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Determination of amino acids in overlapped capillary electrophoresis peaks by means of partial least-squares regression

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Abstract

Amino acid derivatives of 1,2-naphthoquinone-4-sulfonate (NQS) can be separated by capillary electrophoresis at 30 kV in a fused-silica capillary by using a 40 m*M* sodium tetraborate–isopropanol (3:1, v/v) solution as background electrolyte. This procedure was suitable for the most common amino acids. However, the peaks of three amino acids (phenylalanine, isoleucine and tyrosine) were only partially resolved and peaks of histidine and leucine derivatives overlapped completely. Partial least-squares regression (PLS) may overcome the lack of selectivity for these amino acids. Spectroelectropherograms of the corresponding amino acid derivative peaks were monitored with a diode-array spectrophotometer in the range 225 to 540 nm. Both spectra and electropherograms can be used as multivariate data for further analysis. In general, the best predictions were obtained using the time domain.

Keywords: Capillary electrophoresis; Least-squares analysis; Chemometrics; Amino acids; Naphthoquinonesulfonate

1. Introduction

Chemometric methods have been used in liquid chromatography and other separation techniques to check peak purities and to improve the resolution and quantification. Multiple linear regression (MLR) [1] and, especially, partial least-squares regression (PLS) [2,3] have been successfully applied to the quantification of compounds in poorly resolved peaks in high-performance liquid chromatography (HPLC) with diode array detection (DAD). Calibration models have been built from different types of multivariate data. For instance, the chromatographic profiles registered at one (or various) selected wavelength or the cross-section of the spectrochromatograms taken at the maximum of the peaks have been used as valuable multivariate data. Kalman filtering [4,5] and time domain derivative chromatograms [6–9] have improved the resolution of coeluted compounds. Moreover, other chemometric methods take into account the spectral and chromatographic domains simultaneously, which may resolve other more complex systems [10–22].

Amino acids are important in dietetic and pharmaceutical fields [23]. Some of them are essential for humans and animals as they cannot be synthesized by the organism. Thus, amino acids are often added to the diet as food fortifiers. The determination of amino acids is usually carried out by HPLC with pre- or post-column derivatization using ninhydrin, *o*-phthaldialdehyde, phenylisothiocyanate, dansyl chloride, 9-fluorenylmethylchloroformate, etc. [24].

In recent years, capillary electrophoresis (CE) has gained popularity as a separation technique for routine analysis, and its applications are widespread in many fields of analytical chemistry [25]. CE is

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being introduced as a suitable tool for the determination of amino acids [26].

In a previous study, a CE method for the analysis of amino acids based on a pre-capillary derivatization with 1,2-naphthoquinone-4-sulfonate (NQS) was developed [27]. NQS amino acid derivatives were separated in a fused-silica capillary at 25°C using a 40 m*M* sodium tetraborate–isopropanol (3:1, v/v) solution as background electrolyte. The potential applied was 30 kV. This procedure was satisfactory for the most common amino acid derivatives. However, some electrophoretic peaks were not completely resolved. When the physical (electrophoretic) separation of derivatives is not accomplished, chemometric resolution of these compounds is still possible. The mathematical approach may be successful when either spectra or concentration profiles of the compounds are distinguishable from the others. In this study, PLS was proposed to improve the resolution and quantification of strongly coeluted peaks of NQS amino acid derivatives in CE–DAD.

Problems arising from the rather low reproducibility of migrations of analytes are often found in CE. Hence, the analysis and quantification of such compounds are hindered by the experimental variability of the CE peaks. In this study, peak shifts needed a correction in order to have comparable migrations and peak shapes. Some procedures have been proposed for shift adjustment in chromatography and CE. For instance, Kalman filtering [28], Bassel's inequality approach [29], second-order standardization [30,31] and peak alignments [32]. Here, a peak alignment approach was used.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with Milli-Q water. Sodium tetraborate and sodium hydroxide (both Merck, analytical-reagent grade) and isopropanol (Romil, HPLC-grade) were used to prepare the background electrolyte solution consisting of 40 m*M* sodium tetraborate–isopropanol (3:1, v/v).

NQS (Carlo Erba, analytical grade) and 37% (w/w) hydrochloric acid solution (Merck, analytical grade) were used to prepare the reagent solution containing 0.03 *M* NQS–0.1 *M* HCl. The derivatization reaction was developed at pH 10.0 using 0.05 *M* sodium borate–0.09 *M* sodium hydrochloride as buffer solution. A 0.016 *M* sodium dihydrogencitrate–0.038 *M* sodium hydrogencitrate (Merck, analytical grade) solution was used to stabilize the amino acid derivatives after the reaction. Phenylalanine (Phe), isoleucine (Ile), tyrosine (Tyr), histidine (His) and leucine (Leu) were purchased from Merck (all analytical grade).

2.2. Apparatus

A P/ACE Beckman CE system with a diode array spectrophotometric detector was used. Fused-silica capillaries (supplied by Tecknokroma) of 70 cm (effective length 58 cm)×75 μm I.D.×375 μm O.D. were used. Spectroelectropherograms, which were registered in the range 225 to 540 nm, were acquired and processed with a personal computer using Beckman P/ACE station software (version 1.0).

2.3. Capillary electrophoretic conditions

Amino acid derivatives were obtained by mixing equal volumes of sample, reagent and buffer solution, allowing the mixture to react for 5 min at 65°C and pH 10.0. The resulting solution was acidified with a dihydrogencitrate–monohydrogencitrate solution and filtered through a nylon membrane of 0.45 μm pore size.

Samples were injected under pressure at 0.5 p.s.i. for 5 s (1 p.s.i.=6894.76 Pa). Amino acid derivatives were separated at 30 kV with a 40 m*M* sodium tetraborate–isopropanol (3:1, v/v) electrolyte solution. The capillary was thermostatted at 25°C.

Spectra from 225 to 550 nm were acquired at regular steps of 1 s during the electrophoretic run. For wavelength selection, since the spectral bands of derivatives were narrower in the UV range than in the Vis range, the working wavelengths were not chosen at regular steps. Therefore, the width of steps was narrower from 225 to 300 nm (wavelengths were taken every 5 nm), from 300 to 360 was every 10 nm and from 360 to 540 was wider (every 20 nm). From these row data, 31 working wavelengths were finally chosen for analysis, which defined all absorption bands of spectra of derivatives.

Table 1
Composition of the mixtures solutions used: two-component mixtures

Sample	Leu (M)	His (M)
M1	$1.5\cdot10^{-3}$	$1.5\cdot10^{-3}$
M2	$4.76\cdot10^{-4}$	$4.76\cdot10^{-4}$
M3	$1\cdot10^{-3}$	$1\cdot10^{-3}$
M4	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$
M5	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$
M6	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$
M7	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$
M8	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$

Before each run, the column was washed with water for 5 min and dried with air for 10 min. Subsequently, the column was rinsing with water and background electrolyte solution for 10 min. Prior to sample injection, the column was equilibrated by applying a potential of 30 kV for 20 min.

2.4. Data sets

Table 1 shows the composition of the two-component mixtures of Leu and His and Table 2 describes the five-component mixtures. All concentrations lay inside the linear range of the method, which was, at least, up to $3\cdot10^{-3}$ *M* for these amino acids [27].

A suitable strategy when working with small data sets (e.g., eight-sample and 14-sample sets were available for the two- and five-component mixtures, respectively) is predicting each sample using the remaining samples as standards for building the calibration model [33]. Hence, all samples were used for modeling, rank estimation and prediction.

2.5. Software and data processing

Original data corresponding to those wavelengths selected were converted into ASCII files for further mathematical treatment. Matlab for Windows (Version 4.1) was used for calculations [34]. PLS methods were from the PLS_Toolbox [35]. Detailed descriptions of PLS method and other chemometric tools can be found elsewhere [36,37].

3. Theory

3.1. Partial least-squares regression

The PLS algorithm takes into account the information of responses and concentrations simultaneously. The model is built for each analyte by using its concentration vector. Factors from a PLS model are calculated as those variables that describe the maximum amount of relevant information of the spectral or electrophoretic response matrix and of the concentration matrix, as follows:

Table 2
Composition of the mixtures used: five-component mixtures

Sample	Phe (M)	Ile (M)	Leu (M)	His (M)	Tyr (M)
M9	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$
M10	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$
M11	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$
M12	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$
M13	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$
M14	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$
M15	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$
M16	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$
M17	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$4.76\cdot10^{-4}$	$4.76\cdot10^{-4}$
M18	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$4.76\cdot10^{-4}$
M19	$1\cdot10^{-3}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$
M20	$1\cdot10^{-3}$	$1\cdot10^{-3}$	$1\cdot10^{-3}$	$1.5\cdot10^{-3}$	$1\cdot10^{-3}$
M21	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$1\cdot10^{-3}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$
M22	$4.76\cdot10^{-4}$	$1.5\cdot10^{-3}$	$4.76\cdot10^{-4}$	$1\cdot10^{-3}$	$4.76\cdot10^{-4}$

$$\mathbf{R} = \mathbf{TP}^{\mathbf{T}} + \mathbf{E} = \sum \mathbf{t}_k \mathbf{p}_k + \mathbf{E} \quad (1)$$

$$\mathbf{C} = \mathbf{QS}^{\mathbf{T}} + \mathbf{F}' = \sum \mathbf{q}_k \mathbf{s}_k + \mathbf{F}' \quad (2)$$

where, **R** is the response matrix with a dimension NS×NW, **T** (NS×NF) and **P** (NF×NW) are the score and loading matrices associated with the response (the superscript **T** indicates the transposed matrix); **C** the concentration matrix of the analyte (NS×1), **Q** (NS×NF) and **S** (NF×1) the scores and loading of the concentration matrix; and **E** (NS×NW) and **F′** (NS×1) the unexplained information of responses and concentrations, respectively. The corresponding scores and loading for the *k*th factor are $\mathbf{t}_k$, $\mathbf{p}_k$, $\mathbf{q}_k$ and $\mathbf{s}_k$. NS is the number of standards, NW the number of working wavelengths for spectral data or the number of working times for electrophoretic data, and NF the optimum number of latent variables or factors included in the model.

The inner relationship between responses and concentrations in the PLS model is given from their corresponding scores factor by factor:

$$\mathbf{q}_k = \mathbf{b}_k \mathbf{t}_k \quad (3)$$

where $\mathbf{b}_k$ represents the regression coefficients.

Scores and loading of each factor are not calculated at once but one after another, in order to minimize the residuals in the concentration matrix using a mixed relationship which can be generalized for the *k*th factor as:

$$\mathbf{F}_k = \mathbf{F}_{k-1} - \mathbf{t}_k \mathbf{b}_k \mathbf{s}_k^{\mathbf{T}} \quad (4)$$

where $\mathbf{F}_k$ and $\mathbf{F}_{k-1}$ are the residuals of concentrations not explained by a model with k and $k-1$ factors, respectively. Note that for the first factor $\mathbf{E}_0 = \mathbf{C}$.

Once the model is built, it can be used to predict the concentration of unknown samples.

3.2. Estimating the optimum number of latent variables

The optimum number of latent variables for each multivariate calibration method was estimated by leave-one-out cross-validation from the calibration sets [38]. Therefore, the number of latent variables chosen was that which minimized the prediction error of the sum-of-squares (PRESS) function calculated as follows:

$$\mathrm{PRESS}(k) = \sum_{i=1}^{\mathrm{Samples}} \left[c_{i_{\mathrm{true}}} - \hat{c}_{i_{\mathrm{calc}}}(k) \right]^2 \quad (5)$$

where k refers to the number of latent variables considered, $c_{i_{\mathrm{true}}}$ is the real concentration of analyte in the sample i and $\hat{c}_{i_{\mathrm{calc}}}(k)$ is the concentration calculated by multivariate calibration methods using k factors.

3.3. Calculating the prediction error

The prediction error in Tables 3 and 4 for the calibration and prediction steps was calculated using the expression:

$$\text{Prediction error (\%)} = \frac{\sqrt{\sum_{i=1}^{\mathrm{Samples}} (\hat{c}_{i_{\mathrm{true}}} - \hat{c}_{i_{\mathrm{calc}}})^2}}{\sqrt{\sum_{i=1}^{\mathrm{Samples}} (\hat{c}_{i_{\mathrm{true}}})^2}} \times 100 \quad (6)$$

Table 3
Results of the analysis of two-component mixtures using PLS

Amino acid	Type	Calibration			Prediction error (%)
		NF	Cumulative explained *x* variance (%)	Cumulative explained *y* variance (%)	
Leu	Spectra	4	99.99	99.94	21.7
	Electropherograms	3	99.93	99.90	15.5
His	Spectra	4	99.99	99.98	8.6
	Electropherograms	2	99.99	99.98	18.3

Table 4
Results of the analysis of five-component mixtures using PLS

Amino acid	Type	Calibration			Prediction error (%)
		NF	Cumulative explained *x* variance (%)	Cumulative explained *y* variance (%)	
Phe	Spectra	1	99.95	99.92	17.4
	Electropherograms	4	98.49	99.94	14.2
Leu	Spectra	1	99.99	98.97	23.2
	Electropherograms	4	98.69	98.62	11.2
His	Spectra	4	99.99	99.93	9.9
	Electropherograms	3	99.19	99.12	18.0
Ile	Spectra	1	99.98	98.39	23.9
	Electropherograms	4	99.56	99.53	12.1
Tyr	Spectra	3	99.59	99.64	20.2
	Electropherograms	3	98.88	99.85	13.9

3.4. Estimating the similarity of spectral or electrophoretic profiles

The similarity of spectral or electrophoretic profiles is often evaluated as the cosine between a pair of spectral or electrophoretic vectors [39]. These vectors can be obtained from a CE–DAD run of pure standard for each analyte. The cosine, in absolute value, is a number between 0 and 1. When the cosine calculated is 1 or close to 1, the shapes of the two vectors compared are equal or very similar. Conversely, lower cosines are obtained when spectra or electropherograms are significantly different.

4. Results and discussion

Fig. 1 shows the spectroelectropherogram for a five-component mixture as an example. Note that only four peaks can be distinguished since Leu and His derivatives overlapped strongly. The spectra and electrophoretic profiles are shown in Fig. 2. It can be seen that spectra of all derivatives are very similar, while the migration times and peak shapes of electrophoretic profiles are more different (with the exception of profile of His and Leu derivatives). The similarities (cosine value) listed in Table 5 were calculated from these pure data vectors.

4.1. Study of two-component mixtures

The migration time of His and Leu derivatives were very similar, so when both compounds were present in a mixture they coeluted. The cosine of spectra of derivatives taken at the maximum of the peak was 0.990, which indicated that both spectra were almost equal. On the other hand, electrophoretic profiles were more dissimilar (cosine value = 0.909) (see Table 5). No spectral or electrophoretic range with selectivity for the analytes was found. However, the PLS method took advantage of these small differences in the migration profiles and spectra of both derivatives to determine these analytes.

First studies were addressed to ascertain which spectral or electrophoretic data provided the best quantification strategy. Despite the experimental precautions to avoid the variability of the migration time of derivatives, some significant variations in the time domain still arose. Thus, when electropherograms were used as multivariate data a positioning pre-treatment was performed. This procedure consisted of making a data window of 101 channels (measurement time for 100 s) centered on the peak maximum (i.e., peak maximum was set at the 50th time channel). In this way, the maximum of the peak of the analyte was always taken as a reference for its quantification. The procedure is depicted in Fig. 3,

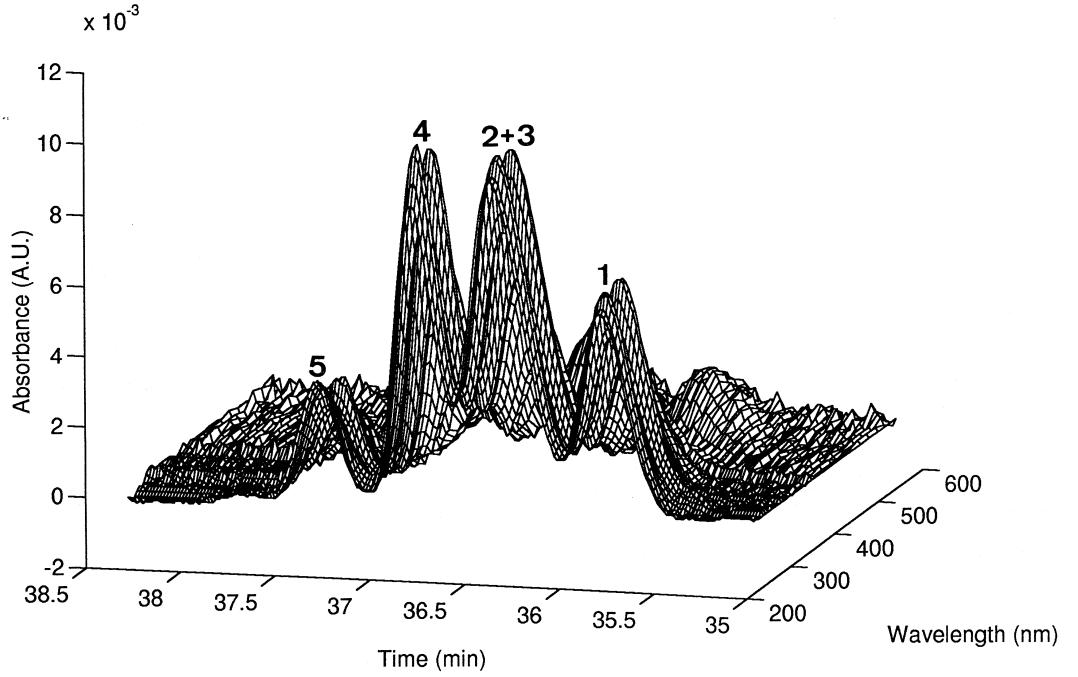

Fig. 1. Three-dimensional plot of the spectroelectropherogram of amino acid derivatives of a five-component mixture solution (sample M14). Species assignment: 1=Phe derivative; 2=His derivative; 3=Leu derivative; 4=Ile derivative; 5=Tyr derivative.

using the electropherograms from three CE–DAD runs as an example.

Each sample was predicted using the remaining samples as standards. The optimum number of factors in the model selected by leave-one-out cross-validation for the spectral and electrophoretic data was between 2 and 4, depending on the case (see Table 3). The explained *x* variances (spectral or electrophoretic profiles variance) and *y* variances (concentration variance) for the different calibration models were higher than 99%. These findings indicated that PLS models kept the relevant information of the data. Prediction results are summarized in Table 3. Leu and His concentrations calculated with PLS were statistically compared with the true values using a *t*-test of paired measurements (its analytical utility has been referenced elsewhere [40]). There were no significant differences between the real Leu or His contents and those from PLS.

4.2. Study of five-component mixtures

This series of mixtures was composed of Phe, Leu, Ile, His and Tyr. Peaks of Phe, Ile and Tyr derivatives were partially resolved while, and, as above, His and Leu derivatives overlapped strongly. Table 5 lists the spectral and electrophoretic similarities of the corresponding NQS derivatives. All spectra strongly overlap with cosine values higher than 0.99. Dissimilarities in the electrophoretic domain were much more noticeable. From this preliminary study, apparently, electrophoretic data seemed to be much more suitable than spectral data as regards resolution and quantification.

As in the binary mixtures, the effect of the variation of the migration time of derivatives on the analysis was minimized by using a 201 channel windows, which contained the peaks of the five analytes. For each analyte, its maximum was set to the same time channel over the time domain.

As in Section 4.1, to predict each sample all the remaining samples were used as standards. Leave-one-out cross-validation analyses found between 1 and 4 factors to be optimal (see Table 4). All calibration models were examined in terms of variance explained. More than 98% of the response and

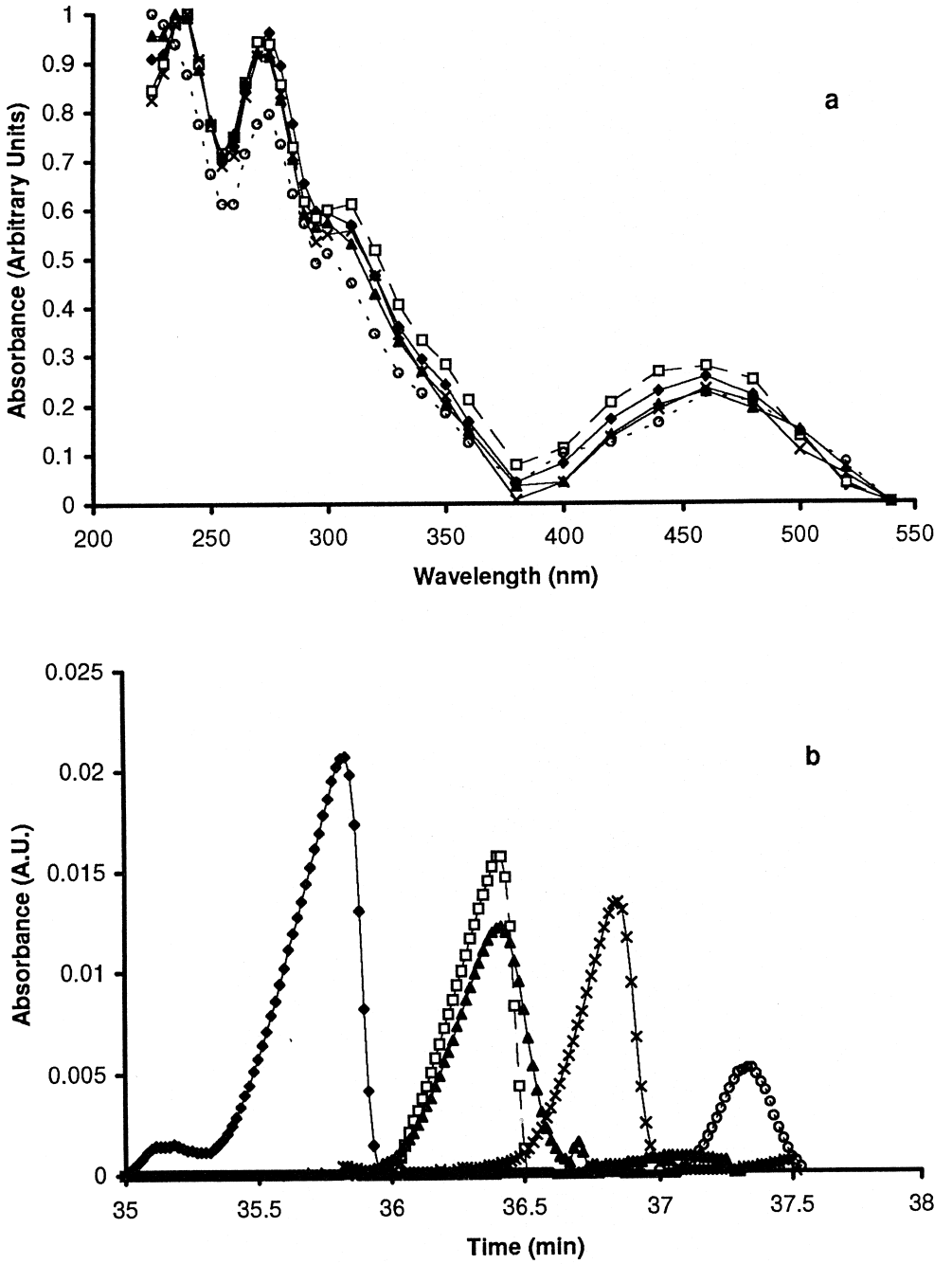

Fig. 2. (a) Spectra and (b) electrophoretic profiles of amino acid derivatives obtained from pure standards. Conditions: $\lambda = 230$ nm; amino acid concentration: $2 \cdot 10^{-3}$ *M* each. ♦ = Phe; □ = Leu; ▲ = His; × = Ile; ○ = Tyr.

Table 5
Study of similarity of electropherograms and spectra (cosine values between pairs of vectors obtained from pure standards for each analyte)

	Leu	His	Phe	Ile
(a) Electrophoretic profiles				
Leu	–	–	–	–
His	0.9091	–	–	–
Phe	0.9327	0.9194	–	–
Ile	0.9479	0.9264	0.9468	–
Tyr	0.9548	0.9225	0.9626	0.9608
(b) Spectral profiles				
Leu	–	–	–	–
His	0.990	–	–	–
Phe	0.9910	0.9920	–	–
Ile	0.997	0.9989	0.9910	–
Tyr	0.9915	0.9947	0.9925	0.9924

concentration variances were modeled in all cases. Subsequently, the calibration models were used to predict each analyte in unknown samples. Results of these determinations are summarized in Table 4. As general comments, prediction errors were between 9.9% and 23.9% when using the spectral information and between 11.2% and 18.0% for electrophoretic data. Consequently, electrophoretic data seemed to be more satisfactory for these analyses. These results are in a reasonable concordance with the actual values, owing to the difficulties for obtaining accurate quantification which are implicit to the CE techniques [41,42]. No significant differences were found when concentrations calculated with PLS were statistically compared with their real values using a *t*-test of paired measurements. As an example, Fig. 4 plots the results of the determination of Phe in these mixtures using the method proposed against the real values; a good correlation between them was observed.

5. Conclusions

CE–DAD provided a huge amount of data suitable for chemometric analysis with PLS. Both spectral and electrophoretic profiles could be used as multivariate data. For each analyte, a calibration model was built. Variations of electrophoretic data on the

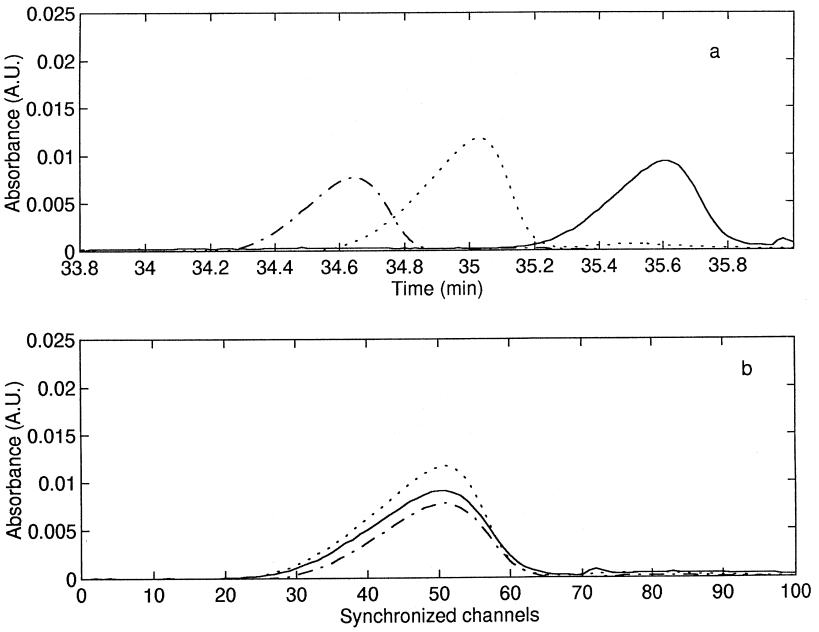

Fig. 3. Scheme of the shift adjustment procedure for the electrophoretic profiles from three two-component CE–DAD runs. (a) Raw electropherograms; (b) synchronized electropherograms: repositioned taking 101 time channels centered on the peak maximum (50 before and 50 after).

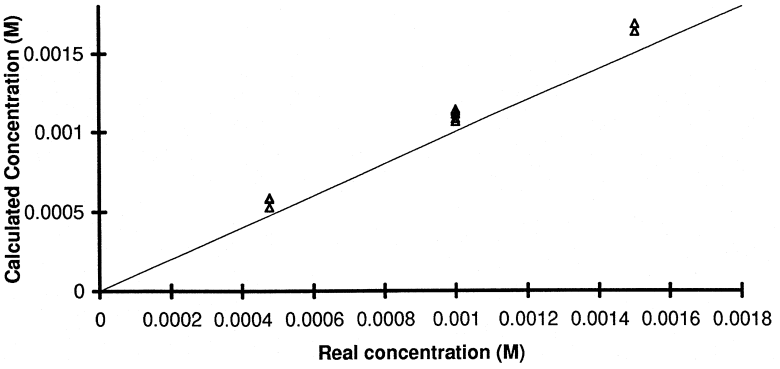

Fig. 4. Comparison between actual and calculated Phe concentrations using PLS in the five-component mixtures using the electrophoretic profiles as data.

time domain were partly corrected using a fixed-size window, which was positioned according to the peak maximum of each analyte. The accuracy was better when using electrophoretic data, owing to the larger dissimilarities on the time profiles among analytes. Since standard deviations of analytical results in CE are typically one order of magnitude greater than those obtained with HPLC, prediction errors in these analyses were considered acceptable. The analysis of the corresponding second-order data (i.e., data matrix arrangements of spectral and time domains simultaneously) with second-order calibration methods may be an attractive possibility for improving the results. Further work is being done in this direction.

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