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Continuous-Flow and Flow Injection pH Gradients for Spectrophotometric Determinations of Mixtures of Nucleic Acid Components

Javier Saurina,* Santiago Hernández-Cassou, Romà Tauler, and Anna Izquierdo-Ridorsa

Department of Analytical Chemistry, University of Barcelona, Diagonal 647, 08028 Barcelona, Spain

A procedure for the rapid determination of mixtures of nucleic acid components from the analysis of spectrophotometric multivariate data obtained with continuous-flow and flow injection pH-gradient systems is proposed. Three flow systems have been developed and assayed in which an on-line pH gradient is generated from the mixing and controlled dispersion of acidic and basic titrant solutions. Quantitative determinations of any particular analyte in the unknown samples in the presence of interferences is performed with a single pure standard for this analyte. They are carried out using an alternating least squares multivariate curve resolution procedure. The methods proposed have been validated using synthetic and real sample mixtures. The results obtained are concordant with the labeled values, and the relative prediction errors are around 5%.

The analysis of mixtures of nucleic acid components is of great interest in biochemical, biomedical, and pharmaceutical fields.[1−3] Apart from the natural nucleic acid components, some derivatives are used extensively as chemotherapeutic and antiviral agents. In particular, azido nucleosides such as 3′-azido-3′-deoxytimidine (AZT) are fundamental in the therapy against the HIV virus.

Liquid chromatography and capillary electrophoresis have been usually used to carry out the analysis of nucleic acid components and their derivatives.[1,2,4−6] However, these techniques may be unsuitable for the analysis of large sets of samples since the separation step is often tedious and time-consuming. In these cases, methods for the simultaneous determination of several analytes without their physical separation and without removing interferences would be preferred. Flow injection analysis and continuous-flow analysis are especially suitable for the fast and easy generation of large amounts of data.[7] These flow methods take advantage of speed, reproducibility, high degree of automation, and low expense of reagents.[7] The data obtained in this way can be arranged in different types of data arrays or matrices, which can be analyzed using chemometric methods.

When the flow methodology provides a vector of data in the analysis of each sample, first-order multivariate calibration methods can be applied. However, these methods require that both unknown and calibration 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 in the calibration set. Besides, when the flow methodology provides data that can be meaningfully arranged in a matrix array (second-order data), second-order multivariate calibration methods can be applied.[8] An important advantage of second-order multivariate calibration methods is that the quantitative determination of the analyte is possible using synthetic standard samples that contain only the analyte of interest (the interferences in the unknown sample do not need to be present in the standard samples).[8,9]

In a previous work, mixtures of nucleic acid components were analyzed with traditional acid−base spectrophotometric titrations, in which the titrant solution was added with a buret to the titration vessel.[10] After each addition, the corresponding pH and spectrum were measured. However, this procedure presents some drawbacks: acid−base titration is the most time-consuming step in the procedure, nucleosides are hydrolyzed in basic medium (pH >9−10), and it is difficult to get pH synchronization between titrations. Flow injection analysis and continuous-flow analysis seem to be good strategies to follow in order to overcome these difficulties.

Several spectrophotometric acid−base titration systems using continuous-flow and flow injection analyses have been proposed in the literature. In continuous-flow methods, the analyte commonly flows at a constant flow rate and mixes with the titrant solution. Titration is often performed either by changing the flow rate of the titrant solution[11−14] or by modifying, on-line, the composition of the titrant solution.[15,16] On the other hand, flow

* Corresponding author: (fax) 34-93-4021233.

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injection titrations usually involve the generation of a pH gradient in the flow injection peak from the controlled dispersion of the acidic and basic solutions.[17−24] The data obtained from these automated spectrophotometric titrations allow distinguishment and resolution of the components of the system according to their different acid−base characteristics. For this purpose, various first-order calibration methods, such as PCR and PLS, have been used successfully.[14,17,18] However, when the data obtained have a second-order structure, an alternative is to analyze the spectrophotometric titration data with second-order multivariate calibration methods,[15,16,21,24] which take advantage of differences in the spectra and the acid−base features of the compounds and allow the determination of the analyte even in the presence of unknown interferences using pure standards of this analyte (second-order advantage[25]).

In the present study, continuous-flow and flow injection pH gradient systems have been developed to carry out fast spectrophotometric determinations of mixtures of nucleic acid components. These methods are based on the generation of a pH gradient in the confluence zone of an acidic and of a basic titrant solution. The second-order spectrophotometric data are subsequently analyzed with a multivariate curve resolution method based on an alternating least squares optimization (MCR-ALS) method.[26−30] This chemometric method takes into account the spectroscopic and the acid−base behavior of the substances simultaneously. The improvements with respect to the manual titrations[10] are that time is saved, no pH measurements are required, synchronized data in the spectral and time orders is provided and instability problems are avoided as nucleosides may hydrolyze in basic medium. Mixtures of four nucleosides (adenosine, cytidine, inosine, and uridine) and a pharmaceutical drug containing a mixture of inosine-5′-monophosphate (a nucleotide) and cytosine (a nucleic base) have been analyzed.

Mixtures of substances with acid−base behavior (such as nucleic acid components) give rank-deficient data matrices when spectrophotometric pH titrations are performed.[10,24,31] A data matrix is rank-deficient when the number of significant contributions to the data variance, estimated by using singular value decomposition or other related factor analysis techniques, is lower than the real number of chemical components present in the system. Therefore, the complete resolution of a rank-deficient data matrix is hindered.[31−33] When the aim of the study is the complete resolution of all the species in the system, the rank-deficiency problem has to be eliminated. However, the total resolution of the system is not necessary for the quantitation of the analyte of interest, and only the partial resolution of the species that have to be quantified is required. As will be shown here, this partial resolution is already achieved when the analysis is performed on an augmented matrix which contains the data matrix of the unknown mixture and that of the standard.

EXPERIMENTAL SECTION

Reagents and Solutions. All chemicals were of analytical grade. Ultrapure water was used for all solutions. Titrant solutions were prepared from 85% (w/w) phosphoric acid solution (Fluka, Budns, Switzerland) and sodium phosphate (Scharlau, Barcelona, Spain) as follows: for the continuous-flow system (see below), the acidic titrant solution was 0.1 M phosphoric acid solution whereas the basic titrant solution was a 0.1 M sodium phosphate solution; for the flow-injection system A (see below), 0.025 M phosphoric acid solution and 0.1 M sodium phosphate solution were used; finally, for the flow-injection system B (see below), 0.025 M phosphoric acid solution and 0.025 M sodium phosphate solution were used. Pure sample solutions were used as standards for the calibration of adenosine, cytidine, inosine, uridine, cytosine, and inosinate solutions, which are designated as A, C, I, U, S, and T, respectively. Binary, ternary, and quaternary mixture solutions of nucleosides used as unknown solutions were: cytidine + uridine, referred to CU; inosine + uridine (IU); cytidine + inosine + uridine (CIU); cytidine + inosine + adenosine (CIA); cytidine + inosine + adenosine + uridine (CIAU). In all cases, the concentration of each compound was 5×10^{-5} M.

A drug (Precurgén from Knoll, Madrid, Spain) containing inosine-5′-monophosphate and cytosine was analyzed. One-fourth g of the drug was dissolved in 200 mL of water, filtered through a Nylon membrane of 0.45 μm pore size, and diluted 20-fold with water. The resulting solution contained 5×10^{-5}mol L^{-1} of inosine-5′-monophosphate and 5×10^{-5} mol L^{-1} of cytosine. This sample solution was designated as Drug.

Apparatus. The spectrophotometric detection was performed with a Hewlett-Packard HP8452A diode array spectrophotometer using a Hellma flow cell of 10-mm path length and 18-μL volume. The acquisition and storage of spectrophotometric data was carried out with Hewlett-Packard software with a Hewlett-Packard Vectra N2 4/50 computer.

Continuous-Flow and Flow Injection pH Gradient Systems. Flow systems enable performance of titrations under the same experimental conditions run to run and allow the generation

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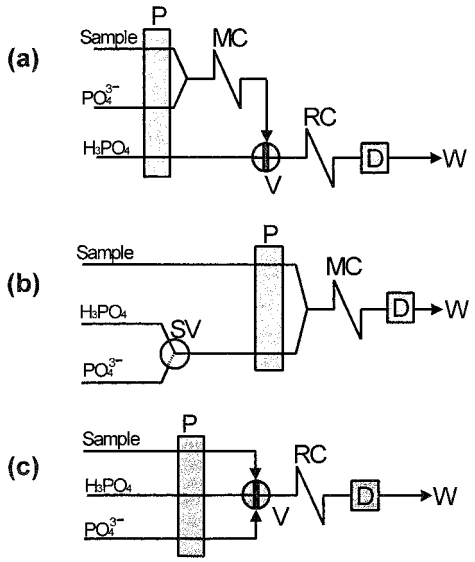

Figure 1. Scheme of the three pH gradient flow manifolds used. P, peristaltic pump; V, injection valve; SV, selection valve; D, detector; MC, mixing coil (200 cm × 0.5 mm i.d.); RC, reaction coil (titration coil); H_3PO_4, acidic titrant solution; PO_4^{3-}, basic titrant solution; W, waste. Flow rates: 0.5 mL/min, each channel. (a) Single injection flow injection titration manifold (FI system A); (b) Continuous-flow titration manifold; (c) Double injection flow injection manifold (FI system B).

of pH gradients in a controlled and reproducible way. In our work, the pH gradients were based on the mixing and dispersion of one acidic and one basic solution. Three different flow manifolds were developed and tested. The use of phosphate solutions as titrants was essential in order to work in a wide range of buffered pH values during the titration, thus increasing the robustness of the process and making more gradual variations of the pH.

In all manifolds, the solutions were pumped with a peristaltic pump Scharlau HP4 by using standard Tygon tubing. T-pieces, connections, and coils were made with Teflon. The samples were injected using an 8-way electrical valve Sirtex VM-8 of variable volume. More detailed descriptions of these manifolds are given below.

Single Injection Flow Injection Manifold (*FI System A*). The single injection flow injection system referred to as system A (see Figure 1a) consisted of a three-channel manifold in which the sample was made alkaline by mixing with the 0.1 M phosphate solution in a 200 cm × 0.5 mm i.d PTFE coil (MC). This basic sample solution was then injected into an acidic carrier of phosphoric acid. The injection volume (300 μL) was large enough to generate an appropriate and gradual pH gradient. The mixing and controlled dispersion between sample and carrier took place in a 200 cm × 0.5 mm i.d. PTFE coil (RC), which was coupled on-line to the detector. The dispersion of the alkaline sample bolus into the acidic stream of the carrier was responsible for the generation of a pH gradient. The shape of this pH gradient was equivalent to that of the dispersed sample. As a result, the pH value was at a minimum ahead of the flow injection peak (with a pH value corresponding to that of the acidic carrier), it increased in the center of the peak up to a maximum and, gradually, decreased in the tail of the flow injection peak until it again reaching the initial acidic pH value. Thus, a central basic region between two acidic regions was obtained.

Continuous Flow Manifold. Continuous-flow titrations were performed by using the assembly shown in Figure 1b. This system consisted of a three-channel manifold for the sample, the acidic (phosphoric acid) solution, and the basic (phosphate) solution, respectively. Initially, the phosphoric acid and the sample solutions were continuously aspirated to the system. As the sample was made acidic, the acidic species of the nucleic acid components were formed. The titration was started once the system achieved a steady state by switching the selection valve (SV) to introduce the phosphate solution into the system. In this way, the sample solution merged with the acidic or basic solutions in a PTFE coil (MC) of 200 cm × 0.5 mm i.d. connected on-line to the spectrophotometer.

Double Injection Flow Injection Manifold (*FI System B*). The flow injection manifold referred to as system B consisted of a carrier channel of phosphoric acid solution in which 175 μL of sample and 500 μL of basic phosphate solution were consecutively injected (see Figure 1c). The mixing and the controlled dispersion of both boluses injected occurred in a 200 cm × 0.5 mm i.d. reaction coil. Therefore, the front of the flow injection peak was made acidic whereas the tail was alkaline.

Data Generation and Data Pretreatment. The titrations were monitored by registering spectra in the range 220−350 nm at regular intervals of 2 s. The whole measurement time was 2 min for the continuous-flow data and 4 min for the flow injection data. Readings over time related directly to the pH gradient; i.e, spectral changes over time were caused by the pH gradient. These flow systems allowed time synchronization, which, in turn, ensured that the pH at each time of measurement was the same for all the samples analyzed (run-to-run pH-gradient synchronization). Since in the acid−base equilibrium each species is defined by the same single spectrum and the same concentration profile, the data obtained have a trilinear structure.[25] This data structure is a feature of great importance for obtaining accurate predictions.

In the flow injection manifolds, the insertion of the sample into a carrier stream of variable composition caused changes in the refractive index over time. This affected the spectrophotometric measurements since it produced an additional contribution to the absorbance. However, such a contribution was almost constant at any wavelength of the spectrum for each measurement time, and consequently, it can be easily corrected by subtracting the absorbance value at 350 nm from the spectrum (none of these nucleic acid components absorb at 350 nm).

Data Sets under Study. Each data set was arranged in data matrices which contained the absorbance data for the different solutions analyzed. They are referred to in the text with the same nomenclature as that used to name the different samples prepared and analyzed (see Reagents and Solutions).

For quantification, the data matrix of an unknown sample mixture has to be analyzed simultaneously with that of a standard sample. To perform this simultaneous analysis with the method proposed (see Data Treatment) the following augmented matrices were built: [**CU;C**], [**CU;U**], [**IU;I**], [**IU;U**], [**Drug;S**], [**Drug;T**], [**CIU;C**], [**CIU;I**], [**CIU;U**], [**CIA;C**], [**CIA;I**], [**CIA;A**], [**CIAU;C**], [**CIAU;I**], [**CIAU;A**], and [**CIAU;U**]. All these matrices were built using one matrix of the sample mixture and one matrix of the standard of the analyte to be quantified. In all cases, the data were arranged in columnwise (wavelengthwise) augmented matrices,

Table 1: Rank Values for the Data Matrices Corresponding to Spectrophotometric Acid−Base Titrations of Mixtures of Compounds, Each One Participating in an Acid−Base Equilibrium

system	R^a	S^b	Rank of the matrices: individual	Rank of the matrices: augmented[c]
1 compound	1	2	2	2
2 compounds	2	4	3	4
3 compounds	3	6	4	5
4 compounds	4	8	5	6

[a] *R*, number of independent reactions. [b] *S*, number of absorbing species. [c] Wavelengthwise augmented data matrix that contains the two following individual matrices: that corresponding to the system analyzed and that including a single compound of those present in the system (it can be considered the matrix of the standard).

placing one matrix on the top of the other and keeping common wavelengths in the same columns.

As an example, [**CIAU;C**] is the columnwise augmented matrix built from the mixture matrix **CIAU** and the standard matrix **C**. In this case, the problem to be solved is the determination of cytidine in the unknown mixture matrix, **CIAU**, using as a standard the matrix of a pure cytidine sample, **C**. Note that no information about interferences present in the **CIAU** mixture matrix (inosine, adenosine, and uridine) is included in the matrix of the standard and that the nature of the components in the mixture sample do not have to be known in advance.

DATA TREATMENT

Data Structure. The number of chemical components ("chemical" or "pseudo" rank of the matrix) is estimated preliminarily by visual inspection of the singular-values plots, as we assume that the chemical components have associated singular values much larger than other possible contributions such as instrumental drift or experimental error. However, the number of species finally chosen is the one that provides a reliable resolution of the system from a chemical point of view.

In closed reacting systems, such as those studied here, in which the different concentration profiles are linearly dependent because of the governing equilibrium reactions (e.g., acid−base reactions), a rank deficiency may appear in the data matrices. It has already been demonstrated[31−33] that in these systems the rank of a data matrix **D** is

$$\text{rank}\,(\mathbf{D}) \leq \min\,(R + 1, S)$$

where *R* is the number of independent reactions and *S* is the number of absorbing species.

Table 1 shows the theoretical rank of the data matrices obtained in the spectrophotometric acid−base titration of mixtures of compounds, each one participating in an acid−base equilibrium and thus yielding two species (one protonated and one deprotonated) that are spectrophotometrically active. With the exception of the matrices with a single compound, the rank of all other data matrices is lower than the total number of absorbing species present (rank deficiency). Rank augmentation of a rank-deficient data matrix can be achieved by matrix augmentation.[31−33] In Table 1, it is shown that rank of the columnwise augmented data matrices increases by one with respect to that of the individual rank-deficient data matrices. In spite of the fact that the rank deficiency hinders the complete resolution of profiles of all the species present in the system, it does not hinder the resolution of the concentration profiles for the analyte of interest.

Application of the MCR-ALS Method. The aim of the present study is the quantitative determination of nucleic acid components in unknown complex mixtures by using a second-order multivariate resolution method. The augmented matrices defined and built for each particular quantitative determination are described in the Experimental Section. The resolution of each augmented-data matrix provided an estimation of the concentration and spectra profiles of the species included in the model using an iterative, constrained alternating least squares (ALS) optimization.[26−30]

Model Selection. The whole data variance could be divided into the data variance due to the analyte and that due to the interferences (which includes all the other compounds present in the sample).

For the flow injection system A, the analyte-data variance was modeled with two components, one for the acidic and another for the basic species, since it presented a single acid−base equilibrium in the working-pH region. It was assumed that the interferences had a chemical behavior similar to that of the analyte, and thus, could be approximately explained by two components (one acidic and one basic). Therefore, a four-component model for the resolution of all augmented matrices of the flow injection system A was chosen.

The continuous-flow and the flow injection system B showed large additional contributions which appeared at the most basic pH region. These contributions could be attributed partly to a high phosphate absorption and partly to a residual contribution by the deprotonated species of the different compounds. A six-component model was used for these systems: three components for the analyte and three more for the interference.

Alternating Least Squares Optimization. The procedure was based on the compliance with a generalized Beer's law. The iterative optimization started with the evaluation of the unknown species spectra using an initial estimation of the $\mathbf{C}_{aug}$ data matrix:

$$\mathbf{S}^T = (\mathbf{C}_{aug})^+ \mathbf{D}^*_{aug} \qquad (1)$$

where $\mathbf{D}^*_{aug}$ is the reproduced data matrix for the number of components considered and $(\mathbf{C}_{aug})^+$ is the pseudoinverse of $\mathbf{C}_{aug}$.

The initial estimation of the $\mathbf{C}_{aug}$ data matrix is obtained in the following way: an estimation of the concentration profiles for the acidic and basic components of the analyte was obtained from the analysis of the matrix of the standard; an estimation of the concentration profiles corresponding to the contribution of the interference is obtained by analyzing the data matrix of the sample with techniques based on the detection of the "purest" variables.[34,35]

In a second stage, the concentration matrix $\mathbf{C}_{aug}$ was updated using the equation:

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$$\mathbf{C}_{aug} = \mathbf{D}^*_{aug} (\mathbf{S}^T)^+ \quad (2)$$

Here, $(\mathbf{S}^T)^+$ is the pseudoinverse of the $\mathbf{S}^T$ matrix.

These steps were repeated until convergence was achieved.

During the ALS optimization of $\mathbf{S}^T$ and $\mathbf{C}_{aug}$ the following were constraints that could be potentially applied to get physically meaningful solutions: (a) the concentration profiles and the pure spectra were nonnegative, (b) the concentration profiles were unimodal (although this condition is not applicable to flow injection system A), (c) correspondence between common species in the different data matrices, (d) the pure spectrum of each species was the same in all runs where that species was present, (e) the concentration profiles of common species present in different runs had equal shapes, and (f) closure constraint for the standard (only in the continuous-flow system). The possible unmodeled data variance in the residuals was evaluated by the lack of fit using the following equation:

$$\% \text{ lack of fit} = 100 \, (\Sigma\Sigma \, (d_{if}^* - d_{if})^2 / \Sigma\Sigma \, d_{if}^{\,2})^{0.5} \quad (3)$$

where d_{if}^* and d_{if} refer to the calculated and the real absorbance data, respectively.

The quantification was performed by comparing the areas below the concentration profiles for the analyte in the standard and in the unknown sample:

$$C_{unk} = (A_{unk}/A_{std}) \, C_{std} \quad (4)$$

Here, C_{unk} and C_{std} are the concentrations of the analyte in the unknown and standard samples, respectively; A_{unk} and A_{std} are the areas below the concentration profiles in the unknown and in the standard samples, respectively.

RESULTS AND DISCUSSION

The three flow systems described here are presented according to the complexity of their models. First, the flow injection system A, in which both analyte and interference are modeled with two contributions, is discussed. Second, the results and particular features of the continuous-flow system are shown. Finally, the flow injection system B is commented upon.

Single Injection Flow Injection System (System A). In this flow injection system the sample was made alkaline before being injected into an acidic carrier (see Experimental Section). Thus, both the beginning and the end of the flow injection peak were more acidic than the maximum of the peak. Rank analysis of the data matrices of the pure analyte standard solutions indicated the presence of two main factors, caused by the acidic and basic species of each nucleoside. Figure 2 shows the spectra obtained for a solution of cytidine using system A. The whole titration was performed in a single flow injection peak and lasted about 250 s. The acidic form of cytidine was detected from the beginning to around 40 s and from around 106 s to the end, while the basic form was predominant around 80 s.

Figure 3 depicts the concentration and spectra profiles recovered in the resolution of the augmented matrix [**CIAU;C**] with the MCR-ALS procedure. The concentration profile recovered for the acidic species of cytidine is a double peak, while for the basic species the concentration profile is unimodal and embedded inside the acidic double peak. The concentration profiles for the analyte in the standard and in the unknown samples are almost identical, thus demonstrating that the MCR-ALS method correctly resolved this compound. Furthermore, the shape of the spectra (Figure 3c) obtained for protonated and deprotonated cytidine agreed with the real ones.[10]

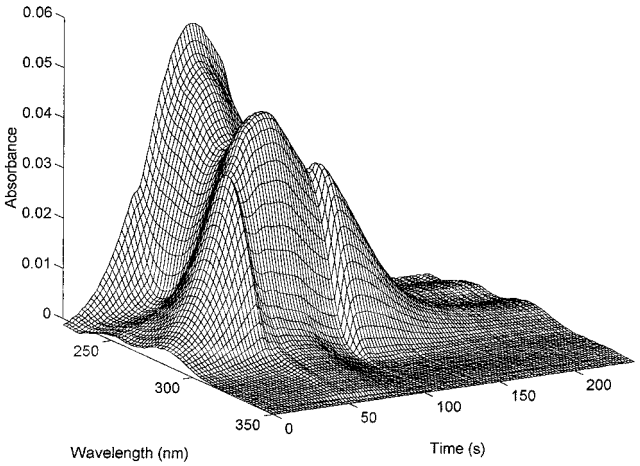

Figure 2. Three-dimensional plot of the experimental data obtained for a 5×10^{-5} M cytidine solution using the FI system A.

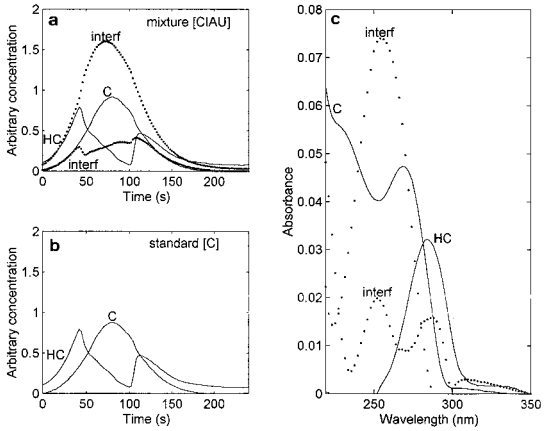

Figure 3. Concentration profiles and spectra recovered in the resolution of the augmented matrix [**CIAU;C**] for the FI system A using the ALS-MCR method. Species assignment: HC, acidic cytidine species; C, basic cytidine species; interf., interference species. (a) Concentration profiles obtained for **CIAU**; (b) Concentration profiles for **C**; (c) Spectra of standard and interference species.

The concentration and spectra profiles for the interfering contribution in the CIAU sample mixture are also shown in Figure 3. These profiles do not belong to any compound present in the mixture but they are probably a linear combination of the concentration and spectra profiles of all interfering species (inosine, adenosine, and uridine).

The determination of each particular analyte can be carried out either from the acidic or from the basic concentration profiles by applying eq 4. As pointed out in a previous study,[10] the quantitation from the acidic profiles was much more accurate than that from the basic ones. This was attributed to the fact that in the acidic zone at the beginning of the titration there was selectivity for the acidic species in the standard samples. On the other hand, the quantitation using the basic species gave higher

Table 2. Determination of Nucleic Acid Components in Unknown Mixtures in the Presence of Interferences Using Pure Standards of the Analyte by the MCR-ALS Method[a]

data matrix	analyte	% prediction error: acidic species	% prediction error: basic species	% lack of fit
[**CU;C**]	cytidine	2.9	0.3	6.68
[**CU;U**]	uridine	1.3	14.4	3.91
[**IU;I**]	inosine	4.1	1.6	3.89
[**IU;U**]	uridine	2.9	3.4	3.33
[**Drug;S**]	cytosine	3.3	3.6	5.31
[**Drug;T**]	inosinate	1.5	0.4	6.55
[**CIU;C**]	cytidine	2.8	3.4	3.93
[**CIU;I**]	inosine	6.8	0.3	3.33
[**CIU;U**]	uridine	1.4	11.7	4.05
[**CIA;C**]	cytidine	14.8	0.5	4.78
[**CIA;I**]	inosine	4.3	0.1	3.54
[**CIA;A**]	adenosine	4.1	5.9	5.55
[**CIAU;C**]	cytidine	1.1	1.3	2.61
[**CIAU;I**]	inosine	0.3	0.2	2.10
[**CIAU;A**]	adenosine	17.5	7.1	3.23
[**CIAU;U**]	uridine	7.3	54.3	2.71

[a] Results from the analysis of the spectrophotometric data obtained with the flow injection system A.

errors, as the experimental conditions of the determinations did not allow a complete formation of the basic species of the analyte, and a significant proportion of the acidic forms were still present. This was especially noticeable for those compounds with a pK_a value of around 9. Therefore, there was no selectivity for the concentration profile of the basic species, which hindered its correct resolution.[27,36] This conclusion is confirmed in the present study (see Table 2), where the prediction error was 5.3% when the acidic species was used for calibration and 9.7% when the basic species was used for calibration. Table 2 gives the results of the determination of each particular analyte in the unknown mixtures from the analysis of the corresponding flow injection spectrophotometric titrations using concentration profiles as initial estimates. There is selectivity for the acidic species of the standard both at the beginning and at the end of the FI peak, and the basic pH value obtained at the peak maximum is not high enough to give selectivity for the basic species. The prediction errors in the quantitation of cytosine and inosinate in the real sample (Drug) were about 2%. The lack of fit ranged between 2.1 and 6.7%, which indicated that the main contributions were successfully modeled.

Continuous-Flow System. Data obtained in the titration of a standard cytidine solution showed that spectral changes associated with the acid−base equilibrium of cytidine occurred in the range of measurement times between 0 and 40 s, whereas in the last part of the pH gradient (from 40 to 130 s) the spectrophotometric changes were attributed to the background variation (caused by the phosphate ions in alkaline medium). This contribution cannot be efficiently removed by background subtraction and, thus, had to be included as an additional factor in the model. This differs from the flow injection system A described above, where background contributions were negligible. Consequently, each nucleic acid compound had to be modeled as three contributions, using three initial estimates, one for the acidic, one for the basic, and one for the background species, respectively. Three interfering contributions were also considered in each case. Therefore, a six-species model was used for the resolution of any augmented matrix, even though there were more than three chemical species from the interference in the ternary and quaternary mixtures (six and nine interfering species, respectively).

The overall prediction error for the acidic species (8.8%) was lower than that for the basic species (19.9%), which indicated that the acidic species led to a better determination of these mixtures. The accuracy in the analysis of cytosine and inosinate in the Drug sample was 5.12% and 4.89%, respectively.

Flow Injection System B. Experimental spectra obtained in the titration of a standard cytidine solution showed three successive zones with predominance of the acidic (0 to 80 s), the basic (80 to 120 s), and the basic background contributions (from 120 s). As in the continuous-flow system, the third contribution was attributed to the absorption of the phosphate ions in high-alkaline medium. The same behaviour was encountered for the other compounds.

As in the other flow titrations, the most accurate quantitations were obtained using the acidic species. The percentage of error in the prediction of binary mixtures was about 5% and increased up to 13.6% when more complex samples were analyzed. The accuracy of the prediction of the components of the Drug sample was about 5.5%.

CONCLUSIONS

Three flow-spectrophotometric systems have been developed and tested. The quantification of each particular analyte can be based on any of the species formed over a pH gradient (the acidic and basic products), although the best accuracy is achieved using the acidic species. The simplest data and the best determinations have been obtained using a single injection flow injection manifold referred to as FI system A, while higher prediction errors were obtained for the continuous-flow and the flow injection system B. The throughput frequency ranges between 2 and 3 min per sample. Additionally, with these flow-titration methods it is possible to analyze unstable compounds which could not be studied with classical spectrophotometric titrations, as they may decompose in the vessel during the titration experiment.

The method is applied to analyze mixtures of different complexity (binary, ternary, and quaternary mixtures, and a drug) with reasonably good quantification errors. At present, second-order chemometric methods are not yet used as routine tools for the analysis of real samples. Consequently, great efforts must be made in this direction in order for these to become a reliable alternative to traditional methods of analysis. These studies are the basis for further analysis of chemotherapeutical and antiviral drugs involving nucleic acid derivatives in much more complex samples such as blood and serum.

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