of a fifth singular, which is interpreted as if the shapes of the pH profiles of the two NQS reagent species in the different experiments are not exactly equal in all the titrations but slightly modified by the reaction with the amino acid. The excess of NQS reagent concentration with respect to the amino acid was not enough to neglect its consumption by the reaction with Phe.

The effect of different constraints on the recovery of pure spectra and pH profiles of the species present has been evaluated (Table 1). In practice, because of random and systematic errors such as baseline drift and variations in the flow rates, it is difficult

Table 2. Effect of Constraints on the Recovery of Pure Species by Multivariate Curve Resolution in the Simultaneous Analysis of Four Continuous-Flow Acid–Base Spectrophotometric Titrations of Several Amino Acid–NQS Reaction Systems[a]

	cosine value				% error		
amino acid	I	II	III	IV	III	IV	total
Pro	1	0.997	0.973	0.940	2.2	1.1	1.8
Lys	1	0.997	0.976	0.961	0.6	9.9	7.2
Asp	1	0.999	0.964	0.775	1.4	41.3	32.8

[a] Constraints applied: closure constraint with respect to both reagent and analyte and equal shape constraint to only the pH profiles of the amino acid derivative species. See Table 1 for information.

to obtain the complete recoveries of these profiles. Thus, in this study, cosine values higher than 0.95 are considered to be related with good recoveries of spectra. In Table 1, a summary of results is given. In all cases, the spectral profiles of the reagent species I and II are correctly recovered, showing clearly that the inclusion of the response matrix of the pure NQS reagent provides the selective information needed for the resolution of these two NQS species. Using equal shapes of the pH profiles constraint and the closure constraint (approaches c, d, and e), the best results are obtained. Option e (closure constraint with respect to both reagent and Phe and equal shape constraint to only pH profiles of Phe derivative species) is the most powerful for the resolution of this reaction system. Figure 3 shows pure spectra and pH profiles obtained with this option. The shapes of the pH profiles recovered for species I are slightly different in the four experiments. This agrees with the fact that NQS is consumed by the reaction with the amino acid.

(C) Study of the Reactions between Pro, Lys, and Asp with NQS. The reaction between NQS and three other amino acids (Pro, Lys, and Asp) was also studied by multivariate curve resolution in the same way as for Phe. For every amino acid, four spectrophotometric titrations were analyzed simultaneously using the proposed method. Table 2 shows a summary of the results obtained, demonstrating the general use of the method. Under the experimental conditions of the spectrophotometric titrations, the formation of the amino acid derivatives is nearly quantitative for Phe, Pro, and Lys, while for Asp it is low. Species IV (Table 2) is the most difficult to resolve, since its pH profile is always embedded below the pH profile of species II. Moreover, the two pure spectra of species IV and II are very similar, with a high degree of overlap. In particular, species IV of the NQS–Asp system is the poorest recovered because, in this case, a very low amount of derivative species is generated in the reaction (<15%). A longer reaction time and higher temperature may be required for completion of the reaction in the case of Asp.

CONCLUSIONS

The continuous-flow titration method proposed in the present work is able to provide highly structured second-order data, thus allowing improved resolution of unknown chemical reaction-based systems. The multivariate curve resolution procedure allows the recovery of important analytical information about these reaction-based systems, such as the pure spectra profiles of the reagent and product species, the pH profiles of all species present in the system, the percentage of analyte that has been reacted, and the optimum pH for the maximum development of the reaction.

Results of the reactions between NQS and amino acids indicate that constraints like closure and equal shape of profiles selectively applied to only some of the products of the reaction (amino acid derivatives) lead to improved recovery of pure spectra and pH profiles. The method proposed can easily be extended to the characterization and analytical application of other types of chemical reactions.

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SUPPORTING INFORMATION AVAILABLE

Two tables and three figures concerning the experimental results (6 pages). See any current masthead page for ordering information.

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