A comparison of chemometric methods for the flow injection simultaneous spectrophotometric determination of aniline and cyclohexylamine

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A flow injection spectrophotometric method for the simultaneous determination of aniline and cyclohexylamine using multivariate calibration methods is proposed. The method is based on the reaction of these amines with 1,2-naphthoquinone-4-sulfonate, yielding spectrophotometrically active derivatives. The data analysed with multivariate calibration methods consisted of the spectra registered in the range 290–590 nm at the maximum of the flow injection peak. Although the spectrum of each derivative was characteristic, overlapping occurred and no selective wavelengths were found. The predictive abilities of principal component regression and partial least-squares regression (PLS), non-linear PLS, locally weighted regression (LWR) and artificial neural networks were examined for the determination of aniline and cyclohexylamine in sample mixtures. The accuracy for cyclohexylamine and aniline quantifications in unknown mixtures was optimum with LWR, providing overall prediction errors of 3.4 and 5.6%, respectively.

Introduction

This paper describes a new method for determining aniline and cyclohexylamine, which are the main impurities found in cyclamate samples. The need to determine these amines arises from the fact that cyclamate salts are widely used as non-caloric sweeteners in diet food and beverages, pharmaceutical products and table top sweeteners. The toxicity of these amines has been extensively studied elsewhere (refs. 1–3 for aniline and 4–6 for cyclohexylamine). Aniline and related compounds have been classified as priority organic pollutants and their contents in environmental, industrial and food samples have been regulated. According to the European Pharmacopoeia, the maximum permissible concentrations of aniline and cyclohexylamine in table top sweeteners are 1 and 10 ppm, respectively.[7] The standard method for the determination of these amines in cyclamate preparations is based on gas chromatography.[8]

Owing to the toxicological significance of these amines in industrial and dye wastes and pesticides, other analytical methods have been proposed, including gas chromatographic,[9–10] liquid chromatographic,[11,12] colorimetric[13] and flow injection[14,15] determinations of aniline and liquid chromatographic[16] and flow injection[17] determinations of cyclohexylamine.

In contrast to the methods mentioned above for the determination of aniline and cyclohexylamine, a chemometric approach[18–20] may constitute a rapid, feasible and attractive alternative for satisfying the demands of control and routine analyses.

In this study, a flow injection spectrophotometric method was developed for the simultaneous determination of aniline and cyclohexylamine using multivariate calibration methods. The method is based on the reaction of aniline and cyclohexylamine with 1,2-naphthoquinone-4-sulfonate to yield spectrophotometrically active derivatives. The derivatization procedure was carried out in a three-channel flow injection manifold. Data consisting of spectra registered along the flow injection peak were analysed with several chemometric methods. The aniline and cyclohexylamine derivatives showed characteristic spectral shapes enabling both compounds to be distinguished, although both spectra overlapped considerably and no selective wavelength was found. Although interference was encountered when determining these amines by classical univariate calibration methods, mixtures of aniline and cyclohexylamine were successfully resolved and quantified on the basis of their spectral differences using multivariate calibration methods. The predictive abilities of several multivariate calibration methods, including principal component regression (PCR), partial least-squares regression (PLS), non-linear PLS, locally weighted regression (LWR) and artificial neural networks (ANN), were therefore investigated.

Experimental

Reagents, solutions and samples

All chemicals were of analytical reagent grade. Solutions were prepared with doubly distilled water. The reagent solution consisted of 2×10^{-3} M sodium 1,2-naphthoquinone-4-sulfonate (NQS) (Carlo Erba, Milan, Italy) in 0.1 M hydrochloric acid (Merck, Darmstadt, Germany). The buffer solution for the development of the reaction at pH 10.0 consisted of 0.1 M sodium carbonate + 0.09 M sodium hydroxide. Aniline hydrochloride (Sigma, St Louis, MO, USA) and cyclohexylamine hydrochloride (Acros Organic, NJ, USA) were used as received; their purities were checked by gas chromatography[7] and no evidence of cyclohexylamine impurity in the aniline sample, and *vice versa*, was found.

Apparatus

Spectrophotometric detection was performed with a Hewlett-Packard (Avondale, PA, USA) HP8452A diode-array spectrophotometer using a Hellma (Mülheim, Germany) flow cell of 10 mm pathlength and 18 μl volume. The acquisition and storage of spectrophotometric data were carried out with Hewlett-Packard software on a Hewlett-Packard Vectra N2

4/50 computer. Further data treatment with multivariate calibration methods was performed on a 150 MHz Pentium PC.

Flow injection system

The flow injection system consisted of a three-channel manifold (Fig. 1). Solutions were pumped using standard Tygon tubing with a Scharlau (Barcelona, Spain) HP4 peristaltic pump. Connectors, T-pieces and coils were made of Teflon. Sample solutions (200 μl) were injected into a water carrier using a variable-volume Sirtek (Barcelona, Spain) MV-8 electrical injection valve. Reagent and buffer solutions merged in a 2 m × 0.5 mm id coil (MC) and then joined the carrier stream. The reaction of amines with NQS took place in a 950 cm × 0.5 mm id coil (RC) thermostated at 80 °C using an SBS (Barcelona, Spain) TFB-3 water-bath.

Data generation and treatment

The spectra were registered in the range 290–590 nm at 2 s intervals in the flow injection peak for 66 s. Spectra, which were originally stored using the standard Hewlett-Packard software, were transformed into ASCII files. Subsequently, these spectra were arranged to obtain suitable files for Matlab (Math Works, Natick, MA, USA) using a laboratory-written Q-Basic program. Each sample was injected in triplicate and the multivariate data obtained for further mathematical analysis consisted of the average spectrum corresponding to the peak maximum for each sample injection. By working with the average spectrum, we were able to shorten the time of analysis as the dimensions of the data matrices were correspondingly smaller. In addition, the average spectrum provided better predictions than the individual spectrum of the maximum of each injection.[21] All transformations described above took about 2 min per sample.

The composition of standard and unknown mixtures of cyclohexylamine and aniline used in assessing the model and for making predictions are described in Table 1. The dimensions of these data matrices were the number of the working wavelengths of each spectrum, *NW* (*NW* = 51 absorbance values taken at a regular interval of 6 nm from 290 to 590 nm), by the number of samples, *NS* (*NS* = 8 for both calibration and prediction sets).

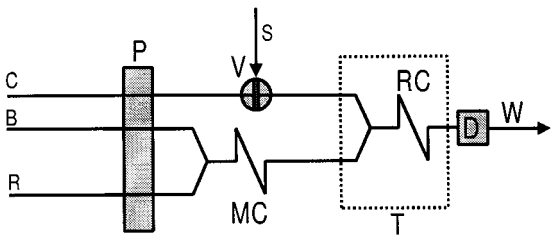

Fig. 1 Flow injection manifold. P = peristaltic pump; V = injection valve; D = detector; T = thermostated bath; RC = reaction coil (950 cm × 0.5 mm id); MC = mixing coil (200 cm × 0.5 mm id) S = sample solution; C = carrier (water); R = reagent solution (2×10^{-3} M NQS + 0.1 M HCl); B = buffer solution (0.1 M Na_2CO_3 + 0.09 M NaOH); W = waste. Flow rates: channel C = 0.85; channel R = 0.85; and channel B = 0.75 ml min^{-1}.

Multivariate calibration methods

For all calculations, Matlab for Windows (Version 4.1)[22] was used. Multivariate calibration methods were carried out with the PLS_Toolbox[23] and the Neural Network Toolbox[24] for use with Matlab. Calculations were carried out using a Pentium PC at 150 MHz. With this computer, calibration and prediction steps took 1–2 s for PCR, PLS, LWR and NL-PLS and between 5 s and a few minutes for ANN, depending on their complexity.

A common requirement for this type of calibration method (*i.e.*, first-order calibration methods) is that unknown samples and standards be of the same nature, in order that all contributions from the analytes, interferences and matrix effects present in the samples are modelled implicitly.[20] Although multivariate calibration methods have been extensively described elsewhere,[18,19,24–29] a brief description is given below.

Principal component regression (PCR)

PCR decomposes the experimental matrix of responses of the calibration set as follows:

$$\mathbf{R} = \mathbf{T}\,\mathbf{P}^{T} + \mathbf{E}$$

R being the response matrix with a dimension *NS* × *NW* (number of standards by number of working wavelengths), **T** the scores matrix (*NS* × *NF*), $\mathbf{P}^{T}$ the loading matrix (*NF* × *NW*) (the superscript T indicates the transposed matrix) and **E** the residual error matrix (*NS* × *NW*), where *NF* is the number of latent variables or factors included in the model which are able to keep the relevant variance of data.

Next, the scores matrix **T** is correlated with the concentration matrix **C** (*NS* × *NA*), where *NA* is the number of analytes, using the expression

$$\mathbf{C} = \mathbf{TB} + \mathbf{E}$$

where **B** is the matrix of regression coefficients which is resolved by using a least-squares procedure.

This model is subsequently applied to predict the concentrations of unknown samples.

Partial least-squares regression (PLS)

The PLS algorithm takes into account the information of responses and concentrations simultaneously. There are two procedures available to solve the system: in PLS1, one model is built for each analyte by using its concentration vector (*e.g.*, **C** is *NS* × 1), whereas in PLS2, all analyte concentrations are simultaneously considered in constructing the calibration model (*e.g.*, **C** is *NS* × *NA*). In this way, factors from a PLS model are calculated as those variables which describe the maximum amount of information for the concentration matrix. Factors

Table 1 Composition of the mixture solutions used as standard (S1–S8) and unknown (U1–U8) samples

	Cyclohexylamine concentration/M			
Aniline concentration/M	5×10^{-5}	7.5×10^{-5}	1×10^{-4}	1.25×10^{-4}
3×10^{-5}	S1	U3	U5	S7
5×10^{-5}	U1	S3	S5	U7
7.5×10^{-5}	U2	S4	S6	U8
1×10^{-4}	S2	U4	U6	S8

containing the relevant information of **R** and **C** were obtained as follows:

$$\mathbf{R} = \mathbf{T}\,\mathbf{P}^{\mathrm{T}} + \mathbf{E}'$$

$$\mathbf{C} = \mathbf{Q}\,\mathbf{S}^{\mathrm{T}} + \mathbf{E}''$$

where, as with PCR, **T** and **P** are the score and loading matrices associated with the response, Q and **S** are the scores and loading of the concentration matrix and E' and E'' are the unexplained information of responses and concentrations, respectively.

The relationship between scores and concentrations is obtained from

$$\mathbf{C} = \mathbf{TB}Q^{\mathrm{T}} + E$$

where **B** is the matrix of the regression coefficients obtained by a least-squares procedure.

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

Non-linear PLS (NL-PLS)

PCR and PLS algorithms have essentially been developed for modelling linear data since they apply inner linear relationships between responses and concentrations. However, they can also be applied to non-linear data. In the latter case, the non-linearity of the response can be considered by including more factors. Alternatively, non-linear PLS procedures have also been proposed, which utilise different types of inner non-linear relationships. Among these, polynomial functions[25] and artificial neural networks[26] are often used.

Locally weighted regression

Locally weighted regression is based on the idea that the prediction of unknown samples can be improved by using close standard samples (*e.g.*, with very similar matrix compositions and concentrations), while the use of further away standards can lead to less efficient models. This method is implemented by weighting in the regression model according to the proximity of the standards to the sample to be predicted. In general, the criterion for measuring the proximity between the sample and each standard is the Mahalanobis distance.[27] However, here, the method has been modified as it gives the same weight to all close standards while rejecting the distant standards according to their PC distribution and the analyst's criterion.

The selection of the optimum number of latent variables for PCR and PLS methods mentioned above was performed by cross-validation.[28]

Artificial neural networks

Artificial neural networks have been successfully applied to the empirical modelling of various types of data without focusing on the actual mathematical relationships between the variables.[29] The neural network is composed of an input layer, which contains the entry data, an output layer which usually contains the quantitative values and one or several hidden layers of neurones which calculate the coefficients (weights) between inputs and outputs using transfer functions. In the calibration step, the network is usually trained by back-propagation using standard samples in order to adjust the weights and thereby minimise the error. Subsequently, in the prediction step, the network is used to make predictions on new unknown samples.

In this case, the network architecture consisted of a three-layered structure in which (i) the input layer values contained spectral information (*e.g.*, band maxima absorbances, scores), (ii) the hidden layer contained eight neurones operating with linear transfer functions and (iii) the output layer contained the concentration values of aniline and cyclohexylamine in the standard mixtures. The network was trained by back-propagation until the percentage error desired (1%) was achieved. The initial weights and biases at the start of the learning process were randomised.

Calculating the prediction error

The prediction error for the calibration and prediction steps was calculated using the expression

$$\text{error (\%)} = \frac{\sqrt{\sum_{i=1}^{\text{sample}} (C_{i\text{true}} - C_{i\text{calc.}})^2}}{\sqrt{\sum_{i=1}^{\text{sample}} (C_{i\text{calc.}})^2}} \times 100$$

where $C_{i\text{true}}$ is the real concentration of analyte in the sample i and $C_{i\text{calc.}}$ is the concentration calculated by multivariate calibration methods.

Results and discussion

Fig. 2 provides an example of a three-dimensional plot of the raw spectrophotometric data obtained from the injection of a 10^{-4} M aniline + 10^{-4} M cyclohexylamine solution. Preliminary studies sought to determine the type of spectral information that was analytically useful for making predictions. It was found that the spectrum of the maximum of the flow injection peak was preferable to the whole spectral information. This was because measurements over time did not help to differentiate between the two analytes. Indeed, the shapes of the concentration profiles for the two analytes were identical. Consequently, including more spectral information did not improve predictions, but rather increased the amount of correlated data and the time of analysis.

Fig. 3 shows the spectra of cyclohexylamine and aniline derivatives in the range 290–590 nm. Each compound showed a characteristic spectrum, the shape of which could be distinguished from the other. However, there was a high degree of overlap and no selective wavelength was found to determine those amines with classical univariate calibration. The estimated similarity of these spectra using the correlation value (cosine value between the two spectra) was 0.946. Consequently, the resolution and simultaneous determination of these amines using multivariate calibration methods are dependent on

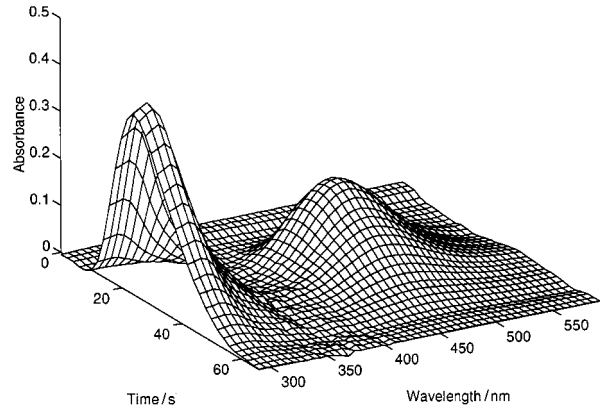

Fig. 2 Three-dimensional plot of the spectrophotometric data for the injection of a 10^{-4} M aniline + 10^{-4} M cyclohexylamine solution into the flow injection system. Flow injection conditions as in Fig. 1.

the particular features of the spectral response of each analyte.

The distribution of the spectra of the standard and unknown samples on the first and second principal components is shown in Fig. 4. The first principal component (PC-1) was highly correlated with the maximum absorbance of each spectrum, which was related to the total concentration of amines in each sample. Thus, samples with a high concentration of amines lay to the right, whereas samples with a low concentration of amines fell to the left of the PC-1 axis. Moreover, samples with the same total concentration scored approximately the same for this PC-1. The PC-2 accounted for the ratio [cyclohexylamine]/[aniline] of the samples; samples with higher ratios were at the top of the graph, samples with ratios close to 1 lay on the middle of the PC-2 axis and samples with lower ratios appeared at the bottom. Additionally, other patterns and clusters could be recognised. All samples with the same concentration of aniline lay on approximately straight lines. A similar behaviour was observed with respect to the cyclohexylamine concentration. These findings indicated a great structure in the distribution of the standard and unknown samples.

The selection of the optimum number of components for calibration with PCR and PLSR was estimated beforehand by cross-validation.[28] Suitable modelling of the data variance and accurate predictions were obtained using two latent variables for aniline and three latent variables for cyclohexylamine. Models built with a larger number of components gave less accurate predictions.

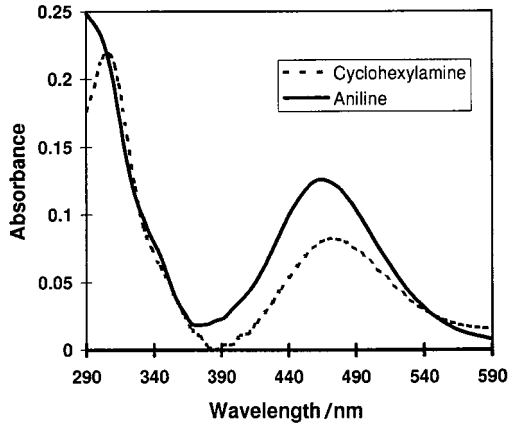

Fig. 3 Spectra of the aniline and cyclohexylamine derivatives recorded at the maximum of the flow injection peak. Aniline concentration = 10^{-4} M; cyclohexylamine concentration = 10^{-4} M. Flow injection conditions as in Fig. 1.

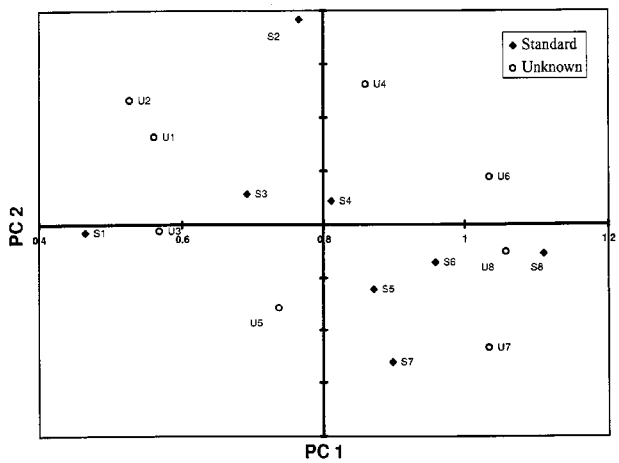

Fig. 4 Distribution plot of standard and unknown samples on the first and second principal components.

In the case of non-linear PLS modelling using polynomials, the optimum degree of the polynomial was two. When higher degrees were tested, data overfitting was observed because although the calibration error decreased, the prediction error increased considerably. In this study, the amount of excess reagent with respect to the analytes seems to be sufficient in order to avoid non-linearities in the responses (non-linearities might appear if the NQS concentration was not sufficient to derivatize both amines quantitatively, for example, as there would be competition for the reagent). For this reason, non-linear algorithms did not improve the prediction given by linear algorithms.

Table 2 summarises the results of the prediction of the sample mixtures with the PCR and PLS methods. The quantification errors were similar for all methods. In all cases, cyclohexylamine was predicted with greater accuracy than aniline, with overall errors of about 4 and 9.5%, respectively. A possible explanation for this finding might be that the spectrum of the cyclohexylamine derivative presented greater selectivity with respect to the background NQS absorption (not apparent for these measurements as it was taken as a reference) and, thus, cyclohexylamine could be more easily modelled.

Various neural network architectures were designed and trained with up to three hidden layers of neurones being used. However, the simplest of these, *i.e.*, those with one hidden layer of neurones, were found to be optimum. Several types of data were analysed, including the use of the whole spectrum for each sample and other spectral information (see below). When whole spectra were used, the network complexity and the learning times increased greatly. Indeed, the network was unsuccessfully trained since the desired fitting error was not reached. Other simpler data inputs (*e.g.*, band maxima absorbances, scores of PC-1 and PC-2 and absorbance values for the purest variables) were efficiently modelled by back-propagation with a relatively small number of epochs (< 2000). The results of these predictions are given in Table 3, where it can be seen that aniline was more accurately determined than cyclohexylamine (with prediction errors around 5–6.5 and 27%, respectively), in contrast to PCR and PLS methods, which gave better results for cyclohexylamine. These results may be explained by the fact

Table 2 Percentage errors in the calibration and prediction steps using PCR and PLS methods

	Error (%)			
	Aniline		Cyclohexylamine	
Method	Calibration	Prediction	Calibration	Prediction
PCR[a]	9.9	9.2	3.7	3.9
PLS1[a]	9.4	9.3	3.5	4.1
PLS2[a]	9.4	9.2	3.7	3.9
NL-PLS[a,b]	6.6	11.0	3.5	6.7

[a] Using two factors for aniline and three for cyclohexylamine. [b] The degree of polynomial is two.

Table 3 Percentage errors in the calibration and prediction steps using neural networks for different types of data

	Error (%)			
	Aniline		Cyclohexylamine	
Type of data	Calibration	Prediction	Calibration	Prediction
Band maxima[a]	0.1	6.5	0.4	27.4
Scores[b]	0.1	6.4	2.0	27.3
Purest variables[c]	0.3	5.0	0.3	27.4

[a] Absorbances at 305 and 480 nm. [b] Scores of the first and second principal components. [c] Absorbances at 296 and 332 nm.

that inputs for the ANN were much more strongly correlated with the aniline concentration in the samples than with that of cyclohexylamine and, therefore, aniline was more accurately quantified.

In this paper, we propose an alternative approach to improve quantification. This consists of the building of local PLS models for the prediction of each sample. Therefore, instead of using all the standard information, a selected subset of standards was considered for each unknown sample. Obviously, local models could be used with the other calibration methods (*e.g.*, PCR or NL-PLS). However, since one is not markedly superior to the others, PLS was chosen for testing. Thus, each sample could be predicted by using the most similar standards (*e.g.*, those which lie closest to its neighbourhood). The proximity between the unknown sample and the standards can be seen from its distribution on PC-1 and PC-2 in Fig. 4. This approach was, in essence, similar to a locally weighted regression,[27] which in predicting gives a high weight to nearby standards and penalises those which are furthest away, in accordance with the Mahalanobis distances. However, the present method gave the same weight to all close standards and rejected the distant ones according to their PC distribution and the analyst's criterion. This method seemed to work better than locally weighted regression, at least in the prediction of the mixtures of cyclohexylamine and aniline proposed here.

Accordingly, samples U1 and U2 were predicted using standards S1, S2, S3 and S4; samples U7 and U8 with standards S5, S6, S7 and S8; samples U3 and U5 with standards S1, S3, S5 and S7; and samples U4 and U6 with standards S2, S4, S6 and S8 (see their distribution in Fig. 4 and compositions in Table 1). The subsets of standard and unknown samples were able to define clusters characterised by low aniline concentration, high aniline concentration, high cyclohexylamine concentration and low cyclohexylamine concentration, respectively.

Fig. 5 shows the results of the determination of these amines in the unknown samples compared with their actual values. It can be seen that there was good agreement between the real and calculated values. The overall prediction errors were 3.4 and 5.6% for cyclohexylamine and aniline, respectively.

Conclusions

We have proposed a new method for the simultaneous determination of aniline and cyclohexylamine using flow injection analysis and multivariate calibration methods. For each sample, the only information required is its spectrum, which is registered at the maximum of the flow injection peak. Several multivariate calibration methods were tested, including PCR, PLS, NL-PLS and ANN. It was found that samples with a high (or low) concentration of a particular analyte were more accurately modelled when using standards of similar characteristics. Consequently, the best option for carrying out this determination seems to involve the building of local calibration models in which standards are selected to predict each particular sample. The criterion for choosing standards is their proximity to the sample that is to be predicted, according to their distribution on principal component plots.

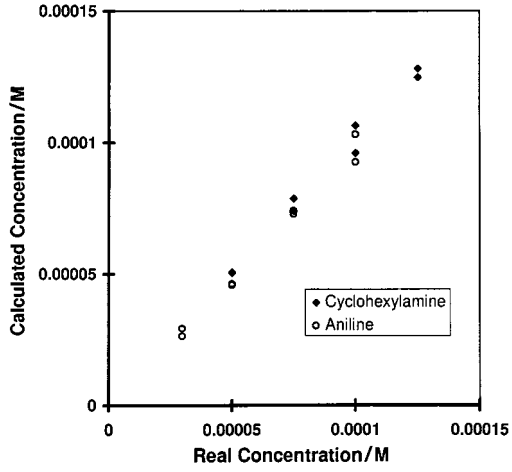

Fig. 5 Comparison between actual and calculated concentrations of aniline and cyclohexylamine in the unknown samples using local PLS models.

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