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The International Journal of Pure and Applied Analytical Chemistry

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Publication information: *Talanta* (ISSN 0039-9140). For 2006, volumes 68–70 are scheduled for publication. Subscription prices are available upon request from the Publisher or from the Regional Sales Office nearest you or from this journal's website (<http://www.elsevier.com/locate/talanta>). Further information is available on this journal and other Elsevier products through Elsevier's website: (<http://www.elsevier.com>). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by standard mail (surface within Europe, air delivery outside Europe). Priority rates are available upon request. Claims for missing issues should be made within six months of the date of dispatch.

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USA mailing notice: *Talanta* (ISSN 0039-9140) is published monthly by Elsevier B.V. (P.O. Box 211, 1000 AE Amsterdam, The Netherlands). Annual subscription price in the USA US\$ 3,628 (valid in North, Central and South America), including air speed delivery. Application to mail at periodical postage rate is pending at Jamaica, NY 11431.

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ASTM clustering for improving coal analysis by near-infrared spectroscopy

J.M. Andrés*, M.T. Bona

Instituto de Carboquímica, CSIC, Procesos Químicos, Miguel Luesma Castán, no. 4, 50018 Zaragoza, Spain

Received 24 November 2005; received in revised form 8 May 2006; accepted 17 May 2006

Available online 3 July 2006

Abstract

Multivariate analysis techniques have been applied to near-infrared (NIR) spectra coals to investigate the relationship between nine coal properties (moisture (%), ash (%), volatile matter (%), fixed carbon (%), heating value (kcal/kg), carbon (%), hydrogen (%), nitrogen (%) and sulphur (%)) and the corresponding predictor variables. In this work, a whole set of coal samples was grouped into six more homogeneous clusters following the ASTM reference method for classification prior to the application of calibration methods to each coal set. The results obtained showed a considerable improvement of the error determination compared with the calibration for the whole sample set. For some groups, the established calibrations approached the quality required by the ASTM/ISO norms for laboratory analysis. To predict property values for a new coal sample it is necessary the assignation of that sample to its respective group. Thus, the discrimination and classification ability of coal samples by Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) in the NIR range was also studied by applying Soft Independent Modelling of Class Analogy (SIMCA) and Linear Discriminant Analysis (LDA) techniques. Modelling of the groups by SIMCA led to overlapping models that cannot discriminate for unique classification. On the other hand, the application of Linear Discriminant Analysis improved the classification of the samples but not enough to be satisfactory for every group considered.

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Keywords: Near-infrared spectroscopy (NIR); Coal analysis; Pre-treatment effects; Soft Independent Modelling of Class Analogy (SIMCA); Linear Discriminant Analysis (LDA)

1. Introduction

On-line coal analysis is of great interest to power station operators as it would allow efficient plant operation. Unfortunately, no single method provides on-line accurate determination of coal properties and a variety of them are currently used in coal industry based on nuclear, capacitance, microwave or ultrasonic techniques [1–3]. Most of them rely on the determination of the inorganic constituents of coal to further extrapolate for the calculation of the other coal properties. While this approach can be useful for coal batches of the same origin, the usual practice of blending coals to fulfil environmental regulations [4] and performance optimisation prevents its widespread use in coal burning power plants.

In this context, Diffuse Reflectance Infrared Spectroscopy in the near-infrared (NIR) range covering the 1100–2500 nm region

can be a technique of choice for analyzing organic minerals like coal. DRIFTS sampling technique gained popularity for the measurement of coals since Fuller and Griffiths [5] described it as a proper technique for rapid identification of powders. Extensively used for the characterization of scattering samples, this technique presents as main advantages, not only a reduced sample time preparation but also high signal-to-noise ratio (SNR) spectra collected in minimal time. Reflection, absorption and scattering light are combined, resulting in a spectrum very similar to a transmission one, except for the relative intensities of the bands.

The first application of DRIFT in this field was the analysis of coal blends. Whereas the determination of binary blended coals was carried out by the method of absorption ratios [6], blends containing more than two coals were analyzed by Fredericks et al. [7] taking larger regions of the mid-FT-IR spectrum and relating the composition of the sample to its infrared spectra with a processing package based on factor analysis. Other applications for quantification of functional groups followed [8] and the full characterization of thermal coals has been attempted applying

* Corresponding author. Tel.: +34 976 73 39 77; fax: +34 976 73 33 18.
E-mail address: jmandres@carbon.icb.csic.es (J.M. Andrés).

multivariate calibration techniques to the mid-IR spectra [9–13] with relative success.

The near-infrared range has been used in several papers where coal is characterized by DRIFT-near-infrared spectroscopy [14,15] and, lately, the modified DRIFT-near IR spectra have been correlated with coal properties (moisture, volatile matter, oxygen content, maximum fluidity temperature and solidification temperature) applying multivariate calibration [16].

In a previous work the use of near-infrared spectroscopy for the analysis of coal was demonstrated [17]. When it was applied to a wide set of coal samples the method showed a relatively good precision though it failed to provide the reproducibility analysis required by the corresponding ASTM/ISO norms for coal. In the same paper, the application of this technique to a homogeneous set of samples of known origin showed that most of the properties could be determined in the way required by the above-mentioned norms.

The logical extension of those results are reported in this paper, in which the diffuse reflectance near-infrared spectroscopy coupled with the Partial Least Squares (PLS) method has been applied to clusters of coal samples obtained by supervised clustering following the corresponding ASTM norm. Once the validity of the approach has been demonstrated, two chemometric methodologies were used: Soft Independent Modelling of Class Analogy (SIMCA) and Linear Discriminant Analysis (LDA) [18].

Although the literature contains several works where coal is characterized by DRIFT-near-infrared spectroscopy, it is the first time that a clustering and classification technique is applied to coal samples in order to correlate spectral data to property analysis.

2. Experimental

One hundred and forty-two samples of raw coal from different suppliers were used. All of them showed a controlled particle size distribution, following the specifications for pulverized coal burners, typically: 98% <300 μm ; 95% <150 μm ; 75% <75 μm . They were analyzed for moisture, ash, volatile matter (VM), fixed carbon (FC), heating value (HV), carbon, hydrogen, nitrogen and sulphur by the usual ISO/ASTM standard methods. The statistical results of the full set of samples analysis are presented in a previous work [17].

An ATI Mattson Infinity Series FTIR spectrometer controlled by WinFIRST 3.5 software was employed for NIR spectra acquisition. All the spectra were acquired with a diffuse reflectance sample cup in absorbance mode, viz. $\log(1/\text{reflectance})$. Spectra obtained were the result of co-adding 32 scans over the range 1100–2500 nm performed at 1 cm^{-1} of digital resolution.

Multivariate calibration and pre-treatment spectra were performed with The Unscrambler v9.1, from CAMO. Two methods were used for supervised pattern recognition technique: SIMCA (Soft Independent Modelling of Class Analogy) method, as implemented by The Unscrambler v9.1 and LDA (Linear Discriminant Analysis) using SPSS v12.0 for Windows.

2.1. Selection of the calibration set and data processing

ASTM D388 norm [19] has been applied to establish the number of clusters. Thus, coal sample grouping was developed according to rank, whereby fixed carbon and gross calorific value to the mineral matter-free basis (Mm-free) were calculated with the Parr formulas. These formulas include sulphur and ash content minimized in a determined factor. The analytical values and the strategy for building the statistical models are described further on.

As it was done for the full set coal samples [17], mathematical pre-treatments were used in order to study the correlation between the modified spectral data and the coal properties in the calibration models.

3. Results and discussion

3.1. Cluster analysis

In a previous work [17], a principal component analysis of the full set of samples revealed the tendency to group the samples into more homogeneous sets. In that case, the study of a group supplied by the same power station led to a considerable improvement of the results. As the origin of the samples is usually unknown, a clustering method is needed to improve the coal analysis.

The clustering method developed in this paper follows the ASTM D388 norm, as it is the most commonly used in coal research. Although this norm classifies coal samples with regard to analytical laboratory values, the aim of this paper is to demonstrate the correlation between spectral data and coal properties for each cluster previously defined.

This norm establishes categories of coal based on gradational properties that depend principally on the degree of metamorphism, covering from lignite to anthracite. These categories indicate ranges of physical and chemical characteristics that are useful in making broad estimates of the behaviour of coal in mining, preparation and use. The basis of this classification is according to fixed carbon and gross calorific value calculated to the mineral matter-free basis. The higher-rank coals are classified according to fixed carbon on the dry basis; the lower-rank coals are classified according to gross calorific value on the moist basis [19].

The clustering process developed in this work could be considered like a hierarchical approximation. As it is shown in Fig. 1, from top to bottom, the totality of the samples were sequentially split following the standard method above-mentioned. This approach led to a number of well-defined clusters. Some of them (labelled in grey), with not enough samples to develop a calibration model, were joined to another cluster highly similar; on the other hand, there is only one anthracite sample that due to its differences in both spectral data and analytical values was removed from the calibration sample set. Notice that the norm defines three more clusters but they are not presented in the diagram because of the lack of samples.

Once the coal samples have been distributed into different clusters in the tree-diagram scheme, it was necessary to verify

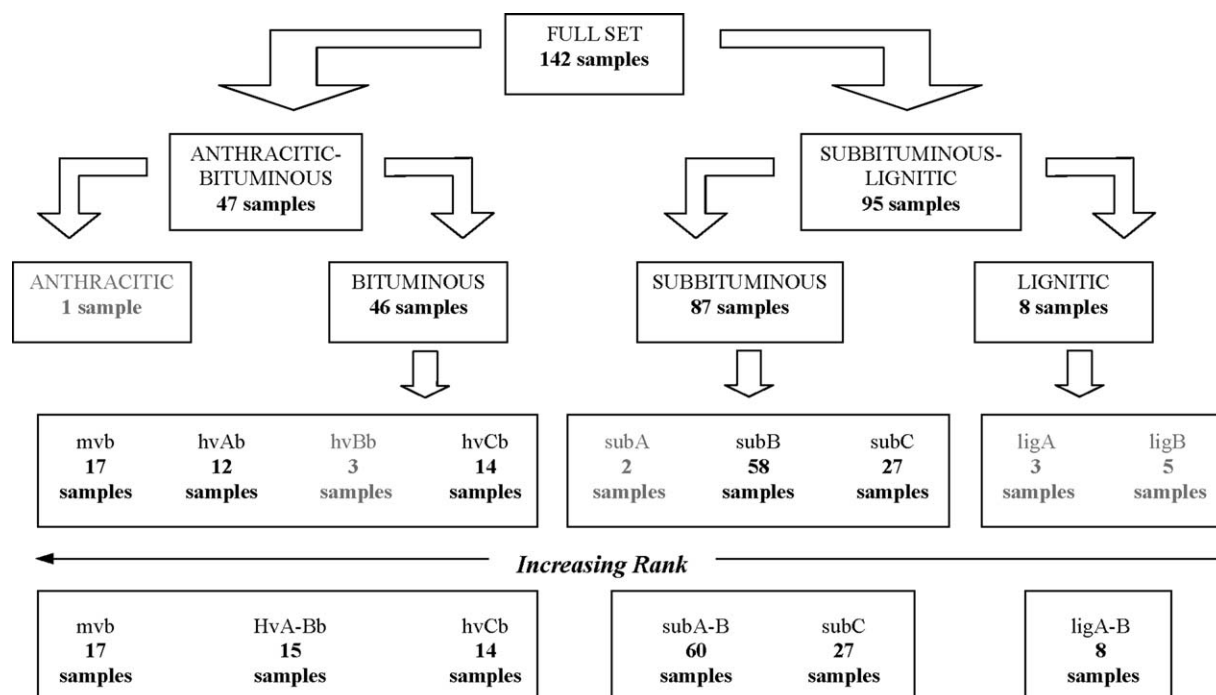


Fig. 1. Classification of coals by rank. mvb: medium volatile bituminous; hvAb: high volatile A bituminous; hvBb: high volatile B bituminous; hvCb: high volatile C bituminous; subA: subbituminous A; subB: subbituminous B; subC: subbituminous C; ligA: lignite A; ligB: lignite B.

the improvement in the calibration accuracy for each division. Because of the results given by the full set of samples [17] considered hydrogen content (%) as the most suitable variable to be correlated to near-infrared spectral features, spectral data – in absorbance mode – and hydrogen content (%) for each cluster was correlated to study precision and accuracy.

The performance of the method was evaluated through the application of Partial Least Squares Regression (PLSR) calibration models with the absolute prediction error determined by the Root Mean Squared Error of Cross-Validation (RMSECV). Fig. 2 shows how the prediction ability improves, leading to two clusters with error values below 0.3.

To compare models of different sizes the F -test has been used to determine the degree of significance along the proposed procedure. The aim of the study will be to discard the null hypothesis, $H_0: s_1^2 = s_2^2$ where $s^2 = \text{RMSECV}^2$ and demonstrate an alternative hypothesis, $H_1: s_1^2 > s_2^2$. For that, the assay estimates $F_0 = s_1^2/s_2^2$ where $s_1^2 > s_2^2$, that will be compared to F_c , given

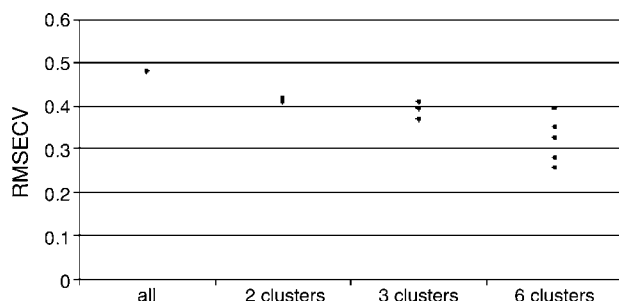


Fig. 2. Root Mean Squared Error of Cross-Validation (RMSECV) obtained for hydrogen (%) content as the number of clusters increases.

by a significance level of $\alpha_c = 0.05$ and τ_1, τ_2 number of degrees of freedom. H_0 will be refused if $F_0 > F_c$.

Once established the methodology, the statistical significance of the splitting process was determined considering s_1 as the original cluster RMSECV and s_2 as the RMSECV of the final one. Table 1 summarizes the results taking the full sample set

Table 1

F -statistics for the corresponding degrees of freedom (τ) along the splitting process considering RMSECV-hydrogen content (%) as cluster variances

Original cluster (τ)	Final cluster (τ)	F_0	F_c
Full sample set (139)	Bituminous (42)	1.37	1.53
	Subbituminous-lignite (95)	1.31	1.34
Bituminous (42)	Medium volatile bituminous (16)	2.14	2.08
	High volatile A–B bituminous (11)	1.59	2.51
	High volatile C bituminous (12)	2.14	2.43
Subbituminous-lignite (95)	Subbituminous (84)	1.29	1.39
	Lignite (6)	1.13	3.27
Subbituminous (84)	Subbituminous A–B (57)	1.12	1.48
	Subbituminous C (25)	2.14	2.08
Full sample set (139)	Bituminous (42)	1.37	1.53
	Subbituminous-lignite (95)	1.31	1.34
Full sample set (139)	Bituminous (42)	1.37	1.53
	Subbituminous (84)	1.68	1.34
	Lignite (6)	1.48	3.69
Full sample set (139)	Medium volatile bituminous (16)	2.94	2.04
	High volatile A–B bituminous (11)	2.18	2.43
	High volatile C bituminous (12)	2.94	2.32
	Subbituminous A–B (57)	1.88	1.44
	Subbituminous C (25)	3.41	1.75
	Lignite (6)	1.48	3.69

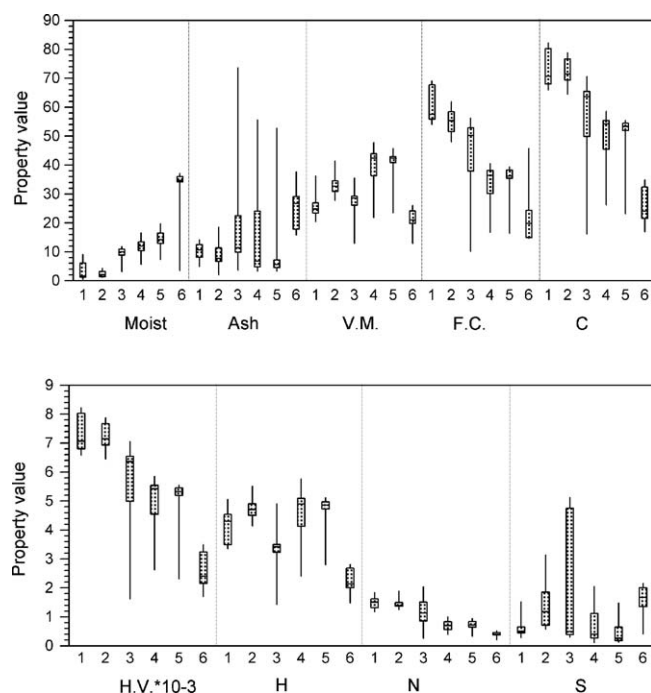


Fig. 3. Statistical results for each cluster and property value (%): 1, medium volatile bituminous; 2, high volatile A–B bituminous; 3, high volatile C bituminous; 4, subbituminous A–B; 5, subbituminous C; 6, lignite.

as original cluster when the system is analyzed as a whole or assigning the corresponding cluster if it is studied sequentially. Results with $F_0 > F_c$ are highlighted in bold type. It can be concluded that although the analysis of the sequential process leads to subbituminous C and medium volatile bituminous clusters as significant splits, the resultant F -test when the original cluster corresponds to the full sample set support the process of splitting with four in six significant final clusters.

After defining the clusters, the statistical results are presented in Fig. 3. The Y-axis represent the property value in percentage (%) and the X-axis shows the different clusters numbered from 1 to 6, corresponding to: 1, medium volatile bituminous; 2, high volatile A–B bituminous; 3, high volatile C bituminous; 4, subbituminous A–B; 5, subbituminous C; 6, lignite. It can be observed that clusters tend to be more homogeneous in property values even though some cases, such as ash content, present values far apart from the median. Further on, these values will be considered as potential outliers in the calibration process.

3.2. Calibration models and effect of the pre-treatments into the clusters

As it was presented previously, several mathematical pre-treatments were applied to spectral data in order to minimize the physical features of the sample. Among all possible pre-treatments, the most commonly used ones to correct the spectral variations were used in this work: baseline offset, range normalization, baseline offset followed by range normalization and full multiplicative scatter correction (MSC). Despite the widespread use in other field applications, in this paper neither first nor sec-

ond derivatives were taken into account due to the bad results obtained previously [17]. All the pre-treatments were applied to the different spectral data group corresponding to each cluster. It is noteworthy that the detection of outliers was done independently for each different cluster. Samples with high leverages, or studentized residual values over ± 3 were some of the criteria considered for the selection of anomalous coal samples. In any case, the deletion of each outlier must be considered subjectively.

All the errors obtained after applying PLS regression are shown in Fig. 4. In this plot the bars correspond to the absolute errors (RMSECV) given by the different spectral data pre-treatment, whereas the line represents the error for the full set of samples. This line shows the errors obtained when baseline offset/normalization pre-treatment was applied, considered the best result for the whole set of samples [17].

Generally, the diagram presents a reduction in the RMSECV values, but it is remarkable the improvement obtained in high volatile C bituminous and subbituminous C clusters for all the properties studied. Although the RMSECV represents the predictive ability of the model, residuals should be analyzed in order to study the error distribution and to verify the reproducibility levels allowed by ASTM/ISO reference methods. The results obtained show that both nitrogen and sulphur content residuals fulfil the parameters established for reproducibility limits, below 0.3%. Nevertheless, neither of them presents a good predictive ability of the model due to coal samples have a mean property value for nitrogen and sulphur quite low. For the rest of the properties two residual distributions have been found. Fig. 5 shows an example considering carbon content as a property to be compared between high volatile A–B and high volatile C bituminous clusters. Although, a priori, the RMSECV results, shown in Fig. 4, revealed better prediction ability for high volatile C cluster, the residual sample distribution do not fulfil the ASTM reproducibility requirements, being quite close to the limits. On the contrary, higher RMSECV values for high volatile A–B cluster lead to a very different residual distribution with 8 of 15 residual samples between the limits allowed by the norm and the rest of the samples quite far from the limits leading to higher global error values. Considering that all of the samples fulfil the calibration criteria related to the studentized residual limits, between ± 3 , the results suggest that a residual distribution highly homogeneous leads to better absolute errors despite being out of the reproducibility limits. This reasoning agrees with subbituminous C cluster behaviour because of the fact that its residual distribution is quite similar to high volatile C bituminous one.

In the same way, when RMSECV values were expressed in percentage error around the cluster mean for comparison purposes, volatile matter, fixed carbon, heating value, carbon content and hydrogen content, both in high volatile C bituminous and subbituminous C present relative error values below 5% as Fig. 6 shows, enough for semi-quantitative on-line analysis.

On the other hand, variables such as nitrogen and sulphur content with successful absolute error values turn into prediction abilities out of usefulness when they are expressed as relative errors.

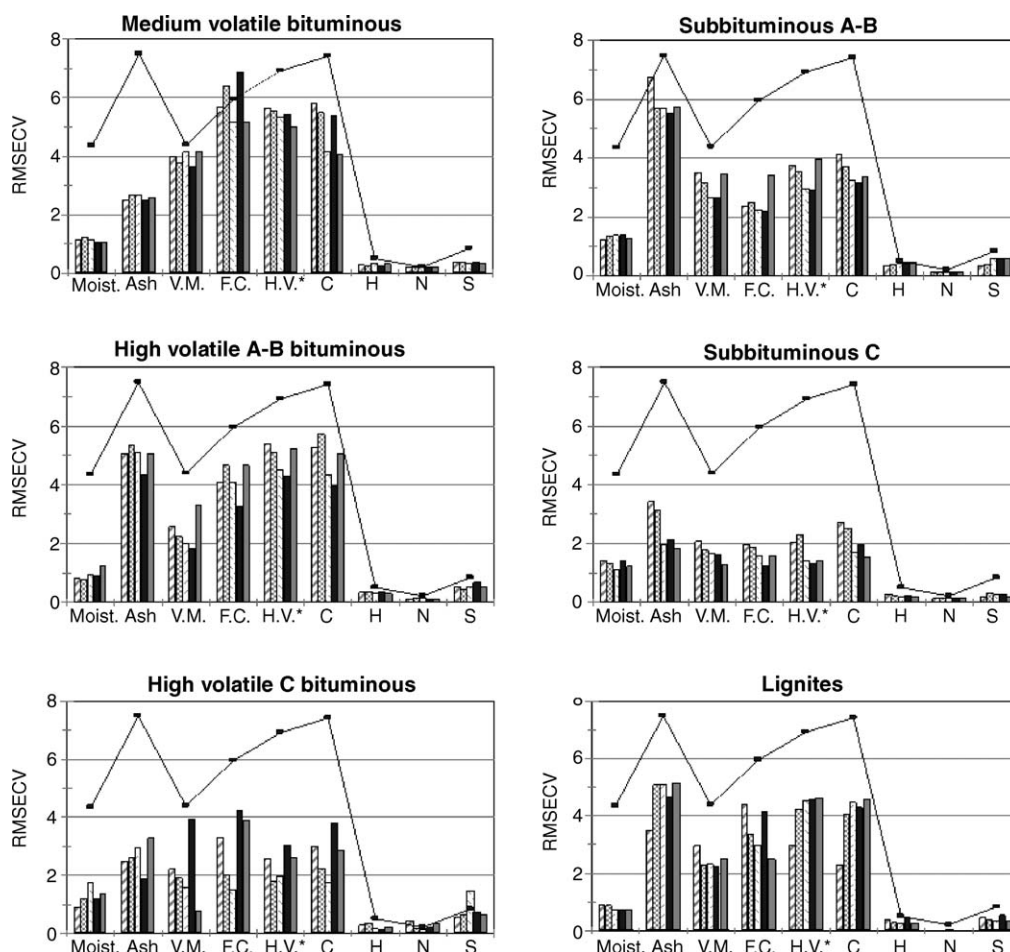


Fig. 4. RMSECV obtained for each cluster and property after applying different pre-treatment: the first bar corresponds to original spectra followed by baseline correction, baseline/normalization, normalization and MSC. The line represents the error with the full set sample. *Values of heating value scaled ($\times 10^{-2}$) for fitting the graph.

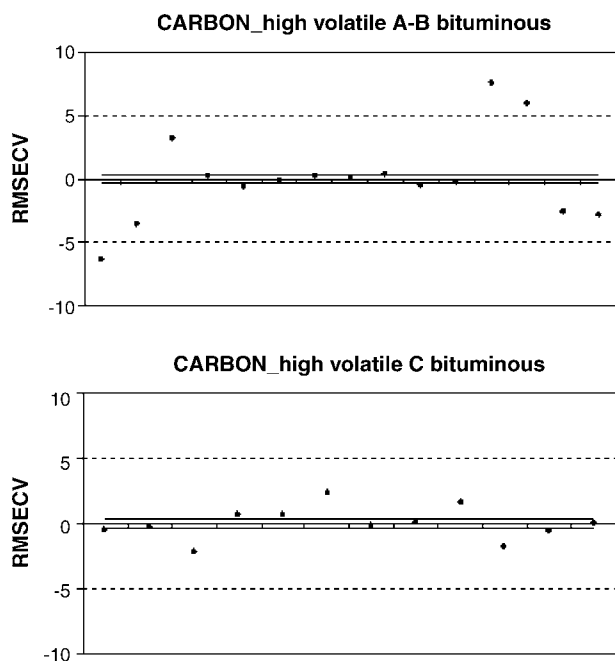


Fig. 5. Carbon content residual sample distribution for high volatile A-B and high volatile C bituminous clusters.

With regard to ash content, the impossibility to absorb inorganic compounds in the NIR region leads to quite high relative error values. Although clustering process improves the results, it is important to take into account that ASTM norm does not consider the influence of the mineral matter content along the clustering process, so the improvement in the ash content calibration results from an indirect measurement of more homogeneous sample sets.

It is worth highlighting the calibration for moisture. As it was explained in a previous work, moisture calibration with the

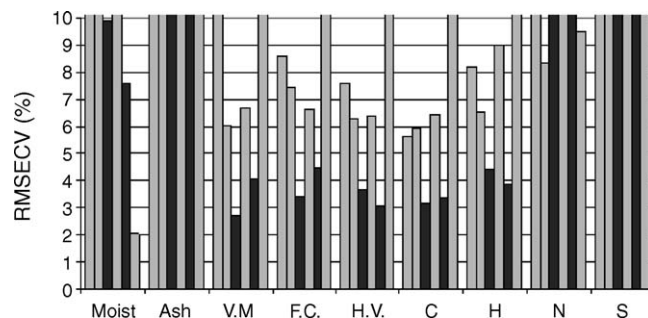


Fig. 6. RMSECV values as percentage errors around the cluster mean.

Table 2
F-statistics in significant models

Cluster	Property	τ_1	τ_2	Pre-treatment	F_0	F_c
High volatile C bituminous	Moisture	13	13	–	3.66	2.55
High volatile C bituminous	Volatile matter	12	11	MSC	4.41	2.79
High volatile C bituminous	Sulphur	12	12	–	6.84	2.69
Subbituminous A–B	Sulphur	59	59	–	2.69	1.51
Subbituminous C	Sulphur	26	25	MSC	2.44	1.92

τ_1 and τ_2 correspond to the number of degrees of freedom for the cluster with baseline/normalization pre-treatment (τ_1) and the alternative one (τ_2).

whole set of samples was not suitable despite the feasibility of the technique. Water is present in coal in different ways and the ISO/ASTM procedures only measure water that evaporates at 105 °C. The improvement of moisture calibration was demonstrated when more homogeneous samples were grouped. In this case, all the clusters show a decrease of the RMSECV, especially lignite samples achieving a relative precision of 2.33% compared to 38.84% with the full set of samples. Lignite rank coal presents higher moisture content, mainly physisorbed, the one measured applying ASTM/ISO procedures when the samples are analyzed. Thus, spectral lignite data seems to be correlated to the measured moisture with high reliability. This is a radically different result than that obtained for the full set of samples and can be explained considering the different states of water in the coal: free, physisorbed, chemisorbed and hydration water in minerals. The determination of moisture measures only water in the first three states while NIR measures the full water content. The clustering of the coal samples produces more homogeneous sets in which the mineral matter is of similar nature for the coal samples in the cluster, so the difference between moisture and NIR determined water is linear with the mineral matter content and can be adequately compensated by the PLS method.

Focusing on the different spectral modifications, it seems to be baseline/normalization the pre-treatment that presents a major improvement in the prediction ability, as it was with the full sample set. In order to ensure the significance level of the model, an F-test was developed when the above-mentioned pre-treatment did not give the lower residual value into the cluster. Only 5 in 54 situations led to better results with a different pre-treatment. The corresponding F values for these cases are shown in Table 2. As it was pointed out, τ_1 and τ_2 refer to the number of degrees of freedom taking baseline/normalization or the alternative pre-processing spectral data, respectively. Summarizing, numerical details about absolute and relative error values for calibration and prediction models, number of principal components selected and outliers removed for the best calibration models are presented in Table 3.

3.3. Classification based on spectral data

Once the clustering process has been detailed, it is necessary for the system to establish a classification method able to assign a new sample to one of the clusters previously defined considering its NIR spectral data as the unique information about the object. Two supervised classification systems are developed to ensure the suitability of the process: SIMCA and LDA.

- *Soft Independent Modelling of Class Analogies (SIMCA)* [20,21] took the x-spectral data in log(1/R) unit for one cluster and carried out a principal component analysis (PCA) to model the shape and position of samples in space for class definition. The number of principal components necessary to describe the modelled variance is determined using cross-validation technique. Typically a very small number of data represent most of the variability in the x-data. This process was repeated for each cluster to get a multidimensional box for each group of coals.

After modelling the clusters, the classification of a new sample would require its NIR spectral data to be compared to the different models previously defined. This procedure reduces enormously the time analysis because it is not necessary to develop the corresponding ASTM norms to characterize the new sample.

To check the feasibility of the process, the full set of samples is considered as a test set. The use of their spectral data will corroborate the efficiency of the ASTM clustering process.

Firstly, it is considered the six clusters previously defined to classify the samples of the test set. The results show that the system is not able to distinguish between the different models leading to a great overlap between the multidimensional boxes.

When clusters are grouped into three larger clusters, labelled as bituminous, subbituminous and lignite, the method assign correctly the test set. The results are shown in a Cooman's plot, Fig. 7, where the orthogonal distances from a sample to two different models are compared at the same time. This plot includes class membership limits, at a 5% significance level, for both models. Green crosses represent the test set whereas blue circles correspond to the samples included in subbituminous model placed along the abscissa axis, and red circles those modelling the lignite group along the ordinate one. In this case, the graphic shows how subbituminous and lignite coal samples are near to the corresponding models whereas the bituminous one are correctly rejected. This fact renders high sensitivity and specificity of the models.

So, SIMCA is not able to differentiate between the six clusters probably because the spectral data similarities make multidimensional boxes construct quite close. Since the classification into three groups does not improve significantly the calibration errors except for subbituminous cluster, as Table 1 shows, it is necessary try to find a suitable classification system to distinguish between the six clusters.

Table 3

Best results for calibration and prediction error obtained for each group

Cluster	Property	Mean	RMSEC	RMSEC (%)	RMSECV	RMSECV (%)	PC	Outliers
Medium volatile bituminous	Moisture	3.40	0.79	23.20	1.14	33.48	2	1
	Ash	10.29	2.26	21.95	2.67	25.94	2	0
	Volatile matter	25.98	3.80	14.62	4.14	15.93	1	0
	Fixed carbon	60.32	4.69	7.77	5.18	8.59	1	1
	Heating value	7060.85	404.12	5.72	535.65	7.59	2	0
	C	73.43	3.55	4.83	4.16	5.67	2	1
	H	4.15	0.26	6.26	0.34	8.19	2	1
	N	1.48	0.18	12.18	0.21	14.21	1	0
High volatile A–B bituminous	S	0.62	0.31	49.68	0.34	54.48	1	0
	Moisture	2.33	0.63	27.06	0.96	41.24	3	0
	Ash	9.69	4.65	48.01	5.09	52.55	1	0
	Volatile matter	33.05	1.36	4.12	2.00	6.05	4	1
	Fixed carbon	54.94	3.51	6.39	4.09	7.44	2	0
	Heating value	7118.59	349.10	4.90	448.50	6.30	4	0
	C	72.41	3.40	4.69	4.32	5.97	4	0
	H	4.73	0.23	4.87	0.31	6.56	3	0
High volatile C Bituminous	N	1.44	0.06	4.17	0.12	8.34	4	0
	S	1.37	0.47	34.23	0.51	37.14	1	1
	Moisture*	9.31	0.65	6.98	0.92	9.82	2	0
	Ash	18.58	0.45	2.42	2.97	15.98	6	0
	Volatile matter*	27.82	0.55	1.96	0.75	2.70	2	2
	Fixed carbon	44.28	0.97	2.19	1.51	3.41	2	2
	Heating value	5403.33	113.31	2.10	196.26	3.63	1	2
	C	56.32	1.22	2.16	3.14	0.99	2	2
Subbituminous A–B	H	3.39	0.08	2.36	0.15	4.42	2	2
	N	1.17	0.01	0.60	0.13	11.15	4	2
	S*	2.20	0.37	16.85	0.56	25.50	3	1
	Moisture	11.99	0.76	6.34	1.38	11.51	11	0
	Ash	14.40	3.23	22.44	5.69	39.52	10	2
	Volatile matter	39.65	1.33	3.34	2.65	6.68	11	2
	Fixed carbon	33.96	1.32	3.89	2.25	6.62	10	2
	Heating value	4637.78	168.54	3.63	296.26	6.39	10	2
Subbituminous C	C	50.22	1.93	3.84	3.25	6.47	10	1
	H	4.56	0.32	7.01	0.41	8.99	7	0
	N	0.70	0.05	7.17	0.11	15.77	11	2
	S*	0.71	0.05	7.04	0.36	50.68	15	1
	Moisture	14.62	0.62	4.24	1.11	7.59	8	1
	Ash	9.37	1.24	13.23	1.98	21.12	7	1
	Volatile matter	40.69	0.83	2.04	1.65	4.05	7	1
	Fixed carbon	35.31	0.40	1.13	1.58	4.47	9	1
Lignites	Heating value	4628.37	93.38	2.02	141.15	3.05	7	1
	C	51.03	1.08	2.12	1.71	3.35	7	1
	H	4.70	0.11	2.34	0.18	3.83	7	1
	N	0.72	0.06	8.36	0.12	16.73	8	1
	S*	0.46	0.06	13.17	0.16	35.12	9	1
	Moisture	35.30	0.32	1.02	0.73	2.33	1	1
	Ash	23.33	2.70	10.74	5.08	20.21	1	1
	Volatile matter	22.31	1.17	5.53	2.31	10.93	1	1
	Fixed carbon	19.04	2.41	10.76	2.98	13.29	1	1
	Heating value	2590.57	235.45	9.63	453.49	18.55	1	1
	C	27.30	2.31	8.88	4.48	17.20	1	1
	H	2.35	0.14	6.24	0.27	12.03	1	1
	N	0.42	0.01	1.51	0.04	10.09	2	1
	S	1.75	0.27	17.09	0.34	21.52	1	0

All data are obtained after baseline/normalization pre-treatment except those with * corresponding to different significant pre-processing (see Table 2).

- *Linear Discriminant Analysis (LDA)* [22] is a supervised pattern recognition technique that, in this case, evaluates and reinforces the capacity to distinguish between the clusters optimized by the ASTM reference method. As LDA cannot

deal with the high number of variables provided by the spectra, a principal component analysis was applied in order to reduce their number. Thus, LDA was developed by taking the PCA scores. Only three principal components were neces-

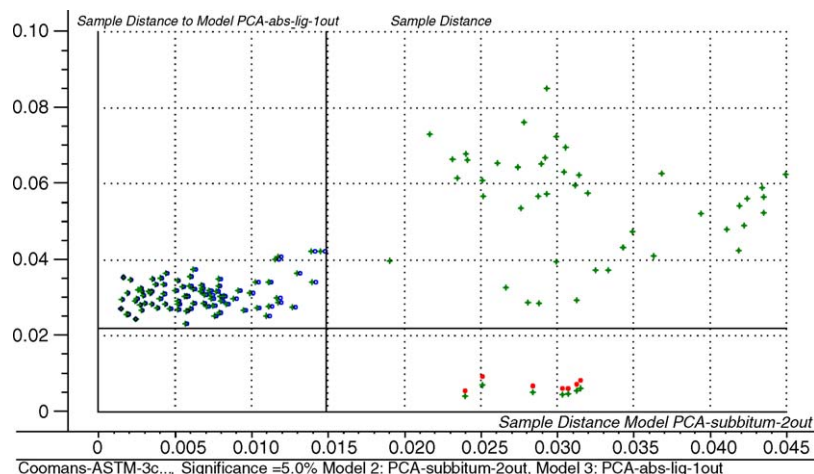


Fig. 7. Cooman's plot considering bituminous, subbituminous and lignite models.

sary to account for 99.86% explained variance (PC1: 99.13%; PC2: 0.52%; PC3: 0.21%) when $\log(1/R)$ scores were studied. The loading plots, presented in Fig. 8, reveal the tendency to correct non-linearities, scatter and spectral features assigned to X–H functional groups. More factors would imply taking into account noise spectral data. Once the discriminant functions were calculated, a representation of the canonical scores, in Fig. 9, shows the distribution of the centroid groups with a main overlap corresponding to subbituminous A–B and subbituminous C. When the samples were classified, the percentage of samples correctly classified increased from 60.3 to 73.8% when within-groups or separate-groups covariance matrix were used computing prior probability from group sizes in both cases. The classification for each cluster was 70.6% for medium volatile bituminous, 60% for high volatile A–B bituminous, 64.3% for high volatile C bituminous, 95% for subbituminous A–B, 33.3% for subbituminous C and 100% for lignite.

Linear Discriminant Analysis distinguishes between clusters characterized by important spectral differences. It could be because of the fact that ASTM norm classifies according to properties that do not correspond to chemical sample nature but a heating assay of the sample. With the idea of discriminating and classifying new samples in a more reliability way, we are implementing a clustering method taking into account both the laboratory variables that characterize the samples and their spectroscopic influence.

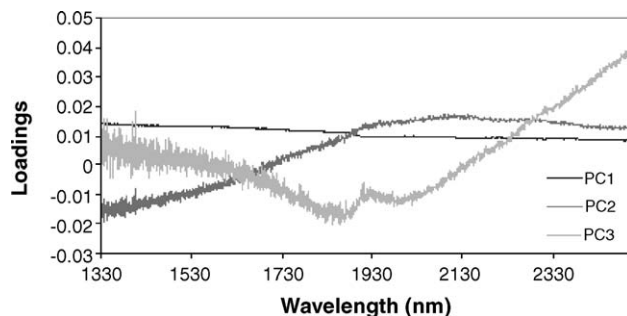


Fig. 8. Loadings of PC1–PC3 corresponding to PCA in $\log(1/R)$ units.

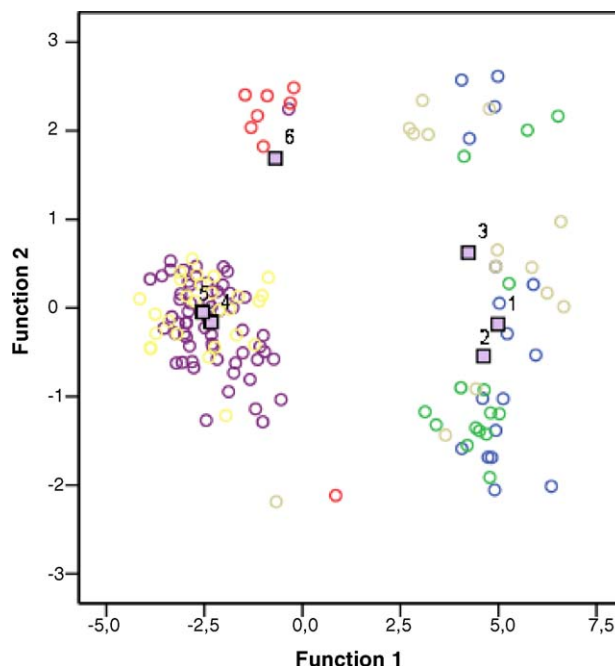


Fig. 9. Canonical scores of LDA: 1, medium volatile bituminous; 2, high volatile A–B bituminous; 3, high volatile C bituminous; 4, subbituminous A–B; 5, subbituminous C; 6, lignite.

4. Conclusions

The determination of coal properties based on its near-infrared spectrum can be done with good precision by grouping the coals in homogeneous sets following the ASTM reference norm for coal classification. The application of PLS to these groups yields a considerable reduction of the prediction error compared with the use of the whole set of samples. In some cases, the prediction error is comparable or close to the reproducibility required by the ASTM/ISO norms for coal analysis and better than those provided by commercial on-line analysers. The pre-treatment to be used for the best calibration results varies with the coal group and reflects the different behaviour of the coals not only in light absorption but in light scattering.

Nevertheless, a significance study suggests that baseline correction followed by normalization could be considered as a general spectral modification.

The application of the developed calibrations to a new sample requires a prior step consisting of the assignation of the sample to one of the coal groups, whereby it is necessary a proper discrimination between clusters. The two techniques used in this work led to poor discrimination ability. SIMCA models overlap between them causing simultaneous assignation to several clusters for a great number of samples while the use of LDA provides better results although its sensitivity and specificity is not enough for the unique and correct assignation of every sample to its corresponding group. More powerful methods for coal classification are required for this analytical method to be applied to coal.

Acknowledgements

The authors are grateful to the European Coal and Steel Community for funding this research within the framework of Project 7220-PR/118. We also thank to ENDESA, RWE Ag, EVN Ag and PPC for providing us coal samples.

References

- [1] M. Nina, Skorupska, IEA Coal Res. 52 (1993).
- [2] Jane.V. Thomas, Anal. Chem. 67 (12) (1995) 317R.
- [3] G.R. Couch, IEA Coal Res. 22 (1996).
- [4] A.M. Carpenter, IEA Coal Res. 81 (1995).
- [5] M.P. Fuller, P.R. Griffiths, Anal. Chem. 50 (13) (1978) 1906.
- [6] M.P. Fuller, I.M. Hamadeh, P.R. Griffiths, D.E. Lowenhaupt, Fuel 61 (1982) 529.
- [7] P.M. Fredericks, R. Kobayashi, P.R. Osborn, Fuel 66 (1987) 1603.
- [8] H.H. Schobert, Coal, The Energy Source of the Past and Future, American Chemical Society, 1987.
- [9] S. Tesch, K.-H. Rentrop, M. Otto, Fresenius J. Anal. Chem. 344 (1992) 206.
- [10] C.E. Alciaturi, M.E. Escobar, R. Vallejo, Fuel 75 (4) (1996) 491.
- [11] C.E. Alciaturi, T. Montero, C. De La Cruz, M.E. Escobar, Anal. Chim. Acta 340 (1997) 233.
- [12] C.E. Alciaturi, M.E. Escobar, C. De La Cruz, R. Vallejo, Anal. Chim. Acta 436 (2001) 265.
- [13] E.A. Cloutis, Fuel 82 (2003) 2239.
- [14] O. Ito, H. Seki, M. Iino, Fuel 67 (1988) 573.
- [15] O. Ito, Energy Fuels 6 (1992) 662.
- [16] M. Kaihara, T. Takahashi, T. Akazawa, T. Sato, S. Takahashi, Spectrosc. Lett. 35 (3) (2002) 369.
- [17] J.M. Andrés, M.T. Bona, Anal. Chim. Acta 535 (2005) 123.
- [18] D. Luc Massart, L. Kaufman, The Interpretation of Analytical Chemical Data by the Use of Cluster Analysis, Wiley–Interscience, 1983.
- [19] ASTM D388-92a, Standard Classification of Coals by Rank.
- [20] T. Naes, T. Isaksson, T. Fearn, T. Davies, A User-friendly Guide to Multivariate Calibration and Classification, NIR Publications, 2002.
- [21] M.A. Sharaf, D.L. Illman, B.R. Kowalski, Chemometric, vol. 195, John Wiley & Sons, New York, 1986.
- [22] W.R. Dillon, M. Goldstein, Multivariate Analysis, John Wiley & Sons Inc., 1984.

Solid phase extraction and HPLC determination of veterinary pharmaceuticals in wastewater

Sandra Babić*, Danijela Ašperger, Dragana Mutavdžić,
Alka J.M. Horvat, Marija Kaštelan-Macan

Laboratory of Analytical Chemistry, Faculty of Chemical Engineering and Technology, University of Zagreb, Marulićev trg 20, HR-10000 Zagreb, Croatia

Received 30 November 2005; received in revised form 27 March 2006; accepted 3 July 2006

Available online 22 August 2006

Abstract

This work focuses on the application of SPE–HPLC analysis of important veterinary pharmaceuticals from different classes in highly complex wastewater matrix. The pharmaceutical investigated included three sulfonamides (sulfamethazine, sulfadiazine and sulfaguanidine), a sulfonamide synergist (trimethoprim), a tetracycline (oxytetracycline), a fluoroquinolone (enrofloxacin) and a β -lactame (penicillin G/procaine). The method involves pre-concentration and clean-up by solid phase extraction (SPE) using Oasis HLB extraction cartridges. Final analysis of the selected pharmaceutical compounds was carried out by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD). Recoveries were ranged from 68.3 to 97.9% with relative standard deviation below 8.4%. Only for sulfaguanidine low recovery was obtained. Limits of quantification were in the range 1.5–100 $\mu\text{g/L}$ depending on pharmaceutical. The described method was applied to the determination of pharmaceuticals in wastewater samples from pharmaceutical industry.

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Keywords: Pharmaceuticals; Wastewater; High-performance liquid chromatography; Diode array detection; Solid-phase extraction

1. Introduction

Until last two decades exposure of pharmaceuticals to environment has not been considered a big concern because they were thought to be safe [1]. Nowadays, however, they are referred to as the emerging contaminants. The fact that these compounds are not in the regulatory list of environmental pollutants resulted in comparatively little attention paid to them. For majority of the emerging contaminants data about their occurrence, related risk assessment and ecotoxicological data are not available. Of all the emerging contaminants antibiotics are the biggest concern since their environmental emissions can increase the occurrence of resistant bacteria in the environment, which may lead to unforeseen consequences [2,3]. Although pharmaceuticals are expected to occur in trace concentrations, because of their continuous use and bacterial resistance attributed to their action, they represent particular problem. Moreover, physico-chemical properties (mostly poor degrad-

ability) of some of these compounds enable their penetration through all natural filters and constructed treatment facilities thus putting drinking water supply systems at the risk. Several investigations have shown some evidence that substances of pharmaceutical origin are often not eliminated during wastewater treatment and also not biodegraded in the environment [3]. Their incomplete removal from the wastewater treatment plants (WWTPs) is referred to as the major source of their release into environment [4–6]. Therefore, multiresidue analytical method is a prerequisite to provide reliable data on the behavior of pharmaceuticals in WWTPs. The information obtained from analysis of influents and effluents of WWTPs may serve to optimize a treatment process or possible pre-treatment step so that the emission of undesired pollutants into the receiving waters is prevented.

At the moment different methods, mainly based on HPLC–MS, GC–MS are being developed [7,8]. Gas chromatography coupled to electron ionisation (EI) mass spectrometry (MS) is both sensitive and selective for the determination of sulfonamides but derivatisation of non-volatile and thermally labile pharmaceuticals is required prior to analysis [9]. This increases the overall analysis time and it may lead to errors to the analytical technique. High-performance liquid chromatography

* Corresponding author. Tel.: +385 145 97 205; fax: +385 1 45 97 250.
E-mail address: sandra.babic@fkit.hr (S. Babić).

(HPLC) is one of the most popular and sensitive method which can separate a wide variety of pharmaceuticals, some of which are not amenable to gas chromatography. Liquid chromatography with fluorescence detection has been reported to have a low limit of detection. However, the technique certainly requires derivatisation to improve the fluorescence properties for detection [10,11]. High-performance liquid chromatography–mass spectrometry (HPLC–MS) and gas chromatography–mass spectrometry (GC–MS) have been used to determinate pharmaceuticals in variety matrices [8,12,13]. However, when analyzing highly contaminated samples, such as wastewater, suppression of electrospray ionization is most likely to occur. A solution to this problem is improved sample clean-up and quantification by internal standards or standard addition method [14,15]. The use of DAD as a detector for HPLC has proved to be a powerful tool in the determination and identification of compounds as it allows the on-line acquisition of their UV spectra. In addition, most of above mentioned methods are for one class of antibiotics only (e.g. tetracyclines, sulfonamides). A challenge is presented in the simultaneous extraction and analysis of multiple classes of compounds.

Fluoroquinolones, sulfonamides, tetracyclines and β -lactams represent classes of antibiotics that are widely used in human and veterinary medicine. Prior studies show that these compounds are only partially eliminated and there is the potential for residues of antibiotics to be released in WWTP effluent into the aquatic environment [16,17]. Sulfonamides are antibacterial agents, commonly used in veterinary prophylaxis of infections, in the treatment of diseases and as growth promoters. These compounds become hazardous if they occur in water at wastewater treatment locations where they might inhibit growth of microorganisms involved in wastewater biotreatment. In addition the risk of developing antibiotics is enhanced, which makes the therapeutic use of similar medicine inefficient. Recent research has detected sulfamethazine as a potential thyroid carcinogenic agent. Sulfonamides have great potential to resist degradation and are hydrophilic enough to be transferred to the aquatic environment [18]. Trimethoprim, a dihydrofolate reductase inhibitor structurally different from sulfonamides, is commonly prescribed in the combination with sulfonamides. Tetracyclines are broad-spectrum antibiotics highly effective against numerous gram-positive and gram-negative bacteria. Due to those properties they are very popular in treating human and animals. Tetracycline antibiotics are widely used in live-stock because of their therapeutic and economic advantages. They are used both in the treatment and in the prevention of animal infections [19]. Usually, tetracycline antibiotics have been determined by microbiological methods or immunoassays. However, these screening techniques usually lack specificity. Alternative to them is thin-layer chromatography (TLC) which has similar sensitivity but much higher specificity and can easily distinguish one tetracycline antibiotic from another [20] but TLC requires large number of standards to be run simultaneously with the sample. Tetracyclines are unstable in basic media and are photodegradable. They have some features in common with sulfonamides (i.e., they are amphoteric and form metal complexes in both acidic and basic media) [18]. β -lactam antibiotics

have been the most widely used as antimicrobial drugs for more than 80 years and still constitute the most important group of antibiotics. Penicillins, one of the most important group of β -lactam antibiotics, are commonly used as the raw material for the production of semisynthetic penicillins and they are extremely effective and still widely used in veterinary applications and in human medicine, so that its indiscriminate release to the environment could eventually reduce its efficiency in these fields by increasing the penicillin resistance of human and animal pathogens. Penicillin G or benzylpenicillin (R: benzyl) which has the greatest antimicrobial activity within all natural penicillins, is highly sensitive to heat, acids and penicillinases [21]. The aim of this work was to develop a method for simultaneous determination of selected pharmaceuticals: sulfonamides (sulfamethazine, sulfadiazine, sulfaguanidine), a sulphonamide synergist (trimethoprim), a tetracycline (oxytetracycline), a fluoroquinolone (enrofloxacin) and β -lactam (penicillin G/procaine) in highly complex wastewater matrix. The method involves sample pre-treatment by solid-phase extraction (SPE) and analytical determination by HPLC coupled with diode array detector (DAD). The described method has been applied to the determination of the selected pharmaceuticals in wastewater from pharmaceutical industry.

2. Experimental

2.1. Pharmaceutical standards and reagents

The pharmaceuticals studied were: trimethoprim (TMP), oxytetracycline (OTC), enrofloxacin (ENRO), sulfaguanidine (SGUA), sulfamethazine (SMETH), sulfadiazine (SDIAZ) and penicillin G/procaine (PGP). All pharmaceutical standards were of analytical grade (>99%) and obtained from Veterina Animal Health Ltd. (Kalinovica, Croatia). The chemical structures of the pharmaceuticals included in this study are shown in Fig. 1.

Stock solution of pharmaceutical mixture was prepared in methanol and stored protected from light at 4 °C. Mass concentration of each pharmaceutical standard in the mixture was 10 mg/L, except ENRO 1 mg/L. The working standard solutions were prepared from this stock by serial dilution. All used solvents were HPLC-grade supplied by Kemika (Zagreb, Croatia).

2.2. Sample preparation and extraction

Water samples were collected in amber glass bottles. Prior to extraction, wellspring water and wastewater used in this study were filtered with Whatman filter paper to eliminate the suspended matter and then filtered with the 0.45 μ m nylon membrane filter. The samples were stored at 4 °C until SPE extraction, which was performed within 24 h in order to avoid any degradation. The spiked wellspring water samples were prepared from pre-filtered wellspring water by addition of 1 mL of working standard solutions of pharmaceuticals mixture to 100 mL of water. The studied pharmaceuticals were extracted from spiked water and wastewater samples and pre-concentrated on 60 mg Waters Oasis HLB cartridges (Waters, Milford, Massachusetts). Before sample application, the cartridges were pre-conditioned

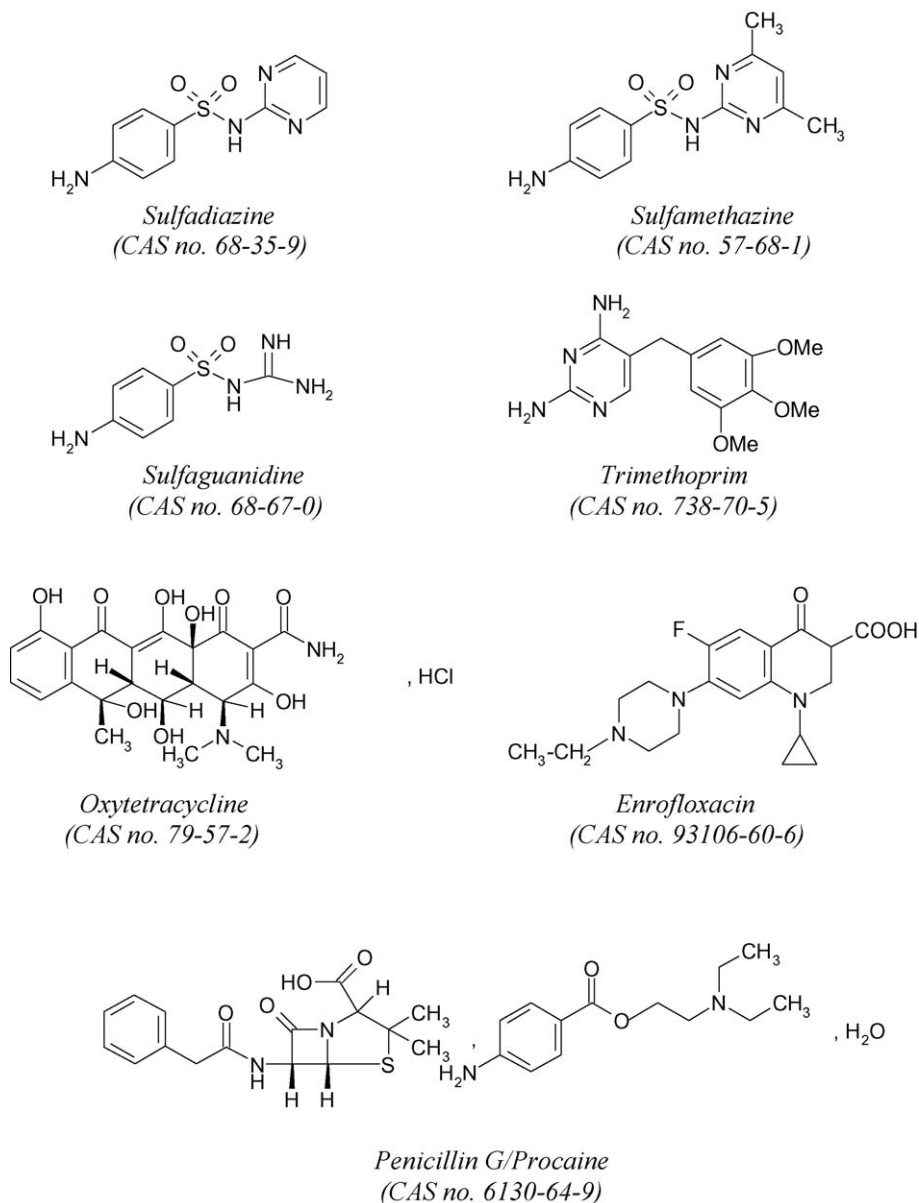


Fig. 1. Chemical structure of the studied pharmaceuticals.

with 5 mL of each methanol, and water. The pH of water for pre-conditioning step was adjusted to 4.0 with HCl. Sample volume of 100 mL was applied to the cartridge. The samples were extracted using a SPE apparatus (Supelco, Bellefonte, PA, USA) at the flow rate no greater than 4 mL/min. A wash step with 2 mL methanol (2%) was applied after the sample loading. The cartridge was air dried for about 5 min under vacuum to remove excess water. The analytes retained were eluted with 2×5 mL of methanol. Following elution, the filtrates were evaporated on a rotary evaporator at 40 °C to dryness and redissolved with 1 mL of methanol obtaining 100-fold preconcentration.

2.3. HPLC–DAD analysis

The LC analyses were performed using a Varian ProStar 500 (Walnut Creek, California, USA) HPLC system consisting of a

ProStar autosampler, ProStar 230 tertiary pump system, ProStar 330 diode array detector, and thermostatted column compartment. The column temperature was set to 30 °C and injection volume was 20 μ L. A LiChrosphere 100 CN, 125 mm $\times$ 4.0 mm, particle size 5 μ m column (Merck, Darmstadt, Germany) was used to separate all compounds in the mixture.

The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (0.01 M oxalic acid) and B (acetonitrile). Simultaneous mobile phase gradient program and flow rate gradient program was used: the elution started with 100% of A which was maintained for 6 min, followed by a 19-min linear gradient to 50% of eluent B and 5-min linear gradient back to 100% of A. Flow rate gradient started with 1.0 mL/min which was maintained for 6 min, followed by a 19-min linear gradient to 0.8 mL/min and 5-min linear gradient back to 1 mL/min. The investigated pharmaceuticals were eluted

within 20 min. A 30-min post time allowed re-equilibration of the column. The separation was monitored at absorbance wavelength of 280 nm.

3. Results and discussion

3.1. SPE–HPLC–DAD

Chromatographic separation was achieved on CN modified chromatographic column. Improved resolution of different components was achieved by manipulating the solvent and additive composition (oxalic acid, acetonitrile and methanol were used), the volume proportion of solvents in mobile phase and the concentration of oxalic acid. Acetonitrile and 0.01 M oxalic acid were selected. Further improvement in separation was obtained by mobile phase gradient and flow rate gradient. Experimental conditions selected enable separation in 20 min.

The critical step in method development is the sample preparation procedure, especially since many pharmaceuticals have amphoteric properties. Literature refers to SPE reverse phase but amphoteric pharmaceuticals bind strongly to free silanol groups, and they cannot be eluted with standard organic solvents. To overcome such problems resulting from amphoteric nature of many pharmaceuticals, new functionalized SPE materials that separate analytes by their hydrophobic and polar properties, e.g. hydrophilic–lipophilic balance cartridges (HLB), appear to be advantageous [22].

The structures of the investigated drugs are shown in Fig. 1. As can be seen from the figure, all sulfonamides investigated have at least two nitrogen functions. The amide attached to the sulfur is referred to as N¹ and is deprotonated at pH 6.5–7.4 (except for sulfaguanidine 11.3). The amine attached to the aromatic cycle is referred to as N⁴ and is protonated at pH 2.5. For this reason, sulfonamides are positively charged at acidic conditions, are neutral between pH 2.5 and 6 and negatively charged at alkaline conditions (see also Fig. 1). Trimethoprim is, also, characterized by two pK_a values corresponding to protonation of the two heterocyclic nitrogen atoms (N¹ and N³) [23]. Fluoroquinolones (ENRO) are cationic in acidic conditions (pK_{a1} = 5.86, pK_{a2} = 8.24) because of deprotonation of the carboxylic acid group and protonation of the piperazinyl amino group (N⁴) [17]. Tetracyclines are cationic at pH less than 3 and as zwitterion above this pH [24]. Since sulfonamides and tetracyclines are amphoteric and tetracycline antibiotics are unstable under strong acid conditions [25] pH of the sample solution is one of the most important parameter to control. Beside pH con-

trol, various organic solvents as eluents were tested (acetonitrile, methanol, and ethanol). On the basis of these preliminary investigations, pH 4 of water samples was chosen as the optimum. In all experiments following pharmaceuticals were extracted from water samples at pH 4.0 using Waters Oasis (HLB) cartridges with methanol as extraction eluent. The extraction efficiency was checked by recovery experiments.

3.2. Method validation

The performance characteristics of the SPE–HPLC method were established by validation procedure with spiked water samples. Selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision and recovery were studied.

For qualitative purpose, the method was evaluated by taking into account the precision in the retention factor (*t_R*-value) and selectivity. A high repeatability in the retention was obtained with R.S.D. values lower than 1.5%. Optimization of the chromatographic separation enabled an efficient separation of pharmaceuticals. The absence of false positive results for all sample blanks and good separation ensure that the signal measured is not influenced by other substances (Fig. 2). These were considered acceptable for selectivity.

Linearity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery were evaluated for quantitative purpose. The linearity was evaluated for each pharmaceutical in the mixture using five concentrations in the range of 1.5–1000 µg/L depending on the pharmaceutical. Calibration curves were prepared for each compound from the spiked water by plotting the peak area versus the analyte concentration. Blanks were also prepared as quality control tool, but not included in regression analysis. The amount of extracted pharmaceuticals was determined by HPLC. The results were analyzed by linear regression method. Coefficients of correlation were higher than 0.9989 thus confirming the linearity of the method. (Table 1.)

Limits of detection (LOD) and quantification (LOQ) were experimentally estimated from the injection of standard solution serially diluted until the signal-to-noise ratio for any single analyte reached a value of ten for LOQ and three for LOD. The limits of detection and quantification are reported in Table 1.

To evaluate the intra- and inter-day repeatability of the method in terms of precision, 100 mL of wellspring water samples were spiked with stock solution of pharmaceutical mixture (10 mg/L each pharmaceutical; ENRO 1 mg/L) extracted with the Oasis HLB cartridges and analysed following the proposed methodology. Intra-day repeatability was determined by analysing three replicates of spiked water samples using same equipment and same analytical procedure in 1 day. Intra-day repeatability was expressed as relative standard deviation (R.S.D.). The results of the intra-day precision experiments are summarised in Table 2. Satisfactory results were achieved for all pharmaceuticals with R.S.D.s lower than 8.4% indicating high measurement repeatability of the SPE–HPLC method. Inter-day reproducibility was evaluated analysing three replicate samples on three different days. As it is shown in Table 2, the R.S.D.s were between 3.3 and 10.1%.

Table 1
Parameters of quantitative determination of pharmaceuticals by SPE–HPLC

Pharmaceutical	<i>r</i> ²	Range (µg/L)	LOD (µg/L)	LOQ (µg/L)
Sulfaguanidine	1.0000	60–1000	40	60
Sulfadiazine	0.9993	20–500	10	20
Sulfamethazine	0.9992	20–500	10	20
Oxytetracycline	0.9989	30–500	10	30
Penicillin G/procaine	0.9989	100–500	30	100
Trimethoprim	0.9991	30–500	10	30
Enrofloxacin	0.9992	1.5–25	0.1	1.5

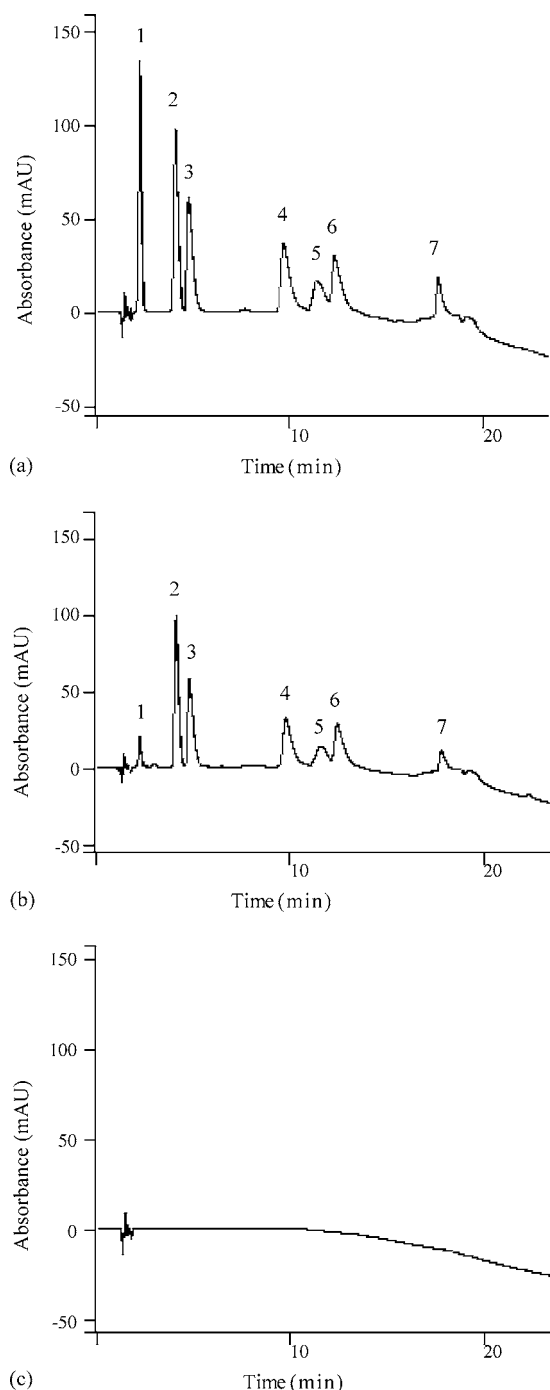


Fig. 2. Chromatograms of: (a) standard pharmaceutical mixture, (b) extracted sample from spiked water and (c) blank extract; 1-SGUA, 2-SDIAZ, 3-SMETH, 4-OTC, 5-PGP, 6-TMP and 7-ENRO.

The recoveries of the analytes from spiked water samples were evaluated by the ratio of the response (peak area) obtained from measurements of extracts to response of the corresponding standard solution. Recoveries were expressed by its average value of three determinations and values between 89.3% and 97.9% were obtained, excepted penicillin G/procaine (68.3%) and sulfaguandine (11.2%). This fact introduces high uncertainties in quantitation of penicillin G/procaine and sulfaguandine at very low concentrations but can allow the determination of their presence or absence. Recovery of sulfaguandine was poor

Table 2

Intra- and inter-day precision

Pharmaceutical	Intra-day precision		Inter-day precision	
	Recovery (%)	R.S.D. (n = 3)	Recovery (%)	R.S.D. (n = 3)
Sulfaguandine	11.2	7.4	11.3	8.9
Sulfadiazine	93.0	1.1	93.1	3.3
Sulfamethazine	94.5	5.2	94.7	7.1
Oxytetracycline	89.3	8.4	89.5	10.1
Penicillin G/ procaine	68.3	6.4	68.4	8.7
Trimethoprim	97.9	2.0	97.9	3.9
Enrofloxacin	95.6	7.5	95.7	9.7

because of low breakthrough volume. Breakthrough was tested by spectrophotometric determination with 3-aminophenol [26]. 3-aminophenol solution was added to filtrate and orange-yellow azo product was formed. This experiment indicates sulfaguandine in filtrate e.g., Oasis HLB extraction material retained sulfaguandine weaker than other drugs investigated. As a consequence of low recovery, limits of detection and quantification for sulfaguandine and penicillin G/procaine were higher than for other studied pharmaceuticals.

3.3. Application to real samples

The described method was applied to the determination of sulfamethazine, sulfadiazine, sulfaguandine, trimethoprim, oxytetracycline, enrofloxacin and penicillin G/procaine in two

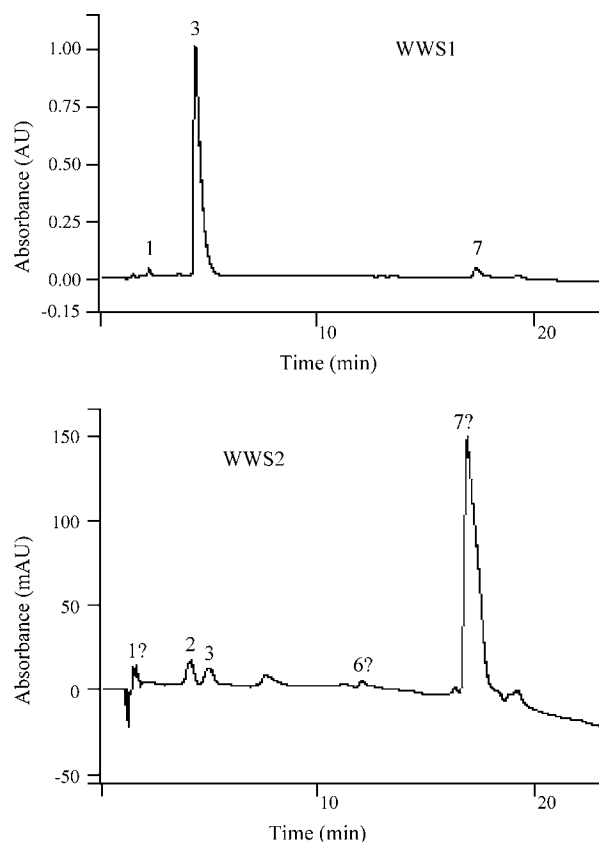
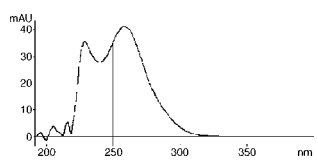
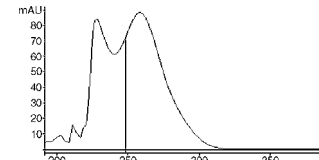
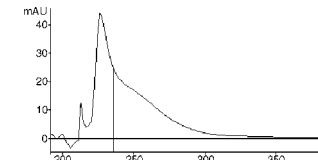
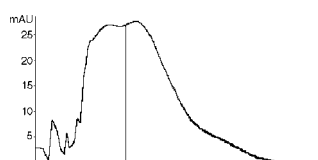
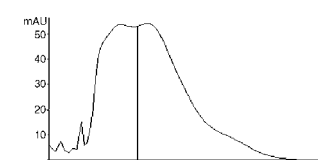
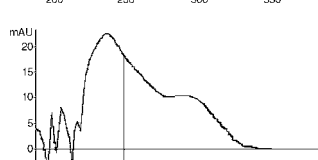
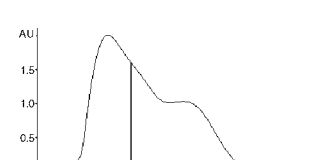
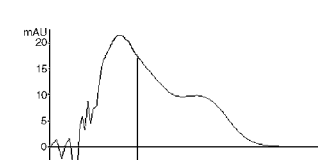
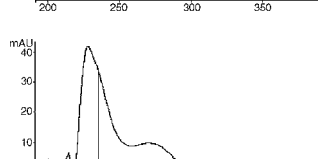
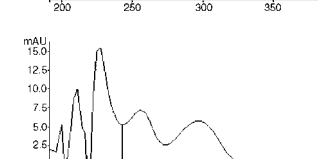
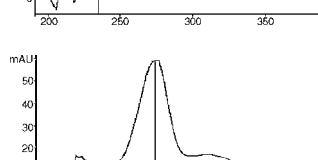
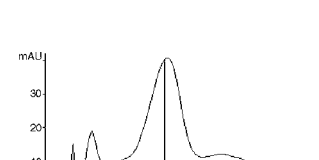
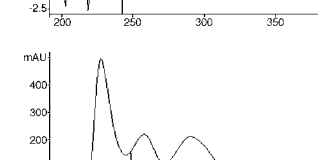


Fig. 3. HPLC–DAD chromatograms of wastewater samples; 1-SGUA, 2-SDIAZ, 3-SMETH, 4-OTC, 5-PGP, 6-TMP and 7-ENRO.

Table 3
Results of wastewater samples analysis

Pharmaceutical	Standards		WWS1			WWS2		
	t_R	UV spectra	t_R	UV spectra	γ ($\mu\text{g/L}$)	t_R	UV spectra	γ ($\mu\text{g/L}$)
Sulfaguanidine	2.187		2.177		211.1	2.143		— ^a
Sulfadiazine	4.017		N.D.	—	—	4.082		111.4
Sulfamethazine	4.967		4.905		>500	4.920		107.1
Trimethoprim	12.567		N.D.	—	—	12.280		— ^a
Enrofloxacin	17.602		17.293		23.7	17.602		— ^a

^a Not quantified because the UV spectra do not match.

wastewater samples from pharmaceutical industry. Wastewater treatment consists of only a primary settlement and plant receive only industrial wastewater. Chemical synthesis processes of the veterinary pharmaceuticals industry produce wastewaters which are variable in character depending on production. Wastewater sample 2 (WWS2) was sampled one month after wastewater sample 1 (WWS1). Wastewater is slightly opalescent to turbid, colourless to gray or light yellow. Before analysis, wastewater samples were filtered (through Büchner funnel, Black Whatman filtering paper and 0.45 µm nylon filter) and acidified to pH 4. Hundred millilitres (100 mL) of thus prepared wastewater samples were applied to Oasis HLB cartridges and filtrates were evaporated to dryness, residues were dissolved in 1 mL of methanol and injected to HPLC system. Chromatograms of wastewater samples obtained with diode array detector are shown in Fig. 3.

The factors used to confirm the presence of the target compounds in these samples were: retention time and UV spectrum. Peaks at the same retention time than SGUA, SMETH and ENRO and SGUA, SDIAZ, SMETH, TMP and ENRO appeared in the chromatograms of WWS1 and WWS2, respectively. In WWS2 only the presence of SDIAZ and SMETH was confirmed by the factors defined above because the UV-spectra of other compounds and pharmaceutical standards do not match. These peaks represent the unknown substances from matrix or peaks of investigated veterinary pharmaceuticals overlapped with the peak of unknowns. OTC and PGP were not detected in analyzed wastewater samples. The results of real sample analysis were summarized in Table 3.

4. Conclusions

A SPE followed by HPLC–DAD determination has been proposed for simultaneous analysis of the pharmaceutical compounds: sulfamethazine, sulfadiazine, sulfaguanidine, trimethoprim, oxytetracycline, enrofloxacin and penicillin G/procaine. The identification of the pharmaceuticals was possible correlating the retention times and UV spectra. The SPE procedures provide enrichment factor of 100-fold and acceptable recoveries, all above 68%, except for sulfaguanidine with 11.2% recovery. SPE–HPLC–DAD gave detection limits ranging from 0.1 to 40 µg/L enabling the determination of pharmaceuticals in highly loaded wastewaters. HPLC–DAD is an inexpensive analytical technique compared to HPLC–MS for routine analysis of pharmaceuticals in wastewater samples. This routine analysis of pharmaceuticals can be useful tool to know the amount of

pharmaceuticals discharged from WWTPs to the aquatic environment and to evaluate the effect of conventional WWTPs in the elimination of pharmaceutical compounds.

Acknowledgement

This work has been supported by EU project EMCO (INCO CT 2004–509188)—Reduction of environmental risks, posed by Emerging Contaminants, through advanced treatment of municipal and industrial wastes and Croatian Ministry of Education, Science and Sport (Project No. 0125055).

References

- [1] L.R. McDonald, E.L. Roberson, in: L.E. McDonald (Ed.), *Veterinary pharmacology and therapeutics*, Iowa State University Press, Ames-Iowa, 1991, pp. 897–902.
- [2] O.A.H. Jones, N. Voulvoulis, J.N. Lester, *Wat. Res.* 36 (2002) 5013.
- [3] T. Heberer, *Tox. Lett.* 131 (2002) 5.
- [4] K. Polestra, W.W. Bakker, P.A. Klok, J.A. Kamps, M.J. Hardonk, D.K. Mijer, *Am. J. Pathol.* 151 (4) (1997) 1163.
- [5] M. Carballa, F. Omil, J.M. Lema, M. Llompart, C. Garcia-Jares, I. Rodriguez, M. Gomez, T. Ternes, *Water. Res.* 38 (2004) 2918.
- [6] D. Daughton, T.A. Ternes, *Environ. Health Perspect.* 107 (6) (1999) 907.
- [7] W.M.A. Niessen, *J. Chromatogr. A* 812 (1998) 53.
- [8] S. Castiglioni, R. Bagnati, D. Calamari, R. Fanelli, E. Zuccato, *J. Chromatogr.* 1092 (2005) 206.
- [9] V.B. Reeves, *J. Chromatogr. B* 723 (1999) 127.
- [10] N. Takeda, Y. Akiyama, *J. Chromatogr. A* 607 (1992) 31.
- [11] P. Vinas, C.L. Erroz, N. Campillo, H.M. Cordoba, *J. Chromatogr. A* 726 (1996) 125.
- [12] S. Porter, *Analyst* 119 (1994) 2753.
- [13] T.M.A. Msagati, M.M. Nindi, *Talanta* 64 (2004) 87.
- [14] T. Reemtsma, *Trends Anal. Chem.* 20 (2001) 533.
- [15] M.D. Hernando, M. Petrović, A.R. Fernandez-Alba, D. Barcelo, *J. Chromatogr. A* 1046 (2004) 133.
- [16] S. Yang, J. Cha, K. Carlson, *J. Chromatogr. A* 1097 (2005) 40.
- [17] J.E. Renew, C.H. Huang, *J. Chromatogr. A* 1042 (2004) 113.
- [18] S. Diaz-Cruz, D. Barcelo, *Trends Anal. Chem.* 24 (7) (2005) 645.
- [19] S. Bogialli, R. Curini, A.D. Corcla, M. Nazzari, R. Samperi, *Anal. Chem.* 75 (2003) 1798.
- [20] I. Choma, *Chem. Anal.* 46 (2001) 1.
- [21] Z. Aksu, Ö. Tunç, *Process Biochem.* 40 (2005) 831.
- [22] D.W. Klopfin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, *Environ. Sci. Technol.* 36 (2002) 1202.
- [23] M.Y. Haller, S.R. Muller, C.S. McArdell, A.C. Alder, M.J.-F. Suter, *J. Chromatogr. A* 952 (2002) 111.
- [24] C.A. Anderson, H.S. Rupp, W.-H. Wu, *J. Chromatogr. A* 1075 (2005) 23.
- [25] Y. Wen, Y. Wang, Y.-Q. Feng, *Talanta* 70 (2006) 153.
- [26] P. Nagaraja, H.S. Yathirajan, C.R. Raju, R.A. Vasanth, P. Nagendra, M.S. Hemantha Kumar, *Il Farmaco* 58 (2003) 1295.

Preface

The First workshop of the European Union (EU) funded project EMCO (Reduction of environmental risks, posed by emerging contaminants, through advanced treatment of municipal and industrial wastes) entitled: analysis and removal of contaminants from wastewaters for the implementation of the water framework directive (WFD) was organized in Dubrovnik, Croatia in collaboration with the EU project SWIFT-WFD (*Screening methods for Water data InFormaTion in support of the implementation of the Water Framework Directive*), Faculty of Chemical Engineering and Technology of the University of Zagreb (Croatia), Consejo Superior de Investigaciones Científicas (CSIC, Barcelona) and Agilent Technologies. The workshop took place at the International Center of Croatian Universities during 20–21st of October 2005.

In the course of the workshop, 28 lectures and 20 posters were presented, being the principal topics legislation and policy in the field of wastewater treatment, analysis of contaminants in wastewaters, wastewater treatment technologies, risk assessment with special emphasis on the use and validation of the yeast assays for estrogenicity and the special session devoted to report the results of the first year of the EMCO project.

During the course of the workshop it was pointed out the urgent need for a European list of emerging contaminants, as possible candidates for the introduction into the WFD list of priority substances. This list is amendable for revision and addition of new contaminants each four years. Such addition or replacement of pollutants will be based on the results of present and future monitoring programs, like the one carried out under the EMCO projects, where a comprehensive list of pharmaceuticals has been monitored for the first time in Bosnia and Herzegovina, Croatia and the State Union of Serbia and Montenegro. Other emerging pollutants identified are personal-care products (PPCP), pesticides, and endocrine disruptors. The resulting combined pure qualitative list will represent an initial step towards a more detailed picture of wastewater treatment plant contamination. Other features are being added—concentrations, frequency of finding, physico-chemical, environmental and ecotoxicological properties.

Adequate monitoring and analytical concepts were pointed out to be necessary. Without appropriate tools it will not be possible to investigate fate and behaviour of emerging pollutants at the wastewater treatment plants and receiving waters. In this sense, analysis of organic pollutants in wastewater is complex basically due to variety of physico-chemical and toxicological properties of compounds included into the same group, like pharmaceuticals.

That is why there is a need to develop and accept when ready, standard procedures of chemical analysis and risk assessment of compounds, which is more urgent for emerging contaminants, together with inter-comparison studies in order to validate the analytical protocols. In this respect in Dubrovnik the results of the first European Union inter-laboratory study on endocrine disruption effect using a recombinant yeast assay (RYA) were reported indicating a good comparison level and the possibility to use RYA as a monitoring tool for the rapid identification of endocrine disruption the wastewater treatment plants.

This special issue of *Talanta* contains selected papers presented at the Workshop. It should be noticed that the papers presented at this conference were divided between two journals, *ABC*, and *Talanta*, so only few of them are included here. Finally we would like to thank the local organizers and our co-workers from the Department of Environmental Chemistry for their help when preparing this workshop. The next EMCO workshop will take place in April 2007 in Belgrade, Serbia. We are looking forward to meeting you there.

Damià Barceló*

Mira Petrovic

IIQAB-CSIC, Barcelona, Spain

* Corresponding author. Tel.: +34 93 400 61 00;
fax: +34 93 204 59 04.

E-mail address: dbcqam@iiqab.csic.es
(D. Barceló)

30 June 2006

Available online 9 August 2006

Selenium speciation in dill (*Anethum graveolens* L.) by ion pairing reversed phase and cation exchange HPLC with ICP-MS detection

Oktaý Cankur¹, Santha K.V. Yathavakilla, Joseph A. Caruso*

Department of Chemistry, McMicken College of Arts and Sciences, University of Cincinnati, 507A Rieveschl Hall,
P.O. Box 210172, Cincinnati, OH 45220, USA

Received 21 September 2005; received in revised form 25 January 2006; accepted 25 January 2006
Available online 9 March 2006

Abstract

In the present work, speciation of selenium in dill (*Anethum graveolens* L.), supplemented with sodium selenite during its growth, was performed using ion pairing reversed phase and cation exchange chromatography. Heptafluorobutyric acid (HFBA) was used as the ion-pairing agent in reversed phase chromatography. In cation exchange chromatography, two different gradient programs were employed for the identification of selenospecies using pyridinium formate as the mobile phase. Low molecular weight selenocompounds were extracted from root, stem and dill leaf with 0.1 M HCl. Enzymatic digestion was used for the extraction of selenospecies related to high molecular weight compounds. The chromatograms obtained from different parts of the plant revealed major differences in the type of selenospecies as well as their concentrations. The major selenospecies found in different parts of the plant is *Se*-methyl-selenocysteine (MeSeCys). Another major *Se* species identified is *Se*-methyl-selenomethionine (MeSeMet), which has the highest relative concentration in the root indicating possible *Se* volatilization from that part of the plant. Selenomethionine (SeMet) is present in minor quantities in all parts of the plant.

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Keywords: Dill (*Anethum graveolens*); IP-RP-HPLC; SCX-HPLC; ICP-MS

1. Introduction

Selenium is an essential trace element for humans and animals. Essentiality of *Se* was established in the observation of some deficiency symptoms, which were reduced after *Se* supplementation [1]. Although the metabolism and requirements of *Se* in the organisms are not yet completely understood, it is known to have both structural and enzymatic roles [2]. At least, 15 selenoproteins and/or selenoenzymes have been identified in biological systems [2,3]. There is strong evidence that *Se* or some of its compounds are effective against different types of cancer [4]. Clark et al. showed that dietary supplementation of 200 µg *Se* day⁻¹ is capable of decreasing the overall cancer morbidity and mortality by nearly 50% with certain tumors [5].

High doses of *Se* have been known to cause major health problems in livestock and humans for more than a century [1].

Therefore, *Se* contaminated soil, which is the primary source for *Se* to enter food chain, must be treated. Phytoremediation has been suggested as a low cost, environmentally friendly and efficient cleanup procedure of *Se* laden/polluted natural bodies [6]. Plants, especially *Se* accumulators, are very effective in removing *Se* from soil and water and metabolize it to volatile, relatively non-toxic selenium forms, such as dimethylselenide (DMS_e) [7]. Zayed and Terry [8] observed that volatilization of *Se* occurs primarily in the roots.

Coupling of inductively coupled plasma mass spectrometry (ICP-MS) and high performance liquid chromatography (HPLC) is an excellent technique for the speciation of *Se* and has been used successfully for a number of different sample types [9,10]. However, molecular information about the compounds is not provided by ICP-MS. Identification of species is achieved by retention time matching with available standards utilized in a standard addition mode.

Green leafy vegetables are good source of minerals as well as vitamins. Dill (*Anethum graveolens* L.) is a green leafy vegetable that belongs to the carrot family and has an attractive flavor. It has been used as a basic component in canning, soups and sauces

* Corresponding author. Tel.: +1 513 556 9306.

E-mail address: joseph.caruso@uc.edu (J.A. Caruso).

URL: <http://www.uc.edu/plasmachem/>.

¹ Present address: University of Ankara, Turkey.

and also flavoring salads and seafood [11,12]. Recently, it has been reported that it is a potential source of antioxidant and also has anti-microbial properties [13]. Due to various above-stated applications of dill, it would be interesting to investigate the selenium species in this plant. The purpose of this study is to investigate selenium species in different parts of dill (*A. graveolens* L.) that is grown in a controlled environment and supplemented with Se in the form of sodium selenite. The current study involves separation of low molecular weight selenium species using ion pairing reversed phase chromatography and cation exchange chromatography. Although there are a few studies on the determination of total Se in dill [14,15] to the best of our knowledge, Se speciation in this plant has not yet been reported.

2. Experimental

2.1. Instrumentation

Chromatographic separation was achieved using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary HPLC pump, a vacuum degasser, an autosampler and a multi wavelength UV detector. The system was coupled to an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan). The ICP-MS was equipped with the Agilent octapole reaction system and H_2 was used as reaction gas. The outlet of UV detector was connected to sample inlet of the ICP-MS nebulizer using a 0.25 mm i.d. polyether ether ketone (PEEK) tubing of 30 cm in length.

Alltima C8 column (Alltech Associates, Deerfield, IL, USA) (250 mm $\times$ 4.6 mm i.d. with 5 μ m particle size) and a Phenomenex (Torrance, CA, USA) silica-based strong cation exchange column (Phenosphere SCX, 150 mm $\times$ 3.2 mm i.d. with 5 μ m particle size) were used for separations.

Total digestions of samples were performed using a closed-vessel CEM discover-explorer microwave digestion system (CEM Corporation, Matthews, NC, USA). Flow injection analysis was employed for the determination of total Se and HPLC pump was used for pumping the carrier solution. A rheodyne injector with a 50 μ L sample loop was used for sample injection. A Sorvall centrifuge (Kenro Laboratory Products, Newtown, CT, USA) was used for the centrifugation of plant extracts.

2.2. Reagents

All reagents used throughout the experiments were of analytical grade. Deionized water (18 M Ω cm) was obtained from Barnstead deionization system (Sybron, Barnstead, Boston, MA, USA). The extractions were carried out using 0.1 M HCl prepared from 36% (w/w) HCl (Fischer Scientific, Fair Lawn, NJ, USA) by diluting with deionized water. Pyridinium formate buffer was prepared by dissolving required amount of pyridine (Fischer Scientific, Fair Lawn, NJ, USA) in water and adjusting pH of the resultant solution to 3.0 by addition of formic acid (J.T. Baker, Phillipsburg, NJ, USA). Methanol (Tedia Company, Fairfield, OH, USA) was added to this solution to give a final methanol concentration of 3% (v/v). This was used as

mobile phase in cation exchange chromatography. In reversed phase experiments, 0.15% (v/v) heptafluorobutyric acid (HFBA) (Sigma, St. Louis, MO, USA) containing 5% (v/v) methanol was used as mobile phase (pH 2.1).

Standard stock solutions were prepared from sodium selenite (ICN Biomedicals Inc., OH), sodium selenate (Aldrich, Milwaukee, MO), MeSeCys and SeMet (Sigma, St. Louis, MO) by dissolving in water and storing at -21°C . Working solutions were prepared from stock solutions by serial dilution with water to obtain a final concentration of 100 ppb. Since MeSeMet and trimethylselenonium iodide (TMeSe^+I^-) are not commercially available, they were synthesized in the laboratory according to the procedure described by Wrobel et al. [16].

2.3. Sample pretreatment

Dill samples, grown in pots, were purchased from a local market. They were kept untreated for the first 2 days and fed with DI water only. From third day onwards, the plants were supplemented with 100 mL of 10 mg L^{-1} selenite solution over a period of 2 weeks. A control sample was also grown under the same conditions as Se treated plants, but watered with DI water only. After harvesting, the plants were washed several times with deionized water to remove the soil and other material from the surface and then separated into root, stem and leaf. They were ground manually under liquid N_2 using mortar and pestle and then stored at -21°C until extraction.

2.4. Total digestion

For determining total Se concentration, the samples were treated using suprapure HNO_3 (Pharmaco, Hartford, CT, USA) and microwave digested. A 0.050 g of different plant compartments (root, stem and leaf) was weighed and placed in a glass digestion vessel with teflon lined caps supplied from CEM. After addition of 1.0 mL of concentrated HNO_3 to remove initially formed gaseous products, the vials were kept uncapped for 0.5 h, capped, and placed in the digestion system. The digestion program involved three steps. In the first step, the temperature was increased to 80°C in 10 min, which was increased to 90°C in 10 min in the second step. Finally in the third step, the temperature was maintained at 90°C for 30 min. During all these steps, a pressure below 250 psi was maintained. The clear digests were diluted with deionized water for the ICP-MS measurements.

2.5. Extraction of Se species

During extraction, about 0.250 g of each of different plant parts were placed into glass vials and 3.0 mL of 0.1 M HCl was added. In the enzymatic extraction, 0.125 g of root was combined with 1.5 mL of deionized water and 0.020 g of Protease XIV. The mixtures of both acid and enzymatic hydrolysis were stirred for 24 h at room temperature. During extraction, the vials were closed to prevent contamination and/or evaporation of extraction solution. The solutions were then centrifuged at 5000 rpm and the supernatant solution was transferred to 1.5 mL eppendorf tubes and centrifuged at 12,000 rpm for 20 min. The centrifugation step was repeated until no observable particulate material

was present at the bottom of the vial and the clear supernatant was used as sample.

2.6. Procedure

HNO₃ digests and 0.1 M HCl extracts of various plant compartments were analyzed for Se concentration by flow injection analysis. DI water was used as the carrier solution at a flow rate of 1.0 mL min⁻¹.

The experimental conditions for ICP-MS and chromatographic separation are given in Table 1. Collision/reaction cell was used to allow Se monitoring at $m/z=80$. H₂ was used as the reaction gas at a flow rate of 4.0 mL min⁻¹. Two different gradient programs were used during the cation exchange (SCX-HPLC) experiments. While the first program consists of a step increase of pyridine concentration from 0.75 mM to 8.0 mM, the second program uses gradient elution using pyridinium at 5.6–50 mM concentrations as given in Table 1. In ion pairing reverse phase (IP-RP-HPLC) experiments, isocratic elution was employed with a mobile phase flow rate of 0.8 mL min⁻¹.

3. Results and discussion

3.1. Total elemental analysis

Se supplemented dill plants produced 10–15% less overall yield than the plants that were left untreated. All the plant compartments (leaf, stem and root) were analyzed separately for total Se. Owing to substantial matrix affect, standard addition method was used to determine the total Se content in the plant.

The results from total digest analysis are given in Table 2. The highest Se concentration was found in root (31.8 µg g⁻¹). The total concentration of Se in stem and leaf were found to be 9.3 µg g⁻¹ and 10.3 µg g⁻¹, respectively. The concentration of Se in control plants was found to be less than 0.3 µg g⁻¹. Therefore, significant Se accumulation occurs when dill plants are enriched with Se. Plant samples were not lyophilized and the results are based on their fresh wet weight.

The HCl extracts were also analyzed for their total Se content in order to evaluate the extraction efficiency. The extraction efficiencies in Table 2 were calculated by dividing the extractable Se concentration (with 0.1 M HCl) by the total concentration of Se in each part. As given in the table, the extraction efficiencies are 31%, 35% and 33% for root, stem and leaf, respectively. These low extraction efficiency values indicate that about 65–70% of total Se in each part of dill cannot be extracted with 0.1 M HCl.

Table 1
Experimental parameters

ICP-MS parameters		
Power	1300 W	
Plasma Ar flow rate	15 L min ⁻¹	
Carrier Ar flow rate	1.16 L min ⁻¹	
Isotopes monitored	Se: 76, 77, 78, 80, 82	
Reaction gas and the flow rate	H ₂ , 4.0 mL min ⁻¹	
Temperature of spray chamber	2 °C	
Dwell time for each isotope	0.1 s	
SCX-HPLC (gradient elution)		
Column	Phenomenex SCX (150 mm × 4.6 mm, 5 μm)	
Mobile phase (Program 1)	(A) 0.75 mM pyridinium formate, 3% (v/v) methanol, pH 3.0 (B) 8.0 mM pyridinium formate, 3% (v/v) methanol, pH 3.0	
Program 1	0–7	100% A
	7–7.1	100% A–100% B
	7.1–28	100% B
	28–28.1	100% B–100% A
	28.1–45	100% A
Mobile phase (Program 2)	(A) 5.6 mM pyridinium formate, 3% (v/v) methanol, pH 3.0 (B) 50 mM pyridinium formate, 3% (v/v) methanol, pH 3.0	
Program 2	0–5	100% A
	5–30	100% A–100% B
	30–35	100% B
	35–45	100% B–100% A
	45–60	100% A
Flow rate	1.0 ml min ⁻¹	
Injection volume	100 μl	
IP-RP-HPLC (isocratic elution)		
Column	Alltech Alltima C8 (250 mm × 4.6 mm, 5 μm)	
Mobile phase	0.15% (v/v) heptafluorobutyric acid, 5% (v/v) methanol, pH 2.1	
Flow rate	0.8 ml min ⁻¹	
Injection volume	50 μl	

To investigate whether these non-extractable species are high molecular weight compounds, enzymatic extraction was applied to dill root. However, enzymatic extract was not analyzed for total Se content. A comparison of total chromatographic peak area of all Se species obtained by enzymatic extraction of dill root was three times higher than that obtained by acid extract, suggesting higher extraction efficiency from roots by enzymatic method. However, this observation is based on the assumption

Table 2
Total and extractable (with 0.1 M HCl) concentration of selenium in dill root, stem and leaf, and the extraction efficiencies

Part of the plant	Total Se concentration (µg g ⁻¹)		Extractable concentration ^a (µg g ⁻¹)	Extraction efficiency (%)
	Supplemented	Control		
Root	31.8 ± 1.3	0.17 ± 0.05	9.8 ± 0.5	31
Stem	9.4 ± 0.5	0.25 ± 0.04	3.3 ± 0.3	35
Leaf	10.3 ± 0.4	0.26 ± 0.04	3.4 ± 0.3	33

^a 0.25 g of sample was extracted with 0.1 M HCl by magnetic stirrer for 24 h at room temperature.

of similar chromatographic recoveries for species extracted by both methods.

3.2. Chromatographic separation of selenospecies

Selenium in the plants may be present as either inorganic or organic Se compounds and these may incorporate into high molecular weight compounds such as proteins. Terry et al. [7] suggested a Se pathway in higher plants. After absorption by roots, Se is metabolized by the plant to form organoselenium compounds, which are either accumulated or volatilized. In the current study, presence of selenospecies in dill compartments was confirmed by reversed phase and cation exchange chromatography.

In speciation analysis, the conservation of species in their original form is important. To minimize loss of molecular identity of species, samples were extracted just 1 day after harvesting and maintained at -21°C until analysis. The extracted solutions thus stored, showed no significant change in the chromatographic peak intensities for a period of 4 weeks.

3.3. IP-RP-HPLC-ICP-MS

The chromatographic separation of selenium species by IP-RP-HPLC has been widely used for Se studies. It has been used successfully for Se speciation analysis in plant samples [9,17]. Since selenoamino acids are too hydrophilic to be retained on typical reversed phase columns such as C8 or C18, separation can only be achieved by using ion-pairing agents, which result in stronger retention [18,19]. Perfluorinated carboxylic acids, like HFBA, give better resolution for different classes of Se compounds [19]. Furthermore, it has been suggested that HFBA allows separation of additional organoselenium species when compared to trifluoroacetic acid (TFA) and pentafluoropropanoic acid (PFPA) [20]. Hence, in this study, 0.15% (v/v) HFBA was selected as the ion-pairing agent for the separation of Se species extracted from different compartments of dill.

The IP-RP-HPLC chromatogram obtained from the solution containing seven Se standards is given in Fig. 1. Baseline resolution was achieved for all standards except inorganic selenium species Se(IV) and Se(VI) which elute close to the dead volume. However, the resolution for these two inorganic selenium compounds was adequate for qualitative purposes. During the preliminary experiments, it was observed that concentration of MeOH in the mobile phase had significant effect on the signal shape of MeSeCys. When 10% (v/v) MeOH was used in the mobile phase, signal obtained from aqueous MeSeCys standard was very broad. This observation was independent of the concentration of MeSeCys injected. Instead, when 3% (v/v) MeOH was used, higher sensitivity and better resolution were obtained with some increase in analysis time (figures not included). Use of 5% (v/v) MeOH in the mobile phase provided adequate sensitivity and resolution for the elution of aqueous standard solutions as depicted in Fig. 1. Hence, 5% (v/v) MeOH was used for all chromatographic runs.

The chromatograms obtained from different parts of plant extracts (root, stem and leaf) are given in Fig. 2. These chro-

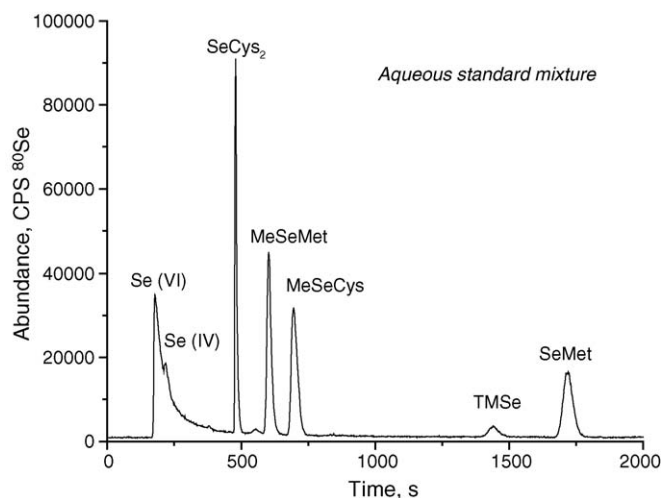


Fig. 1. The chromatogram of aqueous selenium standards by IP-RP-HPLC.

matograms were obtained by diluting the extracts with water at a ratio of 80:20 (extract: DI water). Comparison of the chromatograms for each plant compartment reveals major differences in both the number of species present in each part and their relative concentrations. When the total area of the chromatograms were compared, it was clear that most of the accumulation took place in the root, followed by leaf and stem. The total chromatographic area values of leaf and stem extracts relative to that of root were found to be 0.59 and 0.44, respectively. The ratios of these values are different from their corresponding total Se concentration ratios in the extracts (Table 2). This may be because of the fact that all the species extracted by 0.1 M HCl might not be eluted from the column.

Identification of selenospecies present in the sample extracts was based on the retention time matching with available standards followed by standard addition method. The species that eluted at 730 s (Fig. 2) was identified as MeSeCys, which was the major species in both root and stem with relative percent area

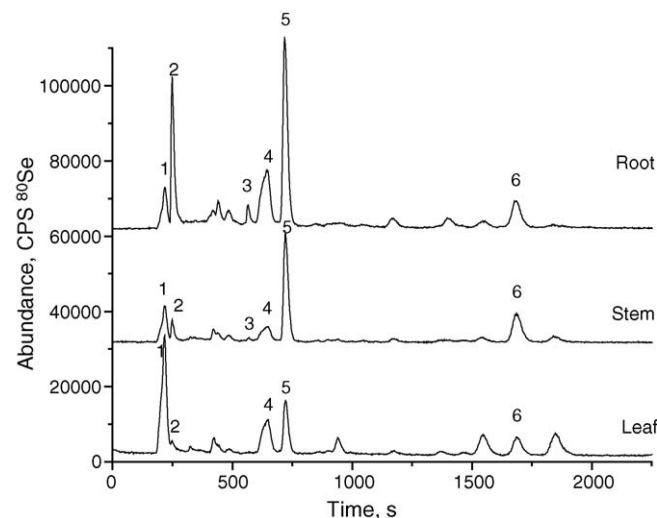


Fig. 2. IP-RP-HPLC-ICP-MS chromatograms of extracts of leaves, roots and stems of dill: (1) Se(VI); (2) Se(IV); (3) SeCys₂; (4) MeSeMet; (5) MeSeCys; (6) SeMet.

values of 32% and 38%, respectively. In leaf extracts, MeSeCys peak corresponds to only 13% of the total chromatographic area. The highest concentration of MeSeCys (250 ng mL^{-1}) was found in root extracts, by standard addition method, followed by stem and then leaf. However, it must be noted that all extractions were carried out with fresh wet samples. In plants, MeSeCys is synthesized from SeCys (produced from glutathione) by selenocysteine methyl transferase enzyme [7]. The percentage of MeSeCys in leaf extracts of Indian mustard (*Brassica juncea*), one of the most studied plants in Se speciation, was also found to be 13% when treated with selenite [9,21]. In the same report when genetically modified plants were studied, an increase in this value to 80% was observed. Similar genetic modifications may be applied to increase the percentage of this species in dill. This may be desirable, since MeSeCys is thought to be a precursor to Se molecules, which show anti-oxidation activity.

The percent area of SeMet in stem extract was calculated to be 19% of the total chromatographic area, which is more than twice as much present in root and leaf extracts (8.6% and 8.2%, respectively). The concentrations of SeMet in root, stem and leaf extracts determined by standard addition method were found to be close to each other, $\sim 80 \text{ ng mL}^{-1}$. To investigate whether SeMet is incorporated in proteins, enzymatic hydrolysis with non-specific enzyme, Protease XIV which can act along the whole protein structure [10,22] was performed. Fig. 3 shows chromatogram obtained from enzymatic extracts of dill root. As seen in this figure, the number of peaks, particularly in the first 10 min increased. Furthermore, the chromatographic peak area of SeMet from enzymatic extraction was approximately 12 times higher than that of 0.1 M HCl extract (compare Fig. 3). This indicates presence of high molecular weight compounds that contain SeMet. Enzymatic hydrolysis yielded a selenospecies that eluted at 1375 s in IP-RP-HPLC chromatogram in Fig. 3, which did not match any of the commercially available standards. However, MeSeCys could not be extracted by enzymatic hydrolysis, which maybe due to its conversion to other forms under the current extraction conditions.

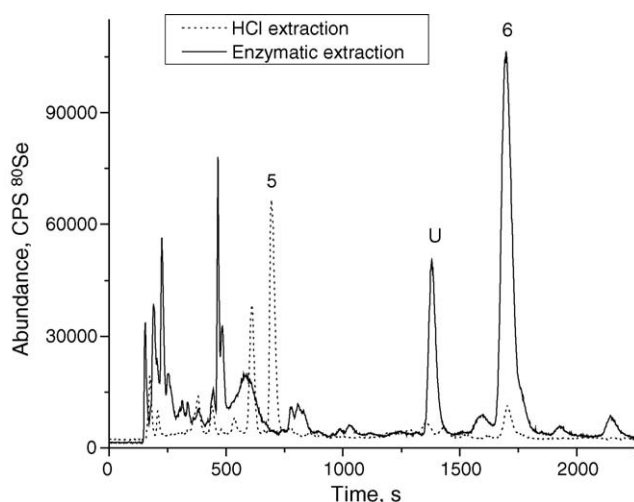


Fig. 3. IP-RP-HPLC-ICP-MS chromatograms of HCl and enzymatic extraction of dill root: (5) MeSeCys; (6) SeMet; (U) unknown.

MeSeMet (eluted at 650 s in the IP-RP-HPLC chromatogram given in Fig. 2) was found at higher concentrations in dill root compared to other plant compartments. The concentration of MeSeMet was found to be highest in root extracts, while the lowest concentration was observed in stem extracts. The peak area values of MeSeMet relative to root, in stem and leaf extracts were 0.27 and 0.63, respectively. MeSeMet, produced by methylation of SeMet, is considered precursor of volatile selenospecies [7]. The presence of volatile selenospecies such as DMSe and DMDSe was reported in *B. juncea*, where the presence of MeSeMet was also confirmed [9,17]. It has been reported that most of the volatilization took place in the roots [8]. Since the highest concentration of MeSeMet was observed in root compartment, dill may have the potential to volatilize Se. Further studies are required to determine volatile Se species from this plant.

Other species present in dill root extracts is SeCys₂, which elutes at 550 s in IP-RP-HPLC (Fig. 2). Although the retention time of SeCys₂ in RP chromatogram of mixed standard is less than 500 s (Fig. 1), spiking experiments with the standard (figure not included) showed that the species eluted at 550 s on RP chromatogram in Fig. 2 is SeCys₂. The sample matrix affected retention time of SeCys₂ considerably, hence spiking experiments were needed for accurate identification. The percent area corresponding to SeCys₂ peak in dill root extract is less than 3% of the total chromatographic area. Its concentration was much lower in the stem and no detectable signal could be obtained from leaf extracts.

The IP-RP chromatograms obtained from extracts showed that two inorganic Se species, Se(IV), whose concentration decreased from root to leaf, while that of Se(VI) showed opposite trend. The only plant compartment where their concentrations were close to each other was stem. Leaf is the only part where inorganic Se is the major Se species, primarily Se(VI). Total inorganic selenium content was lowest in stem. This is consistent with the study on *Brassica* by Zayed et al. [23] where Se(VI) was reported to be easily transported to shoots and leaves than Se(IV) since the latter is rapidly converted to organic selenospecies and retained in the roots.

Three peaks, with retention times at 950 s, 1550 s and 1850 s were detected in leaf extracts which were not present at detectable concentrations in root and stem. Identity of these peaks was not established, but will be investigated as this study expands.

3.4. SCX-HPLC-ICP-MS

Another method widely used for the separation of low molecular weight selenium compounds is cation exchange chromatography [24]. It was reported that cationic selenium species can be separated efficiently using pyridinium formate as the mobile phase [25]. In this study, cation exchange chromatography was used as a complementary technique to IP-RP-HPLC for Se speciation. Methanol (3%, v/v) was added to the mobile phase in order to increase sensitivity of ICP-MS [25]. Two different elution programs, Program 1 and Program 2 were used. The chromatograms obtained, for aqueous standard solution using Program 1 and

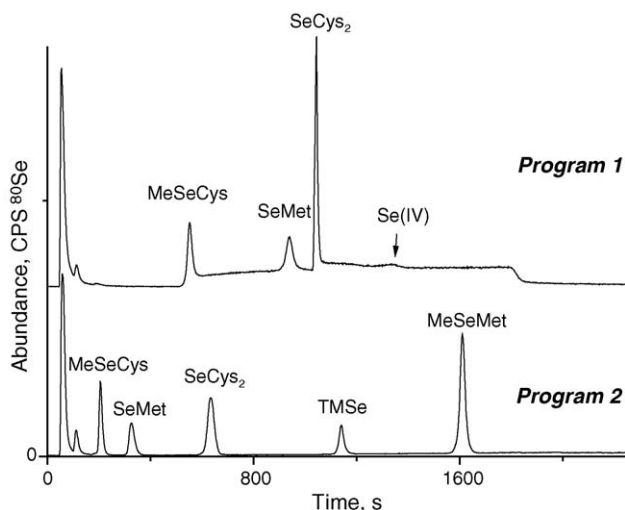


Fig. 4. Cation exchange chromatograms of aqueous standard solution using Program 1 and Program 2 (Table 1).

Program 2 (Table 1), are given in Fig. 4. Program 1 was not suitable for strongly retained cationic species like MeSeMet and TMeSeI, as they were not eluted in a reasonable period of time and dispersed in the column. Although baseline resolution was achieved with Program 2 for aqueous standards, including both early eluting and strongly retained selenospecies, it is not suitable for the identification of early eluting selenospecies such as SeMet and MeSeCys in the sample extracts, since the baseline in the first few minutes of the chromatogram increased due to heavy matrix (Fig. 5). These two programs were not combined in a single program since the difference in the concentrations of pyridinium formate in the starting and ending buffer solutions would have resulted in an unstable plasma conditions. The difference in the concentration of pyridine in the starting and the ending solution in a gradient program is usually an order of magnitude [25]. Therefore, the separation of early eluting

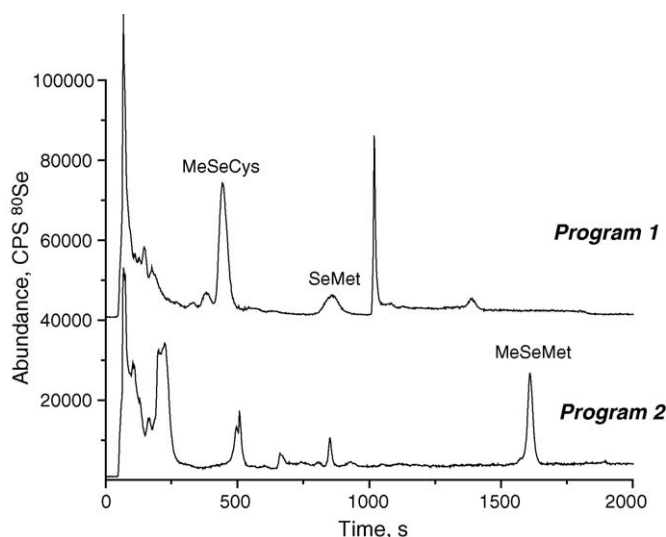


Fig. 5. Cation exchange chromatograms of dill root extract following Program 1 and Program 2 (Table 1).

species was done using Program 1 while Program 2 was used for later eluting species such as MeSeMet and TMeSeI.

For cation exchange chromatography, the root compartment was selected since most of the selenospecies present in the plant were present in this compartment at concentrations sufficient for identification. Furthermore, the concentration of MeSeMet, which overlaps with an unknown selenospecies in RP chromatography, was found to be highest in this compartment as concluded by IP-RP experiments. The presence of this species in root is important as it is the precursor of volatile selenospecies and also volatilization takes place at much higher rates than other compartments of plant. The cation exchange chromatogram for dill root extracted with 0.1 M HCl using Program 1 and Program 2 is given in Fig. 5. As shown in the figure, the presence of MeSeCys and SeMet was also confirmed by SCX chromatography using Program 1, while MeSeMet was identified using Program 2 (Fig. 5).

Cation exchange chromatography is not suitable for the separation of inorganic selenospecies, Se(IV) and Se(VI), as they are present as their oxyanions, SeO_3^{2-} and SeO_4^{2-} , respectively, which elute at the dead volume on SCX columns. Furthermore, at pH 3, selenite (SeO_3^{2-}), a conjugate base of weak selenous acid, is protonated. It was reported that when the pH of the solution is less than four, it is retained on SCX column and eluted as a dispersed plug [26]. As shown in Fig. 4, Se(VI) is eluted in the dead volume without any retention on the column, and Se(IV) is eluted as a broad peak at approximately 1350 s using Program 1.

4. Conclusions

Extraction is one of the critical steps in speciation analysis. Acid hydrolysis with 0.1 M HCl for 24 h at room temperature is found to be effective for the extraction of low molecular weight selenospecies from leaf, stem and root compartments of dill (*A. graveolens* L.). Enzymatic extraction resulted in the appearance of additional Se species, which were not observed in acid hydrolysis extracts. The speciation of selenium in plant samples is useful for the understanding the metabolic pathways in plant physiology as well as the determination of selenospecies, which are important for the prevention of some diseases. This is the first study on the speciation of selenium in dill. It has been found that when supplemented with sodium selenite, dill can accumulate and metabolize Se to some biologically and environmentally important species. The accumulation was mainly in the organic form in all parts of the plant. The major species found in all parts of the plant was MeSeCys, which has been suggested as a precursor of small molecular species that could be the possible active species in some types of cancer chemoprevention. Accumulation of this organic form may also be increased after genetic modification, which has been shown for Indian mustard [9]. Se supplemented dill plants can be used as a source of MeSeCys. Indeed, dill could be grown with the same effect in certain fertile parts of the western U.S., since the soil tends to be seleniferous. The presence of MeSeMet suggests that the plant could volatilize Se, since it is a precursor of DMSe, a volatile and less toxic selenium species. Like *B. juncea* [9], there is

higher amount of MeSeMet in the roots, where volatilization is believed to occur at higher rates compared to other compartments [8]. Taking into account that dill self-seeds readily, it may be used for phytoremediation purposes to clean up selenium contaminated soil. Further experiments to investigate the volatile species from this plant need to be conducted to assess the volatilization capability of the plant. There are unidentified species in leaf, which is the edible part of the plant, which are being further investigated.

Acknowledgements

Dr. Oktay Cankur would like to thank TUBITAK for financial support through NATO-B1 grant and University of Cincinnati for partial support for the extension of his stay. The authors are grateful to NIESH-SBRP for partial support by grant #ES04908. Agilent Technologies and CEM Corporation are recognized for their continuing support.

References

- [1] G.F. Combs, S.B. Combs, *The Role of Selenium in Nutrition*, Academic Press Inc., 1986.
- [2] M.P. Rayman, *Lancet* 356 (9225) (2000) 233.
- [3] Y. Ogra, K.I. Enicar, R. Lobinski, K.T. Suzuki, *Anal. Bioanal. Chem.* 379 (5–6) (2004) 861–866.
- [4] D.G. Barceloux, *J. Toxicol. Clin. Toxicol.* 37 (2) (1999) 145–172.
- [5] L.C. Clark, G.F. Combs, B.W. Turnbull, E.H. Slate, D.K. Chalker, K. Dan, J. Chow, L.S. Davis, R.A. Glover, G.F. Graham, E.G. Gross, A. Krongrad, J.L.J. Leshner, H.K. Park, B.B. Sanders Jr., C.L. Smith, J.R. Taylor, *J. Am. Med. Assoc.* 276 (24) (1996) 1957–1963.
- [6] N. Terry, A. Zayed, in: W.T. Frenkenberger Jr., R.A. E. (Eds.), *Environmental Chemistry of Selenium*, Frankberger, Marcell Dekker Inc., 1998, pp. 633–655.
- [7] N. Terry, A.M. Zayed, M.P.D. Souza, A.S. Tarun, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51 (2000) 401–432.
- [8] A.M. Zayed, N. Terry, *J. Plant Physiol.* 143 (1) (1994) 8–14.
- [9] T.D. Grant, M. Montes-Bayon, D. LeDuc, M.W. Fricke, N. Terry, J.A. Caruso, *J. Chromatogr. A* 1026 (1–2) (2004) 159–166.
- [10] S. Bird, P.C. Uden, J.F. Tyson, E. Block, E. Denoyer, *J. Anal. At. Spectrom.* 12 (7) (1997) 785–788.
- [11] W. Kmiecik, Z. Lisiewska, J. Slupski, *Food Chem.* 86 (2004) 105–111.
- [12] R. Huopalahti, R.R. Linko, *J. Agric. Food Chem.* 31 (1983) 331–333.
- [13] G. Singh, S. Maurya, M.P.D. Lampasona, C. Catalan, *J. Food Sci.* 70 (4) (2005) M208–M215.
- [14] M. Ozcan, *Food Chem.* 84 (3) (2004) 437–440.
- [15] V.D. Zheljazkov, P.R. Warman, *J. Agric. Food Chem.* 52 (9) (2004) 2615–2622.
- [16] K. Wrobel, S.S. Kannamkumarath, J.A. Caruso, *Anal. Bioanal. Chem.* 377 (4) (2003) 670–674.
- [17] M. Montes-Bayon, T.D. Grant, J. Meija, J.A. Caruso, *J. Anal. At. Spectrom.* 17 (2002) 1015–1023.
- [18] K.N. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, *J. Chromatogr. A* 833 (1999) 147–155.
- [19] P.C. Uden, H.T.B.C. Kahakachchi, J.F. Tyson, *J. Chromatogr. A* 1050 (1) (2004) 85–93.
- [20] M. Kotrebai, J.F. Tyson, E. Block, P.C. Uden, *J. Chromatogr. A* 866 (2000) 51–63.
- [21] M. Montes-Bayon, D.L. LeDuc, N. Terry, J.A. Caruso, *J. Anal. At. Spectrom.* 17 (2002) 872–879.
- [22] L.H. Reyes, F.M. Sanz, P.H. Espilez, J.M. Marchante-Gayon, J.I.G. Alonso, A. Sanz-Medel, *J. Anal. At. Spectrom.* 19 (2004) 1230–1235.
- [23] A. Zayed, C.M. Lytle, N. Terry, *Planta* 206 (1998) 284–292.
- [24] P.C. Uden, *Anal. Bioanal. Chem.* 373 (2002) 422–431.
- [25] E.H. Larsen, M. Hansen, T. Fan, M. Vahl, *J. Anal. At. Spectrom.* 16 (12) (2001) 1403–1408.
- [26] W. Goessler, D. Kuehnelt, C. Schlagenhufen, K. Kalcher, M. Abegaz, K.J. Irgolic, *J. Chromatogr. A* 789 (1–2) (1997) 233–245.

Improvement in LC–MS calibration by addition of fragment signals

L.F. Capitán-Vallvey^a, A. Ariza^b, A. Gonzalez-Casado^a, R. Checa^b, N. Navas^{a,*}

^a *Department of Analytical Chemistry, University of Granada, C/Fuentenueva s/n, Granada E-18071, Spain*

^b *Laboratorio Central de Sanidad Animal, Ministerio de Agricultura, Pesca y Alimentación, Camino del Jau s/n, Santa Fe, Granada E-18320, Spain*

Received 27 October 2005; received in revised form 8 May 2006; accepted 17 May 2006

Available online 27 June 2006

Abstract

Liquid chromatography–mass spectrometry has become a powerful analytical tool, with high selectivity and sensitivity. Usually in this technique, the calibration function is estimated from the molecular peak signal. This report describes the improvement in sensitivity when the signals from several fragments in addition to the molecular peak are used to establish the calibration function. The influence of the dwell time has also been analysed as an important instrumental parameter that influences the signal range, and consequently, the sensitivity. The calibration function obtained by adding fragment signals was used to estimate the instrumental detection limit using three different procedures, comparing and discussing the results obtained.

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Keywords: LC–MS; Calibration function; Addition of fragments; Chemometrics; NSAID

1. Introduction

Liquid chromatography–mass spectrometry (LC–MS) is an increasingly important analytical technique. Its development over the last 15 years is related to the development of key ionisation techniques, such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) [1]. Although ESI and APCI normally give little or no fragmentation, modern LC–MS instruments have methods for inducing fragmentation prior to mass separation. Consequently, LC–MS is currently as popular as Gas chromatography–mass spectrometry for quantification and identification [2]. But the LC–MS spectra are not unique for a molecule because the ionisation energy is not constant, making this an instrumental parameter to optimise. Fragmentation largely depends on the geometric configuration of the LC–MS interface and on the chromatographic conditions used. The mass spectra obtained differ with the nature of the eluent and the MS conditions [3]. In practice, when the ionisation energies are the same, LC–MS mass spectra are laboratory and instrument specific [4]. For this reason, the spectrum libraries have not yet been satisfactorily established and different approaches have been proposed [5,6].

This problem has also contributed to the fact that the calibration functions of the LC–MS methods are usually estimated from the base peak in the spectrum, this being the molecular peak in most situations. Nowadays, the intra-laboratory reproducibility of LC–MS spectra can be tested and corroborated, so they can be used for different purposes.

This study, by applying chemometrics tools, examines the possibility of using more than one characteristic fragment from the mass spectrum in order to obtain the calibration functions of the analytical methods with the goal of obtaining better sensitivities. Three non steroidal anti-inflammatory drugs (NSAIDs) were selected to carry out the study: flufenamic acid (FFA) [*N*-(α,α,α -trifluoro-*m*-tolyl) antranilic acid], diclofenac sodium (DIC) [sodium *o*-[(2,6-dichlorophenyl)amino]phenyl]acetate] and hydroxyflunixin (OH-FLU) [5-hidroxy,2-(2-methyl-3-trifluoromethylanilino) nicotinic acid]. Although these compounds belong to the same class of pharmaceutical drugs, they have different molecular structures which makes it possible to obtain different fragmentation patterns. For each compound, three characteristic and significant ions were selected for the study, with one always being the molecular peak. The other two fragments were characteristic fragments.

In addition, the influence of the dwell time, as an instrumental parameter that affects the sensitivity of the method, was also investigated, as well as different ways to estimate the detection limit, since this analytical parameter is calculated from the cali-

* Corresponding author. Tel.: +34 958243388; fax: +34 958243328.
E-mail address: natalia@ugr.es (N. Navas).

bration function. These studies were carried out always using a different number of characteristic ions from the mass spectra.

2. Experimental

2.1. Apparatus and software

The LC system was a 2695 Waters coupled with a Z-Spray Mass Detector Micromass Platform LCZ-4000 with an electrospray interface. The equipment was connected to a Pentium 200 fitted with a MassLynx workstation.

Software programs used for the statistical treatment of the data were the Statgraphics Plus 6.0 software package (Statistical Graphics System Corporation, USA, 1992) and Excel software package (Microsoft Office 97, ver. 8.0, 1997).

2.2. Reagents

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water (purified with a Milli-RO plus Milli-Q station from Millipore) and HPLC quality were used throughout. Methanol, acetonitrile, formic acid and ammonia were supplied by Panreac (Barcelona, Spain). Nitrogen gas (99.999% pure) for the mass spectrometer was purchased from Praxair (Málaga, Spain).

FFA was supplied from Sigma (Barcelona, Spain); DIC and OH-FLU were kindly supplied by the EU Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany).

An individual stock standard solution of every drug containing 1.0 g L^{-1} was prepared by dissolving each pure compound in methanol. These solutions were stored in dark bottles at 4°C and warmed to room temperature before use. Intermediate standard solutions of 10.0 and 1.0 mg L^{-1} were prepared by dilution with methanol and were stored in dark bottles at 4°C . The working standard solutions were prepared daily in vials by diluting the intermediate standard solutions in the mobile phase.

2.3. Chromatographic and mass detection procedure

Ten microliters of the sample was injected by the autosampler. Separation was performed on a C_{18} column $150 \text{ mm} \times 2.1 \text{ mm}$ i.d. and particle size $5 \mu\text{m}$ (Hypersil ODS, Agilent Technologies, Las Rozas, Madrid, Spain). A C_{18} pre-column $10 \text{ mm} \times 2.1 \text{ mm}$ i.d. and particle size $5 \mu\text{m}$ was used to protect the chromatographic column. A gradient was applied with aqueous ammonium acetate buffer 10.0 mM pH 5.5 (A) and acetonitrile (B); the gradient conditions were as follows: from 0 to 5 min, holding 30% B; from 5 to 15 min, ramps to 50% B and from 15 to 15.1 ramps to 30% B. The total run time was therefore 15 min. The flow rate was 0.3 mL min^{-1} . The HPLC system was coupled the mass spectrometer detector with an electrospray interface. No split was necessary to introduce the eluent into the mass spectrometer.

The mass spectrometer was operated in the electrospray ionisation (ESI) with negative ion detection. High-purity nitrogen (5.0 ; 99.999%) was used as the drying gas (gas flow 300 L/h) and as the ESI nebuliser gas, setting its pressure at 200.0 psi .

Table 1

Cone voltage to obtain the molecular ions and their characteristic fragment ions

Compound	Fragment (m/z)	Lost group	Cone voltage (V)
OH-FLU	311 MP^{a}	$[\text{H}]^+$	20
	267	H^+ , CO_2	25
	227	$[\text{HCOOH}]^+$, 2[FH]	50
DIC	294 MP^{a}	$[\text{H}]^+$	25
	250	H^+ , CO_2	35
	214	$[\text{HCOOH}]^+$, Cl	35
FFA	280 MP^{a}	$[\text{H}]^+$	25
	236	H^+ , CO_2	35
	176	$[\text{HCOOH}]^+$, 3[FH]	70

^a Molecular peak.

No auxiliary gas was used. The source block and the desolvation temperature were set at 100 and 350°C , respectively. The capillary voltage was set at 3.00 kV . The instrument was operated in selected ion recording (SIR) mode. Only when necessary was the instrument operated in full scan mode. The dwell time was set at 0.20 , 0.5 and 1.0 s per ion to study its influence on the response. The cone voltages for each molecular ion and their respective fragments are summarised in Table 1. The extraction voltage was optimised at 5.0 V . The tube lens voltage and the ion energy were set at 0.50 and 0.5 V , respectively.

All the mass spectrometer parameters cited above that are related to the flow and composition of the mobile phase, except nitrogen flow, the source block and desolvation temperatures, were optimised by direct injection of the individual standard solutions of $1.0 \mu\text{g L}^{-1}$ of each drug in methanol by the syringe bomb.

3. Results and discussion

3.1. Chromatographic separation

The three compounds studied belong to NSAIDs; FFA is an antranilic acid derivative, DCA is an acetylsalicylic acid derivative and OH-FLU is a nicotinic acid derivative. The polarity differences between them made it possible to achieve complete chromatographic separation by using a C_{18} and applying a gradient with ammonium acetate buffer and acetonitrile in the mobile phase. As OH-FLU presents the highest polarity, it was eluted first with an acetonitrile percentage of 30% at 4.5 min retention time. DIC and FFA were eluted at 9.1 and 14.0 min retention times, respectively, decreasing the polarity of the mobile phase by increasing the acetonitrile percentage from 30% up to 50%.

3.2. Mass spectra

OH-FLU, DIC and FFA were selected to carry out these chemometric studies because of their different chemical structures and their mass spectrum patterns which present different characteristic fragments in addition to the molecular peak. As is well known, many factors influence mass spectra of organic

compounds when analysed by LC–MS [3]; thus, the first step was to test the reproducibility of the mass spectra at the experimental conditions selected.

Fig. 1 shows the mass spectra obtained for each compound in full scan mode with a cone voltage value of: 60 V for FFA, 30 V for DIC and 50 V for OH-FLU. Three ions were selected to carry out the study: the molecular peak and the fragments indicated in Fig. 1. All these ions were in the mass spectrum with a relative intensity >10%. Later, the instrumental mass detector conditions were changed to obtain the maximum signal of each ion selected, as well as the molecular ions as their two characteristic fragments. The voltage difference between the sample cone and the extraction cone establishes the acceleration of the ions in the region called “collision induced dissociation (CID)” in the mass detector. Once the extraction voltage was set, the increased value of the cone voltage produced an increase in the kinetic ion energy and thus, an increase in the number of col-

lisions. This process benefits the ion fragmentations. In order to obtain the maximum signal from each ion, the cone voltage was optimised as was the molecular ion as their two selected characteristic fragments. Once the extraction voltage was tested and set to 4.0 V, the cone voltage was optimised and the values indicated in Table 1 were selected.

For FFA and OH-FLU, the optimised cone voltage was different for each ion; therefore, the reproducibility of three mass spectra was studied for each compound. For DIC, the maximum signals for the two fragment ions were obtained at the same cone voltage for which reason the reproducibility of two spectra was tested.

For each value of the cone voltage, five mass spectra were obtained. The abundance of the ion/base peak ratio was calculated and it was tested that the reproducibility of every one of these ratios, expressed as relative standard deviations, was $\leq 10\%$.

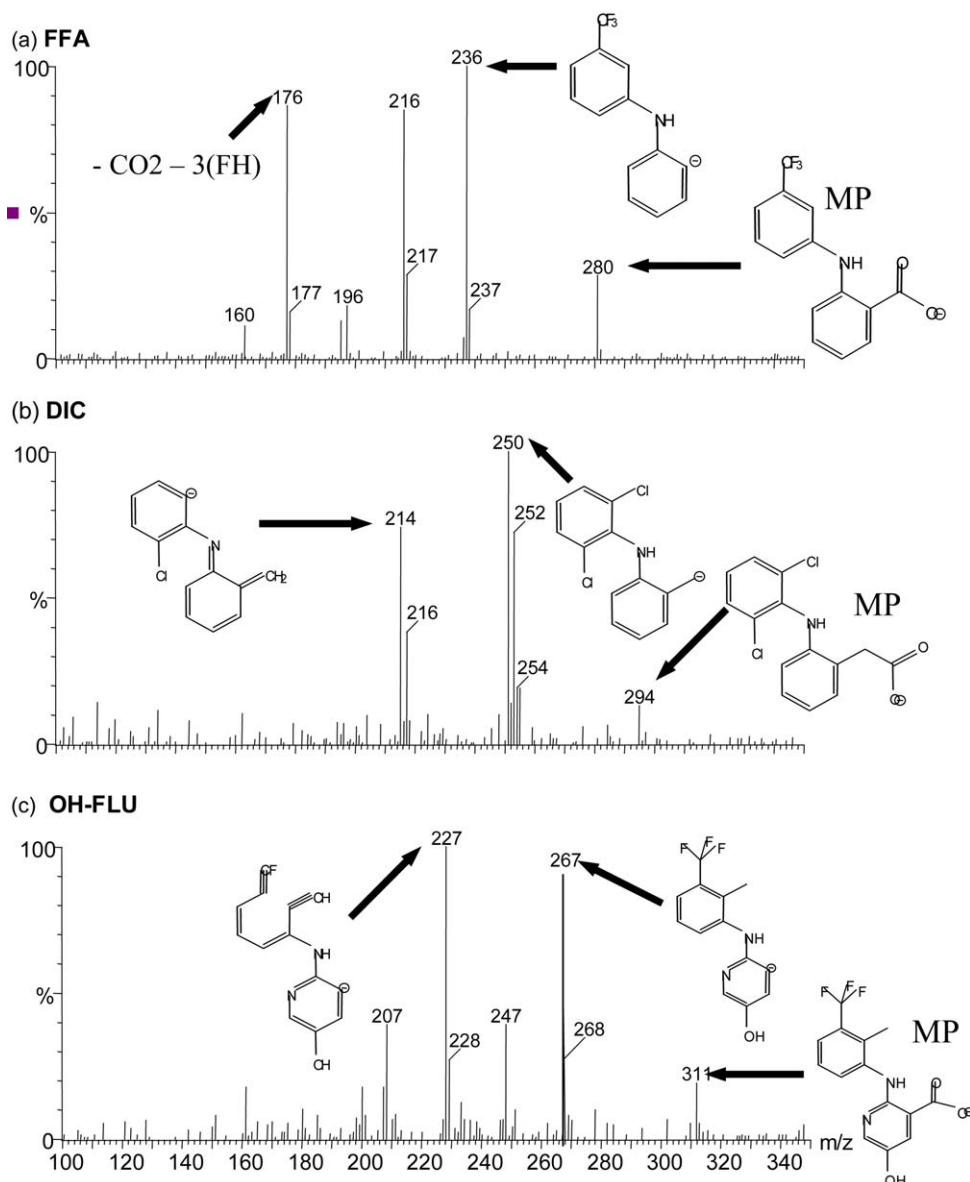


Fig. 1. Mass spectrum in full scan mode of (a) FFA, (b) DIC and (c) OH-FLU, with a cone voltage set at 60, 30 and 50 V, respectively.

3.3. Number of fragments and calibration functions

Different calibration functions were obtained from equally spaced dilutions of the standards. The concentrations tested ranged from 200 to 1000 ng/mL for OH-FLU, from 200 to 2000 ng/mL for DIC and from 300 to 5000 ng/mL for FFA. Two experimental replicates and three injections of each one were carried out. The analytical signal was taken as the area of the chromatographic peak. This area was obtained in SIR mode using the signal of: (a) the molecular ion, (b) the molecular ion plus the first characteristic fragment, (c) and the molecular ion plus two characteristic fragments. The dwell time was set to 500 ms per ion for OH-FLU and to 1000 ms for DIC and FFA, these being the values discussed in the following section. All the chromatographic and the mass parameters were the same except the cone voltage value, which was changed as described above.

The statistical parameters for each calibration line are given in Table 2. The lack-of-fit test was applied to check the linearity of the calibration graphs according to the guidelines of the Analytical Methods Committee [7]. The in-line (R^2) and on-line ($1 - R.S.D._{slope}$) linearity was also checked [8]. “In-line” linearity refers to the straight line fit (goodness-of-fit) of the analytical signal values obtained experimentally in relation to the analyte concentration, that is it describes the absence of curvature on the calibration line. “On-line” linearity indicates the greater or lesser dispersion of the data around the calibration line. Both linearities have to be evaluated at the same time since one depends on the other. A low degree of on-line linearity would indicate

that it is not possible to accurately distinguish the curvature by means of the lack-of-fit test. For each compound, the slopes of the three calibration functions (a , b and c) were compared to learn if they were similar or not. This comparison was carried out using the analysis of covariance (ANCOVA), which takes into account the two possible sources of variation in this comparison: (1) random errors in measuring; (2) the controlled factor or fixed effect, which represents the variability in the measuring process arising from the differentiating factor of the slopes being compared, i.e. in this case, the effect of the number of ions. The statistical test establishes as null hypothesis that the slopes do not present significant differences ($H_0 \equiv \beta_a = \beta_b = \beta_c$), so H_1 implies different slopes. The following reasoning is then applied: if the number of ions used to establish the calibration functions does not introduce systematic variability, their respective slopes will not differ significantly and their variability, arising from the random errors that always occur, will be comparable to the scattering of the slopes for each individual function. Thus, there are two variances that act as measures of this variability, and they are compared by F -test with $(k - 1, n_a + n_b + n_c - 6)$ degrees of freedom, k being the number of slopes to be compared and n is the number of experimental pairs of values for each calibration function:

$$F_{cal} = S_N^2 / S_D^2$$

S_N^2 measures the contribution of the difference observed between the slopes of the three calibration lines and the sum of the squares of the deviations from the linear model (i.e. the variance between the curves), and S_D^2 measures the contribution

Table 2
Statistical parameters of the calibration functions

Compound	Mass (m/z)	Intercept	Slope (sensitivity)	R^{2a} (%)	P -value ^b (%)	Linearity ^c (%)	ANCOVA
OH-FLU	311	19.72	2588.73	99.8	12.04	98.78	$F_{tab} = 3.195$, $F_{cal} = 805.07$ (H_1 , different slopes)
	267 + 311	1.66	4462.29	99.6	91.61	98.52	
	227 + 267 + 311	−10.82	6838.17	99.7	15.53	98.64	
DIC	294	10.91	176.76	99.3	53.45	97.98	$F_{tab} = 3.204$, $F_{cal} = 3561.58$ (H_1 , different slopes)
	250 + 94	19.48	1831.87	99.9	35.73	99.01	
	214 + 250 + 294	41.094	2459.48	99.8	27.17	98.77	
FFA	280	−51.23	2271.93	97.7	19.74	95.73	$F_{tab} = 3.204$, $F_{cal} = 2018.71$ (H_1 , different slopes)
	236 + 280	31.20	1747.10	99.8	26.02	98.94	
	176 + 236 + 280	63.48	9045.52	99.7	43.86	98.56	

^a In-line linearity.

^b Lack-of-fit test.

^c On-line linearity.

Table 3
Sensitivity and dwell time

Dwell time (ms)	Sensitivity								
	OH-FLU			DIC			FFA		
	MP ^a	MP + 1 Frag ^b	MP + 2 Frag	MP ^a	MP + 1 Frag ²	MP + 2 Frag	MP + 2 Frag	MP + 1 Frag ²	MP + 2 Frag
200	994	2648	3419	249	1194	1534	796	5336	7065
500	2589	4462	6838	173	844	1081	1764	4081	5220
1000	2657	3812	5451	177	1832	2459	2272	1747	9046

^a Molecular peak.

^b Fragment.

of the deviations from the linear model, considering the calibration curves independently (i.e. the variance within each curve) [9].

Table 2 shows that in this case, $F_{\text{cal}} > F_{\text{tab}}$, then S_N^2 is much greater than S_D^2 and it can be concluded that, with the confidence level used, the three calibration curves present a slope whose values differ significantly from each other. Thus, there are three different calibration functions for each compound with increasing slopes, and therefore with increasing sensitivity. For the three compounds, the sensitivity increased with the number of ions, with this increment being significant when the molecular peak plus two fragment ions was used: the sensitivity increased twice for OH-FLU, four times for FFA and fourteen times for DIC.

Once the good linearity was corroborated by the relevant statistical parameter (R^2 and the P -value), it could be selected as the calibration function with the greatest sensitivity. In this case, these were those obtained using the molecular peak plus two fragments.

3.4. Dwell time study

The effect of the dwell time was investigated because of its importance as an instrumental parameter that affects the sensitivity achieved. For a given dwell time value, the number of points used to draw a chromatographic peak acquired in SIR mode decreases with the number of mass used to obtained the chromatogram. It has been previously demonstrated that a long dwell time improves the signal to noise ratio [10,11], but it is necessary to obtain an adequate profile of the chromatographic peak with at least six points (Nyquist criterion). Taking this into account, values of 200, 500 and 1000 ms were tested for each signal. As shown in Table 3, the highest sensitivities were not always reached with a long dwell time, but they were always obtained using three mass: the molecular peak plus two fragments. For OH-FLU, the highest sensitivity value was reached for three fragments with a dwell time of 500 ms. For DIC and FFA, the highest value was found using 1000 ms of accumulation time.

3.5. Detection limit estimation

Three different ways to estimate the detection limits were tested. Two of them used the signal assigned to the chromatographic blank. This signal was estimated using a method widely explained in Ref. [12]. The chromatographic blank was estimated by direct integration of the background noise using extrapolated values of the base-peak width at concentrations different to zero. This method has been successfully applied in situations characterised by the absence of analytical signals related to the blank, as occurs with chromatographic techniques, electrophoretic techniques, and differential voltammetric techniques [11,13,14]. Additionally, this method has been used to estimate detection limits in the determination of carbetamide in water by micro liquid–liquid extraction followed by HPLC [15].

Following the IUPAC recommendations [16], the detection limit (DL) was then calculated using chromatographic blank replicates: $DL = \Delta_{\alpha\beta} s_{A0}/b$, being $\Delta_{\alpha\beta} = 3.49$ ($\alpha = \beta = 5\%$, $n = 12$) [17], s_{A0} the standard deviation of 10 chromatographic blank signals, and b is the slope of the calibration function. Another detection limit evaluation was carried out using three chromatographic blank signals for inclusion in the calibration function [18].

Finally, the detection limit was also estimated by the widely used signal to noise ratio ($S/N = 3$), which suffers from the lack of a suitable statistical test to ensure the quality of the measured values.

Table 4 shows the detection limits estimated by these three procedures and for different numbers of fragments. Unexpectedly, the number of fragments does not seem to be significant in the detection limits evaluated using the calibration function obtained by the addition of fragments. The greatest differences appear according to the procedure used to estimate it. As expected, the detection limit values calculated from 10 chromatographic blanks were smaller than those obtained from the calibration function because the former are estimated taking into account only the variability from the background noise assigned to the chromatographic blank (s_{A0}), whereas the latter values, estimated from the calibration function, are evaluated consid-

Table 4
Detection limit calculated by three different procedures

Compound	Number of fragment	Detection limit ^a (ng/mL)		
		IUPAC recommendation (from 12 blank standard deviation ^b)	From $S/N = 3$	From standard calibration
OH-FLU	1	5	6	93
	2	6	21	115
	3	9	17	101
DIC	1	14	56	157
	2	6	36	57
	3	5	44	96
FFA	1	4	20	247
	2	14	10	83
	3	3	18	112

^a Detection limits obtain using the optimised dwell time: 500 ms for OH-FLU and 1000 ms for DIC and FFA.

^b $DL = \Delta_{\alpha\beta} s_{A0}/b$, being $\Delta_{\alpha\beta} = 3.49$ ($\alpha = \beta = 5\%$, $n = 16$) [19], s_{A0} the standard deviation of 12 chromatographic blank signals, and b is the slope of the calibration function.

ering more variability sources, such as the residual (s_{xy}), the slope (s_b) and the intercept (s_a) standard deviations. The values obtained with the signal to noise ratio are not comparable with the others since they are not obtained with a statistical criteria.

Despite the fact that the lowest detection limit values were obtained from 10 chromatographic blank signals, the evaluation of this limit from the standard calibration function is proposed, since it contains the entire variability source in the analytical signal.

4. Conclusion

This study shows the possibility of using the sum of signals coming from different fragments including the molecular peak as is usual in GC–MS methods as an analytical signal to establish the calibration function in LC–MS. In this way, the instrumental analytical sensitivity of LC–MS can be improved. About the dwell time, instrumental parameter that too contributes to the sensitivity, we found that higher values do not always increase sensitivity. Regarding the detection limit, we found that the number of fragments used to establish the calibration function does not affect this analytical characteristic and that the detection limit depends on the procedure used to calculate it.

References

- [1] A.L. Burlingame, R.K. Boyd, S.J. Gaskell, *Anal. Chem.* 70 (1998) 647R.
- [2] VAM Report LGC/VAM/1998/071, Optimisation of the number of ions which are acceptable for identifying different chemical species using LC–MS.
- [3] L. Rivier, *Anal. Chim. Acta* 492 (2003) 69.
- [4] M.J. Bogusz, R.D. Maier, K.D. Kruger, W.J. Frunchnicht, *J. Chromatogr. B* 713 (1998) 361.
- [5] W. Weinmann, A. Wiedermann, B. Eppinger, M. Renzm, M. Svodova, *J. Am. Soc. Mass Spectrom.* 10 (1999) 1028.
- [6] P. Marquet, N. Venisse, E. Lacassie, G. Lachatre, *Analisis* 28 (2000) 925.
- [7] Analytical Methods Committee, *Analyst* 119 (1994) 2363.
- [8] L. Cuadros-Rodriguez, A.M. García-Campaña, J.M. Bosque-Sendra, *Anal. Lett.* 29 (7) (1996) 1231.
- [9] A.M. García-Campaña, L. Cuadros Rodriguez, F. Alés Barrero, C. Jiménez Linares, M. Román Ceba, *J. AOAC Int.* 80 (1997) 657.
- [10] J.L. Eveleigh, C.J. Ducauze, *J. Chromatogr. A* 765 (1997) 241.
- [11] K. Li, M. Fingas, *J. Hazard. Mater.* 102 (2003) 81.
- [12] A. Gonzalez-Casado, L. Cuadros-Rodriguez, E. Alonso-Hernandez, J.L. Vilchez, *J. Chromatogr. A* 726 (1996) 133.
- [13] G. Muñoz-Soto G, A. Gonzalez-Casado, L. Cuadros, E. Alonso, J.L. Vilchez, *J. Capillary Electroph.* 4 (1997) 15.
- [14] R. Blanc, A. Gonzalez-Casado, A. Navalon, J.L. Vilchez, *Anal. Chim. Acta* 403 (2000) 117.
- [15] M.G. Muñoz-Soto, F. Carricondo-Moguel, A. Gonzalez-Casado, E.J. Alonso-Hernandez, J.L. Vilchez, *J. Liquid Chromatogr. Rel. Tech.* 24 (2001) 355.
- [16] IUPAC, L.L.A. Currie, *Pure Appl. Chem.* 67 (1995) 1699.
- [17] Clayton, *Anal. Chem.* 59 (1987) 2506.
- [18] L. Cuadros, A. García-Campaña, C. Linares, M. Román, *Anal. Lett.* 26 (1993) 1243.
- [19] Clayton, *Anal. Chem.* 59 (1987) 2506.

Validation of an enzyme-linked immunosorbent assay (ELISA) for the determination of 4-nonylphenol and octylphenol in surface water samples by LC-ESI-MS

R. Céspedes^a, K. Skryjová^b, M. Raková^b, J. Zeravik^b, M. Fránek^b, S. Lacorte^a, D. Barceló^{a,*}

^a *Department of Environmental Chemistry, IQAB-CSIC, Barcelona, Spain*

^b *Veterinary Research Institute, Brno, Czech Republic*

Received 1 December 2005; received in revised form 22 May 2006; accepted 3 July 2006

Available online 14 August 2006

Abstract

4-Nonylphenol (NP) and octylphenol (OP) were measured by direct ELISA in both laboratory-fortified and surface water samples collected monthly from 10 rivers. In this procedure, samples were concentrated by solid phase extraction (SPE) using Lichrolut RP-18 sorbent with good recoveries obtained for both LC–MS and ELISA, giving a low level of detection (LOD) at the range of low $\mu\text{g L}^{-1}$ and good reproducibility. Analysis of 40 surface water samples demonstrated that the ELISA may be a useful screening tool for the determination of the alkylphenols in surface water matrices. The concentration of NP and OP in surface waters ranged from 0.11 to $6.58 \mu\text{g L}^{-1}$. A good correlation of results obtained by ELISA and LC–MS within the concentration range of 0.08– $6.86 \mu\text{g L}^{-1}$ was found in the river samples [$R^2 = 0.924$, $n = 39$]. The influence of various factors on assay determination was also discussed.

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Keywords: ELISA; Liquid chromatography–mass spectrometry (LC–MS); Nonylphenol; Octylphenol

1. Introduction

Alkylphenol ethoxylates (APEOs) belong to a group of non-ionic surfactants used in agricultural and industrial products; they have been widely used in the last 40 years as detergents, emulsifiers, antifoamers and pesticide adjuvants [1]. Concern over their possible environmental and health impact led to the ban of APEOs from cleaning products in many European countries [2]. Alkylphenols (APs) 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) are the most common degradation products of APEOs. Among others, NP is widely used as a plastic additive and antioxidant, as well as in the tanning industry and in pesticide formulations [3–6]. OP is used as a detergent, emulsifier, solubiliser and wetting agent [7]. The alkyl chain can vary in size, degree of branching and the attachment position to the phenol [8]. As a result of their widespread use, these compounds have been detected in water samples [9–12] and fish tissue [13–16], sediments and sewage sludge [17–19].

Although APEOs are not classified as highly toxic substances, some of their metabolites (APs and short chain APEOs) are more persistent, bio accumulative, lipophilic and less water soluble than their parent compounds [1,19], being highly toxic to aquatic organisms, mammals and birds [20–21]. In addition, *in vitro* [5] and *in vivo* studies [3,6,22–25] have confirmed their estrogenic potential [26–28]. Compounds involved in these mechanisms (called endocrine disrupters) may interfere with the normal endocrine function of both wildlife and humans [29–32]. The biodegradation of NPEOs under aerobic conditions yields mainly short ethoxy chain oligomers (NPE₁O and NPE₂O), whereas under anaerobic conditions fully de-ethoxylated nonylphenol (NP) is also formed. Increasing toxicities were found with decreased ethoxylate chain length [28,33–35]. Due to their endocrine disrupting properties, NP and OP are included in the priority list of the New Framework Directive (Directive 2000/60/EEC) [36]. So far, NP and OP are analysed by gas or liquid chromatography coupled to mass spectrometry. However, low cost, rapid and automated methods are needed for screening purposes [37]. Immunochemical techniques [4,38–42] are among some of the emerging techniques used to discriminate between contami-

* Corresponding author. Tel.: +34 93 400 61 70; fax: +34 93 204 59 04.
E-mail address: dbcqam@cid.csic.es (D. Barceló).

nated and non-contaminated samples. Advantages of ELISA include cheap instrumentation, small sample volumes for analysis and rapid analytical capabilities. However, filtration and thorough clean up steps are required if complex matrices such as waste waters are to be analysed to avoid matrix effects.

Although assays for surfactants started in the eighties [43], most of them have only been reported in the recent years [40–42,44–50]. Concerning the analysis of NP and OP, Goda et al. [40], developed a monoclonal ELISA for the determination of alkylphenol polyethoxylates (APnEOs) and their biodegradation products, whereas Mart'ianov et al. [42] were able to detect NP and NP-related compounds such as OP, alkylphenol ethoxylates, alkylphenol carboxylates and their halogenated derivatives using an ELISA based on polyclonal antibodies. The ELISA result was compared with standard HPLC and LC–MS/MS techniques for river samples [40] and waste waters [40,42].

Fránek et al. developed ELISAs based on both polyclonal and monoclonal antibodies for detection of NP and OP [46,50]. A strong tendency to recognize alkylphenols as a total analyte amount was indicated by the cross-reactivity pattern for ELISA based on polyclonal antibodies [50]. More detailed cross-reactivity testing indicated the assay allowed for reliable recognition of 4-NP and *tert*-OP in a mixture of various APs. No cross-reactivity was observed for linear alkylbenzene sulfonates and phenolic compounds. Specificity of monoclonal antibody-based ELISA enabled sensitive detection of linear long-chain forms of 4-*n*-alkylphenols. Thus, this assay offered the potential for the detection of the linear chain forms of APs that are the minor fraction of NP/OP isomer spectra in real environmental samples [50]. The polyclonal antibodies produced by Fránek's group were incorporated into the Biacore biosensor immunoassay and used for the analysis of shellfish samples [51]. Recently, a very sensitive chemiluminiscent ELISA and simple dipstick test for 4-*n*-NP, based on the above mentioned monoclonal antibody were developed [51]. From a practical point of view, the above polyclonal ELISA being able to detect the total amount of APs (preferably NP and OP), was considered to be a more relevant method of environmental screening than the assay which recognised only linear AP species using monoclonal antibodies. The objective of the present study was to investigate the use of ELISA as a screening technique for the effective monitoring of NP and OP in water samples. For such a purpose, the ELISA performance was (1) compared to conventional HPLC-MS in terms of sensitivity, precision and accuracy using fortified samples and (2) evaluated as a qualitative or semi-quantitative screening tool for determining NP and OP or total APs in 40 samples of surface water.

2. Experimental

2.1. Chemicals and biochemicals

Dimethylsulfoxide (DMSO) was purchased from Sigma (St. Louis, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was pro-

vided by Serva (Heidelberg, Germany). All other chemicals were of analytical grade. The preparation of enzyme conjugate (9-pOHPh-non-HRP) and rabbit antibody (9-pOHPh-non-OVC) are described in a previous paper [46].

4-Nonylphenol (90% technical mix of isomers) was purchased from Sigma (St. Louis, USA). 4-Heptylphenol (4-HP), used as internal standard in the LC–MS analysis, and 4-*tert*-octylphenol, were both obtained from Aldrich (Milwaukee, WI, USA). Stock solutions of individual standards were prepared at 1000 $\mu\text{g L}^{-1}$ in methanol. Working standard solutions were obtained by further dilution of stock solutions with methanol. HPLC-grade solvents (water, methanol, acetonitrile, acetone and dichloromethane) were purchased from Merck (Darmstadt, Germany). Analytical grade sodium acetate was purchased from Panreac (Barcelona, Spain).

2.2. Buffers and solutions

Carbonate buffer (50 mmol L^{-1}), pH 9.6 for coating; 10 mmol L^{-1} phosphate-buffered saline (PBS), pH 7.2 (containing 145 mmol L^{-1} NaCl) for dilution of the peroxidase tracer; 10 mmol L^{-1} phosphate-buffered saline with 0.1% Tween-80 (PBST), pH 7.2 (containing 145 mmol L^{-1} NaCl) for washing the microtitre plate; substrate buffer for HRP was 0.1 mol L^{-1} sodium acetate, pH adjusted to 5.5 by addition of 1 mol L^{-1} of citric acid; substrate solution for HRP was prepared by addition of 1 mL of substrate buffer, 200 μL of 1% (w/v) solution of TMB in DMSO and 20 μL of 6% (v/v) H_2O_2 to 20 mL Milli Q-water; stopping reagent 2 mol L^{-1} H_2SO_4 .

2.3. Sample collection

Ten surface water samples were collected monthly between April and August 2001 (excepting July) from different rivers. Surface water samples were collected in Pyrex borosilicate glass containers. Each bottle was rinsed with tap water and with high purity water, prior to sampling. Samples were stored at 4 °C immediately after sampling.

2.4. Sample preparation

Water samples were filtered through 0.45 μm membrane filters and extracted within 48 h to avoid degradation of target compounds.

Solid phase extraction (SPE) with Lichrolut RP-18 (500 mg, 6 mL) sorbent (Merck, Darmstadt, Germany) was performed using an automated sampler processor automated sample preparation with extraction columns (ASPEC XL) from Gilson (Villiers-le-Bel, France), fitted with a 817 switching device and an external 306 LC pump, as described in a previous paper [52]. SPE cartridges were conditioned by passing 7 mL of methanol, and 3 mL of LC-grade water through the cartridge at a flow rate of 3 mL min^{-1} . Five hundred millilitres of filtered water sample were percolated through the C18 cartridge at a flow rate of 5 mL min^{-1} . After preconcentration, the extraction materials were completely dried (30 min) using a Baker SPE 12 g apparatus (J.T. Baker, Deventer,

Netherlands) connected to a vacuum system (set at -15 psi). Analytes were eluted with ($2\text{ mL} \times 5\text{ mL}$) of $\text{MeOH}:\text{CH}_2\text{Cl}_2$ (9:1) and a five minute delay between the two elutions. After elution, the extracts were evaporated to dryness with a gentle stream of nitrogen and reconstituted with $300\text{ }\mu\text{L}$ methanol.

The methanolic extract was spiked with an internal standard (4-heptylphenol) at $1000\text{ }\mu\text{g L}^{-1}$ prior to measurement with LC–MS. For the ELISA determinations, $50\text{ }\mu\text{L}$ of the methanolic extracts were evaporated under a stream of nitrogen and dissolved in 1 mL PBS and sonicated for 30 min in an ultrasonic bath. The samples were stored at $-18\text{ }^\circ\text{C}$ in glass vials with Teflon lined screw caps.

2.5. ELISA procedure

An automated Strip Washer EL $\times$ 50TM from Bio-Tech Instruments (Vermont, USA) was used for washing microtiter plates. All ELISA experiments were performed in sterile 96-well microplates Maxisorp (Nunc, Roskilde, Denmark) and the absorbance was measured with an EIA iEMS Reader and Software Genesis 3.04 (Labsystems MF, Helsinki, Finland). Milli Q equipment for water purification was obtained from Millipore Corp (Bedford, USA).

Microtiter plates were coated with $200\text{ }\mu\text{L}$ /well of antibody, diluted with coating buffer and incubated at $4\text{ }^\circ\text{C}$ overnight. Unbound components were removed by washing three times with $300\text{ }\mu\text{L}$ of PBST. Nonylphenol standard or sample diluted with PBS ($100\text{ }\mu\text{L}$) and tracer in PBS ($100\text{ }\mu\text{L}$) were added to the wells in triplicate. The plates were shaken on a microtiter plate shaker for 1 min followed by 1 h incubation at $4\text{ }^\circ\text{C}$. After plates were washed three times, $200\text{ }\mu\text{L}$ of the enzyme substrate and chromogen ($\text{H}_2\text{O}_2 + \text{TMB}$) were added. After plates were shaken for 15 min , the enzymatic reaction was stopped by adding $100\text{ }\mu\text{L}$ of $2\text{ mol L}^{-1}\text{ H}_2\text{SO}_4$. The absorbance was measured at 450 nm with an ELISA reader and the AP concentration in water samples calculated from the calibration curve generated during the same run.

2.6. Liquid chromatography–mass spectrometric analysis

LC–MS conditions are described in [52]. Briefly, the HPLC system consisted of an HP 1100 auto sampler having a $100\text{ }\mu\text{L}$ loop and an HP 1090 A LC binary pump both from Hewlett Packard (Palo Alto, CA, USA). The HPLC separation was achieved on a $5\text{ }\mu\text{m}$, $250\text{ mm} \times 4\text{ mm}$ i.d. Cis reversed phase column (LiChrospher 100 RP-18) from Merck (Darmstadt, Germany).

The injection volume was set at $20\text{ }\mu\text{L}$ and the flow rate was 1 mL min^{-1} . The separation was performed in negative ionisation mode (NI) under gradient elution conditions using acetonitrile (A) and water (B). The gradient elution began from 30% A, to 60% A for 15 min , to 70% A for 5 min , to 90% A for 10 min and back to the initial conditions for 3 min . The extraction voltage used was 90 V . Detection was carried out using an LC-MSD HP 1100 mass selective detector, equipped with an electrospray (ESI) interface.

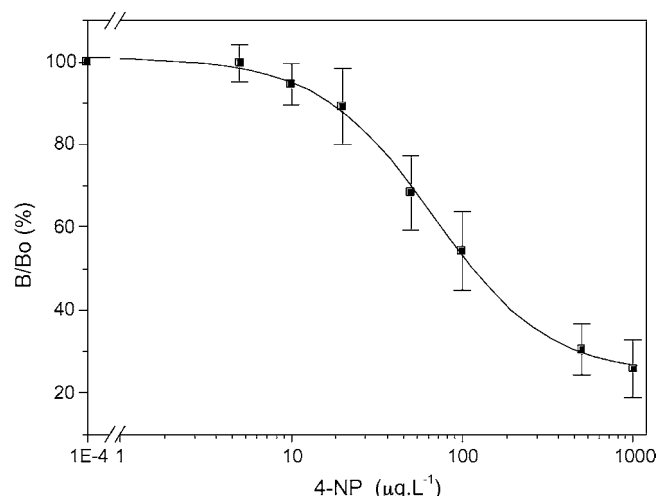


Fig. 1. Dose–response curve for 4-NP calibrator. The bars indicate the standard deviation for six replicate immunoassays. ELISA was based on the antibody 9-pOHPh-non-OV-C and 9-pOHPh-non-HRP reagent combination. The coating was carried out at the antibody dilution 1:1000, the tracer dilution was 1:2000.

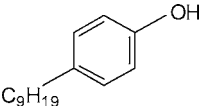
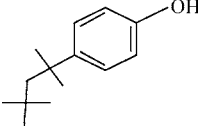
3. Results and discussion

3.1. Validation of ELISA using fortified samples

The calibration curve for NP obtained by ELISA is shown in Fig. 1. The standard dose response is expressed here as B/B_0 , where B = absorbance at a given concentration of analyte and B_0 = absorbance at zero concentration of the analyte. The curves were constructed in the concentration range of $5\text{--}1000\text{ }\mu\text{g L}^{-1}$. As shown in Fig. 1, the stability of the established ELISA procedure was good—the relative standard deviation for each calibration point on 6 curves was within 4–9%. The IC_{50} values, defined as the analyte concentration causing binding inhibition at half-maximum in the dose–response curve were established in the range of $54.1\text{--}65.0\text{ }\mu\text{g L}^{-1}$ for six curves.

The LOD of the ELISA procedure covering the preconcentration step was 6 ng L^{-1} in water samples. With the purpose of comparison between both methods, Table 1 shows structures, detection limits for NP and OP (LOD and IDL, calculated using a signal-to-noise ratio of 3 ($S/N=3$) in selected ion monitoring mode for LC–MS), and the dynamic range of the methods. Both methods detected concentrations within the ng L^{-1} to $\mu\text{g L}^{-1}$ levels, ELISA being 10 times more sensitive than LC–MS, demonstrating its high sensitivity for the target compounds under study. IDLs obtained by LC–MS showed that lower concentrations could be achieved if higher water samples were to be preconcentrated. Both methods present a wide dynamic range, being one order of magnitude broader in the SPE-LC-MS than for the ELISA. This increases the applicability of the method for real water samples, where concentration ranges can increase various orders of magnitude. The robustness of the method is also shown by the good repeatability (0.4–4.3% for LC–MS) obtained by both methods. Throughout the concentration range tested, recoveries obtained permitted the quantitative determination of NP and OP in surface waters.

Table 1
Structures of analytes, detection limits (calculated in selected ion monitoring for LC–MS) and dynamic range for NP and OP determined in water samples by ELISA and LC–MS in negative mode of ionisation

Compound	Structure	LOD ($\mu\text{g L}^{-1}$)		IDL (ng)		Dynamic range ($\mu\text{g L}^{-1}$)	
		ELISA	LC–MS	LC–MS	ELISA	LC–MS	LC–MS
NP (nonylphenol)		0.006	0.07	0.6	5–1000	5–10000	
OP (octylphenol)		0.006	0.06	0.4	5–1000	5–10000	

LOD: detection limit; IDL: instrumental detection limit.

In order to compare the analytical performance of the ELISA and LC–MS methods, ground water samples were spiked with NP and OP, respectively at 0.5, 2, 5, 20 and 50 $\mu\text{g L}^{-1}$; the accuracy of both techniques were determined analysing the same extracts by both methodologies. This range was selected because it reflected the average concentration levels found in surface waters. The accuracy of ELISA was assessed in fortified samples carried through the sample preparation procedure after reconstitution and dilution of the sample with PBS buffer. Because antibody response to NP and OP was different, standard curves using the same antibody were calibrated with technical NP or OP standard to obtain true recovery values for the respective analyte. The recovery of NP added to water sample was quantified by LC–ESI–MS using an external standard calibration using the base peak m/z 219 $[\text{M} - \text{H}]^-$ for NP and m/z 205 $[\text{M} - \text{H}]^-$ for OP, respectively. The results of the recovery experiments of NP and OP are summarized in Table 2. Extracts were first analysed by LC–MS, then evaporated, reconstituted with PBS and then analysed by ELISA, so lower recoveries obtained by ELISA are explained by analyte loss during evaporation. Values of 87–73% for the recovered NP and 117–51% for OP were obtained in the lower range of spiked concentrations (0.5 and 5.0 $\mu\text{g L}^{-1}$). The recoveries found at the higher spiking levels (20 and 50 $\mu\text{g L}^{-1}$)

were only 47 and 40% and 33–40% for NP and OP, respectively (values derived from the respective NP and OP calibration). As expected, the recoveries obtained by the addition of NP alone were higher than the values obtained for the equimolar mixture of NP and OP (30–50%). It should be noted that recovery of the NP+OP mixture in spiked waters was measured using NP calibration and therefore these recovery values were lower due to the weaker OP response compared with NP (cross-reactivity value for OP was 38% versus 66% for NP [50]). Therefore, we observed approximately double values for OP calculated with OP calibration versus OP calculated with NP calibration. Subsequent experiments showed that decreased recovery was observed for highly spiked concentrations and was caused during the sample preparation step. To measure analyte within the working (calibration) range of our ELISA, samples of different analyte concentration were diluted with PBS buffer at different dilution rates. The samples with low analyte concentration (0.5 and 2.0 $\mu\text{g L}^{-1}$) were dissolved in 1 ml of buffer (starting dilution) prior to ELISA detection, whereas the samples of higher concentrations (5–50 $\mu\text{g L}^{-1}$) were diluted using progressive serial dilution (50–500 times) with the buffer. Surprisingly, when sample dilution was carried out using only a single step for the high analyte concentration, the recovery values ranged from 70–90% (unpublished results). It is supposed that the lipophilic character of NP and OP resulted in analyte losses (e.g. adsorption on working materials) within serial dilution steps with regard to the results in Table 2. This indicates that samples suspected to have a high concentration of these compounds should be measured using a single dilution step when more accurate results are required. With respect to the ELISA sensitivity, samples suspected to have high concentrations of these compounds (50 $\mu\text{g L}^{-1}$ or higher) could be analysed directly from the sample, avoiding the preconcentration step. As shown in Table 2, the recoveries determined using the LC–ESI–MS reference method were within the range 69–116% and 67–80% for NP and OP, respectively. These values permit the quantitative determination of NP and OP in surface water samples.

Table 2
Recoveries (%) obtained by ELISA and LC–ESI–MS in ground water samples

C spiked $\mu\text{g L}^{-1}$	ELISA				LC–MS	
	%NP ^a	%NP+OP ^a	%OP ^a	%OP ^b	%NP	%OP
0.5	87	50	50	117	69	67
1	–	–	–	–	105	71
2	75	39	32	71	116	79
5	73	34	24	51	103	80
10	–	–	–	–	99	73
20	47	35	19	40	109	72
50	40	30	15	33	104	68

^a Values derived from NP calibration.

^b Values derived from OP calibration.

Table 3
Concentrations ($\mu\text{g L}^{-1}$) obtained by ELISA and LC-ESI-MS in surface water samples

April	LC-MS			ELISA
	NP	OP	Total	
1	0.15	<LOD ^a	0.15	0.32
2	0.36	<LOD	0.36	0.47
3	0.45	0.10	0.55	0.61
4	0.50	<LOD	0.50	0.49
5	0.25	0.13	0.38	0.39
6	0.28	<LOD	0.28	0.25
7	0.24	0.10	0.34	0.56
8	0.25	<LOD	0.25	0.30
9	0.89	0.11	1.00	0.81
10	0.49	<LOD	0.49	0.43
May	LC-MS			ELISA
	NP	OP	Total	
1	0.23	<LOD	0.23	0.12
2	0.15	<LOD	0.15	0.30
3	0.27	0.65	0.92	0.30
4	0.23	<LOD	0.23	0.44
5	<LOD	<LOD	<LOD	0.60
6	0.15	0.16	0.30	0.30
7	0.16	<LOD	0.16	0.60
8	0.21	<LOD	0.21	0.54
9	0.27	0.63	0.90	2.48
10	0.28	0.30	0.58	0.60
June	LC-MS			ELISA
	NP	OP	Total	
1	0.32	<LOD	0.32	0.24
2	0.28	<LOD	0.28	0.49
3	5.58	<LOD	5.58	2.71
4	0.16	<LOD	0.16	0.30
5	0.15	<LOD	0.15	0.29
6	0.29	0.17	0.46	0.38
7	0.24	<LOD	0.24	0.20
8	0.29	0.09	0.38	0.30
9	2.07	0.13	2.20	2.80
10	0.23	0.09	0.28	0.16
August	LC-MS			ELISA
	NP	OP	total	
1	<LOD	<LOD	<LOD	0.15
2	0.20	<LOD	0.20	0.31
3	6.41	0.17	6.58	6.86
4	<LOD	<LOD	<LOD	<LOD
5	0.11	<LOD	0.11	0.13
6	<LOD	<LOD	<LOD	0.15
7	<LOD	<LOD	<LOD	0.08
8	<LOD	<LOD	<LOD	<LOD
9	1.73	<LOD	1.73	1.50
10	<LOD	0.12	0.12	<LOD

^a <LOD: below detection limit.

3.2. Comparison of ELISA and LC-MS for surface water samples

NP and OP were determined in 40 surface water samples by ELISA and LC-MS for intercomparison purposes (Table 3).

The values of LC-MS represented the individual concentrations of NP and OP respectively, while the values of ELISA represented the total amount of AP in the sample. Fig. 2 shows the chromatograms corresponding to NP and OP detected from site 9 (May) in selected ion monitoring (SIM) mode, which corresponds to concentrations of 0.27 and 0.63 $\mu\text{g L}^{-1}$ for NP and OP, respectively. ELISA values were calculated from a standard curve calibrated with NP (Fig. 1). In general, there was a good agreement between the levels detected with both techniques, being between 0.08 and 6.86 $\mu\text{g L}^{-1}$, which is in accordance with levels found in earlier studies [9,11,12]. The higher levels were found in samples collected from sites 3 and 9 and only in two samples (August, Sample 4 and Sample 8), no traces were detected with either of the techniques. These results demonstrate the reliability of the ELISA technique for screening applications.

Table 3 reports the individual concentrations of NP and OP, the sum of them obtained by LC-MS and the total concentration obtained with ELISA. The ELISA detected 38 positive samples as shown in Table 3. Out of these samples, four of them (10% of the total samples analysed) were false positives, when compared with the LC-MS values. ELISA detected concentrations ranging from 0.08 up to 0.60 $\mu\text{g L}^{-1}$, whereas confirmation by LC-MS showed that neither NP nor OP was present. It should be noted that false positives do not constitute an important problem in immunoassays, since positive results should afterwards be checked using another analytical method (LC-MS in this work) to confirm or reject the positive results obtained.

There was a good correlation between ELISA and LC-MS methods for NP and OP in water within the concentration range of 0.11–6.86 $\mu\text{g L}^{-1}$, reflected with a correlation coefficient (R^2) of 0.924, $n = 39$ and a slope of 0.8936 (Fig. 3). The sample from site 3 (June) with a concentration of 5.58 $\mu\text{g L}^{-1}$ of NP was excluded from correlation evaluation because its concentration was significantly biased according to the mean (mean = 0.68 $\mu\text{g L}^{-1}$, $n = 40$). In some cases, there was an overestimation with ELISA, especially in samples with very low concentrations of NP and OP.

Only one false negative was assigned to the ELISA detection. LC-MS found 0.12 $\mu\text{g L}^{-1}$ of OP from site 10 (August), but ELISA did not detect this compound. The lower sensitivity of ELISA towards OP compared with NP, proves that this ELISA can detect NP or NP and OP simultaneously, but is less reliable in detecting only OP at trace levels. False negative results represent a more critical factor in screening analysis since these samples are not further analyzed. However, the ELISA used in this work was directed to determine NP, and although OP can be detected when it appears in conjunction with NP, the test should not be focused towards the determination of solely OP in waters. In this sense, OP appears at lower concentrations and less frequently in the waters analysed (only 14 positive values ranging from 0.09 to 0.65 $\mu\text{g L}^{-1}$, compared to 33 positive values for NP at 0.11–6.41 $\mu\text{g L}^{-1}$).

The results obtained demonstrate that ELISA constitutes a good method which can be used as a screening tool for rapidly determining concentrations of NP in water samples. The major advantage of the ELISA method is the possibility to analyse many samples rapidly. Approximately 40 samples can be anal-

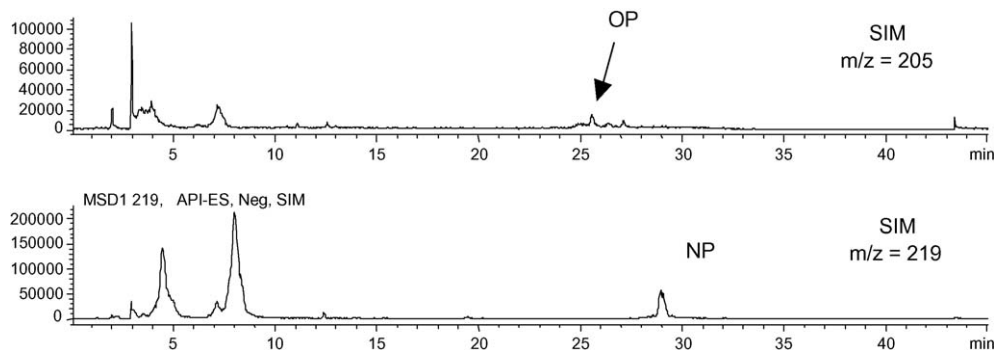


Fig. 2. Ion chromatogram by LC-ESI-MS in selected ion monitoring (SIM) corresponding to site 9 water extract for NP and OP.

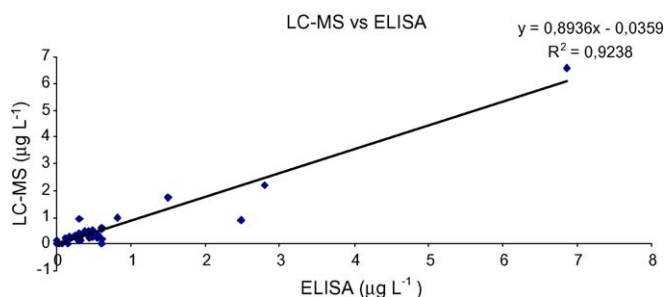


Fig. 3. Correlation between LC-MS and ELISA values ($n = 39$) for NP and OP in surface waters.

used in less than 4 h by ELISA, which considerably exceeds the time effectiveness of the LC-MS method. Considering the fact that four plates can be easily handled simultaneously, the number of samples handled per unit of time can be even more significant. However, chromatographic techniques, preferably with MS, are needed for the final identification and confirmation of all the target analytes.

4. Conclusions

A polyclonal antibody-based ELISA method directed to determine NP and OP in water was validated using fortified and surface water samples. Both ELISA and LC-MS provided good sensitivity and precision, although a preconcentration step was needed to determine such compounds at environmentally relevant concentrations. A good correlation between the ELISA and LC-MS in 39 surface river waters within the concentration range of $0.08\text{--}6.86\ \mu\text{g L}^{-1}$ demonstrated that, ELISA is a convenient tool for screening NP concentrations in water samples. The validated ELISA test permits the determination of NP with high reliability, although it is not able to detect OP with the same assay sensitivity within a single assay performance. Only 1 false negative and 4 false positive findings in 40 analysed samples were detected based on comparison with confirmatory LC-MS. False negative results can be obtained when OP is the only analyte present in water. On the other hand, the four false positives demonstrate the need to perform confirmatory analysis before providing the results. If ELISA is to be applied to more complex matrices, such as waste waters, the sample preparation method will need to be improved to reach standard conditions for both LC-MS and immunological detection.

Acknowledgements

This work has been funded by the EU Project SWIFT WFD (Contract SSPI-CT-2003–502492) and by the Spanish Ministry of Science and Education (CTM2005–24255E). This work reflects only the author's views and the European Community is not liable for any use that may be made of the information contained therein. We thank Roser Chaler, Dori Fanjul and D. Fotjová for excellent technical assistance. Merck is acknowledged for the gift of the LC columns and the SPE cartridges used in this work. The authors are very grateful to Paula Viana, from the Direcao Geral do Ambiente do Portugal, for providing us with water samples from Portugal.

References

- [1] G.G. Ying, B. Williams, R. Kookana, R. Environ. Int. 28 (2002) 215.
- [2] C.G. Naylor, J.P. Mieur, W.J. Adams, J.A. Weeks, F.J. Castaldi, L.D. Ogle, R.R. Romano, J. Am. Oil Chem. Soc. 69 (1992) 695.
- [3] S. Jobling, D. Sheahan, J.A. Osborne, P. Matthiessen, J.P. Sumpter, Environ. Toxicol. Chem. 15 (1996) 194.
- [4] M.C. Estévez-Alberola, M.P. Marco, Anal. Bioanal. Chem. 378 (2004) 563.
- [5] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenstein, Environ. Health Perspect. 92 (1991) 167.
- [6] E.J. Routledge, J.P. Sumpter, J. Biol. Chem. 272 (1997) 3280.
- [7] C.A. Staples, P.B. Dorn, G.M. Klecka, S.T. O'block, L.R. Harris, Chemosphere 36 (1998) 2149.
- [8] S.N. Pedersen, L.B. Christiansen, K.L. Pedersen, B. Korsgaard, P. Bjerregaard, Environ. Sci. Technol. 233 (1999) 89.
- [9] M. Castillo, E. Martínez, A. Ginebreda, L. Tirapu, D. Barceló, Analyst 125 (2000) 1733.
- [10] P.L. Ferguson, C.R. Iden, B.J. Brownawell, Anal. Chem. 72 (2000) 4322.
- [11] M. Petrovic, A. Rodríguez-Alba, F. Borrell, R.M. Marce, E. González-Mazo, D. Barceló, Environ. Toxicol. Chem. 21 (2002) 37.
- [12] R. Céspedes, S. Lacorte, D. Raldúa, A. Ginebreda, D. Barceló, B. Piña, Chemosphere 61 (2005) 1710.
- [13] M. Solé, M.J. Lopez de Alda, M. Castelló, C. Porte, K. Ladergaard-Pedersen, D. Barceló, Environ. Sci. Technol. 34 (2000) 5076.
- [14] T. Tsuda, K. Suga, E. Kaneda, M. Ohsuga, Chemosphere 41 (2000) 757.
- [15] S.A. Snyder, T.L. Keith, C.G. Naylor, C.A. Staples, J.P. Giesy, Environ. Toxicol. Chem. 20 (2001) 1870.
- [16] M. Petrovic, M. Solé, M.J. López de Alda, D. Barceló, Environ. Toxicol. Chem. 21 (2002) 2146.
- [17] A. Marcomini, F. Filippuzzi, W. Giger, Chemosphere 17 (1988) 853.
- [18] M. Petrovic, D. Barceló, Anal. Chem. 72 (2000) 4560.
- [19] (a) M. Ahel, W. Giger, Chemosphere 26 (1993) 1471;
(b) M. Ahel, E. Molnar, S. Ibric, W. Giger, Water Sci. Technol. 42 (2000) 15.

- [20] T. Tsuda, A. Takino, K. Muraki, H. Harada, M. Kojima, *Water Res.* 35 (2001) 1786.
- [21] T. Isobe, H. Nishiyama, H. Takada, *Environ. Sci. Technol.* 35 (2001) 1041.
- [22] R. White, S. Jobling, S.A. Hoare, J.P. Sumpter, M.G. Parker, *Endocrinology* 135 (1994) 175.
- [23] E.J. Routledge, J.P. Sumpter, *Environ. Toxicol. Chem.* 15 (1996) 241.
- [24] A.C. Nimrod, W.H. Benson, *Crit. Rev. Toxicol.* 26 (1996) 335.
- [25] C. Desbrow, E.J. Routledge, G. Brighty, J.P. Sumpter, M. Waldock, *Environ. Sci. Technol.* 32 (1998) 1549.
- [26] S. Jobling, J.P. Sumpter, *Aquat. Toxicol.* 27 (1993) 361.
- [27] A.M. Soto, C. Sonnenschein, K.L. Chung, M.F. Fernández, N. Olea, F.O. Serrano, *Environ. Health Perspect.* 103 (1995) 113.
- [28] S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter, *Environ. Sci. Technol.* 32 (1998) 2498.
- [29] T. Colborn, F.S. Vom Saal, A.M. Soto, *Environ. Health Perspect.* 101 (1993) 378.
- [30] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenstein, *Environ. Health Perspect.* 92 (1991) 167.
- [31] E.J. Routledge, D. Shbahan, C. Desbrow, G.C. Brighty, M. Waldock, J.P. Sumpter, *Environ. Sci. Technol.* 32 (1998) 1559.
- [32] C. Sonnenschein, A.M. Soto, *J. Steroid Biochem. Mol. Biol.* 65 (1998) 143.
- [33] M. Ahel, W. Giger, M. Koch, *Water Res.* 28 (1994) 1131.
- [34] M.R. Servos, *Water Qual. Res. J. Can.* 34 (1999) 123.
- [35] M. Takino, S. Daishima, K. Yamaguchi, *J. Chromatogr. A* 904 (2000) 65.
- [36] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000. Commission of the European Communities, Brussels, 2000.
- [37] E. Trullols, I. Ruisánchez, F.X. Rius, J. Huguet, *Trends Anal. Chem.* 24 (6) (2005) 516.
- [38] M. Castillo, A. Oubiña, D. Barcelo, *Environ. Sci. Technol.* 32 (1998) 2180.
- [39] M. Farré, A. Oubiña, M.P. Marco, A. Ginebreda, L. Tirapu, D. Barceló, *Environ. Sci. Technol.* 33 (1999) 3898.
- [40] Y. Goda, A. Kobayashi, S. Fujimoto, Y. Toyoda, K.-I. Myagawa, M. Ike, M. Fujita, *Water Res.* 38 (2004) 4323.
- [41] Y. Goda, M. Hirobe, A. Kobayashi, S. Fujimoto, M. Ike, M. Fujita, *Anal. Acta Chim.* 528 (2005) 47.
- [42] A.A. Mart'ianov, B.B. Dzantiev, A.V. Zherdev, S. A Eremin, R. Céspedes, M. Petrovic, D. Barceló, *Talanta* 65 (2005) 367.
- [43] S.I. Wie, B.D. Hammock, *Anal. Biochem.* 125 (1982) 168.
- [44] S. Fujimoto, Y. Goda, *Nippon Rinsho* 58 (2000) 2491.
- [45] Y. Goda, A. Kobayashi, K. Fukuda, S. Fujimoto, M. Ike, M. Fujita, *Water Sci. Technol.* 42 (2000) 81.
- [46] M. Franek, J. Zeravik, S.A. Eremin, J. Yakovleva, M. Badea, A. Danet, C. Nistor, N. Ocio, J. Emneus, *Fresenius J. Anal. Chem.* 371 (2001) 456.
- [47] A. Rose, C. Nistor, J. Erne, D. Pfeiffer, U. Wollemborg, *Biosens. Bioelectron.* 17 (2002) 1033.
- [48] M. Badea, C. Nistor, Y. Goda, S. Fujimoto, S. Dosho, A. Danet, D. Barcelo, F. Ventura, J. Emneus, *Analyst* 128 (2003) 849.
- [49] J. Yakovleva, J. Zeravik, I.V. Mchura, A.A. Formanovsky, M. Fránek, S.A. Eremin, *Int. J. Environ. Anal. Chem.* 83 (2003) 597.
- [50] J. Zeravik, K. Skryjová, Z. Nevoranková, M. Franek, *Anal. Chem.* 76 (2004) 1021.
- [51] J.V. Samsonova, M.Y. Rubtsova, M. Fránek, *Anal. Bioanal. Chem.* 375 (2003) 1017.
- [52] R. Céspedes, M. Petrovic, D. Raldúa, U. Saura, B. Piña, S. Lacorte, P. Viana, D. Barceló, *Anal. Bioanal. Chem.* 378 (2004) 697.

Synchronous fluorescence and excitation emission characteristics of transformer oil ageing

Subbiah Deepa^a, R. Sarathi^b, Ashok K. Mishra^{a,*}

^a Department of Chemistry, Indian Institute of Technology Madras, Chennai 600 036, India

^b Department of Electrical Engineering, Indian Institute of Technology Madras, Chennai 600 036, India

Received 29 November 2005; received in revised form 30 January 2006; accepted 30 January 2006

Available online 9 March 2006

Abstract

This paper describes the evaluation of synchronous fluorescence spectroscopy (SFS) and excitation emission matrix fluorescence (EEMF) spectroscopy as means of monitoring transformer oil degradation. When accelerated thermal ageing method is used, the onset of degradation of transformer oil on 17th day and transformer oil with polypropylene and cellulosic paper on 23rd and 27th days is sensitively reflected in the SFS and EEMF fluorescence spectral characteristics.

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Keywords: Transformer oil; Synchronous scan fluorescence; Excitation emission matrix fluorescence; Ageing

1. Introduction

Transformer oil is a derivative of petroleum crude. Mineral oils used for insulation are complex mixtures of linear saturated hydrocarbons (paraffins), cyclic saturated hydrocarbons (naphthenes), aromatic hydrocarbons and a small fraction of non-hydrocarbons, with hundreds of individual compounds. It contains high proportions of polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs) which are fluorescent molecules. Current techniques employed specifically for the analysis of the PAHs in transformer oil are limited to a standard test method for measuring the total mass of polycyclic aromatics in unused oil [1] and a procedure involving analysis of PAHs fingerprint of transformer oil using GC–MS. Both the techniques have lengthy extraction procedures, and although quantitative data can be obtained, the time consumed is long and expense is high. Transformer oils are susceptible to oxidation, which leads to the formation of compounds such as acids, aldehydes, esters, ketones, peroxides and alcohols. The deterioration of oil characteristics under working conditions may be hazardous to the electric equipment and installation. The parameters that accurately indicate the degradation in the transformer

oils are the dielectric breakdown voltage (electrical parameter), water content, neutralization number and oxidation stability of the oils. The oxidation of oil results in the formation of polar (oxygenated) compounds, which are responsible for the increase in acidity of the oil, and consequently reduces the dielectric breakdown voltage and increases the interfacial tension. High voltage power transformers make use of paper-insulated windings immersed in transformer oil. Replacement of traditional insulating materials like paper with commercially available thermosetting polymers has been practised recently. Several thermoplastics like polyetherimide, polyethylenes, polystyrene, Noryl and imidazole cured epoxy polymers were exposed to variation in humidity, ambient air, temperature and their stability was checked by Frost et al. [2]. Polypropylene films are used in capacitor coils and as cable wrap for layer and phase separation in rotating electrical equipment and transformers. During normal operation of the transformer, the temperature of the windings will increase and over a period of time this causes the cellulose chains in the paper to cleave. As a result, the mechanical strength of the paper measured directly or through its degree of polymerization, degrades with time and this will have an adverse effect on the performance of the insulation. A number of diagnostic test methods are available to monitor the insulation of power transformers. These include techniques such as gas chromatography for dissolved gas analysis (DGA) [3] to determine the hydrocarbon and carbon dioxide concentrations, moisture and

* Corresponding author. Tel.: +91 44 2257 4207; fax: +91 44 2257 4202.
E-mail address: mishra@iitm.ac.in (A.K. Mishra).

acidity measurements of the winding insulation, degree of polymerization (DP) measurements on paper samples [4] and high performance liquid chromatography (HPLC) [5] for the analysis of furan, product formed from paper degradation.

Thermal ageing experiments has been performed by many authors over the last few decades [6–8] and the results of these experiments were used to make predictions about the transformer oil lifetime. Effects of oxidation on the ageing of oil–paper insulation have been studied by accelerated ageing experiments on paper wrapped insulated conductors in the presence of air and nitrogen environments over a temperature range of 115–145 °C by Saha et al. [7]. Recently, Palmer et al. [6] have characterized the absorption response of transformer oil to in-service and laboratory aged transformer oils. This study indicated a significant increase in the absorbance over a range of wavelengths for both transformer oil degraded in the laboratory and for transformer oil drawn from transformers that have been in service for extended period of time.

Recently, Neimanis et al. [9] and Gupta [10] have reported the determination of moisture content in oil impregnated paper using near infrared (NIR) spectroscopy. Fourier transform infrared (FTIR) and near infrared (NIR) spectroscopy has been used to characterize the ageing of cellulosic paper [11]. Spectrofluorimetry has been widely used in the characterization of hydrocarbons in environmental analysis [12–16] and in process monitoring applications. Fluorimetry is a powerful detection method used in separation techniques like liquid chromatography and electrophoresis. One of the most important advantages of the fluorescence method is its selectivity; however in its application to complex natural systems, the selectivity of conventional fluorescence techniques appears to be insufficient. Instead, multidimensional fluorescence techniques like synchronous fluorescence (SFS) and EEMF are used in such applications, providing additional information about the samples. For multifluorophoric systems, the importance of SFS and EEMF has been well-established [17–19]. An EEMF spectrum is obtained as a plot of emission intensity as a function of both excitation and emission wavelengths and contains in it all possible information of conventional steady-state fluorimetry. An SF spectrum is obtained by simultaneously scanning both the excitation and emission monochromators in a spectrofluorimeter. The resulting excitation–emission data matrix provides a total intensity profile of the sample over the range of excitation and emission. SFS in particular has been found to be useful for the analysis [20] and identification [21] of oils, but the work has not been focused specifically on oils used primarily for insulation purposes. A recent work on the possible use of fluorescence in monitoring oil quality by SFS technique has been reported [22], which mainly focuses on the chemometric techniques applied to the characterization of transformer oil.

The most critical components of a transformer are immersed in its oil. By monitoring and identifying the condition of oil, the state of the transformer can be diagnosed.

This investigation is aimed at a detailed analysis of fluorescence characteristics of transformer oil and a comparison of the ageing process of transformer oil in presence and absence of insulating materials (polypropylene film and the kraft paper)

towards obtaining a simple and convenient analytical method of monitoring transformer oil degradation.

2. Experimental

2.1. Materials and methods

Oil samples were supplied by Raj Lubricants, Chennai under the trade name of Electrol, with a breakdown voltage of 65 kV. The general life of the transformer oil is much longer than the research duration; these investigations were based on studying the ageing effect with increased speed by thermally accelerated ageing of oils. Three kinds of degradation were designed and performed to investigate the response of transformer oil to various studies. Oil samples were taken in three 500 ml beakers and kept in an air-circulated oven maintained at 100 °C for 31 days. In one 500 ml beaker with oil, kraft paper was immersed and aged thermally. Kraft papers are extensively used in a variety of electrical equipments. The thickness of the paper used for the study was 60 μm . In case of insulation, paper absorbs moisture from the surrounding more rapidly than the pressboard material. Hence, before dipping into transformer oil, papers were placed in an oven maintained at 100 °C for 24 h and then impregnated in oil for ageing studies. In the second beaker, biaxially oriented polypropylene film of 20 μm thickness was used. The percentage of crystallinity was 71, dielectric constant of the material 2.2 and melting point of the material was 178 °C. The breakdown voltage of the film is 380 $\text{V } \mu\text{m}^{-1}$. The third beaker was in the absence of these insulating materials. Studies were carried out on these oils for every 2 days till the 30th day of ageing processes.

2.2. Apparatus

A Perkin Elmer lambda 25 UV–vis spectrophotometer was used for the absorbance measurements. Fluorescence measurements were done by using a Hitachi F 4500 spectrofluorimeter. For SFS measurements, the scan speed was 240 nm s^{-1} and PMT voltage was at 700 V. Excitation and emission slits were 5 nm each. Excitation source was 100 W xenon lamp. SFS was measured in the excitation wavelength range 200–500 nm. The EEMF spectra were measured in the excitation wavelength range of 250–590 nm with an interval of 10 nm and in the emission wavelength range 270–600 nm within an interval of 5 nm, respectively, maintained at a slit width of 5/5 and scan speed 1200 nm s^{-1} . FTIR measurements were done in Perkin Elmer IR spectrophotometer by scanning from 550 to 4000 cm^{-1} . NIR measurements were done to characterize the ageing of cellulosic paper using UV–vis–NIR Cary spectrophotometer.

3. Results and discussion

3.1. UV–vis absorption studies

Transformer oil is a highly absorptive material and the absorbance with the neat sample of oil exceeds the range of the spectrophotometer in the wavelength range 200–400 nm. Trans-

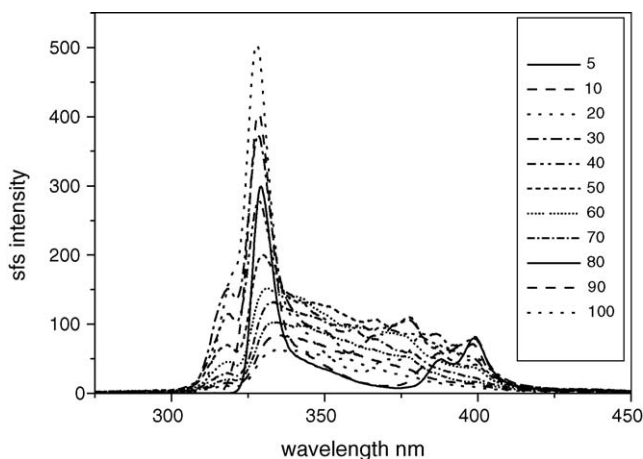


Fig. 1. SFS spectra of raw oil at different $\Delta\lambda$.

former oils are very complex mixtures and may consist of as many as 2900 paraffinic, naphthenic and aromatic hydrocarbon molecule types, 25% of these being aromatic [23]. Oxidation changes the color of the oil, which can be monitored, by the change in the absorbance of the oil by UV–vis spectroscopy [7]. With ageing of oil, a red shift in absorbance was observed. Thus, the raw oil without paper and polypropylene also developed color on ageing, indicating that, the insulating material is not responsible for the formation of the absorbing species and it arises due to oxidation of oil. The degradation of oil was shifted from 17th day in the case of raw oil to 23rd and 27th days in presence of polypropylene and paper insulation.

3.2. Fluorescence studies

In the course of investigation into fluorimetric analysis of multifluorophoric systems at higher concentration [24], it was observed that the use of right-angle geometry is capable of providing important analytical usefulness despite the presence of factors like light attenuation (LA), self-absorption (SA), energy transfer (ET) and collisional quenching (CQ). Kao et al. [25] also observed that for the multifluorophoric samples, right-angle geometry exhibits the widest linear dynamic range and lowest detectable fluorophore concentration. Hence, right-angle geometry was used for SFS and EEMF studies in characterizing the transformer oils.

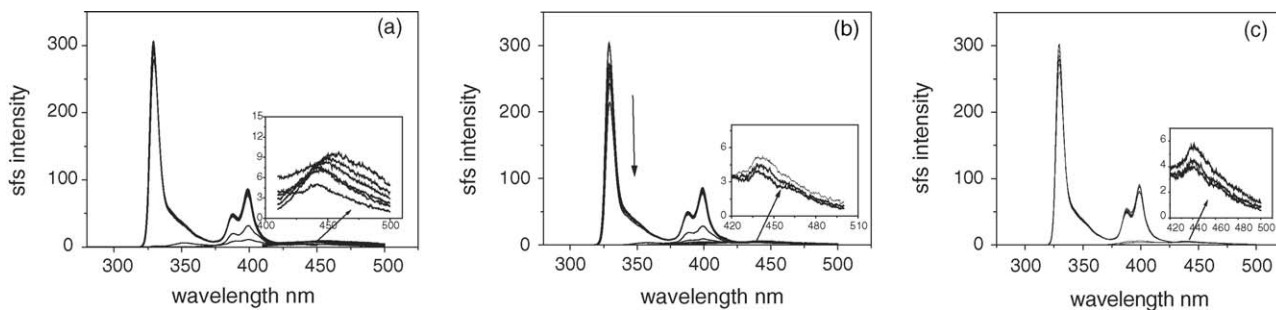


Fig. 2. SFS spectra of raw oil (a) and raw oil with polypropylene (b) and with paper (c) with increasing days: 0 day (Virgin), days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31. The spectra overlapped extensively till day 17th in raw oil and day 23rd in oil with polypropylene and 25th day in oil with paper. $\Delta\lambda = 5$ nm. There was a substantial loss of SF intensity at 330 nm on day 19 in raw oil, 25th day in oil with polypropylene and 27th day in oil with paper.

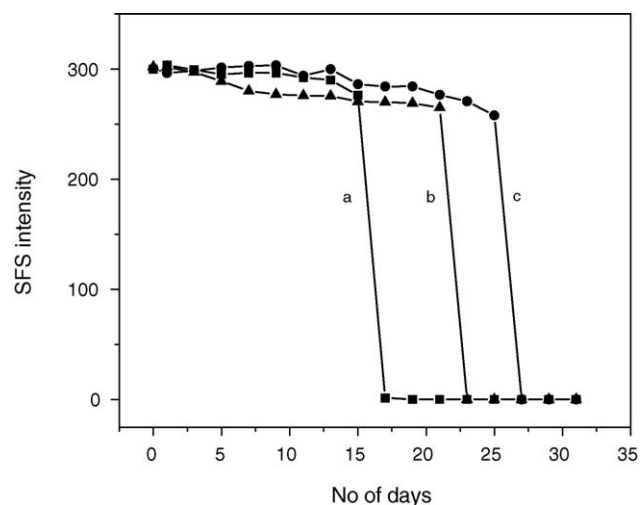


Fig. 3. Variation of SFS intensity with degradation of (a) raw oil, (b) oil with polypropylene and (c) oil with paper at $\lambda_{\max}^{\text{SFS}}$ 300 nm.

Transformer oil is a multifluorophoric system and as expected, the emission spectra keep changing in their shape and intensity, when the excitation wavelength is varied. This lack of uniqueness makes conventional fluorescence useless of monitoring purposes. Hence, SFS and EEMF spectroscopy were used for the analysis of transformer oil monitoring its ageing process.

The crucial parameter in SF spectroscopy is $\Delta\lambda$, the difference in wavelength of the excitation and the emission monochromators when they are scanned simultaneously. When the $\Delta\lambda$ is very low (<2 nm), scattering interferences are expected. Similarly when $\Delta\lambda$ is very large, those fluorophores, which have a very small stokes shift are not expected to be reflected in the SFS spectrum. Thus, for a particular type of a complex multifluorophoric system, the choice of optimum $\Delta\lambda$ varies. For example, in case of motor fuel characterization, an optimum $\Delta\lambda$ 40 nm is known to be more useful [19]. In order to arrive at an optimized $\Delta\lambda$ for transformer oil, the value was progressively varied in the range 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nm and the corresponding SF spectra recorded as shown in Fig. 1. It was found that at $\Delta\lambda$ 5 nm, SF spectra showed better resolved features in the transformer oil spectrum (Fig. 1). For a variety of transformer oil originating from different petroleum crude, the choice of $\Delta\lambda$ for particular transformer oil is not necessar-

ily best at $\Delta\lambda$ 5 nm. However as Fig. 1 shows it is possible to identify an SFS band, which does not change much when $\Delta\lambda$ is varied. In the present case, it is at 330 nm (Fig. 1). The variation in SF spectra at $\Delta\lambda$ 5 nm for raw transformer oil and in presence of insulating polypropylene and paper is shown in Fig. 2a–c, respectively. As is evident from the figures, a prominent SFS band at 330 nm was found to decrease substantially with ageing of transformer oil and changes of this band with regard to the presence of the insulating material could be rationalized in terms of progressive ageing of the oil. A drastic decrease in the SFS intensity of 330 nm band with ageing after 17th day in raw transformer oil and 23rd and 27th days in transformer oil with polypropylene and paper was observed. This is accompanied by the appearance of a new band at 450 nm, (shown in the inset of Fig. 2a–c) which appear to be due to the formation of a

new species on degradation. The SFS band intensity at 330 nm is highly invariant to the degradation of the transformer oil in all the three cases as shown in Fig. 3. As is evident from Fig. 3, the degradation of transformer oil with polypropylene and paper starts from 23rd and 27th days whereas it started at 17th day in raw oil. The SFS intensity at 330 nm is fairly constant till the onset of degradation, i.e. 17th, 23rd and 25th days in raw oil and oil with polypropylene and paper, respectively, which then drastically decreases. It is well known that, by the addition of insulating material to oil, the stability of the oil is increased [10].

In EEMF, the fluorescence intensity is plotted as a function of both the excitation and the emission wavelengths. Fig. 4A–C shows the variation of the contours with time of raw oil samples and in presence of polypropylene and paper, respectively. The EEMF contour maximum for raw transformer oil without degra-

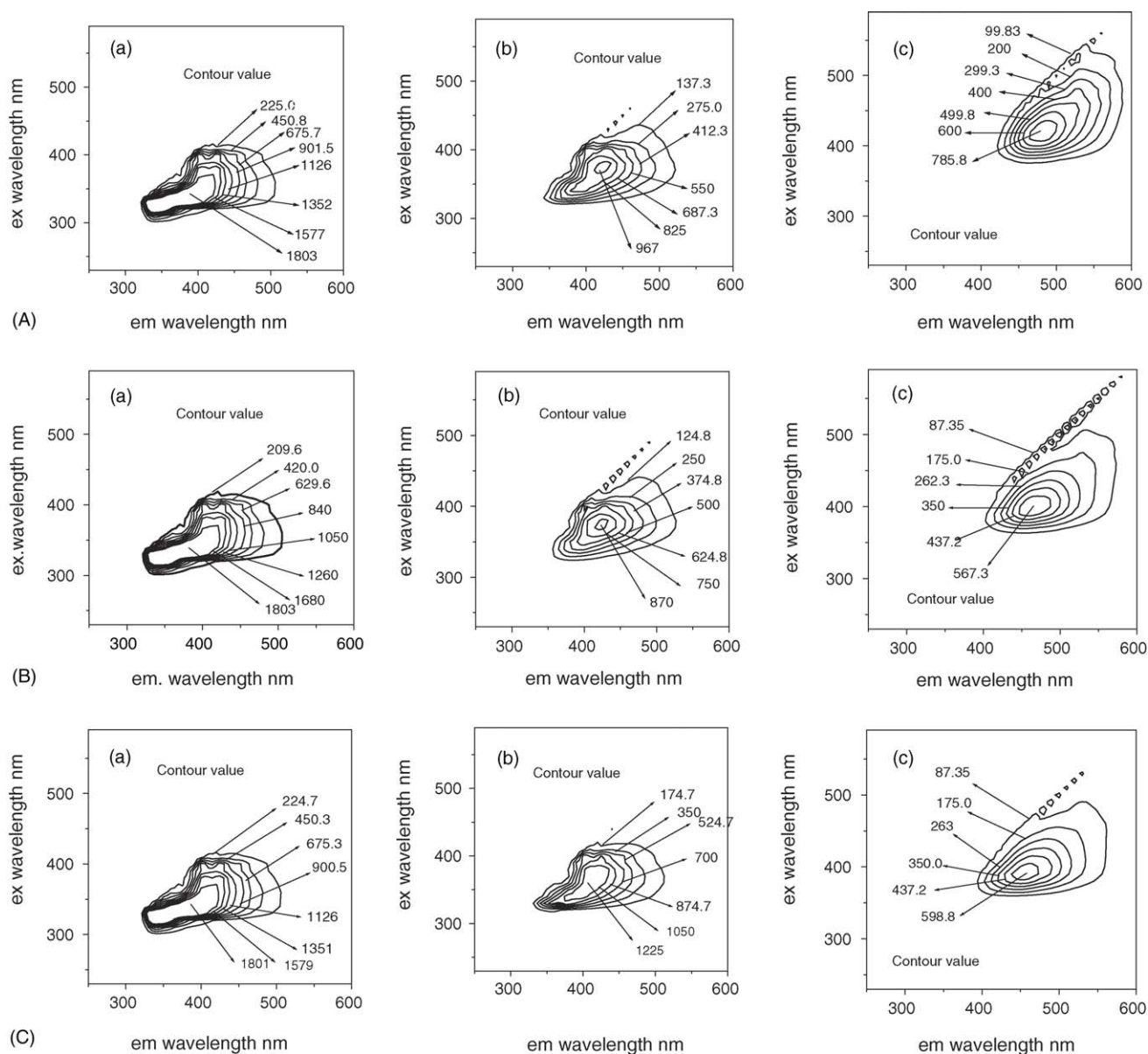


Fig. 4. (A) EEMF contour spectra for raw transformer oil with ageing: (a) raw oil, (b) day 19, (c) day 31. (B) EEMF contour spectra for transformer oil with polypropylene film upon ageing: (a) raw oil, (b) day 25, (c) day 31. (C) EEMF contour spectra for transformer oil with paper upon ageing: (a) raw oil, (b) day 27, (c) day 31.

ation was (370, 415) nm, which progressively shifted to (420, 485) nm at the end of 31 days, accompanied with a decrease in fluorescence intensity (Fig. 4). Fluorescence intensity drastically decreased at the 17th day and then marginally increased upon ageing till the 31st day in the case of the raw transformer oil whereas, in transformer oil with polypropylene and kraft paper, the ageing process started from 23rd and 27th days with a red shifting of contour maximum to (390, 460) nm with a drastic decrease in intensity. A slight increase in fluorescence intensity was observed after 23rd and 27th days as shown in Fig. 2b and c. Thus, the drastic decrease in fluorescence intensity with ageing of transformer oil has striking similarity with the results reported by Saha et al. [7]. They investigated the conditions of aged insulation samples in the presence of air and nitrogen environments over a temperature range of 115–145 °C. With increase in ageing time, the peak maximum return voltage was found to initially increase and then found to decrease drastically due to lowering of the insulation resistance. Both SFS and EEMF technique were able to report this phenomenon sensitively and faithfully. Fig. 5A–C shows the variation of fluorescence excitation, emission maxima and intensity of raw transformer oil and oil in presence of polypropylene and paper. As can be seen, a red shift in the contour maximum is observed with ageing. Thus, the EEMF fluorescence of the transformer oil agrees well with the SFS results.

When transformer oil is degraded, acidity increases, resulting in increase in C=O bond and C=C double bonds. During thermal decomposition, paraffinic compounds dehydrogenate and form naphthenic compounds. The naphthenes further dehydrogenate and form conjugated C=C double bonds and aromatics. Upon thermal oxidation, the unsaturated hydrocarbons in the oil form hydroperoxides, which on subsequent oxidation results in aldehydes and ketones by a free radical mechanism [6]. A kinetic experimental study of the oxidation reactions of naphthenic mineral oil (with and without additives) as a function of temperature has been carried out by Neto et al. [26]. Their results showed that the presence of hindered phenolic antioxidants significantly reduces the formation of oxidation products. Presence of antioxidants in the oil sample inhibits degradation until the antioxidants are consumed [26,27]. This could explain the sudden onset of fluorescence loss on day 17 for raw oil and on days 23 and 27 for oil with polypropylene and paper. There is a distinct possibility that under thermally accelerated ageing conditions in an air circulated oven, there could be volume loss and evaporation of small molecular mass species, which could include fluorophores emitting at shorter wavelengths. Loss of these could result in overall fluorescence loss and a red shift of the fluorescence contour. However, if evaporation of low-molecular mass fluorophores were affecting the SF or EEM spectra, the spectral profiles would have started changing right from the first sample taken after 2 days, would have been a gradual process, and probably with more significant changes during the initial days.

Since transformer oil is a derivative of petroleum crude, the spectral fingerprint and SFS characteristics of transformer oils obtained from different sources may show variations. Thus, no unique fluorescence characteristic can be ascribed to transformer

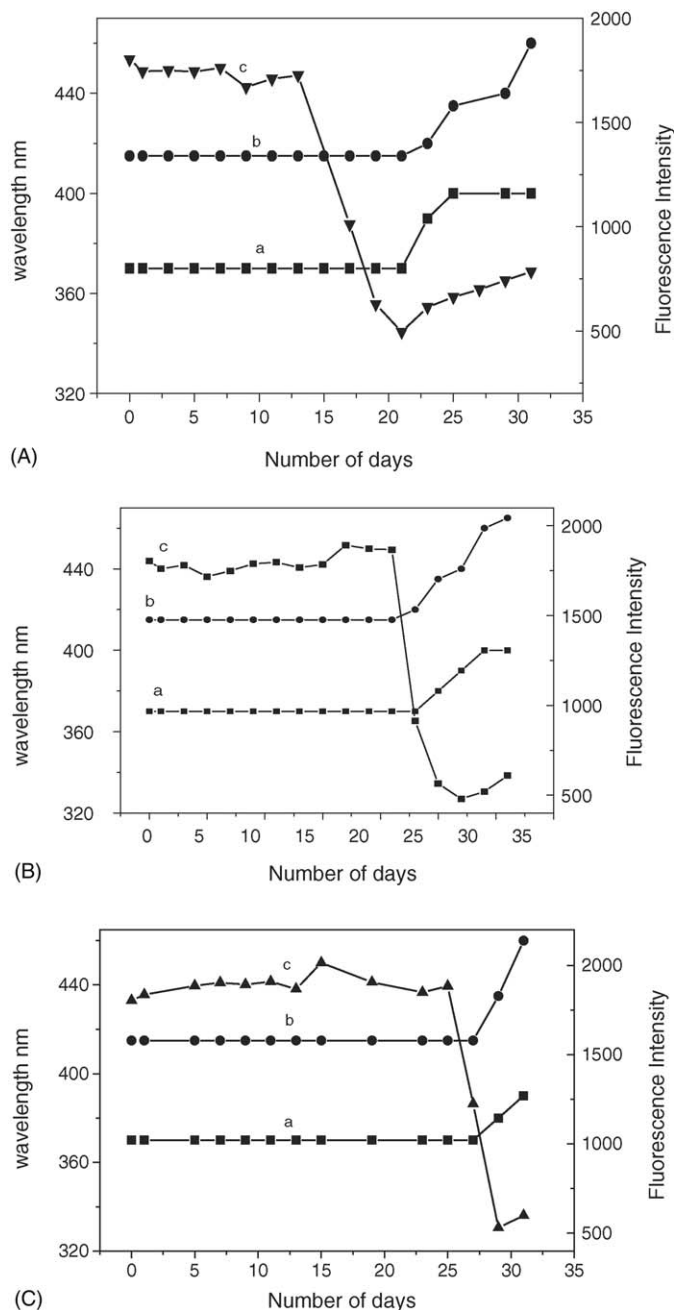


Fig. 5. (A) Variation of: (a) excitation maxima, (b) emission maxima and (c) fluorescence intensity with ageing of raw oil. (B) Variation of: (a) excitation maxima, (b) emission maxima and (c) fluorescence intensity with ageing of oil in presence of polypropylene. (C) Variation of: (a) excitation maxima, (b) emission maxima and (c) fluorescence intensity with ageing of raw oil in presence of paper.

oil in general and monitoring of transformer oil characteristics needs to be done starting with the measurement of SFS and/or EEMF spectra of the virgin oil.

3.3. IR studies on the ageing of transformer oil

In order to confirm the formation of a new carbonyl species on ageing process, IR spectra of raw and aged oil under different conditions were recorded. The vibration frequency correspond-

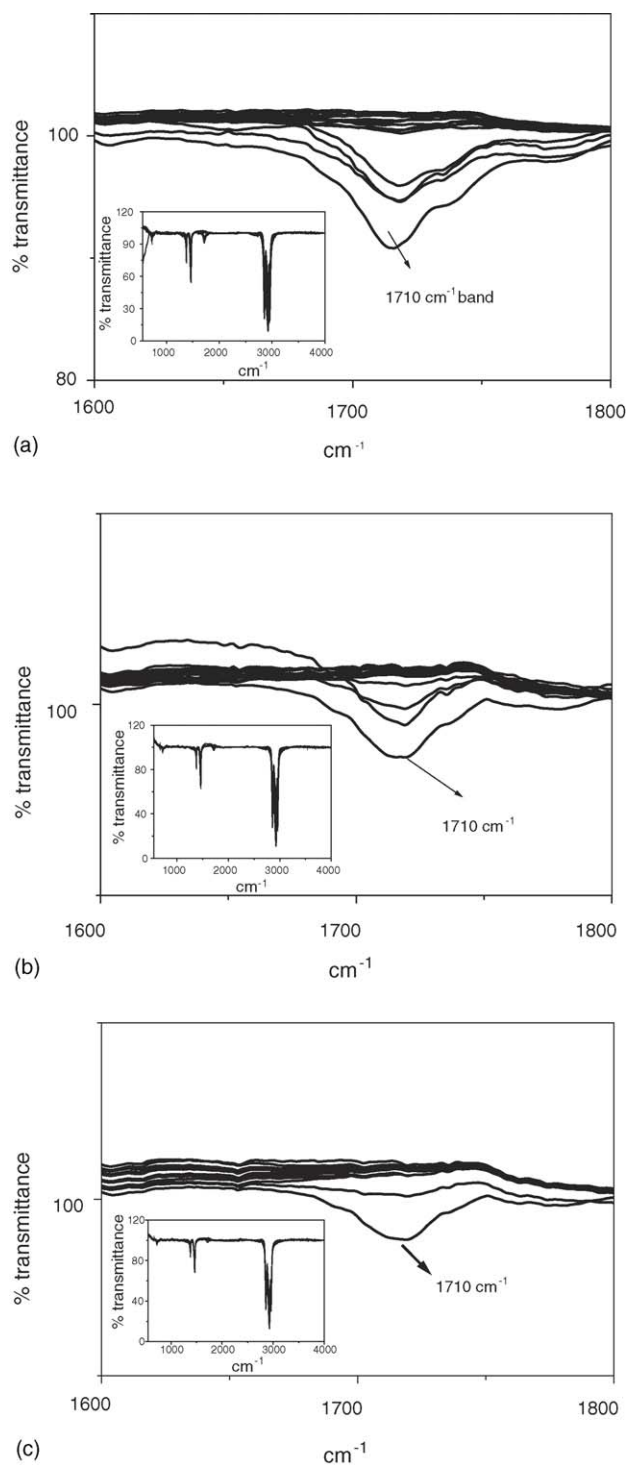


Fig. 6. (a) FTIR spectra of raw oil. (b) FTIR spectra of oil in presence of polypropylene. (c) FTIR spectra of oil in presence of paper.

ing to the carbonyl stretching frequency at 1710 cm^{-1} appeared during the process of ageing, which could be due to the presence of oxidation products of the oil like aldehydes and ketones. Thus, Fig. 6a–c shows the FTIR spectra of raw transformer oil and the polypropylene and paper impregnated oil upon ageing which supports the fluorescence and UV–vis absorption studies.

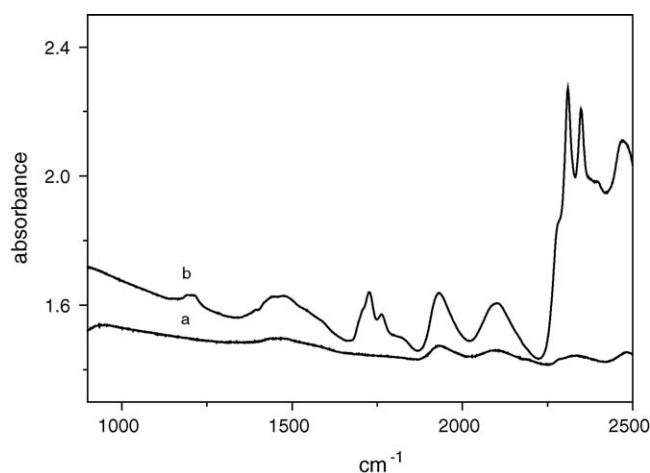


Fig. 7. NIR spectra of (a) raw paper and (b) in oil.

3.4. NIR studies on cellulosic paper

It has been reported by Saha et al. [7] that during ageing, the surface of the paper, which is in direct contact with the transformer oil, undergoes a color change and becomes darker since the oxidation products from the oil were attracted to the surface. A significant concentration of doubly bonded carbons and oxygen present in the surface of the samples had been identified using (X-ray photoelectron spectroscopy) XPS [7]. Ali et al. [11] have reported FTIR and NIR spectroscopy to characterize the ageing of cellulosic paper. Fig. 7 shows the NIR spectra of Kraft paper in air and paper impregnated in transformer oil, which agrees well with the spectra, reported in literature [8]. Water/OH features dominate the NIR spectra but the development of carbonyl/carboxyl overtones was also observed in the region of 1700–1900 nm. The oil impregnated paper showed a well-defined water combination band at about 1950 nm and the O–H stretch first overtone at about 1450 nm.

4. Conclusion

Synchronous excitation spectra and EEMF of oils can be used as a fingerprint and can be applied, together with fluorescence intensities, as a rapid screening tool to investigate similarities of oils. Thus, when accelerated thermal ageing method is used, the onset of degradation of transformer oil on 17th day and transformer oil with polypropylene and cellulosic paper on 23rd and 27th days is sensitively reflected in the SFS and EEMF spectral characteristics. This was supported by the FTIR and NIR studies on the ageing of transformer oil and the cellulosic paper. Fluorescence is a totally unperturbative technique. Delivery of excitation light and collection of fluorescence light can be easily done using optical fibers. Thus, it should be possible to develop in situ monitoring methods for transformer oil degradation.

Acknowledgements

S.D. thanks Council of Scientific and Industrial Research (CSIR), New Delhi for fellowship. We are grateful to Sophisti-

cated Analytical Instrumentation Facility, IIT Madras for FTIR and NIR spectral studies. We thank Raj Lubricants, Chennai for the supply of sample and valuable help.

References

- [1] IP346/92 (BS2000 Pt. 346), Determination of PCA content in unused lubricating base oils and asphaltene-free petroleum fractions—DMSO extraction/refractive index method, The British Standards Institute, 389 Chiswick High Road, London, W4 4AL United Kingdom.
- [2] N.E. Frost, P.B. McGrath, C.W. Burns, *IEEE Trans. Power Deliv.* 11 (1996) 331–334.
- [3] J.J. Kelly, *IEEE Trans. Ind. Appl.* 16 (1980) 777.
- [4] J. Unsworth, F. Mitchell, *IEEE Trans. Electr. Insul.* 25 (1990) 737.
- [5] A.M. Emsley, X. Xiao, R.J. Heywood, M. Ali, *IEEE Proc. Sci. Measur. Tech.* 147 (2000) 116.
- [6] J.A. Palmer, X. Wang, A. Mander, D. Torgerson, C. Rich, Conference Record of the 2000 IEEE International Symposium on Electrical Insulation, Anaheim, CA, USA, 2000, p. 460.
- [7] T.K. Saha, M. Darveniza, Z.T. Yao, D.J.T. Hill, G. Yeung, *IEEE Trans. Power Deliv.* 14 (1999) 1359.
- [8] D.J.T. Hill, T.T. Le, M. Darvenzia, T.K. Saha, *Polym. Degrad. Stability* 49 (1995) 429.
- [9] R. Neimanis, H. Lennholm, R. Kriksson, 1999 Annual Report Conference on Electrical Insulation and Dielectric Phenomena, 1999, p. 162.
- [10] B.K. Gupta, *IEEE Intern. Sympos. Electr. Insulat.*, Arlington, Virginia, USA, 1998, p. 583.
- [11] M. Ali, A.M. Emsley, H. Herman, R.J. Heywood, *Polymer* 42 (2001) 2893.
- [12] J.M. Shaver, L.B. McGown, *Appl. Spectrosc.* 49 (1995) 813.
- [13] M. Picer, N. Picer, *Chemosphere* 24 (1992) 1825.
- [14] M. Picer, V. Hocenski, *Water Res.* 28 (1994) 619.
- [15] L. De domencio, E. Crisafi, G. Magazzu, A. Puglisi, A. Larosa, *Mar. Pollut. Bull.* 28 (1994) 587.
- [16] E.S. Brodskii, S.A. Savchuk, *J. Anal. Chem.* 53 (1998) 1070.
- [17] D. Patra, A.K. Mishra, *Trends Anal. Chem.* 21 (2002) 787.
- [18] D. Patra, A.K. Mishra, *Appl. Spectrosc.* 55 (2001) 338.
- [19] D. Patra, A.K. Mishra, *Talanta* 53 (2001) 783.
- [20] J.B.F. Lloyd, *Nat. Phys. Sci. (Lond.)* 231 (1971) 64.
- [21] P. John, I. Soutar, *Anal. Chem.* 48 (1976) 520.
- [22] C. Bessant, L. Ritchie, S. Saini, B. Pahlavanpour, G. Wilson, *Appl. Spectrosc.* 55 (2001) 840.
- [23] A. Sierota, J. Rungis, *IEEE Electr. Insul. Mag.* 11 (1995) 1.
- [24] D. Patra, A.K. Mishra, *Analyst* 125 (2000) 1383.
- [25] S. Kao, A.N. Asanov, P.B. Oldham, *Instrum. Sci. Technol.* 26 (1998) 375.
- [26] R.C.R. Neto, D.O. Lima, T.D.S. Pinhelro, R.F. Almeida, T.N.C. Dantas, M.S.G. Dantas, M.A.S. Araujo, C.L. Cavaleante, D.C.S. Azevedo, *Ind. Eng. Chem. Res.* 43 (2004) 7428.
- [27] P.R. Krishnamoorthy, S. Vijayakumari, S. Sankaralingam, *IEEE Trans. Electr. Insul.* 27 (1992) 271.

Comparative study of electrospray and photospray ionization sources coupled to quadrupole time-of-flight mass spectrometer for olive oil authentication

J.L. Gómez-Ariza^{*}, A. Arias-Borrego, T. García-Barrera, R. Beltran

Departamento de Química y Ciencias de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Campus de El Carmen, 21007-Huelva, Spain

Received 15 September 2005; received in revised form 24 January 2006; accepted 7 February 2006

Available online 24 March 2006

Abstract

The use of fast and reliable analytical procedures for olive oil authentication is a priority demand due to its wide consumption and healthy benefits. Olive oil adulteration with other cheaper vegetable oils is a common practice that has to be detected and controlled. Rapid screening methods based on high resolution tandem mass spectrometry constitute today the option of choice due to sample handling simplicity and the elimination of the chromatographic step. The selection of the ionization source is critical and the comparison of their reliability necessary. The possibilities of the direct infusion electrospray ionization (ESI) and the recently introduced atmospheric pressure photospray ionization source (APPI), coupled to quadrupole time-of-flight (QqTOF), have been critically studied and compared to control olive oil adulteration. These techniques are very rapid (approximately 1 min per sample) and have high discrimination power to elucidate key components in the edible oils studied (olive, hazelnut, sunflower and corn). Nevertheless, both sources are complementary, being APPI more sensitive for monoacyl- and diacylglycerol fragment ions and ESI for triacylglycerols. In addition, methods reproducibility's are very high, especially for APPI source. Mixtures of olive oil with the others vegetable oils can be easily discriminated which has been tested by using principal components analysis (PCA) with both ESI-MS and APPI-MS spectra. Analogously, linear discriminant analysis (LDA) confirms methods reproducibility and detection of other oils used as adulterants, in particular hazelnut oil, which is especially difficult given its chemical similarity with olive oil.

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Keywords: Atmospheric pressure photoionization; Electrospray ionization; Quadrupole time-of-flight; Olive oil authentication; Fingerprinting

1. Introduction

Olive oil is one of the most important ingredients of the so-called Mediterranean diet, used for frying, roasting and seasoning. Medical and epidemiological research conducted under the auspices of the International Olive Oil Council have demonstrated the benefits of eating olive oil in cardiovascular [1] and diabetes [2] diseases, as well as in bone and nervous system development [3,4]. In addition, it has been proved its antioxidant and anti-aging properties at cell and mitochondrial levels [5], as well as the general favorable action of this food-stuff on the nutrition and diet. For these reasons, olive oil is one of the most consumed edible vegetable oils and it is

particularly expensive, which may present the opportunity for producers to stretch their merchandise by adulterating it with cheaper vegetable oils of lower quality [6]. The situation is especially critical for hazelnut oil adulteration due to its high similitude in chemical composition with olive oil. This fact makes difficult the use of triacylglycerols (TAGs), which are considered to be good fingerprints for adulteration detection purposes [7].

Olive oil fingerprinting is usually accomplished by NMR [8], Raman [9,10], pyrolysis-MS [11], GC-MS [12] or direct HS-MS techniques [12]. However, more rapid, accurate, reliable and robust analytical methodologies are being requested for olive oil authentication, to be suitable for routine analysis.

Recent developments in mass spectrometry makes possible to fulfill these requirements, because of the high resolution reached with tandem mass spectrometry (triple quadrupole QqQ, quadrupole time-of-flight QqTOF and others) avoids chromato-

^{*} Corresponding author. Fax: +34 959219942.

E-mail address: ariza@uhu.es (J.L. Gómez-Ariza).

graphic separation and reduces the analysis time and sample preparation significantly. These analytical tools allow fingerprinting in complex food matrixes as olive oil and provide useful information about all the components present in the sample. Goodacre et al. [13] reported the use of ESI-MS technique in olive oil authentication, showing that information-rich spectra from vegetable and nut oils can be generated without chromatographic separation. This spectral information allows the discrimination of olive oil from others commonly used as adulterants, particularly hazelnut oil. Latterly, Gómez-Ariza et al. [14] described the employ of the atmospheric pressure photoionization (APPI) [15,16] source for this purpose, considering its suitability to ionize analytes with little or no polarity. In addition, the APPI source can suffer less matrix effects because of its specificity towards the analytes which form the molecular radical ion $M^{\bullet+}$. However, both sources are complementary, because the ionization pattern of ESI based in the formation of ammonium adducts $[M-NH_4]^+$ [13] changes in APPI to the production of $[M+H]^+$ and $[M]^{\bullet+}$ ions of key compounds [14], as well as the ion $[R_2CO-H_2O]^+$ formed by the loss of water from the acylium ion. This latter ion is absent in the ESI-MS spectra.

Therefore, a comparison of both sources considering their discrimination capability on several highly consumed edible oils and their mixtures is necessary, especially for olive and hazelnut oils. The use of ESI-MS for the analysis of several oils including corn, grapestone, pomace, olive, extra virgin olive, peanut, soya, sunflower oils and a mixed oil comprising peanut, sunflower and soya oils has been previously considered [13]. However, the analysis of mixtures including olive oil which could provide information about the adulteration level of this food remains unknown. In addition, under our knowledge the system APPI-MS has not been used for this purpose.

The aim of the present work is the application of ESI-MS and APPI-MS systems for the discrimination of edible oils and mixtures to deep insight into the complementary use of both sources.

2. Materials and methods

2.1. Reagents and sample preparation

Oil samples [olive oil (O), hazelnut oil (H), sunflower oil (S) and corn oil (C)] were supplied by Olibeas cooperative (Huelva, Spain) and stored at 4 °C.

The solvents, HPLC-grade, dichloromethane, methanol and toluene, were purchased from Teknokroma (Barcelona, Spain), and the ammonium acetate was supplied by Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

All oils were 1000-fold diluted with a mixture of 60% dichloromethane: 40% 10 mM ammonium acetate in methanol when the ESI source was used. For APPI dilution was performed with dichloromethane/methanol (60:40, v/v).

Oil mixtures were prepared at 90, 70 and 50 (v/v) of olive oil with hazelnut, sunflower and corn oils, separately. Prior to infusion these mixtures were dissolved in the solution described above.

2.2. Instrumentation

2.2.1. ESI-QqTOF

The samples were loaded into a 100 μ L volume Hamilton gastight syringe mounted into an integrated apparatus pump operating at a flow rate of 5 μ L min⁻¹ and introduced directly into an API QSTAR® XL Hybrid system (Applied Biosystems, Foster City, CA, USA).

All data were obtained in positive ion mode (ES⁺). The TAGs were identified by the molecular ion mass spectra. Averaged spectra in full-scan mode were acquired for 1 min in the m/z range 100–2000 with 1.005 s scan time. The resolution for the Q1 region was fixed at unit mass resolution.

The parameters of the QqTOF system were optimized for analysis with the source electrospray ionization (ESI). The ion-spray voltage (IS) was finally set at 3000 V, the source and desolvation temperatures were both 60 °C with a declustering potential (DP) fixed at 76 V and focusing potential (FP) at 230 V (the orifice (OR) and focusing ring (RNG) voltages control the amount of declustering and fragmentation in the orifice-skimmer region of the vacuum interface). The curtain gas (CUR) was set at 1.13 L min⁻¹ and the nebulizer gas (UHP 99.999% nitrogen) was finally optimized at 1.56 L min⁻¹ for signal stability and sensitivity.

The ion energy (IE) was fixed at 2.0 V with a channel electron multiplier (CEM) of 2250 V.

ESI-MS-MS spectra were acquired by using nitrogen as the collision gas, the collision energy was set at 50–60 eV, and both the quadrupole (Q) and TOF analyzers were employed. Other conditions were the same as for the MS analysis described above.

2.2.2. APPI-QqTOF

The instrumental conditions used with this system were described elsewhere [14]. Briefly in this study was used, flow injection analysis (FIA) for sample introduction in the APPI-QqTOF. It was performed using an Agilent 1100 series HPLC provided with a binary pump and autosampler. A model KDS 100 syringe pump from KD scientific (New Hope, PA, USA) was used to deliver the dopant to the APPI ion source. This instrument was connected to an API QSTAR® XL Hybrid system (Applied Biosystems) fitted with an atmospheric pressure photoionization source. This ion source made use of a heated nebulizer (HN), whose heater was maintained at 350 °C for all these experiments. Nitrogen was used to nebulize the liquid eluant (nebulizer gas) and also to transport the finely dispersed sample drops through the heated quartz tube in which they were vaporized (auxiliary gas).

The operation conditions were the following: the flow rate provided by LC pumps was fixed at 0.5 mL min⁻¹ using 50% (v/v) aqueous methanol as LC eluant. A 5 μ L loop was used in the injector of the autosampler. The dopant (toluene) was delivered at 0.05 mL min⁻¹.

All data were obtained in positive ion mode. Averaged spectra in full-scan mode were acquired in the m/z range 100–1100.

The parameters of the QqTOF system were optimized for the analysis with the source APPI. The capillary voltage was finally set at 1500 V, with a DP fixed at 85 V and FP at 300 V. The

IE was at 2.0 V with a CEM of 2300 V. So that, the CUR was finally set at 1.13 L min^{-1} , and the nebulizer gas optimized at 1.50 L min^{-1} with the heater gas at 6.0 L min^{-1} . This gas assists solvent evaporation and increases the ionization of the sample.

2.2.3. Chemometrics

Computations were performed by using the statistical package STATISTICA Version 6.0 (2001) (StatSoft, Tulsa, USA). Principal components analysis (PCA) and linear discriminant

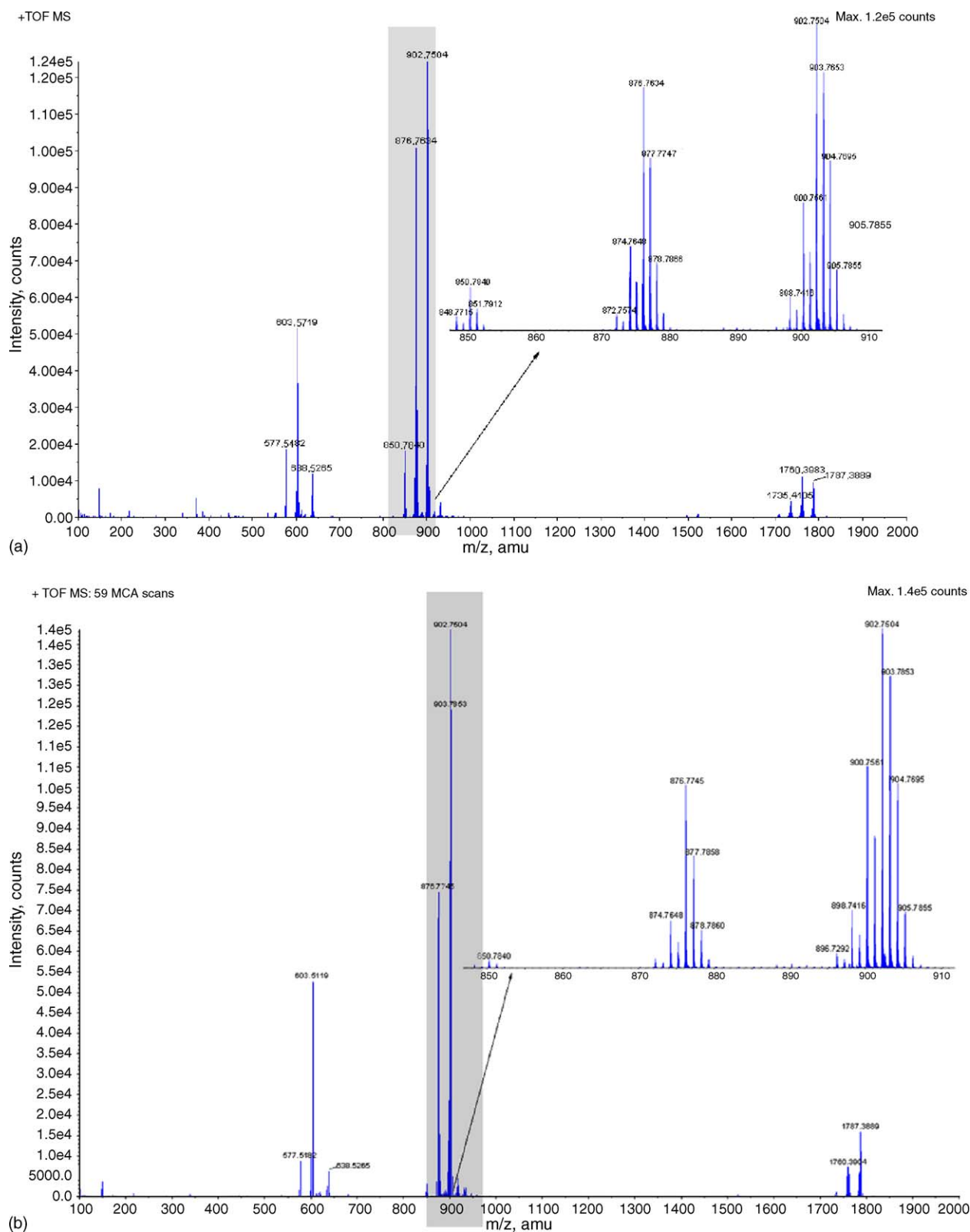


Fig. 1. Full-scan ESI-MS: (a) olive oil and (b) hazelnut oil. The region between m/z 800 and 900 is magnified.

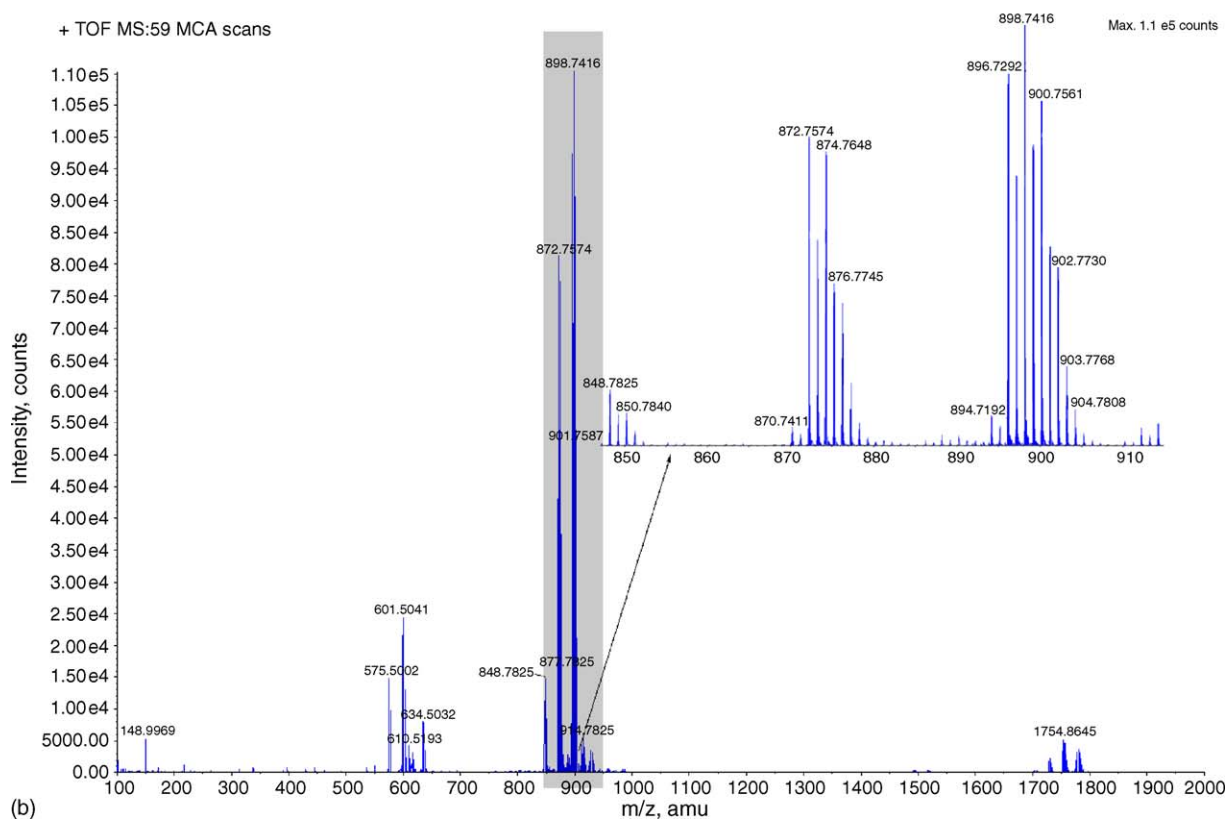
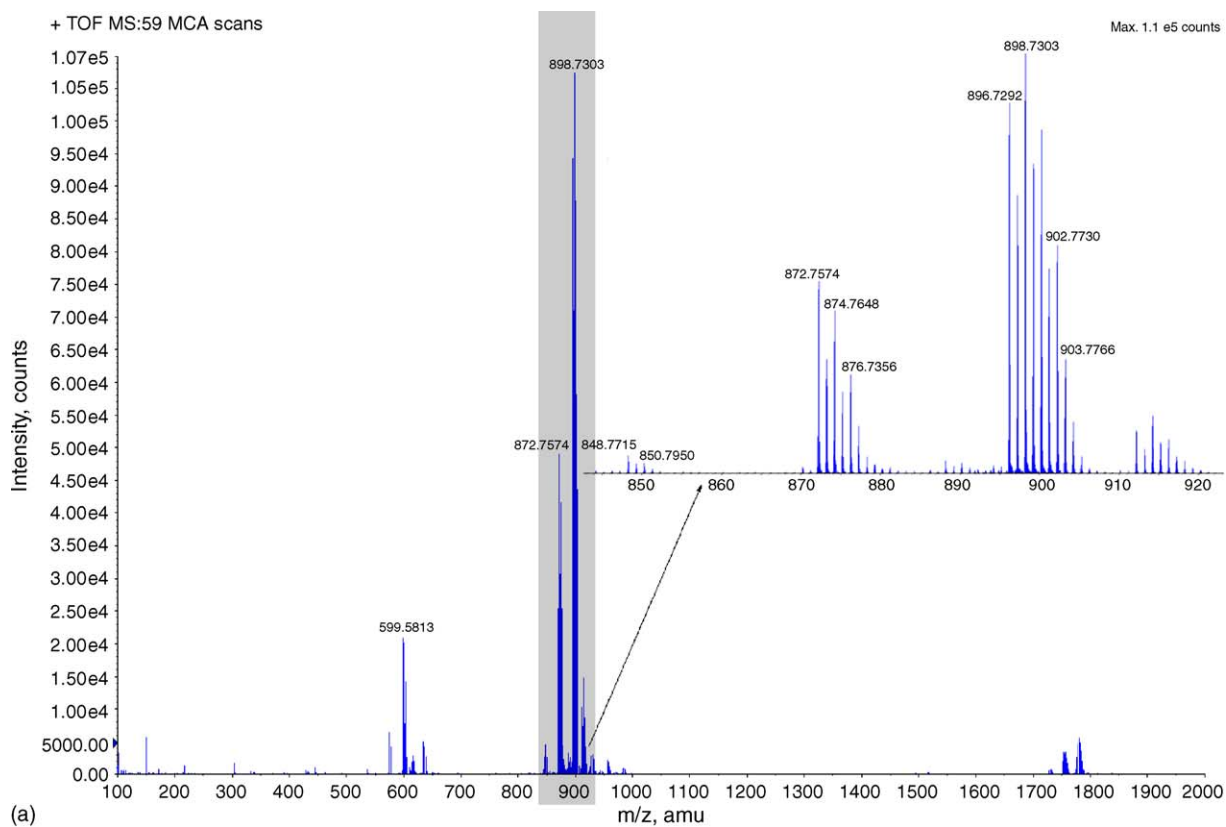


Fig. 2. Full-scan ESI-MS: (a) sunflower oil and (b) corn oil. The region between m/z 800 and 900 is magnified.

analysis (LDA) were applied to the relative abundance of peaks in the mass spectra obtained from both ESI- and APPI-QqTOF.

3. Results and discussion

3.1. ESI and APPI mass spectra

ESI-MS spectrum (Fig. 1) of olive oil is dominated by a peak at m/z 903 that can be matched with a similar peak in the hazelnut oil spectrum. This peak can be attributed to the ammonium adduct $[M + \text{NH}_4]^+$ of triolein (trioleoylglycerol, OOO), which is the most abundant triacylglycerol in both olive and hazelnut oils [13]. The spectra of both edible oils are very similar (Fig. 1), although the peak at m/z 897, caused by trilinoleoylglycerol (LLL), is only detected in hazelnut oil and can be used as marker for olive oil adulteration assessment [17]. For sunflower and corn oils (Fig. 2) the most abundant peak lies at m/z 899 originated by the ammonium adduct of 1,2-dilinoyleyl-3-oleoylglycerol (LLO). Table 1 summarizes the most abundant triacylglycerols in the different oils under study.

Fig. 2 shows that LLL ammonium adducts (m/z 897) are the second prominent peak in the APPI-MS spectra of sunflower and corn oils. An additional difference between these oils is the presence in sunflower of 1,2-dilinoyleyl-3-behenoylglycerol (BLL), m/z 957, Table 1.

The m/z values of significant peaks in the MS/MS spectra from above mentioned ions are shown in Table 2. The major daughter ions from the precursor ion at m/z 903 (OOO) are observed at m/z 603 $[\text{OO}]^+$ and m/z 265 acylium ion of the oleic acid fatty acyl, in both olive and hazelnut oils. The MS/MS spectrum of LLL, characteristic in hazelnut oil shows two peaks at m/z 599 $[\text{LL}]^+$ and m/z 263 acylium ion of the linoleic acid

Table 2

Masses of the characteristic fragment ions A and fragment ions C of triacylglycerols using the ESI-MS/MS

TAGs	Ion A	$[M - \text{RCO}]^+ (m/z)$	Ion C	$[\text{RCO}]^+ (m/z)$
OOO	OO	603	O	265
LLL	LL	599	L	263
LLO	OL	601	P	239
PPO	PO	577	Po	237
POPo	PoO	575	Ln	261
PLO	PL	575	B	323
LLLn	LLn	597	S	267
BLL	BL	659	–	–
SOP	SO	605	–	–

fatty acyl. In sunflower and corn oils the MS/MS spectrum of the precursor ion m/z 899 LLO shows two daughter ions at m/z 601 $[\text{LO}]^+$ and m/z 263 acylium ion previously mentioned. The MS/MS spectrum of BLL in sunflower oil exhibits ions at m/z 660 $[\text{BL}]^+$, m/z 599 $[\text{LL}]^+$ and m/z 263 this latter above mentioned.

Results from the full-scan APPI- and ESI-MS confirm the presence of $[M + \text{H}]^+$, $[M]^{+\bullet}$, $[\text{R}_i\text{CO}]^+$ and $[\text{R}_i\text{CO} - \text{H}_2\text{O}]^+$ ions in the former [14] and $[M + \text{NH}_4]^+$ adducts in the later [13]. The higher abundance of diacylglycerol (DAG) and monoacylglycerol (MAG) in the APPI-MS [14] than in ESI-MS (Fig. 3) is also remarkable.

Fig. 4 shows the full-scan APPI mass spectra for the sunflower and corn oils, which exhibit significant differences with those corresponding to olive and hazelnut oils. The most abundant peak from sunflower and corn oils using the APPI source is that from monoacylglycerol at m/z 337 $[\text{L}]^+$, while in olive and hazelnut oils (Fig. 5), is the m/z 339 $[\text{O}]^+$.

Table 1

Mains TAGs identified in the ESI-QqTOF mass spectra of four vegetables oils

TAGs	$[M + \text{NH}_4]^+ (m/z)$	Olive	Hazelnut	Sunflower	Corn
OOO	903	✓	✓	✓	✓
POO	877	✓	✓	✓	✓
LOO	901	✓	✓	✓	✓
PLO	875	✓	✓	✓	✓
PPO	851	✓	n.d	✓	✓
OOS	905	✓	✓	✓	✓
LLO	899	✓	✓	✓	✓
POPo	848	✓	n.d	✓	✓
LLL	897	n.d	✓	✓	✓
LLP	873	✓	✓	✓	✓
LLLn	895	n.d	n.d	✓	✓
LnLP	871	n.d	n.d	✓	✓
SOP	879	✓	✓	✓	✓
BLL	956	n.d	n.d	✓	n.d

OOO, 1,2,3-trioleoylglycerol; POO, dioleoyl-1-palmitoylelglycerol; LOO, 2,3-dioleoyl-1-linoleoylglycerol; PLO, 1-palmitoyl-2-linoleoyl-3-oleoylglycerol; PPO, 1,2-dipalmitoyl-3-stearoylglycerol; OOS, 1,2-dioleoyl-3-stearoylglycerol; LLO, 1,2-dilinoyleyl-3-oleoylglycerol; POPo, 1-palmitoyl-2-oleyl-3-palmitoylelglycerol; LLL, 1,2,3-trilinoylelglycerol; LLP, 1,2-dilinoyleyl-3-palmitoylelglycerol; LLLn, 1,2-dilinoyleyl-3-linolenylelglycerol; LnLP, 1-linolenyl-2-linoleyl-3-palmitoylelglycerol; SOP, 2-oleyl-3-palmitoyl-1-stearoylglycerol and BLL, 1,2-dilinoyleyl-3-behenoylglycerol. ✓, detected; n.d, not detected.

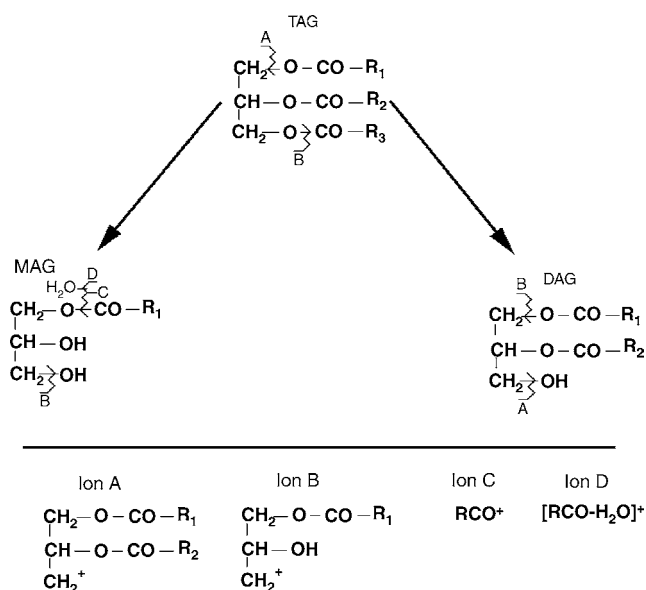


Fig. 3. Fragmentation of triacylglycerols (TAGs), diacylglycerols (DAGs) and monoacylglycerols (MAGs), using positive ion APPI. A–D are the most important fragment ions.

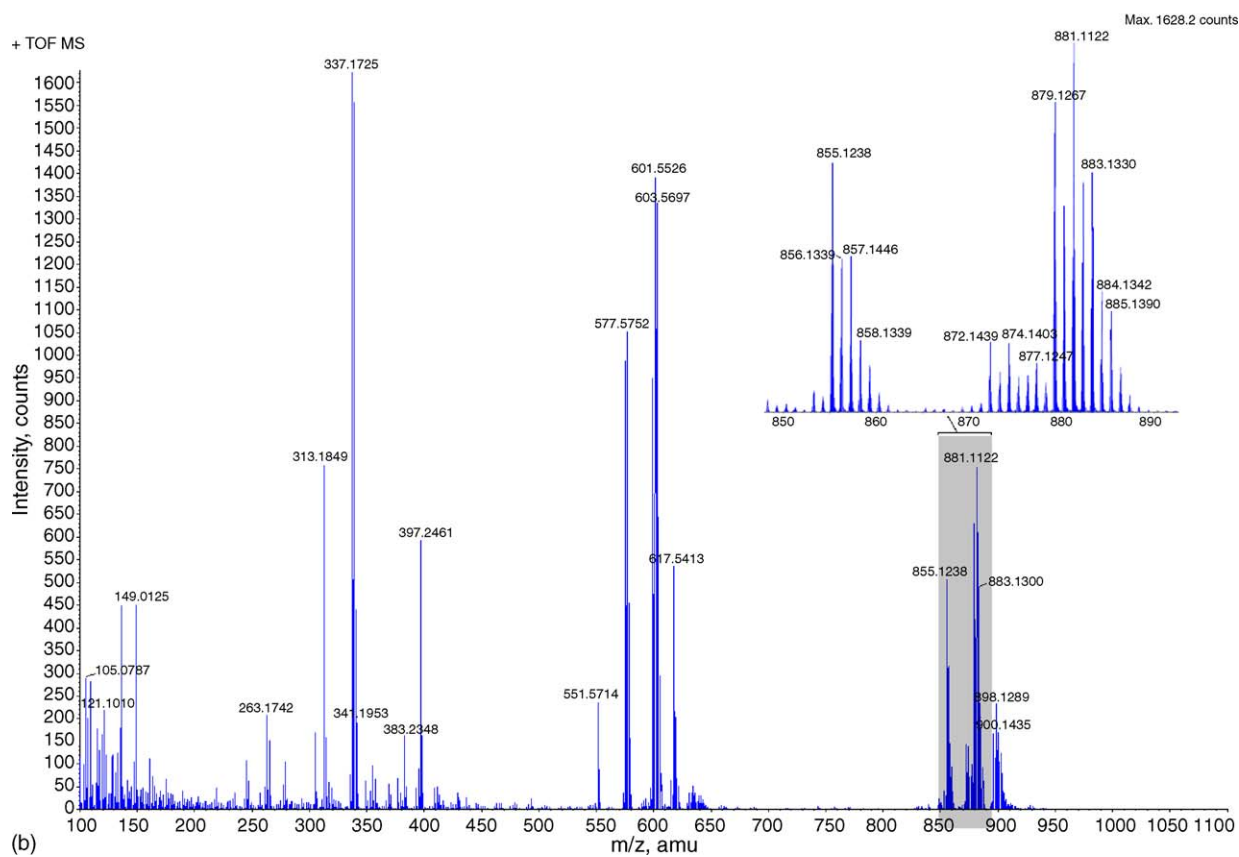
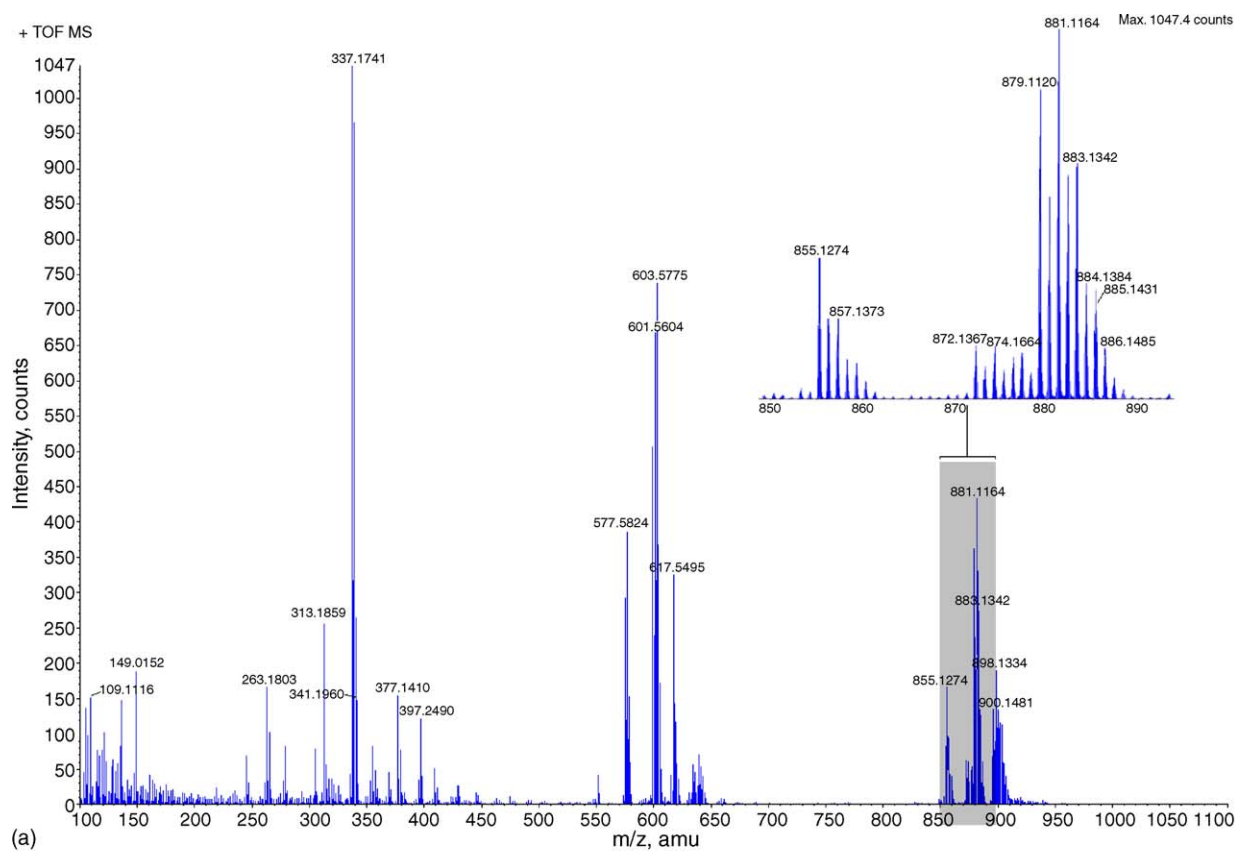


Fig. 4. Full-scan APPI-MS: (a) sunflower oil and (b) corn oil. The region between m/z 800 and 900 is magnified.

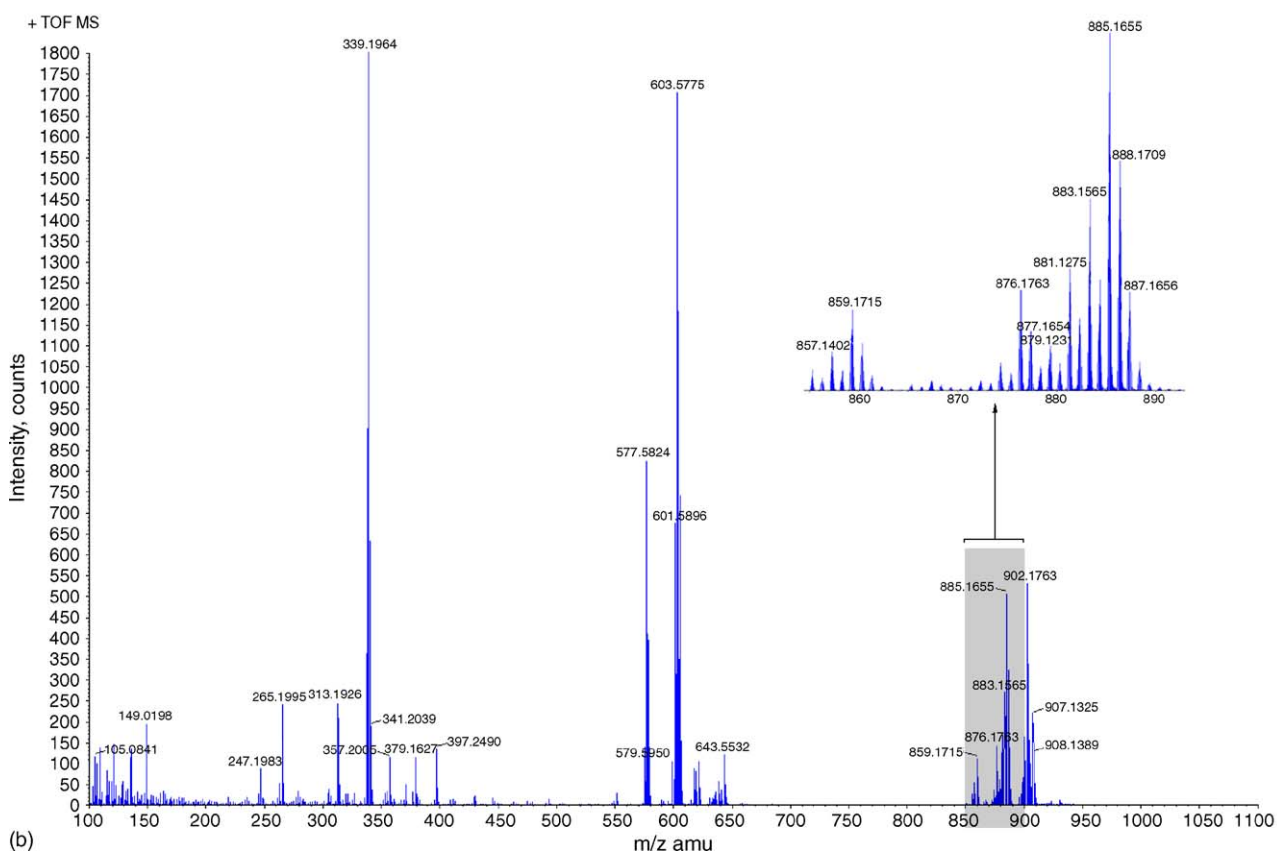
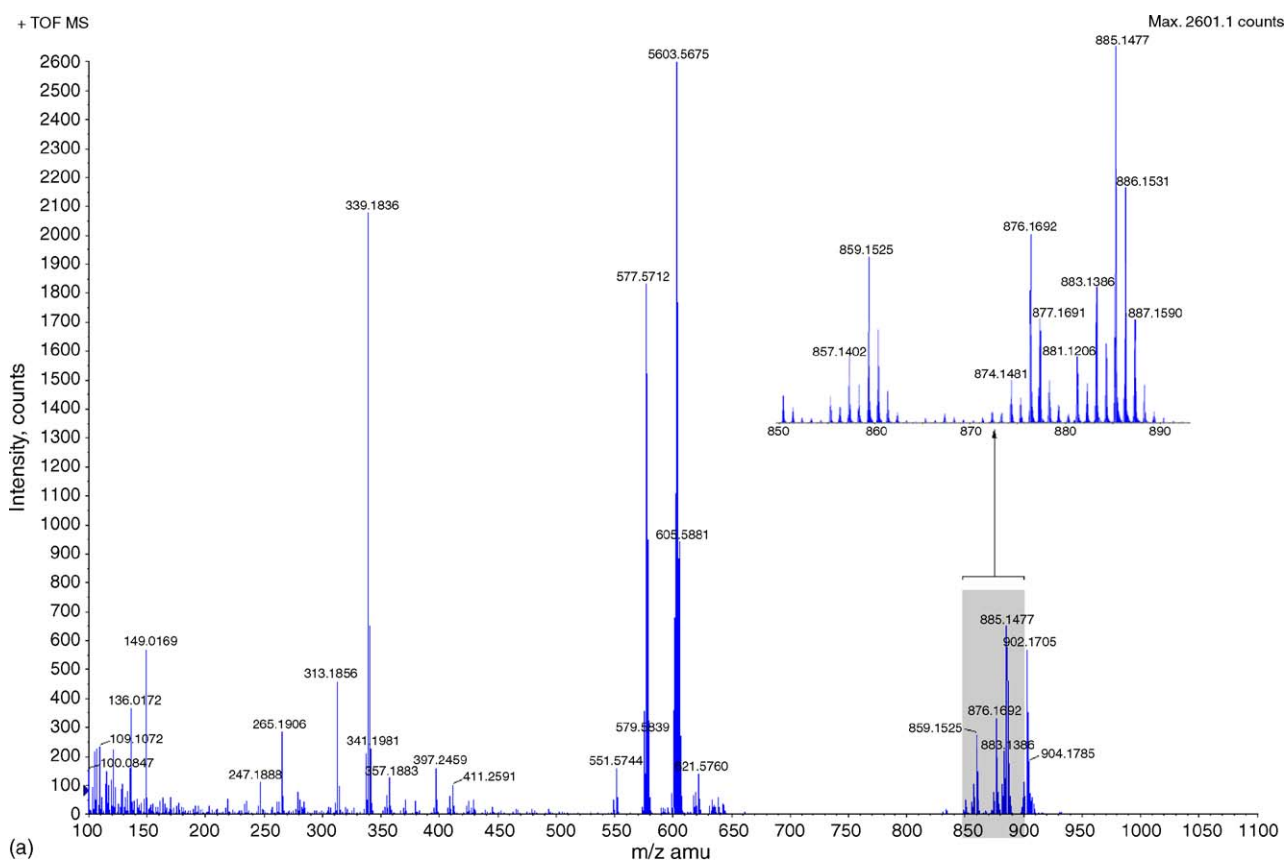


Fig. 5. Full-scan APPI-MS: (a) olive oil and (b) hazelnut oil. The region between m/z 800 and 900 is magnified.

Table 3
Relative abundance TAG (%) of hazelnut, sunflower, corn, olive oil and their mixtures obtained from the ESI-QqTOF

Sample	OOO	POO	LOO	PLO	PPO	OOS	LLO	POPo	LLL	LLP	LLLn	LnLP	SOP	BLL
O	26.92 ± 0.52	21.74 ± 0.19	11.76 ± 0.22	7.77 ± 0.18	3.94 ± 0.07	15.32 ± 0.06	3.13 ± 0.09	1.34 ± 0.03	n.d	1.52 ± 0.02	0.07 ± 0.05	0.16 ± 0.01	5.98 ± 0.31	n.d
H	31.16 ± 0.63	16.57 ± 0.14	18.23 ± 0.20	4.35 ± 0.13	0.34 ± 0.06	16.91 ± 0.19	5.37 ± 0.13	n.d	1.44 ± 0.07	0.89 ± 0.01	0.18 ± 0.01	n.d	3.54 ± 0.18	0.16 ± 0.01
S	11.45 ± 0.03	5.01 ± 0.01	17.31 ± 0.04	8.23 ± 0.04	0.54 ± 0.03	2.59 ± 0.02	21.01 ± 0.13	0.88 ± 0.01	18.71 ± 0.09	9.72 ± 0.08	0.41 ± 0.01	0.33 ± 0.06	0.86 ± 0.07	2.94 ± 0.07
C	7.95 ± 0.23	6.26 ± 0.09	15.12 ± 0.01	12.75 ± 0.14	1.44 ± 0.01	1.66 ± 0.05	18.47 ± 0.30	2.43 ± 0.04	16.32 ± 0.05	13.52 ± 0.10	1.28 ± 0.02	0.80 ± 0.01	0.99 ± 0.03	0.27 ± 0.06
O:H (90:10)	28.64 ± 0.20	19.96 ± 0.48	8.75 ± 0.10	4.65 ± 0.05	2.32 ± 0.02	12.82 ± 0.13	2.18 ± 0.09	0.84 ± 0.01	0.39 ± 0.03	0.99 ± 0.01	n.d	0.10 ± 0.03	4.27 ± 0.01	0.11 ± 0.07
O:H (70:30)	29.87 ± 0.28	18.84 ± 0.23	9.33 ± 0.05	4.26 ± 0.03	2.02 ± 0.02	12.93 ± 0.16	2.33 ± 0.04	0.71 ± 0.02	0.44 ± 0.01	0.89 ± 0.01	n.d	0.10 ± 0.13	4.13 ± 0.19	0.13 ± 0.02
O:H (50:50)	30.21 ± 0.33	16.78 ± 0.29	10.61 ± 0.03	3.77 ± 0.03	1.58 ± 0.02	13.04 ± 0.14	2.58 ± 0.02	0.41 ± 0.14	0.59 ± 0.02	0.80 ± 0.14	n.d	0.35 ± 0.02	3.46 ± 0.08	0.14 ± 0.02
O:S (90:10)	34.58 ± 0.67	18.47 ± 0.07	10.52 ± 0.18	5.34 ± 0.09	2.05 ± 0.02	10.68 ± 0.11	7.04 ± 0.01	0.82 ± 0.04	3.93 ± 0.05	2.22 ± 0.07	n.d	0.14 ± 0.03	3.67 ± 0.01	0.55 ± 0.20
O:S (70:30)	28.17 ± 0.11	14.79 ± 0.18	12.50 ± 0.04	5.60 ± 0.03	1.67 ± 0.05	8.31 ± 0.16	12.35 ± 0.03	0.81 ± 0.01	8.08 ± 0.05	3.48 ± 0.04	0.17 ± 0.01	0.17 ± 0.01	2.88 ± 0.07	1.02 ± 0.03
O:S (50:50)	23.23 ± 0.10	11.59 ± 0.31	14.18 ± 0.07	5.78 ± 0.06	1.28 ± 0.04	6.64 ± 0.10	16.42 ± 0.18	0.73 ± 0.01	11.55 ± 0.13	4.47 ± 0.06	0.21 ± 0.01	0.19 ± 0.01	2.35 ± 0.03	1.37 ± 0.03
O:C (90:10)	34.61 ± 0.27	19.41 ± 0.08	9.74 ± 0.11	6.25 ± 0.06	2.34 ± 0.07	11.18 ± 0.04	5.41 ± 0.03	1.07 ± 0.01	2.80 ± 0.02	2.77 ± 0.03	0.23 ± 0.01	0.20 ± 0.01	3.79 ± 0.10	0.21 ± 0.01
O:C (70:30)	25.44 ± 5.11	14.63 ± 0.19	11.65 ± 0.05	8.25 ± 0.05	1.96 ± 0.05	7.56 ± 0.09	11.52 ± 0.14	1.35 ± 0.03	7.59 ± 0.05	6.08 ± 0.05	0.49 ± 0.05	0.33 ± 0.01	2.72 ± 0.01	0.44 ± 0.12
O:C (50:50)	24.12 ± 0.07	13.58 ± 0.12	12.05 ± 0.01	8.55 ± 0.07	1.85 ± 0.01	6.98 ± 0.04	12.41 ± 0.06	1.35 ± 0.01	8.50 ± 0.07	6.67 ± 0.09	0.54 ± 0.01	0.38 ± 0.02	2.54 ± 0.12	0.48 ± 0.02

Olive oil (O); hazelnut oil (H); sunflower oil (S); corn oil (C); olive oil–hazelnut oil (O:H) 90:10; olive oil–hazelnut oil (O:H) 70:30; olive oil–hazelnut oil (O:H) 50:50; olive oil–sunflower (O:S) 90:10; olive oil–sunflower oil (O:S) 70:30; olive oil–sunflower oil (O:S) 50:50; olive oil–corn oil (O:C) 90:10; olive oil–corn oil (O:C) 70:30; olive oil–corn oil (O:C) 50:50. Average of three determinations ± standard deviation (S.D.). n.d, not detected.

Table 4
Fragment ions of mono-, di-, triacylglycerols and acylium ions $[R_iCO]^+$ relative abundance (%) the mass spectra obtained from APPI-QqTOF, for hazelnut, sunflower, corn, olive oil and their mixture

Sample	OOO	LLL	LLO	OO	OL	LL	OP	L	P	O	Ln	$[RCO]^+ O$	$[RCO]^+ L$	$[RCO-H_2O]^+ O$	$[RCO-H_2O]^+ L$
O	5.14 ± 0.45	n.d	1.84 ± 0.13	23.75 ± 1.17	8.67 ± 0.50	1.64 ± 0.17	16.32 ± 0.70	5.01 ± 0.47	6.24 ± 0.22	25.15 ± 0.02	0.38 ± 0.16	3.22 ± 0.05	0.66 ± 0.04	1.21 ± 0.04	0.41 ± 0.04
H	6.23 ± 0.13	0.57 ± 0.01	1.45 ± 0.04	28.88 ± 0.08	8.43 ± 0.09	1.05 ± 0.06	11.41 ± 0.22	4.57 ± 0.12	3.40 ± 0.18	28.31 ± 0.41	0.19 ± 0.02	3.29 ± 0.03	0.62 ± 0.01	1.22 ± 0.01	0.37 ± 0.01
S	1.95 ± 0.20	4.82 ± 0.07	5.97 ± 0.16	14.56 ± 0.71	12.11 ± 0.53	8.55 ± 0.43	6.68 ± 0.11	17.59 ± 0.53	4.10 ± 0.43	17.32 ± 0.11	0.49 ± 0.53	1.77 ± 0.15	2.22 ± 0.62	0.69 ± 0.02	1.22 ± 0.02
C	1.37 ± 0.09	4.84 ± 0.21	5.95 ± 0.16	11.82 ± 0.42	13.60 ± 0.20	8.70 ± 0.01	9.18 ± 0.36	16.92 ± 0.07	6.75 ± 0.38	15.09 ± 0.18	0.65 ± 0.01	1.41 ± 0.01	2.03 ± 0.01	0.57 ± 0.02	1.11 ± 0.03
O:H1	4.37 ± 0.15	0.38 ± 0.17	1.45 ± 0.09	25.72 ± 0.17	6.17 ± 0.03	1.00 ± 0.09	15.19 ± 0.38	3.68 ± 0.07	5.44 ± 0.01	27.29 ± 0.09	0.30 ± 0.01	2.83 ± 0.01	0.53 ± 0.03	0.95 ± 0.01	0.32 ± 0.01
O:H2	5.15 ± 0.12	0.56 ± 0.01	1.10 ± 0.02	27.06 ± 0.53	6.75 ± 0.18	0.78 ± 0.02	13.87 ± 0.06	3.98 ± 0.03	5.09 ± 0.06	29.12 ± 0.21	0.24 ± 0.01	3.92 ± 0.10	0.62 ± 0.02	1.40 ± 0.03	0.35 ± 0.01
O:H3	5.43 ± 0.03	0.60 ± 0.19	0.99 ± 0.01	29.04 ± 0.19	6.93 ± 0.23	0.83 ± 0.05	13.10 ± 0.35	4.02 ± 0.05	5.02 ± 0.05	30.70 ± 0.35	0.23 ± 0.01	4.32 ± 0.08	0.64 ± 0.01	1.56 ± 0.02	0.38 ± 0.01
O:S1	4.60 ± 0.24	0.44 ± 0.02	1.31 ± 0.01	27.87 ± 0.24	6.62 ± 0.06	1.03 ± 0.02	16.20 ± 0.11	3.57 ± 0.05	5.36 ± 0.15	27.14 ± 0.36	0.27 ± 0.01	3.35 ± 0.02	0.59 ± 0.01	1.28 ± 0.02	0.37 ± 0.03
O:S2	3.68 ± 0.09	2.07 ± 0.06	3.08 ± 0.07	22.02 ± 0.54	8.35 ± 0.25	3.71 ± 0.27	13.92 ± 0.03	8.35 ± 0.36	5.33 ± 0.02	23.40 ± 0.24	0.33 ± 0.06	2.92 ± 0.01	1.08 ± 0.01	1.12 ± 0.05	0.64 ± 0.41
O:S3	3.63 ± 0.35	2.97 ± 0.04	4.04 ± 0.01	20.11 ± 0.70	9.24 ± 0.22	5.01 ± 0.28	12.85 ± 0.10	10.20 ± 0.28	5.08 ± 0.04	20.87 ± 0.31	0.43 ± 0.14	2.54 ± 0.02	1.30 ± 0.01	0.98 ± 0.01	0.73 ± 0.01
O:C1	4.11 ± 0.01	0.53 ± 0.03	1.37 ± 0.04	25.68 ± 0.08	6.83 ± 0.04	1.30 ± 0.03	16.66 ± 0.04	4.10 ± 0.08	6.15 ± 0.03	26.99 ± 0.10	0.31 ± 0.01	3.60 ± 0.02	0.61 ± 0.02	1.36 ± 0.10	0.39 ± 0.10
O:C2	4.10 ± 0.06	1.86 ± 0.11	2.67 ± 0.05	22.98 ± 0.31	8.74 ± 0.18	3.54 ± 0.17	15.39 ± 0.02	7.14 ± 0.25	5.77 ± 0.05	21.92 ± 0.29	0.34 ± 0.01	2.91 ± 0.07	0.98 ± 0.02	1.09 ± 0.02	0.57 ± 0.01
O:C3	3.37 ± 0.09	2.45 ± 0.04	3.36 ± 0.01	20.99 ± 0.24	9.79 ± 0.10	4.65 ± 0.08	14.26 ± 0.07	9.24 ± 0.22	5.89 ± 0.06	20.15 ± 0.17	0.39 ± 0.01	2.56 ± 0.07	1.23 ± 0.04	0.96 ± 0.02	0.70 ± 0.01

Olive oil (O); hazelnut oil (H); sunflower oil (S); corn oil (C); olive oil–hazelnut oil (O:H1) 90:10; olive oil–hazelnut oil (O:H2) 70:30; olive oil–hazelnut oil (O:H3) 50:50; olive oil–sunflower (O:S1) 90:10; olive oil–sunflower oil (O:S2) 70:30; olive oil–sunflower oil (O:S3) 50:50; olive oil–corn oil (O:C1) 90:10; olive oil–corn oil (O:C2) 70:30; olive oil–corn oil (O:C3) 50:50. Fatty acid abbreviations: O, oleic (18:1), L, linoleic (18:2), P, palmitic (16:0), Ln, linolenic (18:3). Average of three determinations ± standard deviation (S.D.). n.d, not detected.

3.2. Oil mixtures study

In order to assess the possible adulteration of olive oil with the previously studied vegetable oils, several mixtures were performed as previously described (Section 2.1). As can be seen in Table 3, several triacylglycerols can be used as markers of the presence of hazelnut in olive oil, like OOO and LOO, when the ESI source is used. These compounds are present in both olive and hazelnut oils, although their relative abundance in the mass spectra is higher in the last (OOO, 31.16%; LOO, 18.23%) than those from olive oil (OOO, 26.92%; LOO, 11.76%). This difference can be appreciated in mixtures with hazelnut oil at percentages lower than 10%. In addition, LLL, which is not present in olive oil, is at 1.44% in the mass spectra from hazelnut oil and for this reason it can also be used to identify this adulteration. The adulteration of olive oil with sunflower and corn oils can be detected on the basis of LLO, which is the most abundant triacylglycerol in the formers. Due to the low relative abundance of LLO in olive oil mass spectra, an increase of this peak can be used for this purpose. In this way, BLL is only present in a measurable abundance in sunflower oil.

When the APPI source is used (Table 4) significant differences can be pointed out between hazelnut and olive oils. Hazelnut oil mass spectra exhibit a higher relative abundance of OOO (6.23%), the fragment ion of DAG [OO]⁺ (28.88%) and of MAG [O]⁺ (28.31%). As commented previously, the presence of LLL in sunflower and corn oils and its absence in olive oil, can also be detected using the APPI source. Analogously, [LL]⁺ and [L]⁺ appear in the mass spectra of sunflower and corn oils with a higher relative abundance. Mixtures of olive oil with them are in good agreement with the previous results for the ESI source.

The APPI source allows more sensitive detection of monoacyl- and diacylglycerol fragment ions than ESI, which confers to the former additional advantages. However, both sources are complementary, since ESI is more suitable for the detection of triacylglycerols.

3.3. Principal component analysis

For a better understanding of the discriminating efficiency of the descriptors selected, a preliminary study based on principal component analysis has been carried out, despite of the a priori knowledge the class membership of the oil samples. PCA finds the maximum variations in the data set and forms new variables known as principal components (PCs) [18], such that each successive PC accounts for as much of the remaining variability as possible and each new variable must be totally independent of all other variables. In this case, PCA was applied to our data calculated according to the relative abundance (percentage) of each peak in the TIC, as a semiquantitative method.

For the ESI source, the abundance of TAG adducts (OOO, POO, LOO, PLO, PPO, OOS, LLO, POPo, LLL, LLP, LLLn, LnLP, SOP and BLL) in mass spectra of the oils were considered as variables, and each type of oil or mixture as a class. From the loadings of original variables in the two first considered principal components (Table 5), the dominant variables in the first principal component (PC1) representing 70.56% of the total variance are POO, LLO, LLL, LLP, OOS and OOO while PPO, POPo and LOO show the lower values of variance. The variables appear at negative and positive values of PC1 and PC2. From this fact, it could be inferred that they provide different type of information. Principal component two (PC2) explains up to 18.04% of the total variance. Examining the loadings PPO and POPo are the most dominant variables in PC2. The first two principal components account for 88.6% of the total variance and were considered to be sufficient for this study.

The resulting ordination plot from PCA on these oils and their mixtures samples with ESI source is shown in Fig. 6. It can be seen even from this simple PCA that clear separation in PCA space occurs between the three oils (hazelnut, sunflower and corn oil), which are clearly separated from olive oil. Moreover, the principal components analysis showed that the mixtures

Table 5
Loadings of the variables for the two first principal components (PC's) using ESI and APPI sources

ESI			APPI		
Variable	PC1	PC2	Variable	PC1	PC2
OOO	−0.292	0.047	OOO	−0.255	−0.149
POO	−0.304	−0.159	LLL	0.277	−0.066
LOO	0.195	0.296	LLO	0.279	−0.031
PLO	0.260	−0.340	OO	−0.272	−0.089
PPO	−0.168	−0.462	OL	0.268	−0.033
OOS	−0.295	0.018	LL	0.280	−0.017
LLO	0.302	0.121	OP	−0.216	0.475
POPo	0.199	−0.483	L	0.279	−0.083
LLL	0.308	0.097	P	0.043	0.793
LLP	0.310	−0.121	O	−0.270	−0.132
LLLn	0.275	−0.243	Ln	0.262	0.252
LnLP	0.248	−0.289	[RCO] ⁺ oleic acid	−0.259	−0.041
SOP	−0.288	−0.185	[RCO] ⁺ linoleic acid	0.277	−0.097
BLL	0.239	0.322	[RCO-H ₂ O] ⁺ oleic acid	−0.252	−0.030
			[RCO-H ₂ O] ⁺ linoleic acid	0.277	−0.083

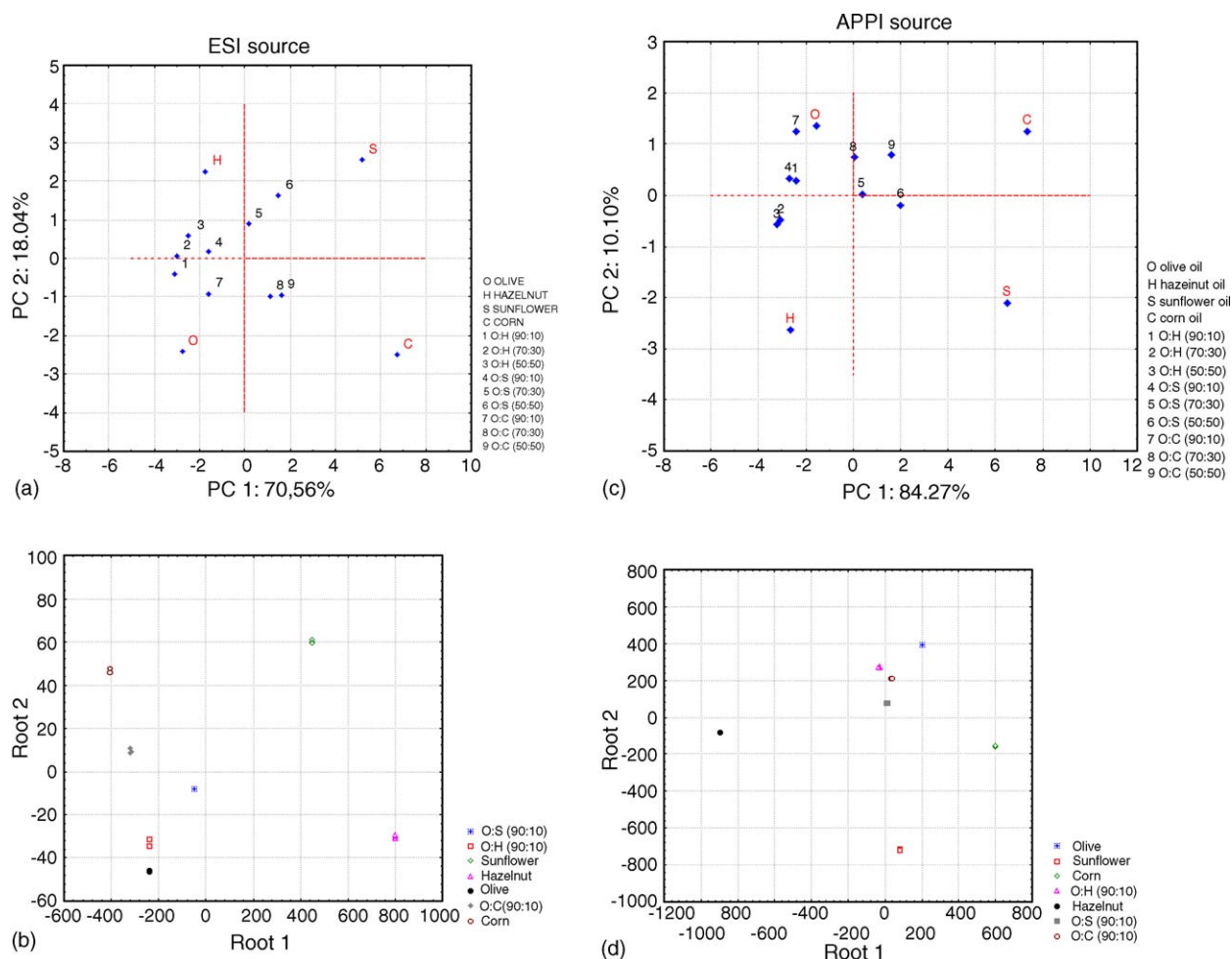


Fig. 6. Principal components analysis for (a) ESI-MS and (c) APPI-MS. Linear discriminant analysis for (b) ESI-MS and (d) APPI-MS.

(90% olive oil with 10% hazelnut and 90% olive oil with 10% corn oil) are the nearest in the PCA space to olive oil.

Similarly, PCA was applied to the data obtained with the APPI source. The relative abundance in the mass spectra corresponding to compounds, fragments and acylium ions (Table 4) from the different oils were considered as variables, and each type of oil or mixtures of them as classes.

Table 5 shows the first two principal components that were extracted. These typically accounted for >94% of the overall variance. Examining the score plot of the objects in the space defined by the two principal components considered (Fig. 6), it can be observed an evident separation of the four oils and their mixtures. These results were in good agreement with those obtained by the ESI source.

3.4. Linear discriminant analysis

Unsupervised techniques only have visualizing capabilities, so a supervised pattern recognition method such as linear discriminant analysis was performed with the purpose of obtaining classification rules to assign categories to samples.

After applying LDA, four discriminant functions were calculated. Fig. 6 shows the scatter plot of oils samples in the plane defined by the discriminant functions DF_1 and DF_2 . Seven differ-

ent classes were correctly separated (olive oil, hazelnut oil, sunflower oil, corn oil and their corresponding mixture 90:10 with olive oil). We analyzed the mixture 90:10 of different oils with olive oil, because this adulteration is the most difficult to detect.

LDA using the relative abundance of TAGs as the variables showed that oils and their mixtures were clearly grouped according to their triacylglycerols composition. These results are consistent with other previously published data related to olive oil [19].

Moreover, the pattern recognition method shows that the reproducibility of the analysis is very high, especially with the APPI source, and that olive oil can be clearly separated from other edible oils which could be used as adulterants.

4. Conclusions

The use of both ESI- and APPI-QqTOF represents a good alternative for edible oils fingerprinting. These couplings involving powerful ionization sources and highly discriminant mass analyzers, namely QqTOF-MS, provide a high resolution for multi-component matrices such as vegetable oils. These instrumental systems enable a very simple sample handling and high sample throughput, because no chromatographic separation is required.

ESI and APPI sources are complementary on the basis of the higher sensitivity of ESI for triacylglycerols and APPI for monoacyl- and diacylglycerol fragment ions. Reproducibility of the analysis is very high but APPI provides better results. In addition, some fragments such as $[R_i\text{CO}-\text{H}_2\text{O}]^+$, corresponding to the lost of water from the acylium ion only can be detected by APPI-MS.

Both ESI- and APPI-QqTOF can be used for olive oil adulteration diagnosis, because the discrimination from commonly used adulterants is allowed. The PCA of olive oil permits a safe evaluation of its authenticity and provides a precise classification of other edible oils and their mixtures. The application of LDA verifies methods reproducibility, especially with the APPI source, and gives additional evidences of the presence of adulterants.

Acknowledgements

The authors are indebted to “Ministerio de Ciencia y Tecnología (MCyT)” for the Grant REN2002-04366-C02-02. We thank Applied Biosystems for technical assistance with APPI-MS.

References

- [1] R. Aparicio, J. Harwood (Eds.), *Handbook of Olive Oil Analysis and Properties*, Aspen Publisher Inc., Gaithersburg, Maryland, 2000.
- [2] C. Rodríguez-Villar, A. Pérez-Heras, I. Mercade, E. Casals, E. Ros, *Diabet. Med.* 21 (2004) 142.
- [3] C. Puel, A. Quitin, A. Agalias, J. Mathey, C. Obled, A. Mazur, M.J. Davicco, P. Lebecque, A.L. Skaltsounis, V. Coxam, *Br. J. Nutr.* 92 (2004) 119.
- [4] K. Tuovinen, *Toxicology* 196 (2004) 31.
- [5] J.R. Huertas, E. Martínez-Velasco, S. Ibanez, M. Lopez-Frias, J.J. Ochoa, J. Quiles, G.P. Castelli, J. Mataix, G. Lenaz, *Biofactors* 9 (1999) 337.
- [6] L. Cercaci, M.T. Rodríguez-Estrada, G. Lercker, *J. Chromatogr. A* 985 (2003) 211.
- [7] R. Aparicio, R. Aparicio-Ruiz, *J. Chromatogr. A* 881 (2000) 93.
- [8] A.D. Shaw, A. Di Camillo, G. Vlahov, A. Jones, G. Bianchi, J.J. Rowland, D.B. Kell, *Anal. Chim. Acta* 348 (1997) 357.
- [9] V. Baeten, M. Meurens, M.T. Morales, R. Aparicio, *J. Agric. Food Chem.* 44 (1996) 2225.
- [10] H. Yang, J. Irudayaraj, *J. Am. Oil Chem. Soc.* 78 (2001) 889.
- [11] G. Vlahov, A.D. Shaw, D.B. Kell, *J. Am. Oil Chem. Soc.* 76 (1999) 1223.
- [12] I.M. Lorenzo, J.L.P. Pavon, M.E.F. Laespada, C.G. Pinto, B.M. Cordero, *J. Chromatogr. A* 945 (2002) 221.
- [13] R. Goodacre, S. Vaidyanathan, G. Bianchi, D.B. Kell, *Analyst* 127 (2002) 1457.
- [14] J.L. Gómez-Ariza, A. Arias-Borrego, T. García-Barrera, *Rapid Commun. Mass Spectrom.*, submitted for publication.
- [15] D.B. Roob, T.R. Covey, A.P. Bruins, *Anal. Chem.* 72 (2000) 3653.
- [16] J.A. Syage, M.D. Evans, *Spectroscopy* 16 (2001) 15.
- [17] J. Parcerisa, I. Casals, J. Boatella, R. Codony, M. Rafecas, *J. Chromatogr. A* 881 (2000) 149.
- [18] S. Wold, K. Esbensen, P. Geladi, *Chemom. Intell. Lab. Syst.* 2 (1987) 37.
- [19] P. Kaufman, *Anal. Chim. Acta* 277 (1993) 467.

Accuracy profiles from uncertainty measurements

A. Gustavo González*, M. Ángeles Herrador

Department of Analytical Chemistry, University of Seville, Spain

Received 19 September 2005; received in revised form 29 December 2005; accepted 8 February 2006

Available online 20 March 2006

Abstract

Accuracy profiles of chemical assays are introduced and derived from the uncertainty of the analytical result. The calculation of accuracy profiles is based on the estimation of the measurement uncertainty of the analytical assay from validation data. For the sake of illustration, a case study dealing with the spectrofluorimetric determination of quinine in tonic water is explained in detail.

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Keywords: Accuracy profiles; Method validation; Measurement uncertainty

1. Introduction

The concept of accuracy profile was first introduced in the papers of Hubert et al. [1] and Boulanger et al. [2]. Recently, the commission on the validation of analytical procedures of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) have used the accuracy profiles for improving the assessment of accuracy in method validation [3–5]. According to the ISO 5725 definition [6], accuracy expresses the closeness or agreement between a test result (Z) and the accepted reference value ($\hat{Z}$), which is an estimation of the true unknown one (μ). Method validation scrutinizes the accuracy of results by considering both systematic and random errors. Accuracy is, therefore studied as an entity with two components: trueness and precision [7,8]. The accuracy of results relates only to the fitness-for-purpose of an analytical system verified by method validation [9]. Instead of assessing trueness and precision independently, it is possible to assess accuracy in a global way according to the concept of acceptability limit λ [3–5]. Thus, the analytical result Z may differ from the estimation of the unknown ‘true value’ $\hat{Z}$ in an extent less than the acceptability limit:

$$|Z - \hat{Z}| < \lambda \quad (1)$$

This limit λ depends on the goals of the analytical procedure: 1% for bulk materials, 5% for determination of active ingredients in dosage forms, 15% in bio/environmental analysis [5]. A procedure can be validated if it is very likely that the requirement given by (1) is fulfilled; that is

$$P(|Z - \hat{Z}| < \lambda) \geq \beta \quad (2)$$

β being the probability that a future determination fall inside the acceptability limits. Mee [10] computed the called ‘ β -expectation tolerance interval’ (β ETI), that is, the interval of future results that met Eq. (2). The estimation of the bias and the reproducibility is performed from suitable validation standards (VS), prepared in the same matrix as the expected for the future samples. Certified or internal reference materials represent the best way to obtain a validation standard, but spiked samples can be considered as a suitable alternative. The analytical result can be related with the estimation of unknown ‘true value’ according to the bias (δ):

$$\delta = Z - \hat{Z} \quad (3)$$

Assuming that the result Z is the overall mean of a nested scheme where a validation standard is analysed n times (replications) at p different conditions (for example, days – intralaboratory validations – or laboratories – intercollaborative trials), the β ETI is given by [10]:

$$\delta \pm Q_t k_s S_R \quad (4)$$

* Corresponding author. Tel.: +34 954557173; fax: +34 954557168.
E-mail address: agonzale@us.es (A.G. González).

Q_t is the β quantile of the Student's t -distribution and k_s is an expansion factor:

$$k_s = \sqrt{1 + \frac{1}{pnB^2}} \quad (5)$$

With

$$B = \sqrt{\frac{R+1}{nR+1}}; \quad R = \frac{S_B^2}{S_r^2} \quad (6)$$

S_B^2 and S_r^2 are the estimates of the between conditions variance and the within conditions variance (repeatability). The reproducibility variance, S_R^2 is obtained as

$$S_R^2 = S_r^2 + S_B^2 \quad (7)$$

It can be easily demonstrated that

$$k_s^2 S_R^2 = S_R^2 + \frac{nS_B^2 + S_r^2}{np} \quad (8)$$

The right term of the second member of Eq. (8) is the estimation of the variance of the overall mean that can be assimilated to the bias uncertainty for a nested design with p conditions for experiments and n replications within each condition. Thus

$$k_s^2 S_R^2 = S_R^2 + S_\delta^2 = u^2(Z) \quad (9)$$

And β ETI can be given now by

$$\delta \pm Q_t u(Z) \quad (10)$$

The sum of the estimated variances for reproducibility precision and bias can be assimilated to the square of the global uncertainty of the result Z (obtained with a given validation standard). When the conditions are laboratories (collaborative trials), the uncertainty is rightly estimated. In case of intralaboratory validation, commonly, the conditions are associated to 'different days'. In this way, S_R corresponds to the intralaboratory reproducibility or intermediate precision. It should be advisable to consider also the variance contribution of the robustness study in order to estimate the uncertainty due to a laboratory transfer.

β ETI can also be easily derived from the definition of measurement uncertainty and bias. Considering that the analytical procedure is validated using suitable validation standards, the measurement uncertainty $u(Z)$ can be evaluated according to any of the well known approaches: GUM approach or bottom-up [11], top-down inter-laboratory validation [12] or from intra-laboratory validation data [13–15]. The final expression of the analytical result will be

$$Z \pm ku(Z) \quad (11)$$

k being the chosen coverage factor. The reference value $\hat{Z}$ is known for the validation standards used and therefore, the interval for the bias is given by

$$(Z \pm ku(Z)) - \hat{Z} = (Z - \hat{Z}) \pm ku(Z) = \delta \pm ku(Z) \quad (12)$$

The last member of (12) is equivalent to (10) by setting $k = Q_t$. Thus the β ETI can be easily calculated from the uncertainty measurement obtained from validation data.

The initial objective of the accuracy profile was to give to the analyst a practical (and visual) decision tool to help them to accept the validity of their analytical methods with a known risk. The β ETI was thus used with this purpose. As Feinberg et al. pointed out [5], a secondary benefit of this interval was the estimation of the uncertainty. However, the present paper is just focused conversely, in the determination of the accuracy profiles from the uncertainty measurement that is previously estimated from the validation data of the analytical assay.

In the following, the computation of the accuracy profiles from the uncertainty measurement of analytical assays is outlined and explained.

2. Calculation of the accuracy profile from the estimation of the uncertainty of the analytical assay

We are interested to estimate the accuracy profile from the uncertainty measurement of the analytical assay from validation data according the LGC/VAM protocol [16] and the ISO/DTS 21748 guide [17]. The basic model for the uncertainty of the measurand Z , here the quinine concentration in the tonic formulation, is given by three terms for intralaboratory measurements:

$$u^2(Z) = S_R^2 + u^2(\delta) + u_{rob}^2(Z) \quad (13)$$

where S_R is the reproducibility standard deviation (intermediate precision), $u(\delta)$ the uncertainty associated with the bias/trueness of the procedure and u_{rob} is the uncertainty coming from a robustness exercise. The precision study, trueness study and robustness study in cases of formulations can be easily performed through validation standards (VS) prepared in the same matrix of the samples [5,16].

2.1. Intermediate precision and trueness study

Both intermediate precision and trueness study can be performed from the prediction of actual concentrations of the VS according to the analytical assay. An ANOVA by considering the different days as the main variation source is then performed for each concentration. Accordingly, for each concentration level we consider results z_{ij} with two indices: i (from 1 to p) corresponding to the different conditions (here, the days) and j (from 1 to n) accounting for the repetitions. From the ANOVA we can obtain easily [18,19] the estimation of the within-condition variance (S_W^2) and of the between-condition variance (S_B^2). The repeatability variance is taken just as the within-condition one: $S_r^2 = S_W^2$. The intra laboratory reproducibility or intermediate precision can be taken as indicated by Eq. (7).

On the other hand, the bias uncertainty can be estimated from the ISO/DTS 21748 guide [17]:

$$u^2(\delta) = \frac{S_R^2(1 - \gamma + (\gamma)/n)}{p} \quad (14)$$

with $\gamma = S_r^2/S_R^2$.

2.2. Robustness study

Robustness tests can be considered as intra-laboratory simulations of inter-laboratory studies, if the alterations introduced in the influential factors are suitably selected. The robustness study is performed according to the procedure reported by González et al. [16], based on the landmark procedure suggested by Youden [20]:

- (1) Identify the influential factors.
- (2) For each factor, define the nominal and extreme values expected in routine work and encode them as follows: nominal value = 0, high value = +1, low value = −1.
- (3) Arrange the experimental design according to a 2^{7-4} Plackett–Burmann matrix.
- (4) Perform the experiments in random order on VS close to the target value.

The Plackett–Burmann matrix is used to study up to seven factors in eight experiments. The eight runs are split into two groups of four runs on the basis of the levels +1 or −1. The effect of every factor x_k is estimated as the difference of the mean result obtained at level +1 from the obtained at the level −1:

$$D(x_k) = \frac{1}{4} \left(\left(\sum_{i=1}^N Z_i \right)_{(x_k=+1)} - \left(\sum_{i=1}^N Z_i \right)_{(x_k=-1)} \right) \quad (15)$$

Once the effects $D(x_k)$ have been estimated, to determine whether variations have a significant effect on the result, a significance t -test is used [16]:

$$t(x_k) = \frac{\sqrt{2}|D(x_k)|}{S_R} \quad (16)$$

The t -value is compared with the 95% confidence level two-tailed critical one with the degrees of freedom coming from the precision study for each concentration: $\nu = pn - 1$. If $t(x_k) < t_{\text{crit}}$, then the procedure is robust against the factor x_k . In this case, the uncertainty on the measurand Z coming from the factor x_k , $u(Z(x_k))$ is evaluated from [16]:

$$u(Z(x_k)) = \frac{t_{\text{crit}} S_R}{1.96\sqrt{2}} \frac{\delta_{\text{real}}(x_k)}{\delta_{\text{test}}(x_k)} \quad (17)$$

Here δ_{real} is the change in the factor level which would be expected when the method is operating under control routine analysis and δ_{test} is the change in the factor level specified in the robustness study. Once the contributions of the influential factors have been estimated, the relative uncertainty of the robustness study is calculated as

$$\text{RSD}_{\text{rob}} = \sqrt{\frac{\sum_k u^2(Z(x_k))}{\bar{Z}^2}} \quad (18)$$

$\bar{Z}$ is the average value of the eight results obtained in the robustness study. By taking this value for any future concentration Z , the uncertainty of robustness is $u_{\text{rob}}(Z) = Z \text{RSD}_{\text{rob}}$. This leads to the final uncertainty budget according Eq. (13). The expanded

uncertainty is obtained as the product of the standard uncertainty $u(Z)$ and the coverage factor k selected as the β quantile of the Student's t distribution.

2.3. Accuracy profiles

Once the uncertainty of Z is obtained, and remembering the conditions (1) and (2), the corresponding βETI is constructed:

$$|\delta \pm ku(Z)| < \lambda \quad (19)$$

When the βETI is evaluated for a number of concentration levels (VS) covering the whole range of analyte concentrations specified in the method scope, the accuracy profile can be constructed according to Feinberg et al. [5]: for each concentration level the βETI is calculated from Eq. (12). The upper and lower tolerance interval limits are then connected by straight lines to interpolate the behaviour of the limits between the levels studied. The quantification limits are located at the intersections between the interpolating lines and the acceptance limits. If we deal with three levels: low ($\hat{Z}_l$), medium ($\hat{Z}_m$) and high ($\hat{Z}_h$), three βETI are computed and expressed as percentage values: $100(\delta_l \pm ku(Z_l))/Z_l$, $100(\delta_m \pm ku(Z_m))/Z_m$ and $100(\delta_h \pm ku(Z_h))/Z_h$. The points corresponding to the upper limits have the coordinates $(\hat{Z}_l, 100(\delta_l + ku(Z_l))/Z_l)$, $(\hat{Z}_m, 100(\delta_m + ku(Z_m))/Z_m)$ and $(\hat{Z}_h, 100(\delta_h + ku(Z_h))/Z_h)$, and they are connected by linear segments. The same procedure is done for the lower limits $(\hat{Z}_l, 100(\delta_l - ku(Z_l))/Z_l)$, $(\hat{Z}_m, 100(\delta_m - ku(Z_m))/Z_m)$ and $(\hat{Z}_h, 100(\delta_h - ku(Z_h))/Z_h)$. The intersections between these two segmented lines with the acceptance limit straight lines $y = \lambda$ and $y = -\lambda$ (in %) give the aforementioned quantification limits. Some typical patterns are shown in Figs. 1 and 2. The domain of valid concentrations for future assays corresponds to the quantification limits L (lowest quantification limit) and U (upper quantification limit).

Some real accuracy profiles are available in the literature for several bioanalytical determinations: Buprenorphine and its N-dealkylated metabolite in human plasma by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS)

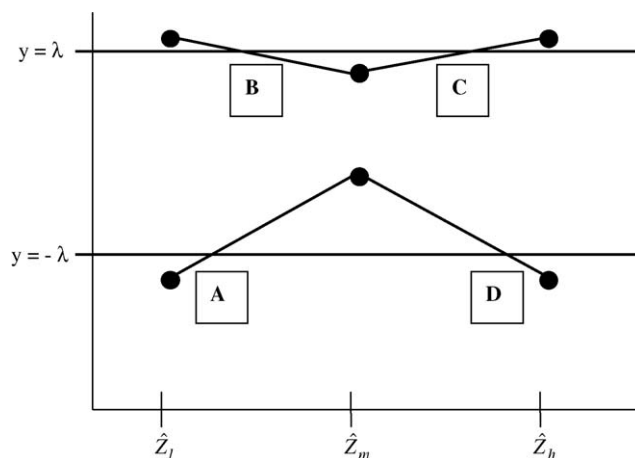


Fig. 1. Accuracy profile showing intersections with the acceptance limit $y = \lambda$ at concentration B and C , and with the acceptance limit $y = -\lambda$ at concentrations A and D . $A < B$ and $C < D$, and consequently, $L = A$ and $U = D$.

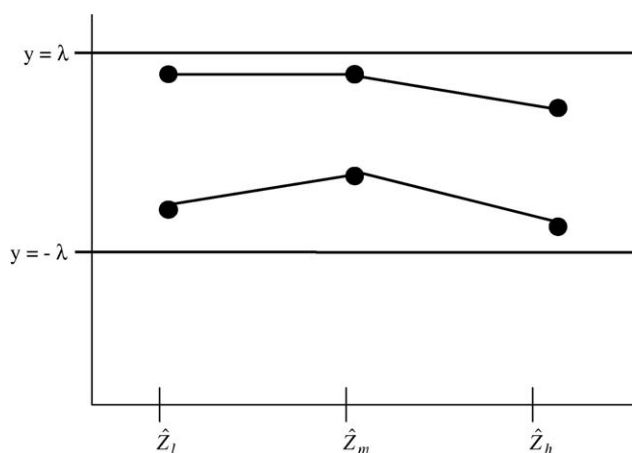


Fig. 2. Accuracy profiles without intersections with the acceptance limits. In this case $L = \hat{Z}_l$ and $U = \hat{Z}_h$.

[21]; cypoterone acetate in human plasma using LC/MS/MS [22] and in human skin by high performance liquid chromatography (HPLC) [23]; aporphine alkaloids in *Cassia filiformis* also by HPLC [24]; pimarane and tachylobane diterpenes in leaves of *Croton Zambesicus* by capillary gas chromatography [25] and loperamide in human plasma by LC/MS/MS [26].

In order to illustrate this topic in a new field, a worked example dealing with the determination of quinine in tonic water is detailed in the following, step by step.

3. Case study: accuracy profile for the determination of quinine in tonic water by fluorescence spectrometry

The quantitation of quinine in tonic water from fluorescence measurements is a well known procedure [27–30]. Solutions containing quinine exhibit in acid medium (0.05 M sulphuric acid) a maximum excitation wavelength at 350 nm and a maximum emission wavelength at 450 nm. Fluorescence intensities are linear against the concentration from 0 to 1.2 mg/L quinine in 0.05 M sulphuric acid with correlation coefficients higher than 0.9999.

Tonic water is a soft drink formulation containing quinine as ingredient, which gives the product its characteristic bitter taste. The FDA requires that tonic water contain no more than 83 mg/L quinine [31]. The procedure for quantitation of quinine

Table 2

Results of the precision and trueness study from one-way ANOVA for each concentration level

Nominal concentration (mg/L quinine)	$Z = \bar{\bar{z}}$	S_r^2	S_B^2	S_R^2	γ	$u^2(\delta)$
66.0	66.38	0.0100	0.7186	0.7286	1.37×10^{-2}	0.1444
83.0	83.24	0.0068	0.7769	0.7837	8.68×10^{-3}	0.1558
100.0	100.04	0.0072	0.8805	0.8878	8.11×10^{-3}	0.1768

The predicted Z value for each level was taken as the ANOVA overall mean ($\bar{\bar{z}}$).

in tonic water is as follows: Pipet 1.00 mL of tonic water (previously degassed by 15 min sonication in an ultrasonic bath) into a 100 mL volumetric flask and dilute to the mark with 0.05 M sulphuric acid. Measure the fluorescence intensity in a suitable fluorescence spectrometer, with 10 mm pathway quartz fluorescence cuvettes at 350 nm excitation wavelength and at 450 emission wavelength. Calculate the concentration of quinine in the tonic water from interpolation in the calibration line.

According to the FDA guidelines, VS were prepared covering approximately the 80%, 100% and 120% of the target amount, 83 mg/L quinine; that is, 66.0, 83.0 and 100.0 mg/L quinine, respectively. Three replicates ($n = 3$) were prepared per concentration level ($m = 3$). The overall preparation step was repeated in a matrix simulating as much possible the future tonic water samples. For this purpose, the matrix was prepared according to the data of the National Public Health Institute of Finland on tonic water formulations [32]. Thus, the formulation matrix contains nearly 9.8 g sucrose, 10.2 mg NaCl, 0.40 mg KCl and 7.80 mg CaCl_2 per 100 g of tonic water. Quinine sulphate ACOPHARMA (Terrasa, Barcelona, Spain) with batch no. 021780 and 99.63% purity was used for spiking “placebos”. VS were prepared in the tonic matrix as indicated above on five days with three concentrations each replicated three times giving a $5 \times 3 \times 3$ experimental design.

Both intermediate precision and trueness study were carried out from the prediction of actual concentrations of the VS according to the procedure for the determination of quinine in tonic water. These data are reported in Table 1. In our case, $m = 3$, $p = 5$ and $n = 3$. The results coming both intermediate precision and trueness study for each concentration level are presented in Table 2.

Table 1
Predicted concentrations of VS, expressed as mg/L and bias

Level	Theoretical concentration	Predicted concentration (bias)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1	66.0	65.33 (−0.67)	66.81 (0.81)	67.44 (1.44)	65.72 (−0.28)	66.61 (0.61)
1	66.0	65.38 (−0.62)	66.79 (0.79)	67.48 (1.48)	65.70 (−0.30)	66.36 (0.36)
1	66.0	65.22 (−0.78)	66.72 (0.72)	67.48 (1.48)	65.88 (−0.12)	66.70 (0.70)
2	83.0	84.49 (1.49)	82.83 (−0.17)	82.65 (−0.35)	82.30 (−0.70)	83.74 (0.74)
2	83.0	84.53 (1.53)	82.77 (−0.23)	82.70 (−0.30)	82.51 (−0.49)	83.82 (0.82)
2	83.0	84.60 (1.60)	82.92 (−0.08)	82.56 (−0.44)	82.48 (−0.52)	83.65 (0.65)
3	100.0	100.25 (0.25)	101.36 (1.36)	99.98 (−0.02)	98.84 (−1.16)	99.60 (−0.40)
3	100.0	100.20 (0.20)	101.44 (1.44)	100.02 (0.02)	98.93 (−1.07)	99.77 (−0.23)
3	100.0	100.32 (0.32)	101.50 (1.50)	99.87 (−0.13)	98.75 (−1.25)	99.82 (−0.18)

Table 3

Influential factors with their level range for the robustness study of the fluorimetric determination of quinine

Factor	Level		
	Low = −1	Nominal = 0	High = +1
x_1	0.04 M	0.05 M	0.06 M
x_2	347 nm	350 nm	353 nm
x_3	447 nm	450 nm	453 nm

Table 4

Plackett–Burmman incomplete matrix and results for the robustness study of the fluorimetric determination of quinine

Runs, N	Factors x_k ($k = 1-3$)			Result, Z_i (mg/L)
	x_1	x_2	x_3	
1	+1	+1	+1	81.65
2	+1	+1	−1	82.85
3	+1	−1	+1	81.17
4	+1	−1	−1	83.05
5	−1	+1	+1	83.25
6	−1	+1	−1	84.06
7	−1	−1	+1	81.80
8	−1	−1	−1	82.72

Table 5

Effect of the influential factors, their significance and their contribution to the measurand uncertainty in the robustness study of the fluorimetric determination of quinine

Factor, x_k	Effect, $D(x_k)$	Significance, $t(x_k)$	Uncertainty contribution $u(Z(x_k))$
x_1	−0.7775	1.09	6.90×10^{-2}
x_2	0.7675	1.07	0.4599
x_3	−1.2025	1.68	0.4599

Robustness tests were carried out by using the VS of 83 mg/L. In our case study, the selected influential factors were the concentration of sulphuric acid (x_1), the excitation wavelength (x_2) and the emission wavelength (x_3). The level ranges for these factors are depicted in Table 3. We have selected extreme values beyond the normally expected ones. Thus, typical concentrated sulphuric acid may vary from 96% to 98% in weight, which may lead to differences against 0.05 M up to 2%, which is from 0.049–0.051 M. The wavelength accuracy of the fluorescence spectrometer is of ± 2 nm, which is from 348 to 352 nm for excitation and from 448 nm to 452 nm for emission. The incomplete

Table 6

Uncertainty budget for the three VS of the fluorimetric determination of quinine

Nominal concentration (mg/L quinine)	$Z = \bar{z}$	S_R^2	$u^2(\delta)$	$u_{\text{rob}}^2(Z)$	$u^2(Z)$	$u(Z)$	$U(Z) = ku(Z)$
66.0	66.38	0.7286	0.1444	0.2764	1.1494	1.0721	2.3157
83.0	83.24	0.7837	0.1558	0.4346	1.3742	1.1723	2.5322
100.0	100.04	0.8878	0.1768	0.6278	1.6923	1.3009	2.8099

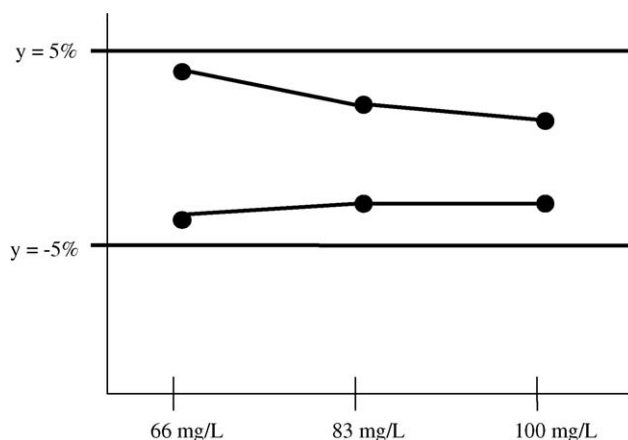


Fig. 3. Accuracy profile for the case study with $L = 66$ mg/L and $U = 100$ mg/L of quinine in tonic water.

Plackett–Burmman matrix for these three factors together with the results obtained for the VS of nominal value 83.0 mg/L quinine is presented in Table 4.

The effect of the factors $D(x_k)$ and the t -values $t(x_k)$ are computed from Eqs. (15) and (16) and are gathered in Table 5. The contribution of each factor to the uncertainty of Z is evaluated from Eq. (17). Here, for x_1 , $\delta_{\text{real}} = 0.051 - 0.049 \text{ M} = 0.002 \text{ M}$, whereas $\delta_{\text{test}} = 0.06 - 0.04 \text{ M} = 0.02 \text{ M}$. For x_2 and x_3 , we have $\delta_{\text{real}} = 4 \text{ nm}$ and $\delta_{\text{test}} = 6 \text{ nm}$. This leads to the contributions indicated also in Table 5.

According to these data, $\text{RSD}_{\text{rob}} = \sqrt{((6.9 \times 10^{-2})^2 + (0.4599)^2 + (0.4599)^2) / ((82.5688)^2)} = 7.92 \times 10^{-3}$.

This leads to the final uncertainty budget presented in Table 6. The expanded uncertainty is computed from a coverage factor taken as the Student's t tabulated value at a 95% significance level, and 14 degrees of freedom, which gives $k = 2.16$. The corresponding expanded uncertainties are also gathered in Table 6.

To obtain the accuracy profile and to build the βETI , first of all, we will choose the acceptability limit λ for the tonic formulation. According to Feinberg et al. [5], a value of 5% can be selected. Then, from the data of Table 6 we can construct the βETI according Eq. (12) and check for the acceptability limit according Eq. (19). These findings are shown in Table 7. As can be observed, results are consistent with Eq. (19) for the three VS and therefore, the acceptability limit holds true for the concentration range of quinine in tonic water from 66 to 100 mg/L. The corresponding plot of the accuracy profile is given in Fig. 3.

Table 7

Accuracy profiles for each concentration level in the fluorimetric determination of quinine

Nominal concentration (mg/L quinine)	$Z = \bar{z}$	$U(Z)$	$\delta = Z - \hat{Z}$	$100(\delta + U(Z))/Z$ (%)	$100(\delta - U(Z))/Z$ (%)	λ (%)
66.0	66.38	2.3157	0.38	4.06	−2.91	±5
83.0	83.24	2.5322	0.24	3.33	−2.75	±5
100.0	100.04	2.8099	0.04	2.85	−2.77	±5

4. Conclusion

The evaluation of accuracy profiles for chemical assays can be easily performed once the uncertainty of the measurand is estimated from the proper validation data.

References

- [1] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, *Anal. Chim. Acta* 391 (1999) 135–148.
- [2] B. Boulanger, P. Chiap, W. Dewé, J. Crommen, Ph. Hubert, *J. Pharm. Biomed. Anal.* 32 (2003) 753–765.
- [3] B. Boulanger, W. Dewé, P. Hubert, Objectives of pre-study validation and decision rules, AAPS conference. APQ Open Forum, 2000. Indianapolis.
- [4] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, *J. Pharm. Biomed. Anal.* 36 (2004) 579–586.
- [5] M. Feinberg, B. Boulanger, W. Dewé, P. Hubert, *Anal. Bioanal. Chem.* 380 (2004) 502–514.
- [6] ISO 5725-1 Accuracy (trueness and precision) of measurement method and results. Part 1. General Principles and Definitions, 1994.
- [7] EURACHEM Guide. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. December 1998. <http://www.eurachem.ul.pt/guides/mval.htm>.
- [8] CX/MAS 01/8. Codex Alimentarius Commission. Codex Committee on Methods of Analysis and Sampling (FAO/WHO), Measurement Uncertainty, Relationship between the analytical result, the measurement uncertainty and the specification in Codex standards. Agenda Item 4a of the 23rd session, Budapest, Hungary, 26 February–2 March 2001.
- [9] I. Taverniers, E. Van Bockstaele, M. De Loose, *Trends Anal. Chem.* 23 (2004) 480–490.
- [10] R.W. Mee, *Technometrics* 26 (1984) 251–253.
- [11] Guide to the Expression of Uncertainty in Measurement, ISO, Geneva, Switzerland, 1993, corrected and reprinted in 1995.
- [12] Analytical Methods Committee, *Analyst* 120 (1995) 2303–2308.
- [13] V.J. Barwick, L.R. Ellison, VAM Project 3.2.1, Development and Harmonisation of Measurement Uncertainty Principles. Part D: Protocol for Uncertainty Evaluation from Validation Data, January 2000. Report No.: LGC/VAM/1998/088.
- [14] A. Maroto, R. Boqué, F.X. Rius, *Anal. Chim. Acta* 391 (1999) 173–185.
- [15] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, *STP Pharma Pratiques* 13 (2003) 101–138.
- [16] A. Gustavo González, M. Ángeles Herrador, Agustín G. Asuero, *Talanta* 65 (2005) 1022–1030.
- [17] ISO/DTS 21748 Guide to the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation. ISO, Geneva, 2003.
- [18] ISO-5725-2 Accuracy (trueness and precision) of measurement methods and results. Part 2. Basic method for the determination of repeatability and reproducibility of a standard measurement method, 1994.
- [19] T. Luping, B. Schouenborg, Methodology of Inter-comparison Tests and Statistical Analysis of Test Results. Nordtest project No. 1483-99, SP Swedish National Testing and Research Institute, SP report 2000:35, Borås 2000.
- [20] W.Y. Youden, *Statistical Techniques for Collaborative Tests*, AOAC, 1967, pp. 29–32.
- [21] A. Ceccato, R. Klinkenberg, Ph. Hubert, B. Streel, *J. Pharm. Biomed. Anal.* 32 (2003) 619–631.
- [22] B. Christiaens, M. Fillet, P. Chiap, O. Rbeida, A. Ceccato, B. Streel, J. De Graeve, J. Crommen, Ph. Hubert, *J. Chromatogr. A* 1056 (2004) 105–110.
- [23] S.H. de Hassonville, P. Chiap, J.F. Liégeois, B. Evrad, L. Delattre, J. Crommen, G. Piel, Ph. Hubert, *J. Pharm. Biomed. Anal.* 36 (2004) 133–143.
- [24] C. Stévigny, M.C. Wautier, J.-L.H. Jiwan, P. Chiap, Ph. Hubert, Q.-L. Joëlle, *Planta Medica* 70 (2004) 764–770.
- [25] S. Block, D. Brkic, Ph. Hubert, Q.-L. Joëlle, *Phytochem. Anal.* 16 (2005) 342–348.
- [26] B. Streel, A. Ceccato, R. Klinkenberg, Ph. Hubert, *J. Chromatogr. B* 814 (2005) 263–273.
- [27] D.C. Harris, *Quantitative Chemical Analysis*, 5th ed., W.H. Freeman, 1999, pp. 97–101, 511–534.
- [28] D.A. Skoog, J.J. Leary, *Principles of Instrumental Analysis*, 4th ed., Saunders College, 1992, pp. 174–191.
- [29] J. O'Reilly, *J. Chem. Educ.* 52 (1975) 610–612.
- [30] D.T. Sawyer, W.R. Heinemann, J.M. Beebe, *Chemical Experiments for Instrumental Methods*, John Wiley & Sons, 1984, p. 272.
- [31] <http://www.wholefoods.com/healthinfo/quinine.html>.
- [32] <http://www.fineli.fi/food.php?foodid=29501&lang=en>.

Development of a multi-residue analytical methodology based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters

Meritxell Gros^a, Mira Petrović^{a,b,*}, Damià Barceló^a

^a *Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona 18-26, 08034 Barcelona, Spain*

^b *Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain*

Received 7 December 2005; received in revised form 18 April 2006; accepted 12 May 2006

Available online 24 July 2006

Abstract

This paper describes development, optimization and validation of a method for the simultaneous determination of 29 multi-class pharmaceuticals using off line solid phase extraction (SPE) followed by liquid chromatography–triple quadrupole mass spectrometry (LC–MS–MS). Target compounds include analgesics and non-steroidal anti-inflammatories (NSAIDs), lipid regulators, psychiatric drugs, anti-histaminics, anti-ulcer agent, antibiotics and β -blockers. Recoveries obtained were generally higher than 60% for both surface and wastewaters, with exception of several compounds that yielded lower, but still acceptable recoveries: ranitidine (50%), sotalol (50%), famotidine (50%) and mevastatin (34%). The overall variability of the method was below 15%, for all compounds and all tested matrices. Method detection limits (MDL) varied between 1 and 30 ng/L and from 3 to 160 ng/L for surface and wastewaters, respectively. The precision of the method, calculated as relative standard deviation (R.S.D.), ranged from 0.2 to 6% and from 1 to 11% for inter and intra-day analysis, respectively. A detailed study of matrix effects was performed in order to evaluate the suitability of different calibration approaches (matrix-matched external calibration, internal calibration, extract dilution) to reduce analyte suppression or enhancement during instrumental analysis. The main advantages and drawbacks of each approach are demonstrated, justifying the selection of internal standard calibration as the most suitable approach for our study. The developed analytical method was successfully applied to the analysis of pharmaceutical residues in WWTP influents and effluents, as well as in river water. For both, river and wastewaters, the most ubiquitous compounds belonged to the group of anti-inflammatories and analgesics, antibiotics, the lipid regulators being acetaminophen, trimethoprim, ibuprofen, ketoprofen, atenolol, propranolol, mevastatin, carbamazepine and ranitidine the most frequently detected compounds. © 2006 Elsevier B.V. All rights reserved.

Keywords: Liquid chromatography–tandem mass spectrometry; Pharmaceuticals; Surface and wastewater analysis; Multi-residue analytical method; Ion suppression

1. Introduction

During the last three decades the impact of chemical pollution has focused almost exclusively on the conventional “priority” pollutants. However, the growing use of pharmaceuticals worldwide, classified as the so-called emerging contaminants, has become a new environmental problem, which has awakened great concern among scientists in the last few years. Even though they are found in very low concentrations, there is still a lack of knowledge about long-term risks that the presence of a large variety of drugs may pose for non-target organisms as well as for human health.

Wastewater treatment plants (WWTPs) are major contributors of pharmaceuticals in the environment. Due to their high consumption, pharmaceuticals along with their metabolites are continuously introduced to sewage waters, mainly through excreta, disposal of unused or expired drugs or directly from pharmaceutical discharges [1–3]. Recently, it has been reported that the elimination of some pharmaceutical compounds during wastewater treatment processes is rather low and as a result, they are found in surface, ground and drinking waters [4–7]. For this reason, Pharmaceuticals may be able to cause the same exposure potential as persistent pollutants, since their high transformation and removal rates can be compensated by their continuous input into the environment.

Consequently, there is a growing need to develop reliable analytical methods, which enable their rapid, sensitive and selective

* Corresponding author. Tel.: +34 934006172; fax: +34 932045904.
E-mail address: mpeqam@cid.csic.es (M. Petrović).

determination in environmental samples, at trace levels. Several analytical methodologies are already available in the literature for the determination of pharmaceutical compounds in surface and wastewaters, and numerous papers reported occurrence of specific pharmaceuticals at levels ranging from ng/L to µg/L [8,15]. Nevertheless, the vast majority of them are focused on specific therapeutic classes, paying special attention to antibiotics which are believed to be the ones of biggest concern among all pharmaceuticals due to their potential for antibiotic resistance [1,16,19]. However, multi-residue analytical methodologies, including multiple-class pharmaceuticals, are becoming the required tools to provide reliable and wider knowledge about their occurrence, as well as for the monitoring of their removal, partition and ultimate fate in the environment [9,20–24]. Simultaneous analysis of several groups of compounds with quite different physico-chemical characteristics generally requires a compromise in the selection of experimental conditions, which in some cases means not obtaining the best performance for each one of the compounds. However, developing a multi-group method is rewarding as it can be applied in routine analysis, providing a large amount of data.

In the literature reported, pre-concentration and isolation of target analytes from water samples is mainly based on off-line solid phase extraction (SPE). However, the majority of multi-group methods generally include more than one extraction

step, using two or more different sorbent materials or different elution solvents, fractionating target compounds in groups according to their physico-chemical properties and pharmaceutical classes, which results in an overall time-consuming sample preparation [9]. Instrumental analysis is performed by gas and liquid chromatography–mass spectrometry (GC–MS) [25–29], (LC–MS) [10,24,30] and LC–MS–MS [2,11,15,20,31] being the latter the method of choice due to its versatility, specificity and selectivity, enabling the detection of target compounds in the low ng/L range. However, matrix effects are one of the major drawbacks of LC–MS–MS, especially when working in electrospray ionization mode (ESI). Matrix effect may result in the suppression or, less frequently, the enhancement of analyte signals, leading sometimes to erroneous results [32–34]. Therefore, an exhaustive study to evaluate matrix effects should be included in the method validation, in order to ensure the reliability of the results obtained.

In light of these concerns, the aim of this work was the development of a sensitive multi-residue analytical method, based on off line SPE followed by LC–ESI–MS/MS (QqQ) for the simultaneous analysis of an extended list of 29 pharmaceuticals in both surface and wastewaters. Within this list different therapeutic classes are incorporated. Target compounds were selected due to their occurrence and ubiquity in the aquatic environment [35,36], according to the information found in the literature reported, as

Table 1
Target analytes and their physico-chemical properties

Therapeutic groups	Compounds	Log K_{ow}	p K_a	MW	P_v (mmHg)	Molecular formula
Analgesic and antiinflammatories	Ketoprofen	3.12	4.45	254.29	3.72E–7	C ₁₆ H ₁₄ O ₃
	Naproxen	3.18	4.15	230.27	1.892E–6	C ₁₄ H ₁₄ O ₃
	Ibuprofen	3.97	4.91	206.23	1.162E–11	C ₁₃ H ₁₈ O ₂
	Indomethacine	4.27	4.5	357.8	9.89E–11	C ₁₉ H ₁₆ ClNO ₂
	Diclofenac	4.51	4.14	296.16	6.14E–8	C ₁₄ H ₁₀ Cl ₂ NO ₂
	Mefenamic acid	5.12	4.2	241.3	4.636E–7	C ₁₅ H ₁₅ NO ₂
	Acetaminophen	0.46	9.38	151.17	7E–6	C ₈ H ₉ NO ₂
	Propyphenazone	1.94	nd	230.31	5.2E–6	C ₁₄ H ₁₈ N ₂ O
Lipid regulators and cholesterol lowering statin drugs	Clofibric acid	nd	nd	214.5	nd	C ₁₀ H ₁₁ O ₃ Cl
	Gemfibrozil	4.77	nd	250.34	nd	C ₁₅ H ₂₂ O ₃
	Bezafibrate	4.25	nd	361.82	nd	C ₁₉ H ₂₀ ClNO ₄
	Pravastatin	3.1	nd	446.00	nd	C ₂₃ H ₃₆ O ₇
	Mevastatin	3.95	nd	390.51	2.19E–12	C ₂₅ H ₃₈ O ₅
Psychiatric drugs	Carbamazepine	2.47	7	236.27	1.84E–7	C ₁₅ H ₁₂ N ₂ O
	Fluoxetine	3.82	8.7	309.33	nd	C ₁₇ H ₁₈ F ₃ NO
	Paroxetine	3.95	nd	329.37	nd	C ₁₉ H ₂₀ FNO ₃
Anti-ulcer agent	Lansoprazole	2.58	8.73	369.36	nd	C ₁₆ H ₁₄ F ₃ N ₃ O ₂ S
Histamine H ₁ and H ₂ receptor antagonists	Loratadine	5.20	nd	382.89	1.56E–9	C ₂₂ H ₂₃ ClN ₂ O ₂
	Famotidine	–0.64	nd	337.43	6.02E–11	C ₈ H ₁₅ N ₇ O ₂ S ₃
	Ranitidine	0.27	nd	314.41	1.2E–7	C ₁₃ H ₂₂ N ₄ O ₃ S
Antibiotics	Erythromycin	3.06	8.8	733.93	2.28E–27	C ₃₇ H ₆₇ NO ₁₃
	Azythromycin	4.02	8.74	748.99	3.9E–27	C ₃₈ H ₇₂ N ₂ O ₁₂
	Sulfamethoxazole	0.89	6.0	253.28	6.93E–8	C ₁₀ H ₁₁ N ₃ O ₃ S
	Trimethoprim	0.91	7.12	290.32	9.88E–9	C ₁₄ H ₁₈ N ₄ O ₃
	Ofloxacin	nd	nd	361.37	nd	C ₁₈ H ₂₀ FN ₃ O ₄
	Atenolol	0.16	9.6	266.34	2.924E–10	C ₁₄ H ₂₂ N ₂ O ₃
β-Blockers	Sotalol	0.24	nd	272.37	5.3E–9	C ₁₂ H ₂₀ N ₂ O ₃ S
	Metoprolol	1.88	9.68	267.37	2.88E–7	C ₁₅ H ₂₅ NO ₃
	Propranolol	nd	nd	259.80	nd	C ₁₆ H ₂₁ NO ₂

well as their high human consumption worldwide [37]. Target compounds, classified by groups according to their therapeutical effect and their physico-chemical properties are listed in Table 1. The developed method consists of a single extraction step for all target compounds, simplifying considerably sample preparation. Moreover, an extensive study to evaluate the suitability of several approaches to compensate matrix effects, including standard addition, internal standard calibration and dilution of sample extracts, has been performed and included in the method validation. Analyte identification and confirmation was performed in compliance with the EU regulations (EU Commission Decision 2002/657/EC [38]). The developed method was successfully applied to the analysis of pharmaceutical residues in WWTP as well as in river water samples.

2. Experimental

2.1. Chemicals and reagents

All pharmaceutical standards used were of high purity grade (>90%). Ibuprofen, naproxen, ketoprofen, diclofenac and gemfibrozil were kindly supplied by Jescuder (Rubí, Spain). Indomethacine, acetaminophen, mefenamic acid, clofibrac acid, bezafibrate, mevastatin, azythromycin dihydrate, erythromycin hydrate, carbamazepine, fluoxetine hydrochloride, lansoprazole, loratadine, famotidine, ranitidine hydrochloride, sulfamethoxazole, trimethoprim, ofloxacin, atenolol, metoprolol, propranolol hydrochloride and sotalol hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Propyphenazone, pravastatin and paroxetine hydrochloride were from LGC Promochem (London, UK). Isotopically labelled compounds, used as internal standards, were ^{13}C -phenacetin obtained from Sigma–Aldrich, mecoprop- d_3 from Dr. Ehrenstorfer (Augsburg, Germany), ibuprofen- d_3 , atenolol- d_7 and carbamazepine- d_{10} from CDN Isotopes (Quebec, Canada).

Individual stock standard solutions were prepared on a weight basis in methanol and stored at -2°C . A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol–water (25:75, v/v) before each analytical run and were used as working standard solutions. Stock solutions of ofloxacin, pravastatin and sulfamethoxazole were renewed monthly before their use due to their limited stability. Stock solutions of internal standards were also prepared in methanol and were stored at -2°C . A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions in methanol.

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL and 200 mg, 6 mL) from Waters Corporation (Milford, MA, USA). Other cartridges tested were Oasis MCX (150 mg, 6 mL) also from Waters Corp., C_{18} (500 mg, 3 mL) and ENV+ (200 mg, 6 mL) from Isolute (Mid Glamorgan, UK). $1\ \mu\text{m}$ glass fiber and $0.45\ \mu\text{m}$ nylon membrane filters were purchased from Whatman (UK).

HPLC-grade methanol, acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany). Hydrochloric acid 37%, NH_4Ac and HAc were from Merck (Darmstadt, Ger-

many). Nitrogen for drying 99.995% of purity was from Air Liquide (Spain).

2.2. Sample pre-treatment and solid phase extraction optimization

The method was optimized using ground water, WWTP influent and effluent. The latter were collected from a WWTP located in Rubí (Barcelona, Spain), which receives urban, domestic and industrial wastewaters. Amber glass bottles pre-rinsed with ultra-pure water were used for sample collection. Wastewaters were vacuum filtered through $1\ \mu\text{m}$ glass fiber filters followed by $0.45\ \mu\text{m}$ nylon membrane filters (Teknokroma, Barcelona, Spain). Otherwise, well waters were only filtered with 0.7 and $0.45\ \mu\text{m}$ filters. 500 mL of well water, 200 of effluent and 100 mL of influent wastewaters were measured. In order to optimise the extraction method, the performances of different SPE materials were compared, testing the following cartridges: polymeric Oasis HLB (60, 3 mL), Isolute ENV+ (200 mg, 3 mL), Octadecylsilica Isolute C_{18} (500 mg, 3 mL) and finally, Strong cation Exchange Oasis MCX (150 mg, 6 mL). For this purpose, water samples were spiked, prior to the extraction, with appropriate concentrations of the standard mixture of target analytes. Once the sorbent yielding the highest recoveries was selected, an evaluation if any sample pH adjustment was required, prior to extraction, was performed. For this purpose, recoveries of target analytes from samples with pH adjusted with HCl 1 M to pH 2 were compared with the ones obtained without any pH adjustment (neutral pH).

For the preconcentration step, a Baker vacuum system (J.T. Baker, The Netherlands) was used. Firstly, SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of deionized water (HPLC grade) at neutral pH (or pH 2 in the tests where water samples were acidified), at a flow rate of 1 mL/min. After the conditioning step, water samples were percolated through the cartridges at a flow rate of 10 mL/min. Afterwards the cartridge was rinsed with 5 mL of HPLC-grade water and the cartridge was then dried under vacuum for 15–20 min, to remove excess of water. Elution was performed with $2\ \text{mL} \times 4\ \text{mL}$ of methanol at 1 mL/min. The extract was evaporated under a gentle nitrogen stream and reconstituted with 1 mL of methanol–water (25:75, v/v). Finally, $10\ \mu\text{L}$ of a $10\ \text{ng}/\mu\text{L}$ standard mixture of the internal standards mecoprop- d_3 , ibuprofen- d_3 , for the analysis in negative ion (NI) mode and ^{13}C -Phenacetin, atenolol- d_7 and carbamazepine- d_{10} , for the analysis in positive ion (PI) mode, were added in the extract for internal standard calibration and to compensate possible matrix effects.

2.3. LC–ESI–tandem MS analysis

LC analysis was performed using a Waters 2690 HPLC system (Milford, MA, USA) coupled to a Waters Micromass Quattro triple quadrupole mass spectrometer, equipped with a Z-spray ESI interface (Manchester, UK). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column ($125\ \text{mm} \times 2.0\ \text{mm}$, particle size $5\ \mu\text{m}$) and a C_{18} guard column, both supplied by Merck (Darmstadt, Germany). For the

analysis in NI mode, eluent A was methanol and eluent B was water at a flow rate of 0.2 mL/min. The elution gradient started with 20% of eluent A, increasing to 80% in 20 min, raising to 90% in a 4 min gradient and then, back to initial conditions within 3 min. The column was re-equilibrated for 15 min before another injection. The analysis in PI mode was performed using as eluent A a mixture of acetonitrile–methanol (2:1) and as eluent B a buffer consisting in NH_4Ac 5 mM/HAc at pH 4.7, also at a flow rate of 0.2 mL/min. The elution gradient started with 15% of eluent A, keeping isocratic conditions for three minutes. Then, eluent A increased to 95% in 22 min and was held for 7 min. Finally, initial conditions were reached again in five minutes, with a re-equilibration time of 15 min in order to restore the column. The sample injection volume was set at 10 μL .

MS parameters for the analysis were the following: ESI source block and desolvation temperature: 150 and 350 $^\circ\text{C}$, respectively; capillary voltage: 2.8 kV; argon collision gas 2.5×10^{-3} mbar; cone nitrogen and desolvation gas flow: 43 and 636 L/h. After the selection of the precursor ions for each analyte, product ions were obtained with a combination of colli-

sion energies and cone voltages, parameters that were previously optimized. Instrument control, peak detection and integration were carried out using Masslynx NT software (version 3.4). For increased sensitivity and selectivity, data acquisition was performed working in multiple reaction monitoring mode (MRM). According to the performance characteristics defined in the EU Commission Decision 2002/657/EC for the confirmation and identification of Pharmaceuticals when using LC–tandem MS as the instrumental technique, a minimum of three identification points (IP) are required. According to this LC–MS–MS (QqQ) analysis using two MRM transitions is sufficient to confirm the identity of the compound. The MRM ratio, calculated as the relation between the abundances of both transitions and the LC retention time are criteria also used to confirm the presence of an analyte in the samples. Therefore, in this study transitions between a precursor ion and the two most abundant fragment ions were chosen for each analyte when working in MRM mode, earning four identification points, enough to accomplish the EU directive aforementioned. In the cases where compounds showed poor fragmentation only one transition could be moni-

Table 2

MS/MS parameters for the analysis of target analytes by MRM negative and positive ionization mode

Target compounds	R_t window (min)	R_t (min)	Precursor ion	CV–CE	MRM1	CV–CE	MRM2	MRM ratio
Compounds analyzed by NI mode								
Clofibric acid	0–15.5	11.93	213 $[M - H]^-$	20–10	213 > 127	20–15	213 > 85	4.95
Naproxen		13.89	229 $[M - H]^-$	26–10	229 > 169	26–20	229 > 185	1.40
Mecoprop- d_3	15.5–30	13.91	217 $[M - H]^-$	20–20	217 > 145	–	–	–
Ketoprofen		14.71	253 $[M - H]^-$	30–10	253 > 209	30–15	253 > 197	–
Bezafibrate		16.02	360 $[M - H]^-$	20–20	360 > 274	20–20	360 > 154	5.79
Ibuprofen- d_3		17.99	208 $[M - H]^-$	25–10	208 > 164	–	–	–
Ibuprofen		18.04	205 $[M - H]^-$	20–10	205 > 160	–	–	–
Diclofenac		18.11	240 $[M - H]^-$	20–20	294 > 250	20–15	294 > 214	5.75
Mefenamic acid		19.00	240 $[M - H]^-$	20–20	240 > 196	20–30	240 > 180	16.17
Indomethacine		20.03	356 $[M - H]^-$	20–20	356 > 297	20–20	356 > 312	3.47
Gemfibrozil		21.19	249 $[M - H]^-$	20–30	249 > 121	–	–	–
Compounds analyzed by PI mode								
Atenolol- d_7	0–8	3.06	274 $[M + H]^+$	30–20	274 > 190	–	–	–
Atenolol		3.09	267 $[M + H]^+$	30–20	267 > 190	20–30	267 > 145	1.05
Ranitidine	8–16	3.49	315 $[M + H]^+$	20–20	315 > 176	20–30	315 > 130	1.66
Famotidine		3.58	338 $[M + H]^+$	20–20	338 > 189	20–10	338 > 259	1.64
Sotalol		3.82	273 $[M + H]^+$	20–10	273 > 255	20–20	273 > 213	1.22
Acetaminophen		4.34	152 $[M + H]^+$	25–15	152 > 110	30–20	152 > 93	6.44
Trimethoprim		9.14	291 $[M + H]^+$	30–20	291 > 230	30–30	291 > 261	1.95
Metoprolol		14.07	268 $[M + H]^+$	25–20	268 > 159	30–30	268 > 133	1.05
Sulfamethoxazole		14.12	254 $[M + H]^+$	20–25	254 > 92	20–15	254 > 156	1.14
^{13}C -Phenacetin		15.55	181 $[M + H]^+$	20–15	181 > 139	–	–	–
Azythromycin	16–25	16.14	749 $[M + H]^+$	30–30	749 > 591	30–30	749 > 158	2.12
Pravastatin		17.14	447 $[M + \text{Na}]^+$	30–20	447 > 327	–	–	–
Propranolol		17.34	260 $[M + H]^+$	25–20	260 > 183	25–20	260 > 116	1.78
Ofloxacin		18.20	362 $[M + H]^+$	20–15	362 > 316	20–20	362 > 318	1.50
Carbamazepine- d_{10}		18.59	247 $[M + H]^+$	20–15	247 > 204	–	–	–
Erythromycin		18.62	734 $[M + H]^+$	20–20	734 > 576	20–20	734 > 558	4.96
Carbamazepine		18.71	237 $[M + H]^+$	20–20	237 > 194	25–20	237 > 192	4.09
Propyphenazone		19.34	231 $[M + H]^+$	20–20	231 > 189	20–25	231 > 201	3.04
Lansoprazole	25–32	20.00	370 $[M + H]^+$	20–15	370 > 252	20–20	370 > 205	2.88
Paroxetine		20.44	330 $[M + H]^+$	30–20	330 > 192	30–30	330 > 123	4.51
Fluoxetine		21.26	310 $[M + H]^+$	25–10	310 > 148	20–10	310 > 44	2.88
Mevastatin		27.91	391 $[M + H]^+$	20–15	391 > 185	20–30	391 > 159	1.18
Loratadine		27.94	383 $[M + H]^+$	30–20	383 > 337	30–30	383 > 259	3.07

CV–CE: Cone voltage and collision energy.

tored. However, it only happened for three compounds, and their confirmation in the samples was performed matching their LC retention time with the ones obtained in the standards. Shifts in retention time were less than 3%, so the confirmation was considered accurate enough. For internal standards, only one transition was selected, as they are isotopically labelled compounds which are not likely to be found in environmental samples. In order to increase sensitivity, MRM transitions were classified in different elution time window. In almost each window one internal standard was included. MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table 2. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

2.4. Method validation

Extraction recoveries of target compounds were determined for ground water, WWTP effluent and influent using samples spiked at two concentration levels (50 ng/L and 1 µg/L for ground water, 100 ng/L and 1 µg/L for WWTP effluent and 1 and 10 µg/L for WWTP influent). For each matrix, recoveries were determined comparing the concentrations obtained, calculated by internal standard calibration, with the initial spiking levels. In each case, samples were analyzed in triplicate. As both surface and wastewaters spiked contained target compounds, blanks (no-spiked samples) were analysed in order to determine their concentrations, which were afterwards subtracted to the spiked waters. Matrix effects and the efficiency of internal standard calibration against standard calibration and sample extract dilutions were evaluated for each compound, and the results obtained are discussed in a specific section below.

The precision of the method was determined by repeated intra-day and inter-day analysis (five successive injection of a standard solution in 1 day and in five successive days, respectively), expressing it as the relative standard deviation (R.S.D.) of these replicate measurements. Method detection limits (MDL)

and method quantification limits (MQL) were determined from spiked water samples, as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. The instrumental detection limits (IDL) were estimated from the injection of a standard solution successively diluted until reaching a concentration level corresponding to a signal-to-noise ratio of 3.

3. Results and discussion

3.1. Sample pre-treatment and solid phase extraction

The performance of different solid phase extraction materials was tested, including two polymeric sorbents (Lichrolut ENV+ and Oasis HLB), a mixed polymeric and cation exchange sorbent (Oasis MCX) and a non polar one (C₁₈). In Fig. 1 the performance of the materials tested is summarized. Only the results obtained for the most representative compounds of each therapeutic group in surface waters are illustrated. The analytes not shown, belonging to the same medicinal classes, as well as in other matrices (effluent and influent wastewaters) followed a similar pattern. As it can be observed, quite good recoveries were obtained for acidic compounds when using Oasis MCX, whereas basic and neutral compounds, except carbamazepine, were poorly recovered. This could be attributed to the fact that Oasis MCX is a mixed reversed phase-cation exchange cartridge which, at low pH values, it can efficiently extract acidic, basic and neutral compounds, since the cation exchanger binds the basic compounds and the reversed phase can retain both acidic and neutral ones. As our experiment was performed at neutral pH and analytes were subsequently eluted with pure methanol, only acidic and neutral compounds were recovered. In order to extract efficiently basic analytes, samples should have been adjusted at low pH values and eluted afterwards with methanol–ammonia.

Lichrolut ENV+ was only effective for few compounds. Generally, this cartridge is recommended for the extraction of polar

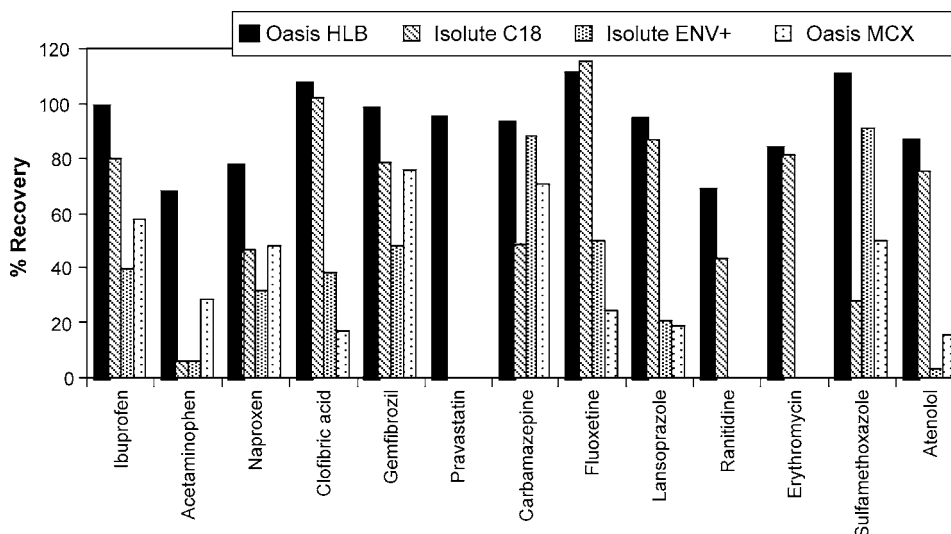


Fig. 1. Recoveries obtained for the extraction of selected Pharmaceuticals in 500 mL of river water, spiked at 1 µg/L without sample pH adjustment using different SPE materials.

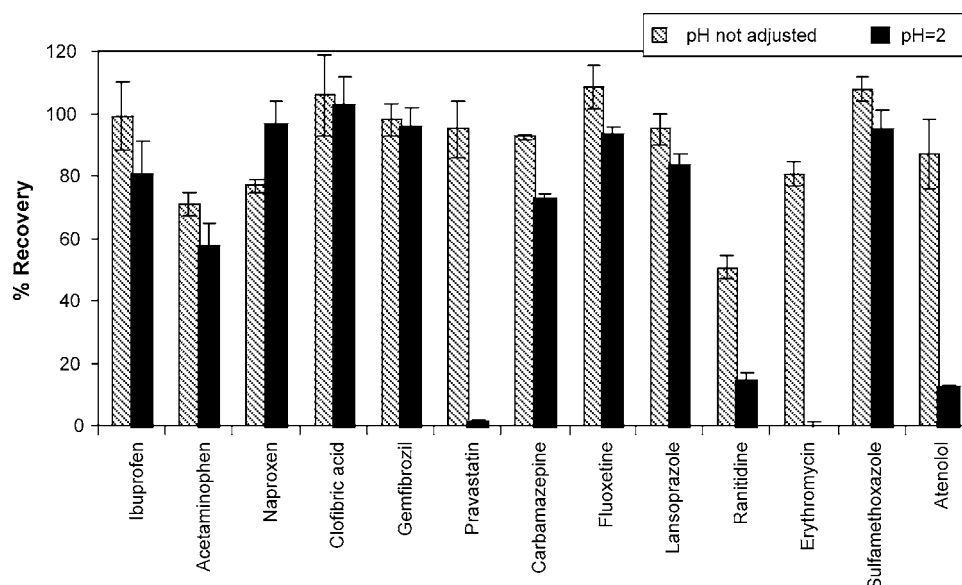


Fig. 2. Influence of pH adjustment, (pH 2 and no pH adjustment), on the recoveries obtained using Oasis HLB SPE cartridges (matrix: river water and spiking level 1 µg/L).

Table 3

Recoveries obtained for target analytes in surface and wastewaters at the two spiking levels tested for each type of water

Therapeutic group	Compound	Recoveries at high spiking level (1 and 10 µg/L for river, effluent and influent wastewater) (R.S.D.%)			Recoveries at low spiking level (50,100 ng/L 1 µg/L for river, effluent and influent wastewaters) (R.S.D.%)		
		Surface waters	WWTP effluent	WWTP influent	Surface waters	WWTP effluent	WWTP influent
Analgesics and anti-inflammatories	Ketoprofen	51 (8)	61 (2)	89 (7)	121 (9)	53 (12)	52 (4)
	Naproxen	77 (2)	51 (1)	60 (1)	73 (6)	81 (9)	34 (5)
	Ibuprofen	99 (11)	90 (8)	63 (9)	70 (12)	87 (7)	111 (9)
	Indomethacine	64 (2)	60 (4)	79 (2)	50 (12)	50 (13)	51 (5)
	Diclofenac	102 (3)	78 (2)	80 (2)	81 (12)	60 (3)	89 (4)
	Mefenamic acid	nd	nd	90 (3)	93 (5)	65 (4)	nd
	Acetaminophen	71 (5)	45 (1)	48 (3)	60 (5)	50 (3)	47 (1)
	Propyphenazone	nd	nd	71 (4)	60 (8)	60 (15)	nd
Lipid regulators and cholesterol lowering statin drugs	Clofibric acid	106 (13)	32 (4)	62 (11)	79 (5)	30 (12)	56 (4)
	Gemfibrozil	98 (5)	61 (2)	88 (3)	80 (4)	71 (11)	80 (9)
	Bezafibrate	78 (15)	88 (13)	82 (4)	62 (2)	107 (3)	79 (5)
	Pravastatin	95 (10)	93 (4)	85 (6)	80 (8)	70 (5)	113 (8)
	Mevastatin	60 (9)	34 (3)	60 (11)	60 (5)	50 (11)	34 (2)
Psychiatric drugs	Carbamazepine	93 (1)	97 (5)	98 (3)	67 (6)	93 (12)	105 (10)
	Fluoxetine	105 (6)	60 (2)	108 (4)	74 (12)	74 (2)	67 (12)
	Paroxetine	95 (5)	65 (3)	96 (1)	110 (12)	76 (12)	84 (4)
Antiulcer agent	Lansoprazole	75 (13)	86 (4)	77 (5)	80 (7)	75 (4)	87 (5)
Histamine H ₁ and H ₂ receptor antagonists	Loratadine	72 (9)	86 (12)	115 (3)	76 (8)	93 (12)	91 (3)
	Famotidine	50 (5)	61 (4)	50 (4)	40 (7)	104 (5)	50 (3)
	Ranitidine	51 (8)	109 (12)	88 (4)	94 (6)	86 (11)	38 (1)
Antibiotics	Erythromycin	81 (5)	116 (4)	48 (3)	70 (4)	50 (13)	98 (9)
	Azythromycin	104 (7)	93 (3)	40 (5)	30 (7)	30 (15)	63 (2)
	Sulfamethoxazole	108 (4)	50 (5)	80 (4)	90 (6)	50 (3)	101 (3)
	Trimethoprim	96 (9)	88 (7)	111 (1)	93 (6)	83 (15)	106 (2)
	Ofloxacin	93 (3)	106 (2)	95 (4)	80 (5)	75 (3)	106 (3)
β-Blockers	Atenolol	87 (12)	92 (5)	82 (5)	96 (6)	96 (5)	97 (2)
	Sotalol	50 (6)	43 (3)	111 (5)	115 (11)	71 (7)	56 (5)
	Metoprolol	60 (8)	103 (5)	104 (4)	103 (5)	114 (6)	86 (12)
	Propranolol	102 (3)	85 (4)	55 (1)	81 (11)	44 (4)	70 (6)

Nd: not calculated.

organic compounds at low pH values, but it can also retain neutral drugs at pH 7, such as carbamazepine and macrolide antibiotics, through hydrophobic interactions.

C₁₈ provided good results for the majority of the compounds. However, comparing to Oasis HLB, the latter was much more efficient, yielding high recoveries for all target compounds. This sorbent, with the combination of the hydrophilic–lipophilic polymer, can extract acidic, neutral and basic analytes at a wide range of pHs, including neutral pH.

In the next step, the effect of prior acidification of the sample is evaluated. Fig. 2, shows the recoveries obtained using Oasis HLB with and without pH adjustment. Basic and neutral pharmaceuticals yielded significantly higher recoveries without acidifying the sample. However, for acidic compounds results were quite similar, except for clofibric acid, ketoprofen, naproxen and mevastatin. As the aim of this work was to extract all target analytes in one single step and recoveries obtained were good without sample acidification, this protocol was selected as the optimum one for further experiments. Recoveries (mean of three replicates $\pm$ R.S.D.) are given in Table 3 and discussed in the method validation section.

3.2. LC–ESI–tandem MS analysis

To optimize the chromatographic separation, a series of preliminary experiments were performed, testing different mobile phases consisting of methanol, acetonitrile or mixture of acetonitrile and methanol as an organic phase and water with different mobile phase additives, such as ammonium acetate and acetic acid at various concentrations. The optimal separation of 22 compounds detected in PI mode was achieved using 5 mM aqueous NH₄Ac/HAc (pH 4.8) and a mixture of acetonitrile/methanol (2:1, v/v). For the 10 compounds detected in NI mode, the best separation was obtained using methanol–water. Representative chromatograms of a 1 μ g/mL standard mixture of the compounds analyzed in NI and PI mode and the internal standards fitted in each retention time window are illustrated in Fig. 3.

The optimization of MS parameters (cone voltage and collision energy) was performed by flow injection analysis (FIA) for each compound. The analgesics and anti-inflammatories ibuprofen, diclofenac, mefenamic acid, ketoprofen, naproxen, indomethacin and the lipid regulators gemfibrozil, bezafibrate and clofibric acid were more sensitive in NI mode, whereas the analysis of the rest of compounds was carried out in PI mode.

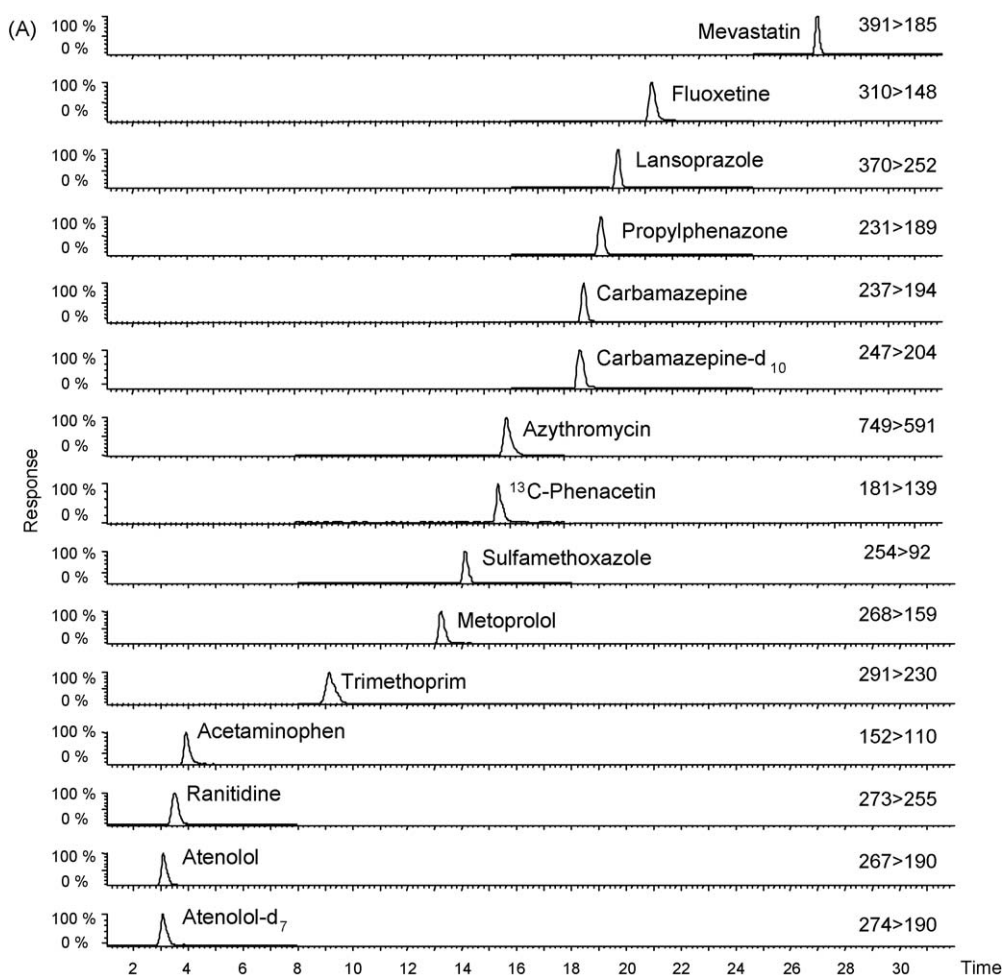


Fig. 3. Example of a MRM chromatogram of a standard mixture at 1 ng/ μ L for target compounds analyzed by (A) positive ionization mode and (B) negative ionization mode.

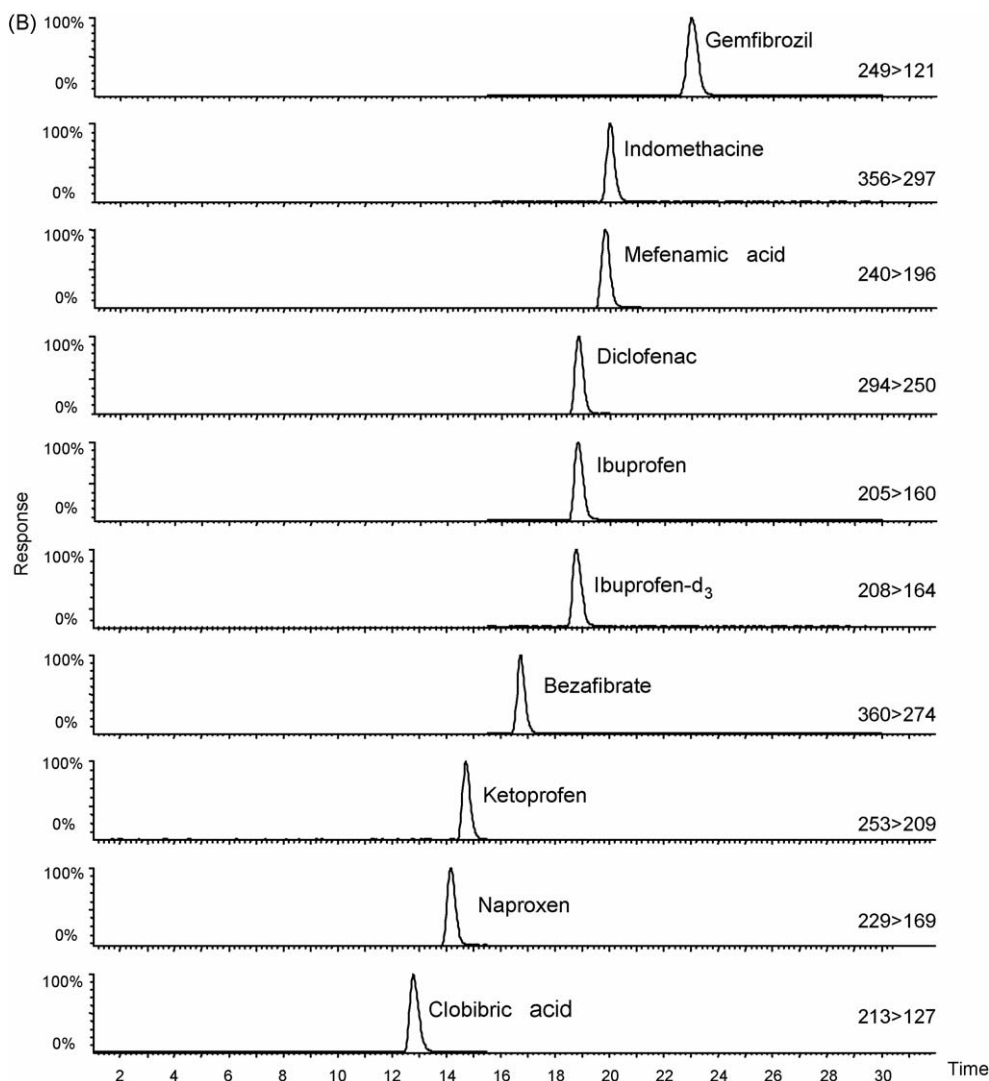


Fig. 3 (Continued).

In order to select precursor ions for each analyte, chromatograms were recorded in full scan mode. In all cases, $[M - H]^-$ for NI mode, and $[M + H]^+$ for PI mode were selected, with only exception being pravastatin which formed the sodium adduct. Once precursors were selected, product scans were recorded, testing different values of cone voltage and collision energies. Cone voltages were selected according to the sensitivity of precursor ions, whereas collision energies were chosen to give the maximum intensity of the fragment ions obtained. Product ions observed for each target compounds are indicated in Table 2 and were in good agreement with the ones reported in the literature [2,15,20].

3.3. Method validation

Sensitivity, linearity, recoveries, precision and the study of matrix effects were considered as the criteria for the validation of the analytical methodology developed. Validation data, determined for each matrix studied, are presented in Tables 3 and 4. Calibration curves were generated using linear regression analy-

sis and over the established concentration range (0.01–1 $\mu\text{g/mL}$) gave good fits ($r^2 > 0.99$). Five-point calibration was performed daily, and the possible fluctuation in signal intensity was checked by injecting a standard solution at two concentration levels after each 8–10 injections.

Recoveries achieved for all target compounds ranged from 60 to 102% and from 50 to 116% for surface and wastewaters, respectively. Only ranitidine, sotalol and famotidine, as well as mevastatin and clobibric acid, in the case of wastewater samples, showed lower recovery rates (50 and 35%, respectively). Nevertheless, as other performance data, such as repeatability and sensitivity, were good, the low recovery was not considered to be an obstacle for their reliable determination.

Detection limits obtained are reported in Table 4. MDL ranged from 1 to 30 ng/L and from 1 to 60 ng/L for surface and wastewaters, respectively. MQL were from 3 to 160 ng/L for both matrices. To ensure correct quantification, precision of the method was studied by analysing five replicates of a 100 $\mu\text{g/L}$ standard. Results are indicated in Table 4 for each compound, showing a precision from 0.2 to 6% and

Table 4
Linearity (regression coefficient), instrumental detection limits (IDL), detection and quantification limits for the method (MDL, MQL), repeatability and reproducibility of the LC–MS/MS method without SPE

Therapeutic group	Compounds	Linearity (r^2)	IDL (pg injected)	MDL (ng/L)			MQL (ng/L)			Repeatability % R.S.D. ($n = 5$)	Reproducibility % R.S.D. ($n = 5$)
				Surface waters	WWTP effluent	WWTP influent	Surface waters	WWTP effluent	WWTP influent		
Analgesics and anti-inflammatories	Ketoprofen	0.9985	60	30	21	28	70	73	95	1	6
	Naproxen	0.9924	30	7	9	9	20	32	32	1	5
	Ibuprofen	0.9989	60	8	12	12	42	20	20	3	9
	Indomethacine	0.9986	60	6	7	7	20	20	20	1	7
	Diclofenac	0.9997	42	2	10	10	5	30	30	1	3
	Mefenamic acid	0.9997	2	0.5	1	3	2	20	26	0.5	3
	Acetamino phen	0.9997	29	17	10	10	40	58	58	3	3
Lipid regulators and cholesterol lowering statin drugs	Propyphenazone	0.9999	11	3	10	7	23	9	30	1	1
	Clofibrac acid	0.9996	6	1	2	2	3	6	6	1	5
	Gemfibrozil	0.9996	21	1	1	3	3	3	9	2	11
	Bezafibrate	0.9998	5	1	2	9	4	6	31	5	7
	Pravastatin	0.9994	200	47	60	60	160	160	160	1	1
Psychiatric drugs	Mevastatin	0.9938	11	7	20	18	24	70	60	1	3
	Carbamazepine	0.9999	6	2	10	18	10	40	61	4	6
	Fluoxetine	0.9959	39	20	20	35	66	70	100	2	2
Antiulcer agent	Paroxetine	0.998	18	8	7	6	20	26	22	3	5
	Lansoprazole	0.9999	62	5	8	14	16	20	47	4	5
Histamine H ₁ and H ₂ receptor antagonists	Loratadine	0.9965	18	2	3	4	7	10	11	0.2	4
	Famotidine	0.9974	12	5	10	12	18	20	26	1	4
	Ranitidine	0.9967	23	2	20	24	5	70	80	1	8
Antibiotics	Erythromycin	0.9946	50	4	6	6	14	20	20	2	6
	Azythromycin	0.9989	27	1	3	7	3	10	23	1	1
	Sulfamethoxazole	0.9976	27	5	20	42	16	60	120	2	9
	Trimethoprim	0.9962	13	1	10	25	4	40	82	3	4
	Ofloxacin	0.999	52	16	43	43	56	110	110	1	5
β-Blockers	Atenolol	0.9954	10	9	10	42	30	40	141	1	6
	Sotalol	0.9975	10	18	60	29	60	100	97	4	9
	Metoprolol	0.9966	34	3	10	12	12	30	36	6	2
	Propranolol	0.9986	16	2	7	12	7	20	40	1	8

from 1 to 11% from intra- and inter-day analysis, respectively.

3.3.1. Matrix effects

One significant drawback in ESI MS quantitative analysis is what is known as matrix effect. It occurs because the ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement leading to erroneous results. There are several strategies to reduce matrix effect, e.g. selective extraction, effective sample clean-up after the extraction, or improvement of the chromatographic separation. Sometimes, these approaches are not the appropriate solutions because they could lead to analyte losses as well as long analysis times [39]. Other plausible and effective strategies, reported in the literature [30,33,40], consist in the use of suitable calibration approaches, such as external calibration using matrix-matched samples, standard addition or internal standard, as well as the dilution of sample extracts. Standard addition is a reliable method, but it is time-consuming. On the other hand, appropriate internal standard (structurally similar unlabeled compound or isotopically labelled standard) are not always commercially available or they are expensive. Therefore, in case of lack of matrix-matched materials and isotopically labelled standards, sample extract dilution is an effective alternative solution. In this work the efficiencies of these three strategies have been extensively studied for each analyte in WWTP effluent and influent samples, in order to select the best approach for quantitation purposes.

In order to evaluate the degree of ion suppression and in what extent the target compounds were sensitive to it calibration curves were prepared using WWTP effluent and influent extracts. Blanks (samples with no addition of the standards) were analyzed simultaneously in order to subtract the levels of target compounds present in the samples. The calibration curve obtained was compared to the one achieved for the same standards in methanol–water (25:75). When both curves are parallel and totally overlapped, compounds are not subjected to ion suppression. Fig. 4 shows an example of this approach, giving calibration curves for ketoprofen and ranitidine, analyzed by NI and PI mode, respectively. The curve above corresponds to the signals of the analyte in the solvent, whereas the second one represents the signals in a spiked influent extract. As it can be observed, curves obtained in a spiked influent extracts have lower slope, which indicates that compounds are susceptible to signal suppression. Signal suppression measured for compounds analyzed under NI conditions (analgesics and anti-inflammatories and some lipid regulators) ranged between 15 and 50% for effluent samples and from 40 to 60% for influent wastewaters, respectively. For the compounds analyzed under PI conditions, ion suppression ranged from 40 to 60% for effluent wastewaters, and over 60% for influent samples, reaching for some compounds values of 80% (ranitidine) and 90% (atenolol).

In order to evaluate the efficiency of the internal standards used in this work for the correction of these signal losses, internal calibration curves in methanol–water (25:75, v/v) and in spiked matrix extracts were compared. The overlapping of both curves means that signal losses experienced by the analytes are cor-

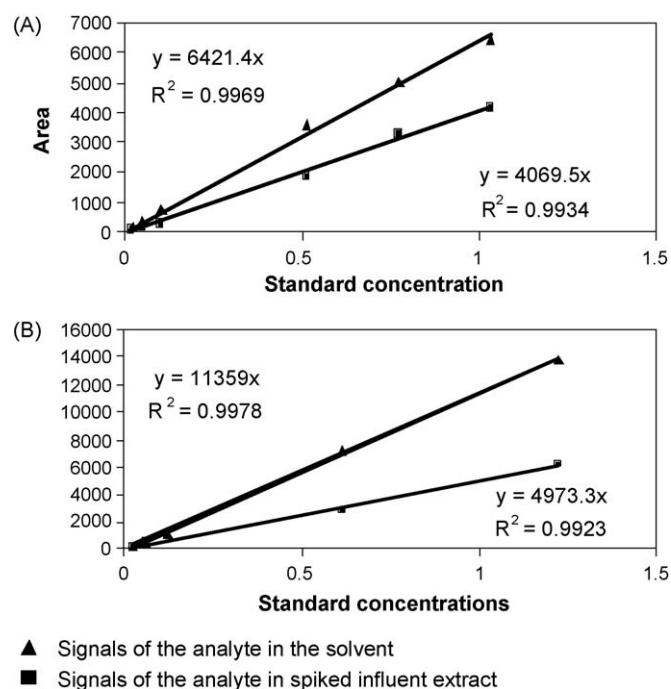


Fig. 4. Calibration curves in solvent vs. those prepared in spiked influent extracts for (A) ketoprofen and (B) ranitidine.

rected by the internal standards. On the other hand, when internal standards are not able to compensate signal losses for a particular compound, curves are not parallel, showing a difference in slopes. Fig. 5 shows an example for clofibric acid, atenolol and carbamazepine. Internal standards used were mecoprop- d_3 (for clofibric acid), atenolol- d_7 and carbamazepine- d_{10} , respectively. It is clearly depicted that internal standards corrected in a great extent signal losses due to ion suppression. Similar results were observed for the rest of the compounds, except for diclofenac, indomethacin, mefenamic acid, azithromycin, metoprolol, erythromycin and ofloxacin, where this correction was not so successful. When signal losses are successfully corrected by internal standards, the difference of slopes between both curves is reduced to a value close to 1, as they are overlapped. Even though signal suppression was considerably reduced for these substances, a difference ranging from 27 to 60% was still observed, which means that the internal standard used does not successfully correct signal losses. These results justify the use of internal standards, since they reduce and efficiently compensate signal losses.

In addition, the efficiency of sample extract dilutions was studied. For this purpose, the signals obtained after sequential dilution of a WWTP effluent and influent extract, (1:2, 1:4 and 1:8), were compared with the ones obtained for the corresponding concentrations of the standards in solvent. It is considered that matrix effects have been corrected when both signals are equal. An example is illustrated in Fig. 6 for bezafibrate and atenolol. For effluent wastewaters, dilution 1:2 was enough to avoid the signal decrease for the compounds analyzed by both NI and PI mode, whereas for influent wastewaters, dilution 1:4 was required to solve this problem. However, for WWTP influents, dilution 1:4 could lead to a considerable decrease in sensitiv-

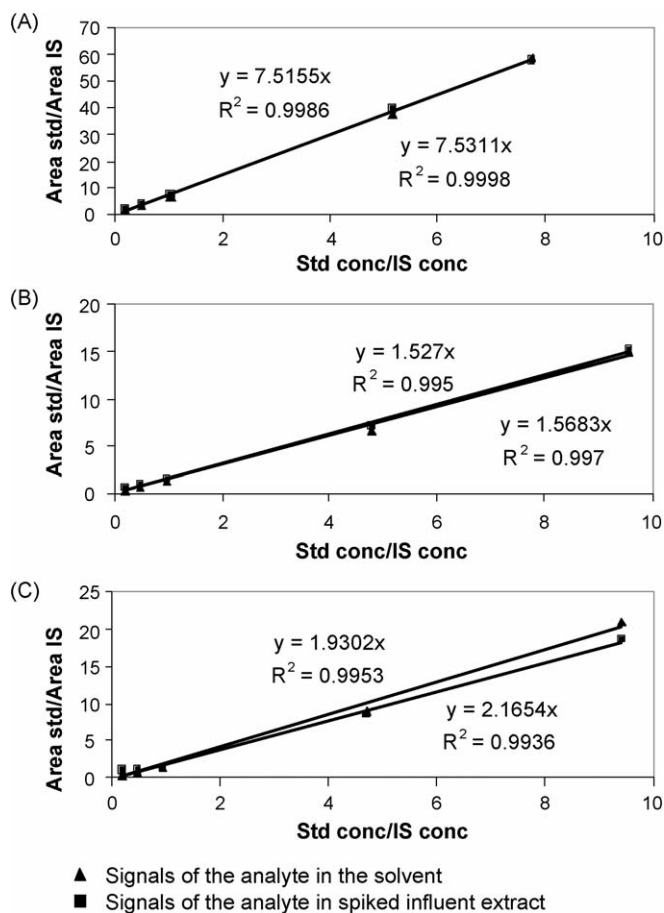


Fig. 5. Internal standard calibration curves in solvent and in spiked influent extracts for (A) clofibric acid (IS – mecoprop-*d*₃), (B) atenolol (IS – atenolol-*d*₇) and (C) carbamazepine (IS – carbamazepine-*d*₁₀).

ity, which is an important drawback that should be taken into account. According to these results, internal standard calibration is as efficient approach to assess the loss of signal intensity and therefore, can be used for quantitation purposes, with the advantage that it is simpler and not so time-consuming as matrix-matched calibration or standard addition.

Only for the compounds that internal standards are not able to compensate ion suppression, the other strategies should be used for quantitation. In our study the approach selected was the dilution of sample extracts.

3.4. Analysis of real surface and wastewater samples

To demonstrate the applicability of the developed method, 10 river waters from the Ebro river basin in Spain, and samples from 5 WWTP (influent and effluent) in Croatia were analyzed. Results obtained are summarized in Table 5. Anti-inflammatories and analgesics, lipid regulators, β -blockers and some antibiotics are the major groups detected in WWTP and among them acetaminophen, ketoprofen, ibuprofen, diclofenac, mevastatin, atenolol, propranolol, sulfamethoxazole and trimetoprim were the most abundant, with concentrations in high ng/L or low μ g/L levels. Maximum concentrations were detected for acetaminophen (paracetamol), with average con-

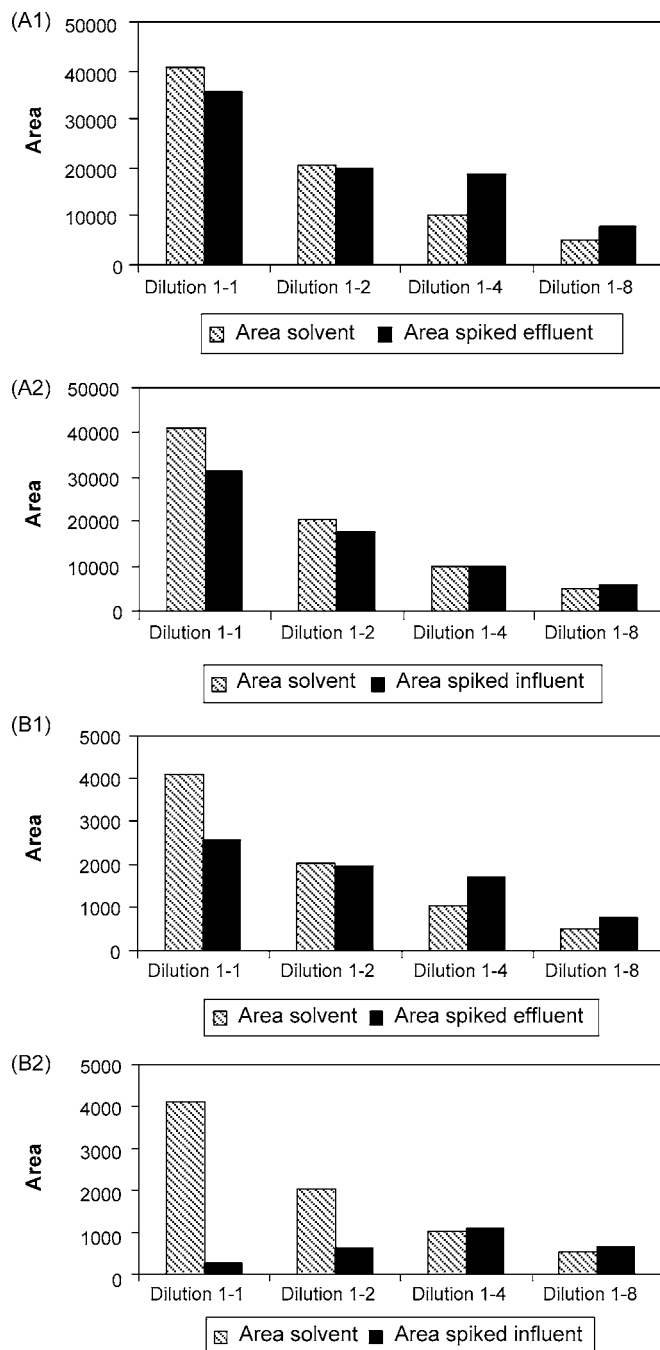


Fig. 6. Sample extract dilutions for (A1) bezaifibrate in WWTP effluent and (A2) bezaifibrate in WWTP influent; (B1) atenolol in WWTP effluent and (B2) atenolol in WWTP influent.

centration of 10 μ g/L in WWTP influent and 2.1 μ g/L in effluent and for antimicrobial trimethoprim (1.1 and 0.29 μ g/L in WWTP influents and effluents, respectively). Other compounds frequently detected in WWTP samples were carbamazepine and ranitidine, with average concentrations of 400 ng/L for carbamazepine in both influent and effluent samples (no elimination) and 188 and 135 ng/L for ranitidine in influent and effluent, respectively. In general, levels detected in Croatian WWTP are similar to those previously reported for WWTP in Spain, Germany, Italy and USA [7,10,20,24,25]. In the Ebro

Table 5

Range of concentrations and average level (expressed in brackets) detected for target Pharmaceuticals in influent and effluent wastewaters from five Croatian urban WWTP and in 10 points from the Ebro river basin

Therapeutic groups	Target compounds	Minimum–maximum concentrations (ng/L)		
		Surface waters	Effluent wastewaters	Influent wastewaters
Analgesics and anti-inflammatories	Ketoprofen	bld	130–620 (318)	160–970 (451)
	Naproxen	bld–50 (33)	bld–160 (108)	blq–190 (99)
	Ibuprofen	bld–150 (60)	40–800 (266)	nd–900 (516)
	Indomethacine	bld–10	bld	bld
	Diclofenac	bld–60 (29)	bld–390 (215)	50–540 (250)
	Mefenamic acid	bld–3 (2)	bld–10 (7)	bld–5 (5)
	Acetaminophen	bld–250 (42)	blq–5990 (2102)	130–26090 (10194)
Lipid regulators and cholesterol lowering statin drugs	Propyphenazone	bld	bld	bld
	Clofibrilic acid	10–20 (11)	20–30 (28)	bld–110 (72)
	Gemfibrozil	bld–60 (46)	bld–320 (120)	bld–360 (155)
	Bezafibrate	bld–10 (8)	bld–10	bld–50 (23)
	Pravastatin	nd	nd	nd
Psychiatric drugs	Mevastatin	bld	bld–800 (383)	bld–1170
	Carbamazepine	bld–110 (30)	bld–630 (410)	bld–950 (420)
	Fluoxetine	bld	bld	bld
Antiulcer agent	Paroxetine	bld	bld	bld
	Lansoprazole	bld	bld	bld
Histamine H ₁ and H ₂ receptor antagonists	Loratadine	bld–20	bld	bld
	Famotidine	bld	bld	bld
	Ranitidine	bld–10	bld–200 (135)	bld–290 (188)
Antibiotics	Erythromycin	bld–30 (17)	bld	bld
	Azythromycin	bld–20 (8)	50–210 (96)	blq–300 (152)
	Sulfamethoxazole	bld–<blq	blq–820 (390)	bld–870 (590)
	Trimethoprim	bld–20 (11)	70–310 (290)	bld–4220 (1172)
	Ofloxacin	bld	bld	bld
β-Blockers	Atenolol	bld–250 (72)	bld–1150 (400)	bld–740 (395)
	Sotalol	bld–70	bld–210 (185)	120–200 (167)
	Metoprolol	bld	bld	bld
	Propranolol	bld	100–470 (290)	80–290 (168)

bld: below limit of detection; blq: below limit of quantification.

river water concentration of compounds positively identified rarely exceeded 100 ng/L, with average concentrations (for 10 samples collected along the river) in the low ng/L range.

4. Conclusions

The multi-residue analytical method developed, based on SPE–LC–MS/MS allowed the simultaneous extraction of 29 multiple-class Pharmaceuticals in a single extraction step, simplifying considerably sample preparation. Recoveries obtained for all target compounds, using Oasis HLB cartridges were higher than 60%, except for ranitidine, sotalol, famotidine (50%) and mevastatin (34%). The application of LC–MS/MS operating in the MRM mode, with two transitions (if available) monitored for each compound, provided good sensitivity and selectivity of detection according to the EU Commission Decision 2002/657/EC requiring a minimum of 3 IP for the confirmation of Pharmaceuticals in environmental samples. Only for keto-profen, ibuprofen, gemfibrozil and pravastatin the number of IP required were not achieved due to their low fragmentation. The method yielded detection limits in the low ng/L range for both river and wastewater thus providing a reliable and robust tool that

can be used for routine analysis of multi-class Pharmaceuticals in aqueous samples.

Acknowledgements

This work was financially supported by the European Union EMCO project (INCO-CT-2004-509188) and by the Spanish Ministerio de Ciencia y Tecnología (EVITA project – CTM2004-06265-C03-01). Waters Corporation (USA) and IST (UK) are gratefully acknowledged for providing the SPE cartridges. Merck (Germany) is acknowledged for providing the SPE cartridges and HPLC columns. R. Chaler and D. Fanjul are also acknowledged for their excellent assistance in LC–MS troubleshooting.

References

- [1] C.G. Daughton, T. Ternes, Environ. Health Perspect. 107 (1999) 907.
- [2] M. Petrovic, M.D. Hernando, M.S. Diaz-Cruz, D. Barcelo, J. Chromatogr. A 1067 (2005) 1.
- [3] S. Díaz-Cruz, D. Barcelo, Handbook Environ. Chem. 5 (2004) 227.
- [4] M. Petrovic, S. Gonzalez, D. Barcelo, Trends. Anal. Chem. 22 (2003) 685.

- [5] M. Carballa, F. Omil, J.M. Lema, M. Llompарт, C. García-Jares, I. Rodríguez, M. Gómez, T. Temes, *Water Res.* 38 (2004) 2918.
- [6] C. Zwiener, F.H. Frimmel, *Sci. Total Environ.* 309 (2003) 201.
- [7] T.A. Ternes, *Water Res.* 32 (1998) 3245.
- [8] T. Ternes, M. Bonerz, T. Schmidt, *J. Chromatogr. A* 938 (2001) 175.
- [9] F. Sacher, F.T. Lang, H.J. Brauch, I. Blankenhorn, *J. Chromatogr. A* 938 (2001) 199.
- [10] M. Farré, M. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, D. Barcelo, *J. Chromatogr. A* 938 (2001) 187.
- [11] B.J. Vanderford, R.A. Pearson, D.J. Rexing, S.A. Snyder, *Anal. Chem.* 75 (2003) 6265.
- [15] M.D. Hernando, M. Petrovic, A.R. Fernandez-Alba, D. Barcelo, *J. Chromatogr. A* 1046 (2004) 133.
- [16] R. Hirsch, T. Ternes, K. Haberer, K.L. Kratz, *Sci. Total Environ.* 225 (1999) 109.
- [19] A. Gentili, D. Perret, S. Marchese, *Trends Anal. Chem.* 24 (2005) 704.
- [20] S. Castiglioni, R. Bagnati, D. Calamari, R. Fanelli, F. Zucatto, E. Zucatto, *J. Chromatogr. A* (2005).
- [21] A.A.M. Stolker, W. Niesing, E.A. Hogendoorn, F.M. Versteegh, R. Fuchs, U.A.T. Brinkman, *Anal. Bioanal. Chem.* 378 (2003) 955.
- [22] T.A. Ternes, M. Bonerz, N. Herrmann, D. Löffler, E. Keller, B.B. Lacida, A.C. Alder, *J. Chromatogr. A* 1067 (2005) 213.
- [23] S. Kim, K. Carlson, *Trends Anal. Chem.* 24 (2005) 635.
- [24] J.D. Cahill, E.T. Furlong, M.R. Burkhardt, D. Kolpin, L.G. Anderson, *J. Chromatogr. A* 1041 (2004) 171.
- [25] S. Öllers, P. Heinz, P. Fässler, R. Müller, *J. Chromatogr. A* 911 (2001) 225.
- [26] I. Rodriguez, J.B. Quintana, J. Carpinteiro, A.M. Carro, R.A. Lorenzo, R. Cela, *J. Chromatogr. A* 985 (2003) 265.
- [27] V. Koutsouba, T. Heberer, B. Fuhrmann, K. Schmidt-Baumler, D. Tsipi, A. Hiskia, *Chemosphere* 51 (2003) 69.
- [28] M.D. Hernando, M. Mezcua, M.J. Gomez, O. Malato, A. Agüera, A.R. Fernandez-Alba, *J. Chromatogr. A* 1047 (2004) 129.
- [29] T.A. Ternes, R. Hirsch, J. Mueller, K. Haberer, *Fresenius J. Anal. Chem.* 362 (1998) 329.
- [30] A. Kloepper, J.B. Quintana, T. Reemtsma, *J. Chromatogr. A* 1067 (2005) 153.
- [31] T. Ternes, *Trends Anal. Chem.* 20 (2001) 419.
- [32] A. Jahnke, J. Gandrass, W. Ruck, *J. Chromatogr. A* 1035 (2004) 115.
- [33] T. Benijts, R. Dams, W. Lambert, A. De Leenheer, *J. Chromatogr. A* 1029 (2004) 153.
- [34] J. Zrostlikova, J. Hajslova, J. Poustka, P. Begany, *J. Chromatogr. A* 973 (2002) 13.
- [35] K.V. Thomas, M.J. Hilton, *Mar. Pollut. Bull.* 49 (2004) 436.
- [36] H. Sanderson, D.J. Johnson, T. Reitsma, R.A. Brain, C.J. Wilson, K.R. Solomon, *Reg. Toxicol. Pharmacol.* 39 (2004) 158.
- [37] TID Index, www.rxlist.com.
- [38] Commission Decision (2002/657/EC) of 12 August 2002 Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities L221, Brussels, Belgium, pp. 8–36.
- [39] M. Stüber, T. Reemtsma, *Anal. Bioanal. Chem.* 378 (2004) 910.
- [40] L. Alder, S. Lüderitz, K. Lindtner, H.J. Stan, *J. Chromatogr. A* 1058 (2004) 67.

Further reading

- [12] X.-S. Miao, B.G. Koenig, C.D. Metcalfe, *J. Chromatogr. A* 952 (2002) 139.
- [13] S. Marchese, D. Perret, A. Gentili, R. Curini, F. Pastori, *Chromatographia* 58 (2003) 263.
- [14] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K.L. Kratz, *J. Chromatogr. A* 815 (1998) 213.
- [17] S. Yang, C.Y.K. Carlson, *J. Chromatogr. A* 1097 (2005) 40.
- [18] S. Díaz-Cruz, D. Barceló, *Trends Anal. Chem.* 24 (2005) 645.

Mineral salts removal and lithium traces determination in highly concentrated solutions and natural brines

Hichem Hamzaoui, Adel M'nif*, Ridha Rokbani

Institut National de recherche scientifique et Technique, B.P. 95, 2050, Hammam Lif, Tunisia

Received 5 September 2005; received in revised form 17 January 2006; accepted 7 February 2006

Available online 29 March 2006

Abstract

The usual methods, of lithium traces determination, refer in particular to the aqueous solutions whose dry residues do not exceed 7 g L^{-1} . Flame spectrometry or atomic absorption is used for the determination of the considered element.

In the case of natural brines, the total dissolved salt ranges from 250 to 350 g L^{-1} . Consequently the determination of lithium traces in this kind of solutions, by the above mentioned spectroscopic methods, may be disturbed due to interferences phenomena. So, a preparatory processing step of the samples is needed.

In order to prevent the mentioned phenomena, the total or partial removal of mineral salts, by adding appropriate organic solvent, is carried out. Accordingly, it is significant to make sure that the total quantity of lithium remains in the solution.

Therefore, the experimental conditions (sulfate ion removal, solvent ratio and solid–liquid contact time) are optimized. After that the different experimental steps are arranged to make sure the lithium determination, by flame spectrometry, practicable.

Finally, the established method is applied firstly to determine Li^+ in some natural brines and secondly to estimate the lithium reserves in the Tunisian natural brines deposits.

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Keywords: Brine; Lithium traces determination; Flame spectrometry

1. Introduction

Natural brines include lithium ions as traces [1]. Despite of their low Li concentrations, the worldwide reserves of lithium content in salt Lakes are estimated to be 60% [2]. Thus, the lithium extraction from brines is completed in USA, Chile, Bolivia and Argentina.

Lithium is usually recovered, from natural brines, as a by-product in the fabrication of other salts [2]. As for Tunisian brines, many studies are led to recover potassium and magnesium salts and it is carefully planned to supplement this task with respect to lithium. To develop such an extraction, it is important to control the Li traces determination in highly concentrated solutions.

The lithium traces determination is well-known for the aqueous solutions whose dry residues do not exceed 7 g L^{-1} [3], and

it is presumed that the Tunisian brines (40–50 times more concentrated than the previously mentioned solutions) may present some difficulties linked to interferences phenomena. In order to palliate to these phenomena, adjusting an appropriate lithium determination method becomes essential.

In addition, in a former study, we established and evaluated the interferences phenomena created due to oceanic salts cations during lithium flame photometry determination [4]. We essentially proved that the ratio $\text{Li}/\sum X$ ($\sum X = \sum [\text{cations}]$) in the brines is very low ($\sim 10^{-4}$), which explains the interference phenomena observed during the lithium quantitative determination.

The influence of the ions Na^+ , K^+ , Ca^{2+} and Mg^{2+} on the determination of lithium is studied. A depressive effect on the lithium determination is observed. This effect depends on the interfering ion and increases in the following order Ca, K, Na and Mg. The depressive effect raises parallel to the increase in the content of the interfering ions.

Furthermore, we ascertain that the standard additions method associated with flame spectrometry solves only partially the preceding problem [5].

* Corresponding author. Tel.: +216 71430470; fax: +216 71430934.
E-mail address: mnif.adel@inrst.nrnt.tn (A. M'nif).

We found that the ratio value $[Li]/\sum[X] = 9.6 \times 10^{-4}$ constitutes a good limit to define the barrier below which the application of the standard additions method is compromised.

Finally, we proved that these interferences are inhibited when the cations contents are reduced to a pre-established level ($[Na] \leq 200$ ppm, $[K] \leq 300$ ppm, $[Mg] \leq 400$ ppm) [6,7].

This led us to undertake a study of the lithium determination in the brines. The objective of this study is to remove a part of the mineral salts from the brine sample in order to determine its lithium content by means flame spectrometry.

Consequently, we succeeded in reducing the salt concentrations, in the studied medium, with less than 500 ppm and developing the protocol of a method for the determination of lithium traces.

2. Experimental

In view of the complexity of the studied brines, we adopted a two-stage investigation method: First, on synthetic brines (chemically similar to natural ones) and thereafter on natural brines.

The brine samples are preliminary treated in order to:

- remove the sulfate ions and avoid therefore the lithium sulfate (Li_2SO_4) precipitation throughout the isopropanol treatment;
- precipitate chloride salts by adding a controlled quantity of isopropanol [6,7];
- precipitate Mg^{2+} ions as carbonate in case of rich magnesium solutions ($[Mg^{2+}] > 20 \text{ g L}^{-1}$) [8].

All the above-mentioned treatments are experimentally optimized so as to avoid disturbing the lithium content in the studied sample.

2.1. Synthetic brine preparation

The included reagents are: anhydrous potassium chloride (+98% Fluka), sodium chloride (+99.5% Prolabo), potassium sulfate (+99.5% Prolabo), hexahydrated magnesium chloride (+97% Prolabo), lithium carbonate (+99% Fluka), barium chloride (+99% Acros), sodium carbonate (+99% Fluka), calcium chloride dihydrates (+99.5% Fluka) and isopropanol (+99.5% Fluka).

Three synthetic solutions S_I , S_{II} and S_{III} , having the compositions mentioned in Table 1, were prepared via dissolving in distilled water, aliquot quantities of sulfates and chlorides salts. These solutions contain, respectively, 10, 40 and 80 mg L^{-1} of lithium. This choice was made to control the prospective influence of the magnesium ion on the determination of Li. Indeed

Table 2
Lithium retention by $BaSO_4$

Mixture no.	Stirring time (min)	[Li ⁺] (ppm) in filtrates	
		Before washing	After washing
1	1	8.46	10.10
2	3	8.87	10.09
3	5	8.87	10.05
4	10	8.87	10.10
5	30	8.46	10.07

in a former work we showed that the removal of magnesium chloride by adding isopropanol is trivial [6].

2.2. Sample treatments

2.2.1. Sulfate ion removal

In a first stage the sulfate ions were crystallized as $BaSO_4$ per addition of a $BaCl_2$ solution. The produced solid phase ($BaSO_4$) might retain a part of the existing lithium in the studied solution. Subsequently we have to make sure that the lithium is not disturbed. Consequently mixtures of 10 mL of the studied solutions and 1 g of freshly prepared $BaSO_4$ are stirred during 1–30 min to optimize the contact time. Finally Li^+ contents are chemically analyzed in the filtrate, prior to and following a washing step of the solid phase. Table 2 shows the analysis results.

These results confirm our hypothesis about the partly retained lithium by $BaSO_4$ solid phase. However, a washing step using distilled water can liberate it. This washing step increases the volume of the sample. Therefore, it is necessary to bring it back, to the initial volume, by evaporation. This procedure is essential to limit the isopropanol consumption during the following treatment steps [8].

2.2.2. Salts precipitation

Oceanic salts precipitation using various individual or binary solvents (methanol, ethanol, isopropanol and acetone) was studied. It was established that isopropanol gave the best precipitation ratios [6–8]. Hereafter the optimal isopropanol volume to be used for chlorides salts precipitation, was studied on synthetic solution (S_I). Fig. 1 represents the obtained results. Particularly we set up that a volume of 30 mL of isopropanol enables the elimination of 96% of chlorides salts contained in 2.5 mL of the studied solution.

Furthermore, in a former work [6] we established that $LiCl$ do not precipitate under isopropanol action. Nevertheless, a washing step with the used solvent ($3 \times 2 \text{ mL}$) is carried out to make sure that the total lithium remains in the filtrate. Moreover, to avoid disturbing the lithium content determination by flame

Table 1
Synthetic solutions composition

Solutions	Li (mg L^{-1})	Na (g L^{-1})	K (g L^{-1})	Mg (g L^{-1})	Ca (g L^{-1})	Cl (g L^{-1})	SO_4 (g L^{-1})
S_I	10	88	3.6	12.6	0.3	150	37
S_{II}	40						
S_{III}	80						

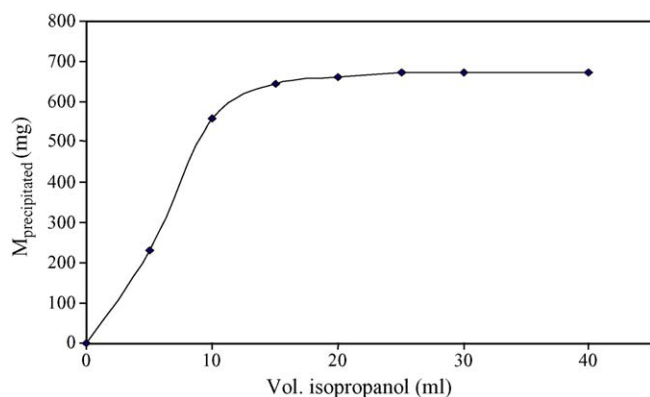


Fig. 1. Isopropanol optimal volume to be used for chlorides precipitation.

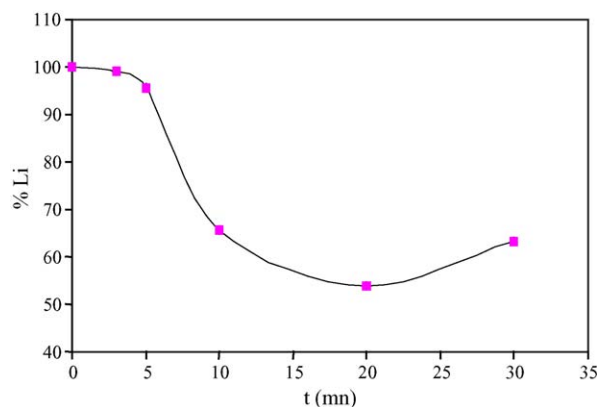


Fig. 2. % Li remaining in solution vs. contact time.

spectrometry, the organic solvent is removed through a distillation step at a temperature between 81 and 82 °C [9]. Lastly, the volume of the resulting filtrate is adjusted to 50 mL taking into account the Li content of the prepared solutions and the measurable range by flame spectrometry (0, 5–10 ppm).

2.3. Lithium determination on synthetic brine

To check the validity of the previous treatment steps, we considered to be opportune, their application on the synthetic brines S_I, S_{II}, S_{III}.

The determinations carried out, after the sulfate ions removal and isopropanol treatments, led to the results recapitulated in Table 3.

The deviations between the measured and initial values indicate that the preceding treatments affect the Li⁺ content.

Consequently we thought that lithium could be retained by the salts precipitated following the addition of isopropanol. A complementary study was then undertaken to elucidate the effect of the contact time between the precipitated salts and the solution.

2.4. Influence of the contact time between the precipitated salts and solutions

Considering solution S_{III}, we studied the effect of contact time (solid–liquid) for the range 3–20 min. The results are given in Fig. 2.

We deduce that a contact time less than 3 min does not affect the Li⁺ content in the studied solution.

Thanks to the obtained results, it is possible to outline in a preliminary approach the following procedure for the determi-

nation of Li⁺ traces in natural brines and strongly concentrated solutions:

- Removal of sulfate ions from the sample (elimination of SO₄²⁻) followed by an evaporation step to concentrate the solution and come back to the initial volume.
- Isopropanol treatment of the sample. During this step it is important not to exceed the optimum contact time between the precipitated solid and the solution.
- Precipitated salts separation.
- Organic phase distillation.
- Lithium determination in the aqueous phase.

2.5. Case of a very magnesian brine

In a former work we established initially that magnesium ion interferes in a depressive way in the determination of lithium content and thereafter that the isopropanol does not precipitate the magnesium compounds well [6]. Consequently in the case of the strongly magnesian brines ([Mg] > 20 g L⁻¹) an additional step would be envisaged in order to reduce its Mg²⁺ content. The adopted technique consists in precipitating Mg²⁺ as carbonate by adding a 70 g L⁻¹ solution of Na₂CO₃. It is important to notice that we checked that the earlier precipitated MgCO₃ does not retain lithium [14]. In the above-mentioned procedure, the Mg²⁺ removal operation is to be carried out right after the sulfate one.

2.6. Final recommended scheme

The procedure to be adopted is described below:

- 1 Measure a volume $V_p = 2.5$ mL of the solution to be analyzed.
- 2 Add an excess of a BaCl₂ solution 1 M (v mL), stir the mixture and separate on filter funnel porosity 3 and finally wash three times the solid phase BaSO₄ with 2 mL of distilled water and recover the filtrate F_1 . The following step depends on the Mg²⁺ content in the initial solution. If Mg²⁺ > 20 g L⁻¹ go to step 3 and if Mg²⁺ < 20 g L⁻¹ go to step 4.
- 3 Add v' mL of a 0.5 M Na₂CO₃ solution, stir the mixture and separate on filter funnel porosity 4 and wash three times the

Table 3
Lithium determination in prepared synthetic brines

Synthetic solutions	[Li] (mg L ⁻¹)		
	Initial	Measured	Deviation (%)
S _I	10	5.6	44.0
S _{II}	40	31.0	22.5
S _{III}	80	52.6	34.25

solid phase (MgCO_3) with 2 mL of distilled water and recover the filtrate F'_1 .

- 4 Concentrate the filtrate F_1 or F'_1 until $V \sim V_p$. Cool the sample to ambient temperature.
- 5 Add 30 mL of isopropanol to the previous sample and mix during 3 min. Separate on filter funnel porosity 4 and wash three times the solid phase with 2 mL of isopropanol and recover the filtrate F_2 .
- 6 Distill the filtrate F_2 until 100°C . After cooling to ambient temperature recover the aqueous solution and rinsing water.
- 7 Dilute the recovered solution to 50 mL and go to the lithium determination by flame spectrometry.

All the preceding steps are recapitulated in Fig. 3 chart.

To confirm this experimental protocol, we carry out the study of its validity for Li^+ contents ranging from 10 to 200 mg L^{-1} . This choice is based on the fact that the natural or evaporated brines have a lithium content included in this interval [1,10–13].

3. Discussion and application of the procedure

3.1. Method applicability

The previous protocol was applied to a batch of synthetic solutions S_x . These samples contain a range of concentrations similar to S_1 with respect to major ions and variable lithium contents [$x \text{ ppm}$]. The results in Table 4 show that the measured lithium contents correspond to those initially introduced. It is important to mention that all the indicated values represent the arithmetic average of at least three experimental results and that the error is less than 1%.

Table 4
Li analysis in synthetic brines

S_x	Li (ppm)		Na (ppm)		K (ppm)		Mg (ppm)		Ca (ppm)		Total remaining cations (ppm)
	I	M	I	M	I	M	I	M	I	M	
S_{10}	10	9.95	4400	130	180	75	630	280	15	10.25	495.25
S_{20}	20	20.10	4400	136	180	70	630	275	15	10.30	491.30
S_{30}	30	30.11	4400	129	180	78	630	278	15	10.50	495.50
S_{40}	40	39.89	4400	133	180	73	630	283	15	10.40	499.40
S_{50}	50	50.14	4400	130	180	75	630	279	15	10.55	494.55
S_{60}	60	60.16	4400	132	180	69	630	270	15	10.35	481.35
S_{70}	70	69.87	4400	135	180	76	630	275	15	10.25	496.25
S_{80}	80	80.50	4400	134	180	72	630	278	15	10.25	494.25
S_{200}	200	201.4	4400	137	180	77	630	277	15	10.35	501.35

I: initial; M: measured (remaining after treatment).

Table 5
Pondered rates of the major elements and lithium in brines of the Tunisian south

Situation	Surface (km^2)	Total salinity average (g L^{-1})	Total reserves in situ ($\times 10^6 \text{ m}^3$)	$[\text{Li}^+]$ ($\times 10^{-3} \text{ g L}^{-1}$)	Li reserves (tonnes)
S. El Adhibate	125 ^a	290	245 ^a	16.00	3920
S. El Briga	22 ^a	330	78 ^a	9.67	754
S. El Mellah	150 ^a	335	600 ^a	15.06	9000
Chott. Jerid	5000 ^b	330	5000 ^b	29.00	145000

^a ONM results.

^b CPG results [1].

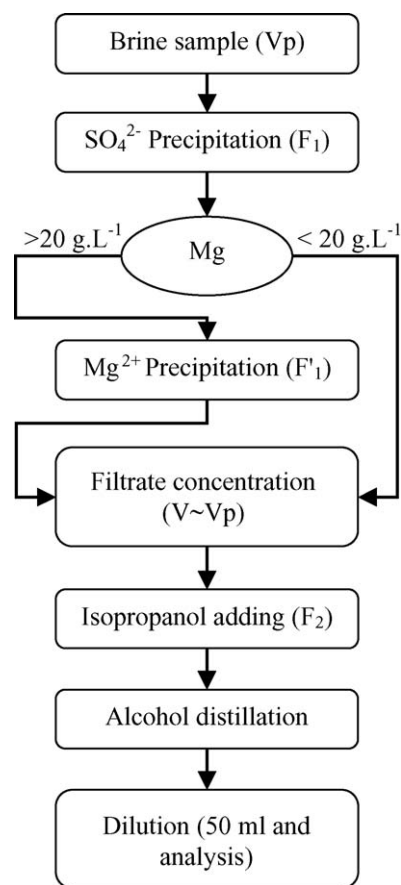


Fig. 3. Recommended method flow chart.

3.2. Application to natural brines

The discussed determination method was applied to the lithium reserves evaluation in the Sebekhat and Chott of the Tunisian south. Table 5 summarizes the results thus obtained.

Chott El Jerid could constitute a considerable reserve of lithium. “Sebekhat El Adhibate” and “El Malah de Zarzis” contain less significant quantities given that this group of deposits accounts only for the tenth of the brine salt lakes Tunisian reserves. However, taking into consideration the best accessibility and pumping conditions of their brines, these two sites represent a really economic interest. On a purely comparative basis, a sample taken from Dead Sea (Jordan), was chemically analyzed. The measured lithium content is 70 mg L^{-1} .

4. Conclusion

The natural brines chemical composition is complex. In addition their lithium content is as traces. As a result, the determination of this element is very difficult to achieve when using the classic and simple flame spectrometry method. We start by verifying the interferences phenomena which appear between the Li traces and major ions, and then we deduce the necessity of investigating a treatment method to make possible the lithium; determination using flame spectrometer.

The recognized procedure is rather complex but by using this classic and simple technique, it allowed the Li^+ determination and consequently the evaluation of lithium reserves enclosed in the Sebkhat and Chotts of the Tunisian south.

Also, this determination method was used to emphasize the lithium retention phenomena by removed salts during the brine

solar evaporation. We showed in particular that 50% of initial Li^+ content might be retained by crystallized salts [15].

Lastly, it is obvious that this method will contribute largely to the development of a lithium extraction process starting from natural brines.

References

- [1] Rapport sur les essais préliminaires et «bench scale» avec la saumure de Zarzis “Sulmag/ONM 1982”.
- [2] R. Perrin, J. Scharff, Chimie industrielle, 2nd ed., DUNOD, 1999, pp. 213–215.
- [3] F. Ecrement, F.P. Burelli, Analusis 3 (1975) 146.
- [4] A.H. Hamzaoui, A. M'nif, R. Rokbani, J. Soc. chim. Tunisie 4 (1999) 419.
- [5] A.H. Hamzaoui, A. M'nif, R. Rokbani, Phys. Chem. News 5 (2002) 107.
- [6] A.H. Hamzaoui, A. M'nif, R. Rokbani, J. Soc. chim Tunisie 4 (1999) 431.
- [7] A. M'nif, Contribution à la valorisation des saumures du sud Tunisien, Habilitation, Fac. Sc. Sfax, 2001.
- [8] A.H. Hamzaoui, Contribution à la détermination de la teneur du lithium et à son extraction dans les saumures, Thèse de Doctorat de spécialité, Fac. Sc. Tunis, 1999.
- [9] R.C. Weast, M.J. Astli, W.H. Beyer, Hand Book of Chemistry and Physics, 65th ed., CRC Press, 1984–1995.
- [10] L. Dieulafait, Ann. Chem. Phys. 5 (1879) 377.
- [11] R. Fresenius, Fresenius Mineralwasser Analysen (1900–1908).
- [12] A. Darapsky, Chem. Ztg. 125 (1930) 1.
- [13] M. Pinta, Spectrometrie d'absorption Atomique, Ed. Masson, Tome 1, 1971.
- [14] A.H. Hamzaoui, A. M'nif, H. Hammi, R. Rokbani, Desalination 158 (2003) 221.
- [15] A. M'nif, A.H. Hamzaoui, R. Rokbani, Asian J. Chem. 13 (2001) 27.

A novel uranyl membrane sensor with potentiometric anionic response

Saad S.M. Hassan*, Amr M.Y. Attawiya

Department of Chemistry, Faculty of Science, Ain Shams University, Cairo, Egypt

Received 31 October 2005; received in revised form 4 February 2006; accepted 7 February 2006

Available online 29 March 2006

Abstract

A novel potentiometric uranyl membrane sensor with a divalent anionic response is developed, characterized and used for determination of uranyl ion. The sensor incorporates triethylenetetramine (TETA) as an ionophore in poly(vinyl chloride) matrix membrane (PVC) plasticized with *o*-nitrophenyloctyl ether (*o*-NPOE). In strong sulphate test solutions, UO_2^{2+} ion forms a highly stable $[\text{UO}_2(\text{SO}_4)_2]^{2-}$ anion, extractable in TETA as $\{(2\text{TETAH})^{2+} [\text{UO}_2(\text{SO}_4)_2]^{2-}\}$ complex. Formation of the complex is confirmed and characterized by elemental analysis, mass spectrometry and infrared spectrometry. Sensor based on this system displays at pH 2.5–3.8 a linear response over the concentration range of 1.0×10^{-1} – $3.5 \times 10^{-5} \text{ mol l}^{-1}$ uranium with a near-Nernstian calibration slope of $-26.5 \pm 0.3 \text{ mV decade}^{-1}$. The lower limit of detection is $\sim 5 \mu\text{g ml}^{-1}$, the lifetime is 12 weeks and negligible interferences are caused by most common cations. Validation of the assay method reveals excellent performance characteristics in terms of sensitivity, selectivity, fast response and potential stability. The sensor is used for the determination of 0.01–7.09 wt% uranium in naturally occurring and certified ore samples. The results show an average recovery of 97.6% and compare fairly well with data obtained using X-ray fluorescence technique.

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Keywords: Uranyl sensor; Potentiometry; Triethylenetetramine (TETA) ionophore; PVC membrane; Uranium ores; Uranyl sulphate

1. Introduction

Many potentiometric uranyl $[\text{UO}_2^{2+}]$ sensors have been described and tested for uranium assessment. Most of these incorporate phosphorus, nitrogen or oxygen based ion exchangers or neutral ionophores dispersed in plasticized polymeric membranes. Phosphorus based ionophores include di(2-ethylhexyl) phosphate (DEHP) [1], tri(2-ethylhexyl) phosphate (TEHP) [2], tri-*n*-octyl phosphine oxide (TOPO) [3], bis[di(4-(1,1,3,3-tetramethyl butyl))] phenyl phosphate [4,5], tetraphenyl-*o*-xylyldiphosphine dioxide [6], acyclic oligoethers with terminal phosphonate groups [7], phosphine oxide–phosphoric acid ester bi-functional compounds [8] and tris(chloroethyl or propyl) phosphate [9].

Nitrogen based uranyl ionophores have been suggested including 2,2'-[1,2-ethanediyl bis(nitriloethylidene)] bis(1-naphthalene) [10], 5,6,7,8-tetrahydro-8-thioxopyrido-[4',3',4,5] thieno[2,3-*d*]pyrimidine-4(3H)one (TTPTP) [11], *N,N'*-diheptyl-*N,N'*, 6,6-tetramethyl-4,8-dioxaundecane diamide

(ETH 295) [12], bis(acetylacetone)-*p*-phenylenediamine [13] and 2-hydroxyacetophenone oxime thiourea trioxine resin [14]. Oxygen compounds such as 18-crown-6, 24-crown-8 and their derivatives [15–18] or complexes with phosphotungstic acid [19] and calixarene derivatives [20–23] have been utilized as uranyl ionophores. Sensors based on all these ionophores display the expected cationic response for UO_2^{2+} with slopes ranging from 24 to 30 mV decade⁻¹. Limitations of these sensors are the significant interference by many cations and anions such as Ba^{2+} , Cr^{3+} , Cu^{2+} , Fe^{3+} , Th^{4+} , Pb^{2+} , V^{4+} , Ce^{3+} , Ni^{2+} , Al^{3+} , Mn^{2+} , Sr^{2+} , Ca^{2+} , F^- , Cl^- , NO_3^- , PO_4^{3-} , I^- , ClO_3^- [1–4,6,8–10,14–17,20–22], narrow response range (down to less than $10^{-4} \text{ mol l}^{-1}$) [1,3,4,6,8,9,15] and short lifetime.

It has been reported that uranyl ions in sulphate solutions form highly stable anionic species of the type $[\text{UO}_2(\text{SO}_4)_2]^{2-}$ extractable in aliphatic amines [24–26]. Since the mechanism of potential response of most liquid and liquid polymeric membrane sensors is based on ion exchange equilibria and analyte extraction process at the membrane interface, attempts were made to test uranyl/sulphate/amine system for developing a uranyl sensor. In this work, we report the first uranyl sensor with anionic response. The sensor is based on the use of tri-

* Corresponding author. Fax: +20 2 682 2991.

E-mail address: saadsmhassan@yahoo.com (S.S.M. Hassan).

ethylenetetramine (TETA) as an ionophore, *o*-nitrophenyloctyl ether as a plasticizer and PVC as matrix membrane. This sensor displays enhanced selectivity and sensitivity for uranyl ion in the presence of many cationic species. Determination of low concentration levels of U^{6+} (down to 0.01 wt%) in certified and natural uranium ores gives results compare fairly well with data obtained using X-ray fluorescence technique.

2. Experimental

2.1. Equipment

An Orion (Model 940) pH/mV meter was used for all potentiometric measurements at $\pm 25^\circ\text{C}$ unless otherwise stated. An Orion double junction Ag/AgCl reference electrode (Model 90-02) containing 10 wt% potassium chloride in the outer compartment was used. An Orion Ross combination glass electrode (Model 81-02) was used for all pH measurements. Philips X-ray fluorescence (X unique II spectrometer) was used for X-ray measurements.

2.2. Reagents and materials

All chemicals were of analytical reagent grade unless otherwise stated, and de-ionized double distilled water was used throughout. Poly(vinyl chloride) powder (PVC), of high molecular weight, dibutylsebacate (DBS), di-octylphthalate (DOP) and *o*-nitrophenyloctyl ether (*o*-NPOE) were obtained from Aldrich (Ave, USA). Tetrahydrofuran (THF) was purchased from Tedia (OH, USA) and freshly distilled prior to use. Triethylenetetramine and dioctylphenylphosphonate (DOPP) were obtained from Across, Janssen Pharm. (Belgium), and uranyl sulphate, nitrate and chloride were obtained from Merck (Germany).

Stock 0.1 mol l^{-1} solutions of uranyl nitrate, chloride and sulphate were prepared by dissolving 5.02 g of $UO_2(NO_3)_2 \cdot 6H_2O$, 3.4 g of UO_2Cl_2 and 4.2 g of $UO_2SO_4 \cdot 3H_2O$ in 50 ml of double distilled deionized water and completed to 100 ml with water. Dilute solutions (1.0×10^{-6} to $1.0 \times 10^{-2}\text{ mol l}^{-1}$) were prepared by appropriate dilutions with water and brought to pH 3.0 by addition of 1 mol l^{-1} HNO_3 , HCl and H_2SO_4 solutions to uranyl nitrate, chloride and sulphate solutions, respectively.

All metal solutions examined in the selectivity experiments were prepared from sulphate salts of high purity. Silver, calcium, strontium and lead nitrates were also used.

2.3. Preparation of uranyl PVC membrane sensor

A 20 mg portion of triethylenetetramine, 400 mg of dibutylsebacate, dioctylphthalate, dioctylsebacate (DOS), *o*-nitrophenyloctyl ether or dioctylphenylphosphonate and 137 mg PVC were dissolved in about 5 ml of tetrahydrofuran. The mixture was poured into a glass ring with an inner diameter of 26 mm resting on a smooth glass plate. The ring was covered with a filter paper for about 24 h to allow slow solvent evaporation. A transparent membrane was obtained (average thickness of $\sim 0.3\text{ mm}$).

A 7 mm diameter piece was cut out with a cork borer and glued to the polished end of a PVC tube (5 mm diameter and 5 cm long) by means of THF. The tube was attached to a sensor glass body as previously described [27–29]. The internal filling solution was a mixture of an equal volume of $10^{-3}\text{ mol l}^{-1}$ uranyl sulphate and potassium chloride solutions. A silver/silver chloride wire (0.3 mm diameter) was used as an inner reference electrode. The sensor was soaked in $10^{-3}\text{ mol l}^{-1}$ UO_2SO_4 solution for at least 24 h before use and stored in the same solution when not in use. The electrochemical cell used was: Ag/AgCl sat KCl/test solution//membrane//internal filling solution (pH 3)/sat. KCl, AgCl/Ag.

2.4. Potentiometric measurements

Aliquots (10 ml) of 10^{-1} to $10^{-6}\text{ mol l}^{-1}$ uranyl sulphate solutions were transferred to 50 ml beakers. The uranyl PVC membrane sensor in conjunction with a double junction Ag/AgCl reference electrode was immersed in the solutions. The potentials were recorded, at a constant stirring, when stabilized to $\pm 0.2\text{ mV}$ and plotted versus uranyl ion concentrations on a semi-logarithmic paper. The graph was used for subsequent determination of unknown uranium concentrations.

2.5. Sensor selectivity

Aliquots (10 ml) of $10^{-3}\text{ mol l}^{-1}$ uranyl sulphate solutions were adjusted to pH 3.0, the sensor was immersed in the solution and the potential was measured. Solutions containing $10^{-3}\text{ mol l}^{-1}$ of sodium or potassium salts of I^- , Cl^- , NO_3^- , NH_4^+ and nitrate salts of Ag^+ , K^+ , Li^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Co^{2+} , Sn^{2+} , Be^{2+} , Pb^{2+} , Sr^{2+} , Cd^{2+} , Fe^{3+} , Al^{3+} , Y^{3+} , Ce^{3+} , Ti^{4+} , V^{4+} , Th^{4+} , Nb^{5+} , V^{5+} and Mo^{6+} were adjusted to pH 3.0 with dilute H_2SO_4 and the potential reading of each was measured. The selectivity coefficients were determined using the separate solutions method [30].

2.6. Determination of uranium in ores

A 2.0 g portion of dried natural or certified ore was accurately weighted, grinded to -200 mesh size and transferred to a 100 ml Teflon beaker. A mixture of 25 ml of concentrated HF (48%), 5.0 ml of concentrated H_2SO_4 (98%) and 10 ml of concentrated HNO_3 (70%) was added. The mixture was heated on a hot plate under a fumehood. A 2.0 ml portion of 1 mol l^{-1} KF, 1.0 ml of 2 mol l^{-1} $Al(NO_3)_3$ and 2 ml of 5% diethylenetriaminepentaacetic acid (DTPA) were added during digestion. Heating was continued till a moistly precipitate was obtained. The mixture was cooled and dissolved in a 100 ml portion of hot $3\text{--}4\text{ mol l}^{-1}$ H_2SO_4 to form anionic uranyl sulphate species $[UO_2(SO_4)_2]^{2-}$.

An aliquot of uranium digest solution (10.0 ml) was adjusted to pH 3.0 ± 0.5 using 1 mol l^{-1} NaOH solutions and TETA-based membrane sensor in conjunction with Ag/AgCl double junction reference electrode was immersed in the test solution. The potential was measured when the potential became stable to $\pm 0.2\text{ mV}$. The concentration of uranium in the solution was calculated using a standard calibration curve. Other uranyl salts

Table 1

Effect of the membrane plasticizer on uranyl TETA based membrane sensor at pH 3.0

Membrane no.	Membrane composition (wt%)							Potential response	
	PVC	TETA	DBS	DOP	DOS	<i>o</i> -NPOE	DOPP	Slope (mV decade ⁻¹)	Linear range (mol l ⁻¹)
1	24.6	3.6	71.8	–	–	–	–	15.3	2.0×10^{-4} – 1.0×10^{-2}
2	24.6	3.6	–	71.8	–	–	–	6.2	Unstable
3	24.6	3.6	–	–	71.8	–	–	Erratic	Unstable
4	24.6	3.6	–	–	–	71.8	–	26.5	3.5×10^{-5} – 1.0×10^{-1}
5	24.6	3.6	–	–	–	–	71.8	9.3	1.0×10^{-2} – 1.0×10^{-1}

(uranyl chloride and uranyl nitrate) were similarly determined after sulphuric acid digestion.

3. Results and discussion

3.1. Nature of the ionophore

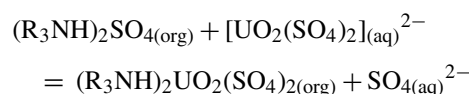
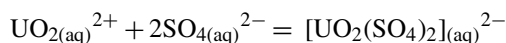
Uranyl sulphate in sulphuric acid medium forms anionic complex of the type $[\text{UO}_2(\text{SO}_4)_2]^{2-}$ extractable in aliphatic amines. In this study, uranyl triethylenetetramine complex was prepared, isolated in the solid form and characterized. Elemental analysis data reveal the formation of a complex with the molecular formula $\text{C}_{12}\text{H}_{32}\text{N}_8\text{UO}_2$ (molecular mass = 558) (calculated C = 25.3%, H = 5.5% and N = 20.0%; found C = 25.8%, H = 5.7% and N = 20.1%). The mass spectrum of the complex displays a molecular ion peak at *m/e* 557 for $(M-1)^+$ which further confirms the proposed structure. The infrared spectrum of the complex shows stretching vibration bands for O–U–O at 912 and 845 cm⁻¹ [31] and symmetric stretching of =N–H groups at 3450–3250 cm⁻¹.

3.2. Potential response of TETA based membrane sensor

Sensors with membranes incorporating triethylenetetramine in plasticized poly(vinyl chloride) in the ratio of 3.6 wt% TETA, 71.8 wt% plasticizer and 24.6 wt% PVC, respectively, were prepared, soaked in uranyl sulphate solution and tested for uranyl response. Potentiometric responses of membranes plasticized with DOP, DOS, DBS and DOPP solvent mediators are either unstable, with weak calibration slopes (6.2–15.3 mV decade⁻¹), or cover a limited concentration range (Table 1). Membranes plasticized with *o*-NPOE, however, display a linear response over the concentration range 1.0×10^{-1} to 3.5×10^{-5} mol l⁻¹ uranyl sulphate with an anionic slope of -26.5 ± 0.3 mV decade⁻¹ and a detection limit of 3×10^{-5} mol l⁻¹ ($\sim 5 \mu\text{g ml}^{-1}$). Optimal potential response is obtained by using 10^{-3} mol l⁻¹ uranyl sulphate as an internal filling solution. Sensors with membrane ingredients deviated $\pm 15\%$ of the above composition give similar results. The general performance characteristics of the sensor is shown in Table 2.

The unusual anionic response of TETA based membrane sensor is probably due to extraction of uranyl ion from sulphuric acid solution in the form of anionic complex $[\text{UO}_2(\text{SO}_4)_2]^{2-}$ [25] through the formation of an amine ion pair complex. Extraction mechanism of uranium anionic species in sulphuric acid medium with some aliphatic amines has been previously reported [26]

and known to proceed as follows:



Extraction of uranyl ion from the aqueous test solution to the organic phase of the sensor's membrane is believed to take place by a similar mechanism involving an anion-exchange equilibrium reaction. The divalent anionic response of TETA based membrane sensor supports the mechanism and complex structure shown in Fig. 1, where donation of the ion pairs of electrons from the –NH₂ primary amino groups is favored over that from the sterically hindered secondary amino groups.

It can be seen that the nature of aqueous test solution plays a crucial role in the membrane response. Anionic divalent near-Nernstian response is only noticed by using uranyl sulphate solution in sulphuric acid medium. In the presence of uranyl nitrate or chloride, however, weak response is obtained (Fig. 2). Formation and extraction of amine-uranyl binary complexes proceed only in sulphuric acid media [25,26]. In nitric and hydrochloric acid solutions, a little tendency to form anionic complexes has been reported and no extraction of uranyl ion by the amine system takes place. Thus, sulphate ion forms the strongest anionic complex with uranyl ion. The complexing constant of $[\text{UO}_2(\text{SO}_4)_2]^{2-}$ at 25 °C has been reported to be 7.1×10^2 compared to 8.8×10^{-1} and 2.4×10^{-1} for uranyl chloride and uranyl nitrate, respectively [24]. Further-

Table 2

Performance characteristics of uranyl TETA based membrane sensor

Parameter	Value
Slope (mV decade ⁻¹)	-26.5 ± 0.3
Working acidity range, pH	2.5–3.8
Response time for 10^{-3} M UO_2^{2+} (s)	30
Linear concentration range (mol l ⁻¹)	3.5×10^{-5} – 1.0×10^{-1}
Lifetime (week)	12
Lower linear range (mol l ⁻¹)	3.6×10^{-5}
Lower limit of detection (mol l ⁻¹)	2.0×10^{-5}
Interfering ions	Fe^{3+} , Th^{4+}
Accuracy (%)	97.6
Correlation coefficient, <i>r</i>	0.9920
Standard deviation (%)	± 0.8
Between-day variability, CV _b (%)	± 1.1
Within-day variability, CV _w (%)	± 0.8

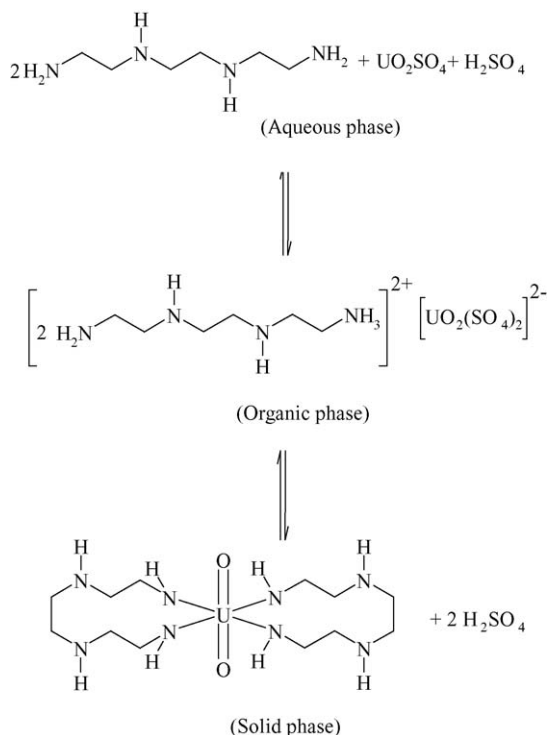


Fig. 1. Structure of uranyl-TETA complex ($C_{12}H_{32}N_8UO_2$, molecular mass = 558).

more, uranyl sulphate complex present in the organic phase is more highly charged than uranyl nitrate in nitric acid or uranyl chloride in hydrochloric acid solutions [32]. This increases the electrical conductivity of the membrane. The sensor responds to

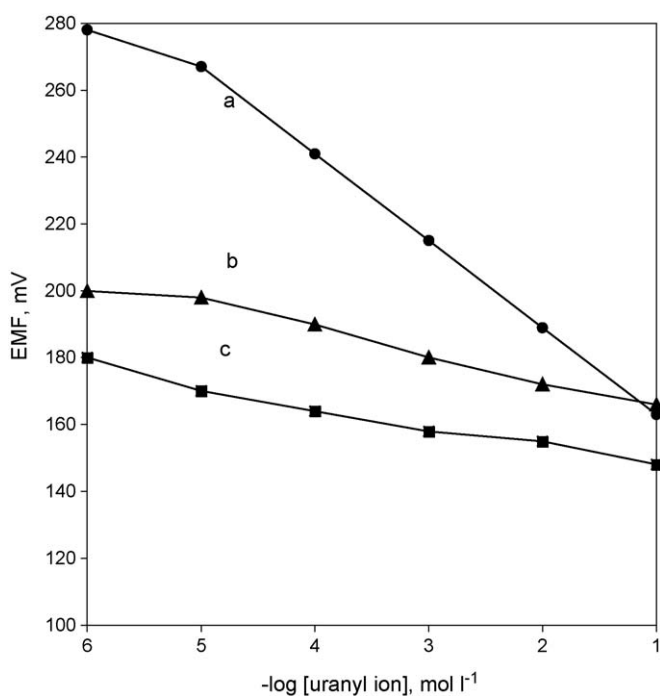


Fig. 2. Effect of uranium anion counterparts on the potential response of TETA based membrane sensor for: (a) uranyl sulphate, (b) uranyl nitrate and (c) uranyl chloride.

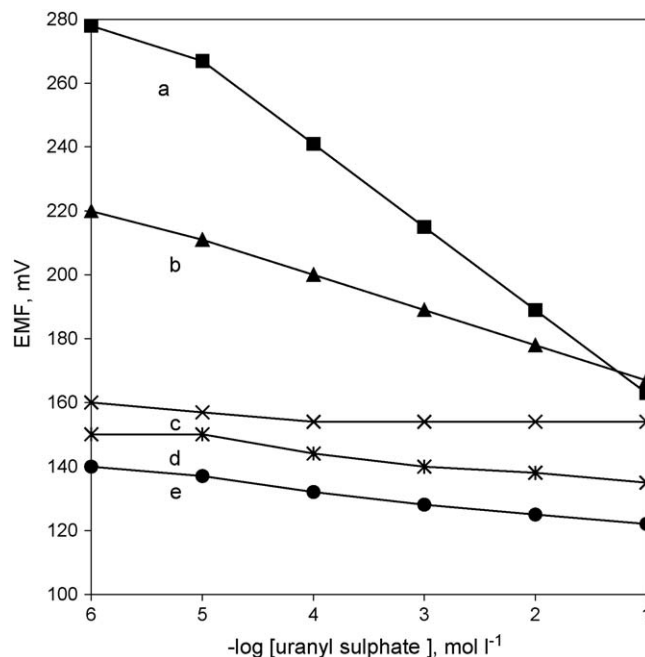


Fig. 3. Effect of membrane plasticizer on the response of TETA based sensor for uranyl sulphate at pH 3.0: (a) *o*-NPOE, (b) DBS, (c) DOS, (d) DOP and (e) DOPP.

uranyl nitrate and chloride test solutions only when these solutions are subjected to a prior digestion with sulphuric acid and converted into uranyl sulphate.

3.3. Effect of membrane plasticizer

As shown in Fig. 3 and Table 1, it is clear that sensor based on triethylenetetramine with *o*-NPOE plasticizer (membrane no. 4), gives a good response for uranyl sulphate with a slope of $-26.5 \pm 0.3 \text{ mV decade}^{-1}$ and a detection limit of $2.0 \times 10^{-5} \text{ mol l}^{-1}$. With DBS plasticizer (membrane no. 1), the performance of the sensor is inferior to that containing *o*-NPOE, the calibration slope is $-15.3 \text{ mV decade}^{-1}$ and the potential readings fluctuates. Sensors with DOP, DOS and DOPP plasticizers (membranes 2, 3, 5) exhibit negligible or no response for uranyl ion probably due to the differences in the carrier mechanisms [33]. It seems that *o*-NPOE improves the membrane selectivity due to its high dielectrical constant ($\epsilon = 24$), affects considerable dissolution of uranyl ions within the membrane and consequently enhances the complex formation and its partition coefficient in the membrane.

3.4. Response time and potential stability

The dynamic response of TETA based membrane sensor is determined by immersion of the sensor in 1.0×10^{-1} – $1.0 \times 10^{-5} \text{ mol l}^{-1}$ uranyl sulphate solutions. The time required to achieve a steady state potential within $\pm 0.2 \text{ mV}$ after increasing or decreasing the uranyl concentrations is short. In general, the response time is more rapid when proceed from diluted to concentrated solutions. A 90% of the final response potential is attained within $\sim 30 \text{ s}$ for uranyl

solutions of concentrations $\leq 10^{-5} \text{ mol l}^{-1}$ and within 15 s for solutions with concentrations $\geq 10^{-4} \text{ mol l}^{-1}$.

3.5. Effect of pH

Dependence of the potential response of TETA based membrane sensor for different concentrations (10^{-2} , 10^{-3} and $10^{-4} \text{ mol l}^{-1}$) of uranyl sulphate over various pH values shows that the potential is pH independent between pH 2.5 and 3.8. Above pH ~ 3.8 , there is a change in the measured potential especially at higher uranyl ion concentrations ($>10^{-3} \text{ mol l}^{-1}$), probably due to the hydrolysis of uranyl ion into $[(\text{UO}_2)_2(\text{OH})_2]^{2+}$, $(\text{UO}_2\text{OH})^+$ and $[(\text{UO}_2)_3(\text{OH})_5]^+$ ions [34], on one hand, and gradual increase of the $[\text{OH}]^-$ ions which causes a decrease in the potential of the sensor on the other hand. However, below pH ~ 2.5 , the sensor potential raises, due to interference's by hydrogen ion. The effect of pH on the sensor response is not only influenced by hydrolysis and complex formation of uranyl ion in aqueous test solution but also depends on the interaction of the membrane components with the solution ions.

3.6. Sensor lifetime

It has been reported that the lifetime of potentiometric sensors based on ionophores in solvent polymeric membranes depends on the distribution coefficient of the electroactive compound and plasticizer between aqueous test solution and organic membrane phases. The lifetime of the sensor depends also on the matrix of the test solution and the analyte measured by the sensor. The lifetime of TETA based membrane sensor for uranyl sulphate is about 3 months. During this time the detection limit of the sensor remains almost constant and the linear regression slope is $-26.5 \pm 0.3 \text{ mV decade}^{-1}$.

3.7. Sensor selectivity

Selectivity coefficients obtained by the separate solutions method [30] recommended by IUPAC using aqueous $10^{-3} \text{ mol l}^{-1}$ solutions of uranyl sulphate and the interfering ions at pH 3.0 are given in Table 3. From these data it can be seen that TETA based membrane sensor is highly selective towards uranyl ion in sulphate medium with respect to monovalent, divalent, trivalent and tetravalent cations. However, Fe^{3+} causes some interference probably due to membrane poisoning. If Fe^{3+} is present at high concentrations and not removed from the test solutions prior to uranium measurement, the membrane becomes inactive and is not easily regenerated. Interferences by Fe^{3+} and Th^{4+} , however, are eliminated by addition of 5 wt% diethylenetriaminepentaacetic acid and 1 mol l^{-1} KF to the test solution prior to uranyl ion measurements.

3.8. Determination of uranyl ions

Validation of the proposed assay method was performed (according to the quality control/quality assurance standards [35] by measuring the range, lower detection limit (LOD), accu-

Table 3
Selectivity coefficients of uranyl TETA based membrane sensor

Interfering ion, J^a	$K_{I,J}^{\text{pot}}$
I^-	8×10^{-4}
NO_3^-	6×10^{-4}
Cl^-	7×10^{-4}
K^+	5×10^{-4}
Li^+	4×10^{-4}
NH_4^+	7×10^{-4}
Ag^+	5×10^{-4}
Ca^{2+}	2×10^{-3}
Mg^{2+}	3×10^{-3}
Cu^{2+}	3×10^{-3}
Co^{2+}	4×10^{-3}
Pb^{2+}	6×10^{-4}
Cd^{2+}	4×10^{-4}
Sn^{2+}	6×10^{-3}
Be^{2+}	5×10^{-4}
Sr^{2+}	3×10^{-4}
Fe^{3+}	8×10^{-1}
Ce^{3+}	7×10^{-4}
Y^{3+}	8×10^{-4}
Al^{3+}	5×10^{-3}
Ti^{4+}	6×10^{-4}
Th^{4+}	2×10^{-1}
V^{4+}	9×10^{-4}
Nb^{5+}	6×10^{-4}
Mo^{6+}	7×10^{-4}

^a All measurements were made using $10^{-3} \text{ mol l}^{-1}$ solutions of both uranyl and the interfering ions.

racy (recovery), precision or repeatability (CV_w), between-day variability (CV_b), linearity (correlation coefficient) and sensitivity (slope). Six batches (six determinations each) with standard $20 \mu\text{g ml}^{-1}$ uranyl sulphate were made. The results (Table 4) show an average recovery of 97.6% and a mean standard deviation of 0.8%. Certified and natural ore samples containing 0.01–7.09 wt% uranium show results deviate 0.8–4.7% from those obtained by X-ray fluorescence method or the certified assigned values.

Table 4
Determination of uranium in some certified and natural ores using uranyl TETA based membrane sensor

Sample label	Uranium (wt%)		Deviation (%)
	Certified value	Found ^a , TETA sensor	
206 GB37	0.1480	0.1370	4.5
206A BL5	7.0900	6.9800	1.5
206A BL4A	0.1250	0.1190	3.5
206A BL3	1.0200	0.9920	2.7
206A BL2A	0.4260	0.4050	3.7
206A BL1	0.0220	0.0211	4.0
206A DH1A	0.2629	0.2398	3.7
206A DL1A	0.0116	0.0111	4.0
Sample A	0.1130 ^b	0.1121	0.8
Sample B	0.6870 ^b	0.6600	4.1
Sample C	3.2100 ^b	3.0000	3.5
Sample D	1.2900 ^b	1.1900	4.7

^a Mean of three measurements.

^b Obtained by X-ray fluorescence spectrometry.

The calculated student's t -value at 95% confidence level does not exceed the theoretical value, confirming good accuracy of the obtained results. An F -test reveals that there is no significant difference between the means and variances of the two sets of results obtained by the proposed sensor and X-ray fluorescence method. Control charts (R and $\bar{X}$) prepared over 1 month [34] for monitoring an internal control uranium sample ($20 \mu\text{g ml}^{-1}$) indicate that the assay method with the proposed sensor during this period is under statistical control.

3.9. Determination of uranium in ores and rocks

Uranium in different ores was determined after acid digestion and elimination of the effects of co-existing interfering ions (e.g. Fe^{3+} , Th^{4+}). Determination of uranium in natural ore samples directly, after digestion, with the proposed sensor was subject to complications due to the complex matrix of these samples. The feasibility of using TETA based membrane sensor for direct determination of uranium in some ores with different compositions was tested. Certified ores containing uranium at concentration levels of 0.01–7.09 wt% were examined. Some other naturally occurring silicate rocks containing

0.11–3.21 wt% uranium were similarly analysed by both X-ray fluorescence spectrometry and the proposed sensor.

Direct potentiometric determination of uranium in some certified ores after sample dissolution shows erratic results due to the interferences of high concentration levels of Fe^{3+} and Th^{4+} ions. Trials to eliminate the effect of these ions were made using some masking agents. Addition of diethylenetriamine-pentaacetic acid circumvents the effect of the interferents and enhances the selectivity of the method. DTPA is known to mask more than 30 elements [36], due to the formation of complexes with higher stability constants than those formed with EDTA or CDTA [37]. Table 4 shows the results obtained using the proposed sensor for potentiometric determination of uranium in some ores after digestion and treatment with DTPA.

3.10. Comparison with other methods

The performance of the proposed potentiometric sensor was compared with some of the previously suggested instrumental methods. Determination of uranium by inductively coupled plasma [38], atomic absorption spectrometry [39], spectrophotometry [40], fluorimetry [41], X-ray-fluorescence spectrometry

Table 5
General performance characteristics of some potentiometric uranyl membrane sensors

Sensor ionophore	Range (mol l^{-1})	Slope (mV decade^{-1})	Lower limit of detection (mol l^{-1})	Interfering ions	References
Di(2-ethylhexyl) phosphate	10^{-4} to 10^{-1}	+24	3×10^{-4}	Cu^{2+} , Fe^{3+} , I^- , ClO_4^- , PO_4^{3-} , NO_3^-	[1]
Tri(2-ethylhexyl) phosphate	10^{-5} to 10^{-1}	+25	1×10^{-5}	Fe^{3+} , Th^{4+} , F^- , Ca^{2+} , V^{4+} , Ni^{2+}	[2]
<i>O</i> -(1,2-Dihydro-2-oxo-1-pyridyl)- <i>N,N,N',N'</i> -bis(tetramethyl) uronium hexafluorophosphate	10^{-5} to 10^{-1}	+27.5	2×10^{-4}	Fe^{3+} , Th^{4+} , F^-	[2]
Trioctyl phosphine oxide	10^{-4} to 10^{-2}	+59	4×10^{-4}	Ni^{2+}	[3]
Bis[di(4-(1,1,3,3-tetramethyl butyl))] phenyl phosphate	10^{-3} to 10^{-1}	+26	3×10^{-3}	Fe^{3+}	[4]
Tetraphenyl- <i>o</i> -xylyldiphosphine	10^{-4} to 10^{-1}	+26	6×10^{-4}	Alkaline earth metals	[6]
<i>O</i> -Methyldihexyl phosphineoxide, <i>O'</i> -hexyl-2-ethylphosphoric acid	10^{-4} to 10^{-2}	+25–31	6×10^{-4}	Fe^{3+} , Al^{3+} , Mn^{2+} , F^- , PO_4^{3-}	[8]
Tri(chloroethyl or propyl) phosphite	10^{-4} to 10^{-1}	+29	6×10^{-4}	Cr^{3+} , Fe^{3+} , Ce^{4+} , U^{4+}	[9]
2,2'-[1,2-Ethanediy] bis(nitriloethylidene)] bis(1-naphthalene)	10^{-7} to 10^{-1}	+28	7×10^{-7}	Fe^{3+} , Cu^{2+}	[10]
5,6,7,8-Tetrahydro-8-thioxopyrido-[4',3',4,5] thieno[2,3- <i>d</i>]pyrimidine-4(3H)one	10^{-5} to 10^{-1}	+30	2×10^{-5}	NR	[11]
<i>N,N'</i> -Diheptyl- <i>N,N'</i> , 6,6-tetramethyl-4,8-dioxauundecane diamide (ETH 295) and 4-(octa-decylamino) azobenzene (ETH5315)	10^{-7} to 10^{-4}	+28	3×10^{-7}	NR	[12]
2-Hydroxyacetophenone oxime thiourea trioxine resin	10^{-5} to 10^{-2}	+26	5×10^{-5}	F^- , I^- , Cl^- , PO_4^{3-}	[14]
1,11-Bis(2-benzylox-5-formylphenoxy)-3,6,9-trioxaundecane	10^{-4} to 10^{-1}	+21.6–39.4	5×10^{-4}	Fe^{3+}	[15]
1,18-Diaza-3,4,15,16-dibenzo-5,8,11,14,21,24-hexaoxacyclohexaicosane-2,17-dione	10^{-6} to 10^{-3}	+27	2×10^{-6}	Sr^{2+} , Pb^{2+}	[16]
2,3,11,12-Dicyclohexano-1,4,7,10,13,16-hexaoxacyclo-octadecane	10^{-5} to 10^{-1}	+27	6×10^{-5}	Fe^{3+} , F^-	[17]
15-Crown-5 phosphotungstate	10^{-5} to 10^{-1}	+30	5×10^{-5}	Alkaline earth metals	[19]
Calixarene	10^{-5} to 10^{-1}	+27	4×10^{-5}	Fe^{3+} , Th^{4+} , F^-	[20]
Calixarene [5,11,17,23-tetrabutyl-25,27-bis(hydroxyl-26-ethoxy carbonyl methoxy)-28-(diethyl carbamoylmethoxy) calix(4)arene]	10^{-5} to 10^{-1}	+28	6×10^{-5}	Fe^{3+} , F^-	[21]
4-Tetra-butylcalix(6)arene	10^{-5} to 10^{-1}	+29	4×10^{-5}	F^- , I^- , Cl^- , PO_4^{3-}	[22]
Triethylenetetramine	10^{-5} to 10^{-1}	–26.5	3.5×10^{-5}	Fe^{3+} , Th^{4+}	This work

NR, not reported.

[42], ion chromatography [43] and capillary zone electrophoresis [44] requires rather expensive equipment, high running cost, involves prior time-consuming separation, extraction or derivatization steps, applicable to a narrow range of concentration and suffers from severe interferences by many ions.

The proposed sensor, however, offers the advantage of short response time (<30 s), low detection limit ($3.0 \times 10^{-5} \text{ mol l}^{-1}$), simple construction, low cost and applicability over at least three orders of magnitude of concentration without pretreatment steps. Furthermore, the response of the sensor for most common interfering cations is low (selectivity coefficients 10^{-3} to 10^{-4}). Many ions including Cu^{2+} , Pb^{2+} alkaline earths, Ce^{3+} , V^{4+} , Al^{3+} , I^{-} , Cl^{-} and NO_3^{-} which seriously interfere with other potentiometric PVC membrane sensors [1,2,6,8–10,14,16,19,22] have negligible effect. The linear range of the proposed sensor is at least one order of magnitude wider than those previously described [1,3,4,6,8,9,15] with enhanced lower limit of detection. Table 5 presents the main analytical features of some potentiometric uranyl membrane sensors.

4. Conclusions

Uranyl potentiometric sensor with anionic response is developed and characterized. In strong sulphuric acid media, a poly(vinyl chloride) membrane plasticized with *o*-nitrophenyloctyl ether and incorporate triethylenetetramine exhibits a near-Nernstian divalent anionic response for uranyl ion. A calibration slope of $-26.5 \text{ mV decade}^{-1}$ over the concentration range of 1.0×10^{-1} – $3.5 \times 10^{-5} \text{ mol l}^{-1}$ and an improved selectivity in the presence of many commonly associated ions are obtained. The response mechanism is based on the formation and extraction of $\{(2\text{TETAH})^{2+} [\text{UO}_2(\text{SO}_4)_2]^{2-}\}$ species in the membrane organic phase. The sensor displays good sensitivity (down to $5 \mu\text{g ml}^{-1}$), long lifetime (12 weeks), short response time (15–30 s) and high reproducibility ($\pm 0.8\%$). Uranium in naturally occurring ores (0.01–7.09 wt%) is satisfactorily determined and results compare fairly well with those obtained by X-ray fluorescence spectrometry are obtained.

References

- [1] D.L. Manning, J.R. Stokely, D.W. Magouyrk, *Anal. Chem.* 46 (1974) 116.
- [2] S.S.M. Hassan, M.M. Ali, A.M. Attawiya, *Talanta* 54 (2001) 1153.
- [3] C.S. Luo, F.C. Chang, *Anal. Chem.* 54 (1982) 2333.
- [4] G.J. Moody, J.M. Slater, J.D.R. Thomas, *Analyst* 113 (1988) 699.
- [5] N.S. Nassory, *Fresenius' J. Anal. Chem.* 337 (1990) 377.
- [6] M.B. Saleh, *Indian J. Chem.* 31A (1992) 12.
- [7] A.N. Khamorov, A.R. Garifzyanov, V.F. Toropova, *Z. Analiticheskoi Khim.* 49 (1994) 1124.
- [8] A. Florido, I. Casas, J. Garcia-Raurich, R. Arad-Yellin, A. Warshawaky, *Anal. Chem.* 72 (2000) 1604.
- [9] I. Goldberg, D. Meyerstein, *Anal. Chem.* 52 (1980) 2105.
- [10] M. Shamsipur, M. Saeidi, G. Azimi, H. Naeimi, H. Sharghi, *Bull. Korean Chem. Soc.* 25 (2004) 629.
- [11] M.B. Saleh, S.S.M. Hassan, A.A. Abdel Gaber, N.A. Abdel Kream, *Sens. Actuators B* 94 (2003) 140.
- [12] M. Lerchi, E. Reitter, S. Wilhelm, *Fresenius' J. Anal. Chem.* 348 (1994) 272.
- [13] H.A.M. Arida, A.M. Elsaied, *Anal. Lett.* 36 (2003) 1079.
- [14] A.K. Jain, V.K. Gupta, U. Khurana, L.P. Singh, *Electroanalysis* 9 (1997) 857.
- [15] N.S. Nassory, *Talanta* 36 (1989) 672.
- [16] S. Johnson, G.J. Moody, J.D.R. Thomas, F.H. Kohnke, J.F. Stoddart, *Analyst* 114 (1989) 1025.
- [17] M. Shamsipur, P.A. Soleyman, M. Akhond, H. Sharghi, *Talanta* 58 (2002) 237.
- [18] A.K. Jain, V.K. Gupta, L.P. Singh, *Anal. Proc.* 32 (1995) 263.
- [19] J. Shih, D. Wang, *Jiemian Kexue Huizhi* 14 (1991) 13.
- [20] D.M. Duncan, J.S. Cockayne, *Sens. Actuators B* 73 (2001) 228.
- [21] J. Ramkumar, B. Maiti, *Sens. Actuators* 96 (2003) 527.
- [22] Y. Kubo, S. Maeda, M. Nakamura, S. Tokita, *J. Chem. Soc., Chem. Commun.* 14 (1994) 1725.
- [23] V.K. Gupta, R. Mangla, U. Khurana, P. Kumar, *Electroanalysis* 11 (1999) 573.
- [24] R.A. Day Jr., R.M. Powers, *J. Amer. Chem. Soc.* 76 (1954) 3895.
- [25] D. Banarjee, K.K. Tripathi, *J. Inorg. Nucl. Chem.* 18 (1961) 199.
- [26] C. Deptula, S. Minc, *J. Inorg. Nucl. Chem.* 29 (1967) 221.
- [27] T.S. Ma, S.S.M. Hassan, *Organic Analysis Using Ion Selective Electrodes*, vol. I, Academic Press, London, 1982.
- [28] S.S.M. Hassan, E.M. Elnemma, A.H.K. Mohamed, *Talanta* 66 (2005) 1034.
- [29] S.S.M. Hassan, A.H. Abou Ghalia, A.F. Amr, A.H.K. Mohamed, *Talanta* 60 (2003) 81.
- [30] IUPAC Analytical Chemistry Division Commission on Analytical Nomenclature, *Pure Appl. Chem.* 72 (2000) 1851.
- [31] J.I. Bullock, *J. Inorg. Nucl. Chem.* 29 (1967) 2257.
- [32] H.G. Swope, *Treatment of radioactive wastes*, in: *Ion-Exchange Technology*, Academic Press, New York, 1956 (Chapter 17).
- [33] E. Pungor, K. Toth, *Anal. Proc.* 22 (1985) 352.
- [34] F.A. Cotton, G. Wilkinson, *Advanced Inorganic Chemistry*, third ed., Wiley, Eastern, New Delhi, 1972, p. 1103.
- [35] J.K. Taylor, *Quality Assurance of Chemical Measurements*, CRC Press, Florida, 1987.
- [36] F.W.E. Strelow, T.N. Van-Derwalt, *Talanta* 26 (1978) 507.
- [37] J. Inczedy, *Analytical Applications of Complex Equilibria*, Ellis Horwood, Chichester, 1976.
- [38] O. Fujino, S. Umetani, E. Ueno, K. Shigeta, T. Matsuda, *Anal. Chim. Acta* 420 (2000) 65.
- [39] M. Kumar, D.P.S. Rathore, A.K. Singh, *Mikrochim. Acta* 137 (2001) 127.
- [40] Y. Hirano, Y. Ogawa, K. Oguma, *Anal. Sci.* 19 (2003) 303.
- [41] C. Moulin, P. Decambox, L. Trecani, *Anal. Chim. Acta* 321 (1996) 121.
- [42] J. Stas, R. Al-Merey, J. Karjou, *Radioanal. Nucl. Chem.* 250 (2001) 525.
- [43] E.H. Borai, A.S. Mady, *Appl. Radiat. Isot.* 57 (2002) 463.
- [44] B. Liu, L. Liu, J. Cheng, *Talanta* 47 (1998) 291.

The investigation of the behavior of a long period grating sensor with a copper sensitive coating fabricated by layer-by-layer electrostatic adsorption

J. Keith, L.C. Hess, W.U. Spendel, J.A. Cox, G.E. Pacey*

Miami University Center for Nanotechnology, Department of Chemistry and Biochemistry, Miami University, Hughes Hall, Oxford, OH 45056, United States

Received 17 January 2005; received in revised form 31 January 2006; accepted 31 January 2006

Available online 7 March 2006

Abstract

Sensors based on changes of refractive index in response to sorption of an analyte on the coating or film of a long period grating fiber (LPG) fiber have recently been reported. In most prior work the coating or film swelled during interaction with the analyte. The swelling mechanism produced a kinetic response that slowed both the sensor's time for steady-state measurement and the reversibility of the sensor. Here, the analytical utility of fabricating these nanometer thin films using the layer-by-layer (LBL) electrostatic assembly method is evaluated using Cu^{II} as the test analyte and Cibacron Blue as the reagent immobilized in the LBL assembly; a generation-4 poly(amidoamine) dendrimer served as the spacer in the assembly. Detection of $1.3 \text{ mg Cu}^{\text{II}} \text{ L}^{-1}$ was observed when six bilayers comprised the coating. The stable response was achieved with 0.6 mg L^{-1} in less than 1 min. When 0.1 M HCl was used as the rinsing solution, this LPG sensor was reversible and the signal to similar concentrations of Cu^{II} reproducible.

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Keywords: Long period gratings; Sensors; Dendrimer; Copper

1. Introduction

The introduction of long period gratings (LPG) in optical fibers provides a completely new approach to the fabrication of highly selective, evanescent-wave chemical and biosensors [1–6,11–16]. These systems are extensions of the photoinduced Bragg gratings that are elegant fiber sensors suitable for multiplexing and distribution applications [17]. Unfortunately, sensors based on Bragg gratings are seriously limited by temperature and strain-induced spectral displacements. It is often necessary to use complex interferometric techniques to detect shifts. In contrast to the manufacturing process of other evanescent wave sensors, where the surface plasmon must be carefully constructed from a waveguide substrate that is polished and coated with a thin film of precise thickness, LPGs rely on the formation of gratings on the core of a communication grade optical fiber.

To understand how the LPG system works, we need to start with some basic concepts. The LPG phase-matching condition is given by Eq. (1) that describes the function of mode indices of the core, η_{0l}^{co} , and the cladding η_{0p}^{cl} :

$$\lambda_p = (\eta_{0l}^{\text{co}} - \eta_{0p}^{\text{cl}})\Lambda \quad (1)$$

where Λ is the period of the grating, p the mode number, and λ_p is the wavelength of the p th order resonance peak. The apparent indices of the core and the cladding modes are given as functions of the propagating wavelength, the geometry of the fiber, and the refractive indices of the core and the cladding materials. In other words, since the fiber core and fiber cladding around the core are unchanged only changes in the refractive index of the outer cladding, or in this case the analyte sensing coating, will be observed as a change position of the spectrum.

The change in the spectrum is in fact a spectral loss or “hole” in the spectral profile of the source light that passes through the fiber core; that is, there is a minimum in the plot of refractive index versus wavelength. Moreover, the position of this minimum shifts in direct proportion to the analyte con-

* Corresponding author. Tel.: +1 513 529 2875; fax: +1 513 529 5715.

E-mail address: paceyge@muohio.edu (G.E. Pacey).

centration in the coating or film in direct contact with the fiber.

Sensors based on LPG fibers have been used previously for both chemical and physical measurements. Examples of the latter were the measurement of temperature and strain [3–11]. For chemical measurements, the LPGs are used bare [12] or are modified to selectively react with a targeted analyte [13–16]. Allsop et al. measured the concentration of xylene in heptane using a bare fiber LPG. The minimum detectable change in the binary mixture was 0.04% [12]. DeLisa et al. demonstrated a LPG biosensor [16]. Goat anti-human IgG was immobilized on the surface of the LPG. A change in refractive index was produced by the antibody–antigen binding. Human IgG binding was observed over a range of 2–100 $\mu\text{g/ml}$.

In the previously reported chemical applications of LPG-based sensors, the coating thicknesses usually were at least 100 μm . Given that the diffusion distance, x , is related to $(Dt)^{1/2}$ and that the diffusion coefficient, D , in a film or coating can be significantly less than the value in solution, which is typically in the range $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ to $10^{-6} \text{ cm}^2 \text{ s}^{-1}$, the imbalance between x -values and coating thicknesses can lead to slow response and recovery times, especially when conditions are optimized for high sensitivity. Indeed, in a collaborative study, we demonstrated that a commercially developed LPG sensor for Cu^{II} exhibited a slow response at low concentration [17]. Specifically, steady state was reached for 10 mM, 0.1 mM, and 0.01 mM $\text{Cu}(\text{II})$ concentrations on the order of seconds, minutes, and hours, respectively. For coated LPG sensors to respond in a steady-state manner in a practical time scale, significantly thinner deposits are required. Moreover, coatings with a macroporous structure in which D -values will approach those of the analyte in a liquid will facilitate response and recovery. A hypothesis of this study is that layer-by-layer (LBL) electrostatic assembly of macromolecules on LPG fibers will provide coatings that potentially can yield duty cycles in the millisecond range. The thickness of such coatings can be controlled in the regime of less than 100 nm and that have effective pore diameters in the nanometer range.

With the LBL method, the substrate is coated with a charged monolayer, such as by adsorption or self-assembly [18,19]. By a procedure of dip-coating, rinsing, and drying, layers of molecules with alternating charge are electrostatically deposited. Examples of assemblies on a substrate to which the initial layer was bound by simple adsorption are reports by Kuhn and Anson [20] and Ingersoll et al. [21] where polyoxometalates (POMs) were adsorbed to carbon and LBL coatings of POMs and $\text{Os}(\text{bpy})_3^{2+}$ were fabricated. Films with up to 20 layers were achieved. We modified a gold substrate with a self-assembled monolayer of 4-aminothiophenol (4-ATP); subsequently an LBL assembly of a POM, $\text{PMo}_{12}\text{O}_{40}^{3-}$, and generation-4 poly(amidoamine) dendrimer (G4-PAMAM) was fabricated [22]. A film comprising 12 bilayers with $3.6 \times 10^{-11} \text{ mol PMo}_{12}\text{O}_{40}^{3-} \text{ cm}^{-2}$ in each bilayer was achieved, whereas with the analogous direct adsorption method, the initial layer of the POM had a greater quantity than the subsequently deposited ones.

An important factor in employing LBL assemblies as coatings is that limiting the number of reactive sites will not necessarily compromise the sensitivity, which is related to the change in refractive index with analyte concentration. The basis of this prediction is that the depth of penetration of the evanescent wave is about $\lambda/10$. In the present study, the light source has a continuous spectrum with a λ_{max} ca. 800 nm, so the penetration of the evanescent wave is only about 80 nm. Any additional thickness in thin film or coating will not improve the LPG sensitivity but will contribute to the above-mentioned imbalance between diffusional distance and coating thickness.

The analyte that was used to evaluate the LPG with the LBL assembly was copper(II) ion, and the trapping agent included in the coating was Cibracron Blue. The selection of copper ion was based on the need to develop a sensor that was suited for its determination in jet fuel [17].

2. Experimental

2.1. Reagent

The following chemicals were purchased from Aldrich: generation 4-poly(amidoamine) dendrimer as a 10% solution in methanol, poly(diallyldimethylammonium chloride) (PDDA), methanol, and acetonitrile. Sodium hydroxide, copper(II) nitrate, sulfuric acid, hydrochloric acid, ethanol (95%), and hydrogen peroxide were purchased from Fisher, and Cibacron Blue (CB) was from Sigma. All chemicals were used without further purification. Water was triple distilled from an in-house system.

2.2. Instrumentation

The LPG instrument used for measurements was a Lunascan III from Luna Innovations (Blacksburg, VA). The instrument includes a LED light source (centered at 820 nm) and a Fabry–Perot interferometer. The optical fiber has a grating written on its core. The actual sensing area is about 3 mm long and is enclosed in a plastic cover with four $3 \text{ mm} \times 0.25 \text{ mm}$ slits to allow the solution to contact the fiber. The hardware is controlled and the data acquisition is achieved with a laptop computer using Luna proprietary software [6]. The intensity of the peaks is plotted versus the wavelength of minimum absorbance. This wavelength value is determined using an algorithm within the Luna Innovations LPG software.

2.3. LBL assembly procedure

The multilayer assemblies that contained G4-PAMAM and CB were fabricated initially on quartz slides; subsequently, the procedure was adapted to modifying the surface of long period grating fibers. The quartz slides were cleaned by soaking in a 7:3 (volume) mixture of concentrated H_2SO_4 :30% H_2O_2 at 80 °C for 10 min and, after rinsing with water and ethanol, sonicating in ethanol for 3 min. After drying with a stream of N_2 , the quartz surface was modified by placing it overnight in 1 wt.% PDDA, after which the aminated surface was rinsed with water and

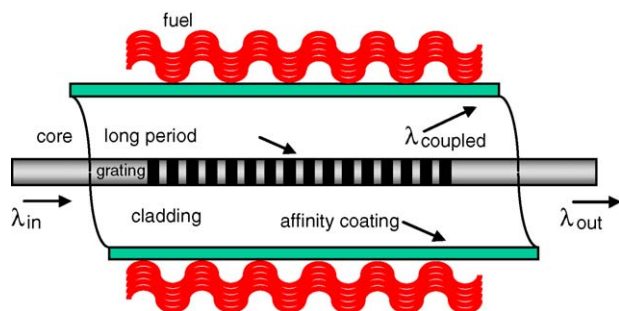


Fig. 1. Optical fiber LPG-based chemical sensing platform.

dried with N_2 . Alternating layers of CB and G4-PAMAM were deposited on this surface by dipping the substrate for 30 min in 1.0 mM CB and in 1 wt.% G4-PAMAM. After each immersion, the surface was rinsed with water and dried with N_2 . The process was monitored by measuring the absorbance at 630 nm using a HP Diode Array Spectrophotometer. The concentration of Cu(II) in solution was determined by inductively coupled plasma emission spectrometry using a Liberty 150 system (Varian, Chicago, IL). Data were fitted to a matrix-matched calibration curve.

Before a LPG fiber was modified by the LBL method, it was dipped in acetonitrile overnight. The fiber was then removed and cleaned using the following procedure: it was ultrasonicated in water for 10 min, placed in piranha solution (7:3 ratio of sulfuric acid and hydrogen peroxide) for 10 min at 60–70 °C, rinsed with water, dipped in ethanol and ultrasonicated for 3 min, then rinsed with ethanol, dipped in water and ultrasonicated for 3 min, rinsed with water, and dried. An initial spectrum of the fiber was taken. The fiber was then dipped in a 1 wt.% PDDA overnight. After rinsing and drying another spectrum was taken. The sensor was then placed in 1.0 mM CB for 30 min, rinsed, and dried. A background spectrum was obtained. The next layer was added by placing the sensor in the 1% PAMAM solution for 30 min. After rinsing and drying, another spectrum was taken. By repeating the last two steps, three bilayers were fabricated.

3. Results and discussion

Fig. 1 is a schematic diagram of a LPG fiber sensor. The main elements are: the fiber core on which the grating is written on,

the fiber cladding that surrounds the core, and the thin film or coating in contact with the cladding. The thin film or coating may or may not contain a functionality that traps the analyte. Initial experiments were performed to establish the background response of a bare LPG fiber. Fig. 2a and b shows spectra of the LPG fiber in contact with air and water, respectively. The combination of the light source and the fiber that were used is sensitive to changes in refractive index over the 1.28–1.45 range; therefore, the minimum that results from spectral loss only appears in Fig. 2b. Subsequent work was with fibers that were modified by the LBL method, the conditions for which were established by studies on quartz slides with UV–vis absorption as the monitoring method.

The LBL fabrication process for the CB-containing assembly was developed using a quartz slide as the base. The verification that the layers were being formed was accomplished by spectrophotometry at the 630 nm $\lambda_{\max}$ of CB. After each CB/G4-PAMAM bilayer was deposited as described in Section 2, the absorbance was measured. The addition of each bilayer increased the absorbance. A least squares fit of absorbance units (AU) versus bilayer number, n , yielded a slope of $3.8 \times 10^{-3} \text{ AU } n^{-1}$; the r^2 value for the fit was 0.98 for the assembly with six bilayers. The linearity of this fabrication procedure is consistent with previous results we obtained for a similar system [22].

A quartz|PDDA|(CB, G4-PAMAM)₆ surface was exposed to 2.5 mL of 5.0 μM Cu(II) for 30 min, and the uptake was quantified by ICP spectrometry. The Cu(II) concentration was in that solution was determined before and after immersion of the modified quartz in the solution. The result was that $(3.5 \times 10^{-9}) \pm (1.2 \times 10^{-10}) \text{ mol cm}^{-2}$ were sorbed. Given that a typical monolayer contains on the order of $10^{-10} \text{ mol cm}^{-2}$ and that both CB and G4-PAMAM can complex Cu^{2+} , this result is reasonable for a six bilayer assembly. Increasing the immersion time to 60 min did not change the result, which suggests that the sites in the assembly were saturated with Cu(II).

A PDDA|(CB, G4-PAMAM)₃ was fabricated on an LPG fiber by the same method as that used to modify quartz. With a 640 mg Cu L^{-1} sample, the response of the LPG|PDDA|(CB, G4-PAMAM)₃ system was tested. A steady-state shift in the minimum was observed in 20 s, which was a significant improvement over that observed with the previously used LPG system

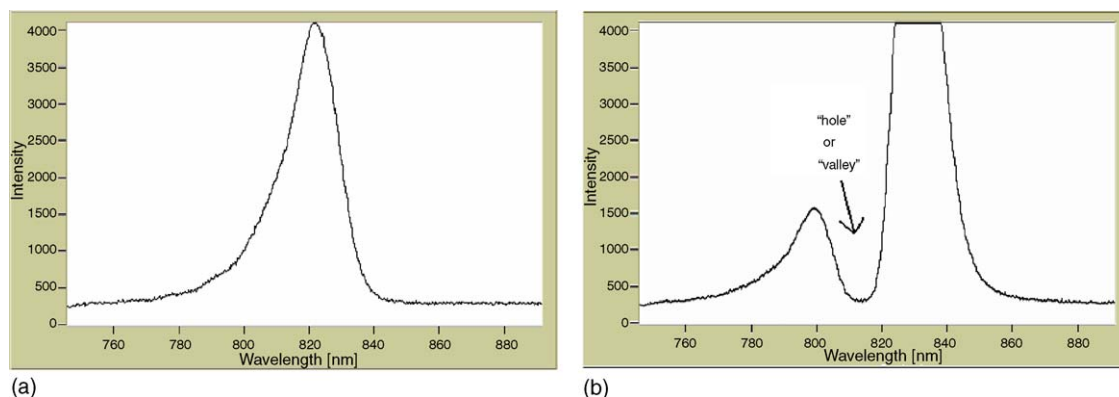


Fig. 2. Bare LPG fiber spectrum in: (a) air and (b) water.

with a Cu(II) affinity coating that is described in Section 1 [18]. With a freshly prepared assembly, the shift in the minimum was 2.01 ± 0.05 nm. The algorithm allows measurement of changes in the position of the minimum of 10^{-4} nm at a relative standard deviation of 5%, so the sensitivity achieved with this LPG|PDDA|(CB, G4-PAMAM)₃ system is sufficient for practical measurement. Moreover, the sensitivity varies from fiber to fiber. As noted by data presented below, the fiber used for this preliminary measurement was from a batch that yielded a low sensitivity.

In our earlier study with an affinity coated fibers [18], the uptake of Cu(II) was reversed in 0.005 M EDTA at pH 9. Here, this method failed. Apparently, CB is a sufficiently strong ligand for Cu(II) relative to EDTA that competitive binding was not useful for removal of the metal from the LBL assembly. An alternative method for removing the Cu(II) from the PDDA|(CB, G4-PAMAM)₃ assembly was studied, namely lowering the effective binding constant by protonating the CB in the film. For this purpose, 0.1 M HCl was used as the stripping solution. After contact with 0.1 M HCl for 60 min, the PDDA|(CB, G4-PAMAM)₃ was treated with 0.1 M NaOH for 60 min before being used to extract Cu(II) from another sample. Without this treatment in base, the shift of the minimum of the spectrum with the LPG|PDDA|(CB, G4-PAMAM)₃ in contact with a Cu(II) solution was not reproducible. Short cycling times of about 5 min were used during the experiments used to determine the calibration line and detection limit.

Even with the treatment in base, the position of the minimum of the LPG spectrum did not return to its initial value, 830 nm; instead, after five to six cycles, the value stabilized at 840 nm. Nevertheless, the steady-state response to 64 mg Cu L⁻¹ remained at 2.0 ± 0.1 nm. The factor that limits the establishment of a stable baseline is probably a change in the structure of the LBL assembly. In this regard, these electrostatic assemblies are somewhat sensitive to ionic strength. This matrix effect will, in turn, cause the refractive index of the assembly to change because the interlayer distances are a function of the matrix. Hence, the refractive index of the LPG|PDDA|(CB, G4-PAMAM)₃ system in the absence of Cu(II) will vary until ion-strength annealing is complete.

The sensitivity of this assembly was constant for about 20 measurements, after which the shift in the position of the minimum upon exposure to Cu(II) diminished. This loss of response was not related to characteristics of the LPG fiber, but rather it was from deterioration of the LBL assembly from cycling in 0.1 M HCl and 0.1 M NaOH to remove the Cu(II) after a measurement. Hence, the response was restored by removing the (CB, G4-PAMAM)₃ and re-coating the LPG|PDDA. Once again the annealing process was observed. However, the background values for the position of the minimum agreed to within 0.2 nm, and the sensitivity was similar to the value for the prior LBL coating reported above.

An important characteristic of LBL assemblies in terms of applicability as coatings on LPGs is the potential ability to match the thickness of the film to the penetration depth of the evanescent wave, about 80 nm with the source in this study that has a λ_{max} of 820 nm. Based on the estimated diameters of CB

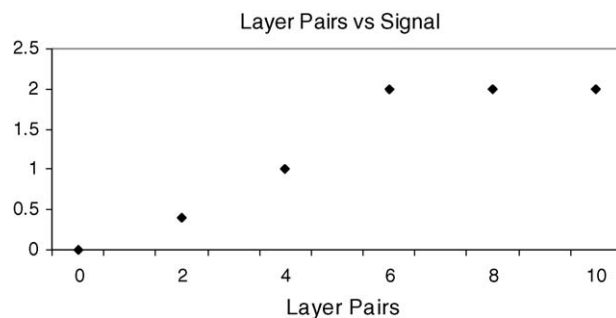


Fig. 3. LPG response vs. number of |PDDA|(CB, G4-PAMAM)_n layers.

and G4-PAMAM, the estimated penetration depth was about 10 bilayers. An assembly with a lower number was not expected to provide maximum sensitivity; a higher number was expected to decrease response time without increasing sensitivity. The result of a study of response wavelength shift versus bilayer number, n , is shown in Fig. 3. The maximum response was reached with $n=6$. Given the uncertainty in the thickness of an electrostatically bound bilayer, the agreement between the data in Fig. 3 and the prediction is reasonable.

One of the issues with LPG fibers is that the optical properties generated by the grating manufacturing process are not uniform or reproducible among fibers. A calibration line using a moderately sensitive LPG fiber was generated using an LPG|PDDA|(CB, G4-PAMAM)₃ assembly with concentrations over the range 3.2–128 mg Cu(II) L⁻¹ (using a 128 mg CuSO₄ L⁻¹ standard solution). A linear least squares fit of the data yielded the equation, $y=0.00936x+0.0176$, where y is the wavelength shift in the minimum and x is the Cu(II) concentration in mg L⁻¹. The correlation coefficient of the fit was 0.9987. The detection limit was determined using a very sensitive fiber where the calibration line used is $y=0.0988x-0.021$, the detection limit was estimated from results with 6.4 mg Cu L⁻¹. A set of 10 trials yielded a shift of 0.65 ± 0.05 in the minimum. Using the criterion of the concentration that gives a signal of three times the standard deviation, the detection limit was 1.3 mg Cu L⁻¹.

4. Conclusion

The goal of this investigation was to demonstrate that the LPG signal could be manipulated using a coating that exhibited a non-swelling response to the presence of analyte. The data presented above are from initial experiments and show that fibers coated with PAMAM and Cibacron Blue by the technique of LBL show very good potential in detecting copper. There are many other reagent combinations that could be used to enhance the detection limit of the LBL–LPG system. The multiple LPG fibers can be placed in a microfluidics channel and provide the redundancy needed for this measurement.

Acknowledgements

The work was supported primarily by the National Science Foundation through grant ECS-0304297 to G.E.P. and J.A.C.

Partial support was from a DAGSI grant, MU-PR-01-16, to G.E.P.

References

- [1] J. Widera, R.F. Reich, G.E. Pacey, S.D. Puckett, C.E. Bunker, J.R. Gord, Prepr. Am. Chem. Soc. Div. Pet. Chem. 47 (2002) 231.
- [2] J. Keith, S. Puckett, G.E. Pacey, Talanta 61 (2003) 417.
- [3] X. Shu, T. Allsop, B. Gwandu, L. Zhang, I. Bennion, IEEE Photon. Technol. Lett. 13 (2001) 818.
- [4] S. Yin, K.-W. Chung, X. Zhu, Opt. Commun. 188 (2001) 301.
- [5] S. Khalilq, S.W. James, R.P. Tatum, Meas. Sci. Technol. 13 (2002) 792.
- [6] A.A. Abramov, A. Hale, R.E.S. Windeler, T.A. Strasser, Electron. Lett. 35 (1999) 81.
- [7] V. Bhayia, D. Campbell, R.O. Claus, A.M. Vengsarkar, Opt. Lett. 22 (1997) 648.
- [8] Y. Liu, L. Zhang, I. Bennion, Electron. Lett. 35 (1999) 661.
- [9] L. Zhang, Y. Lui, J.A.R. Williams, I. Bennion, IEEE J. Sel. Top. Quantum Electron. 5 (1999) 1371.
- [10] C.-Y. Lin, L.A. Wang, G.W. Chern, J. Lightwave Technol. (2001) 1159.
- [11] L.A. Wang, C.Y. Lin, G.W. Chern, Meas. Sci. Technol. 12 (2001) 793.
- [12] T. Allsop, L. Zhang, I. Bennion, Opt. Commun. 191 (2001) 181.
- [13] X. Shu, D. Huang, Opt. Commun. 171 (1999) 65.
- [14] R. Falciai, A.G. Mignani, A. Vannini, Sens. Actuators B 74 (2001) 74.
- [15] S. Khalilq, S.W. James, R. Tatam, Opt. Lett. 26 (2001) 1224.
- [16] M.P. DeLisa, Z. Zhang, M. Shiloach, S. Plievar, C.C. Davis, J.S. Sirkis, W.E. Bentley, Anal. Chem. 72 (2000) 2895.
- [17] G.E. Pacey, S.D. Puckett, L. Cheng, S. Khatib-Shahidi, J.A. Cox, Anal. Chim. Acta 533 (2005) 135–139.
- [18] J. Widera, G.E. Pacey, C. Bunker, J.R. Gord, V.R. Katta, S.W. Buckner, Appl. Opt. 44 (2005) 1011–1017.
- [19] G. Decher, Science 277 (1997) 1232.
- [20] A. Kuhn, F.C. Anson, Langmuir 12 (1996) 5481.
- [21] D. Ingersoll, P.J. Kulesza, R.F. Faulkner, J. Electrochem. Soc. 141 (1994) 140.
- [22] L. Cheng, G.E. Pacey, J.A. Cox, Electrochim. Acta 46 (2001) 4223.

Development of a fluorescence-based microtiter plate method for the measurement of glutathione in yeast

Kamil Lewicki^{a,b}, Stéphanie Marchand^a, Lydia Matoub^a,
Janina Lulek^b, Joël Coulon^a, Pierre Leroy^{a,*}

^a *Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, UMR 7564 CNRS UHP Nancy 1, Faculté de Pharmacie, BP 80403, F 54001 Nancy Cedex, France*

^b *Department of Inorganic and Analytical Chemistry, University of Medical Sciences, Poznan, Poland*

Received 22 September 2005; received in revised form 4 January 2006; accepted 7 February 2006

Available online 24 March 2006

Abstract

The present work was aimed to the development of a fluorescence assay using the universal 96-well microplate format, for the measurement of reduced glutathione (GSH) in yeast cells. The method relies upon the reaction between GSH and a highly selective fluorogenic probe, i.e. naphthalene-2,3-dicarboxaldehyde (NDA). The optimization of the method included the extraction step of GSH from cultured yeast cells in a cold perchloric acid solution, derivatization conditions (10-min reaction at pH 8.6 and at $20 \pm 2^\circ\text{C}$ in darkness) and stability studies of the resulting fluorescent adduct. Full selectivity was observed versus other endogenous thiols (except for γ -glutamylcysteine), glutathione disulfide (GSSG) and enzymatic reducing reagents of GSSG. Linearity was verified in the range $0.3\text{--}6.5\ \mu\text{M}$ ($R^2 > 0.98$) and limits of quantification and detection were 0.3 and $0.05\ \mu\text{M}$, respectively. Relative standard deviation corresponding to repeatability ($n = 3$) and inter-day precision ($n = 5$) were 2.8 and 6.1%, respectively. Mean GSH recovery from cell extracts was 95%. The method appeared highly correlated ($R^2 = 0.96$) with a previously reported HPLC method.

The method was then applied to the monitoring of GSH in the yeast strain *Kluyveromyces lactis* during its growth period and in the presence of an inhibitor of GSH biosynthesis. The method presents the main advantage of a high throughput for the measurement of biological samples. The extent of the method to the study of the redox couple GSSG/GSH by including an enzymatic reduction step and the enhancement of the fluorescence signal using cyclodextrins were discussed.

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Keywords: Glutathione; Yeast; Naphthalene-2,3-dicarboxaldehyde; Fluorescence; Microplate assay

1. Introduction

Reduced glutathione (γ -glutamylcysteinylglycine; GSH) is the major intracellular antioxidant in most of prokaryotic and eukaryotic cells [1]. Its monitoring is of main importance in studies related to oxidative stress [2–5], detoxification processes [6] and related human diseases [7]. Most methods which have been reported for GSH measurement in biological fluids are separative methods mainly relying upon high-performance liquid chromatography (HPLC) [8,9]; they offer both selectivity and low detection levels but time-consuming handling and low sample throughput due to the sequential mode of analytical runs. New trends in the development of methods devoted to GSH

monitoring are aimed toward (1) its labeling in living cells to permit its localization using flow cytometry and microscopy and (2) use of non-separative techniques authorizing a high sample throughput with the improvement of practicability. Selective fluorogenic probes have to be selected in both cases. Among them, the dialdehydic aromatic reagents, *ortho*-phthalaldehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA), present a chemical reaction pathway which leads to a high selectivity of GSH detection: they react as heterobifunctional reagents with both sulfhydryl and amino groups of GSH [10,11] (Fig. 1); OPA-GSH and NDA-GSH adducts present an excitation wavelength in the UV and visible range, respectively. The latter has the main advantage to correspond to the emission line of the argon laser, which is often used in confocal microscopy, flow cytometry, and capillary electrophoresis (CE). Since the first report of OPA as a fluorogenic reagent for GSH assay [12], OPA has mainly been used for GSH derivatization in pre-column [13] or post-column

* Corresponding author. Tel.: +33 3 83 68 21 55; fax: +33 3 83 68 21 54.
E-mail address: pierre.leroy@pharma.uhp-nancy.fr (P. Leroy).

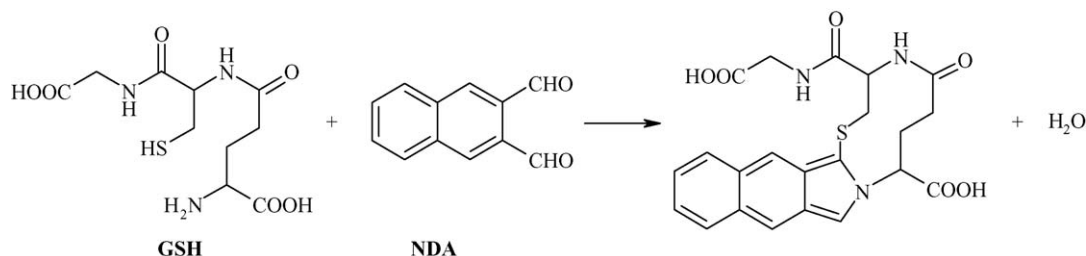


Fig. 1. Reaction scheme between reduced glutathione (GSH) and naphthalene-2,3-dicarboxaldehyde (NDA).

[2–4,10] HPLC systems. NDA is preferentially used in CE coupled with laser induced fluorescence detection [11,14,15]; NDA is also a promising probe for in situ labeling of GSH [16].

Among high-throughput analytical methods, many microplate systems are developed to support and enhance numerous techniques, such as spectrophotometry, fluorescence, solid-phase extraction, and filtration [17]. Microplate systems have been introduced for high throughput screening processes, in which automation and short sample handling are essential; they also allow decreasing costs by minimizing solvents and reagents consumption.

Microplate assays devoted to GSH measurement rely upon either the enzymocolorimetric method of Tietze [18] or related ones [19]. NDA was used in microplate assays to analyze amino acids [20] and to measure the activity of γ -glutamylcysteine ligase (E.C. 6.3.2.2), a key enzyme in GSH biosynthesis, which is located in the cytosolic part of cells [21].

The present work is devoted to the optimization of a microplate assay for GSH measurement in cultured yeast cells. Yeast was selected as a model because these microorganisms have both a plasmic membrane and a wall, like bacteria, needing a particular care for extraction step. Moreover, GSH metabolism in yeast is close to that described in mammalian cells [1]. Thus, the extent of the method to bacteria and mammalian cells seems easier by presently selecting yeast as a cell model. The main points investigated in this work are (1) optimization of extraction of GSH from cultured yeast cells, and derivatization and detection steps, (2) validation of the method including a comparison study with a previously reported HPLC method [10], and (3) the ability of the method to monitor cellular GSH variations under different culture conditions.

2. Experimental

2.1. Reagents and chemicals

All chemicals and solvents were of analytical or HPLC reagent grade and were used without further purification. Reduced glutathione (GSH) and glutathione disulfide (GSSG), various thiols, glutathione reductase (EC 1.6.4.2.; presently used as a crude extract of baker's yeast) and NADPH were obtained from Sigma (Saint-Quentin Fallavier, France); γ -glutamylcysteine (γ -GluCys) and cysteinylglycine (CysGly) were purchased from Bachem (Voisins-le-Bretonneux, France), NDA and dithionite (DT) from Fluka (Saint-Quentin Fallavier, France), dithiotreitol (DTT) from Merck (Darmstadt, Ger-

many), tris-2-carboxyethyl-phosphine hydrochloride (TCEP, HCl) from Molecular Probes (Leiden, The Netherlands), β - and γ -cyclodextrins hydrate from Acros Chemicals (Geel, Belgium) and hydroxypropyl- β -cyclodextrin from Rhône-Poulenc Research Center (Aubervilliers, France).

Phosphate buffer saline (PBS) solution contains 120 mM NaCl, 2.7 mM KCl, and 10 mM K_2HPO_4 , and was adjusted to pH 7.0 with 1 M hydrochloric acid before sterilization.

The stock solution of NDA was prepared in ethanol at a concentration of 5.43 mM (1 mg mL^{-1}) and stored at -80°C . The stock solutions of GSH and GSSG were prepared at a concentration of 3.250 and 1.625 mM, respectively, in 0.1 M hydrochloric acid containing 0.02 M EDTA and were kept at -80°C in darkness for a maximum period of 3 months. During handling, dilutions of stock solutions were kept at 4°C for no longer than 12 h. A calibration curve (8 points) was performed daily with further dilutions ranging from 0.3 to $6.50 \mu\text{M}$ in 3.3% (v/v) perchloric acid.

2.2. Cell culture and extraction conditions

Two yeast strains, *Kluyveromyces lactis* 5a (non-flocculent strain) and *Kluyveromyces lactis* 5c (flocculent strain) were used for experiments, as previously described [22]. Yeast cells were obtained from a preculture and were grown in Sabouraud medium at 25°C under stirring at 300 rpm, as previously described. The culture growth was monitored by measuring the optical density (OD) at 620 nm versus the cell free culture medium. For optimization and validation steps, the *K. lactis* 5a strain was used at a 24-h growth period, which corresponds to the stationary phase of culture growth (the OD ranged between 1.1 and 1.2). For monitoring variations of GSH cellular content during different growth periods (0, 3, 6, 9, and 24 h), the *K. lactis* 5c strain was used. After incubation, 20 mL of cells suspension was centrifuged at $400 \times g$ for 10 min at 4°C ; cell pellets were then washed twice by resuspending in 10 mL of PBS solution and centrifuged as above.

For GSH measurement, the final pellets were resuspended in 10 mL of 3.3% (v/v) perchloric acid, previously cooled, and were shaken vigorously with a vortex for 2 min. After centrifugation at $14,000 \times g$ for 15 min at 4°C , the resulting supernatants were immediately frozen at -80°C until analysis.

2.3. Instrumentation and operating conditions

Ninety-six-well microplates (FIA black microplate model, Greiner Bio-One, Courtaboeuf, France) were used. The

microplate reader (FL 600 model, Bio-Tek, Winooski, VT, USA) was equipped with filters selecting excitation and emission wavelengths at 485 ± 10 nm and 530 ± 12.5 nm, respectively.

The HPLC system consisted of a low-pressure gradient solvent delivery pump (model PU 980, Jasco, Nantes, France), an autosampler equipped with a 20- μ L sample loop, a cooling sample device and a column oven (model AS-300, Thermo Quest, Les Ulis, France), a spectrofluorimetric detector (model FP-920, Jasco, Nantes, France), and a data processing software (model AZURTM V 3.0, Datalys, Saint-Martin d'Herès, France). The tray compartment containing sample vials was cooled at 4 °C. A guard column (8 mm $\times$ 4 mm i.d.) and an analytical column (125 mm $\times$ 4.6 mm i.d.) were packed with Spherisorb ODS-2, 5 μ m (Waters, St Quentin-en-Yvelines, France). GSH measurements were operated as previously described using OPA post-column derivatization coupled with fluorescence detection [10].

2.4. Reduced glutathione microplate assay

The derivatization process was realized as follows: sample was rapidly defrosted in a water bath at 37 °C, 100 μ L of either the cell extract neutralized by adding 25 μ L of NaOH 2 M or the standard GSH solution freshly prepared was transferred into a well of the microplate; 75 μ L of 0.4 M borate buffer pH 9.2 and 20 μ L of NDA stock solution were added to each well. Each sample was measured in triplicate. A blank containing 100 μ L of 3.3% perchloric acid instead of the sample was realized on each microplate. The microplate was shaken for 10 min at 500 rpm in darkness, at 20 ± 2 °C. The fluorescence intensity was immediately measured. The value obtained for the blank was subtracted from each value corresponding to standards and samples. When the concentration was higher than the highest limit of the linearity range, samples were diluted in 3.3% (v/v) perchloric acid.

3. Results and discussion

3.1. Optimization of the derivatization and detection step

Optimization steps were realized by measuring the fluorescence signal in the batch reaction mixture. According to the previously reported fluorescence spectra of the NDA-GSH adduct (λ_{exc} at 472 nm and λ_{em} at 528 nm) [11], the band-pass of the filters selected for excitation and emission were 485 ± 10 nm and 530 ± 12.5 nm, respectively; they were the most appropriate among those available on the instrument presently used.

The first optimized parameter was pH of the reaction medium. As a matter of fact, the reaction between GSH and either OPA or NDA needs an alkaline medium to occur, and a borate buffer of pH value close to 9 is most frequently used for that purpose. Some authors claimed that the fluorescence signal obtained after reaction between NDA and GSH or γ -GluCys was increased at higher pH values [21]. However, this fact can counteract the full selectivity of the reaction, especially versus oxidized glutathione (GSSG) which reacts with the fluorogenic reagent after cleavage of the disulfide bond in highly alkaline medium [23–25]. The optimum pH range to obtain the maximum fluorescence signal

of the OPA-GSH adduct has been reported to be between 8 and 12, as well in a batch solution [12] as in a pre-column derivatization HPLC system [13]. Concerning the reaction between GSH and NDA, Orwar et al. [11] reported a faster reaction and a higher fluorescence signal at pH 9.1 (borate buffer) than at pH 7.4 (HEPES buffer).

We have presently tested three pH values for the reaction medium: 8.6, 9.8, and 12.2 (reaction time: 10 min) and we have observed only a slight increase (approximately 5%) of the fluorescence signal in this pH range. Testing GSSG instead of GSH at two pH values (8.6 and 12.2), we measured no signal at pH 8.6 and 67% of the signal obtained with the same GSH concentration at pH 12.2. Thus, the lowest pH value was retained for further experiments to avoid any GSSG interference.

Derivatization *temperature* was then studied. The derivatization reaction was held at pH 8.6 for 15 min in darkness at 4 and 20 °C, and fluorescence intensity was measured immediately after. The highest fluorescence signal was obtained at 4 °C ($108.6 \pm 5.6\%$ of the fluorescence signal measured at 20 °C). As this increase does not significantly improve the limit of quantification of the method, a temperature of 20 ± 2 °C, which is more convenient in a practical point of view, was selected for further experiments.

Concerning the influence of *reaction time*, an increase of fluorescence signal (approximately 10%) between 5 and 25 min at 20 °C was observed. However, the precision decreased (relative standard deviation ($n = 5$) increasing from 3.1 to 11.1%), probably due to side reactions uncontrolled in the present operating conditions. Thus, a 10-min derivatization period appeared to be the most convenient for further experiments.

3.2. Stability studies of the NDA-GSH adduct

The degradation of OPA [26] and NDA [27] -amino acid-thiol adducts is regarded to be of a first order kinetics process. Presently, we observe a linear decay of the NDA-GSH adduct fluorescence as a function of time (in the 0–75 min period), rather indicating a zero order kinetics (Fig. 2). The *daylight exposure* accelerated the adduct degradation: the fluorescence signal loss reached 48% (reaction mixture stored in darkness) and 73% (reaction mixture exposed to daylight) of the initial value, after 105 min. The *temperature* influence was then evaluated: a significant increase in adduct stability (only 21% of signal loss at 105 min) was achieved at 4 °C, which indicates that thermal degradation occurs along with photolytic and chemical reactions.

In conclusion, it is essential to provide obscurity conditions during handling and to perform the fluorescence measurement no later than 5 min after the 10-min derivatization period. Low temperature conditions should enable a less strict handling approach.

3.3. Influence of fluorescence enhancers

Two kinds of compounds, well-known as general *enhancers of fluorescence* yield, i.e. cyclodextrins (CD) and surfactants at concentrations higher than their critical micellar concentration

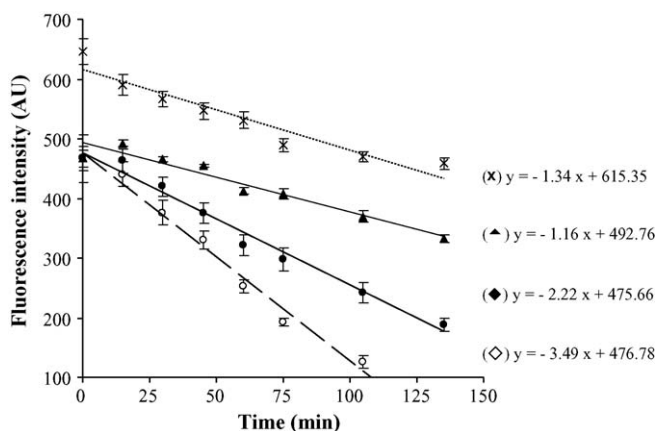


Fig. 2. Influence of daylight, temperature and the presence of β -cyclodextrin on NDA-GSH adduct stability. Measurements were performed after 10-min derivatization in darkness using the optimized operating conditions of the microplate assay (GSH concentration tested: $3.25 \mu\text{M}$), followed by storage ($\diamond$) in daylight at $20 \pm 2^\circ\text{C}$, ($\blacklozenge$) in darkness at $20 \pm 2^\circ\text{C}$, ($\blacktriangle$) in darkness at 4°C , ($\times$) derivatized in presence of 10 mM β -cyclodextrin and stored in darkness at $20 \pm 2^\circ\text{C}$ (five independent experiments).

(CMC), have been tested in the case of NDA-GSH adduct. The aim of these experiments is to verify whether the incorporation of GSH-NDA adduct in a hydrophobic phase (inside surfactants micelles or CD cavity) could increase the fluorescence signal, as a general rule in fluorescence states.

First, three surfactants, i.e. sodium dodecylsulfate (SDS), cetyltrimethylammonium bromide (CTMABr) and Brij-35 were selected because they present different charges (negative, positive, and neutral, respectively). They have been tested at a concentration equal to 120% of their CMC (8.1 , 0.9 , and 0.1 mM , respectively) and no influence on the fluorescence signal was noted. Trials with a higher concentration of surfactants failed due to resulting sample turbidity.

Next, β -CD, its hydroxypropyl form and γ -CD were tested. If considering that stoichiometry of these kinds of host–guest complexes is either 1:1 or 2:1 and that stability constants are low (generally less than 100) [26], a CD concentration excess upper than 10^3 has to be used to move the equilibrium toward complexes formation. Only β -CD induced an increase of fluorescence (+11%) when added to the reaction medium at the concentration of 0.3 mM . However, when a 10.0 mM concentration was tested, an increase in fluorescence signal was observed for both β -CD (+61%) and its hydroxypropyl form (+41%). A similar increase of fluorescence was observed when γ -GluCys was tested instead of GSH with β -CD and its hydroxypropyl form; it seems to show that the presence of the glycine residue in GSH does not play a role in the inclusion process giving rise to the fluorescence signal increase.

Two previous reports have noted an influence of CDs on fluorescence signal of related adducts: (1) Bantan-Polak et al. [20] reported that the fluorescence of fifteen amino acids–cyanide–NDA adducts was enhanced 70% by addition of β -CD at a 10.0 mM final concentration; (2) Wagner and Mc Manus [26] observed a three-fold fluorescence signal increase of the glycine-OPA adduct in the presence of 10 mM of

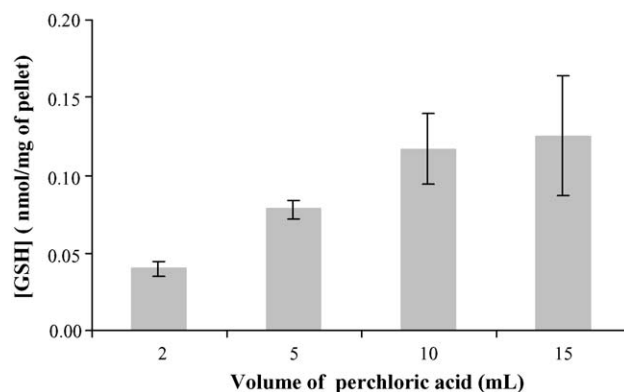


Fig. 3. Variation of GSH concentration in the yeast extract (corresponding to a wet biomass of ca. 25 mg) as a function of perchloric acid volume (five independent experiments).

hydroxypropyl- β -CD. The latter CD is much more water-soluble than β -CD (five-fold), thus it should be interesting to increase its concentration in order to verify if a higher increase is obtained in the fluorescence signal. At our knowledge, this work is the first report of the effect of β -CD on fluorescence signal of the NDA-GSH adduct.

A significant influence of β -CD on NDA-GSH adduct stability was also noted: the fluorescence signal loss after 1 h was 18% with 10 mM β -CD versus 32% without β -CD (Fig. 2). When testing β -CD in the GSH concentration range of 0.3 – $6.5 \mu\text{M}$, a 67% increase of the slope of the equation of the regression line was observed. Thus, the use of CDs seems promising to enhance the assay sensitivity.

3.4. Optimization of the extractive steps for GSH measurement in yeast cultures

Most of reported procedures for extraction of unbound GSH in both prokaryotic [2,3] and eukaryotic [28] cells use a cold acidic solution to homogenize samples, to precipitate proteins from the mixture and to minimize oxidative changes. Presence of walls in bacteria can imply additional treatment as use of an ultrasonic probe [2,3]. Presently, the extractive volume of 3.3% (v/v) perchloric acid in which was resuspended the cell pellets obtained from the *K. lactis* cultures, has been optimized (Fig. 3). The increase in GSH recovery when perchloric acid volume increases indicates that the ratio of acid volume/biomass has to be precisely defined according to the operating conditions. A perchloric acid volume of 10 mL cooled at 4°C has been selected for further experiments using a wet biomass which did not exceed 25 mg .

3.5. Validation of the method

3.5.1. Selectivity

The optimized procedure was applied to a series of thiols at a concentration of $6.5 \mu\text{M}$. Only two thiols, i.e. GSH and γ -GluCys, provide a significant fluorescence signal when reacting with NDA (Table 1). γ -GluCys is the precursor of GSH in its de novo synthesis; its cellular levels are extremely low in eukaryotic

Table 1

Relative fluorescence intensity (RFI%) of different thiols (6.5 μM) and reducing reagents (6.5 and 130 μM) and glutathione disulfide (GSSG) (3.25 μM) derivatized with NDA as referred to NDA-GSH signal (five independent experiments)

	RFI $\pm$ S.D. (%)
GSH	100.0 $\pm$ 3.2
CysGly	4.3 $\pm$ 2.2
γ -GluCys	76.0 $\pm$ 4.0
Cys	0.7 $\pm$ 2.0
Hcy	0.6 $\pm$ 2.2
Thioglycolic acid	1.5 $\pm$ 1.9
DTT	0.0 $\pm$ 1.1
DTT (20 $\times$ excess)	9.3 $\pm$ 0.6
DT	0.0 $\pm$ 0.6
DT (20 $\times$ excess)	0.0 $\pm$ 2.6
TCEP	10.2 $\pm$ 1.0
TCEP (20 $\times$ excess)	169.2 $\pm$ 8.5
Glutathione reductase + NADPH	1.0 $\pm$ 2.3
GSSG	0.0 $\pm$ 0.9

cells, thus its presence will not significantly influence the GSH measurement.

With an aim of evolving the method to the quantification of both reduced glutathione and its disulphide form, the interference of reducing reagents has also been tested at two concentrations: 6.5 and 130 μM (20-fold excess). The most commonly reagents used for the disulphide bond cleavage are thiols themselves, e.g. 2-mercaptoethanol, DTT, and DT. Presently, DTT and DT exhibit no fluorescence signal when they were used at a concentration of 6.5 μM ; the signal of DTT increases very slightly when used in a 20-times excess. DTT has already been reported to interfere in the GSH assay using OPA because of the presence of amino acids in biological extracts [30]. Other commonly used reductants are trialkylphosphines, especially tris-2-carboxyethyl-phosphine hydrochloride (TCEP). Trialkylphosphines do not contain a free thiol group. They are powerful reagents that need only a slight molar excess to reduce the disulphide. TCEP cleaves disulphide in a wide pH range: 1.5–8.5 [29]. None of the different buffers tested (3.3%, v/v) perchloric acid pH 0.4, 0.1 M acetic buffer pH 5.0, 0.1 M phosphate buffer pH 7.5 and 0.1 M Tris buffer pH 8.0) were appropriate to TCEP performance, the signal being dramatically increased by its presence. The resulting interference was confirmed by HPLC, using the previously reported system [16]. With equal concentrations, the fluorescence of a TCEP-containing sample represents 10% of the fluorescence obtained with GSH itself. Thus, the use of TCEP as reducing agent has to be excluded in the present assay.

The enzymatic reduction with glutathione reductase was at last tested as previously described [23]. No significant influence of reagents (the enzyme and its cofactor NADPH in 50 mM phosphate pH 7.8) on the fluorescence signal was observed in the operating conditions. Desired levels of reduction were achieved when this enzymatic reaction was used. Fluorescence intensity of the GSSG sample reduced in presence of glutathione reductase and NADPH was >98% of the expected value.

Main validation parameters of the assay are summarized in Table 2.

Table 2

Main validation parameters of the method

Calibration curve	
Linearity range/coefficient of determination	0.3–6.5 μM /0.98 < R^2 < 1.00
Slope (mean $\pm$ S.D.); $n = 3$	1103.4 $\pm$ 47.6
Intercept (mean $\pm$ S.D.); $n = 3$	–66.1 $\pm$ 39.6
Limit of quantification	0.30 μM
Limit of detection	0.05 μM
Precision	
Repeatability (mean $\pm$ S.D.); $n = 3$	3.8 $\pm$ 0.1 μM
Intermediate precision (mean $\pm$ S.D.); $n = 5$	1.3 $\pm$ 0.1 μM
Accuracy	$y = 0.95x + 0.03$ $R^2 = 0.94$

3.5.2. Linearity range/limit of quantification/limit of detection

The calibration curve was built with standard solutions of GSH corresponding to eight concentrations ranging from 0.3 to 6.5 μM , and prepared in triplicates. The coefficient of determination R^2 of the equation of the regression line was higher than 0.98. The limits of detection (LoD) and of quantification (LoQ) were calculated from the slope (a) and intercept values (b) of the linearity curve, and the standard deviation of the intercept (S.D. b). Limits were expressed, respectively, as: $\text{LoD} = (b + 3 \text{ S.D.}b)/a$ and $\text{LoQ} = (b + 10 \text{ S.D.}b)/a$. LoD and LoQ were 0.05 and 0.3 μM , respectively, and seem convenient for the present assay of intracellular GSH in yeast cultures.

3.5.3. Precision

The repeatability of the method was calculated by triplicate quantification of GSH in a cell suspension resulting from a *K. lactis* 5a strain culture (24-h growth period). For a median concentration value (3.8 μM), the relative standard deviation corresponding to the repeatability was 2.8%, which looks acceptable held accounts that they are biological samples.

To assess the inter-day precision, a GSH standard solution was measured five times over a period of 2 weeks with a calibration curve prepared daily. For a low GSH concentration (1.3 μM), the relative standard deviation corresponding to the inter-day precision was 6.1%.

The accuracy was evaluated by recovery experiments. Four different amounts were added to two distinct cellular extracts of a *K. lactis* 5a culture. The eight supplemented samples and the two virgin extracts were submitted to the assay. The calculated concentrations (y) were compared to the theoretical ones (x). The coefficient of determination between the two sets of data is $R^2 = 0.94$, the slope of the line of regression is close to 1 and the intercept close to 0. Thus, the concentrations measured in biological samples are representative of the expected ones.

3.5.4. Comparison with an HPLC method

A set of 22 biological samples, resulting from extraction of *K. lactis* cultures and representative of the wide studied range, was analysed independently by the present microplate assay and the previously reported HPLC method [10] (Fig. 4). Both linear regression analysis and Bland–Altman plot correlation show the equivalence between the two methods.

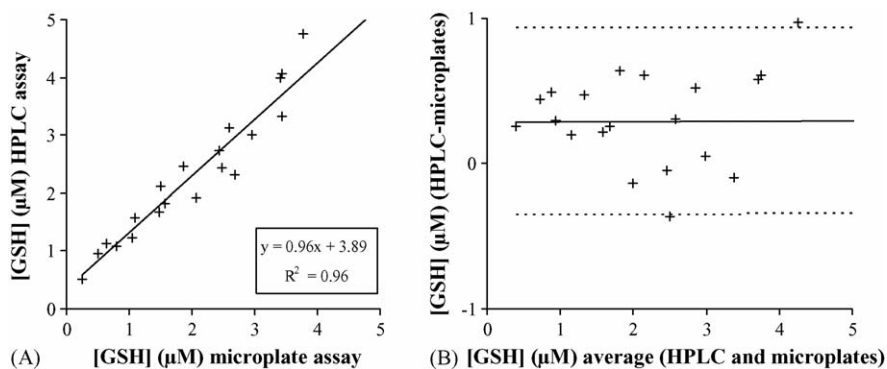


Fig. 4. Comparison of GSH concentrations measured in yeast extracts ($n=22$): (A) by microplate assay (x) and HPLC (y) with linear regression analysis and (B) by Bland–Altman plot correlation which shows the difference in GSH results between the two methods as a function of their value with mean difference (solid line) and limits of agreement corresponding to mean ± 2 S.D. (dashed lines).

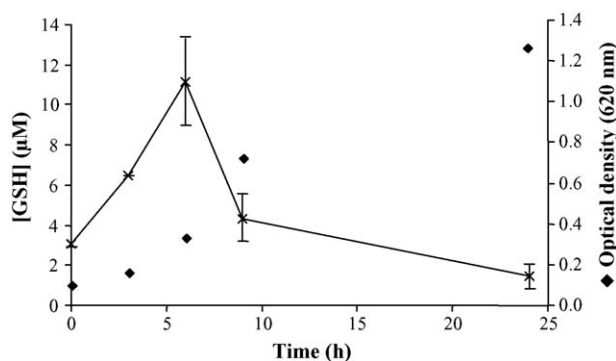


Fig. 5. Variations of GSH concentration in an extract of the yeast strain *Kluyveromyces lactis* 5c during different growth periods (≤ 9 h: exponential phase; >14 h: stationary phase); (three independent experiments).

3.6. Biological applications

The presently optimized and validated method is at our knowledge, the first report of a fluorescence microplate assay devoted to GSH measurement in yeast cells. It was applied to the monitoring of cellular GSH concentrations during growth of the strain *K. lactis* 5c (Fig. 5). The GSH concentration increases at the beginning of the exponential phase, as most cellular components do, then decreases at the end of the exponential phase to reach a low level during the stationary phase (cells no more dividing).

Next, L-buthionimine-S,R-sulfoximine (BSO), a specific inhibitor of γ -glutamylcysteine ligase, the key enzyme in GSH biosynthesis, was added to the culture medium at 6-h growth at a concentration of 5 mM, and cellular GSH concentration was measured 3 h later. The 6–9-h period was included in the exponential phase of growth. A decrease in cellular GSH content of 76% was observed versus control (no BSO added).

4. Conclusions

The newly developed and validated microplate assay affords high selectivity for the measurement of cellular GSH in yeast cells. What is more the method is cost saving, as the reagents and solvents consumption has been significantly reduced, when

compared with commonly used HPLC system with spectrofluorimetric detection. The present method uses the universal 96-well microplate format, thus enables high sample throughput and opens the path towards laboratory automation. In example, an analytical run for a single sample using the HPLC system needs approximately 10 min, whereas the present method enables to analyze 96 samples (or even more if a 264-well design is introduced) within less than 3 min. The present microplate assay offers a similar LoQ value than a previously reported fluorescence assay [21] and a 10-times lower one than enzymo-colorimetric methods [18,19]. At last, the present method can be easily extended to the measurement of GSSG using glutathione reductase and its cofactor NADPH for the reducing step and the fluorescence signal can be enhanced using cyclodextrins.

Acknowledgements

The authors thank Pr Gérard SIEST and Dr Sophie VISVIKIS-SIEST (INSERM Unit U 525; University Henri Poincaré-Nancy 1) to have authorized the use of the microplate fluorescence reader in their laboratory during this study.

References

- [1] M. Penninckx, FEMS Yeast Res. 2 (2002) 295–305.
- [2] S. Saby, P. Leroy, J.C. Block, Appl. Environ. Microb. 65 (1999) 5600–5603.
- [3] M.A. Dziurla, P. Leroy, G.W. Strükmann, M. Sahli, D.U. Lee, P. Camacho, V. Heinz, J.A. Müller, E. Paul, Ph. Ginestet, J.M. Audic, J.C. Block, Water Res. 38 (2004) 236–244.
- [4] M.A. Dziurla, M. Sahli, P. Leroy, E. Paul, Ph. Ginestet, J.C. Block, Water Res. 39 (2005) 2591–2598.
- [5] A. Pompella, A. Visvikis, A. Paolicchi, V. De Tata, A.F. Casini, Biochem. Pharmacol. 66 (2003) 1499–1503.
- [6] A. Pastore, G. Federici, E. Bertini, F. Piemonte, Clin. Chim. Acta 333 (2003) 19–39.
- [7] D.M. Townsend, K.D. Tew, H. Tapiero, Biomed. Pharmacother. 57 (2003) 145–155.
- [8] K. Shimada, K. Mitamura, J. Chromatogr. B 659 (1994) 227–241.
- [9] E. Camera, M. Picardo, J. Chromatogr. B 781 (2002) 181–206.
- [10] P. Leroy, A. Nicolas, M. Wellman, F. Michelet, T. Oster, G. Siest, Chromatographia 36 (1993) 130–134.
- [11] O. Orwar, H.A. Fishman, N.E. Ziv, R.H. Scheller, R.N. Zare, Anal. Chem. 67 (1995) 4261–4268.
- [12] V. Cohn, J. Lyle, Anal. Biochem. 14 (1966) 434–440.

- [13] C. Cereser, J. Guichard, J. Draï, E. Bannier, I. Garcia, S. Boget, P. Parvaz, A. Revol, *J. Chromatogr. B* 752 (2001) 123–132.
- [14] C. Parmentier, M. Wellman, A. Nicolas, G. Siest, P. Leroy, *Electrophoresis* 20 (1999) 2938–2944.
- [15] J. Qin, N. Ye, L. Yu, D. Liu, Y. Fung, W. Wang, X. Ma, B. Lin, *Electrophoresis* 26 (2005) 1155–1162.
- [16] L. Diez, E. Martenka, A. Dabrowska, J. Coulon, P. Leroy, *J. Chromatogr. B* 827 (2005) 44–50.
- [17] R.E. Majors, *LC/GC Mag.* 18 (2005) 70–76.
- [18] S. Allen, J.M. Shea, T. Felmet, J. Gadra, P.F. Dehn, *Methods Cell Sci.* 22 (2001) 305–312.
- [19] C. Neumann, Boubakari, R. Grünert, P.J. Bednarski, *Anal. Biochem.* 320 (2003) 170–178.
- [20] T. Bantan-Polak, M. Kassai, K.B. Grant, *Anal. Biochem.* 297 (2001) 128–136.
- [21] C.C. White, H. Viernes, C.M. Krejsa, D. Botta, T.J. Kavanagh, *Anal. Biochem.* 318 (2003) 175–180.
- [22] M. El-Behhari, J.N. Ekomé, J. Coulon, B. Pucci, R. Bonaly, *Appl. Microbiol. Biotechnol.* 49 (1998) 16–23.
- [23] C. Coutelle, *Ann. Biol. Clin.* 50 (1992) 71–76.
- [24] A.P. Senft, T.P. Dalton, H.G. Shertzer, *Anal. Biochem.* 280 (2000) 80–86.
- [25] J.P. Hissin, R. Hilf, *Anal. Biochem.* 74 (1976) 214–226.
- [26] B.D. Wagner, G.J. McManus, *Anal. Biochem.* 317 (2003) 233–239.
- [27] D.P. Manica, J.A. Lapos, A.D. Jones, A.G. Ewing, *Anal. Biochem.* 322 (2003) 68–78.
- [28] C. Thioudellet, T. Oster, P. Leroy, A. Nicolas, M. Wellman, *Cell Biol. Toxicol.* 11 (1995) 103–111.
- [29] E. Burmeister Getz, M. Xiao, T. Chakrabarty, R. Cooke, P.R. Selvin, *Anal. Biochem.* 273 (1999) 73–80.
- [30] R.C. Scatudo, *Anal. Biochem.* 174 (1988) 265–270.

Kinetic determination of cysteine on flow injection system by utilizing catalytic complexation reaction of Cu(II) with 5,10,15,20-tetrakis (4-*N*-trimethylammino-phenyl) porphyrin

Jianhua Liu, Jun-ichi Itoh *

Department of Applied and Environmental Chemistry, Kitami Institute of Technology, 165 Koen chyo, Kitami 090-8507, Japan

Received 5 December 2005; received in revised form 25 January 2006; accepted 25 January 2006

Available online 28 February 2006

Abstract

A kinetic method for the determination of cysteine on flow injection system is described. Cysteine was found as a catalyst for the complexation reaction of Cu(II) with an ultra sensitive colorimetric reagent of 5,10,15,20-tetrakis (4-*N*-trimethylammino-phenyl) porphyrin (TTMAPP) ($\epsilon = 4.8 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ at 433 nm), which was used as the indicator reaction in this paper. Soret band of either the porphyrin or the produced complex ($\epsilon = 4.6 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ at 411 nm) can be used for detection. The continuous flow injection system greatly enhanced the analytic precision and efficiency of the kinetic method, giving a relative standard deviation of 0.73% for a $0.1 \mu\text{g ml}^{-1}$ cysteine with 10 injections at a throughput of 30 h^{-1} . The detection limits (3S/N) in this case was 15 ng ml^{-1} and the working dynamic range was over 25 ng ml^{-1} to $1 \mu\text{g ml}^{-1}$. Sugars, organic acids and amino acids that are possible in coexistence with cysteine could be tolerated at high concentrations. This method was critically compared with the Ellman's reagent in the determinations of cysteine contents of three pharmaceutical injections for hepatic diseases and one permanent wave agent and showed better applicability in the respect of matrix interferences.

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Keywords: Porphyrin; Flow injection analysis; Kinetic method; Cysteine

1. Introduction

Cysteine is a non-essential amino acid and of great interest in biological, pharmaceutical and nutrition researches. Compared with the large numbers of the electrochemical [1–4] or fluorimetric methods [5–8] for the determination of cysteine, spectrophotometric methods were less reported although they are attractive from the view of simplicity and low cost [9,10]. Low sensitivity is one of the drawbacks of these methods due to the poor absorption intensity of the color reagents used, among which the complex of 3-(2-pyridyl)-5,6-bis(4-sulfophenyl)-1,2,4-triazine with Fe(III) shows the highest molar absorptivity ($2.9 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$) [9]. Stoichiometrical redox reaction of cysteine with a certain oxidant forms the common basis of these methods, thus reductive constituents involved in the sam-

ple matrices would possibly cause interferences making these methods lack of selectivity.

Kinetic method for the determination of a catalyst can achieve high sensitivity and in some cases has a low probability to receive interferences. A kinetic method based on the induction effect of cysteine on iodine-azide reaction was reported with a great improvement in the sensitivity [11], but such traditional batchwise procedure requires the experimental conditions to be strictly controlled, thus making the method laborious and lack of precision. Flow injection analysis (FIA) presents an important concept of total automation of solution handling, offering the advantages of speedy analysis, stable experimental conditions, low contamination risk and low consumption of reagents. Therefore, a kinetic method incorporated into a proper FIA system, particularly a continuous flow injection system, can be expected to exhibit sensitive, precise, simple and rapid characteristics. Unfortunately, recent catalytic kinetic methods were mainly performed on stopped-flow systems [12–16], and the applications of continuous flow system in this field were seldom reported, although continuous flow system offers incomparable

* Corresponding author.

E-mail address: itojun@chem.kitami-it.ac.jp (J.-i. Itoh).

advantages from the view of either precision or simplicity of the procedures [17,18].

Porphyrins are a group of chemical analogues possessing a macrocycle, which consists of four pyrrole rings linked by methine bridges and gives rise to their characteristic spectrum (Soret band) with intense absorption in visible region (400–450 nm). Although extensive studies have been done to explore the analytical applications of porphyrins, most of these studies are only focused on the analyses of a limited group of metals and there is no remarkable development in this field in recent years [19–24]. In 1981, we developed a spectrophotometric method for the ultra trace determination of Cu(II) in human serum by using 5,10,15,20-tetrakis(4-*N*-trimethylammino-phenyl) porphyrin (TTMAPP) [25], which holds a Soret band with absorption intensity comparable to anionic porphyrins ($\epsilon = 4.8 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and also shows good water solubility. Cysteine was found in the determination as one of the substances showing accelerating effect on the metal incorporation to the porphyrin core.

In this paper, the catalytic effect of cysteine on the complexation of Cu(II) with TTMAPP was elucidated and utilized further to develop a catalytic kinetic method for the determination of itself on a continuous flow injection system. The advantages of kinetic method and FIA technique were successfully combined making the method sensitive, simple, fast and precise. This kinetic method was also compared with the spectrophotometric method of the Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) [26] in determinations of cysteine contents of three pharmaceutical injections used for hepatic diseases and one permanent wave agent and the results show that this proposed method is more robust against matrix interferences.

2. Experimental

2.1. Instrumentation

Fig. 1 shows a schematic diagram of the FIA system, which consists of a chromatography pump (CCPM, Toyosoda Industries, Japan), a peristaltic pump (Gilson miniplus3, M&S Instruments Trading Inc.), reaction coils (PTFE, 0.5 mm i.d.) of various lengths, a photometric detector (UV8011, Tosoh

Co., Japan) and a recorder (type 3066, Yokogawa Electronics, Japan). An injection valve (Rheodyne 7125) equipped with a 10 μl sample loop was attached to the chromatography pump for sample injection. Carrier was delivered by the chromatography pump at a flow rate of 0.6 ml min^{-1} , and other reagents by the peristaltic pump at a flow rate of 0.06 ml min^{-1} each. Temperature of the reaction coil (RC1) was controlled with a thermostatic water bath ($\pm 0.1^\circ\text{C}$, Model CTR-220, Komatsu Electronics Inc., Japan). A spectrophotometer (UV8200, Shimadzu Co., Japan) was used for a photometric titration to standardize the concentration of home-synthesized TTMAPP and the reference method of DTNB to determine cysteine contents of the samples.

2.2. Reagents and samples

Cysteine (>98%) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Japan). Other reagents, except for TTMAPP, were purchased from Wako Pure Chemical Industries (Japan) and are of analytical grade. Tetratoluensulfonate salt of TTMAPP was synthesized and purified in our laboratory following a procedure described before [25]. Stock solution of TTMAPP was prepared by dissolving the synthesized powder (0.04 g) into a flask (250 ml) with nitric acid (0.1 M) and was standardized as $1.1 \times 10^{-4} \text{ M}$ photometrically with a standard Cu(II) nitrate solution by measuring the absorbance change of TTMAPP at 433 nm ($\epsilon = 4.8 \times 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$) or of Cu(II)–TTMAPP complex at 411 nm ($\epsilon = 4.6 \times 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$).

Reagents used for flow system were prepared as follows: carrier was prepared as a buffer (0.025 M acetic acid/0.028 M sodium acetate) containing 40% (v) methanol and was degassed before use. Cu(II) and TTMAPP of appropriate concentrations were prepared as a mixture in 0.1 M nitric acid by direct dilution of their stock solutions. Stock solution of standard cysteine ($1000 \mu\text{g ml}^{-1}$) was prepared freshly in acetic acid (0.1 M) before experiment and was kept at 4°C during experimental periods. Working standards were prepared by diluting the stock solution with carrier solution, which was also used to prepare the solutions of interference substances and samples. Distilled and deionized water was used throughout.

2.3. Procedures

2.3.1. Spectrophotometric study on the catalytic effect of cysteine

The catalytic effect of cysteine on the complexation reaction of Cu(II) with TTMAPP was investigated following a batch-wise procedure as below: at room temperature, water (17 ml), acetic acid (2.5 M, 0.25 ml), sodium acetate (2.5 M, 0.5 ml), TTMAPP ($1.1 \times 10^{-4} \text{ M}$, 0.35 ml) and cysteine ($0.1 \mu\text{g ml}^{-1}$ to $20 \mu\text{g ml}^{-1}$, 1.0 ml) were added in sequence into a flask (25 ml); reaction time was measured when an aliquot of standard Cu(II) solution ($1.57 \times 10^{-4} \text{ M}$, 0.25 ml) was added into the flask; at a proper time interval, an aliquot of nitric acid (0.25 ml) was added (final pH 1.0) to quench the reaction and shift Soret band of TTMAPP (411 nm $\rightarrow$ 433 nm); water was added to fill up the flask and the absorbance of the solution was measured at 411 nm (Soret band of Cu(II)–TTMAPP).

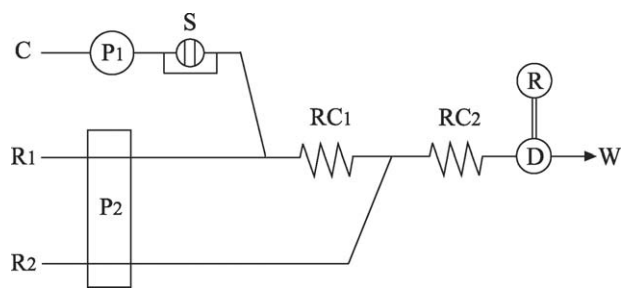


Fig. 1. Schematic diagram of flow injection system. P1, HPLC pump with flow rate of 0.6 ml min^{-1} ; P2, peristaltic pump with flow rate of 0.07 ml min^{-1} ; S, sample injector; RC1 and RC2, reaction coils; D, detector; R, recorder (0.5 mV cm^{-1}); W, waste. C, carrier, aqueous solution of methanol 40% (v) and acetic-acetate (0.025 M/0.028 M) buffer (pH 5.0); R1, mixed solution of Cu(II) and TTMAPP in 0.1 M nitric acid; R2, 1 M nitric acid.

2.3.2. Flow injection analysis

Referring to Fig. 1, carrier (C), mixed solution of Cu(II) and TTMAPP (R1) and nitric acid (R2) were flowed continuously and joined sequentially at RC1 and RC2. The stream was buffered to pH 5.0 at RC1 and acidified to pH 1.0 at RC2, where the spectrum of TTMAPP was shifted from 411 to 433 nm. Cysteine was injected into the FIA system from S and began to catalyze the indicator reaction at RC1. At RC2, nitric acid (R2) quenched the reaction and shifted the spectrum of remaining TTMAPP to 433 nm. The absorbance increase owing to the formed Cu(II)–TTMAPP was detected out as FIA signal of cysteine at 411 nm.

3. Results and discussion

3.1. The cysteine-catalyzed complexation reaction of Cu(II) with TTMAPP

The optimum pH value for the cysteine-catalyzed complexation reaction of Cu(II) with TTMAPP is over the range of pH 4.6–5.4, where TTMAPP takes a free base form of H_2P (P represents porphyrin and the positive charges of side-chains are ignored) and the maximum absorption of its Soret band appears at 411 nm. The spectrum overlaps fully with that of the produced Cu(II)–TTMAPP complex in the pH range, thus direct utilization of Soret band is impossible. By employing strong acid (pH 1.5–0.7), H_2P is acidified to a dicationic form of H_4P^{2+} and its Soret band is shifted to 433 nm due to the total protonation of all the four inner nitrogen atoms of porphyrin core. Such acidification has no influences on the complex of Cu(II)–TTMAPP, thus its Soret band still appears at 411 nm. H_4P^{2+} has no complexing ability with Cu(II), therefore the acidification also serves to quench the complexation reaction.

Following the spectrophotometric procedure (Section 2.3), the catalytic effect of cysteine on the complexation formation of Cu(II) with TTMAPP was investigated over a concentration range of 4.3×10^{-8} to 8.5×10^{-6} M (Fig. 2). Based on Beer's law ($A = \epsilon lc + b$, where the apparent molar absorptivity Cu(II)–TTMAPP $\epsilon = 4.6 \times 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$,

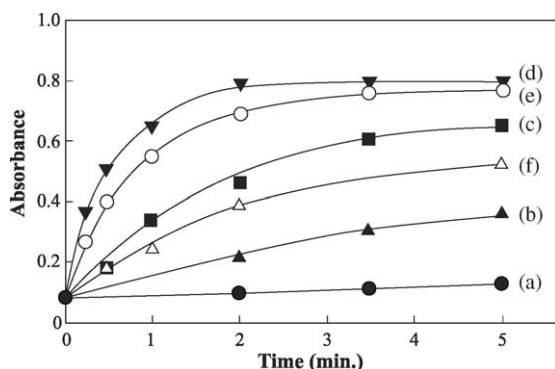
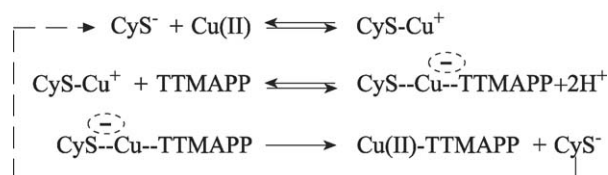


Fig. 2. Formation curves of cysteine-catalyzed complexation reaction of TTMAPP with Cu(II). [Cys] (a) 0 M; (b) 4.3×10^{-8} M; (c) 2.1×10^{-7} M; (d) 2.1×10^{-6} M; (e) 4.3×10^{-6} M; and (f) 8.5×10^{-6} M; [Cu] 2.0×10^{-6} M; [TTMAPP] 2.0×10^{-6} M, pH 5.0 (acetic-acetate buffer); temperature, 25 °C; detection wavelength, 411 nm.



Scheme 1. Pathway outline of cysteine-catalyzed complexation reaction of TTMAPP with Cu(II).

$c = 1.54 \times 10^{-6} \text{ l mol}^{-1} \text{ cm}^{-1}$ and background absorption $b = 0.085 \text{ abs}$) the complete complexation of Cu(II) with TTMAPP should give an absorbance of 0.793 abs. It can be observed that the complexation reaction proceeded only to a slight extent in the absence of cysteine (curve a), and the additions of trace amount of cysteine (curve b with 1/50 and curve c with 1/10 of the amount of TTMAPP) could catalyze the complexation to higher ratios. Cu(II)–TTMAPP formation curve reached to a maximum and stable absorbance (c.a. 0.793 abs) in 2 min when cysteine was added in equal amount of TTMAPP (curve d). Further increases of cysteine concentration did not bring any increase of the reaction rate but inhibited the reaction unexpectedly (curves e and f).

This figure verified the catalytic effect of cysteine on the complexation of Cu(II) with ttmapp but showed meanwhile such catalysis was conditional at least in the respect of cysteine concentration. Referring to a study on the interactions of cysteine with Cu(II) [27], a mechanism was proposed for this cysteine-catalyzed complexation reaction (Scheme 1).

Cysteine takes a proton-disassociated form of CyS^- at pH 5.0, which is favorite to form an intermediate complex with Cu(II). With lowered electronic density the cysteine-complexed Cu(II) becomes easy of access to porphyrin core, but its increased ion radius makes the incorporation difficult only forming an intermediate with a so called “sitting-atop” structure [28]. Such access of the positive charge causes the deprotonation of porphyrin core concurrently, after which Cu(II) incorporation proceeds to completion and CyS^- disassociates achieving the catalytic cycle. When cysteine presents in high concentration, Cu(II) tends to form a stable complex of $(\text{Cys})_2\text{-Cu(II)}$ with cysteine, therefore reaction rate slows down as what had been observed in Fig. 2(e and f).

3.2. Effects of flow system variables

Methanol was added in carrier solution mainly to solve the adsorption of porphyrins to inner walls of PTFE flow tube, which would prolong the total analysis time on the flow system. The preferable methanol ratio in carrier solution was found about 30–70% (v), over which no apparent variance of the signal height was observed on. The addition of methanol retarded the catalytic effect of cysteine to some extent but showed certain effect on suppressing the spreading of the FIA signals by comparison with the carrier of aqueous solution. Carrier of 40% (v) methanol was

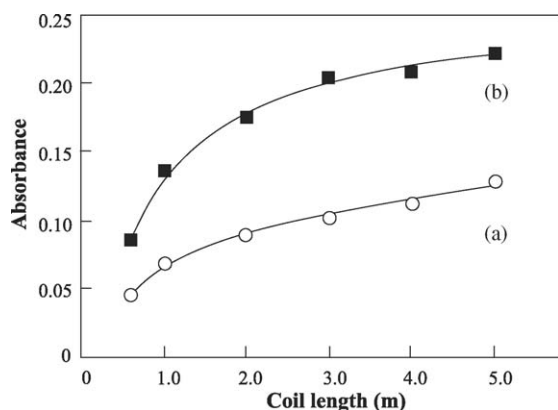


Fig. 3. Effect of reaction coil length (RC1). [Cysteine] (○) $0.1 \mu\text{g ml}^{-1}$; (□) $0.2 \mu\text{g ml}^{-1}$; [Cu] $1.6 \times 10^{-4} \text{ M}$; [TTMAPP] $3.6 \times 10^{-5} \text{ M}$; temperature, 40°C ; detection wavelength, 411 nm.

adopted in our study on the considerations of both sampling rate and experiment cost.

The automated solution-handling of FIA facilitates the control of reaction conditions, thus help to improve the analytical precision greatly. This lessens the necessity to pursue a linear relationship between the detectable parameters and catalyst concentration by solving kinetic equations as in traditional batchwise procedures, and instead the observed FIA signals can be directly linked to catalyst concentration with a non-linear graph generated for calibration. Therefore, catalyst over a certain concentration range should be investigated for the optimization of system variables to obtain a correct estimation. Cysteine of 0.1 and $0.2 \mu\text{g ml}^{-1}$ were used for the following studies.

Reaction coil length of RC1 was investigated over a range of 0.6–5.0 m (Fig. 3). The obtained response curves can be viewed as the formation curves of Cu(II)–TTMAPP complex on the flow system under the catalysis of cysteine injected. The signal height increased in both cases by using longer coils and with a 5-m coil, cysteine of $0.2 \mu\text{g ml}^{-1}$ showed response of 0.22 abs by comparison with 0.13 abs for cysteine of $0.1 \mu\text{g ml}^{-1}$. The reaction rate of the complexation reaction, which can be estimated from the slopes of these curves, decreased in both cases and to a greater extent under the catalysis of cysteine of $0.2 \mu\text{g ml}^{-1}$. At a coil length of 3 m, the reaction rates become equal and a maximum

absorbance difference of 0.10 abs was observed between the two curves. Therefore, a 3-m coil was selected as the optimum for the analysis of cysteine below $0.2 \mu\text{g ml}^{-1}$.

The effect of Cu(II) concentration on the signals was studied over a range of 4.0×10^{-5} to $2.4 \times 10^{-4} \text{ M}$ (Fig. 4a) with TTMAPP being fixed at $2.4 \times 10^{-5} \text{ M}$. Either the maximum absorbance of each curve or the absorbance difference between them was observed at $1.6 \times 10^{-4} \text{ M}$ of Cu(II), above which absorbances of both signals decreased. Such catalytic poisoning of cysteine is possibly caused by its autooxidation due to the excessive presence of Cu(II). Using $1.6 \times 10^{-4} \text{ M}$ of Cu(II), the effect of TTMAPP concentration on the signals was studied over a range of 2.4×10^{-5} to $6.0 \times 10^{-5} \text{ M}$ (Fig. 4b). The signal of $0.1 \mu\text{g ml}^{-1}$ cysteine reached to a maximum around $4.8 \times 10^{-5} \text{ M}$ of TTMAPP, above which no further change was observed. For the signal of $0.2 \mu\text{g ml}^{-1}$ cysteine, the absorbance still showed an increasing tendency at $6.0 \times 10^{-5} \text{ M}$ of TTMAPP. The absorbance difference between two curves did not vary significantly over the TTMAPP concentration range of 3.6×10^{-5} to $6.0 \times 10^{-5} \text{ M}$ ($\Delta A \approx 0.02 \text{ abs}$), thus TTMAPP of $3.6 \times 10^{-5} \text{ M}$ was used as the optimum mainly from an economic consideration.

The effect of temperature on the signals was investigated in a range of 20 – 40°C and both of the signals show linear correlation against temperature by following the equations: $A = 0.023T + 0.01$ for $0.1 \mu\text{g ml}^{-1}$ cysteine and $A = 0.043T + 0.03$ for $0.2 \mu\text{g ml}^{-1}$ cysteine, respectively, where A is the observed absorbance of the cysteine signal and T represents temperature ($20^\circ\text{C} \leq T \leq 40^\circ\text{C}$). Compared with the kinetic method based on batchwise procedure, the sensitivity can be easily improved by employing higher temperature on such continuous flow system, because the reaction magnitude is much easier to be controlled on flow system through adjusting coil length than the reaction time control in the batchwise kinetic method. Temperature of this flow system could be further raised depending on the practical requirement.

Based on the investigations above, a non-linear calibration graph was generated for cysteine over a dynamic concentration range of 25 ng ml^{-1} to $1.0 \mu\text{g ml}^{-1}$ (Fig. 5). Cysteine with concentrations higher than $1.0 \mu\text{g ml}^{-1}$ were detected out with decreased absorbances due to the formation of stable

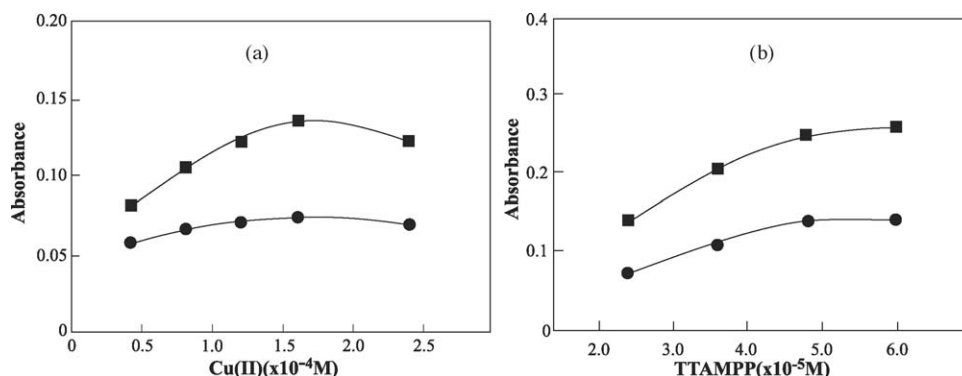


Fig. 4. Effect of indicator reagents concentration. [Cysteine] (○) $0.1 \mu\text{g ml}^{-1}$; (□) $0.2 \mu\text{g ml}^{-1}$; (a) [TTMAPP] $2.4 \times 10^{-5} \text{ M}$; [Cu(II)] 0.4 – $2.4 \times 10^{-4} \text{ M}$ and (b) [Cu(II)] $1.6 \times 10^{-4} \text{ M}$; [TTMAPP] 2.4 – $6.0 \times 10^{-5} \text{ M}$; temperature, 40°C ; detection wavelength, 411 nm.

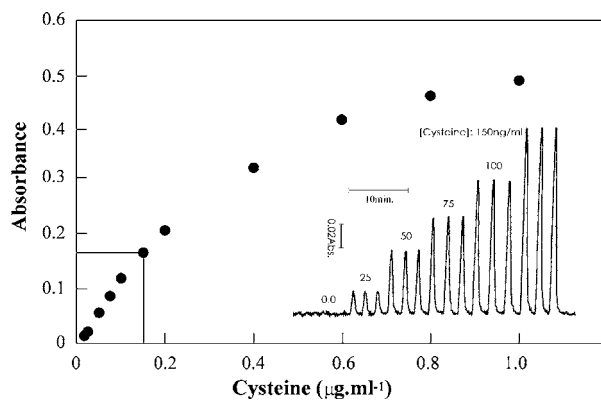


Fig. 5. Calibration graph and FIA signals. [Cu] 1.6×10^{-4} M; [TTMAPP] 3.6×10^{-5} M; temperature, 40 °C; detection wavelength, 411 nm.

(Cys)₂-Cu(II) complexes as has been observed in (Fig. 2). The practically obtained FIA signals of cysteine below 150 ng ml⁻¹ were shown in the inserted figure. Analytical precision was tested by injecting a 0.1 μg ml⁻¹ cysteine 10 times at a throughput of 30 h⁻¹, giving a relative standard deviation of 0.73%. Detection limits in this case was 15 ng ml⁻¹ by taking the signal/noise as 3, and it could be further improved when higher temperature or longer coil length was adopted.

3.3. Interference study and applications

Interferences of substances that are possible in coexistence with cysteine were investigated and results are summarized in Table 1. Organic species like sugar, organic acids and amino acids could be tolerated at high concentration levels except for ascorbic acid, which would interfere seriously due to its similar catalytic effect on the complexation reaction of Cu(II) with TTMAPP. Most metal ions would not bring any influences to this method because TTMAPP is highly specific for Cu(II) under the employed reaction conditions [25].

This kinetic method was compared with the spectrophotometric method of DTNB in the determinations of cysteine contents in three pharmaceutical injections and one permanent

Table 1

Tolerance limits^a of diverse substances on cysteine signal of 100 ng ml⁻¹

Substance	Molar ratio
Glycine	3000
Methionine	1000
Leucine	1000
Phenylalanine	500
Tryptophan	250
Alanine	200
Tyrosine	200
Valine	200
Fructose	500
Mannose	2000
Glucose	70
Folic acid	100
Zn(II)SO ₄	200
Mg(II)SO ₄	500
Pyrocatechol	50

^a Tolerance limits was taken as the concentration causing an error of 5%.

wave agent (Table 2). The pharmaceutical injections have similar constituents, among which sodium sulfite showed the same reactivity as cysteine in the DTNB method. Therefore, the values obtained by DTNB method were corrected by subtracting the absorbances of sodium sulfites of the maker claimed amounts (800 μg ml⁻¹ for brand 1 and 350 μg ml⁻¹ for brands 2 and 3) from the observed absorbances. In the case of the proposed kinetic method, these samples were diluted 2×10^4 -fold for calibration below 100 ng ml⁻¹. The interference of the sample matrices was investigated by adding *N*-ethylmaleimide (NEM), a thiol inhibitor [29] to complex cysteine in the samples, and no signals were detected out after the injection of the NEM-added sample. This result shows that this kinetic method is completely free of the matrix interferences.

The permanent wave agent used is a cysteine type, in which cysteine is required to be involved in a range of 3.0–7.5% (w) and another reducing ingredient of ammonium thioglycolate should be lower than 1% (w). Ammonium thioglycolate was tested and showed the same reactivity as cysteine with DTNB but in the proposed method, the sample was diluted 1×10^6 -fold for calibration and the interference of ammonium thioglycolate was not

Table 2

Determinations of cysteine contents in pharmaceutical injections and permanent wave agent

Samples	Constituents and amounts labelled		Brand no.	DTNB ^a		Proposed method	Difference (%)
				Observed	Corrected ^b		
Pharmaceutical injections (g ml ⁻¹)	Cysteine	770	1	1434	882	837	-5.1
	Sodium sulfite						
	Brand 1	800	2	962	724	755	4.3
	Brands 2 and 3	350					
	Ammonium glycyrrhizic acid	2650	3	926	688	699	1.6
Permanent wave agent (mg ml ⁻¹)	Glycin	20,000					
	Cetostearyl alcohol, sodium laureth sulfate, monoethanolamine, ammonium thioglycolate, cysteine	Amount unknown	1	29.8	—	28.5 ^c	-4.3

^a DTNB, spectrophotometric determination with 5,5'-dithiobis-2-nitrobenzoic acid; the values are the averages of three replicates.

^b Values obtained by subtracting absorbances of sodium sulfite of the labelled amounts.

^c The weight concentration is calculated as 3.5%.

observed at a weight ratio of 1:2 with cysteine. Therefore, the value obtained by this kinetic method is more reliable.

4. Conclusion

The complexation reaction of Cu(II) with an ultra sensitive color reagent of TTMAPP was incorporated into a flow injection system for a kinetic determination of cysteine over a dynamic concentration range of 15 ng ml⁻¹ to 1.0 µg ml⁻¹. The flow system offered stable reaction conditions and speedy solution handling, making the kinetic method highly precise and efficient at a low cost. Most of the common substances that may coexist with cysteine could be tolerated at high concentrations. The comparison with the DTNB method at the determinations of the real samples shows that this kinetic method is robust against the complex matrices of cysteine thus has good applicability.

References

- [1] J. Zen, A.S. Kumar, J. Chen, *Anal. Chem.* 73 (2001) 1169.
- [2] S. Shahrokhian, *Anal. Chem.* 73 (2001) 5972.
- [3] D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel, Rene Kizek, *J. Chromatogr. A* 1084 (2005) 134.
- [4] S.M. Al-Ghann, *Il Farmaco* 59 (2004) 331.
- [5] S. Liang, H. Wang, Z. Zhang, Y. Zhou, H. Zhang, *Fresenius J. Anal. Chem.* 370 (2001) 1117.
- [6] F. Tanaka, N. Mase, C.F. Brabas III, *Chem. Commun.* (2004) 1762.
- [7] S.C. Liang, H. Wang, Z.M. Zhang, X. Zhang, H.S. Zhang, *Spectrochim. Acta A* 58 (2002) 2605.
- [8] S. Pelletier, C.A. Lucy, *J. Chromatogr. A* 992 (2002) 221.
- [9] M.A. Eid, *Mikrochim. Acta* 129 (1998) 91.
- [10] V. Amarnath, K. Amarnath, *Talanta* 56 (2002) 745.
- [11] M.L. Lunar, S. Rubio, D. Perez-Bendito, M.L. Carreto, C.W. McLeod, *Anal. Chim. Acta* 337 (1997) 341.
- [12] B. Tang, H. Zhang, Y. Wang, *Anal. Chim. Acta* 511 (2004) 17.
- [13] B. Tang, H. Zhang, Y. Wang, *Anal. Chim. Acta* 525 (2004) 305.
- [14] G.A. Crespo, F.J. Andrade, F.A. Iñón, M.B. Tudino, *Anal. Chim. Acta* 539 (2005) 317.
- [15] Z. Gong, X. Zhang, G. Chen, X. Xiao, *Talanta* 66 (2005) 1012.
- [16] B. Tang, H. Zhang, Y. Wang, *Spectrochim. Acta A* 61 (2005) 2239.
- [17] R. Kadowaki, S. Nakano, T. Kawashima, *Talanta* 48 (1999) 103.
- [18] T. Shiobara, N. Teshima, M. Kurihara, S. Nakano, T. Kawashima, *Talanta* 49 (1999) 1089.
- [19] M. Biesaga, K. Pyrynska, M. Trojanowicz, *Talanta* 51 (2000) 209.
- [20] Y. Li, J. Hu, J. Yang, B. Zheng, Y. Ha, *Anal. Chim. Acta* 461 (2002) 181.
- [21] K. Kilian, K. Pyrynska, *Talanta* 60 (2003) 669.
- [22] S. Igarashi, N. Ide, Y. Takagai, *Anal. Chim. Acta* 424 (2000) 263.
- [23] M. Trojanowicz, M. Biesaga, J.O. Gawrys, *Anal. Sci.* 17 (2001) i587.
- [24] Q. Hu, G. Yang, J. Yin, Y. Yao, *Talanta* 57 (2002) 751.
- [25] T. Makino, J. Itoh, *Clin. Chim. Acta* 111 (1981) 1.
- [26] G.L. Ellman, *Arch. Biochem. Biophys.* 82 (1959) 70.
- [27] G.A. Bagiyani, I.K. Koroleva, N.V. Soroka, A.V. Ufimtsev, *Kinet. Catal.* 45 (2004) 372.
- [28] E.B. Fleischer, J.H. Wang, *J. Am. Chem. Soc.* 85 (1960) 3498.
- [29] J.D. Gregory, *J. Am. Chem. Soc.* 77 (1955) 3922.

Electronic tongue based on an array of metallic potentiometric sensors

Larisa Lvova^{a,*}, Eugenio Martinelli^b, Emiliano Mazzone^{b,d}, Andrea Pede^d,
Roberto Paolesse^{a,c}, Corrado Di Natale^{b,c}, Arnaldo D'Amico^{b,c}

^a Department of Chemical Science and Technology, University of Rome "Tor Vergata", Italy

^b Department of Electronic Engineering, University of Rome "Tor Vergata", Italy

^c CNR-IMM, Rome, Italy

^d Smartronix s.r.l., Rome, Italy

Received 10 August 2005; received in revised form 23 December 2005; accepted 7 February 2006

Available online 31 March 2006

Abstract

An electronic tongue system based on the array of six metallic potentiometric sensors (metallic wires) was developed and utilized for discrimination of foodstuffs: several types of vinegar and fruit juices. Copper, tin, iron, aluminum, brass and stainless steel wires were included in the array and supplemented by pH glass electrode. The response of potentiometric metallic sensors towards various organic acids has been studied and possible sensitivity mechanisms were discussed. Overall potential changes of metallic sensors were exanimate as complex mixed signals influenced by several components presenting in analyte employing chemometric approach. The multisensor array of such a type can be useful for several applications since of simplicity in handling, low cost of sensors and easy measure procedure.

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Keywords: Potentiometric metallic sensors; Electronic tongue; Acidic analytes identification

1. Introduction

In the last few years the need to control both the safety and the quality of environmental and food samples has becoming more and more urgent. Chemical sensors are an advantageous solution to these demands, since they can allow both on line and remote control of different environments. A major key component of chemical sensors is the sensing material and a great effort has been done for the rational design and preparation of receptors selective for target analytes, with criteria inspired by the "lock and key" principle. This approach is necessary when a single chemical compound should be detected, but in several applications it is not useful or possible to find an analyte able to characterize the overall sample. Inspired by the working mechanism of the mammalian "chemical" senses, olfaction and taste, an alternative approach has been proposed, where sensors should not be necessarily specific for a single analyte, but they can be broadly sensitive, with the only necessary requisite

to behave differently from each other, i.e. to furnish not correlated responses. These sensors should be integrated in an array, and supplemented by suitable pattern recognition procedure in charge to elaborate the response of the device. Sensor arrays for liquid phase analysis have been called "electronic tongues", to underline the attempt to mimic the natural sense. Several tongues based on electrochemical sensors have been devised [1–4], and among them potentiometric were the most widely used ones [5–7].

The sensing mechanism of the most of potentiometric sensors is based on a membrane, made on inorganic (polycrystalline and chalcogenide glass [8,9]) or organic (plasticized polymeric matrix doped with membrane active components [10,11], Langmuir–Blodgett [12] or electropolymerised films [13], etc.) materials. The membrane is the main active component but in the same time it is also one of the weak points of the sensor, because most of drawbacks, such as reproducibility and long-term stability, depend on the membrane. For this reason we have been interested in the development of a different approach, where the presence of the selective membrane is avoided. An attention has been turned to the exploitation of metallic wires (so-called first kind electrodes) as potentiometric sensors for multicomponent analysis. Metallic wires have been widely used recently for the

* Corresponding author. Present address: Biological Research Institute, St. Petersburg State University, St. Petersburg, Russia. Tel.: +7 812 427 70 61; fax: +7 812 427 73 10.

E-mail address: llvova@hotmail.com (L. Lvova).

voltammetric multicomponent analysis and an electronic tongue developed at Linköping University, consisted by an array of different metal electrodes and based on pulsed voltammetry has been used for analysis of liquid samples. The utility of such device has been shown for monitoring the quality of water in a production plant for drinking water [14], monitoring mold growth in liquids [15] and discrimination between the different rinses from household washing machine runs with four different prerequisites [16]. At the same time there are still few reports on the application of potentiometric metallic sensors for multivariate analysis in electronic tongues, namely metallic wires were used in multisensor array for discrimination of vinegars [17], both wires and conducting surfaces in thick-film technology were utilized as potentiometric electrodes for qualitative analysis of natural waters [18]. In this paper, we report the development of the electronic tongue based on the array of metallic sensors made of: copper, tin, iron, aluminum, brass (alloy of Cu and 20 wt% Zn) and stainless steel. The potentiometric response of metallic sensors towards several organic acids has been studied and possible sensitivity mechanisms has been discussed. The resulting device has been exploited for analysis of foodstuffs: vinegars, fruit juices and nectars, since a large amount of organic acids can be found in these samples.

2. Experimental

Citric, L-tartaric, L-malic, L-lactic, L-ascorbic and succinic acids were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Acetic acid was from Carlo Erba Reagents (Rodano, MI, Italy). All chemicals were analytical grade and were used without further purification. All solutions were prepared by using distilled water. Bundles of metallic wires were purchased from BDH Chemicals Ltd., Poole, England. Metallic rods of about 10 cm length were cut out, buffed, cleaned in ultrasonic bath, rinsed with methanol and dried in drying chamber. Pre-treated in this manner wires were used as potentiometric sensors. To prevent a contamination of working surface metallic sensors were mechanically cleaned with an emery-paper, rinsed with methyl alcohol and distilled water before each measurement. The Saturated Calomel Electrode (SCE) was the reference electrode, pH glass electrode was used to control the pH of analyzed solutions, and both sensors were from AMEL, Italy. Data were collected with a PC equipped high-impedance eight-channel analog-to-digital converter designed by Smartronix, Italy.

The potentiometric responses of sensors towards single component aqueous solutions of organic acids were examined in a well-stirred unbuffered 0.01 M KCl background (conductivity 752 ppm) by varying the concentration of acids stepwise from 10^{-6} to 10^{-2} M at every 100 s. A content of dissociated/non-dissociated form of acid in solution was calculated according to the first dissociation constant K_a and pH. Two replicas with all metallic sensors were performed, for every material three individual identical sensors were evaluated at one time, in total number of repetitions was $n = 6$.

Three different types of vinegar (white and red vinegars made of white and red wine correspondingly, balsamic vinegar) and nine fruit juices and nectars available in local stores in Rome,

Italy, were analyzed. Pure vinegars and those diluted 10 and 100 times with tap water were measured. The ionic strength of analytes is an important parameter which may significantly influence discrimination of pure and diluted samples. It is a common practice to use an inert background electrolyte of moderate concentration (normally KCl, 0.01 M, see above) to keep constant solution ionic strength while analyte concentration is varied. Since the aim of present study was to distinguish pure and diluted vinegar samples, simulating in such a way a possibility of fake products distribution on market (false or diluted products of bad quality), simply tap water was chosen for analyzed samples dilution. Tap water, used in present study was hard, which allowed assuming a possibility to keep the condition of ionic strength similarity for all the analyzed samples, pure and diluted ones. To ensure the conformity of samples, a conductivity of tap water (900 ppm), diluted (810–860 ppm) and pure vinegars (850–880 ppm) were controlled prior to potentiometric measurements by portable conductometer DIST WP 1 by HANNA Instruments, UK. pH of vinegar samples varied in a range 2.8–3.4 units; tap water: 6.8 units. Three replicas of each sample were run. Tap water served also as a reference sample. Between samples measurement the sensors were washed with distilled water, polished with an emery paper, rinsed with methyl alcohol and dried.

Fruit juices and nectars produced by four manufactures (A: “Santal” by Parmalat[®] [19], B: “Skipper” by Zuegg[®] [20], C: “Bravo” by Rauch[®] [21] and D: Yoga by Conserve Italia Soc. Coop. a r.l.[®], Italy [22]) were following: four pure 100% juices (Orange A and B, Grapefruit A and Pineapple A), two nectars (Orange C and Pineapple C), two peach juices made of puree (Peach A and D), 80% grapefruit juice (Grapefruit B). Analyzed juices composition (indicated by manufactures): Orange A and B, Grapefruit A and Pineapple B contained 100% pure juices and made of concentrated natural juices without sugar addition; Orange C and Pineapple C contained minimum 50% of pure juice, added water, sugar, citric acid, Ca and Mg citrates; Peach A and D were made of peach puree, added water, sugar, glucose syrup, citric and L-ascorbic acids; Grapefruit B contained minimum 80% of pure juice, water, concentrated rectified grape juice, L-ascorbic and citric acid. Two sets of measurement in fruit juices and nectars were performed. First set was run without any pretreatment immediately after opening the new package and the second one, of the already opened package, 24 h later. Between the measurements juice packages were stored in a refrigerator. Every set consisted of two synchronous measurements. The same like during vinegar analysis, the sensors were washed with distilled water, polished with an emery paper, rinsed with methyl alcohol and dried. Samples were measured in a random order. Conductivity of juices and nectars were controlled by portable conductometer and varied in a range 1500–1650 ppm. Data were treated with Principal Component Analysis (PCA), Unscrambler (v. 7.8, 2002, CAMO ASA) software was used.

3. Results and discussion

The equilibrium potential of metallic electrode depends on the concentration of a primary ion in solution according to the

well-known Nernst equation [23]. For metallic electrode operating in open circuit potentiometric mode anodic and cathodic partial exchange currents corresponding to the oxidation and reduction processes on surface, are equal with the opposite magnitudes and a net current on the electrode is, hence, equal to zero. In reality, in the absence of the primary ion, the apparent steady-state potential of metallic electrode immersed in analyzed solution is determined by several concurrent electrochemical processes. The overall electrode potential change can be explained by mixed potential phenomenon, discussed by several authors [24–25]. Mixed (or corrosion) potential is considered to occur when a non-equilibrium state exists at the electrode surface involving two or more electrochemical processes. Typically different oxidation and reduction reactions take place on the electrode simultaneously. Moreover, the material of metallic wire can undergo complexes formation, physisorption or chemisorption/desorption processes of a number of chemical species, coming from electrode reaction or complexation products, or other solution constituents. As it was pointed above, under open circuit conditions, the algebraic sum of the partial currents for each reaction at the electrode, must be equal to zero:

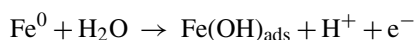
$$i_{\text{total}} = i_{\text{cathodic}} + i_{\text{anodic}} = \sum_{i=1}^n i_{\text{cathodic},i} + \sum_{j=1}^n i_{\text{anodic},j} = 0;$$

but the presence in analyte species that change the rate of cathodic or anodic reactions will change the apparent steady-state electrochemical potential E_m of the electrode; hence, one may suppose the possibility to detect such species with potentiometric metallic electrode. Thus, it has been shown by Xiao et al. [26], and then discussed by Meruva and Meyerhoff [27], that the presence of phosphate ion influence the potential of Co metallic rod potentiometric sensor, whereas the other anions (Cl^- , NO_3^- , AcO^- , etc.) do not have the same effect. While Xiao explained such response of Co metallic electrode by first oxidation of Co and formation of non-stoichiometric $\text{Co}_{1-\alpha}\text{O}$ film on metallic cobalt rod and then by an intercalation of dihydrophosphate particles H_2PO_4^- in cavities, formed by Co oxide; in ref. [27] it was proved, that the response of Co metallic electrode towards inorganic phosphate ions is due to the mixed corrosion potential aroused from the partial oxidation–reduction of the metal and then due to the formation of hardly soluble $\text{Co}_2(\text{PO}_4)_3$. It was shown, that the presence of phosphate enhances the corrosion of Co metallic potentiometric electrode shifting the potential of Co^0 oxidation to Co^{2+} to more negative potentials, which results in negative shift in the mixed potential value E_m of the system upon the addition of phosphate ions. While the presence of phosphate influences anodic electrode reaction, the reduction of oxygen influence the cathodic electrode reaction that contributes to steady-state open circuit mixed potential.

We first studied the potentiometric responses of metallic wires in single component aqueous solutions of acetic, citric, tartaric, malic, lactic, ascorbic and succinic acids. These organic acids are analytes of interest due to their wide-spread occurrence as, e.g. constituents in food and beverage samples and their determination is one of important task of food analysis.

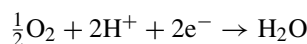
Acetic acid is a one of the main components of vinegar, ascorbic (Vitamin C), L-malic, L-lactic, L-tartaric, succinic and citric acid occur in a large amount in fruits and natural fruit juices, where their amount may serve as an index of juice quality, since it varies significantly due to the production and storage stages. In addition, citric and ascorbic acid are common acidifier and antioxidant additives of the commercial fruit juices. The standard methods for organic acids determination are ion-exchange or ion exclusive chromatography, HPLC and capillary electrophoretic (CE) method. These methods are well developed for routine applications, but there are also several disadvantages for most of them, like a high cost of equipment and time consuming sample pretreatment. In the last few years an application of electrochemical sensors, namely amperometric ones, for analysis of organic acids became a promising alternative due to the low cost and robustness. Thus, amperometric sensors on the base of porous $\text{SiO}_2/\text{SnO}_2$ matrix with immobilized Cu(II) were applied for electrochemical detection of Vitamin C in tablets [28]. Screen-printed ruthenium dioxide electrode was used for ascorbic acid analysis [29]. An ion-chromatographic method combined with electrochemical detection at a copper-based chemically modified glassy carbon electrode (Cu-GC) was shown to provide a simple analytical approach for the determination of some common organic acids in alkaline medium [30]. Amperometric biosensors are widely used for analysis of vitamins, namely ascorbic acid and amino acids [31], among them the glassy carbon [32] and gold microdisc [33] working electrodes without immobilized enzymes modified with various polymers. Along with several advantages, the main drawback of voltammetry and application of amperometric sensors is their generally more complex procedures than potentiometric measurements. Due to the simplicity of potentiometric technique, in the present study we focused on evaluation of performance of potentiometric metallic sensors in acidic analytes in order to apply an array of potentiometric metallic sensors for acidic samples discrimination.

The main source of potential change for non precious metallic sensors, so-called first kind electrodes, immersed in aqueous ionic solution is an electrochemical corrosion due to a slow anodic oxidation of metal and cathodic reduction of other particles. Due to the defects in metallic lattice caused by strain, presence of impurities or surface mechanical treatment the areas with a different potential arise on electrode and a local electrochemical cell is produced. In the presence of electrolyte this cell becomes active and the current flows through metallic electrode. For instance, on iron electrode in aqueous solutions, the oxidation of Fe happens in anodic area. Iron oxidation is a complex cascade of several step processes, thus, on the first step:

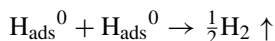
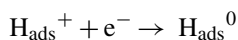


$\text{Fe}(\text{OH})_{\text{ads}}$ covers electrode surface and may slow down the corrosion process evolution in a first moment, but then oxidation to Fe^{2+} occurs. In practice in solution the Fe(II) is present as the complex ion $[\text{Fe}(\text{OH})_6]^{2+}$. If oxygen is present, some of the Fe(II) may oxidizes to Fe(III).

At the same time oxygen reduces in metallic electrode cathodic area:



Other important reducing agent participating in metal corrosion is H^+ . In acid solutions hydrogen ions, adsorbed on the metal surface from the solution reduce producing hydrogen gas:



In case if other species, able to reduce, are present in solution, the dominating electrode reaction will be influenced with those demanding the lowest electrode overvoltage for their formation. Therefore, a direct potentiometry of primary ion with first kind metallic electrodes in multicomponent solutions may face several difficulties due to the huge amount of possible simultaneous processes on the electrode surface. Such processes can be hardly separated and evolved. In the present work, the response of metallic sensor was examined as a complex mixed signal, influenced by several components presenting in analyte. An attempt to found correlations between overall potential change of metallic sensors of first kind and presence of several components in solution in order to identify analyte has been performed employing chemometric approach.

It has been supposed, that the depending on the acid and metal nature the overall apparent steady-state potential E_m of metallic potentiometric sensors will vary in different manner. The potentiometric responses of Fe, Sn, Cu and brass electrodes in aqueous solutions of various organic acids in background of 0.01 M KCl are shown in Fig. 1. Amount of acids' dissociated form in solution were calculated according to the first dissociation constant K_1 and pH. R.S.D. for all sensors potential values were calculated for six repetitions and is showed in Fig. 1 as error bars. As can be seen from Fig. 1, iron undergoes to corrosive oxidation by all the evaluated acids at high concentration but the process has different intensity depending on acid nature. The Fe electrode exhibits a linear response to the logarithm of dissociated acids concentration over the range of 10^{-5} to 5×10^{-3} M with slopes ranging from -60 to -240 mV/decade. Sn electrode exposed to more weak lactic, succinic, ascorbic and acetic acids showed cationic response with a slope in a range from $+6$ to $+22$ mV (Fig. 1, Sn (a)), while the increase of tartaric, citric, malic and acetylsalicylic acids concentration shifted the value of Sn electrode to more negative values (slope in a range -40 to -180 mV/decade) (Fig. 1, Sn (b)). The calibration plots of Cu and brass wires had two different regions. In tartaric, succinic and acetic acids electrodes' potential first slowly increased with an acid concentration growth (we attribute this to a surface polarization due to acid chemisorption, slope varied from $+12$ to $+34$ mV/decade), then at high acid content electrodes showed an anionic response in a linear range -5×10^{-4} to 10^{-3} M with slopes ranging from -14 to -130 mV/decade. According to the information on solubility constants of metal ion complexes [34], tartaric, succinic and acetic acids may form several stable complexation products with copper ions. Adsorbing on electrode surface these products may cause overall electrode potential

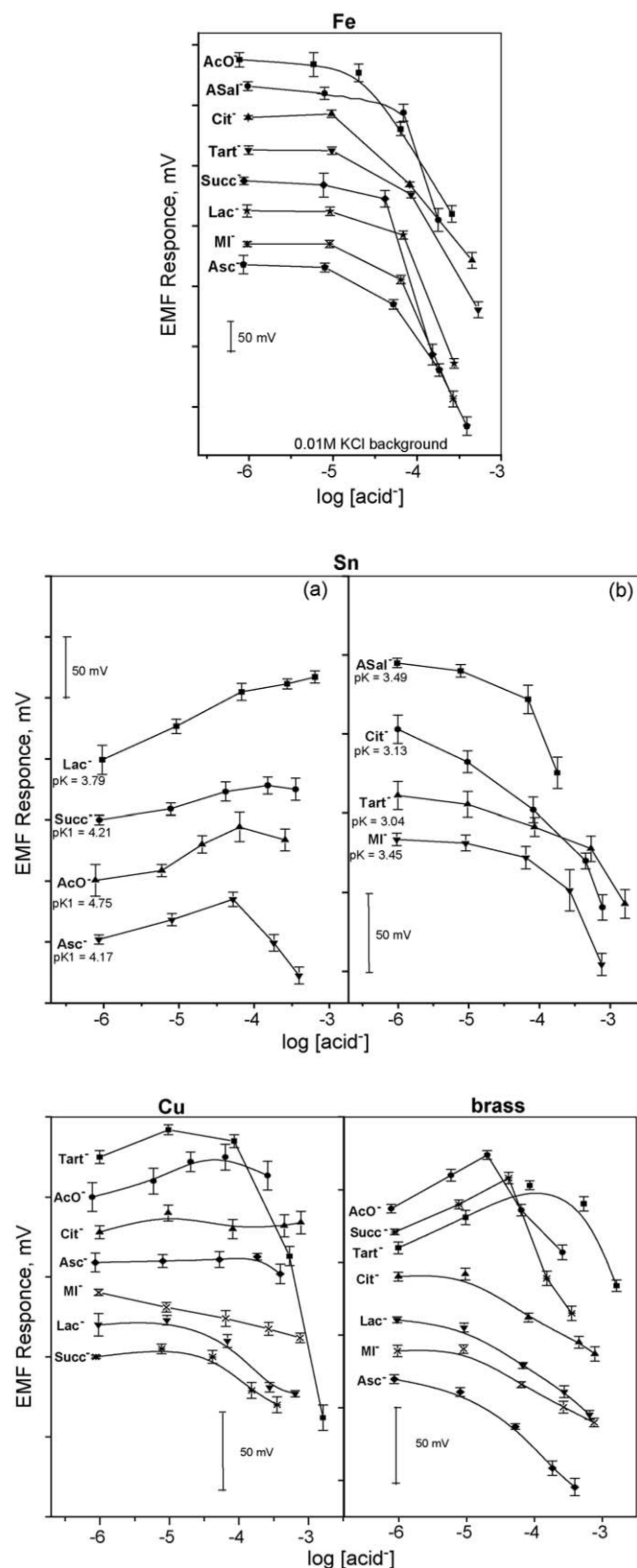
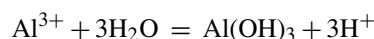
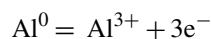


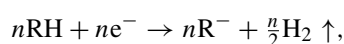
Fig. 1. Potentiometric response of the Fe, Sn (a and b), Cu and brass electrodes to several organic acids aqueous solutions in 0.01 M KCl background, on graphs—Lac⁻: lactate, AcO⁻: acetate, Tart⁻: tartrate, MI⁻: malate, Asc⁻: ascorbate, Succ⁻: succinate, Cit⁻: citrate, ASal⁻: acetyl-salicylate.

decrease. In general, as it can be seen from Fig. 1, brass being an alloy of Cu and 20 wt% of Zn undergoes to more significant corrosive destruction by organic acids, then pure copper electrode. This is an expected result, since an additive of more electronegative Zn decreases the standard potential of brass sensor and its stability in acidic media. Stainless steel in all the acids exhibited a linear response to the logarithm of dissociated acids concentration over the range of 10^{-6} to 10^{-3} M with slopes ranging from +23 to +60 mV/decade, which may be attributed to a partial pH response.

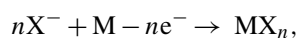
In aqueous solutions of organic acids in 0.01 M KCl background Al wire showed cationic response with a slope in a range from +10 to +60 mV/decade depending on the acid, except acetyl-salicylic acid, where hardly soluble Al(ASal)^+ ($K_{\text{sp}} = 8 \times 10^{-15}$) [34] end-product apparently formed on the surface and shifted the Al electrode E_m potential to the more negative values (data are not shown). The presence in solution of aggressive anions Cl^- activates the pitting corrosion process of such stable material like Al due to the high affinity of Cl^- to adsorb on metallic surface and high solubility of metal chlorides. Pits are initiated at weak sites in the aluminium oxide by chloride attack and propagate according to the reactions:



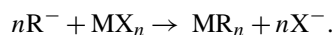
As a pit propagates, the environment inside it (anodic area) changes. Since pH in a pit will decrease, to balance the positive charge produced by Al dilution chloride ions will migrate into the pit. The resulting HCl formation inside the pit causes accelerated pit propagation. More generally, the influence of presented in analyzed sample halide ions X^- on potentiometric response of first type metallic electrodes may be explained by a complex formation with a material of metallic anode [35a]. While a weak acid is reduced at the cathodic area:



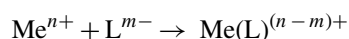
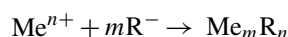
a metal halide is formed on anode:



and a final product forms according to the reaction:

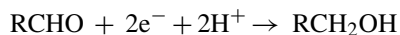


As it was previously mentioned secondary chemical interactions often accompany a metal corrosion process. Insoluble oxide films may form on metal surface passivating corrosion process evolution. The presence of organic acid anions in solution may cause a formation of hardly soluble end products—metal salts or complexes on electrode surface:



These coupling reactions may shift the equilibrium of the net electrochemical reactions, alternating the steady-state mixed potential of the electrode [27].

Moreover, at highly negative metallic electrode potential (Al or Sn) in the acid medium a reduction of organic acid through aldehyde to alcohol may be supposed [35b]:



Indeed, the reduction of ascorbic acid on Al cathode in three electrode cell with Pt auxiliary and SCE reference under applied potential -0.8 V (potential of Al wire during potentiometric measurements) takes place (data are not shown). A study to elucidate the exact response mechanisms of metallic electrodes towards organic acids is now in progress, while only some preliminary assumptions on possible processes were discussed here.

As stated above, food samples like vinegars and fruit juices have a high content of several organic acids. Depending on a kind of the product, its quality, manufacture and storage details, the amount of various components in product may vary significantly. Since it has been shown that overall change of apparent steady-state potential of almost all metallic potentiometric sensors is influenced by several organic acids in their singular aqueous solutions, it has been assumed that electronic tongue system based on array of such sensors may be applied for identification of acidic foodstuffs. Hence, on the second stage of the present study, a discrimination of three types of vinegars, fruit juices and nectars have been performed by means of metallic electronic tongue.

Vinegars production is big branch of food industry, related also with a wine industry (wine utilization for vinegar acidification). Analysis of complex samples such as vinegars is important due to the effort to achieve an adequate quality of production, to ensure uniformity within a brand, to avoid falsifications. The main problem while vinegar analysis is a high acidity of the analyte. A strong dilution of analyzed vinegars is required prior the analysis to protect gaging equipment. Unfortunately, such a strong dilution decrease in the same time an amount of minor components which may be specifically responsible for product brand. Hence, a robust, simple to handle analytical system, which does not require sample pretreatment may be useful for vinegars identification and quality control. The result of vinegars discrimination is given in Fig. 2. A PCA score plot represents 100% of total system variance and the clear separation is observed between all the measured samples. According to the first two principal components (PC1, 86%; PC2, 13%), two main areas can be indicated on the plot, one of them includes all the pure vinegar samples, while the other embraces tap water and diluted vinegar samples. Simultaneously with qualitative discrimination, metallic electronic tongue system was able to observe the direction of quantitative dilution of vinegars, Fig. 3. The highest loadings (contribution of sensor into the discrimination process) for vinegar analysis had Fe, Al and Cu wires due to their easy oxidation/reduction in acidic media (see results of metallic electrodes potentiometric response evaluation in singular organic acids solutions).

A preliminary PCA discrimination result (data are not shown) of nine fruit juices and nectars indicated an ability of electronic tongue based on metallic potentiometric sensors to distinguish

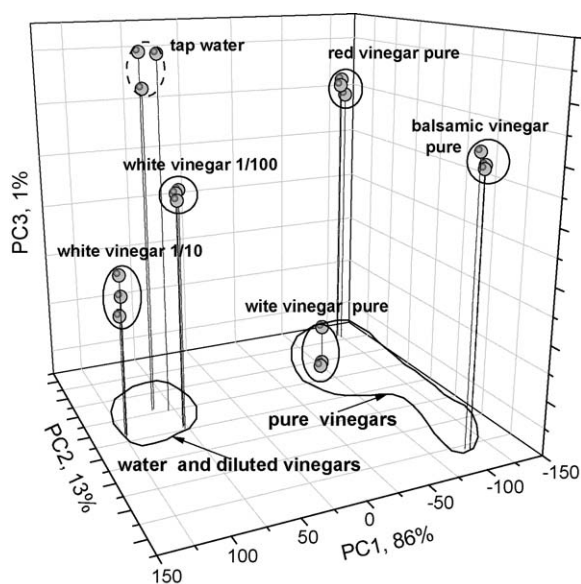


Fig. 2. PCA classification of diluted and pure vinegars.

the groups corresponding to the juices of different tests: peach, orange, pineapple, while grapefruit juices A and B did form well defined cluster. The juices, produced by the same manufacture, namely A (Santal) and B (Skipper) could be unified in two separate groups according to the common features due to the similar production procedure. The main contribution to the fruit juices discrimination gave Al, Fe, brass, Sn and stainless steel wires.

Based on preliminary PCA result, an attempt to classify juices produced by two different manufactures has been performed by means of Soft Independent Modeling of Class Analogy (SIMCA). SIMCA method is based on making a PCA model for each class in the training set. Unknown samples are then compared to the defined class models and assigned to classes according to their analogy to the training samples. Samples were judged according to the membership to the model A (Santal) and model B (Skipper). Fig. 4A illustrates the result of juices classification on Coomans' plot where the orthogonal distances from

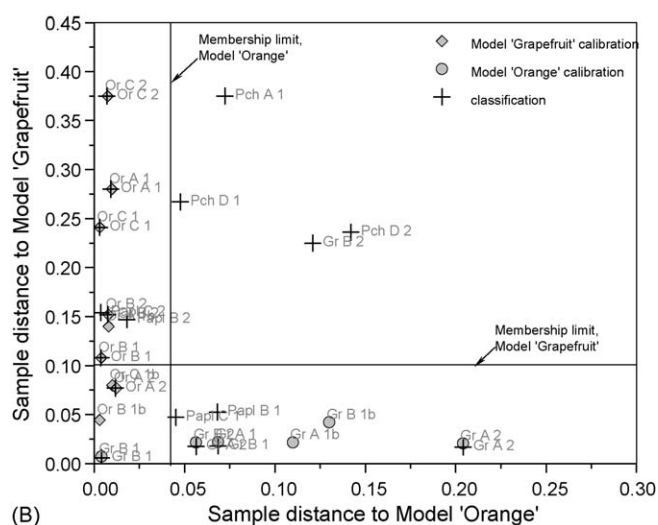
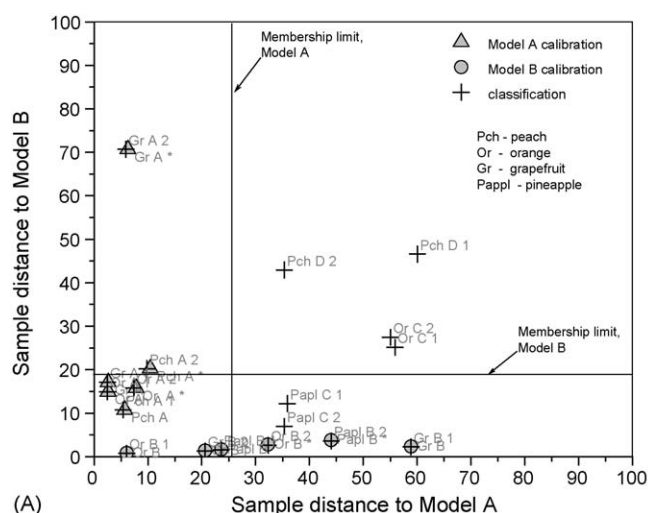


Fig. 4. SIMCA classification (Coomans' plot) of fruit juices and nectars according to (A) production procedure features and (B) membership of 'Orange' and 'Grapefruit' juices. On the graph: indexes 1 and 2 in juices legends correspond to the first and second measurement sets, while index (*) indicates data, used to build two models. Averaged data of two sets of measurements (freshly opened packages and those stored in refrigerator for 24 h) were concurrently used.

the analyzed juice samples to the models A and B are shown. Membership limits mirror the significance level (25%) used in the classification. If samples fall within the membership limits, they belong to the class of that membership. It has been found, that almost all the juices produced both by manufactures A and B could be properly classified according to the production features. Except an outlying Pineapple C nectar, which was falsely classified as belonging to the model B, all the other samples, produced by manufactures C and D are laying out of the both models borderlines and clearly can be indicated as not A or B trade mark products. Fig. 4B illustrated the result of SIMCA classification of fruit juices and nectars according to the membership of two models, based on potentials of metallic sensors in orange (model 'Orange') and grapefruit (model 'Grapefruit') juices. A part of analyzed orange (9 samples) and grapefruit (6 samples) juices were used for models calibration and following cross-validation, while the rested 21 samples were classified. Twenty-five percent

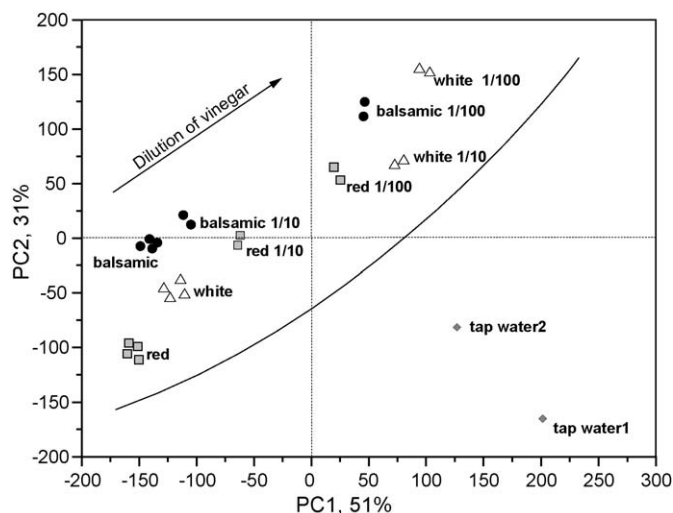


Fig. 3. An observation of vinegars dilution using metallic electronic tongue.

significance level has been used in the classification. As it can be seen, all analyzed orange and grapefruit juices lies within the membership limits. Less acidic peach juices are laying out of the both models borderlines, while pineapple juices were classified as very similar to citrus juices. During two days of measurements juices oxidation process took place in opened packages, even if they were stored in refrigerator. The biggest changes and hence, juice oxidation took place for all the analyzed orange and grapefruit juices. Nevertheless, these changes were averaged of 0.4 units of pH for 24 h and did not influence much the sensors reproducibility and juices discrimination with potentiometric metallic electronic tongue system (see Fig. 4).

4. Conclusion

Potentiometric metallic sensors of so-called first kind (metallic wires) have been applied in electronic tongue system array for analysis acidic liquid foodstuffs. Developed system successfully discriminated various types of vinegar and fruit juices. Simultaneously, electronic tongue was able to observe quantitatively the dilution of vinegar samples and evolve common features of fruit juices according to the manufacture procedure. Since a large amount of organic acids presenting in fruit juices and vinegars, an exploration of metallic sensors response towards several organic acids has been studied and possible sensitivity mechanisms have been discussed. An application of metallic potentiometric sensors for multicomponent analysis of complex analytes has several benefits over common membrane-based sensors due to their robustness, low cost, simplicity in handling and easy measure procedure. Metallic potentiometric sensors-based electronic tongue shows a good promise as an analytical instrument for fast discriminative analysis of acidic analytes.

Acknowledgments

This work has been supported by GOOD FOOD Integrated project by EU. Authors would like to acknowledge the students of electronic engineering department of Tor Vergata University, Rome, Italy, namely Giorgio De Angelis, Cristina Montieri, Tatiyna Primadei and Stefano Serafini for a contribution to this work.

References

- [1] K. Toko, *Sens. Actuators B* 64 (2000) 205–215.
- [2] K.J. Albert, N.S. Lewis, C.L. Schauer, G.A. Sotzing, S.E. Stitzel, T.P. Vaid, D.R. Walt, *Chem. Rev.* 100 (2000) 2595–2626.
- [3] F. Winquist, P. Wide, I. Lundström, *Anal. Chim. Acta* 375 (1997) 21–31.
- [4] A. Legin, A. Rudnitskaya, Yu. Vlasov, in: S. Alegret (Ed.), *Integrated Analytical Systems, Comprehensive Analytical Chemistry XXXIX*, Elsevier, 2003, pp. 437–486.
- [5] L. Lvova, S.S. Kim, A. Legin, Yu. Vlasov, J.S. Yang, G.S. Cha, H. Nam, *Anal. Chim. Acta* 468 (2002) 303–314.
- [6] R. Paolesse, C. DiNatale, M. Burgio, E. Martinelli, E. Mazzone, G. Palleschi, A. D'Amico, *Sens. Actuators B* 95 (2003) 400–405.
- [7] A. Legin, A. Rudnitskaya, L. Lvova, Yu. Vlasov, C. Di Natale, A. D'Amico, *Anal. Chim. Acta* 484 (2003) 33–44.
- [8] C. Di Natale, F. Davide, J.A.J. Brunink, A. D'Amico, Yu.G. Vlasov, A.V. Legin, A.M. Rudnitskaya, *Sens. Actuators B* 34 (1996) 539–542.
- [9] Yu.G. Mourzina, J. Schubert, W. Zander, A. Legin, Yu.G. Vlasov, H. Lüth, M.J. Schöning, *Electrochim. Acta* 47 (2001) 251–258.
- [10] E. Bakker, P. Bühlmann, E. Pretsch, *Chem. Rev.* 97 (1997) 3083–3132.
- [11] B. Adhikari, S. Majumdar, *Prog. Polym. Sci.* 29 (2004) 699–766.
- [12] A. Arrieta, M.L. Rodriguez-Mendez, J.A. De Saja, *Sens. Actuators B* 95 (2003) 357–365.
- [13] G.G. Wallace, M. Smyth, H. Zhao, *Trends Anal. Chem.* 18 (1999) 245–251.
- [14] C. Krantz-Rülcker, M. Stenberg, F. Winquist, I. Lundström, *Anal. Chim. Acta* 426 (2001) 217–226.
- [15] C. Söderström, H. Borén, F. Winquist, C. Krantz-Rülcker, *Int. J. Food Microbiol.* 83 (2003) 253–261.
- [16] P. Ivarsson, M. Johansson, N.E. Höjer, C. Krantz-Rülcker, F. Winquist, I. Lundström, *Sens. Actuators B* 108 (2005) 851–857.
- [17] L. Lvova, G. DeAngelis, C. Montieri, T. Primadei, E. Martinelli, E. Mazzone, A. Pede, R. Paolesse, C. DiNatale, A. D'Amico, *Proceedings of the 3rd IEEE Sensors Conference*, Vienna, Austria, October 23–27, 2004, p. 71.
- [18] R. Martínez-Máñez, J. Soto, E. García-Breijo, L. Gil, J. Ibáñez, E. Llobet, *Sens. Actuators B* 104 (2005) 302–307.
- [19] <http://www.parmalat.com/>.
- [20] <http://www.zuegg.it/>.
- [21] <http://www.rauch.cc/>.
- [22] <http://www.succhiyoga.it/>.
- [23] D. Harvey, *Modern Analytical Chemistry*, first ed., The McGraw-Hill Press, 2000, p. 468.
- [24] J. Koryta, J. Dvorak, V. Bochakova, *Electrochemistry*, Methuen and Co., London, 1970, p. 318.
- [25] P.B. Hahn, D.C. Johnson, M.A. Wechter, A.F. Voigt, *Anal. Chem.* 46 (1974) 553–558.
- [26] D. Xiao, H.-Y. Yuan, J. Li, R.-Q. Yu, *Anal. Chem.* 67 (1995) 288–291.
- [27] R.K. Meruva, M.E. Meyerhoff, *Anal. Chem.* 68 (1996) 2022–2026.
- [28] L.A. Kurihara, S.T. Fujiwara, R.V.S. Alfaya, Y. Gushikem, A.A.S. Alfaya, S.C. de Castro, *J. Colloid Interface Sci.* 274 (2004) 579–586.
- [29] J. Wu, J. Suls, W. Sansen, *Electrochem. Commun.* 2 (2000) 90–93.
- [30] I.G. Casella, M. Gatta, *J. Chromatogr. A* 912 (2001) 223–233.
- [31] M. Sanz Alaejos, F.J. Garcia Montelongo, *Chem. Rev.* 104 (2004) 3239–3265.
- [32] J.-J. Sun, D.-M. Zhou, H.-Q. Fang, H.-Y. Chen, *Talanta* 45 (1998) 851–866.
- [33] A.-M. Yu, D.-M. Sun, H.-Y. Gu, H.-Y. Chen, *Anal. Lett.* 29 (1996) 2633–2640.
- [34] J. Bjerrum, G. Schwarzenbach, L.G. Sillen, *Stability Constants of Metal-Ion Complexes*, first ed., The Chemical Society, London, 1957–1958.
- [35] (a) M. Baizer, in: M.M. Baizer, H. Lund (Eds.), *Organic Electrochemistry. An Introduction and a Guide*, vol. 1, Marcel Dekker Inc., New York/Basel, 1985 (Chapter 20);
(b) L. Eberson, J. Atly, in: M.M. Baizer, H. Lund (Eds.), *Organic Electrochemistry. An Introduction and a Guide*, vol. 1, Marcel Dekker Inc., New York/Basel, 1985 (Chapter 11).

Short communication

Synthesis of an immobilized *Bombyx mori* pheromone-binding protein liquid chromatography stationary phase[☆]Armenak Margaryan^{a,b}, Ruin Moaddel^a, Jeffrey R. Aldrich^b, Jennifer M. Tsuruda^c,
Angela M. Chen^c, Walter S. Leal^c, Irving W. Wainer^{a,*}^a Gerontology Research Center, National Institutes in Aging, National Institutes of Health, Baltimore, MD 21224-6825, USA^b USDA-ARS Chemicals Affecting Insect Behavior Laboratory Beltsville, MD 20705, USA^c Department of Entomology, University of California, Davis, CA, USA

Received 20 December 2005; received in revised form 30 January 2006; accepted 30 January 2006

Available online 3 March 2006

Abstract

The pheromone-binding protein from the silkworm moth, *Bombyx mori* (BmorPBP) has been covalently bonded to a liquid chromatographic stationary phase. The resulting column was evaluated using radiolabeled bombykol and the immobilized protein retained its ability to bind this ligand. The data also demonstrate that the BmorPBP column was able to distinguish between four compounds, and rank them in their relative order of affinity for the protein from highest to lowest: bombykol > bombykal > 1-hexadecanol > (Z,E)-5,7-dodecadien-1-ol, and that the immobilized BmorPBP retained its pH-dependent conformational mobility.

The results of this study demonstrate that pheromone-binding protein from the silkworm moth, *Bombyx mori* and an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatus* have been immobilized on a silica support with retention of ligand-binding activity. The data indicate that proteins from non-mammalian organisms can be used to create liquid chromatography affinity columns.

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Keywords: Affinity chromatography; Screening; Conformational mobility; Odorant binding protein

1. Introduction

Life on the insect scale is a chemical dominion; therefore, insects rely on pheromones to communicate, and their senses of smell and taste dominate the search for both plant and animal hosts. Pheromone and odorant binding proteins (PBPs and OBPs) are a family of small soluble proteins (15 kDa) containing six α -helices enclosing a hydrophobic ligand-binding pocket [1]. These proteins appear to be the major link between the external universe of chemical signals to which insects are sensitive, and the odorant receptors (ORs) embedded in antennal neurons [2,3].

The identification and characterization of OBPs has led to speculation that these proteins may be fruitful research targets in the design of novel insect control tactics, particularly against

disease-transmitting mosquitoes [1]. One approach to the development of a screen for potential attractants, pheromones, and repellents is the development of liquid chromatographic stationary phases containing the immobilized OBP or PBP target. The resulting chromatography columns can then be used in online screens. This approach has been suggested by the use of immobilized protein-based liquid chromatography columns in protein-binding studies, which have been prepared from mammalian serum albumins and α_1 -acid glycoprotein (for a recent review see Ref. [4]). Drug receptors and transporters have also been immobilized and used in affinity screens [5]. However, to our knowledge, insect-derived binding proteins have not been immobilized and used in affinity chromatography studies.

This manuscript reports the initial synthesis and characterization of a liquid chromatography stationary phase containing an immobilized PBP obtained from the silkworm moth, *Bombyx mori*, the BmorPBP. A second insect-derived protein, an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatus*, has also been immobilized with retention of binding activity.

[☆] Mention of commercial products does not constitute an endorsement by USDA.^{*} Corresponding author. Tel.: +1 410 558 8498; fax: +1 410 558 8498.
E-mail address: Wainerir@grc.nia.nih.gov (I.W. Wainer).

2. Experimental

2.1. Reagents

2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride), 3-aminopropyltriethoxysilane (APTS), sodium phosphate monohydrate, potassium chloride and glycine ethyl ester were obtained from Aldrich Chemical Company (Milwaukee, WI). Dibasic sodium phosphate (Anhydrous Sigma Ultra) was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Expression of pheromone and odorant binding proteins

The BmorPBP was produced by recombinant techniques following a previously described method [6] and the odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatus* was also obtained following previously described procedures [7].

2.3. Immobilization of BmPBP

The immobilization was accomplished following a previously described procedure used with bovine serum albumin [8] in which 50 mg of silica (5 μ m, 300 Å) from Chrom Expert (Sacramento, CA, USA) was activated with cyanuric chloride, packed in a HR-5/2 glass column to yield a 3 mm $\times$ 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA, USA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μ l/min for 1 h, followed by 1.6 mg of BmorPBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 8 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, in order to cap any unreacted groups. A second batch of activated silica was only capped with glycine ethyl ester and used to create a control column.

2.3.1. Immobilization of OBP

The immobilization was carried out following the protocol for the BmPBP. The cyanuric acid activated silica was packed in a HR-5/2 glass column to yield a 3 mm $\times$ 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μ l/min for 1 h, followed by 1.5 mg of OBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 4 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, to cap any unreacted groups and sequentially washed with 5 ml of each of the following solutions: phosphate buffer (50 mM, pH 7.0); phosphate buffer (25 mM, pH 7.0) containing 25 mM NaCl; deionized water; phosphate buffer (50 mM, pH 7.0). The column was then stored at 4 °C until further use.

2.4. Protein assay

A Micro BCA Protein Assay Kit[®] from Pierce (Rockford, IL, USA) was used to determine that 1.63 nmol of BmPBP protein

had been immobilized per mg of silica and 1.21 nmol of OBP protein had been immobilized per mg of silica.

2.5. Frontal chromatographic studies

The BmorPBP column (or the control column) was placed in a chromatographic system consisting of a LC-10AD isocratic HPLC pump purchased from Shimadzu (Columbia, MD, USA) and an IN/US system β -ram Model 3 on-line scintillation detector from IN/US (Tampa, FL, USA) with a dwell time of 2 s and running Laura Lite 3 software. The mobile phase consisted of phosphate buffer (50 mM, pH 7.0) delivered at 0.2 ml/min at room temperature. Between every injection the column was washed for 18 h with phosphate buffer (50 mM, pH 4.5) containing 50 mM potassium chloride and 7% methanol, followed by an 18 h wash with phosphate buffer (50 mM, pH 7.0).

The compounds used in this study were (*Z,E*)-10,12-hexadecadien-1-ol (bombykol), 1-hexadecanol and (*Z,E*)-5,7-dodecadien-1-ol which were provided by Dr. Ashot Khimian (USDA-ARS CAIBL, Beltsville, MD, USA), (*Z,E*)-10,12-hexadecadien-1-al (bombykal) which was synthesized from bombykol as previously described [9] and [³H]-bombykol which was obtained from the reduction of bombykal by tritium containing reagents at Amersham Life Sciences (Piscataway, NJ, USA). The binding affinity of [³H]-bombykol was determined with a series of concentrations: 80, 100, 120, 150, 200, 250, and 300 pM.

2.6. Zonal chromatographic studies

The studies were carried out with CquiOBP column using the chromatographic system Series 1100 liquid chromatography/mass selective detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G 1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20 μ l injection loop, a mass selective detector, MSD (G1946 B) supplied with atmospheric pressure ionization electrospray (API-ES) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 250 Mhz Kayak XA computer (Hewlett–Packard, Palo Alto, CA, USA) running ChemStation software (Rev B.10.00, Hewlett–Packard). The mobile phase was deionized water for chiral separation of D,L-lactic acid and ammonium acetate buffer (50 mM, pH 5.0–8.0) for pH dependence and ammonium bicarbonate 10 mM for displacement studies, respectively. The flow rate was 0.2 ml/min, and the experiments were carried out at 37 °C.

3. Results and discussion

In the current study, BmorPBP was initially recycled through the activated column for 4 h and 1.07 nmol of BmorPBP was immobilized per mg of silica support (53% of starting material) which was similar to what was obtained with BSA in which 1.4 nmol of BSA were immobilized in 2 h [8]. In this study, when the recycling time was increased to 8 h the amount of protein immobilized increased to 1.63 nmol per mg of silica

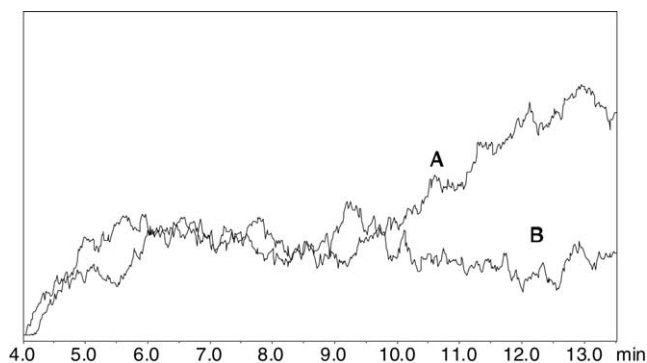


Fig. 1. Frontal chromatogram of a 10 ml sample of 80 pM [^3H]-bombykol in phosphate buffer (50 mM, pH 7.0) at 0.2 ml/min at room temperature on the β -RAM radioflow on the BmorPBP column (A) and control column (B).

support (81% of starting material); additional reaction time did not improve the results. The results indicate that the kinetics of the immobilization reaction was slower with BmorPBP relative to BSA and that protein immobilizations must be individually optimized.

Frontal affinity chromatography techniques, which have been recently reviewed [5], were used to study the binding of the marker ligand, [^3H]-bombykol, and test ligands to the immobilized protein.

The chromatographic trace produced by the application of 80 pM [^3H]-bombykol on the BmorPBP column contained both front and plateau regions (Fig. 1A). The midpoint of the breakthrough curve occurred at an 11.5 min. When the same sample was chromatographed on the control column, the resulting trace did not contain front and plateau regions, and the retention time, calculated at half-height to detector saturation, was 2.8 min (Fig. 1B). The results indicate that non-specific retention due to the chromatographic backbone is not a significant factor in the retention of [^3H] bombykol on the BmorPBP column and that the data obtained with this column reflects binding interactions between bombykol and the immobilized BmorPBP.

The sequential application of increasing concentrations of [^3H]-bombykol produced a series of frontal curves that were used to calculate the binding affinity of [^3H]-bombykol for the immobilized BmorPBP, expressed as the dissociation constant (K_d), as well as the number of active binding sites [P] on the immobilized protein. The data was processed using a one-site non-linear regression model [5] and the calculated K_d value was 80 ± 22 pM, $r^2 = 0.925$ with [P] = 271 ± 29 fmol. No displacement of [^3H]-bombykol was observed on the control column.

Competitive displacement studies with 200 pM [^3H]-bombykol as marker ligand were carried out to determine the selectivity of the immobilized BmorPBP. The retention time of the marker alone was 9.2 min. The addition of 1 μM bombykol to the mobile phase displaced the retention of the marker by 5.9 to 3.3 min, the dead volume of the column. Using the effect of bombykol as the relative standard, 1 μM concentrations of three test compounds were added to the mobile phase, bombykal, 1-hexadecanol, and (Z,E)-5,7-dodecadien-1-ol. The greatest effect was produced by bombykal, which reduced the retention of [^3H]-bombykol by 3.2 to 6.0 min, about 50% of

the effect produced by 1 μM bombykol. This is consistent with the observations by Pophof, where bombykal was shown to elicit some binding to the BmorPBP [10]. The addition of 1-hexadecanol, a saturated bombykol, produced a displacement of [^3H]-bombykol, albeit by only 1 to 8.2 min, indicating that 1-hexadecanol had a lower affinity for BmorPBP relative to bombykal. The addition of (Z,E)-5,7-dodecadien-1-ol to the mobile phase resulted in no change in the retention of [^3H]-bombykol indicating that this compound had no affinity for the BmorPBP. This is consistent with the fact that (Z,E)-5,7-dodecadien-1-ol is the main component of the Siberian moth pheromone [11]. These results demonstrate that the BmorPBP column was able to distinguish between four compounds, and rank them in their relative order of affinity for the protein from highest to lowest: bombykol > bombykal > 1-hexadecanol > (Z,E)-5,7-dodecadien-1-ol, which was the expected order based upon structure and previous binding studies (unpublished data).

The data from this study demonstrate that the BmorPBP has been successfully immobilized onto a silica-based stationary phase through a covalent linkage. The immobilized protein retained its ability to bind a known ligand, bombykol, and to differentiate between compounds with some or no affinity for the BmorPBP. To our knowledge this is the first report of the development of a liquid chromatography affinity support containing an immobilized protein obtained from an insect.

However, the results also demonstrate that it takes about 36 h to regenerate the bombykol binding affinity of the immobilized BmorPBP and that the initial wash required lowering the pH of the mobile phase to 4.5 followed by a second wash at pH 7.0. These steps are consistent with the observation that a pH 4.5 the BmorPBP exists in a conformational state that does not bind ligands and at pH ≥ 6.5 the protein exists in a second conformation to which ligands can bind [1,6,12]. The chromatographic data suggests that the immobilized BmorPBP is capable of undergoing the same conformational changes, albeit at a much slower rate.

The regeneration of the binding affinity of the immobilized BmorPBP is the rate-limiting step in the use of this column for online screening of chemical and biological libraries. A key step in the optimization of this column is to reduce the time required to regenerate the binding affinity. Initial studies of the effect of the ionic strength of the mobile phase on the binding of bombykol to BmorPBP have demonstrated that an increase in ionic strength from 50 to 200 mM decreased the retention of bombykol (data not shown). This approach as well as the addition of organic modifiers to the mobile phase is currently under investigation and the results will be reported elsewhere.

The general applicability of this approach has been tested using an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatus* [12]. The OBP column contained 1.21 nmol protein per mg silica (60% of the applied OBP) and the binding activity of the immobilized OBP was assessed using L-lactic acid, which has been established as a mosquito attractant [13]. Zonal chromatographic studies using D,L-, D- and L-lactic acid established that the OBP column enantioselectively retained L-lactic acid (Fig. 2), indicating that

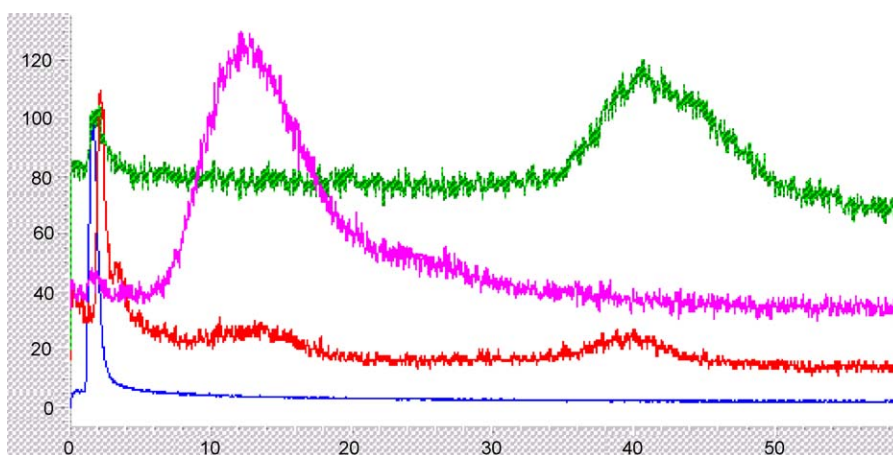


Fig. 2. Zonal chromatographic studies carried out on a column containing an immobilized odorant binding protein obtained from the female mosquito *Culex quinquefasciatus* where the red chromatographic trace was produced by 500 nM D,L-lactic acid; green trace by 250 nM L-lactic acid; purple trace by 250 nM D-lactic acid and blue trace by the injection of 500 nM D,L-lactic acid on a control column not containing the immobilized odorant binding protein. Studies were carried out using a Series 1100 liquid chromatography/mass selective detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a 20 μ l injection loop, the mobile phase was deionized water, the flow rate was 0.2 ml/min, and the experiments were carried out at 37 °C.

the immobilized protein retained the ability to selectively bind ligands.

The data from these studies demonstrate that insect-derived odorant and pheromone-binding proteins can be immobilized on silica-based liquid chromatographic stationary phases with retention and used as affinity screens. The optimization of these columns and the expansion of their applications will be reported elsewhere.

Acknowledgement

This work was supported in part by funding from the Intramural Research Program of the National Institutes of Health.

References

- [1] W.S. Leal, Top. Curr. Chem. 240 (2005) 1.
- [2] P.J. Clyne, C.G. Warr, M.R. Freeman, Y. Lessing, J. Kim, J.R. Carlson, Neuron. 22 (1999) 327.
- [3] L.B. Vosshall, H. Amrein, P.S. Marozov, A. Rzhetsky, R. Axel, Cell 96 (1999) 725.
- [4] C. Bertucci, M. Bartolini, R. Gotti, V. Andrisano, J. Chromatogr. B 797 (2003) 111.
- [5] R. Moaddel, I.W. Wainer, Anal. Chem. Acta (published online, 19 October 2005).
- [6] H. Wojtsaek, W.S. Leal, J. Biol. Chem. 274 (1999) 30950.
- [7] Y. Ishida, A.J. Cornel, W.S. Leal, J. Chem. Ecol. 28 (2002) 867.
- [8] Q. Zhang, H. Zou, H. Wang, J. Ni, J. Chromatogr. A. 866 (2000) 173.
- [9] J.S. McElfresh, J.G. Millar, J. Chem. Ecol. 25 (1999) 687.
- [10] B. Pophof, Chem. Senses 29 (2004) 117.
- [11] A. Khimian, J.A. Klun, Y. Hijji, Y.N. Baranchikov, V.C. Mastro, M.H. Kramer, J. Agric. Food Chem. 50 (2002) 6366.
- [12] D. Lee, F.F. Damberger, G. Peng, R. Horst, P. Guntert, L. Nikonova, W.S. Leal, K. Wuthrich, FEBS Lett. 531 (2002) 314.
- [13] F. Acree Jr., R.B. Turner, H.K. Gouck, M. Beroza, N. Smith, Science 161 (1968) 1346.

Occurrence of betablockers in effluents of wastewater treatment plants from the Lyon area (France) and risk assessment for the downstream rivers

Cécile Miège*, Maxime Favier, Corinne Brosse, Jean-Pierre Canler, Marina Coquery

Cemagref, Water Quality and Pollution Control Research Unit, 3 bis quai Chauveau, CP 220, F-69336 Lyon, France

Received 30 November 2005; received in revised form 18 May 2006; accepted 3 July 2006

Available online 17 August 2006

Abstract

Five betablockers (oxprenolol, metoprolol, propranolol, bisoprolol, betaxolol) were analysed in effluents collected over a 3-month period from wastewater treatment plants (WTP) from the Lyon area in France. The analytical protocol consisted of solid phase extraction of the dissolved aqueous phase on HLB cartridges and analysis by gas chromatography coupled with mass detection (GC–MS) after derivatization. Concentrations of metoprolol, propranolol and bisoprolol varied from 45 to 2838 ng/L whereas oxprenolol and betaxolol were never detected in these effluent samples. A high variability of betablockers concentrations and fluxes was observed between WTP effluents and within each WTP over the time period studied. Considering a flux per person for a dry weather period, Fontaine plant was pointed out as the less efficient WTP, which might be explained by its type of treatment (biological aerated filters). But we need additional analysis of effluent and influent waters to confirm this hypothesis. A tentative approach of local environmental risk assessment of propranolol based on the calculation of PEC/PNEC (predicted environmental concentration/predicted non effect concentration) ratio approach lead us to conclude on a negligible risk for the downstream rivers (Rhône river at Ternay and Saône river at Couzon Mt d'Or).

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Keywords: Betablockers; Wastewaters; GC–MS analysis; Local risk assessment

1. Introduction

The beta-adrenergic receptor blocking compounds (betablockers) are used to treat angina and hypertension. These pharmaceuticals are widely used in variable amounts depending on the country. For example, 35 t of propranolol were distributed in 1999 in France and only 3 t in 1995 in Germany [1]. Once ingested, these pharmaceuticals are not completely eliminated in the human body and they can be excreted into wastewaters [2]. Laurent et al. showed the presence of beta-adrenergic receptors in some fish and hypothesized that there might be a risk of induced dysfunctions on reproduction and growth [3].

In France, it is difficult to obtain data on annual prescription of betablockers. The last available data are from 1999: the three betablockers most prescribed were acebutolol, propranolol and metoprolol (10–45 t/year); prescriptions were much lower for

the other betablockers, such as bisoprolol, oxprenolol, nadolol and betaxolol (less than 2 t) (from the “Agence Française de Sécurité Sanitaire des Produits de la Santé” (AFSSAPS), personal communication). From 1991 to 2001, the prescription of these pharmaceuticals in France has increased by a factor of 50% (from the AFSSAPS, personal communication).

Very few data are available in the literature on the concentration of betablockers in wastewaters. Ternes showed for a German municipal wastewater treatment plant (WTP) using activated sludge process that their removal efficiency was high but incomplete (up to 96% for propranolol and 83% for metoprolol) and decreased after rain events [4]. But these results refute those from Paxeus, who concluded to low removal efficiencies (<10%) with activated sludge process for basic compounds such as betablockers (atenolol and metoprolol) [5]. Betablockers in dissolved aqueous compartment have been measured from the ng/L to the µg/L range in WTP effluents at various locations in Europe and United States [2,4–8]. The contamination of surface waters (in dissolved phase) downstream various WTP varied from 10 to 2900 ng/L according to the betablockers and the site location [4,7–11]. Wiegel et al. showed an increasing concentra-

* Corresponding author. Tel.: +33 4 72 20 87 44; fax: +33 4 78 47 78 75.
E-mail address: cecile.miege@cemagref.fr (C. Miège).

Table 1
Characteristic of the studied wastewater treatment plants (WTP) in the urban area of Lyon

WTP	Population equivalent (PE)	Hospital effluents (number)	Designed capacity (m ³ /d)	Type of treatment
St Fons	1037000	Yes (8)	335000	Primary settling medium-rate activated sludge biological aerated filters (1/4 of the effluent)
Pierre Bénite	700000	Yes (9)	180000	Primary settling low-rate activated sludge (extended aeration)
Fontaines s/Saône	30000	Yes (1)	9670	Primary settling with physico-chemical treatment Biological aerated filters
Neuville s/Saône	20000	No	6000	Primary settling medium-rate activated sludge
St Germain au Mont d'Or	3000	No	900	Primary settling medium-rate activated sludge

tion of metoprolol along the Sale river (Germany) due to inputs from various tributaries (Ilm, Unstrut, Weiße Elster) and WTPs [9]. Thomas et al. quantified propranolol in five estuaries from England with a median value of 13 ng/L [12]. Betablockers have not yet been detected in drinking waters [13,14] although sotalol was found in three ground waters (over a total of 105 samples analyzed) in Baden–Württemberg in Germany [15]. Betablockers have not yet been analysed in suspended particulate matters. Few publications have dealt with solid matrices: Zuccato et al. did not detect any atenolol in sediments from three rivers (Lambro, Po and Adda) in Lombardy (Italy), with a limit of detection of 24 ng/kg [10].

Further investigations are clearly needed to improve our knowledge on the extent of this contamination, which might vary widely according to the geographical location, the season, rainfall events and the type of WTP. The objective of this work was to quantify the concentrations of five betablockers in effluents of WTPs from the Lyon area (France) and to evaluate their temporal variability (over a 3-month period of sampling). We also present a preliminary evaluation of the local environmental risk for the downstream rivers by calculating the ratio PEC/PNEC using measured field concentrations and PNEC data from the literature [16]. The five betablockers (oxprenolol, metoprolol, propranolol, bisoprolol, betaxolol) were chosen because of their occurrence in previous studies [2,4,6] and their compatibility with analysis by gas chromatography coupled with mass detection (GC–MS). Our sampling effort was relatively large: 24 composite samples (24 h integrated samples) for five WTPs over a 3-month period. The WTPs were selected according to their varying characteristics (size and treatment type).

2. Experimental section

2.1. Chemicals

N-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and *N*-methyl-*N*-(trifluoroacetamide) (MBTFA), both from derivatization grade were obtained from Sigma–Aldrich (Lyon, France). Certified (2*RS*)-1-ethylamino-3-[4-(2-methoxyethyl)-phenoxy]propan-2-ol (a metoprolol like molecule, internal standard no. 1) was obtained from Mikromol, (Luckenwalde, Germany). Certified ε -hexachlorohexane (ε -HCH, inter-

nal standard no. 2) and certified betablockers (propranolol hydrochloride, oxprenolol hydrochloride, betaxolol hydrochloride, bisoprolol fumarate and metoprolol) were purchased from Cil–Cluzeau (Ste Foy la Grande, France). Methanol pestipur grade was purchased from SDS (Peypin, France). Ultrapure water was obtained from a Milli-Q water system (Millipore, Molsheim, France).

2.2. Characteristic of sewage treatment plants and sampling method

From October 15th 2004 to January 14th 2005, we have collected 24 composite effluent samples from five WTPs located in the urban area of Lyon (France) (Fig. 1). These WTPs have various capacities, influent origins and treatment processes and their characteristics are presented in Table 1.

Effluent sampling was carried out over 24 h using a flow proportional automatic sampler. The samples were stored in amber glass bottles at 4 °C then extracted within 3 days in order to limit biotic and abiotic degradations.

2.3. Extraction of the dissolved aqueous phase

Prior to extraction, wastewater samples were filtered on Durapore membrane filters (polyvinylidene fluoride, 0.45 μ m pore size, HA type, from Millipore, Molsheim, France) and the internal standard no. 1 was added to the dissolved phase at a concentration of 100 ng/L.

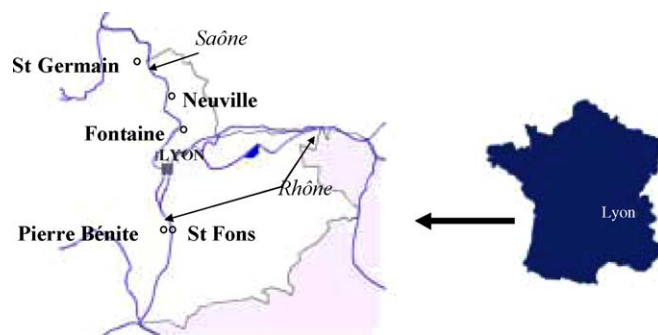


Fig. 1. Location of the five WTPs along the Saône and Rhône rivers in the Lyon area, France.

Table 2

Limit of detection (LD) and mean extraction recoveries (*R*) of betablockers on Oasis® HLB for river waters spiked at 100 ng/L

(n = 5)	LD (rivers)	River water	
		<i>R</i> (%)	R.S.D. (%)
Oxprenolol	2	103	13
Metoprolol	3	100	9
Propranolol	2	94	9
Betaxolol	2	102	11
Bisoprolol	3	103	13
Internal standard no. 1 (metoprolol like molecule)	3	90	12

The pH of filtered water samples was adjusted to 7.5 and 500 mL of wastewater were extracted on SPE cartridges (Oasis HLB cartridges, 3 cc, 60 mg, from Waters, St Quentin en Yvelines, France) as follows: the SPE phase was conditioned by successive percolations of 6 mL of methanol then 6 mL of ultra-pure water. Water samples were percolated through the SPE cartridges at 20 mL/min, then the cartridges were dried under nitrogen flow for 1 h. The elution was achieved using 1 mL of methanol deposited in contact with the SPE phase for 10 min and repeating this procedure 4 times.

SPE recoveries for river water samples spiked at 100 ng/L level are reported in Table 2: they ranged from 90 to 103% depending on the betablocker with relative standard deviation (R.S.D.) below 13%. The efficiency of the extraction step for the effluent samples were controlled by the internal standard no. 1, a metoprolol like molecule. Matrix effects were checked in one effluent from Pierre Bénite WTP by standard addition of 100 and 200 ng/L of the five betablockers.

Limits of detection (LD) were calculated from the equation of the calibration curve ($y = ax + b$, with five concentration levels and $n = 5$ for each concentration level) and the extraction recoveries (*R*) obtained for river waters (Table 2):

$$LD = \frac{3 \cdot s_b}{a} \times \frac{V_{fin}}{V_{wat}} \times R$$

with s_b : standard deviation of b ; V_{wat} : volume of water sample, V_{fin} : volume of final extract.

The LD are reported in Table 2: they are around 3 ng/L for river waters. When comparing the results for river and effluent samples, we observe a decrease of the signal to noise ratio by approximately 5, thus LD for effluent samples are around 15 ng/L.

2.4. Derivatization

Before derivatization, the extracts were evaporated to dryness under nitrogen flow. Derivatization consisted of a trimethylsilylation (with MSTFA) followed by a trifluoroacetylation (with MBTFA). First, 50 μ L of MSTFA were added to the dried residue. After 30 min at room temperature, the samples in closed vessel were heated at 60 °C for 10 min. Then, 20 μ L of MBTFA were added and samples were heated again at 60 °C for 15 min and evaporated nearly to dryness. Finally, 10 μ L of internal stan-

dard no. 2 (ϵ -HCH, at 10 mg/L) were added and the extracts were filled to 1 mL with isoctane for chromatographic analysis.

The efficiency of the derivatization step was controlled in standard solutions: linearity was verified from 1 to 400 μ g/L with correlation coefficients higher than 0.95 and repeatability was found satisfactory with R.S.D. lower than 10% at 50 and 200 μ g/L concentration levels ($n = 5$). The efficiency of the derivatization step for real effluent samples is controlled by the presence of internal standard no. 1, a metoprolol like molecule.

2.5. GC–MS analysis

The gas chromatographic system consisted of a HP6890 GC coupled to a HP5973 mass spectrometer from Hewlett Packard (France). Injections (1 μ L) were performed at 250 °C in the splitless mode with a PTV injector. A HP5 ms (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μ m film) column was used (Hewlett Packard, France). The temperature program was set as follows (total time of 24.5 min): a linear temperature gradient from 50 °C to 250 °C at 25 °C/min, a step at 250 °C for 10 min, then a linear temperature gradient from 250 °C to 280 °C at 20 °C/min and a final step at 280 °C for 5 min.

Mass spectrometry was performed in the selected ion monitoring (SIM) mode. The selected m/z ions were: 284/129 for metoprolol, bisoprolol, oxprenolol, betaxolol and propranolol, 270/129 for internal standard no. 1 and 181/219 for internal standard no. 2.

3. Results and discussion

3.1. Concentrations and temporal variations of betablockers in WTP effluents

Mean and maximum concentrations of individual betablockers in WTP effluents are presented in Fig. 2. Oxprenolol and betaxolol were not detected in the 24 samples analysed. Metoprolol, propranolol and bisoprolol were quantified with a mean concentration of 509, 416 and 791 ng/L, respectively and a maximum concentration of 1774, 1111 and 2838 ng/L, respectively.

In comparison, these three betablockers were also found in 29 German WTP effluents (in dissolved phase) by Ternes at higher concentrations than the other betablockers studied (nadolol,

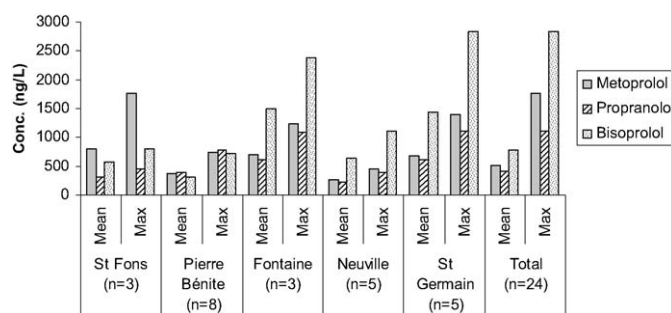


Fig. 2. Mean and maximum concentrations of individual betablockers (ng/L) in five WTP effluents near Lyon, France. (n = number of individual samples) (Oxprenolol and betaxolol were never detected in these samples).

timolol, betaxolol and carazolol) [4]. Huggett (2003) analysed metoprolol, nadolol and propranolol in six WTP effluents in United States from October 2000 to March 2001 (34 samples): propranolol was also found in all the effluent samples with a mean concentration of 117 ng/L and maximum concentration of 1900 ng/L; metoprolol was quantified in 30 samples with a mean concentration of 74 ng/L and a maximum concentration of 1200 ng/L [2]. Andreozzi (2003) studied WTP effluents from various countries (Sweden, Italy, Greece and France, seven samples, from February to March 2001) and similarly to our study, he did not find betaxolol but he detected oxprenolol with a mean concentration of 20 ng/L, which is relatively close to our LD, and a maximum concentration of 50 ng/L. Furthermore, he detected maximum concentrations of propranolol and metoprolol at 90 and 390 ng/L, respectively [6]. Propranolol was also detected in all the samples analysed by Hilton (2003, three WTP effluents) and Ashton (2004, five WTP effluents) [7,8].

The relatively high effluent concentrations of metoprolol and propranolol measured in our study could be explained by their high prescription in France per year (10.8 and 34.6 t, respectively, in 1999), whether the non detection of oxprenolol and betaxolol could originate from their low annual quantities prescribed per year (1 and 0.5 t, respectively, in 1999) (from the AFSSAPS, personal communication). However, the relatively low annual prescription of bisoprolol in France in 1999 (1.6 t) does not allow to explain the high effluent concentration of this compound. Perhaps the annual prescription of bisoprolol has increased since 1999. Unfortunately, these prescription data are unavailable. We know that bisoprolol belongs to the top 200 prescriptions for 2004 in United States together with atenolol, metoprolol and propranolol (www.rsl.com/top200.htm).

Metabolization of pharmaceuticals in human beings reduces their presence in urine, raw waters, WTP and downstream rivers. Lipophilic betablockers (propranolol, metoprolol, bisoprolol, oxprenolol, betaxolol, ...) are extensively metabolized (metabolization higher than 80%) principally in liver, while hydrophilic ones (atenolol, nadolol, sotalol, ...) are excreted mainly unmetabolized (metabolization lower than 20%). From our data on, we have verified that highly metabolized lipophilic betablockers can be found in high concentration in effluents if they are highly produced (except bisoprolol which is present at high concentration but little prescribed).

Concentrations of organic contaminants in effluents are sometimes explained by their removal rate in WTP which depends on their physico-chemical properties (mainly hydrophobicity which affects their ability to be sorbed on sludges or suspended particulate, and volatility which affects their elimination in the atmosphere) and degradation (by photolysis, hydrolysis, or biological degradation). But very little literature data is available on the persistence of betablockers in WTP and the conclusions are sometimes contradictory: Ternes found high removal efficiency in one German municipal activated sludge WTP for propranolol and metoprolol (up to 96 and 83%, respectively) [4] whereas Paxeus concluded to low removal efficiencies (<10%) in 10 activated sludge WTP for basic compounds such as atenolol and metoprolol [5].

The five selected betablockers in our study are all basic, low to moderately hydrophobic (with log K_{ow} from 1.69 for bisoprolol to 3.56 for propranolol [17]) and thus likely to be partially eliminated from liquid effluent by moderate sorption onto sludge, but no data is presently available on the concentration of betablockers in sludge.

Predicting the (bio)degradation from the chemical structure is notoriously difficult and depends on the concentration of the contaminant [18]. Andreozzi [6] studied the photodegradation of propranolol in aquatic environments with photodegradation half life time varying from 1.5 days (in summer at latitudes 50°N) to 16.8 days (in winter at latitudes 50°N) and observed an enhanced photodegradation with humic acids (at 5 mg/L) that act as photosensitizers. This only study available on betablockers degradation cannot allow to explain degradation in WTP.

We also studied the temporal variations of the betablockers concentrations in effluents from Pierre Bénite WTP and from Neuville WTP over the 3-month sampling period in winter (Fig. 3). For Pierre Bénite WTP, we measured a temporal variation up to a factor of 14. For Neuville WTP, these temporal variations were lower: up to 5 times for metoprolol and propranolol and up to 8 times for bisoprolol.

Contrary to antibiotics that are more consumed in winter or X-ray contrast media that are used predominantly from Monday to Friday, betablockers are used continuously during the year. Thus, we should not expect a temporal variation of effluent concentration due to their consumption. The dilution of the influents cannot explain the variations of effluent concentrations. We have

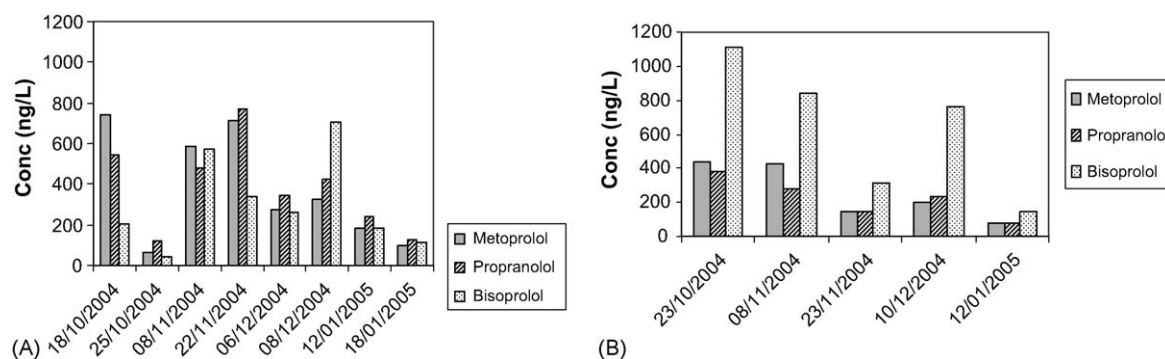


Fig. 3. Temporal variations of the concentrations of individual betablockers (ng/L) for the WTP from (A) Pierre Bénite and (B) Neuville.

verified that the lowest effluents concentrations (Pierre Bénite: 25/10/2004, 12/01/2005 and 18/01/2005, Neuville: 12/01/2005) coincide with the highest influent flow rates. However, for the complete data set, large variations in effluents concentrations remain after the correction by dilution of the influents. Huggett (2003) also studied the variation of effluent concentrations for metoprolol, nadolol and propranolol: over 8 h to 12 day-periods, the variations were explained partly by varying human activities; over 4-week periods, the concentration of propranolol varied from 25 to 120 ng/L without any specific explanation [2].

3.2. Comparison of the fluxes of betablockers discharged by the five WTPs

Knowing that a person produce in average 150 L of wastewater per day (during dry weather) and 110 g of chemical oxygen demand (COD) per day, it is possible to calculate a theoretical number of persons connected to each WTP. This number is more representative than the population equivalent number (reported in Table 1), which was used to design the WTPs before their construction. The calculated theoretical number is equal to 1,850,000 for St Fons WTP, 1,000,000 for Pierre Bénite WTP, 40,000 for Neuville WTP, 13,000 for Fontaine WTP and 2500 for St Germain WTP.

In order to compare the contaminant fluxes released by the five WTPs, we calculated a flux per person using this theoretical number and the effluent concentrations measured during a dry weather period (identified by a chemical oxygen demand, COD, around 700 mg/L in the influent). As we did not have dry weather period samples for St Fons and Neuville plants (COD around 350 mg/L for actual samples), we have corrected the fluxes per person by a factor of two corresponding to the assumed dilution of these two effluents.

Results illustrated in Fig. 4 show that the highest flux of betablockers per person was found for Fontaine WTP, which has only one hospital effluent connected to it. In Fontaine WTP, the fluxes per person were equal to 235, 208 and 455 $\mu\text{g}/\text{person}/\text{d}$ for metoprolol, propranolol and bisoprolol respectively. Fluxes were at least 2 times lower for Pierre Bénite and St Fons WTPs, although 9 and 8 hospital effluents, respectively are connected to these two WTPs. Moreover, we obtained comparable fluxes of betablockers per person for Pierre Bénite and St Germain effluents, whereas St Germain effluents are exclusively domestic and those of Pierre Bénite are a mixture of domestic and hospital effluents. Therefore, the knowledge of the wastewater origin is not sufficient in order to explain the variation of betablock-

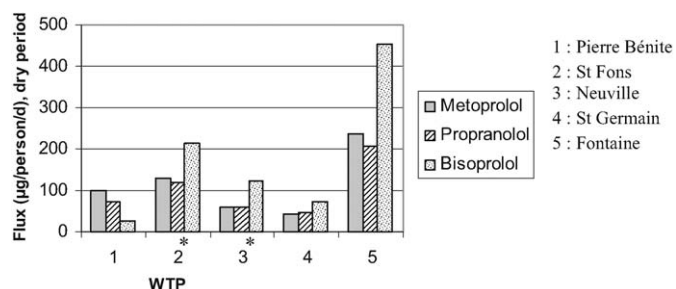


Fig. 4. Comparison of the fluxes of individual betablockers ($\mu\text{g}/\text{person}/\text{d}$) from the five WTPs for a dry weather period. (*) no dry weather period samples available (extrapolated values).

ers concentration in these effluents. This is not surprising since betablockers are mainly used outside hospitals. We would need to take into account the efficiency of betablockers removal inside the WTP, which depends, among other factors, on the type of treatment process. Indeed, a possible explanation for the highest concentration measured in Fontaine effluents could be the lower removal efficiency of this WTP, as it is the only one to use only biological aerated filters (Table 1). However, additional data are needed to confirm this hypothesis.

3.3. Tentative risk assessment for the downstream rivers

Environmental risk assessment aims to evaluate the potential impact of individual substances on the environment by examining both exposure and effects on the ecosystem of their emissions [19]. Risk quotients are calculated between the predicted environmental concentration (PEC) and the predicted non effect concentration (PNEC) which is the concentration of the substance for which adverse effects are not expected to occur. PNECs are derived from results based on standardized acute or chronic toxicity tests performed on several test organisms (i.e. algae, invertebrates and fishes). If the exposure concentration equals or exceeds the effect concentration ($\text{PEC}/\text{PNEC} \geq 1$), then an ecological risk is suspected.

PNEC evaluation: Ferrari et al. [16] estimated a PNEC (chronic) at 10 ng/L for propranolol by dividing the lowest NOEC (chronic non observed effect concentrations) value from fish with a safety factor of 50 (depending of the quality of the data as recommended in the technical guidance document [19]). We have not found PNEC for other betablockers (due to a lack of ecotoxicological data), thus we will concentrate our evaluation on propranolol only.

Table 3
Tentative risk assessment for propranolol in the downstream rivers of five WTPs near Lyon, France

WTP	Maximum fluxes (g/d), propranolol	5 years lowest water flow discharges (m^3/s)	Location	PEC (ng/L)	PEC/PNEC
St Fons	117	300	Rhône (Ternay)	4.5	0.45
Pierre Bénite	78			3.0	0.30
Neuville	2.3			0.7	0.07
Fontaine	3.4	40	Saône (Couzon Mt d'Or)	1.0	0.10
St Germain	1.0			0.3	0.03

With PNEC = 10 ng/L from [16].

PEC evaluation: for this calculation, we assume a realistic worst case. Maximum fluxes of propranolol for each of the five WTPs were calculated using the maximum measured concentrations and wastewater flow. Results are presented in Table 3 and show that St Fons WTP releases about 117 g of propranolol per day, which is about 1.5 times more than Pierre Bénite WTP, 34 times more than Fontaine WTPs, 50 times more than Neuville, and 115 times more than St Germain WTP. These variations in the total fluxes are mainly explained by the number of population equivalent (Table 1). The 5 years lowest water flow discharges (m^3/s) for the downstream rivers measured at Ternay for the Rhône river (connected to effluents from St Fons and Pierre Bénite) and at Couzon Mt d'Or for the Saône river (connected to effluents from Neuville, Fontaine and St Germain) are reported in Table 3. From these values, we have calculated the PEC for propranolol which varies from 0.3 ng/L for St Germain WTP to 4.5 ng/L for St Fons WTP (Table 3).

Considering the Rhône river downstream Pierre Bénite and St Fons WTPs, we estimated a PEC/PNEC ratio for propranolol equal to 0.45 and 0.3, respectively. When summing the PEC for Pierre Bénite and St Fons WTPs, we obtained a PEC/PNEC ratio for propranolol of 0.75 for the Rhône river at Ternay. Considering the Saône river downstream St Germain, Neuville and Fontaine WTPs, we estimated a PEC/PNEC ratio equal to 0.03, 0.07 and 0.1, respectively. When summing the PEC for these three WTPs, we obtained a PEC/PNEC ratio for propranolol of 0.2 for the Saône river at Couzon Mt d'Or.

Using the results of these campaigns performed over a 3-month period, it appears that the risk induced by propranolol and evaluated at a local level would be negligible when using this PEC/PNEC simplified risk assessment approach. Similar conclusions were obtained by Ashton who calculated a PEC/PNEC ratio of 0.002 for propranolol using the results of analysis of five WTP effluents in United Kingdom [8]. But we have to keep in mind that propranolol is present in effluents and downstream rivers with other betablockers and organic contaminants (mixtures) and they can have additive or synergistic effects.

4. Conclusion

Metoprolol, propranolol and bisoprolol were quantified at concentrations varying from 45 to 2838 ng/L in 24 effluent samples from five different WTPs near Lyon, France, whereas oxprenolol and betaxolol were never detected in these samples. High variability of betablockers concentrations and fluxes were observed between WTP effluents and within each WTP over the time period studied (3 months). Considering the betablocker flux per person, Fontaine plant was pointed out as the less efficient WTP, which might be explained by its type of treatment (biological aerated filters). But we need additional analysis of effluents and influents to confirm this hypothesis. A preliminary risk assessment of propranolol based on a PEC/PNEC approach at a local scale lead us to conclude on a negligible risk for the

downstream rivers (Rhône river at Ternay and Saône river at Couzon Mt d'Or).

To complete this study we plan to: (i) quantify the removal of betablockers within WTPs by analysing concentrations both in liquid influents and effluents, (ii) test the effect of rainfall events, the type of process and the operating conditions applied in the WTP, on the removal efficiency of the WTP, (iii) analyse suspended particulars matter and sludge as they might contain significant concentrations of betablockers, especially the more hydrophobic ones (such as propranolol). Moreover, the use of LC–MS–MS detection will allow us to analyse several more polar and hydrophilic betablockers (atenolol, sotalol, acebutolol) and to improve our limits of detection.

Acknowledgements

Laurent Carles from Waters (France) is thanked for his advices on SPE optimization. We also thank Rhône Alpes district for financial support, Grand Lyon for assistance on effluent sampling and Elodie Vidal (Cemagref) for the SPE optimization.

References

- [1] J. Beausse, *Trends Anal. Chem.* 23 (10–11) (2004) 753.
- [2] D.B. Huggett, I.A. Khan, C.M. Foran, D. Schlenk, *Environ. Pollut.* 121 (2) (2003) 199.
- [3] P. Laurent, S. Holmgren, S. Nilsson, *Comparative Biochemistry and Physiology – A Physiology* 76 (3) (1983) 525.
- [4] T.A. Ternes, *Water Res.* 32 (11) (1998) 3245.
- [5] N. Paxeus, *Water Sci. Technol.* 50 (5) (2004) 253.
- [6] R. Andreozzi, M. Raffaele, P. Nicklas, *Chemosphere* 50 (10) (2003) 1319.
- [7] M.J. Hilton, K.V. Thomas, *J. Chromatogr. A* 1015 (1–2) (2003) 129.
- [8] D. Ashton, M. Hilton, K.V. Thomas, *Sci. Total Environ.* 333 (1–3) (2004) 167.
- [9] S. Wiegel, A. Aulinger, R. Brockmeyer, H. Harms, J. Löffler, H. Reincke, R. Schmidt, B. Stachel, W. von Tumpling, A. Wanke, *Chemosphere* 57 (2) (2004) 107.
- [10] E. Zuccato, D. Calamari, M. Natangelo, R. Fanelli, *Lancet* 355 (9217) (2000) 1789.
- [11] F. Sacher, S. Gabriel, M. Metzinger, M. Wenz, *Chimia* 57 (1–2) (2003) 29.
- [12] K.V. Thomas, M.J. Hilton, *Marine Pollut. Bull.* 49 (5–6) (2004) 436.
- [13] S. Webb, T. Ternes, M. Gibert, K. Olejniczak, *Toxicol. Lett.* 142 (3) (2003) 157.
- [14] T. Ternes, J. Stuber, N. Herrmann, D. McDowell, A. Ried, M. Kampmann, B. Teiser, *Water Res.* 37 (8) (2003) 1976.
- [15] F. Sacher, F.T. Thomas Lange, H.J. Brauch, I. Blankenhorn, *J. Chromatogr. A* 938 (2001) 199.
- [16] B. Ferrari, R. Mons, B. Vollat, B. Fraysse, N. Paxeus, R. Lo Giudice, A. Pollio, J. Garric, *Environ. Toxicol. Chem.* 23 (5) (2004) 1344.
- [17] A. Detroyer, Y. Vander Heyden, S. Carda-Broch, M.-C. Garcia-Alvarez-Coque, D.-L. Massart, *J. Chromatogr. A* 912 (2001) 211.
- [18] D.L. Sedlak, K.E. Pinkston, *Water Res. Updat.* 120 (2001) 56.
- [19] EC Technical guidance document on risk assessment, (TGD). Part II, Technical Report, Institute for Health and Consumer Protection, European Chemicals Bureau, European Commission (EC), 2003.

Analysis of enantiomers in biological matrices by charged cyclodextrin-mediated capillary zone electrophoresis in column-coupling arrangement with capillary isotachophoresis

Peter Mikuš*, Peter Kubačák, Iva Valášková, Emil Havránek

Department of Pharmaceutical Analysis and Nuclear Pharmacy, Faculty of Pharmacy, Comenius University, Odbojárov 10, SK-832 32 Bratislava, Slovak Republic

Received 23 August 2005; received in revised form 22 December 2005; accepted 7 February 2006

Available online 15 March 2006

Abstract

The possibility to apply charged chiral selector as buffer additive in capillary zone electrophoresis (CZE) on-line coupled with capillary isotachophoresis (CITP) was studied. Enantioseparations and determinations of trace (ng/ml) antihistaminic drugs [pheniramine (PHM), dimethindene (DIM), dioxopromethazine (DIO)] present in samples of complex ionic matrices (urine) served as model examples. A negatively charged carboxyethyl- β -cyclodextrin (CE- β -CD) was used as a chiral selector in analytical CZE stage following upon a sample pretreatment by CITP (preconcentration of the analytes from 5 to 20-times diluted urine samples, partial sample clean up removing macroconstituents from the sample matrices). A high recognition capability of the oppositely charged CE- β -CD was demonstrated by enantioselective retardation of the drugs in presence of micro- and semi-macroconstituents migrating in CZE stage and detectable by UV detector. In this way, enantiomers of the drugs could be easily separated and determined. Due to lack of interferences between the drugs and sample-matrix constituents in presence of charged CE- β -CD, demands on both spacers in CITP step and multiple column-switching were minimized. CITP-CZE method with charged selector appeared to be a useful analytical approach for the trace enantiomers in complex ionic matrices as it combined enhanced separation selectivity and sample loadability with high separation efficiency and provided favorable performance parameters including sensitivity, linearity, precision, accuracy/recovery and robustness with minimal demands on sample preparation. Analysis of urine sample taken from a patient treated by PHM, showing concentration profile of PHM enantiomers and their metabolites, illustrated potentialities of the method in clinical research.
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Keywords: Capillary isotachophoresis; Capillary zone electrophoresis; Column-coupling electrophoresis; Pheniramine; Dimethindene; Dioxopromethazine; Drug enantiomers; Charged cyclodextrin; Chiral analysis; Urine

1. Introduction

In pharmaceutical research, including chiral purity control, pharmacokinetic studies etc., analytical methods are requested to provide high resolution power, high separation efficiency and high sensitivity. The separation of enantiomers is currently carried out by using high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and capillary electrochromatography (CEC) [1–3].

CE is a very rapidly growing microseparation technique and this is mainly due to the following advantages: (i) high separa-

tion efficiency, (ii) short analysis time, (iii) very small buffer and sample volumes are required, (iv) environmentally friendly technique due to minor organic solvent consumption, (v) CE techniques are easily on-line combinable, (vi) diverse application range. It is well known that separations in CE are predominantly driven by efficiency while in HPLC by selectivity. A common approach to enhance separation selectivity in CE, approved in many cases, is based on the use of selector migrating in opposite direction towards analytes. In this way, separation window is significantly spread and resolution can be increased [1].

Cyclodextrins (CDs) have been used extensively in separation science because they have been shown to discriminate between positional isomers, functional groups, homologues and enantiomers [4]. This property as well as transparency of CDs in UV region of optical spectrum makes them one of the most

* Corresponding author. Tel.: +421 2 50 117 248; fax: +421 2 50 117 100.
E-mail address: mikus@fpharm.uniba.sk (P. Mikuš).

useful agents for a wide variety of separations and therefore this group of chiral selectors was also used in our present work. Charged CD derivatives have been used as chiral selectors in CE for the first time by Terabe [5] and separation effect of charged CDs in CE has been explained in detail by Chankvetadze et al. [6]. Very recently we demonstrated high effectivity of negatively charged carboxyethyl- β -cyclodextrin (CE- β -CD) for the separation of pheniramine and dioxopromethazine enantiomers comparing separation effect of the charged and native form of β -cyclodextrin (β -CD) [7,8]. However, CZE in a single column-configuration has several limitations so that its application for more complex samples is usually depend on a sample preparation.

Main limitation of CZE with a UV absorbance detector is the relatively low sample concentration detection limit so that many trace components in real samples cannot be analyzed by this technique directly. The limit of detection (LOD) can be improved by several orders of magnitude when more sensitive detectors such as laser-induced fluorescence are used; as only a small number of analytes exhibit native fluorescence, derivatization has to be carried out in most cases. Another way to increase the separation capability, sensitivity and detectability of CZE is the use of on-line pre-concentration techniques, e.g. by performing the CITP-CZE combination in two coupled capillaries [9–14]. The coupled column arrangement is characterized by isotachopheresis in the first capillary, serving as an efficient pre-separation and concentration stage, followed by the on-line transfer of the sample cut into the second capillary where analytical zone electrophoresis proceeds as the second stage. Besides the pre-concentration of the analytes, the CITP step has several other specific features that are advantageous for CZE, such as the high sample load (depend on the internal diameter of the pre-separation capillary), transfer of a well-defined fraction of the sample into CZE, and an ideal sample injection for CZE (injection of a short stack of sharp zones of interest).

CZE separations of enantiomers with on-line CITP sample pretreatment have been carried out using neutral chiral selectors in separation electrolytes so far. As examples are given the separations of tryptophan enantiomers spiked into complex ionic matrices (90-component model mixture, urine) using native α -cyclodextrin [15,16]. The results from the runs with urine samples showed that only the CITP-CZE combination with a post-column on-line coupled CITP sample clean-up (responsible for a removal of more than 99% of the sample anionic constituents migrating in the on-line coupled CITP stack and detectable in the CZE stage) provided a universal alternative for the detection and quantitation of the model analyte [15].

The aim of the present work was to demonstrate potentialities of on-line coupled CITP-CZE method including charged chiral selector as buffer additive in analytical (CZE) stage. An enhancement of separation selectivity in comparison with a neutral selector-modified CE was expected maintaining other benefits of coupled CE methods (enhanced sensitivity, minimization of sample pretreatment, etc.). A simplification of working conditions (use of CITP spacers, multiple column switching) due to minimization of interferences between sample matrix constituents and the analytes transferred from CITP into CZE was

expected too. An influence of separating conditions (pH, concentration of chiral selector, sample matrix concentration) on the enantioresolution of various drugs (pheniramine, PHM, dime-thindene, DIM, dioxopromethazine, DIO), serving as model analytes, in urine, serving as complex ionic matrices, was investigated. Performance parameters of the proposed CITP-CZE method were evaluated and application example was shown.

2. Experimental

2.1. Instrumentation

A CS isotachophoretic analyzer (Villa-Labeco, Spišská Nová Ves, Slovak Republic), assembled in the column-coupling configuration of the separation unit, was used in this work. The separation unit consisted of the following modules: (i) an injection valve with a 30 μ l internal sample loop; (ii) an CITP column provided with a 800 μ m I.D. capillary tube made of fluorinated ethylene-propylene copolymer (FEP) and an on-column conductivity sensor; its total length was 90 mm; (iii) a CZE column provided with a 300 μ m I.D. capillary tube made of FEP of 210 mm total length (160 mm to the photometric detector); (iv) a bifurcation block for an on-line coupling of the CITP and CZE columns; (v) a counter-electrode compartment with a hydrodynamically (membrane) closed connecting channel to the separation compartment. The columns were assembled in plexiglass cartridges for better dissipation of Joule heat.

The CZE column was provided with a LCD 2083 on-column photometric detector with variable wavelengths, 190–600 nm (Ecom, Praha, Czech Republic). In this work the photometric detector was set at 265 nm (PHM, DIM) or 240 nm (DIO) detection wavelengths. The signals from the detectors were led to a PC via a Unilab data acquisition unit (Villa-Labeco). ITP Pro32 Win software (version 1.0) obtained from KasComp (Bratislava, Slovak Republic) was used for data acquisition and processing.

Prior to the use, the capillary was not particularly treated to suppress an electroosmotic flow (EOF). A dynamic coating of the capillary wall by means of a 0.2% methylhydroxyethylcellulose (m-HEC 30 000; Serva, Heidelberg, Germany) in leading and background electrolyte solutions served for this purpose [17]. CITP and CZE analyses were carried out in cationic regime of the separation with direct injections of the samples. The experiments were performed in constant current mode [18] at 20 °C. The driving currents applied were 250 μ A (CITP) and 120 μ A (CZE) and the corresponding driving voltages were 1–2 kV (CITP) and 5–6 kV (CZE).

2.2. Chemicals and samples

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, USA). All chemicals used were of analytical grade or additionally purified by the usual methods.

The solutions of the electrolytes were filtered before use through disposable membrane filters (a 1.2 μm pore size) purchased from Sigma (St. Louis, MO, USA).

Pheniramine maleate, *N*-[3-phenyl-3-(2-pyridyl)propyl]-*N*, *N*-dimethylamine maleate (PHM) was obtained from ICN Biomedicals (Eschwege, Germany), dimethindene maleate, *N,N*-dimethyl-3-[1-(2-pyridinyl)ethyl]-1*H*-indene-2-ethanamine maleate (DIM) from USP (USP Convention, Rockville, MA, USA), dioxopromethazine hydrochloride (DPZ) from Intermo (Netherlands). All drugs were present as racemic mixtures. Commercial pharmaceutical preparation, granulous powder Fervex[®], containing 25 mg of racemic PHM per dose, is product of UPSA (Agen, France). Carboxyethyl- β -cyclodextrin (DS 3, CE purity), CE- β -CD, is a commercial product of Cyclolab (Budapest, Hungary).

2.3. Procedures for sample and standard solution preparations

2.3.1. Standard solutions

Pure standard stock solutions of PHM and DIO were prepared in demineralized water at a 10 mg/ml concentration while DIM at 1 mg/ml in diluted acetic acid and the stock solutions were stored at -8°C in the freezer. Working solutions were made by appropriate dilution of the stock solutions with demineralized water so that the concentrations of the drugs in the injected model samples were in ng/ml– $\mu\text{g}/\text{ml}$ range.

2.3.2. Urine samples

Urine samples were obtained from healthy adults with different diet habits (sample I–IV). For CE experiments they were spiked with the drugs from the stock solutions and appropriately diluted (see legends to Figs.). For calibration graph (i) and recovery test (ii), spiked urine samples were diluted 10-times with demineralized water yielding concentrations of enantiomers (i) 10, 25, 50, 75, 100 ng/ml and (ii) 10, 50 and 100 ng/ml. The samples prepared in this way were immediately analyzed or stored in a deep freezer at -20°C . The frozen samples were melted at a room temperature and filtered before the injection into the CE equipment.

The clinical sample of urine was obtained from patient treated by Fervex (three doses administrated in six-hour intervals). Urine was taken 48 h after administration of the third dose, 10-times diluted with demineralized water and directly injected into the CE equipment. A blank urine sample was taken from the same patient 1 day before administration of the first dose, 10-times diluted with demineralized water and directly injected into the CE equipment.

3. Results and discussion

3.1. CITP-CZE separating conditions

The electrolyte systems used for the enantioseparations of the drugs in the CITP-CZE combination are given in Table 1. The buffer constituents and the driving current in the CITP stage (250 μA) were chosen as an experimental optimum considering a rapid CITP separation, sharp zone boundaries and the disturbing role of thermal effects. CZE separating conditions, previously optimized in a single column with respect to enantioresolutions of PHM and DIO [7,8], were adapted to the column-coupling arrangement in this work. The same conditions as used for PHM appeared to be suitable also for DIM.

3.2. Separation possibilities with ionizable chiral selector

In experiments to investigate the capabilities of ionizable chiral selector (CE- β -CD) for the enantioseparation of trace drugs (PHM, DIO, DIM, illustrative figures are with PHM) in the presence of complex ionic matrices (urine), CITP provided conditions for simple removal of macroconstituents (mainly inorganic cations) migrating in front of drug zone and transferring of drug with the rest of micro- and semi-macroconstituents (mostly organic compounds, potential interferents), surrounding drug zone, into CZE stage (Fig. 1). The partial sample clean-up prevented an overloading of the CZE capillary by macroconstituents from a large volume (30 μl) of concentrated urine sample (5–20-times diluted urine) and significantly shortened overall analysis time. Moreover, CITP provided

Table 1
Electrolyte systems^a

Parameter	ITP		Parameter	CZE	
	ITP1	ITP2		CZE1	CZE2
Solvent	Water	Water	Solvent	Water	Water
Leading cation	K ⁺	K ⁺	Carrier cation	GLY	EACA
Concentration (mmol/l)	10	10	Concentration (mmol/l)	25	25
Counter ion	HAc	HAc	Counter ion	HAc	HAc
pH	4.75	4.75	pH	3.2	4.5
EOF suppressor	m-HEC	m-HEC	EOF suppressor	m-HEC	m-HEC
Concentration (% w/v)	0.2	0.2	Concentration (% w/v)	0.2	0.2
Terminating cation	GLY	EACA	Complexing agent	CE- β -CD	CE- β -CD
Concentration (mmol/l)	5	5	Concentration (mmol/l)	0–15	0–5.5
Counter ion	HAc	HAc			
pH	3.2	4.5			

^a HAc = acetic acid; EOF = electroosmotic flow; m-HEC = methylhydroxyethylcellulose; GLY = glycine; EACA = ϵ -aminocaproic acid; CE- β -CD = carboxyethyl- β -cyclodextrin.

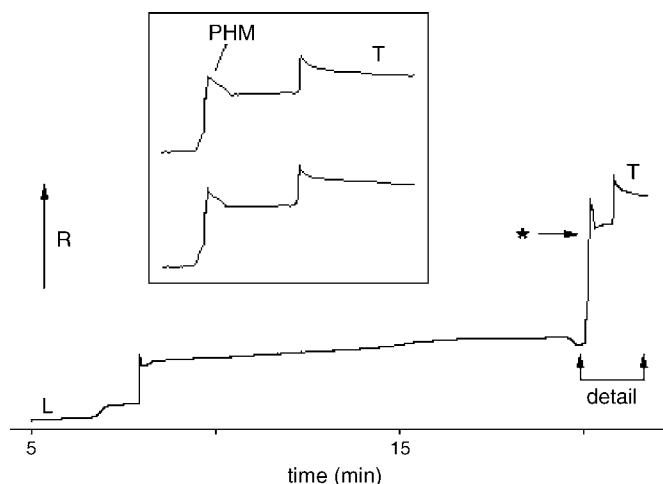


Fig. 1. Typical isotachopherogram as obtained from the separation of urine sample in CITP stage of the CITP-CZE combination. The separation, based on differences in acid-base properties of separated constituents, was carried out in electrolyte system ITP2. Detail traces were obtained under the same conditions, with (upper trace) and without (lower trace) PHM. PHM was present in the sample (10-times diluted urine) at a 20 $\mu\text{g/ml}$ concentration. The asterisk indicates a beginning of the fraction transferred into the CZE stage. L, T = leading and terminating zones, respectively.

concentration of analyte into a short zone serving as an effective sample injection technique for CZE.

Separating conditions in analytical CZE stage were chosen to illustrate an influence of charge of the chiral selector on the separation selectivity. The experiments revealed that CE- β -CD with low effective charge (pH 3.2) was not effective in separation of the drugs from the matrix constituents migrating in CZE stage (Fig. 2). Moreover, its enantioresolution power decreased with increased concentration of the sample matrix constituents, e.g. PHM enantiomers were only partially resolved ($R \sim 0.2$) analyzing 20-times diluted urine at a 15 mg/ml concentration of CE- β -CD. On the other hand, separation capability of the selector considerably increased with its effective charge. This was demonstrated by separation of the drugs at pH 4.5 assuming extensive ionization of CE- β -CD (for ionization of carboxyalkyl- β -CDs see e.g., Refs. [19,20]). For example, Fig. 3 shows that under such conditions PHM enantiomers could be easily resolved each other ($R > 1.5$) and completely separated from micro- and semi-macroconstituents migrating in CZE stage and detectable by UV detector. Similar results were obtained also for DIM and DIO (Fig. 4). The migration velocities of the drugs were strongly influenced by concentration of the selector (2.5–5.0 mg/ml) while velocities of other migrants were influenced only marginally. On the other hand, at this pH the enantioresolution power of CE- β -CD was not significantly influenced by concentration changes of the sample matrix constituents (5–20-times diluted urine was tested). These facts can be utilized separating drug enantiomers in urine samples differing in quantity of matrix constituents. An extremely high effectivity of extensively charged CE- β -CD is illustrated by Fig. 5 where the complete separation and determination of trace (20 ng/ml) PHM enantiomers present in five-times diluted urine was achieved using even a 2.5 mg/ml concentration of

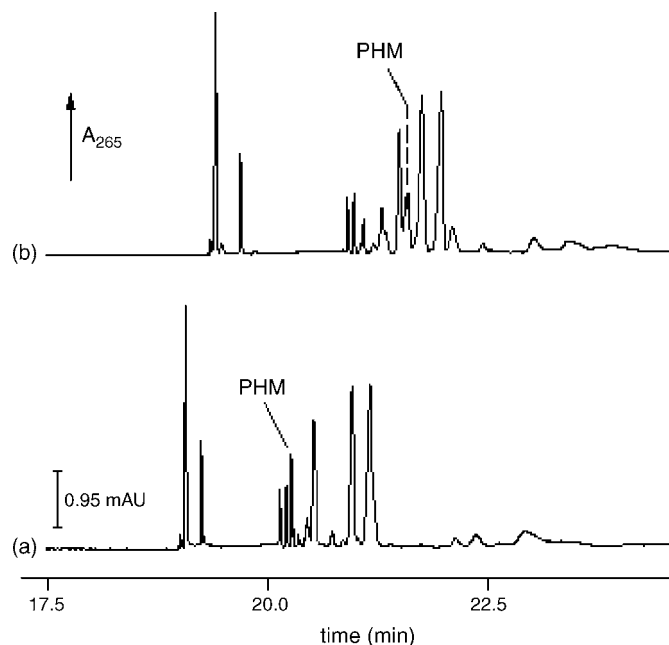


Fig. 2. Electropherograms showing effect of slightly ionized chiral selector, CE- β -CD, on the CZE separation of PHM enantiomers in presence of sample matrix constituents transferred (as in Fig. 1) from CITP stage into the CZE stage of the CITP-CZE combination. The separations were carried out in the electrolyte system CITP1-CZE1 (a) without CE- β -CD (reference trace), (b) with a 15 mg/ml concentration of CE- β -CD. PHM was present in the sample (20-times diluted urine) at a 150 ng/ml concentration.

CE- β -CD. Under such conditions, relatively short analysis time (< 50 min) and high separation efficiency ($\sim 8 \times 10^4$ theoretical plates) were obtained without any sample preparation (except for dilution).

3.3. Performance parameters

The working conditions, chosen for evaluation of performance parameters of CITP-CZE method with charged selector, were based on ITP2 and CZE2 (2.5 mg/ml CE- β -CD for PHM and DIM and 5.5 mg/ml CE- β -CD for DIO) separation systems and 10-times diluted urine samples (Section 2). In all calculations the peak areas of the peaks were corrected to their migration times to compensate for their differential detector residence times.

The detection limits (estimated as 3σ) of PHM, DIM and DIO enantiomers were at ~ 4.8 , 1.1 and 3.2 ng/ml concentrations, respectively, for a 265 nm (PHM, DIM) and 240 nm (DIO) detection wavelengths of the UV detector employed in the CZE stage and for a 30 μl sample volume, while the quantitation limits (estimated as 10σ) were at ~ 16.0 , 3.7 and 10.7 ng/ml concentrations, respectively. The concentrations of the analytes in tested samples, corresponding to the quantitation limits, were determined with acceptable precisions (RSD values ranged in interval 3.95–4.54%, $n = 5$) and accuracies (relative errors ranged in interval 5.84–6.22%, $n = 5$) under the stated conditions.

The linearity of detector response (peak area) for PHM was assessed over the range 10–100 ng/ml for each enantiomer. This

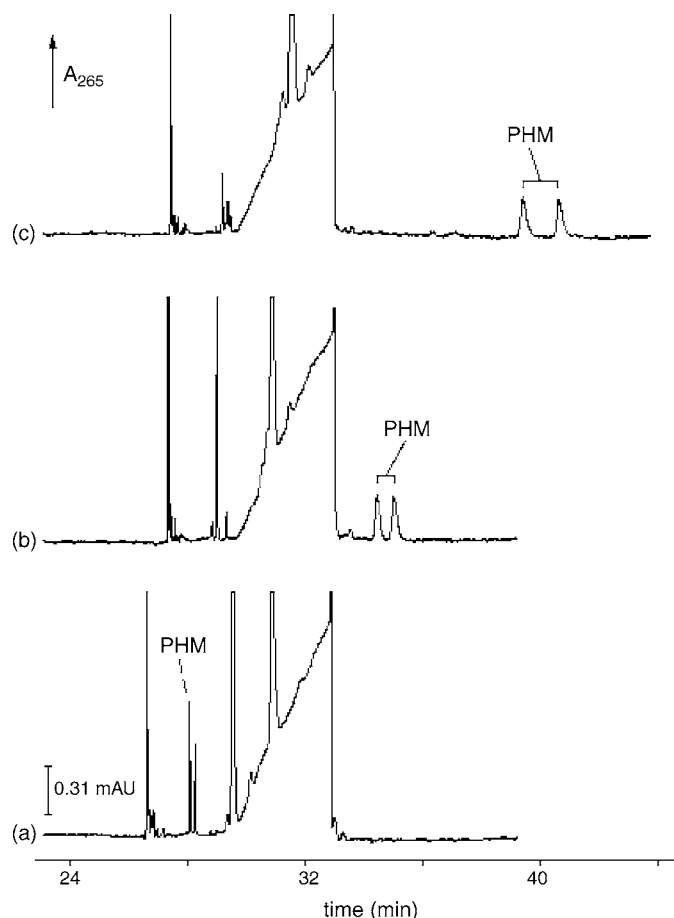


Fig. 3. Electropherograms showing effect of extensively ionized CE- β -CD on the CZE separation of PHM enantiomers in presence of sample matrix constituents transferred (as in Fig. 1) from CITP stage into the CZE stage of the CITP-CZE combination. The separations were carried out in the electrolyte system CITP2-CZE2 (a) without CE- β -CD (reference trace), (b) with a 2.5 mg/ml concentration of CE- β -CD, (c) with a 5.0 mg/ml concentration of CE- β -CD. PHM was present in the sample (10-times diluted urine) at a 150 ng/ml concentration.

represented an interval that was suitable for evaluation of the drug in clinical sample analyzed in this work. The straight-line equations for PHM1 and PHM2 were $y = 2.43 \cdot 10^{-5} + 2.04 \cdot 10^{-3} x$ and $y = 3.74 \cdot 10^{-5} + 1.97 \cdot 10^{-3} x$ (x , ng/ml), respectively, and corresponding determination coefficients were $R^2 = 0.9988$ and 0.9982 , respectively.

The method was validated by evaluation of precision including repeatability, intermediate precision and reproducibility. The results given in Table 2 clearly indicated that CE separations in a hydrodynamically closed system provided highly reproducible migration and response data.

The results from the recovery test (Table 3) outlined a practical use of the proposed CE method. The recoveries were determined by adding known amounts of drug reference substance to the urine samples (I–IV) taken from four healthy adults. For example, amounts of PHM added into urine before its appropriate dilution (10-fold) corresponded to concentrations that could be found in urine after oral administration of ca. 30 mg PHM dose [21]. The mean relative errors indicated by the recovery tests, characterizing accuracy of the

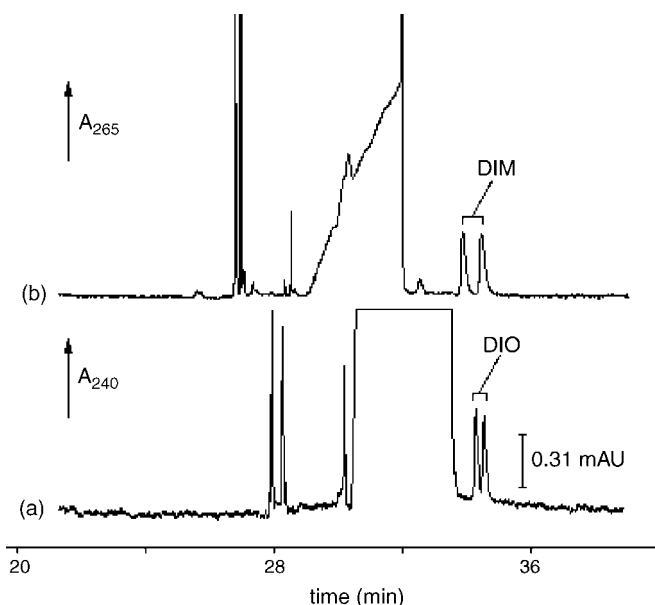


Fig. 4. Electropherograms showing potentialities of charged CE- β -CD employed in CITP-CZE combination for the enantioseparations of various anti-histaminic drugs in urine samples. The separations were carried out in the electrolyte system CITP2-CZE2. (a) DIO was separated using a 5.0 mg/ml concentration of CE- β -CD, (b) DIM using a 2.5 mg/ml concentration of CE- β -CD. The drugs were present in the samples (10-times diluted urine) at ~ 50 – 60 ng/ml concentrations.

method for PHM, DIM and DIO, were 4.5, 4.8 and 3.6%, respectively.

Robustness test examined the effect that deliberate variations in operational parameters (concentration of selector and carrier ion) had on the analysis results (here, enantioresolution of the drugs). Altering concentrations of CE- β -CD in a range of 4.5–5.5 mg/ml and EACA in a range of 20–30 mmol/l, the fluctuations of R were less than 5% of the value obtained under the standard conditions (for this test, ITP2 and CZE2 with 5.0 mg/ml CE- β -CD, and 10-times diluted urine samples were used), except for DIO influenced more significantly by changes of CE- β -CD concentration.

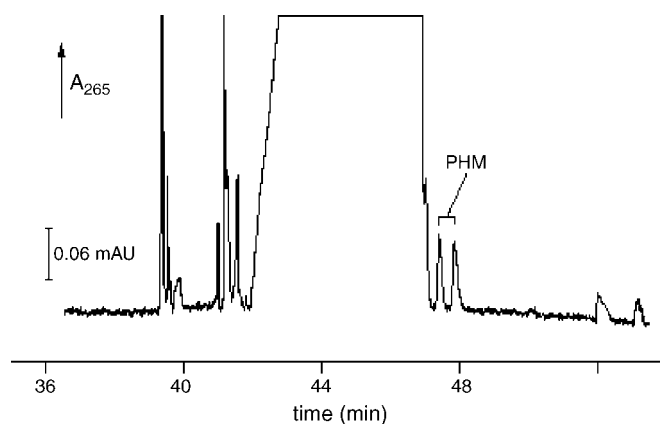


Fig. 5. Electropherogram showing potential of charged CE- β -CD to separate trace (30 ng/ml) PHM enantiomers in concentrated urine samples (urine:water, v/v, 1:4) under the electrophoretic conditions as in Fig. 3b.

Table 2

Precision data (RSD (%), $n = 5$) from the CITP-CZE analysis of drug enantiomers^a

Factor	Repeatability			Intermediate precision ^b			Reproducibility ^c		
	PHM	DIM	DIO	PHM	DIM	DIO	PHM	DIM	DIO
Migration time	0.38	0.38	0.32	0.57	0.69	0.66	1.44	1.56	1.71
Peak area	0.90	0.86	1.15	1.44	1.35	1.83	2.72	2.65	3.23

^a The analyses of urine sample (10-times diluted) spiked with PHM, DIM and DIO (at a 200 ng/ml concentration) using ITP2-CZE2 separation system (2.5 mg/ml CE- β -CD for PHM and DIM and 5.5 mg/ml CE- β -CD for DIO). Each value in this table is average of two enantiomers.

^b The data were collected in 5 successive days.

^c Reproducibility corresponded to conditions where test results were obtained from five repeated injections with the same method and composition of model sample but in different laboratory using modified equipment [a CS Isotachopheretic Analyzer ZKI 01-1 (Villa-Labeco) and a LCD 2083 on-column photometric detector (Ecom)].

Table 3

Recoveries of drugs from urine^a

Sample (number)	Added (ng/ml)									Mean recovery (%)		
	10			50			100					
	PHM	DIM	DIO	PHM	DIM	DIO	PHM	DIM	DIO	PHM	DIM	DIO
Found (I) (ng/ml)	9.5	9.5	9.6	48.7	47.9	48.5	98.0	96.3	97.4	96.8	95.7	96.8
Found (II) (ng/ml)	9.2	9.3	9.6	46.9	47.4	48.0	97.4	95.5	96.5	94.4	94.4	96.2
Found (III) (ng/ml)	9.4	9.4	9.5	47.5	46.8	48.8	98.3	96.5	97.2	95.8	94.7	96.6
Found (IV) (ng/ml)	9.4	9.6	9.4	47.1	47.5	48.4	97.0	96.4	97.2	95.1	95.8	96.0

^a The analyses of urine samples (10-times diluted) spiked with PHM, DIM and DIO using ITP2-CZE2 separation system (2.5 mg/ml CE- β -CD for PHM and DIM and 5.5 mg/ml CE- β -CD for DIO).

3.4. Analysis of clinical sample

The method with evaluated performance parameters was applied for analysis of clinical urine sample taken from a patient treated by PHM. The resulting electropherogram is in Fig. 6. Five compounds, PHM enantiomers and three PHM metabolites, were detected in the sample. Faster migrating pheniramine

enantiomer could be reliably determined. It was present at a 25 ng/ml concentration (250 ng/ml in undiluted urine). RSD for five consecutive determinations was 1.78%. However, lack of standards of PHM metabolites prevented their identification and determination as well as further designed optimization of the separation. In this work, basic separation conditions for several antihistaminic drugs in urine are given. These conditions can be used in clinical analyses as they are or can be further optimized depending on type of drug and its metabolite(s).

4. Conclusion

Experiments with urine samples performed in the CITP-CZE equipment with the column-coupling configuration of the separation unit and charged cyclodextrin as chiral selector in CZE stage showed that this analytical approach provided favorable conditions for separation and determination of trace drug enantiomers in complex ionic matrices. It was demonstrated that chiral selector with charge can provide significantly different affinity towards the analytes on one hand and sample matrix constituents on the other hand, enabling the analytes can be transferred into analytical stage without spacers and multiple column-switching even if accompanied by a part of sample matrix constituents detectable in analytical stage. Besides enhanced (enantio) selectivity, the present analytical approach involves advantages typical for CITP-CZE column-coupled systems (high sensitivity and separation efficiency, enhanced sample loadability, direct analysis of the sample without the need of its preparation). Favorable performance parameters of the proposed method, including sensitivity, linearity, precision, accuracy/recovery and robustness, indicate its potentialities in

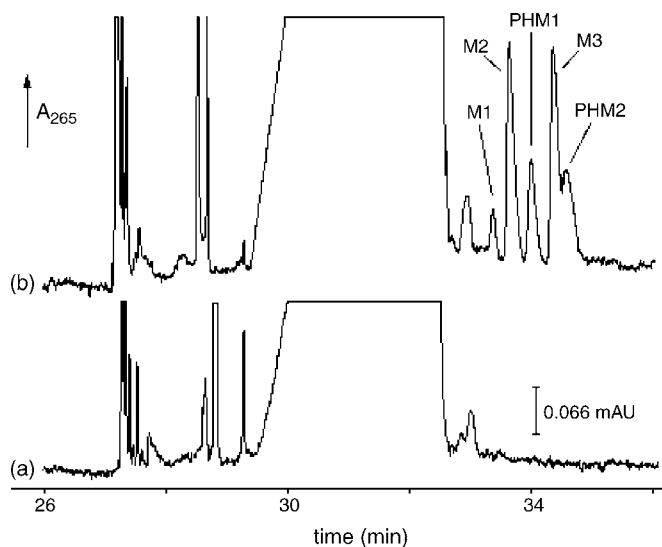


Fig. 6. Analysis of a human urine sample after oral administration of PHM. Electropherogram of (a) blank sample, (b) sample taken from a patient treated by PHM (for sample preparation Section 2). Analyses were carried out under the electrophoretic conditions as in Fig. 3b. Peaks: M1, M2, M3 = pheniramine metabolites, PHM1 = pheniramine enantiomer 1, PHM2 = pheniramine enantiomer 2.

pharmaceutical and clinical analysis. The proposed working conditions may be considered as starting conditions for such applications. Analysis of urine sample obtained from patient treated by PHM can serve as an example.

Acknowledgements

This work was supported by grants from the Slovak Grant Agency for Science under the project No. 1/1196/04 and 1/2310/05.

References

- [1] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (2001) 309–363.
- [2] S. Fanali, Z. Aturki, C. Desiderio, Forensic Sci. Int. 92 (1998) 137–155.
- [3] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357–372.
- [4] E. Schneiderman, A.M. Stalcup, J. Chromatogr. B 745 (2000) 83–102.
- [5] S. Terabe, Trends Anal. Chem. 8 (1989) 129–134.
- [6] B. Chankvetadze, G. Endresz, G. Blaschke, Electrophoresis 15 (1994) 804–807.
- [7] P. Mikuš, I. Valášková, E. Havránek, J. Pharm. Biomed. Anal. 38 (2005) 442–448.
- [8] P. Mikuš, I. Valášková, E. Havránek, J. Pharm. Biomed. Anal. 33 (2003) 157–164.
- [9] F. Foret, V. Šustáček, P. Boček, J. Microcolumn Sep. 2 (1990) 229–233.
- [10] D. Kaniansky, J. Marák, J. Chromatogr. 498 (1990) 191–204.
- [11] D. Kaniansky, J. Marák, V. Madajová, E. Šimuničová, J. Chromatogr. 638 (1993) 137–146.
- [12] D. Kaniansky, F. Iványi, F.I. Onuska, Anal. Chem. 66 (1994) 1817–1824.
- [13] D. Kaniansky, I. Zelenský, A. Hybenová, F.I. Onuska, Anal. Chem. 66 (1994) 4258–4264.
- [14] D. Kaniansky, Capillary isotachopheresis, capillary zone electrophoresis and their on-line coupling. D.Sc. Thesis, Comenius University, Bratislava, 2002.
- [15] M. Danková, D. Kaniansky, S. Fanali, F. Iványi, J. Chromatogr. A 838 (1999) 31–43.
- [16] S. Fanali, C. Desiderio, E. Ölvecká, D. Kaniansky, M. Vojtek, A. Ferancová, HRC J. High Resolut. Chromatogr. 23 (2000) 531–538.
- [17] D. Kaniansky, M. Masár, J. Bielčíková, J. Chromatogr. A 792 (1997) 483–494.
- [18] D. Kaniansky, J. Marák, M. Masár, F. Iványi, V. Madajová, E. Šimuničová, V. Zelenská, J. Chromatogr. A 772 (1997) 103–114.
- [19] J. Szemán, K. Ganzler, A. Salgó, J. Szejtli, J. Chromatogr. A 728 (1996) 423–431.
- [20] M. Fillet, I. Bechet, P. Chiap, P. Hubert, J. Crommen, J. Chromatogr. A 717 (1995) 203–209.
- [21] P.U. Witte, R. Irmisch, P. Hajdu, Int. J. Clin. Pharmacol. Ther. Toxicol. 23 (1985) 59–62.

Enantiospecific resolution of rabeprazole by liquid chromatography on amylose-derived chiral stationary phase using photo diode array and polarimetric detectors in series[☆]

R. Nageswara Rao^{*}, A. Narasa raju, D. Nagaraju

HPLC/UV Group, Division of Analytical Chemistry, Discovery Laboratory Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India

Received 28 October 2005; received in revised form 28 January 2006; accepted 28 January 2006

Available online 3 March 2006

Abstract

A simple and rapid liquid chromatographic method for enantioselective separation and determination of *R*-(+) and *S*-(-) enantiomers of rabeprazole in drugs and pharmaceuticals using photo diode array (PDA) and polarimetric detectors connected in series was developed. Chiralpak AD-H (250 mm × 4.6 mm) 5 μm column packed with amylose tris(3,5-dimethylphenyl carbamate) as a stationary phase and the mobile phase containing *n*-hexane:ethanol:2-propanol(75:15:10, v/v/v) in an isocratic mode has yielded baseline separation with resolution greater than 3.0 at 40 °C. Effects of ethanol, 2-propanol and temperature on separation were studied for optimum resolution. Lansoprazole sulphone was used as an internal standard (IS) for quantitative determination of individual enantiomers in bulk drugs as well as pharmaceutical formulations. The method was validated in terms of accuracy, precision and linearity according to ICH guidelines. The linearity of the method was studied in the range of 0.5–50 μg/ml and the r^2 was >0.9997. The inter- and intra-day precision of assay were determined (R.S.D. < 1%) and the recoveries were in the range of 99.63–100.22% with <1% R.S.D. The limits of detection (LOD) and quantification (LOQ) were 0.02 μg/ml and 0.07 μg/ml for both the enantiomers, respectively.

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Keywords: Rabeprazole; Amylose tris(3,5-dimethylphenylcarbamate); Enantiospecific; PDA; Polarimetric detection

1. Introduction

Rabeprazole, 2-[[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl] methyl] sulfinyl]-1H-benzimidazole (Fig. 1) is one of latest proton-pump inhibitors developed for suppression of gastric acid secretion by inhibition of the H^+/K^+ -ATPase [1]. It is one of the most potent therapeutic agents used for treatment of several gastrointestinal disorders such as gastric and duodenal ulcers [2]. Like other proton-pump inhibitors viz., omeprazole, lansoprazole and pantoprazole, it too has an asymmetric sulfoxide function resulting in a pair of enantiomers of non-superimposable mirror images. However, for therapeutic applications it is administered as a racemic mixture of *R*-(+) and

S-(-) enantiomers orally. Owing to the existence of pharmacological and toxicological differences between stereoisomers, chiral recognition has now become an integral part of drug research and development. Nevertheless, regulatory authorities demand experimental proof of enantiomeric ratio and bioequivalence for registration of new drugs. Analytical methods for separation and determination of the enantiomers of chiral drugs are highly needed. In a policy guidelines presented by the US Food and Drugs Administration (FDA), it was stated that it is essential that a stereo specific assay be used from the beginning of the development of chiral drugs. For a drug product, the FDA released a policy statement that requires the submission of a stereo chemically specific identity test. If an identity test is to be a specific, it must distinguish between enantiomers, or between an enantiomer and the racemate for good manufacturing practices (GMP). Therefore, chiral chromatography has become an important analytical tool for the separation of enantiomers in a racemic mixture and for determining if the “inactive” enantiomer is present in an enantiomerically pure drug or finished product.

[☆] IICT Communication No. 050307.

^{*} Corresponding author. Tel.: +91 40 27193193; fax: +91 40 27160387.

E-mail addresses: rnrao@iictnet.org,
rnrao55@yahoo.com (R. Nageswara Rao).

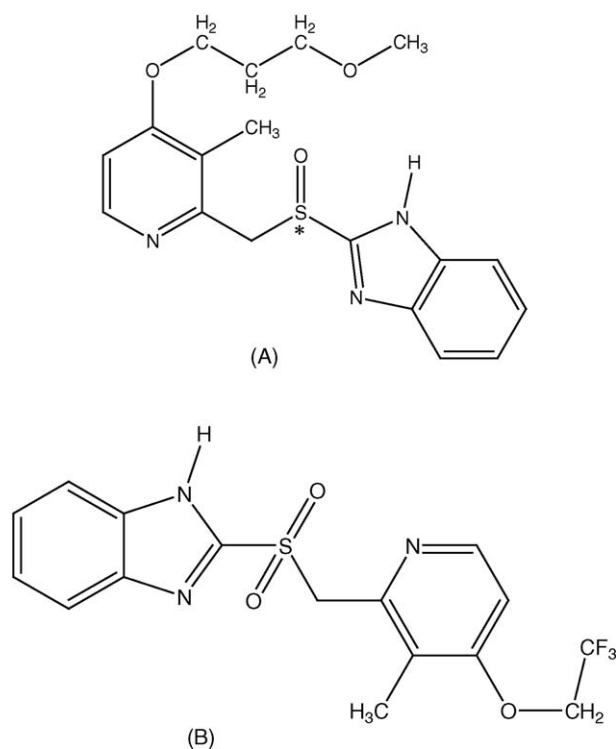


Fig. 1. (A) Rabeprazole; (B) lansoprazole sulphone (internal standard).

The classical method of formation of diastereoisomers to resolve chiral sulfoxides is not easy to perform due to the difficulty of their derivatisation. Polarimetry is also not the method of choice for quantification, mainly because of its low sensitivity. Thus the development of methods for direct chromatographic separation of enantiomers is of growing importance. Among the chromatographic techniques, high-performance liquid chromatography with chiral stationary phases is widely employed for analytical and preparative scale separation of several enantiomeric drugs and pharmaceutical preparations.

A thorough literature search has revealed that a few liquid chromatographic methods involving the use of direct injection and column switching techniques were reported for quantification of rabeprazole in plasma using UV and MS/MS as detectors [3–8]. Non-aqueous capillary electrophoresis was employed for its analysis in biological fluids [9]. El-Gindy et al. [10] have proposed both spectrophotometric and chromatographic methods to investigate the acidic and photo oxidative degradants of rabeprazole in aqueous solutions. However, to the best of our knowledge, so far there were no chiral HPLC methods reported for determination of the enantiomers of rabeprazole in bulk drugs and pharmaceutical formulations.

Several HPLC methods for enantiomeric resolution of asymmetric sulfoxides on different types of chiral stationary phases including the derivatives of cellulose and amylose were reported [11–13]. The enantiomeric separation of omeprazole, lansoprazole and pantaprazole was accomplished using LC and CE on stationary phases derived from proteins and polysaccharides [14–20]. Nevertheless, chiral separation of rabeprazole on some type of chromatographic techniques is not yet reported. Recently, Nozal et al. have studied the use of supercritical fluid

chromatography for enantiomeric separation of several benzimidazole derivatives containing asymmetric sulfoxide function viz., omeprazole, lansoprazole, pantaprazole and rabeprazole on amylose based Chiralpak AD column using SFC grade CO₂ containing alcohol-type organic modifiers [21]. In the present investigation, we describe for the first time a simple and rapid stereo specific HPLC method using Chiralpak AD-H column containing tris(3,5-dimethylphenyl carbamate) amylose as a stationary phase for separation and determination of the rabeprazole enantiomers in bulk drugs and pharmaceutical preparations.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-grade *n*-hexane, 2-propanol (S.D.Fine chem., Mumbai, India) and ethanol (Changshu Yangyuan Chemical, China) were used. Rabeprazole ($\pm$ RS), and Lansoprazole sulphone (2-[[[3-methyl-4-(2,2,2-trifluoro-ethoxy)-2-pyridinyl]methyl]sulfonyl]-1H-benzimidazole) were gifted by M/s Vishnu Chemicals Pvt. Ltd. (Hyderabad, India). Membrane filters of 0.45 μ m were procured from M/s Pall Pharmalab Filtration Pvt. Ltd. (Mumbai, India).

2.2. Apparatus

The HPLC system was composed of two LC-10AT VP pumps, an SPD-M10A VP PDA detector, SIL-10AD VP auto injector, and SCL-10A VP system controller attached with thermostat (all from Shimadzu, Kyoto, Japan). Polarimetric detector (IBZ Messtechnik GMBH, Hannover, Germany) was connected to PDA in series. Chiralcel OD-H (250 mm $\times$ 4.6 mm) 5 μ m column attached with Chiralcel OD (10 mm $\times$ 4.6 mm) guard column and Chiralpak AD-H (250 mm $\times$ 4.6 mm) 5 μ m column attached with Chiralpak AD (10 mm $\times$ 4.6 mm) guard column (Daicel Chemical Industries, Tokyo, Japan) were used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

Chromatographic separation was achieved on Chiralpak AD-H (250 mm $\times$ 4.6 mm) 5 μ m column attached with Chiralpak AD (10 mm $\times$ 4.6 mm) guard column using a mobile phase consisting of *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v) at a flow rate of 1.0 ml/min at 40 °C. Polarimetric detector was used for identification of the enantiomers while the detection was done at 284 nm using PDA.

2.4. Preparation of stock and standard solutions

Stock solutions were prepared by dissolving 50.1 mg of racemic rabeprazole and 49.87 mg of lansoprazole sulphone (internal standard) in the mobile phase in 100 ml volumetric flasks. The flasks were wrapped with aluminum foil and kept in

a refrigerator at 5 °C. Samples and standards were prepared by diluting appropriate volumes of working solutions in 25 ml volumetric flasks with the mobile phase. Standards for calibration were prepared in the range 0.5–50 µg/ml. The specified concentration of each enantiomer and IS were taken as 25 µg/ml and 15 g/ml, respectively.

3. Results and discussion

Rabeprazole is one of the chiral sulfoxides of considerable importance not only in asymmetric synthesis but also as an anti-ulcer drug. Its chirality is due solely to the presence of an asymmetric sulfoxide function resulting in a pair of enantiomers. Development of methods for resolution of these enantiomers are quite important.

3.1. Column selectivity

Preliminary experiments were carried out on a C₁₈ column using β-cyclodextrin as a chiral selector dissolved in mobile phase containing methanol and water. However, these trails could not result in any possible separation of the enantiomers. Later, two different polysaccharide based CSPs were investigated. Polysaccharide derivatives in particular 3,5 dimethylphenyl carbamates of amylose (Chiralpak AD-H) and cellulose (Chiralcel OD-H) show a broad applicability in resolving a number of racemic compounds. Initially, the Chiralcel OD-H column with a mobile phase containing ethanol and 2-propanol as organic modifiers in *n*-hexane was studied for separation. The retention and the resolution data are summarized in Table 1. It could be seen from Table 1 that the enantiomers were retained longer on this column with 2-propanol when compared to ethanol. This is because of the stronger elution strength of ethanol than 2-propanol on polysaccharide derived stationary phases. The enantiomers were separated but the peak shapes were quite broad with poor resolution. So in an attempt to improve the separation and find the best compromise of retention and resolution of enantiomers, amylose based Chiralpak AD-H column was selected. The enantiomers were separated with sharp peaks and good resolution. Thus it was continued for further optimization.

Table 1
Effect of organic modifier on retention and resolution of rabeprazole enantiomers on Chiralcel OD-H column at 25 °C

Mobile phase composition (A:B:C, v/v/v)	k'_1	α	R_s
70:00:30	2.49	1.28	1.74
75:00:25	2.83	1.26	1.76
75:10:15	2.61	1.26	1.96
75:15:10	2.22	1.25	1.93
75:25:00	1.51	1.24	1.51
80:00:20	4.18	1.31	3.12
80:10:10	3.21	1.27	2.19
80:20:00	2.07	1.24	2.13
85:15:00	3.38	1.27	2.44

k'_1 : capacity factor of (+)-rabeprazole; R_s : resolution; α : selectivity; (A:B:C) *n*-hexane:ethanol:2-propanol.

3.2. Mobile phase selectivity

The amount of ethanol in the mobile phase was varied and different compositions were prepared and tried to know its effect on the resolution of enantiomers. The enantiomers were separated with higher retention times. Later 2-propanol was tried as an organic modifier in place of ethanol. Good resolutions were still observed with decreased retention times. An unusual exception was noticed and it was not in accordance with the general retention phenomenon on polysaccharide derived stationary phases. In general, the mechanism of chiral recognition on this type of phases is not well established and an empirical approach based on experimentation is always necessary. However, it is known to some extent that the chiral resolutions were achieved through different hydrogen bonding, π – π , dipole–dipole induced interactions on such stationary phases [22–24]. The electronegative atoms such as nitrogen and oxygen along with the aromatic rings of rabeprazole might participate in hydrogen bonding and dipole–dipole induced interactions with –CO– and –NH– groups of carbamate moieties of amylose-derived stationary phase. Furthermore, there could be π – π interactions of different magnitudes between the substituted phenyl moieties of carbamate and the aromatic rings of rabeprazole enantiomers. Both the solute molecules and organic modifier compete for the active sites of the chiral stationary phase. This phenomenon could be explained by the difference in the steric bulkiness around the hydroxyl moiety of the mobile phase modifier. The less bulky alcohols could be inserted in to the cavity of the CSP more easily than bulkier alcohols. The insertion of the mobile phase modifier into the chiral cavities of the CSP could induce changes in the dominant chiral recognition mechanism leading to formation of more stable diastereomeric complexes with the two enantiomers. So in the presence of ethanol rabeprazole enantiomers might interact strongly with the stationary phase, leading to higher retention on the column. In another attempt to improve the separation and find the best compromise of retention and resolution, different combinations of ethanol and 2-propanol in *n*-hexane were studied. The strength of *n*-hexane was kept constant at 75% and the proportions of 2-propanol and ethanol (for the remaining 25%) were varied to study its effect on resolution and selectivity (Table 2). The effects on capacity factors (k') and resolution (R_s) are shown in Figs. 2 and 3, respectively. It could be seen from Fig. 2 that, as the percentage of ethanol was increased, the retentions were also increased. But the resolution was increased only up to 15% of ethanol. Later the resolution was found to decrease with further increase of ethanol. The optimum resolution was observed with the mobile phase composition of *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v).

3.3. Effect of temperature

The effect of temperature on retention as well as resolution was carried out in the range of 25–40 °C and the results are shown in Fig. 2. As the column temperature was increased, retentions were decreased as expected. These results could be attributed to the fact that the analytes on a molecular level have small adsorption at higher temperatures and migrate fast

Table 2

Effect of temperature and organic modifier on selectivity of rabeprazole enantiomers on Chiralpak AD-H column

Mobile phase composition (A:B:C, v/v/v)	Temperature (°C)			
	25	30	35	40
70:00:30	1.23	1.17	1.17	1.15
75:00:25	1.15	1.17	1.16	1.15
75:05:20	1.15	1.14	1.12	1.15
75:07:18	1.20	1.14	1.14	1.13
75:10:15	1.17	1.16	1.14	1.13
75:15:10	1.09	1.16	1.17	1.28
75:20:05	1.08	1.15	1.13	1.16
75:25:00	1.07	1.13	1.11	1.10
80:00:20	1.17	1.17	1.13	1.14

(A:B:C) *n*-hexane:ethanol:2-propanol.

through the column. The combined effect of temperature and organic modifiers on the resolution of enantiomers is shown in Fig. 3. The results show that an increase in temperature decreases the resolution for all the mobile phase compositions except for *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v)

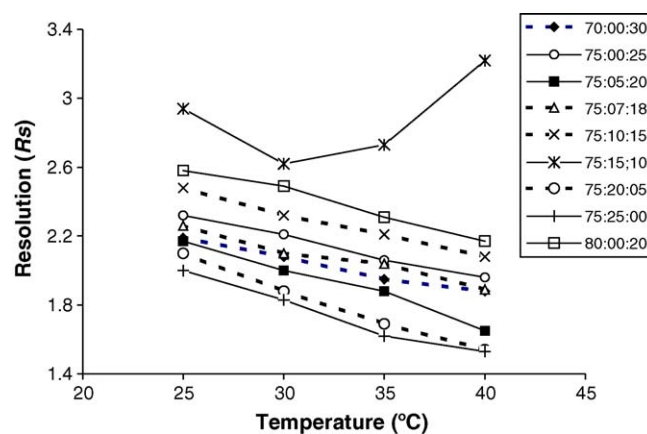


Fig. 3. Effect of temperature and organic modifier on resolution of rabeprazole enantiomers on Chiralpak AD-H column. Note: The ratio values in the inserted box represent the mobile phase composition *n*-hexane:ethanol:2-propanol (v/v/v).

at which the resolution was found to increase ($R_s > 3.0$) with decreased retention at 40 °C. Thus a mobile phase containing *n*-hexane:2-propanol:ethanol (75:10:15, v/v/v) was chosen for the separation of rabeprazole enantiomers on Chiralpak AD-H column maintained at 40 °C. The flow rate was kept at 1.0 ml/min throughout the analysis. Lansoprazole sulphone (IS) was used as an internal standard for quantification of each enantiomer. The chromatographic separation of rabeprazole ($\pm RS$) and IS in the optimized conditions is shown in Fig. 4. The chromatogram showing the identification of the two enantiomers based on their optical rotation using polarimetric detector is given in Fig. 5. The method was validated in terms of accuracy, precision and linearity as per ICH guidelines.

3.4. System suitability

The solution of rabeprazole (10 $\mu\text{g/ml}$) was prepared in the mobile phase and used for system suitability studies. The column was stabilized for 30 min in the optimized conditions and three replicate injections were made. The system was deemed to be suitable if the resolution between the rabeprazole enantiomers was not less than 3.0 and tailing factor was not more than 1.10 (at 10% base). The system suitability data is recorded in Table 3.

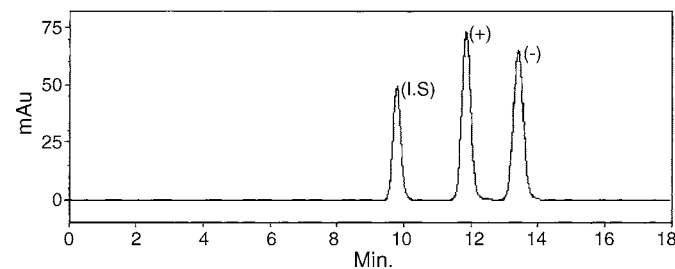


Fig. 4. Typical chromatogram of (+)-rabeprazole, (-)-rabeprazole and internal standard (IS) on Chiralpak AD-H column with *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v) as mobile phase at 40 °C using PDA as a detector.

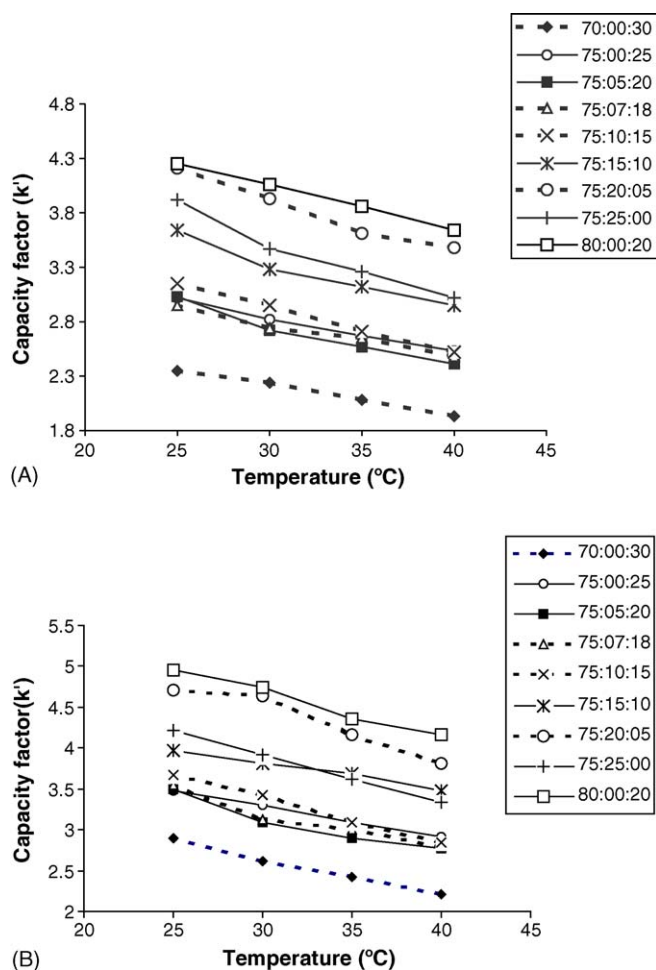


Fig. 2. Effect of temperature and organic modifier on retention of (A) (+)-rabeprazole and (B) (-)-rabeprazole on Chiralpak AD-H column. Note: The ratio values in the inserted box represent the mobile phase composition *n*-hexane:ethanol:2-propanol (v/v/v).

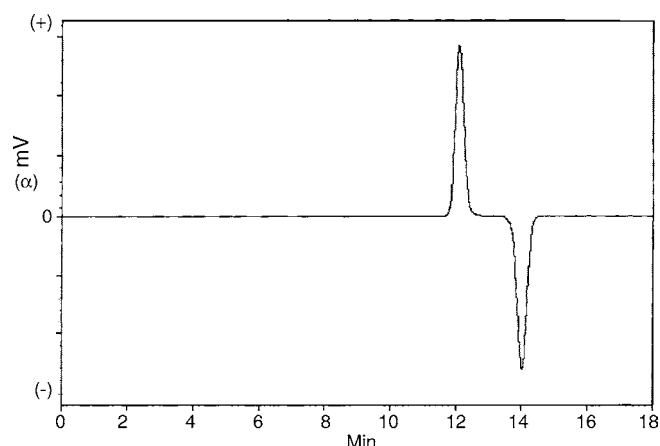


Fig. 5. Typical chromatogram of (+)-rabeprazole and (–)-rabeprazole on Chiralpak AD-H column with *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v) as mobile phase at 40 °C using polarimetric detector.

3.5. Accuracy

The accuracy of the method was determined by analyzing spiked solutions of rabeprazole at five levels in the range 50–150% with respect to the specified concentration of 25 µg/ml in triplicate. The recoveries were in between 99.63 and 100.22% with R.S.D. less than 1%.

3.6. Precision

Precision of the method was tested by preparing six individual solutions of rabeprazole and making triplicate injections for each. The assays were calculated from the peak areas for both the enantiomers and the R.S.D. were found to be less than 0.32%. Inter- and intra-day assay precision was performed by analyzing the solutions for five times in a day for three days and the R.S.D. of the assays were less than 0.41% for both the enantiomers.

3.7. Linearity

Calibration graphs were drawn in the range of 0.5–50 µg/ml of rabeprazole solutions prepared freshly for three days. Curves were linear with $r^2 > 0.9997$ and the regression equations for (+)-rabeprazole and (–)-rabeprazole were $55086x + 1619$ and $55196x - 398$, respectively.

3.8. Robustness

Robustness of the method was checked by making small deliberate changes in the contents of ethanol and 2-propanol in

Table 3
System suitability data using Chiralpak AD-H column with *n*-hexane:ethanol:2-propanol (75:15:10, v/v/v) as mobile phase at 40 °C

Compound	k'	α	R_s	As	N
Rabeprazole (+)	2.95	1.18	3.24	1.07	15036
Rabeprazole (–)	3.47			1.08	15255
Lansoprazole Sulphone (IS)	2.26			1.07	21000

k' : capacity factor; α : selectivity; R_s : resolution; As: tailing factor; N : theoretical plates/meter.

Table 4
Results of analysis in pharmaceutical formulations

Formulation	Claimed value (mg)	(+)-Rabeprazole		(–)-Rabeprazole	
		Found (mg)	Assay (%)	Found (mg)	Assay (%)
Brand-I	20	9.97	49.85	10.03	50.15
Brand-II	20	9.98	49.92	10.02	50.08
Brand-III	20	10.02	50.11	9.98	49.89

the mobile phase. It did not effect the resolution except a small change in retention times. Effect of temperature was also studied by analyzing the sample at 40 ± 2 °C. The resolution remained still above 3.0. Effect of flow rate was studied by analyzing the samples with 0.9 ml/min and 1.1 ml/min flow rates. In both the cases resolution was found to be above 3.0.

3.9. LOD and LOQ

The limits of detection (LOD) and quantification (LOQ) were determined by measuring signal/noise (S/N) ratios. LOD was taken as the concentration of the analyte where S/N was 3 and it was found to be 0.02 µg/ml for both the enantiomers. LOQ was taken as the concentration of analyte where S/N was 10 and it was found to be 0.07 µg/ml for both the enantiomers.

3.10. Determination of enantiomeric ratio of rabeprazole in pharmaceutical formulations

Different tablet formulations containing rabeprazole sodium (equivalent to 20 mg of the active ingredient) were analyzed for determination of enantiomeric ratio by the proposed method of HPLC. The two enantiomers were very well separated in the developed conditions and there was no interference from the excipients in determining the ratio of enantiomers. Three different pharmaceutical formulations were analyzed and the results are recorded in Table 4. A typical chromatogram showing the separation of enantiomers in the formulation is shown in Fig. 6. The interference of excipients such as starch, microcrystalline cellulose and cyclodextrins on separation and determination of rabeprazole enantiomers in pharmaceutical formulations has been evaluated by two different procedures: (i) checking the purity of the enantiomeric peaks at 11.92 and 13.82 by PDA and (ii) recovery after spiking with known amounts of the pure rabepra-

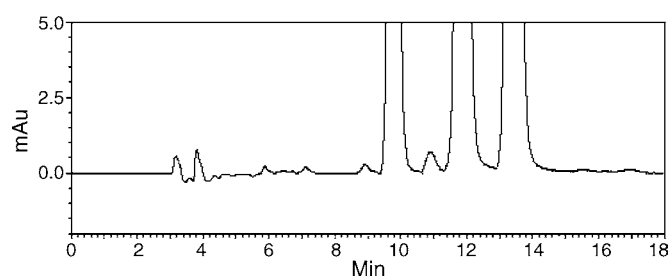


Fig. 6. Typical chromatogram shows well resolved impurities with the both the enantiomers of rabeprazole in pharmaceutical formulations.

Table 5
Recovery studies in pharmaceutical formulations

Amount added (μg/ml)	(+)–Rabeprazole			(–)–Rabeprazole		
	Claimed value (μg/ml)	Found (μg/ml)	Recovery (%) (± R.S.D.)	Claimed value (μg/ml)	Found (μg/ml)	Recovery (%) (± R.S.D.)
10	4.95	4.97	100.40 ± 0.24	5.05	5.01	99.21 ± 0.37
20	9.97	9.95	99.80 ± 0.38	10.03	10.01	99.80 ± 0.45
30	15.02	15.05	100.20 ± 0.72	14.98	15.03	100.33 ± 0.57
40	20.05	20.07	100.10 ± 0.82	19.95	20.06	100.55 ± 0.29
50	24.96	24.99	100.12 ± 0.78	25.04	24.98	99.76 ± 0.94

zole to the formulation. The on-line PDA spectra were recorded and peak purity was checked. The peak purity was found to be 0.9996 and 0.9994 for (+)-rabeprazole and (–)-rabeprazole peaks, respectively. It indicates that the excipients do not interfere with the separation of enantiomers in the formulations. Further, their interference on quantitative determination has been evaluated by recovery studies by spiking known amounts of rabeprazole enantiomers to formulations at different levels in the range of 10–50 μg/ml. The analytical results thus obtained by the developed method are recorded in Table 5. The recoveries were in the range of 99.21–100.55% with R.S.D. less than 0.94%. These results also suggest that there is no interference from excipients in determining the rabeprazole enantiomers in formulations. Thus the developed method was found to be quite simple, rapid and reliable for determination of enantiomeric ratio and the assay of rabeprazole in tablet formulations.

4. Conclusions

The enantiospecific resolution of rabeprazole, a relatively new proton-pump inhibitor on polysaccharide based chiral stationary phases was studied. The amylose-derived Chiralpak AD-H has shown better resolution when compared to the cellulose based Chiralcel OD-H column. The effects of organic modifiers viz., ethanol and 2-propanol and the temperature on the resolution and retention of enantiomers were evaluated. The optimized conditions were validated in terms of accuracy, precision and linearity as per ICH guidelines. The developed method was found to be quite simple, rapid, and sensitive for enantioselective separation and determination of rabeprazole enantiomers in bulk drugs and pharmaceuticals.

Acknowledgement

The authors thank Dr. J.S. Yadav, Director, Indian Institute of Chemical Technology for encouragement and permission to communicate the results for publication.

References

- [1] M. Morii, H. Takata, H. Fujisaki, N. Takeguchi, *Biochem. Pharmacol.* 39 (1990) 661–667.
- [2] M. Rabinson, J. Horn, *Drugs* 63 (2003) 2739–2754.
- [3] H. Nakai, Y. Shimamura, T. Kanazawa, S. Yasuda, M. Kayano, *J. Chromatogr. B* 660 (1994) 211–220.
- [4] S. Takakuwa, S. Chiku, H. Nakata, T. Yuzuriha, N. Mano, N. Asakawa, *J. Chromatogr. B* 673 (1995) 113–122.
- [5] N. Mano, Y. Oda, S. Takakuwa, S. Chiku, H. Nakata, N. Asakawa, *J. Pharm. Sci.* 85 (1996) 903–907.
- [6] N.V.S. Ramakrishna, K.N. Vishwottam, S. Wishu, M. Koteswara, S. Suresh Kumar, *J. Chromatogr. B* 816 (2004) 209–214.
- [7] Y. Zhang, X. Chen, D. Qi Gu, Zhong, *Anal. Chim. Acta* 523 (2004) 171–175.
- [8] S.S. Singh, M. Jain, H. Shah, H. Gupta, P. Thakker, R. Shah, B.B. Lohray, *J. Chromatogr. B* 813 (2004) 247–254.
- [9] A. Tivesten, S. Folestad, V. Schonbacher, K. Svensson, *Chromatographia* 49 (1999) S7–S11.
- [10] A. El-Gindy, F. El-Yazby, M.M. Maher, *J. Pharm. Biomed. Anal.* 31 (2003) 229–242.
- [11] S.A. Matlin, M.E. Tiritan, A.J. Crawford, Q.B. Cass, D.R. Boyd, *Chirality* 6 (1994) 135–140.
- [12] S.A. Matlin, M.E. Tiritan, Q.B. Cass, D.R. Boyd, *Chirality* 8 (1996) 147–152.
- [13] Q.B. Cass, F. Batigalha, *J. Chromatogr. A* 987 (2003) 445–452.
- [14] R.M. Orlando, P.S. Bonato, *J. Chromatogr. B* 795 (2003) 227–235.
- [15] H. Stenhoff, A. Blomqvist, P. Lagerstrom, *J. Chromatogr. B* 734 (1999) 191–201.
- [16] A.M. Cairns, H.-Y. Rita, J.D. Chiou, J.L. Rogers, Demetriades, *J. Chromatogr. B* 666 (1995) 323–328.
- [17] M. Miura, H. Tada, T. Suzuki, *J. Chromatogr. B* 804 (2004) 389–395.
- [18] H. Katsuki, A. Hamada, C. Nakamura, K. Arimori, M. Nakano, *J. Chromatogr. B* 757 (2001) 127–133.
- [19] D. Eberle, R.P. Hummel, R. Kuhn, *J. Chromatogr. A* 759 (1997) 185–192.
- [20] Q.B. Cass, A.L.G. Degani, N.M. Cassiano, J. Pedrazolli Jr., *J. Chromatogr. B* 766 (2001) 153–160.
- [21] M. Jesus del Nozal, L. Toribio, L. Jose, C. Bernal, Alonso, J. Juan, Jimenez, *J. Sep. Sci.* 27 (2004) 1023–1029.
- [22] I.W. Wainer, M.C. Alembic, *J. Chromatogr.* 358 (1986) 85–93.
- [23] I.W. Wainer, R.M. Stiffin, *J. Chromatogr.* 411 (1987) 139–151.
- [24] C. Yamamoto, E. Yahshima, Y. Okamoto, *Bull. Chem. Soc. Jpn.* 72 (1999) 1815–1825.

A principal component analysis based method to discover chemical differences in comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC $\times$ GC-TOFMS) separations of metabolites in plant samples

Karisa M. Pierce^{*}, Janiece L. Hope, Jamin C. Hoggard, Robert E. Synovec

Department of Chemistry, Box 351700, University of Washington, Seattle, WA 98195, USA

Received 7 December 2005; received in revised form 25 January 2006; accepted 25 January 2006

Available online 28 February 2006

Abstract

Comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GC $\times$ GC-TOFMS) provides high resolution separations of complex samples with a mass spectrum at every point in the separation space. The large volumes of multidimensional data obtained by GC $\times$ GC-TOFMS analysis are analyzed using a principal component analysis (PCA) method described herein to quickly and objectively discover differences between complex samples. In this work, we submitted 54 chromatograms to PCA to automatically compare the metabolite profiles of three different species of plants, namely basil (*Ocimum basilicum*), peppermint (*Mentha piperita*), and sweet herb stevia (*Stevia rebaudiana*), where there were 18 chromatograms for each type of plant. The 54 scores of the m/z 73 data set clustered in three groups according to the three types of plants. Principal component 1 (PC 1) separated the stevia cluster from the basil and peppermint clusters, capturing 61.84% of the total variance. Principal component 2 (PC 2) separated the basil cluster from the peppermint cluster, capturing 16.78% of the total variance. The PCA method revealed that relative abundances of amino acids, carboxylic acids, and carbohydrates were responsible for differentiating the three plants. A brief list of the 16 most significant metabolites is reported. After PCA, the 54 scores of the m/z 217 data set clustered in three groups according to the three types of plants, as well, yielding highly loaded variables corresponding with chemical differences between plants that were complementary to the m/z 73 information. The PCA data mining method is applicable to all of the monitored selective mass channels, utilizing all of the collected data, to discover unknown differences in complex sample profiles.

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Keywords: Principal component analysis; Complex sample analysis; GC $\times$ GC-TOFMS; Metabolites

1. Introduction

Researchers in the field of metabolomics aim to qualitatively and quantitatively describe the collection of all low molecular weight molecules found in a cell that support general cell life functions and participate in metabolic reactions [1–3]. To this end, researchers strive to provide methods for analyzing all metabolites present in a sample using a single comprehensive analytical technique. Common techniques include UV, IR, and FTIR spectroscopy [4], nuclear magnetic resonance [5,6], liquid chromatography [7], capillary electrophoresis [8], and liquid

chromatography or gas chromatography combined with mass spectrometry (GC–MS) [9,10].

In 2004, Weckwerth et al. used the multivariate analysis techniques principal component analysis (PCA) and hierarchical cluster analysis to distinguish GC–MS separations of metabolite extracts from different *Arabidopsis* plants [11]. This technique provided objective classification and quantification of metabolite profiles within a single species from GC–MS chromatograms. These researchers used LECO Pegasus software to deconvolute and identify 652 metabolites for 19 chromatograms. However, deconvolution of an entire chromatogram requires a large amount of computation time, and that time requirement is multiplied for large data sets of numerous chromatograms.

In general, metabolite researchers currently spend time deconvoluting all overlapped peaks in the entire chromatogram

^{*} Corresponding author. Tel.: +1 206 616 2918.

E-mail address: piercek1@u.washington.edu (K.M. Pierce).

as the very first step in the analysis process. However, in 2004, Jonsson et al. made it a point to use non-processed GC-TOFMS data for the comparison of a large series of aspen extract chromatograms (84 chromatograms) [12]. Jonsson et al. showed metabolic differences between aspen tree leaves at three different developmental stages grown under two different photoperiods by using supervised partial least squares discriminant analysis (PLS-DA) on GC-TOFMS fingerprints of the aspen tree leaf samples. Jonsson demonstrated the alternative to skip the time consuming step of deconvoluting all the data and instead start with multivariate analysis of the raw data, and then deconvolute only select areas of interest as targeted by the multivariate analysis.

Recently, researchers have begun to apply third order instrumentation to metabolomics problems. The GC $\times$ GC-TOFMS instrument can selectively and sensitively determine a large number of substances in a single analysis of a very complex sample. In 2004, Sinha et al. demonstrated that GC $\times$ GC-TOFMS also provides trilinear data that is well suited for analyzing complex samples using the chemometric technique parallel factor analysis (PARAFAC) for deconvoluting overlapped subregions [13]. PARAFAC analysis of the GC $\times$ GC-TOFMS separations of Huilmo (*Sisyrinchium striatum*) grass metabolite extracts provided quantitative information for pure metabolites of interest in very complex regions of the chromatogram where peaks were overlapped. In a second study, Sinha et al. again employed the trilinear nature of GC $\times$ GC-TOFMS separations of human infant urine metabolite extracts in order to use a dot-product mass spectral matching algorithm to rapidly locate and quantify target analytes for disease profiling [14]. At the same time, Hope et al. demonstrated that GC $\times$ GC-TOFMS was amenable to the PARAFAC analysis of rye grass metabolite responses to pre- and post-harvest trauma [15].

Then, in 2005, Welthagen et al. used GC $\times$ GC-TOFMS to analyze metabolite profiles of obese NZO and lean control mouse spleen samples for biomarker identification [16]. These researchers used LECO ChromaTOF software for deconvolution and peak identifications as well as intensive manual comparisons to create a list of identified and quantified metabolites for the obese and lean control mice. This resulted in elucidation of biomarkers for obesity.

In the work reported herein, GC $\times$ GC-TOFMS instrumentation is combined with the multivariate analysis technique PCA to quickly analyze and compare a large number of complex chromatograms and objectively extract information that describes the chemical differences between samples. Using this PCA method to find and deconvolute subregions of interest can be done in a matter of minutes to a couple of hours while the conventional deconvolution of entire chromatograms often requires several hours. PCA works by translating the chromatographic data from n -dimensional variable (chromatographic) space into principal component (PC) space where there is one score describing each chromatogram in the data set in the new, much lower dimensional, PC space [17,18]. The PCs are sorted based on their eigenvalues and oriented such that they span the greatest amount of variation inherent in the scores. The eigenvalue is the magnitude of each PC determined by the sum of squares of the scores

on that PC. Thus the eigenvalue quantifies the amount of variance captured by the PC. Then the PCs that capture the greatest variation in the chromatograms (PCs with the greatest eigenvalues) are retained while the PCs that contain only noise are discarded (PCs that have the smallest eigenvalues). The eigenvalue for each PC is also commonly converted to a percentage of the total sum of squares of the entire dataset so the user can decide how many PCs to use for modeling significant chemical (class-to-class) variations and discarding the noise (within-class) variations. Also, the cosine of the angle between each original variable axis and each new PC (loadings value) is calculated. For variables (in chromatographic space) that varied the most, those particular variables had the greatest influence on the orientation of the PCs and the loadings values were greatest in magnitude. PCA provides a scores plot that displays the chemical differences between chromatograms while providing a loadings plot that correlates with how much variation was found in each variable. The aim in the current work is to use PCA data mining of GC $\times$ GC-TOFMS chromatograms to objectively and quickly discover the natural chemical differences of organic acid extracts of basil (*Ocimum basilicum*), peppermint (*Mentha piperita*), and sweet herb stevia (*Stevia rebaudiana*). PARAFAC deconvolution is then used for accurate quantification of the analytes of interest.

2. Experimental

2.1. Sampling, extraction, derivatization

Organic acid extractions of the plant tissues were performed using the procedure described in detail by Hope et al. [15]. Two basil plants (*Ocimum basilicum*), two peppermint plants (*Mentha piperita*), and two sweet herb stevia plants (*Stevia rebaudiana*) were obtained (Molbak's, Woodinville, WA, USA) where the plants were grown on commercial potting soil. For each plant, a leaf from the top, a leaf from the middle, and a leaf from the bottom of the stem were cut and immediately quenched and homogenized in liquid nitrogen. Three 100 mg aliquots of each leaf tissue (2 plants $\times$ 3 leaf positions $\times$ 3 aliquots = 18 total samples) were mixed with 1 ml extraction solution (chloroform/methanol/water; 1:3:1, all chemicals from Sigma Aldrich, St. Louis, MO, USA) and sat at room temperature for 1 h, then sonicated for 10 min. The samples were centrifuged to form a pellet of particulate matter and 200 μ l of the supernatant was evaporated to dryness. Next, 30 μ l of methoxyamine hydrochloride (15 mg/ml) in pyridine was added to the sample. After sitting 1 h at room temperature, the sample was trimethylsilylated for another hour at room temperature by adding 30 μ l of BSTFA with 1% TMCS. After trimethylsilylation where the trimethylsilyl (TMS) groups replace highly polar hydroxyl groups, 30 μ l of heptane was added. This procedure resulted in the extraction of TMS-derivatized carboxylic acids, TMS-derivatized carbohydrates, and TMS-derivatized amino acids.

2.2. GC $\times$ GC-TOFMS

The TMS-derivatized samples were analyzed using a LECO Pegasus 4D GC $\times$ GC-TOFMS instrument (LECO Corporation,

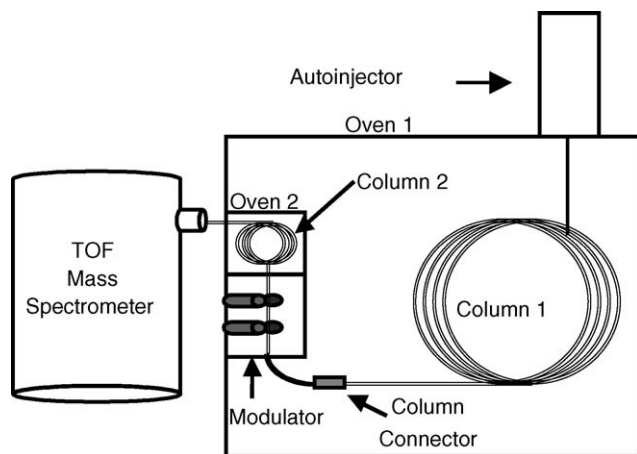


Fig. 1. Schematic of the GC $\times$ GC-TOFMS.

St. Joseph, MI, USA) equipped with a cryogenic modulator, shown in Fig. 1. The first dimension chromatographic column (column 1) was 20 m $\times$ 250 μ m $\times$ 0.5 μ m DB5 (Alltech, Deerfield, IL, USA). The second dimension chromatographic column (column 2) was 2 m $\times$ 180 μ m $\times$ 0.2 μ m Rtx200 (Restek, Bellefonte, PA, USA). Ultra high purity helium (0.8 ml/min) was used as the carrier gas in constant flow mode. The Agilent 7683 autosampler (Agilent, Palo Alto, CA, USA) injected 0.2 μ l of sample splitless in a 270 $^{\circ}$ C inlet onto column 1. The column 1 oven was held at 70 $^{\circ}$ C for 2 min, then ramped at 10 $^{\circ}$ C/min to 270 $^{\circ}$ C and held for 5 min. The column 2 oven was a constant 20 $^{\circ}$ C higher than the column 1 oven. No mass spectra were collected during the solvent delay for the first 7 min of each run. Chromatographic runs were 20 min long. The modulation period (P_M) was 1 s [19]. Each of the 18 samples were run in triplicate resulting in 54 GC $\times$ GC-TOFMS data files. The transfer line was at 290 $^{\circ}$ C and the ion source set point was 200 $^{\circ}$ C. The detector voltage was -1600 V and the filament bias was -70 V. Mass spectra were collected from m/z 60 to 600 at 100 spectra/s.

2.3. Data processing

Raw data were exported as comma separated value (.csv) files and loaded into Matlab 6.5 (The Mathworks, Natick, MA, USA) for data processing. The GC $\times$ GC-TOFMS produces a three-dimensional array of data for each sample run but PCA requires a row vector representation for each sample. Thus, by using only one mass channel at a time, the three dimensional array is reduced to a two-dimensional matrix and this matrix is then unfolded into the row vector representation for each sample prior to PCA. PCA was applied to mass channel (m/z) 73 chromatograms. All of the chemical compounds that underwent trimethylsilylation produce a fragment ion of m/z 73. PCA was also applied to m/z 217 chromatograms. Mass spectral searches were performed using the full mass spectrum. Mass spectral similarity searches were performed using NIST MS search 2.0 (NIST/EPA/NIH Mass Spectral Library, NIST 02). The NIST main library and a library built in-house were used. Mass spectral match values are the average of the match values given by ChromaTOF software for sample replicates. The m/z chromatograms

were individually baseline corrected and normalized to the sum of the total ion current signal. The chromatograms were submitted to comprehensive two-dimensional retention time alignment in Matlab prior to PCA to get rid of slight retention time variations, but the original peak retention times were referred to in the ChromaTOF files for mass spectral information [20]. The PCA algorithm was from the PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA) and was operated in Matlab. Quantification of component peak volumes was achieved by PARAFAC peak deconvolution and quantification software from the PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA) in order to address the possibility of overlapping compounds biasing the concentration ratios. The unimodality and non-negativity constraints were used for PARAFAC. The number of PARAFAC components to model was determined by choosing the model with the number of components with the smallest residuals out of possible models with one to four components. An in-house written Matlab program was used to locate chromatographic peaks in the two-dimensional data array where peaks were defined as locations in the chromatogram where the derivative of the chromatographic signal was equal to zero, the zero crossing was at a local maximum, and the peak signal was greater than five times the standard deviation of the baseline noise for m/z 73 data.

3. Results and discussion

Fig. 2A is a typical GC $\times$ GC-TOFMS separation of basil at m/z 73. The chemical components of the basil extract were widely distributed throughout the chromatographic plane. Many components were present in this natural sample and the chromatographic peak heights ranged over three orders of magnitude. Fig. 2B is an apex plot of the basil chromatogram. Each of the 54 m/z 73 chromatograms used in this study contained over 1400 peaks according to the program used to find local peak maxima. It will be shown that PCA data mining can be used as a first step to successfully compare such complex chromatograms and locate metabolites that significantly differed between species for the 54 chromatograms.

PCA scores plots were used to objectively compare metabolite profiles. The scores plot obtained from submitting the combined 18 basil, 18 peppermint, and 18 stevia m/z 73 chromatograms to PCA is shown in Fig. 3A. The scores clustered in three groups according to the three types of plants. Principal component 1 (PC 1) separated the stevia cluster from the basil and peppermint clusters and captured 61.84% of the total variance. Principal component 2 (PC 2) separated the basil cluster from the peppermint cluster and captured 16.78% of the total variance. To a smaller extent, PC 2 separated the stevia scores from the peppermint and basil scores. The eigenvalues for the first 20 PCs are plotted in Fig. 3B. The first two PCs had the greatest eigenvalues and thus contained the chemically relevant variance, so PC 1 and PC 2 were used for further data analysis. The remaining PCs had eigenvalues that contained a significantly smaller portion of the total variance and thus captured noise variations.

Locations of highly loaded variables corresponded with locations of chromatographic peaks that most influenced the clus-

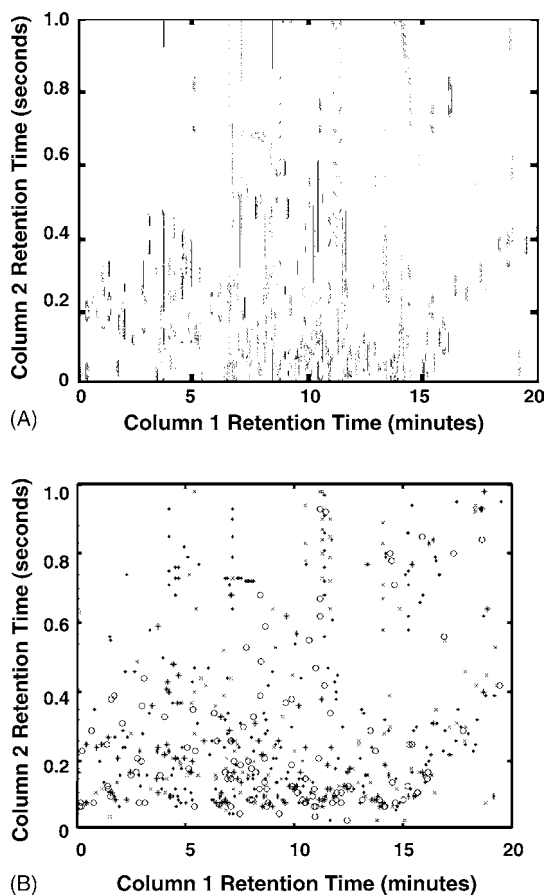


Fig. 2. Typical GC $\times$ GC-TOFMS separation of basil. (A) Components of the basil extract are widely distributed throughout the chromatographic plane in this contour plot of a GC $\times$ GC-TOFMS basil chromatogram. (B) Over 1400 component peaks appear in each of the GC $\times$ GC-TOFMS separations. Peak locations in the m/z 73 chromatogram are displayed in this peak apex plot, generated with a program that identifies local peak maxima: (*) indicates peaks with heights 80–100% of the height of the largest peak in the chromatogram (i.e. maximum); (○) indicates peaks of heights 60–80% of maximum; (×) indicates peaks of heights 40–60% of maximum; (·) indicates peaks 15 σ to 40% of maximum.

tering in the PCA scores plot. This means that the highly loaded variables indicated the chemical compounds that distinguished sample types. Fig. 4A is a contour plot of the loadings on PC 1 for PCA of the m/z 73 chromatograms. In Fig. 4A, the top 10 most highly loaded variables on PC 1 were marked by ovals. These were the locations of sample components that most distinguished stevia from both basil and peppermint. Fig. 4B is a contour plot of the loadings on PC 2, calculated for the scores plot in Fig. 3A. A square indicated the location of the top ten most highly loaded variables on PC 2. These were the locations of metabolites that most distinguished basil from peppermint, and to a lesser extent, stevia from the other two plants. The most highly loaded variables marked by ovals and squares were later used as a “template” that was projected onto the original 54 m/z 73 chromatograms to objectively and quickly locate sample components that distinguished the plants from each other.

PCA can, in principle, be applied to all selective mass channels. Here, we demonstrate the PCA method for the use of two mass channels in order to describe the concept. The m/z 217

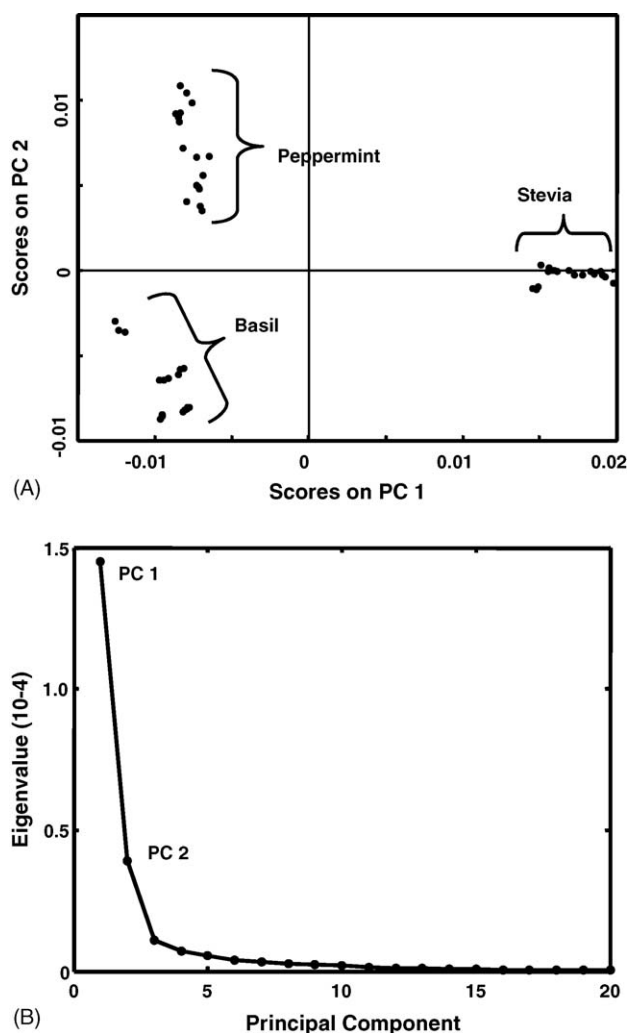


Fig. 3. (A) The scores plot obtained from PCA of 18 basil, 18 peppermint, and 18 stevia GC $\times$ GC-TOFMS m/z 73 chromatograms demonstrates differentiation between-species based on metabolite profiles. PC 1 captured 61.84% of the variance and PC 2 captured 16.78% of the variance. (B) Eigenvalues for the first 20 PCs. PC 1 and PC 2 capture the significant chemical variations.

chromatograms were extracted from the 54 original GC $\times$ GC-TOFMS replicates and submitted to PCA. PCA results of the m/z 217 chromatograms are shown in Fig. 5A as a plot of scores on PC 1 versus scores on PC 3. PC 1 captured 67.80% of the total variance, PC 2 captured 16.21% of the variance, and PC 3 captured 6.05% of the variance. PC 1 separated both the peppermint and basil scores from the stevia scores. This means that the highly loaded variables on PC 1 mostly described metabolites that made stevia different from both peppermint and basil. PC 3 separated the peppermint scores from the basil scores, so the highly loaded variables on PC 3 correspond with metabolite differences between basil and peppermint. The eigenvalues for the first 20 PCs are plotted in Fig. 5B. The first three PCs had the greatest eigenvalues and thus contained the chemically relevant variance, so PC 1 and PC 3 were used for data mining. The remaining PCs had eigenvalues that contained a significantly smaller portion of the total variance and thus captured noise variations. Fig. 5C is a contour plot of the loadings on PC 1 for

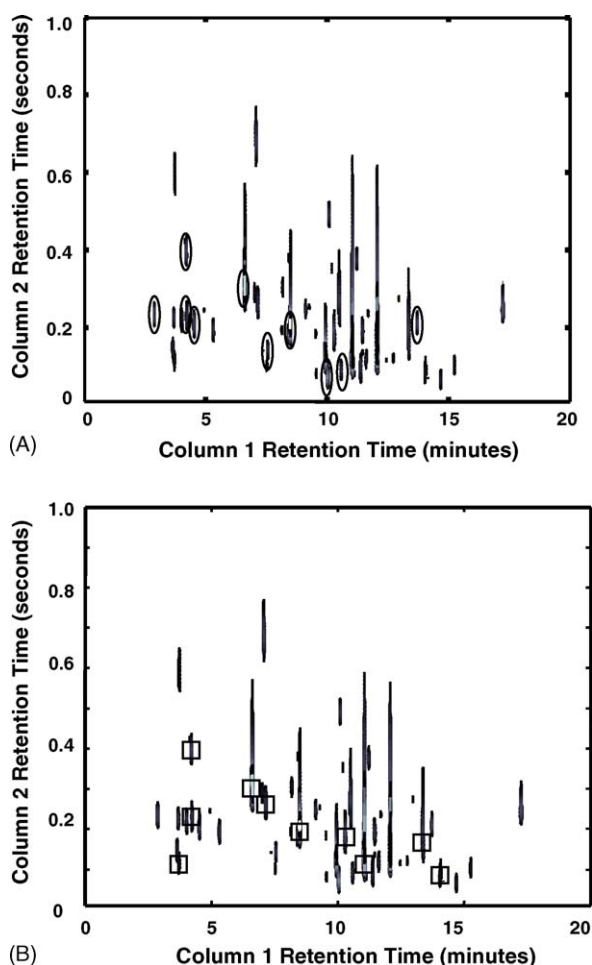


Fig. 4. Loadings plots obtained from PCA of the 18 basil, 18 peppermint, and 18 stevia GC $\times$ GC-TOFMS chromatograms. (A) Contour plot of a subset of the loadings on PC 1 provided by PCA of the 54 m/z 73 chromatograms. The loadings shown have an absolute value greater than 0.005. Ten of the most highly loaded variables are marked by an oval and are listed in Table 1. (B) Contour plot of the loadings on PC 2 provided by PCA of the 54 m/z 73 chromatograms. The loadings shown have an absolute value greater than 0.005. Ten of the most highly loaded variables are marked by a square and are listed in Table 2.

submission of the m/z 217 chromatograms to PCA. The loadings for m/z 73 in Fig. 4A were different than the loadings for m/z 217 in Fig. 5C. This implies that m/z other than the derivatization fragment ion m/z may contain selective information that is beneficial for discovering other significant chemical components or to target families of sample components. The remainder of this report describes the PCA method applied to only m/z 73, but this method is applicable to all m/z for full utilization of the entire set of data that was collected. Although it has never been demonstrated for GC $\times$ GC-TOFMS metabolites data, a method called hierarchical PCA could conceivably be applied to this type of GC $\times$ GC-TOFMS data where other mass channels that give complementary information to the derivatization fragment can be added into the model [21]. Then by doing several PCA models based on a subset of all the mass channels, the scores from all the PCA models could be combined to form a new matrix, upon which a final PCA model is based yielding abstract representations of the original variables that distinguished the samples. The work reported herein, however, strives to start with the initial step of showing that the PCA information in selective mass channels directly identifies the metabolites that statistically differ among the GC $\times$ GC-TOFMS profiles.

In Fig. 6A, the template of highly loaded variables (from Fig. 4A and B) was projected onto a contour plot of a typical basil m/z 73 chromatogram. The template located chromatographic peaks that were significantly different among the sample replicates. Two of the peaks marked by ovals had very low intensity in basil but had very high intensity in stevia. For this reason, peaks at the following column 1 retention time (^1t_R) and column 2 retention time (^2t_R) were not visible in the m/z 73 chromatogram of basil (Fig. 6A), but the peaks are much more intense and visible in the m/z 73 chromatogram of stevia (Fig. 6B): $^1t_R = 10.0$ min and $^2t_R = 0.08$ s and $^1t_R = 13.7$ min and $^2t_R = 0.21$ s. The highly loaded peaks were identified by mass spectral library matching. The 10 peaks that were most highly loaded on PC 1 are listed in Table 1 along with the average mass spectral match value where the match value could range from 0 to 999, with 999 being the best match between the observed spectrum and a library spectrum. Also listed in Table 1 is the average abundance of each metabolite in stevia relative to

Table 1
Highly loaded component peaks for between-species metabolite profiling

^1t_R (min)	^2t_R (s)	Component	MS match value	PC 1 loadings	Relative abundance stevia/basil (%RSD)
2.9	0.20	Valine,(TMS)	960	0.0179	0.32 (4.2)
4.2	0.20	Glycine,bis(TMS)	950	0.0181	0.33 (3.9)
4.2	0.40	Succinic acid	955	0.0135	0.45 (5.4)
4.5	0.18	Propanoic acid,bis(TMS)	934	0.0172	0.53 (3.7)
6.6	0.30	Malic acid,tris(TMS)	943	0.0440	0.43 (9.1)
7.3	0.11	Threonic acid,tetra(TMS)	890	0.0143	0.71 (3.7)
8.5	0.20	Tartaric acid,bis(TMS)	946	0.0466	0.26 (6.6)
10.0	0.08	Carboxylic acid,tris(TMS)	892	0.0186	1.29 (1.9)
10.9	0.09	Quinic acid,(TMS)	846	0.0394	5.32 (1.6)
13.7	0.21	d-Xylopyranose,tetra(TMS)	813	0.0183	1.41 (1.3)

List of highly loaded component peaks on PC 1 for PCA of 18 basil, 18 peppermint, and 18 stevia GC $\times$ GC-TOFMS chromatograms. Average signals of metabolites are listed for stevia samples relative to basil samples. The metabolites are quantified by averaging the deconvoluted total chromatographic peak signal over the top leaf replicate runs for each plant species ($n=6$).

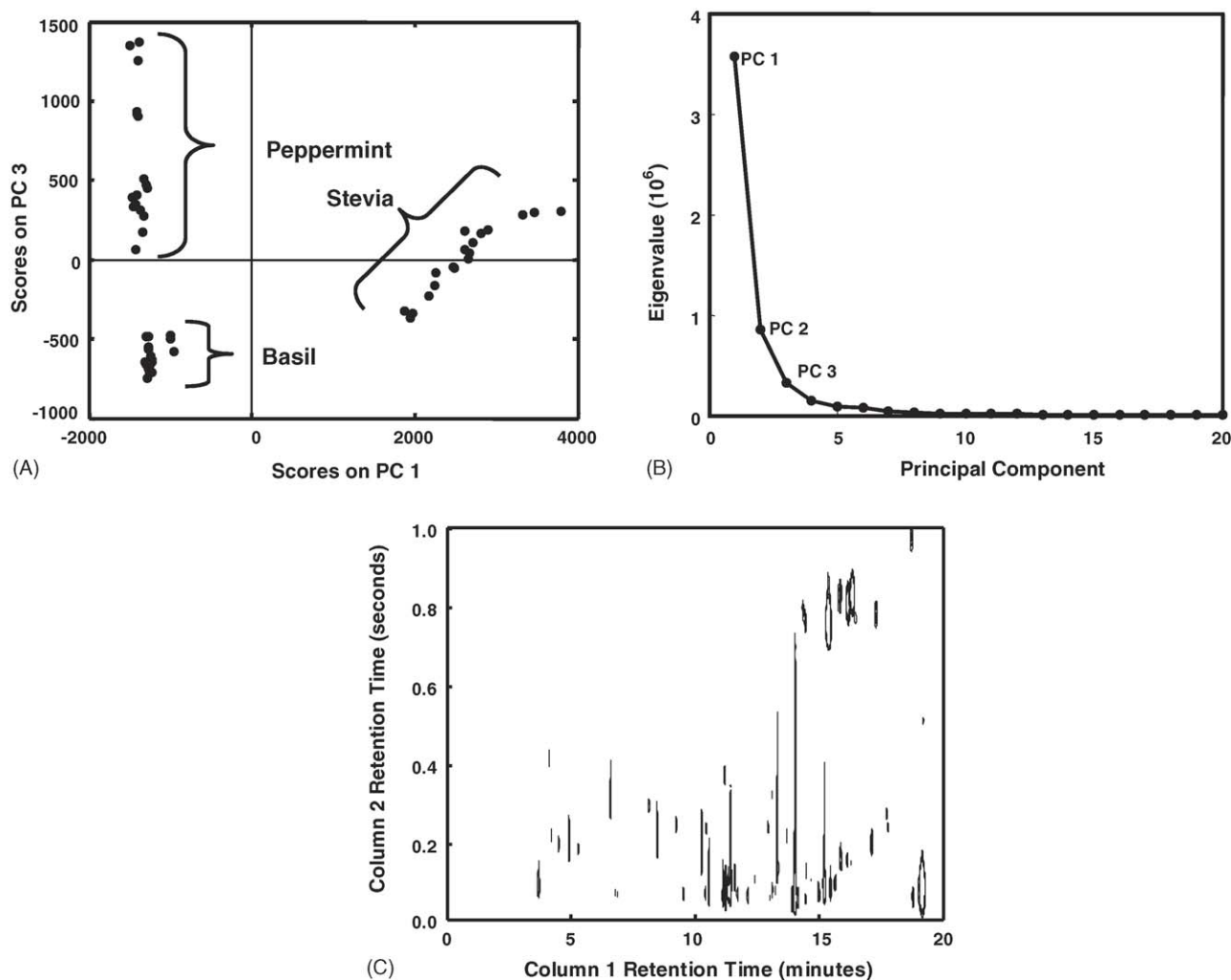


Fig. 5. PCA for m/z 217. (A) Scores plot from PCA of the 54 m/z 217 chromatograms. (B) Eigenvalues for the first 20 PCs. PC 1, PC 2, and PC 3 capture the significant chemical variations. (C) Contour plot of the loadings on PC 1 provided by PCA of the 54 m/z 217 chromatograms. The loadings shown have an absolute value greater than 0.005.

basil. Note that all metabolites were separated and analyzed as TMS derivatives. The 10 peaks that were most highly loaded on PC 2 are listed in Table 2. Also listed in Table 2 is the average abundance of each metabolite in peppermint relative

to basil. For both PC 1 and PC 2, the highly loaded metabolites responsible for the between-species variations included amino acids, sugars, and carboxylic acids. Four of the top ten most highly loaded chemical compounds on PC 2 were also in the

Table 2
Highly loaded component peaks for between-species metabolite profiling

¹ <i>t</i> _R (min)	² <i>t</i> _R (s)	Component	MS match value	PC 2 loadings	Relative abundance pepper/basil (%RSD)
3.7	0.19	Leucine,bis(TMS)	939	0.0299	0.47 (1.9)
4.2	0.40	Succinic acid	943	0.0346	1.32 (1.7)
4.2	0.20	Glycine,bis(TMS)	928	0.0344	0.37 (1.5)
6.6	0.30	Malic acid,(TMS)	895	0.0345	1.45 (1.8)
7.1	0.27	Butanoic acid,bis(TMS)	897	0.0312	0.30 (1.8)
8.5	0.20	Tartaric acid,bis(TMS)	947	0.0509	0.53 (1.7)
10.2	0.17	Citric acid,propanetricarboxylic(TMS)	917	0.0410	1.50 (0.7)
10.9	0.09	Quinic acid,(TMS)	785	0.0418	3.88 (1.4)
13.3	0.14	Myoinositol,hexa(TMS)	894	0.0353	1.70 (1.6)
14.1	0.10	Glycoside,tetra(TMS)	837	0.0303	1.89 (1.2)

List of highly loaded component peaks on PC 2 for PCA of 18 basil, 18 peppermint, and 18 stevia GC × GC-TOFMS chromatograms. Average signals of metabolites are listed for peppermint samples relative to basil samples. The metabolites are quantified by averaging the deconvoluted total chromatographic peak signal over the top leaf replicate runs for each plant species ($n = 6$).

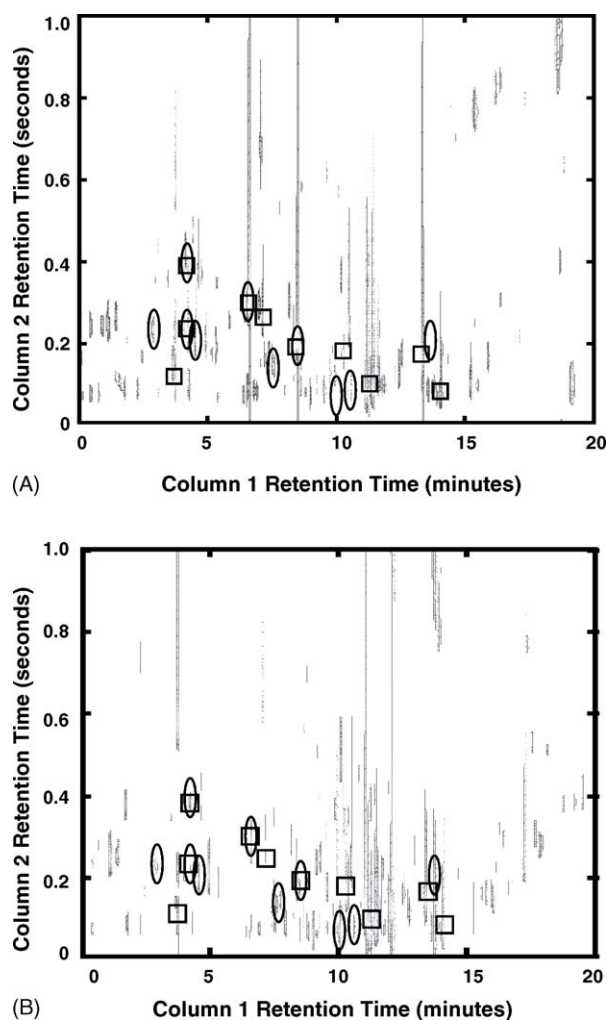


Fig. 6. Highly loaded variable locations projected onto (A) a typical basil m/z 73 chromatogram and (B) a typical stevia m/z 73 chromatogram. The highly loaded variable locations are used to identify the sample components that had the most influence on the clustering in the scores plot in Fig. 2. These metabolites cover a wide range of the separation space and are responsible for differentiating the three plants.

top ten list for highly loaded chemical compounds on PC 1. For demonstration purposes, note that out of over 1400 readily apparent chemical components in each of 54 separations, the PCA method presented herein objectively and quickly discovered the 16 most significant compounds that differentiated the three samples.

The chemical differences between the basil, peppermint, and stevia samples that were used to illustrate the PCA method were large since three different species were compared. In the future, the limitations of the PCA method in terms of how similar the samples can be before the method fails should be rigorously studied. For example, Jeong et al. quantified metabolite differences between GC–MS separations of aspen leaves at three different developmental stages (young, expanding, mature) [22]. The metabolites they quantified were amino acids, dicarboxylic organic acids, and sugars. Most of the amino acids they found decreased in concentration as tissues grew from “young”

to “mature.” In 1998, Fischer et al. also reported that young spinach leaves had more amino acids and fructose compared to mature spinach leaves using HPLC data [23]. The trend of having higher concentrations of metabolites in younger leaf tissues than in older leaf tissues is found in other specific types of plants [23–25]. In the future it will be interesting to quantify such amino acids, sugars, and organic acids using the PCA method applied to GC $\times$ GC-TOFMS separations of plant tissues from different developmental stages. Given suitable tissue samples, the extraction and derivatization method described in our current work would be appropriate for GC $\times$ GC-TOFMS separations.

4. Conclusions

The 54 m/z 73 chromatograms each contained over 1400 peaks. The PCA data mining method produced a list of the metabolites that significantly differentiated the samples from this large volume of data. The PCA data mining method is applicable to all selective m/z . This allows the analyst to utilize the entire set of data that was collected to quickly locate analytes of interest for deconvolution, quantification and discovery of the significant differences of complex samples.

References

- [1] W.B. Dunn, D.I. Ellis, *Trends Anal. Chem.* 24 (2005) 285.
- [2] L.W. Sumner, P. Mendes, R.A. Dixon, *Phytochemistry* 62 (2003) 817.
- [3] A. Guttman, M. Varoglu, J. Khandurina, *Drug Discovery Today* 9 (2004) 136.
- [4] S.G. Oliver, M.K. Winson, D.B. Kell, F. Baganz, *Trends Biotechnol.* 16 (1998) 373.
- [5] R. Bligny, R. Douce, *Curr. Opin. Plant Biol.* 4 (2001) 191.
- [6] J.B. Bundy, D.J. Spurgeon, C. Svendsen, P.K. Hankard, *FEBS Lett.* 521 (2002) 115.
- [7] E. von Roepenack-Lahaye, T. Degenkob, M. Zerjeski, M. Franz, U. Roth, L. Wessjohann, J. Schmidt, D. Scheel, S. Clemens, *Plant Physiol.* 134 (2004) 548.
- [8] A. Garcia, C. Barbas, R. Aguilar, M. Castro, *Mol. Diagn. Genet.* 44 (1998) 1905.
- [9] A. Barsch, T. Patschkowski, K. Niehaus, *Funct. Integr. Genomics* 4 (2004) 219.
- [10] M. Tetsuo, C. Zhang, H. Matsumoto, I. Matsumoto, *J. Chromatogr. B* 731 (1999) 111.
- [11] W. Weckwerth, K. Wenzel, O. Fiehn, *Proteomics* 4 (2004) 78.
- [12] P. Jonsson, J. Gullberg, A. Nordstrom, M. Kusano, M. Kowalczyk, M. Sjostrom, T. Moritz, *Anal. Chem.* 76 (2004) 1738.
- [13] A.E. Sinha, J.L. Hope, B.J. Prazen, C.G. Fraga, E.J. Nilsson, R.E. Synovec, *J. Chromatogr. A* 1056 (2004) 145.
- [14] A.E. Sinha, J.L. Hope, B.J. Prazen, E.J. Nilsson, R.M. Jack, R.E. Synovec, *J. Chromatogr. A* 1058 (2004) 209.
- [15] J.L. Hope, B.J. Prazen, E.J. Nilsson, M.E. Lidstrom, R.E. Synovec, *Talanta* 65 (2005) 380.
- [16] W. Welthagen, R.A. Shellie, J. Spranger, M. Ristow, R. Zimmermann, O. Fiehn, *Metabolomics* 1 (2005) 65.
- [17] R.G. Brereton, *Chemometrics: Data Analysis for the Laboratory and Chemical Plant*, Wiley, New York, 2003.
- [18] K.R. Beebe, *Chemometrics: A Practical Guide*, Wiley, New York, 1998.
- [19] P. Schoenmakers, P. Marriott, J. Beens, *LCGC Eur.* 1 (2003) 1.
- [20] K.M. Pierce, L.F. Wood, B.W. Wright, R.E. Synovec, *Anal. Chem.* 77 (2005) 7735.

- [21] S. Wold, N. Kettaneh, K. Tjessem, J. Chemometr. 10 (1996) 463.
- [22] M.L. Jeong, H. Jiang, H.S. Chen, C.J. Tsai, S.A. Harding, Plant Physiol. 136 (2004) 3364.
- [23] E.S. Fischer, G. Lohaus, D. Heineke, H.W. Heldt, Physiol. Plantarum 102 (1998) 16.
- [24] A.G. Roberts, S.S. Cruz, P. Boevink, I.M. Roberts, N. Sauer, K.J. Oparka, Scottish Crop Research Institute, Annual Report, 1999, p. 76.
- [25] B.K. Singh, Plant Amino Acids, Marcel Dekker, Inc., New York, 1999.

Worldwide interlaboratory study on the determination of ochratoxin A in different wine type samples

N. Ratola^a, P. Barros^b, T. Simões^c, A. Cerdeira^d, A. Venâncio^e, A. Alves^{a,*}

^a LEPAE, Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

^b IVV, Instituto da Vinha e do Vinho, Rua de Serpa Pinto 571–1, 4400-307 Vila Nova de Gaia, Portugal

^c IVDP, Instituto dos Vinhos do Douro e Porto, Rua de Ferreira Borges 27, 4050-253 Porto, Portugal

^d CVRVV, Comissão de Viticultura da Região dos Vinhos Verdes, Rua da Restauração 318, 4050-501 Porto, Portugal

^e Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Received 15 January 2006; received in revised form 8 May 2006; accepted 17 May 2006

Available online 3 July 2006

Abstract

Interlaboratory studies are decisive tools to help the validation of a specific analytical methodology or to assess the reproducibility of the use of different methods to analyze a given compound or compounds in certain sample matrices. In this work, homogeneous samples of two white wines (“White Wine” and “White Liqueur Wine”) and one red wine (“Red Fortified Wine”) from Portugal with different production techniques and characteristics, namely in alcohol strength (10.5%, 16.0% and 19.0% ethanolic content, respectively), were analyzed for their contents in ochratoxin A (OTA), a mycotoxin generated from fungal contamination. White Liqueur Wine was naturally contaminated, whereas the other two wine type were spiked with ethanolic OTA solutions. The participation of 24 laboratories from 17 countries of five continents was ensured for this study. Although with no restrictions in terms of analytical methodology to employ, 75% of the laboratories resorted to immunoaffinity columns clean-up followed by high performance liquid chromatography with fluorescence detection (HPLC-FD), most of them in accordance with the European Standard EN 14133. For White Wine samples, the general mean OTA concentration was 1.96 µg/l (two outliers) with interlaboratorial standard deviation (s_L) of 0.53 µg/l; for White Liqueur Wine, mean of 1.59 µg/l (one outlier), with s_L = 0.59 µg/l; and for Red Fortified Wine, mean of 2.73 µg/l (no outliers), with s_L = 0.96 µg/l. Outliers were determined by Cochran and Grubbs tests. The Horrat index, recommended by the Association of Official Analytical Chemists (AOAC) for the quality assurance of the collaborative study was, on average, 1.7. This study proved that OTA determination in wines is reproducible, regardless of the methodology employed.

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Keywords: Ochratoxin A; Wines; Interlaboratory study; z -Scores; Outliers

1. Introduction

Ochratoxin A (OTA) is a mycotoxin (fungal contamination-derived metabolites) produced by strains of *Aspergillus* and *Penicillium*, and recently detected in several food matrices, including alcoholic beverages. Since first reported in wines by Zimmerli and Dick in 1995 [1], the pressure for seeking food safety urged OIV to recommend a maximum limit of 2 µg/l for safe intake in wines [2]. The European Union recently adopted the value of 2.0 µg/kg as maximum residue level of OTA for wine (red, white and rosé) [3]. OTA has been classified as pos-

sessing nephrotoxic, carcinogenic, teratogenic, genotoxic and immunotoxic properties [4], amongst other hazardous effects. The main problem is that the routes of OTA intake for humans come from several food sources rather than from a specific acute one. Nevertheless, various data have been published in literature reporting OTA levels in wines ranging from a few nanograms to several micrograms per litre worldwide [5–18].

Different analytical methodologies have been proposed for OTA screening, mainly comprising liquid–liquid or immunoaffinity extraction steps, previous to liquid chromatographic analysis with fluorescence detection (HPLC-FD) [19,20]. Other detection methods used include mass spectrometry (MS/MS) [19,21] and photodiode array detection (PDA) [17]. Different clean-up procedures such as anion exchange [22] and molecular imprinted polymers (MIP) [23] were also

* Corresponding author. Tel.: +351 22 5081883; fax: +351 22 5081449.
E-mail address: aalves@fe.up.pt (A. Alves).

recently reported. A reference method has been approved in 2003 by the European Committee for Standardization (CEN)—EN 14133 [24]. In general, immunoaffinity columns are recommended as clean-up procedure of OTA from wines and beers, prior to HPLC-FD analysis. The official method adopted by the Association of Official Analytical Chemists (AOAC) is also based on these techniques [25].

Interlaboratory studies aiming the validation of the reference method or focusing on proficiency objectives (ring-tests) have been performed for wine matrices, especially when alcoholic contents round up to 12% (v/v).

A proficiency test has been conducted on FTIR wine analysis with the participation of six laboratories [26]. The wine sample had an average 12% in alcohol strength. Reproducibility was evaluated for several analytical determinations such as density, ethanolic content, dry extract, total sugars, total acidity, pH, and total polyphenolic index. An interlaboratory study featuring six laboratories compared the determination of ethyl carbamate in alcoholic beverages (red, white and fortified wines, brandy and wine spirit) by HPLC–FLD and GC–MS [27].

The European Standard EN 14133:2003 reports the results of an interlaboratory test according to AOAC guidelines for collaborative study procedures to validate characteristics of a method of analysis for OTA quantification in wines [24]. The study was conducted in 1999, with the participation of 16 laboratories, strictly applying the aforementioned standard. There is no information concerning the type of wines analysed, apart from the citation that the samples were white wine, red wine or beer, spiked at three concentration levels around 0.1–0.2 ng/ml, 0.9–1.1 ng/ml and 2.0–3.0 ng/ml, and one naturally contaminated sample (mean concentration of 0.283 ng/ml for white wine and 1.690 ng/ml for red wine).

The Bureau InterProfessionnel d'Etudes Analytiques (BIPEA) periodically organizes proficiency tests on several wine determinations, following ISO Guide 43:1997 [28]. It is probably the organization that covers a wider range of wine types [29]. For physico-chemical analysis, red wines, dry white wines, sparkling wines, dessert wines, rosé wines, aromatized wines have been included. Contaminants such as pesticides and mycotoxins are among the determinations. However, statistical reports are sent to the participating laboratories and therefore no statistical data is divulged.

Other matrices of study and reference materials were also reported in literature as a target for similar interlaboratory collaborations, such as sewage sludge [30], wastewater [31], water [32], saliva [33], ambient air [34], plants [35] or other food products [36–38]. A review on interlaboratory studies applied to analytical chemistry aiming their different purposes and evaluation methods was published by Hund et al. [39].

The importance of such interlaboratory studies, beyond the validation of an analytical method, is nowadays seen as an important contribution to the estimation of the global uncertainty associated to the results. Concerning the complex nature of many modern methods of analysis, proficiency testing schemes allowing laboratory specific standard operating procedures (SOPs), are more to the point than method-evaluating schemes like ISO 5725:1994 [40]. Several authors describe the calculation of the

uncertainty with the inclusion of the interlaboratory variability [41–44].

In view of these foundations, in this work it is proposed to study three different wine matrices, which have undergone dissimilar vinification processes and therefore having different alcohol strength:

- Red Fortified Wine and White Liqueur Wine, in which fermentation is arrested before completion by alcohol distillate addition, allowing sugar and alcohol content to be higher (around 150 g/l total sugars and 18–19% alcohol strength (v/v)).
- White Wine, in which fermentation is complete, thus having lower sugar and alcoholic content (less than 0.3 g/l sugars and 9% alcohol strength). In the specific case of these samples, the acidity is slightly higher than in common table wines.

Being aware of the importance of interlaboratory studies to assess the fiability of OTA determination methods in alcoholic beverages, two Portuguese entities, LEPAE—Process Engineering, Environment and Energy Laboratory (a research group from the Faculty of Engineering of the University of Porto, FEUP) and ALABE, an Association of the Laboratories of Enology, organised such a study. ALABE acted as the guarantee of confidentiality of the process and LEPAE/FEUP performed all statistical treatment of data.

2. Experimental

2.1. Organization of the study

The main steps of this Interlaboratory Study are presented in Table 1.

2.2. Samples analyzed

The study was designed to comprise different types of wines and different levels of OTA contamination, both natural and spiked. As aforementioned, three different wines were chosen. In order for the participating laboratories to analyze all samples in triplicate, the necessary amount of each sample was sent, varying from 250 ml to 750 ml, according to the requirements of the methodologies employed by the different participants. Spiking was done with 9% and 18% ethanolic solutions of OTA.

Table 1
Steps of the ochratoxin A (OTA) interlaboratory study

Step	Action	Time frame
1	Last call for participants	May 2004
2	Setting of participating entities	May–June 2004
3	Samples preparation	June 2004
4	Samples delivery	June 2004
5	Experimental analysis	June–October 2004
6	Reception of the results	June–October 2004
7	Statistic analysis	November 2004
8	Final report/presentation of the results	December 2004

Table 2
Participating laboratories and responsible, sorted alphabetically by country

Participating laboratories	Country	Responsible/contact person
Food Science Australia	Australia	Peter Varelis
Institute of Analytical Chemistry University of Vienna	Austria	Norbert Maier Wolfgang Lindner
Laboratory of Food Analysis Faculty of Pharmaceutical Sciences Ghent University	Belgium	Sarah De Saeger Carlos Van Peteghem
Instituto Adolfo Lutz Seção de Química Biológica	Brazil	Myrna Sabino Sandra Navas Valter Rivieri
ITAL, Núcleo de Microbiologia	Brazil	Marta Taniwaki
Liquor Control Board of Ontario, QA Laboratory	Canada	Carol Drummond
Departamento de Bromatología, Nutrición y Dietética Facultad de Farmacia Universidad de Concepción	Chile	Mario Vega Gisela Rios Roberto Saelzer
Unit of Toxicology Institute for Medical Research and Occupational Health	Croatia	Ana-Marija Domijan
Alko Inc., Alcohol Control Laboratory	Finland	Pekka Lehtonen
Laboratoire de Génie Chimique UMR 5503 CNRS/INPT/UPS)	France	Ahmed Lebrihi
Landesuntersuchungsamt, Institut für Lebensmittelchemie	Germany	Paul Majerus
Department of Microbiology Faculty of Sciences University of Szeged	Hungary	János Varga József Téren
Department of Postharvest Science ARO The Volcani Center	Israel	Amnon Lichter
Centro de Engenharia Biológica Universidade do Minho	Portugal	Armando Venâncio
INETI, LIA (Laboratório de Indústrias Alimentares)	Portugal	Luísa Calhau Cristina Saldanha
Instituto dos Vinhos do Douro e do Porto	Portugal	Tomás Simões
LABIAGRO, Laboratório Químico, Agroalimentar e Microbiológico, Lda	Portugal	Cristina Tendinha
Laboratório de Engenharia de Processos, Ambiente e Energia (LEPAE) Faculdade de Engenharia Universidade do Porto	Portugal	Arminda Alves Nuno Ratola
Department of Analytical Chemistry Faculty of Chemistry and Chemical Technology University of Ljubljana	Slovenia	Irena Kralj Cigić Matija Strlič
Centro Tecnológico Nacional de la Conserva	Spain	Esther Matencio
Departamento de Bromatología, Tecnología de Alimentos y Toxicología Facultad de Farmacia Universidad de Navarra	Spain	Adela López de Cerain
Department of Analytical Chemistry Faculty of Pharmacy University of Anadolu	Turkey	Muzaffer Tunçel Göksel Altiokka
Central Science Laboratory	UK (England)	Susan MacDonald
R-Biopharm Rhone Ltd.	UK (Scotland)	Carol Donnelly Martin Gallagher

The spiked amounts were targeted to the vicinity of the OIV recommended limit.

2.3. Participating institutions

Following the Call for Participants, 24 entities from all over the world kindly presented their will to contribute to this study. The list of the participating laboratories is presented in Table 2.

2.4. Analytical methods employed

As the purpose of this study was not the validation of a given method, several analytical procedures were employed by the different laboratories to analyze the content of OTA in the wine samples. The initially proposed method was the reference method described by the European Standard EN 14133 [24], but the participants were free to choose another. The important thing to notice is the principles of the method, rather than the specific nuances of minor differences in operating values. In this case, the reference method basically consists in the dilution of the wine samples with a solution containing polyethylene glycol (PEG) and sodium hydrogen carbonate, followed by filtration and clean-up by immunoaffinity columns (IAC). OTA is eluted with methanol and quantified by reversed-phase HPLC (mobile phase: water–acetonitrile–acetic acid, 99:99:2) with fluorescence detection (FD) at 333 nm (excitation) and 460 nm (emission). This method was chosen by 50% of the partici-

pants. Another 25% employed the same principle, only using a phosphate buffer solution (PBS) instead of PEG. One of the participants using this method quantified at 225 nm (emission). The remaining six laboratories used somewhat different procedures. Table 3 indicates the methods employed and the number of laboratories using them, with some remarks when major modifications to the EN 14133 strategy were made.

2.5. Statistic analysis

The statistic analysis of the results, namely the detection and elimination of outliers, the precision parameters evaluation (repeatability and reproducibility), means and *z*-scores were based in the standard ISO 5725:1994 [40], the Guide ISO 43:1997 [28] and the Guide EURACHEM “Use and interpretation of proficiency testing (PT) schemes by laboratories” [45] and comprised several steps now detailed.

2.5.1. Assessment of consistency and outliers

This procedure was done to find out and deal with the presence of inconsistent values, outliers or other irregularities. Global mean and respective confidence interval (at 95%) were calculated for each level and parameter for all laboratories before the scrutiny for outliers, performed by numerical tests. First, an upper-tail Cochran test is repeatedly performed, for the comparison of the interlaboratory variances, and consequent elimination of laboratories with interlaboratory variability significantly

Table 3
Methodologies employed by the laboratories in the analyses of OTA

Method employed	Number of laboratories	Remarks
EN 14133 PEG-IAC-HPLC/FD)	12	
PBS-IAC-HPLC/FD	6	PBS, phosphate buffer solution One lab at 225 nm (emission)
SPE-HPLC/FD	2	SPE, solid-phase extraction One lab using octadecylsilyl (ODS) cartridges for extraction; quantification at 385 nm (emission) with reverse-phase Ultracarb(30) column; mobile phase 1% acetic acid in 50% aqueous acetonitrile (0.65 ml/min flowrate); pH of column effluent adjusted to 11; injection volume: 50 µL One lab determined OTA with 3 standard additions; Hypersil ODS 5 column; injection volume: 20 µL
MIP-SPE-HPLC/FD	1	MIP, molecular imprinted polymer First 1 ml-RP-SPE cartridge (100 mg) and then SPE cartridge packed with OTA-specific MIP (100 mg) Reverse-phase HPLC with Synergy Max-RP 80A column; mobile phase: water/methanol/acetic acid (57:43:2, v/v/v) at 0.80 ml/min; injection volume: 50 µL
SPE-HPLC/PDA	1	PDA, photo diode array SPE with C18 cartridges and quantification with C18 column
DI-IS-HPLC	1	DI, direct injection IS, internal standard (diflunisal) Direct injection of samples after addition of diflunisal; C18 column with mobile phase acetonitrile/water/acetic acid (40:60:1, v/v/v) at 0.4 ml/min
SAX-LC/MS-MS	1	SAX, strong anion exchange columns Elution with 5 ml ethylacetate (1% formic acid) MS, mass spectrometry detection Triple quadrupole MS operating in positive electrospray ionization with multiple reaction monitoring; Alltima C18 column; mobile phase: gradient mixtures of 0.3% formic acid in acetonitrile and 0.3% formic acid in water; injection volume: 20 µL

superior to the mean (within laboratory consistency). Grubbs tests for single and double outliers are then applied to check if there are laboratories with means significantly different from the remaining, and eliminate them (between-laboratory variability). Care was taken as to rule out only a number of laboratories up to 2/9 of the total, meaning not more than 5 in this study, for each of the three samples analyzed.

Cochran test is unilateral, once only the laboratories with variability above the mean are ruled out, and not the ones below [46]. The Grubbs tests allow the detection of anomalous mean values and are performed using the values retained from the within-laboratory consistency evaluation. In the case of the single outlier test, the occurrence of one outlying result, either the smallest or the largest, is evaluated [46].

As always throughout this study, laboratories with stragglers were maintained, whereas laboratories with outliers were ruled out.

2.5.2. Evaluation of precision and general mean

After the elimination of outliers for each wine sample type, values for general mean, $\hat{m}$, and variances S_L^2 (interlaboratory), S_r^2 (repeatability) and S_R^2 (reproducibil-

ity), were calculated according to standard ISO 5725:1994 [40].

2.5.3. Evaluation of laboratory performance (z-scores)

Considering all participant laboratories and to have an indication on their performance on this study, z-scores were calculated [40] and graphically displayed for each of the three different sample types.

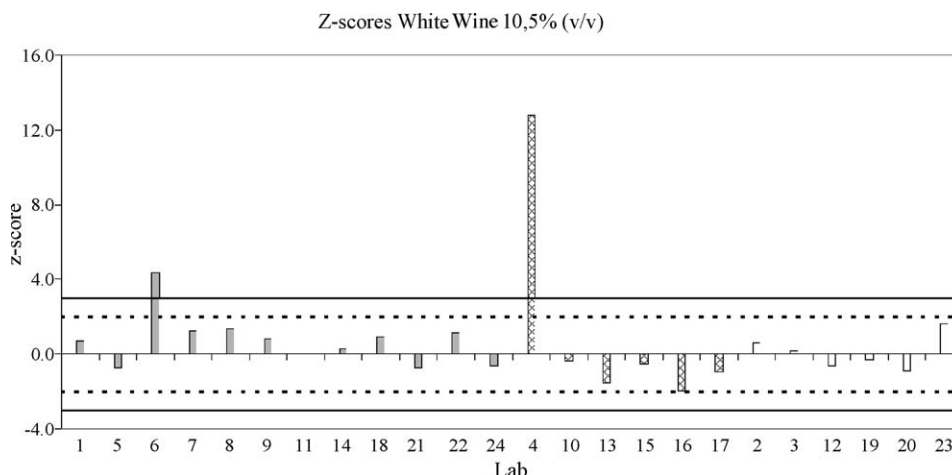
3. Results and discussion

The organisation of the interlaboratory study results was planned to emphasise the comparison of the values obtained by the 24 participant laboratories, for the analysis of OTA in the three types of wine samples conveyed (White Wine, White Liqueur Wine and Red Fortified Wine). The confidentiality of the results was assured by ALABE.

Following the elimination of outliers, the parameters for precision, the means and respective standard deviations in the OTA quantification were calculated for each sample type. Individual and mean results are presented for each laboratory considered for each sample type, as well as the stan-

Table 4
Statistical parameters regarding OTA quantification, for White Wine samples (spiked)

Laboratory	Assay 1 ($\mu\text{g/l}$)	Assay 2 ($\mu\text{g/l}$)	Assay 3 ($\mu\text{g/l}$)	Method	Mean ($\mu\text{g/l}$)	Standard deviation	z-Score	Outlier
1	2.19	2.40	2.52	EN 14133	2.37	0.17	0.8	
2	2.27	2.30	2.29	SPE-HPLC/FD	2.29	0.02	0.6	
3	2.10	2.04	2.07	MIP-SPE-HPLC/FD	2.07	0.03	0.2	
4	9.85	8.56	8.50	PBS-IAC-HPLC/FD	8.97	0.76	12.8	Cochran
5	1.60	1.66	1.42	EN 14133	1.56	0.12	-0.7	
6	4.43	4.48	4.18	EN 14133	4.36	0.16	4.4	Grubbs
7	2.70	2.70	2.50	EN 14133	2.63	0.12	1.2	
8	2.70	2.70	2.70	EN 14133	2.70	0.00	1.4	
9	2.49	2.51	2.20	EN 14133	2.40	0.17	0.8	
10	1.70	1.70	1.90	PBS-IAC-HPLC/FD	1.76	0.12	-0.3	
11	2.07	1.71	2.15	EN 14133	1.98	0.23	0.0	
12	1.64	1.59	1.65	DI-IS-HPLC	1.63	0.03	-0.6	
13	1.11	1.09		PBS-IAC-HPLC/FD	1.10	0.01	-1.6	
14	2.13	2.23	2.00	EN 14133	2.12	0.11	0.3	
15	1.70	1.64	1.73	PBS-IAC-HPLC/FD	1.69	0.05	-0.5	
16	0.80	0.80	1.00	PBS-IAC-HPLC/FD	0.87	0.12	-2.0	
17	1.53	1.51	1.35	PBS-IAC-HPLC/FD	1.46	0.10	-0.9	
18	2.50	2.45	2.46	EN 14133	2.47	0.03	0.9	
19	1.93	1.82	1.63	SAX-LC/MS-MS	1.79	0.15	-0.3	
20	1.52	1.56	1.42	SPE-HPLC/PDA	1.50	0.07	-0.8	
21	1.53	1.29	1.90	EN 14133	1.57	0.31	-0.7	
22	2.58	2.60	2.60	EN 14133	2.59	0.01	1.2	
23	3.00	2.60	2.90	SPE-HPLC/FD	2.83	0.21	1.6	
24	1.56	1.60	1.76	EN 14133	1.64	0.11	-0.6	
Accepted laboratories					22 out of 24			
General mean					1.96			
Standard deviation of the general mean					0.53			
Relative standard deviation (%)					27.30			
Maximum value (accepted laboratories)					2.83			
Minimum value (accepted laboratories)					0.87			
s_L (interlaboratorial standard deviation)					0.53			
s_r (repeatability standard deviation)					0.13			
s_R (reproducibility standard deviation)					0.55			

Fig. 1. z -Scores White Wine 10.5% (v/v).

dard deviation of means and respective performance indicator (z -score). Other parameters displayed comprise the global mean, relative and absolute standard deviation for the different samples, variances (interlaboratorial, repeatability and reproducibility), as well as maximum and minimum values.

3.1. White Wine (10.5%, v/v)

Table 4 shows the different statistical parameters for all laboratories, for White Wine samples. Outliers are also referred, and the test used to assess them.

As can be seen, two laboratories were excluded in this case, and the general mean of the accepted ones was $1.96 \mu\text{g/l}$, with relative standard deviation (R.S.D.) of 27.3%.

Fig. 1 depicts the z -scores for all laboratories. Notice that, unlike Table 4, results are divided by three different groups of methods. Grey columns represent EN 14133 (12 labs), crossed columns stand for PBS-IAC-HPLC/FD (6 labs) and finally, the remaining methods were joined in another group, named “other methods” (white columns,

6 labs). Furthermore, the mean ($2.35 \mu\text{g/l}$) and the confidence intervals are referred to all participants, before outlier tests.

As expected, the outliers (labs 4 and 6) show the worst results and are considered incorrect according to the z -scores test. There seems to be no clear relation between the results and the methods employed. Only with some effort, and before the outlier tests, the most deviant results can be globally attributed to the PBS-IAC-HPLC/FD group.

When removing the outliers, the mean values match the parameters of Table 4 above, and it is noticeable in Fig. 2 that EN 14133 laboratories produce the highest percentage of results outside the confidence intervals. Considering the individual mean of each of the three groups, the PBS-IAC-HPLC/FD has the most deviant behaviour comparing to the general mean (-29.8%), followed by the EN 14133 ($+11.5\%$) and the other methods ($+3.0\%$).

The relative standard deviation for reproducibility (27.3%) was slightly higher than the 14.3% published in the European Standard 14133 for white wines [24], probably because several alterations or completely different analyti-

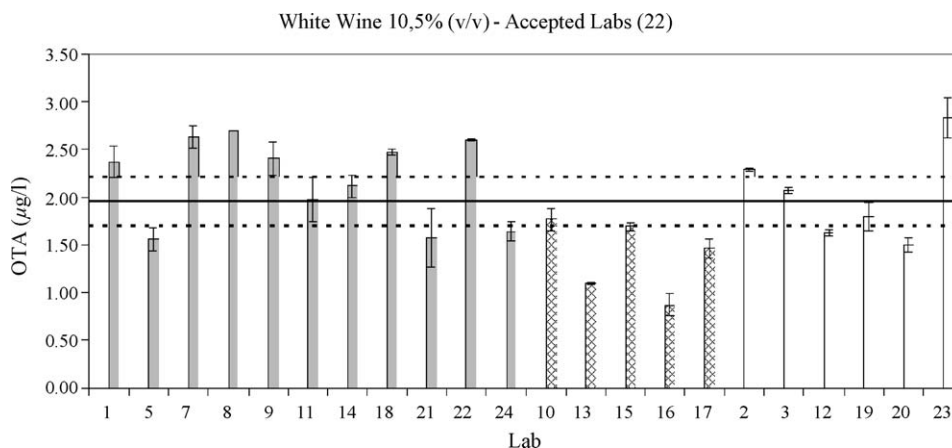


Fig. 2. White Wine 10.5% (v/v)—accepted labs (22).

Table 5
Statistical parameters regarding OTA quantification, for White Liqueur Wine samples

Laboratory	Assay 1 (µg/l)	Assay 2 (µg/l)	Assay 3 (µg/l)	Method	Mean (µg/l)	Standard deviation	z-Score	Outlier
1	1.24	1.33	1.59	EN 14133	1.38	0.18	−0.3	Cochran
2	2.02	1.97	2.04	SPE-HPLC/FD	2.01	0.04	0.7	
3	1.63	1.68	1.70	MIP-SPE-HPLC/FD	1.67	0.04	0.1	
4	6.32	4.95	4.85	PBS-IAC-HPLC/FD	5.37	0.82	6.3	
5	0.86	1.14	0.90	EN 14133	0.97	0.15	−1.0	
6	3.29	3.35	3.10	EN 14133	3.25	0.13	2.8	
7	2.00	2.00	2.10	EN 14133	2.03	0.06	0.7	
8	2.00	2.00	2.00	EN 14133	2.00	0.00	0.7	
9	1.86	1.92	1.75	EN 14133	1.84	0.09	0.4	
10	1.53	1.62	1.60	PBS-IAC-HPLC/FD	1.58	0.05	0.0	
11	1.34	1.19	1.20	EN 14133	1.24	0.08	−0.6	
12	2.13	2.24	2.17	DI-IS-HPLC	2.18	0.06	1.0	
13	0.87	0.79		PBS-IAC-HPLC/FD	0.83	0.06	−1.3	
14	1.68	1.68	1.78	EN 14133	1.71	0.06	0.2	
15	1.26	1.26	1.23	PBS-IAC-HPLC/FD	1.25	0.02	−0.6	
16	0.60	0.70	0.70	PBS-IAC-HPLC/FD	0.67	0.06	−1.5	
17	1.01	0.61	0.93	PBS-IAC-HPLC/FD	0.85	0.21	−1.2	
18	2.01	1.96	1.89	EN 14133	1.95	0.06	0.6	
19	1.12	0.93	1.04	SAX-LC/MS-MS	1.03	0.10	−0.9	
20	1.54	1.27		SPE-HPLC/PDA	1.41	0.19	−0.3	
21	1.38	1.23	1.39	EN 14133	1.33	0.09	−0.4	
22	1.74	1.84	1.79	EN 14133	1.79	0.05	0.3	
23	2.00	2.50	2.30	SPE-HPLC/FD	2.27	0.25	1.1	
24	1.32	1.20	1.30	EN 14133	1.27	0.06	−0.5	
Accepted laboratories						23 out of 24		
General mean						1.59		
Standard deviation of the general mean						0.58		
Relative standard deviation (%)						36.79		
Maximum value (accepted laboratories)						3.25		
Minimum value (accepted laboratories)						0.67		
s_L (interlaboratorial standard deviation)						0.59		
s_r (repeatability standard deviation)						0.11		
s_R (reproducibility standard deviation)						0.60		

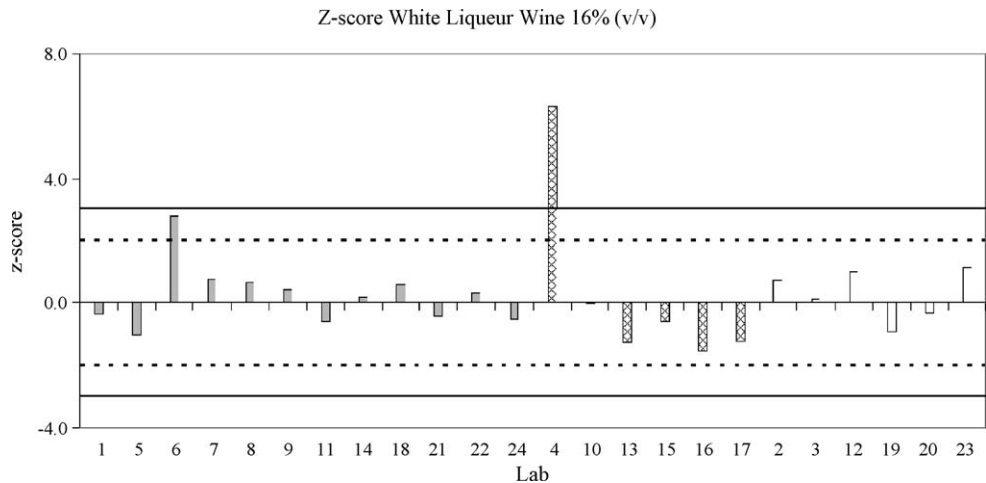


Fig. 3. z-Scores White Liqueur Wine 16% (v/v).

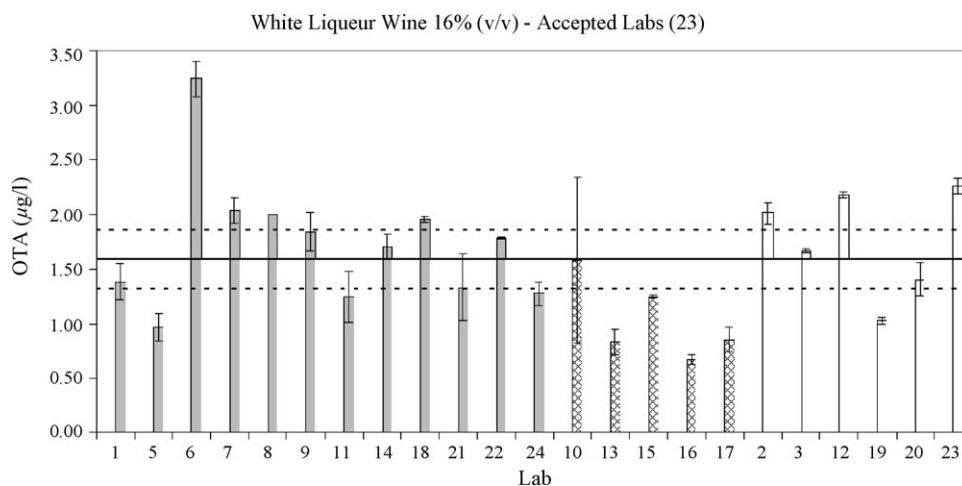


Fig. 4. White Liqueur Wine 16% (v/v)—accepted labs (23).

cal methodologies were included and also because a different wine type was analyzed. Even so, if the EN 14133 group is considered separately and without its outlier (lab 4), the R.S.D. is closer (20.0%) to the one in the standard.

3.2. White Liqueur Wine (16%, v/v)

In Table 5 the different statistical parameters for all laboratories, for White Liqueur Wine samples, are shown. One outlier is referred, by the Cochran test.

Table 6
Statistical parameters regarding OTA quantification, for Red Fortified Wine samples (spiked)

Laboratory	Assay 1 (µg/l)	Assay 2 (µg/l)	Assay 3 (µg/l)	Method	Mean (µg/l)	Standard deviation	z-Score
1	2.24	2.85	2.71	EN 14133	2.60	0.32	−0.1
2	2.84	2.83	2.86	SPE-HPLC/FD	2.84	0.02	0.1
3	3.44	3.54	3.49	MIP-SPE-HPLC/FD	3.49	0.05	0.7
4	4.50	4.46	5.63	PBS-IAC-HPLC/FD	4.86	0.66	2.0
5	2.34	2.29	2.29	EN 14133	2.31	0.03	−0.4
6	5.22	4.84	3.87	EN 14133	4.64	0.70	1.8
7	3.20	3.30	3.50	EN 14133	3.33	0.15	0.6
8	1.60	1.60	1.60	EN 14133	1.60	0.00	−1.1
9	3.89	3.25	3.60	EN 14133	3.58	0.32	0.8
10	2.55	2.63	2.60	PBS-IAC-HPLC/FD	2.59	0.04	−0.1
11	2.00	1.63	2.80	EN 14133	2.14	0.60	−0.6
12	2.78	2.87	2.77	DI-IS-HPLC	2.81	0.06	0.1
13	1.50	1.26		PBS-IAC-HPLC/FD	1.38	0.17	−1.3
14	2.63	2.55	2.45	EN 14133	2.54	0.09	−0.2
15	1.32	1.48	1.48	PBS-IAC-HPLC/FD	1.43	0.09	−1.2
16	1.10	1.00	1.20	PBS-IAC-HPLC/FD	1.10	0.10	−1.5
17	2.21	1.87	1.69	PBS-IAC-HPLC/FD	1.92	0.26	−0.8
18	3.64	3.41	3.45	EN 14133	3.50	0.12	0.7
19	3.89	3.59	3.52	SAX-LC/MS-MS	3.67	0.20	0.9
20	1.65	1.62		SPE-HPLC/PDA	1.64	0.02	−1.0
21	2.06	2.72	2.31	EN 14133	2.36	0.33	−0.3
22	3.33	3.39	3.47	EN 14133	3.40	0.07	0.6
23	4.30	3.70	3.90	SPE-HPLC/FD	3.97	0.31	1.2
24	2.16	1.87	1.65	EN 14133	1.89	0.26	−0.8
Accepted laboratories						24 out of 24	
General mean						2.73	
Standard deviation of the general mean						1.02	
Relative standard deviation (%)						37.43	
Maximum value (accepted laboratories)						4.86	
Minimum value (accepted laboratories)						1.10	
s_L (interlaboratorial standard deviation)						0.96	
s_r (repeatability standard deviation)						0.28	
s_R (reproducibility standard deviation)						1.00	

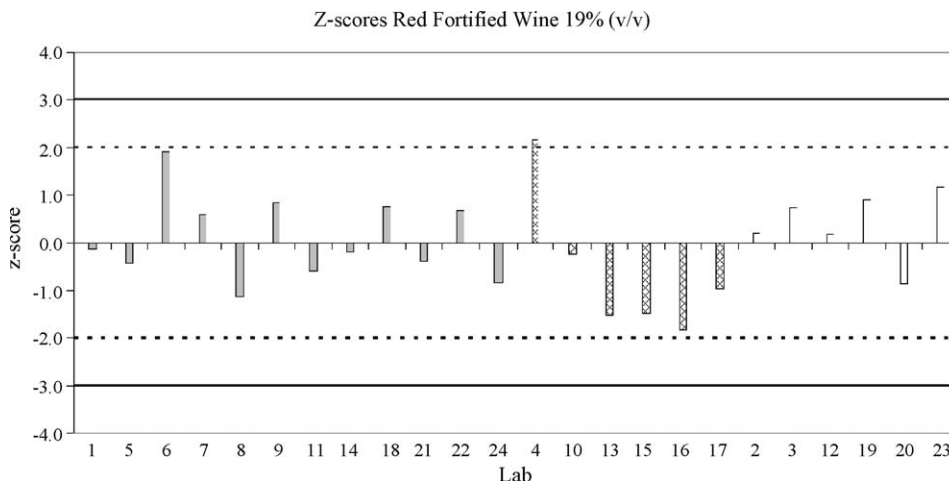


Fig. 5. z-Scores Red Fortified Wine 19% (v/v).

For these samples, only one laboratory was excluded. The general mean of the accepted ones was $1.59 \mu\text{g/l}$, with R.S.D. of 36.79%, somewhat higher than for the White Wine samples, with a much lower alcohol content.

Fig. 3 illustrates the z-scores for all laboratories, with the same grouping by method, before the outlier tests (mean = $1.75 \mu\text{g/l}$).

Again, the outlier (lab 4) had the worst result and was considered incorrect according to the z-scores test. The most sensible variability seems to happen in the PBS-IAC-HPLC/FD group. When removing the outlier, the mean values match the parameters of Table 5, and these findings are confirmed, both in number of laboratories with results outside the confidence intervals and in the deviation of the individual group mean from the general mean (−34.8%, followed by other methods with +10.8% and EN 14133 with 8.8%), as seen in Fig. 4.

There is no information concerning other collaborative studies with this type of wines and therefore results for reproducibility could not be compared, assuming that the “white wines” analyzed in the European Standard 14133 [24] are not liqueurs.

3.3. Red Fortified Wine (19%, v/v)

For Red Fortified Wine samples, the different statistical parameters for all laboratories can be seen in Table 6. No outliers were found in this case.

The general mean of all laboratories was $2.73 \mu\text{g/l}$, with R.S.D. similar to White Liqueur Wine (37.43%). In the study depicted in the European Standard EN 14133 [24], red wines yielded a value of 12.9% R.S.D., but again probably dealing with different types of wine, namely regarding sugar and alcohol content and acidity. Considering the EN 14133 group separately, the R.S.D. (30.9%) approaches, although to a lesser extent than for White Wine, the one in the standard. Nevertheless, these samples, which had the highest alcohol content (19%), were the most consistent, when subjected to the statistical tests.

Fig. 5 shows the z-scores for all laboratories, grouped by methods. Although having no outliers, lab 4 had the worst result, but was only qualified as questionable, according to the z-scores

screening. The trends found for the White Liqueur Wine samples were confirmed in this case, as the PBS-IAC-HPLC/FD method group mean was the most deviant comparing to the general mean (−18.9%, followed by other methods with +12.4% and EN 14133 with +3.4%).

3.4. Overall results and quality assurance of the study

AOAC demands specific requirements for laboratory validation of chemical methods [47]. The protocol for interlaboratory studies requires the use of a minimum of eight laboratories and the acceptable range for the methods variability by the Horrat index may provide useful information to assure the quality of the study.

The Horrat index (HORRAT_R) is defined as the ratio between the values found for the R.S.D. (%) of the reproducibility and the R.S.D. (%) calculated from the Horwitz equation [48]. Acceptable values of this ratio are typically 0.5–2.

$$\text{HORRAT}_R = \frac{\text{R.S.D.}_R (\text{found})}{\text{R.S.D.}_H (\text{Horwitz})}$$

The Horwitz equation was statistically derived from data of more than 10,000 collaborative studies [49], and even considering the inherent limitations from the differences between analytes, methods or matrices are not strictly accounted for, it is a valuable estimation when interlaboratory studies cannot be provided. The R.S.D. calculation from the Horwitz equation is $\text{R.S.D.}_H = 2^{(1-0.5 \log C)}$, where C is the concentration of the analyte in the sample as a decimal fraction, such as $1 \text{ mg/kg} = 10^{-6}$.

Table 7 presents the Horrat index evaluated in this study and the one calculated for the results published in the European Standard 14133. Horrat indexes in this study were, on average, within the recommended range and it should be noticed that its evaluation supposes the same analytical methodology. However, the evaluation for the results of the laboratories that strictly used the EN 14133 in the present study was slightly higher, reaching the maximum recommended of 2, on average, with a value above that limit for the Red Liqueur Wine samples (2.7). The average Horrat index calculated using the results published in

Table 7

The Horrat index calculated from the Horwitz equation and the Horwitz equation corrected by Thompson

Wine type	OTA concentration		R.S.D. found (%)	R.S.D. Horwitz (%)	Horrat index (Horwitz)	Horrat index (Thompson)
	μg/l	%				
All analytical methods included (this study, 24 labs)						
White	1.96	0.000020	27.3	20.3	1.3	1.2
White liqueur	1.59	0.000016	36.8	20.9	1.8	1.7
Red liqueur	2.73	0.000027	37.4	19.3	1.9	1.7
Average					1.7	1.5
EN 14133 (this study, 12 labs)						
White	2.37	0.000024	31.8	19.7	1.6	1.4
White liqueur	1.73	0.000017	34.2	20.7	1.7	1.6
Red liqueur	2.14	0.000021	53.1	20.0	2.7	2.4
Average					2.0	1.8
Results extracted from EN 14133 [21] (16 labs)						
White	0.105	0.000001	15.9	31.5	0.5	0.7
White	0.998	0.000010	13.3	22.4	0.6	0.6
White	1.764	0.000018	13.1	20.6	0.6	0.6
White	0.283	0.000003	14.6	27.1	0.5	0.7
Red	0.186	0.000002	11.9	28.9	0.4	0.5
Red	0.813	0.000008	12.5	23.2	0.5	0.6
Red	2.53	0.000025	13.6	19.5	0.7	0.6
Red	1.69	0.000017	13.6	20.7	0.7	0.6
Average					0.6	0.6

the EN 14133 standard is within the range of acceptance of the reproducibility (0.6).

There could be two main reasons for these punctual deviations. The first is that the level of training and experience as well as quality assurance/quality control (QA/QC) procedures was not discriminated among the laboratories, giving a more realistic view of the worldwide production of OTA results. The second is that wines different from those for which the standard was validated were chosen and matrix effects can be important, probably not affecting significantly the accuracy of the methodology but increasing the interlaboratorial variability. It should also be mentioned that the Horwitz approach has its limitations and is not unquestionably accepted, as reported by Ritter et al. [50] for analysis in plastics and by van der Veen [51] in doping control in sport, which pointed towards a possible underestimation of the uncertainty. Furthermore, Thompson [52] stated that this expression could not be valid in all cases, proposing some modifications for the highest and the lowest concentration ranges, with the following expressions in terms of σ_R (standard deviation of the reproducibility):

$$\sigma_R = 0.22C, \quad \text{for } C < 1.2 \times 10^{-7}$$

(in mass fraction, which corresponds to 120 mg/kg)

$$\sigma_R = 0.02^{(1-0.5 \log C)}, \quad \text{for } < 1.2 \times 10^{-7} \leq C \leq 0.138$$

(the Horwitz equation, in absolute value)

$$\sigma_R = 0.01C^{0.5}, \quad \text{for } C > 0.138$$

(in mass fraction, which corresponds to 138 g/kg)

The ranges of this study fall under the first expression, which forecasts a value of R.S.D. of 22%. Correcting the Horrat index under this assumption, the results are slightly better, as can be seen in Table 7. There is only one value outside the recommended range (Red Liqueur Wine for EN 14133, with 2.4) and the average values for the three situations studied fall clearly within that range (1.5, 1.8 and 0.6 for all laboratories, laboratories employing EN 14133 in this work and results reported in the European Standard EN 14133, respectively).

Taking into account that the current study rather than validate one method aims to compare different methods for the same analytic quantification, which is more prone to some inconsistencies, as stated by Lisinger et al. [53], the worldwide and method-free character of the work could have caused additional problems. Instead, the results show that, in this case and despite all their possible sources, they can be meaningless, supporting the reliability of the conclusions.

4. Conclusions

The participation of a large number of laboratories (24), from almost all continents of the world was very important to have a general assessment of the current methods employed to analyze ochratoxin A in wines and their global performance. As was seen, the results were consistent, with low variability amongst different laboratories. In the three sample types, only two outliers (White Wine), one (White Liqueur Wine) and none (Red Fortified Wine) were found, when the maximum number of laboratories that could be excluded was five.

When comparing the results by methods, the differences were not very clear, meaning that the use of different methods had no

relation with the global variability. Even the use of methods so distinct from the standard EN 14133 such as LC/MS-MS did not present significant variability. When comparing the individual mean of each group with the global mean for each wine, the six laboratories adopting the PBS-IAC-HPLC/FD approach were the ones showing the most deviant behaviour and the lowest values in OTA content. If we report to the number of laboratories with results outside the confidence intervals, the EN 14133 and the PBS-IAC-HPLC/FD share the least accomplished results. However, these findings cannot constitute a rule of statistical significance, due to the overall proximity between the results of all groups. Furthermore, it should be pointed out that the EN 14133 and the PBS-IAC-HPLC/FD approaches have in common the use of immunoaffinity columns, which may contribute to a rise in the variability of the results, either by its production process or the experience of the operator.

The choice of three different types of wine to analyze, with distinct alcohol contents (10.5%, 16% and 19%) meant to test even further the flexibility of the OTA analysis. The most noticeable aspect was the fact that the relative standard deviation of the laboratories means (without outliers) was lower in the first case (White Wine, 27.30%) than in the other two (White Liqueur Wine and Red Fortified Wine, with 36.49% and 37.43%, respectively). It is, however, difficult to assume indisputably that the alcohol content is directly linked to this phenomenon. Considering the results of the EN13144 group alone, the R.S.D. are closer to the ones referred in the European Standard, especially for White Wine (20.0% versus 14.3%). The average Horrat index (1.7 with the Horwitz equation and 1.5 after correction by Thompson) fell within the range recommended by the AOAC for the quality assurance of collaborative studies (0.5–2).

It can be safely stated that all the overall results yielded by this Interlaboratory Study were very good. Only two laboratories (labs 4 and 6) produced outliers at some point, and were removed from the statistical analysis when such event took place. The analysis and quantification of OTA is, therefore, reproducible worldwide, for the type of wine samples included in this work.

Acknowledgments

LEPAE/FEUP and ALABE wish to acknowledge the financial support of the INIAP (Portugal), through the Program AGRO, contract number 255 and the effort and cooperation of all participant laboratories and people involved in this interlaboratory study.

References

- [1] B. Zimmerli, R. Dick, J. Chromatogr. B 666 (1995) 85.
- [2] Office International de la Vigne et du Vin (OIV), Reduction de L'Ochratoxine A dans les Vins, Resolution CST 1/2002, Paris, France, 2002.
- [3] Commission regulation (EC) no. 123/2005 of 26 January 2005 amending regulation (EC) no. 466/2001 as regards ochratoxin A, Off. J. Eur. Union L25 (2005) 3.
- [4] E.E. Creppy, J. Toxicol.-Toxin Rev. 18 (1999) 273.
- [5] M. Ospital, J.-M. Cazabeil, A.-M. Betbeder, C. Tricard, E. Creppy, B. Medina, Ver. Fr. Oenol. 169 (1998) 16.
- [6] Y. Ueno, Mycotoxins 47 (1998) 25.
- [7] S. MacDonald, P. Wilson, K. Barnes, A. Damant, R. Massey, E. Mortby, M.J. Shepherd, Food Addit. Contam. 16 (1999) 253.
- [8] I. Festas, P. Herbert, L. Santos, M. Cabral, P. Barros, A. Alves, Am. J. Enol. Vitic. 51 (2000) 150.
- [9] N. Ratola, L. Martins, A. Alves, Anal. Chim. Acta 513 (2004) 319.
- [10] A. Pietri, T. Bertuzzi, L. Pallaroni, G. Piva, Food. Addit. Contam. 18 (2001) 647.
- [11] R. Saelzer, M. Vega, A. Retamal, G. Ríos, E. Herlitz, Noticias Técnicas del Laboratorio 2 (2002) 6.
- [12] E.H. Soufleros, C. Tricard, E.C. Bouloumpasi, J. Sci. Food Agric. 83 (2003) 173.
- [13] G.S. Shephard, A. Fabiani, S. Stockenström, N. Mshicileli, V. Sewram, J. Agric. Food Chem. 51 (2003) 1102.
- [14] N. Bellí, S. Marín, A. Duaigües, A.J. Ramos, V. Sanchis, J. Sci. Food Agric. 84 (2004) 591.
- [15] J. Blesa, J.M. Soriano, J.C. Moltó, J. Mañes, J. Chromatogr. A 1054 (2004) 397.
- [16] C.A.R. Rosa, C.E. Magnoli, M.E. Fraga, A.M. Dalcer, D.M.N. Santana, Food Addit. Contam. 21 (2004) 358.
- [17] G.J. Soleas, J. Yan, D.M. Goldberg, J. Agric. Food Chem. 41 (2001) 2733.
- [18] J. Varga, Z. Kozakiewicz, Trends Food Sci. Technol. 17 (2006) 72.
- [19] A. Leitner, P. Zöllner, A. Paolillo, J. Stroka, A. Papadopoulou-Bouraoui, S. Jaborek, E. Anklam, W. Lindner, Anal. Chim. Acta 453 (2002) 33.
- [20] J.M. Sáez, Á. Medina, J.V. Gimeno-Adelantado, R. Mateo, M. Jiménez, J. Chromatogr. A 1029 (2004) 125.
- [21] P. Zöllner, A. Leitner, D. Lubda, K. Cabrera, W. Lindner, Chromatographia 804 (2000) 818.
- [22] M. Reinsch, A. Töpfer, A. Lehmann, I. Nehls, U. Panne, Food Chem. 100 (2007) 312–317.
- [23] N.M. Maier, G. Buttinger, S. Welhartzki, E. Gavioli, W. Lindner, J. Chromatogr. B 804 (2004) 103.
- [24] European Committee for Standardization (CEN), Foodstuffs – determination of ochratoxin A in wine and beer – HPLC method with immunoaffinity column clean-up, EN 14133:2003, Brussels, Belgium, 2003.
- [25] Association of Official Analytical Chemists (AOAC), Determination of ochratoxin A in wine and beer, Official Method 2001.01, AOAC International, 2002.
- [26] J.L. Moreira, A.M. Marcos, P. Barros, Ciência Téc. Vitiv. 17 (2002) 41.
- [27] S.M. Abreu, A. Alves, B. Oliveira, P. Herbert, Anal. Bioanal. Chem. 382 (2005) 498.
- [28] ISO Guide 43:1997, Proficiency testing by interlaboratory comparisons, International Organization for Standardization, Genève, Switzerland, 1997.
- [29] Accessed on 30/12/2005 <http://www.bipea.org/en/essais.htm>.
- [30] C. Lindig, Chemosphere 37 (1998) 405.
- [31] M. Farré, F. Arranz, J. Ribó, D. Barceló, Talanta 62 (2004) 549.
- [32] R. Hoogerbrugge, S.M. Gort, E.G. van der Velde, P. Van Zoonen, Anal. Chim. Acta 388 (1999) 119.
- [33] A.H. Garde, Å.M. Hansen, T.B. Nikolajsen, Accred. Qual. Assur. 8 (2003) 16.
- [34] T.F. Bidleman, S. Cussion, L.M. Jantunen, Atmos. Environ. 38 (2004) 3713.
- [35] E. Eljarrat, M. Guillamón, J. Seuma, B.B. Mogensen, I.S. Fomsgaard, A. Olivero-Bastidas, F.A. Macías, A. Stochmal, W. Oleszek, O. Shakaliene, D. Barceló, J. Chromatogr. A 1047 (2004) 69.
- [36] J. van Loock, S.P.J. van Leeuwen, P. Roos, S. Carbonnelle, J. de Boer, L. Goeyens, H. Beernaert, Talanta 63 (2004) 1169.
- [37] J.-P. Villeneuve, S. de Mora, C. Cattini, Trends Anal. Chem. 23 (2004) 469.
- [38] F.J. Santos, E. Barceló-Barrachina, F. Toribio, L. Puignou, M.T. Galceran, E. Persson, K. Skog, C. Messner, M. Murkovic, U. Nabinger, A. Ristic, J. Chromatogr. B 802 (2004) 69.
- [39] E. Hund, D.L. Massart, J. Smeyers-Verbeke, Anal. Chim. Acta 423 (2000) 145.
- [40] ISO 5725:1994, Accuracy (trueness and precision) of measurement methods and results, International Organization for Standardization, Genève, Switzerland, 1994.
- [41] H. van der Voet, J.A. van Rhijn, H.J. van de Wiel, Anal. Chim. Acta 391 (1999) 159.

- [42] I. Taverniers, E. van Bockstaele, M. de Loose, *Trends Anal. Chem.* 23 (2004) 490.
- [43] P. Dehouck, Y. Vander Heyden, J. Smeyers-Verbeke, D.L. Massart, R.D. Marini, P. Chiap, P. Hubert, J. Crommen, W. Van de Wauw, J. De Beer, R. Cox, G. Mathieu, J.C. Reepmeyer, B. Voigt, O. Estevenon, A. Nicolas, A. Van Schepdael, E. Adams, J. Hoogmartens, *J. Chromatogr. A* 1010 (2003) 63.
- [44] G. Becher, L.S. Haug, C. Thomsen, *Talanta* 63 (2004) 1122.
- [45] N. Boley (Ed.), *EURACHEM Guide, Selection, Use and Interpretation of Proficiency Testing (PT) Schemes by Laboratories*, 1.0th ed., EURACHEM, Teddington, UK, 2002.
- [46] Y. vander Heyden, J. Saevels, E. Roets, J. Hoogmartens, D. Decolin, M.G. Quaglia, W. van den Bossche, R. Leemans, O. Smeets, F. van de Vaart, B. Mason, G.C. Taylor, W. Underberg, A. Bult, P. Chiap, J. Crommen, J. de Beer, S.H. Hansen, D.L. Massart, *J. Chromatogr. A* 830 (1999) 3.
- [47] W. Horwitz, AOAC requirements for single laboratory validation of chemical methods, Draft 2002-11-07, AOAC, 2002 (accessed on 30/12/2005) http://www.aoac.org/Ag_Materials/additives/aoac_slv.pdf.
- [48] W. Horwitz, *Anal. Chem.* 54 (1982) 67A.
- [49] W. Horwitz, *J. AOAC Int.* 86 (2003) 109.
- [50] A. Ritter, V.R. Meyer, *Polym. Test.* 24 (2005) 988.
- [51] A.M.H. van der Veen, *Accred. Qual. Assur.* 8 (2003) 334.
- [52] M. Thompson, *Analyst* 125 (2000) 2020.
- [53] T.P.J. Lisinger, W. Kandler, R. Krska, M. Grasserbauer, *Accred. Qual. Assur.* 3 (1998) 322.

Simultaneous determination of ascorbic acid, uric acid and neurotransmitters with a carbon ceramic electrode prepared by sol–gel technique

Abdollah Salimi*, Hussein MamKhezri, Rahman Hallaj

Department of Chemistry, Kurdistan University, P.O. Box 416, Sanandaj, Iran

Received 18 July 2005; received in revised form 4 January 2006; accepted 7 February 2006

Available online 24 March 2006

Abstract

A sol–gel carbon composite electrode (CCE) has been prepared by mixing a sol–gel precursor (e.g. methyltrimethoxysilane) and carbon powder without adding any electron transfer mediator or specific reagents. It was demonstrated that this sensor can be used for simultaneous determination ascorbic acid, neurotransmitters (dopamine and adrenaline) and uric acid. Direct electrochemical oxidation of ascorbic acid, uric acid and catecholamines at a carbon composite electrode was investigated. The experimental results were compared with other common carbon based electrodes, specifically, boron doped diamond, glassy carbon, graphite and carbon paste electrodes. It was found that the CCE shows a significantly higher of reversibility for dopamine. In addition, in comparison to the other electrodes used, for CCE the oxidation peaks of uric acid, ascorbic acid and catecholamines in cyclic and square wave voltammetry were well resolved at the low positive potential with good sensitivity. The advantages of this sensor were high sensitivity, inherent stability and simplicity and ability for simultaneous determination of uric acid, catecholamines and ascorbic acid without using any chromatography or separation systems. The analytical performance of this sensor has been evaluated for detection of biological molecules in urine and serum as real samples.

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Keywords: Electroanalysis; Carbon composite electrode; Sol–gel; Dopamine; Adrenaline; Ascorbic acid; Uric acid; Real samples

1. Introduction

Dopamine (DA) and other catecholamines play important roles in central nervous system, cardiovascular, renal and hormonal systems functioning as well as in drug addiction and Parkinson disease [1]. Similarly ascorbic acid (AA) has been used for prevention and treatment of common cold, mental illness, infertility, cancer, and in some clinical manifestations of HIV infections [2]. Uric acid (UA) is the primary product of purine metabolism in the human body. It has been shown that extreme abnormalities of UA levels are symptoms of several diseases (e.g. gout, hyperuricaemia and Lesch–Nyhan syndrome) [3,4]. Therefore, it is essential to develop simple and rapid methods for determination these biological molecules in routine analysis. The development of voltammetric sensors for the determination of neurotransmitters, uric and ascorbic acids in

human fluid such as urine and serum has received considerable interest in recent years. Neurotransmitters, ascorbic and uric acids are electrochemically active compounds co-existing in body fluids and can be determined by electrochemical techniques. A major problem is that AA, UA and neurotransmitters are oxidized at nearly same potential with poor sensitivity at solid (unmodified) electrodes which results in overlapped voltammetric responses making their discriminations highly difficult [5]. Simultaneous detection of neurotransmitters, especially dopamine, ascorbic and uric acids is a problem of critical importance not only in the field of biomedical chemistry and neurochemistry but also for diagnostic and pathological research. Moreover the solid electrodes very often suffer from the fouling effect due to the accumulation of oxidized product on the electrode surface, which results in rather poor selectivity and sensitivity. Electrochemical techniques with modified electrodes have received great interest for uric and neurotransmitters determination in the presence of ascorbic acid as they are more selective and less time consuming than those based on other colorimetric or spectrophotometric methods. Different

* Corresponding author. Fax: +98 871 6660075.
E-mail address: absalimi@yahoo.com (A. Salimi).

modified electrodes such as DNA modified graphite powder microelectrode [6], layer by layer assembled carbonnanotubes [7], highly oxidized electrodes [8], self-assembled monolayers of hetroaromatic thiols [9], exfoliated graphite electrode [10], poly-phenosafranine modified electrodes [11], gold electrode modified with *N*-acetylcysteine self-assembled monolayer [12], poly-sulfosalicylic acid modified glassy carbon electrode [13], choline and acetylcholine modified glassy carbon electrode [14], self-assembled bilayer membrane modified glassy carbon electrode [15], cationic self-assembled monolayers [16], clay modified electrode [17], glassy carbon electrode modified with 2,2-bis(3-amino-4-hydroxyphenyl) hexafluoropropane [18], tetrabromo-*p*-benzoquinone modified carbon past electrode [19] and carbon paste electrode modified with iron(II) phthalocyanine complexes [20], have been used for simultaneous detection of catecholamines and ascorbic acid or uric acid and ascorbic acid. Most of these studies demonstrated the individual determination of both catecholamines and uric acid as well as ascorbic acid by eliminating the others. Also, most modified electrodes used for catecholamines, uric and ascorbic acids detections have certain disadvantages, such as considerable leaching of electron transfer mediators and poor long-term stability. Furthermore the preparation methods are more expensive and difficult or the need for renewal of the surface after each measurement. In addition the irreversible adsorption behaviour renders the routine analysis difficult and some of them are not sensitive enough for real sample analysis. Finally the oxidation peaks of biomolecules are not well separated.

Sol-gel technology has aroused great interest in the design and application of electrochemical sensors due to its simplicity, physical rigidity, transparency, porosity and permeability, versatility and flexibility in the fabrication procedure [21–23]. Silver and copper dispersed composite electrodes prepared by sol-gel derived ceramic graphite containing 3-mercaptopropyl trimethoxysilane have been used for simultaneous determination of ascorbic acid and dopamine [24,25]. A fluorescent sol-gel biosensor has been also used for uric acid determination [26]. The modified electrodes prepared with sol-gel technique have some disadvantageous such as low detection sensitivity and consuming specific and expensive reagents and materials for their preparation. Therefore, it is necessary to develop an electrochemical sensor based on sol-gel technique, which is free from the above problems. To our best knowledge, none of the modified electrodes prepared by sol-gel technique show better performance in peak separation and simultaneous determination of AA, UA and neurotransmitters. In an effort to construct an efficient electrochemical sensor based on sol-gel technique and their applications in electroanalysis of important analytes such as insulin, NADH, hydrazine, hydroxylamine, sulphite, L-cysteine, glutathione and sulphur oxoanions modified carbon ceramic electrodes were employed in our group [27–36]. Application of nafion coated glassy carbon electrode modified with catechin-hydrate and basal plane pyrolytic graphite electrode modified with immobilization of multiwall carbon nanotubes for voltammetric and amperometric detection of dopamine and epinephrine in the presence of ascorbic acid was reported in our recent work [32,35]. In this work we present the application of

carbon ceramic electrode prepared by mixing graphite powder and methyltrimethoxysilane without adding any electron transfer mediator or specific reagents for simultaneous determination of catecholamines (dopamine and adrenaline), uric and ascorbic acid. Finally the analytical performance of this sensor for simultaneous determination of dopamine, ascorbic and uric acids in urine and human serum samples is evaluated by square wave voltammetry.

2. Experimental

2.1. Chemical and reagents

Methyltrimethoxysilane (MTMS) and nujel oil were purchased from Aldrich. Dopamine, adrenaline, ascorbic acid, uric acid, and high purity graphite powder (50 μ M diameter) were received from Merck and used without purification. All other chemicals and reagents were of analytical grade. Double distilled water was used to prepare all solutions. Buffer solutions (0.1 M) were prepared from phosphoric acid (H_3PO_4), sodium acetate (CH_3COONa), di-sodium hydrogen phosphate (Na_2HPO_4), hydrogen chloride (HCl) and sodium hydroxide (NaOH) for the pH range 1–10. Solutions were deaerated by bubbling high purity (99.99%) of argon gas through them prior to the experiments. All electrochemical experiments were carried out at room temperature $25 \pm 0.1^\circ\text{C}$.

2.2. Apparatus and procedures

The Metrohm electrochemical system (757 VA Computrace Metrohm, The Netherlands) equipped with a (757 VA Computrace software, Metrohm, The Netherlands) was used for voltammetric and amperometric measurements. A three-electrode cell, including glassy carbon 0.0314 cm^2 , carbon paste 0.0314 cm^2 , boron doped diamond 0.25 cm^2 , graphite 0.0314 cm^2 and carbon ceramic 0.0314 cm^2 , electrodes as working electrode, an Ag/AgCl (saturated KCl) and a platinum wire were used as reference and counter electrode respectively. Glassy carbon (GC), Graphite, Pt wire and reference electrodes were from Metrohm and boron doped diamond (BDD) was from (BDD, DeBeers Industry Diamond Division Ascot, supplied via Windsor Scientific, Slough, UK). The carbon ceramic electrode (CCE) was prepared by sol-gel technique. The preparation procedure is explained in the following section. A Metrohm drive shaft for rotating working electrode was used in amperometric measurements. A personal computer was used for data storage and processing. The following instruments parameters were used to record the square wave voltammograms: square wave amplitude: 35 mV, frequency 25 Hz and step potential 4 mV.

2.3. Preparation of the carbon ceramic electrode

The carbon ceramic electrode was prepared through the conventional method described by the Lev and co-workers [37], by mixing 0.2 ml (MTMS), 0.3 ml methanol and 10 μ l hydrochloric acid (11 M). This mixture was magnetically stirred for 5 min after which 0.3 g graphite powder was added and the resultant

mixture shaken for additional 3 min. Then, the carbon sol–gel paste was packed into one end of a Teflon tube (with 2 mm inner diameter and 5 cm length, and the length of composite material in the tube was about 0.5 cm) and dried under ambient conditions (25 °C) for 48 h. A copper wire was inserted through the composite end to establish electrical contact. The electrodes was polished with polishing paper and subsequently rinsed with distilled water. In amperometric measurements the CCEs rotated by contact them with a Metrohm drive shaft.

3. Results and discussions

3.1. Electrochemical oxidation of ascorbic acid, dopamine and uric acid

The cyclic voltammograms recorded for ascorbic acid, dopamine and uric acid at glassy carbon, carbon paste, basal plain pyrolytic graphite electrode, boron doped diamond and carbon ceramic electrodes, are shown in Fig. 1. As can be seen at the surface of all electrodes, the oxidation of uric and ascorbic acids are irreversible with large overpotentials although peak potentials for both molecules at less positive values at BPPGE and CCE in compared to other electrode materials. These results indicate the slow electron transfer rate for the oxidation of these biomolecules at utilized electrodes. Such sluggish electron transfer kinetics is related to the electrode fouling caused by the deposition of AA, UA and their oxidation products.

For dopamine, the cathodic peak was observed at the surface all electrodes but peak potentials separation are 0.35, 0.45 and 0.6 V at GC, CP and BDD electrodes, respectively. At the surface pyrolytic graphite electrode a quasireversible behaviour ΔE_p (150 mV) was observed for oxidation of dopamine. In comparison to above electrodes at CCE the dopamine oxidation is reversible with peak separation 30 mV and cathodic to anodic peak current ratio unity. To examine the ability of different electrodes to oxidation of other neurotransmitter derivatives, cyclic voltammogram for adrenaline oxidation at above electrodes were recorded (not shown). The irreversible oxidation peak for adrenaline was observed at the same dopamine oxidation peak potential for all electrodes. Similar electrochemical behaviour was observed for oxidation of dopamine and other catecholamine at glassy carbon electrode modified with single wall carbon nanotubes [38] and microcylinder polymer modified electrodes [39]. The effect of potential scan rate on the oxidation response of dopamine and adrenaline were examined in the 20–200 mV s⁻¹ range of scan rates (not shown). For dopamine the ratio of cathodic to anodic peak currents is about one, but for adrenaline only at scan rate above 50 mV s⁻¹ the cathodic peak is observed. The oxidation currents for both catecholamines, uric and ascorbic acids are increasing with the square root of scan rate, indicating that the oxidation currents were diffusion controlled, which is the ideal case for quantitative measurements. Fig. 2 shows the dependence of the voltammetric response of the CCE on the dopamine and

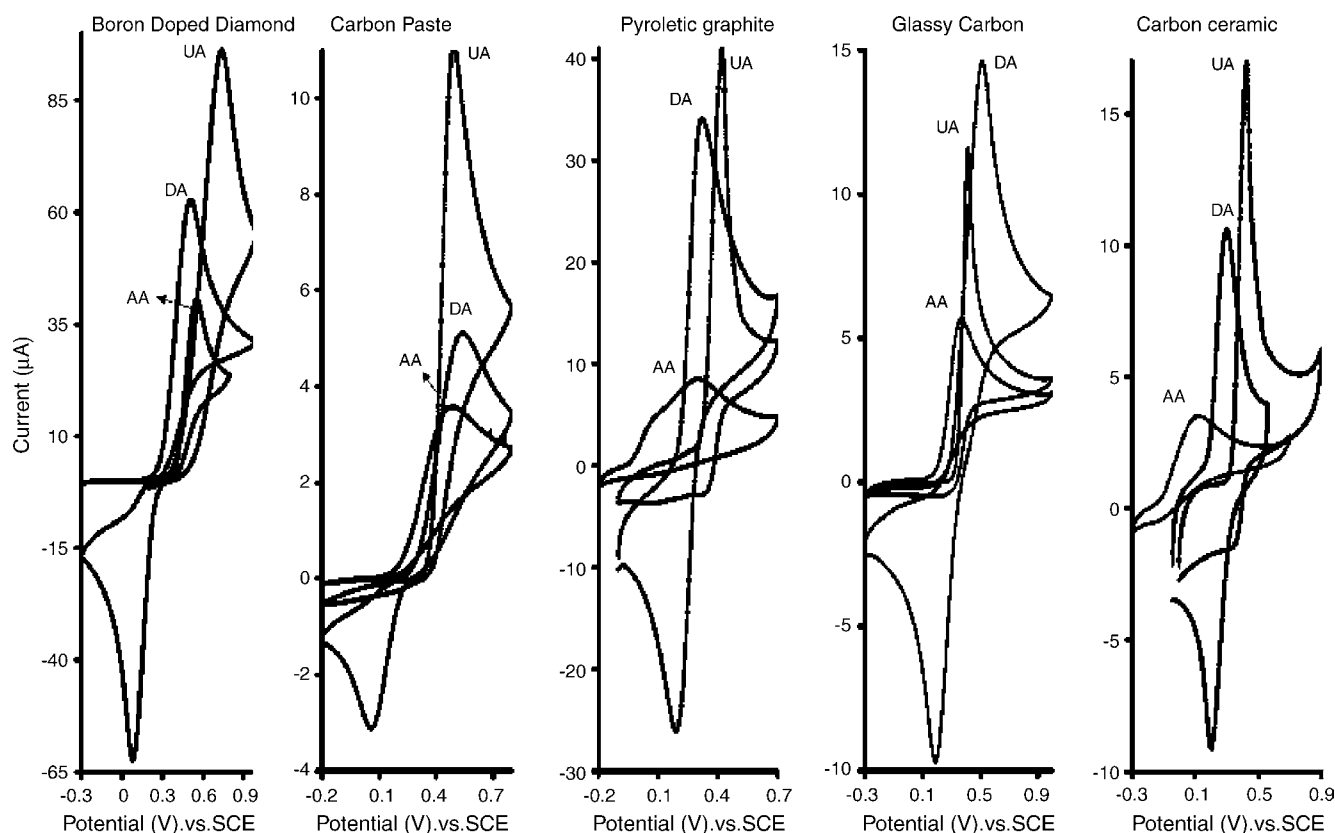


Fig. 1. Cyclic voltammograms of 5 mM AA, 1 mM UA and 1 mM DA at the surface different modified electrodes in phosphate buffer solution (pH 5) at scan rate 20 mV s⁻¹.

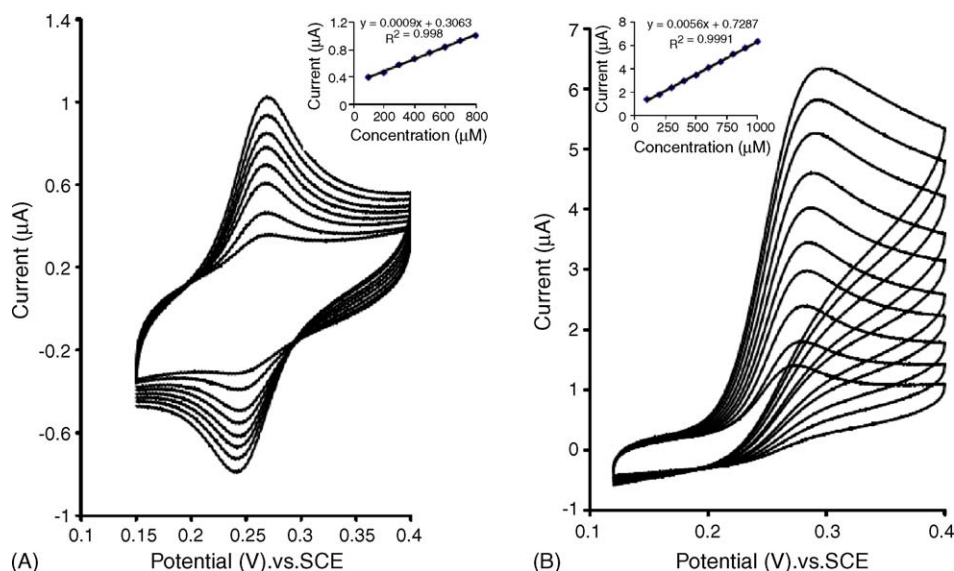


Fig. 2. Cyclic voltammograms of a CCE in 0.1 M phosphate buffer solution (pH 5) at scan rate 20 mV s^{-1} with various concentration of: (A) dopamine from inner to outer 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mM; (B) adrenaline from inner to outer 0.1 to 1 mM. Insets: the plots of peak currents vs. biomolecules concentration.

adrenaline concentration with addition of these biomolecules. Using a constant scan rate of 20 mV s^{-1} the variation in the peak currents were analysed as a function of added dopamine and adrenaline concentration. The response was found to produce a linear response over the entire concentration range of 0.1–1 mM ($I_p/\mu\text{A} = 0.0056[\text{adrenaline}]/\text{mM} + 0.728 \mu\text{A}$, $R^2 = 0.9991$) and 0.1–0.80 mM ($I_p/\mu\text{A} = 0.009 [\text{dopamine}]/\text{mM} + 0.306 \mu\text{A}$, $R^2 = 0.998$) with a limit of detections (signal to noise = 3) to the 30 and $20 \mu\text{M}$ for adrenaline and dopamine, respectively. For uric and ascorbic acids the cyclic voltammograms were recorded at different concentration (not shown). The relationships between concentration and peak currents were linear over the concentration range, 0.2–0.8 mM

($I_p/\mu\text{A} = 0.0108[\text{uric acid}]/\text{mM} + 4.47 \mu\text{A}$, $R^2 = 0.9996$) and 0.1–1 mM ($I_p/\mu\text{A} = 0.0014[\text{ascorbic acid}]/\text{mM} + 1.31 \mu\text{A}$, $R^2 = 0.9997$) with a limit of detections (signal to noise = 3) to the 15 and $25 \mu\text{M}$ for uric acid and ascorbic acid respectively.

Since the electrochemical behaviour of above compounds are pH dependent, we investigated the oxidation activity of dopamine, adrenaline, uric and ascorbic acids on the CCE in buffers with pH values 1–10. Fig. 3 shows the recorded cyclic voltammograms of 2.0 mM dopamine in solution with pH values ranging from 1 to 10. As can be seen in the inset of Fig. 3, E_p decreased by increasing the pH value, and current response for dopamine oxidation is high at pH range 4–7, clearly showing that protons are involved in the oxidation process. The slope

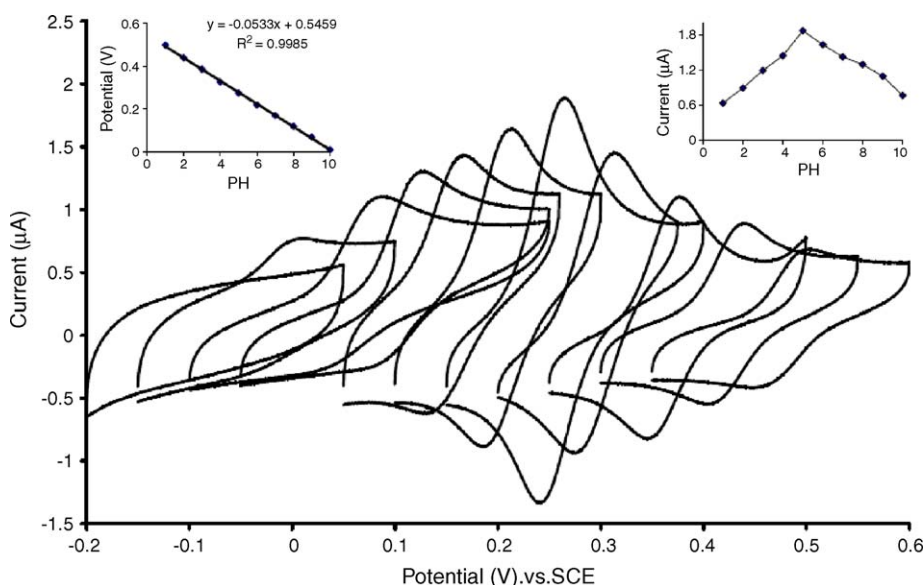


Fig. 3. Cyclic voltammograms of 2.0 mM dopamine recorded at different pH values from right to left 1 to 10, scan rate 20 mV s^{-1} , Insets: the plots of peak potentials and peak currents vs. pH.

of E_p versus pH is 55 mV/pH unit, which is very close to the anticipated Nernstian value for a two-electron two-proton process. A similar behaviour was observed for other compounds, whereas for adrenaline a small current changes were produced in the entire pH range. Based on both, high peak current and low possible peak potential, pH 5 was selected as the working pH value to carry out the square wave voltammetry and amperometric determination of these compounds.

3.2. Cyclic voltammetry of a mixture of ascorbic acid, uric acid, dopamine or adrenaline

Since uric acid, dopamine, adrenaline and ascorbic acid have similar oxidation potential at most solid electrodes and many modified electrodes, separate determination of these biomolecules is a great problem due to overlapped signals. The main objective of the present study is to simultaneously determine AA, UA, DA or adrenaline using carbon ceramic electrode prepared by sol–gel technique. To evaluate the sensitivity and selectivity of this sensor for simultaneous determination of above biomolecules, cyclic voltammograms mixture of these species are recorded at the surface CCE and compared with other electrode materials (Fig. 4). We can see the peak potentials for dopamine, uric and ascorbic acids are indistinguishable at a GC, BDD, and carbon paste electrodes. At the surface pyrolytic graphite electrode separate peaks for oxidation dopamine and uric acid was observed, however, the broad overlapped voltammetric peak for ascorbic acid oxidation is a serious obstacle

in mixture determination. Fortunately at CCE the overlapped voltammetric peaks is resolved into three well defined CV peaks (Fig. 4) at about 0.1, 0.3 and 0.45 V corresponding to the oxidation of AA, DA, and UA, respectively.

3.3. Simultaneous determination of AA, UA, DA or adrenaline by square wave voltammetry technique

Since square wave voltammetry has much higher current sensitivity and ability for peak separations than cyclic voltammetry it was employed to estimate the lower limit of detection and simultaneous determination AA, UA and DA or adrenaline. It is well known that AA is a major interference in the voltammetric detection of UA and neurotransmitters (vice-versa) as they are oxidized at about the same potentials. Also the electrochemical sensing of the other biomolecules using solid unmodified electrodes suffers from the interference of AA that is present at milimolar level in biological fluids. In addition the fouling effect of the AA and its oxidation products decreases the reproducibility of analytical method for biomolecules detection. One of the main objectives of this study is to detect AA, UA and DA or adrenaline simultaneously. Fig. 5A shows the SQWV of a mixture of ascorbic acid, dopamine and uric acid. Fig. 5B shows the SQW voltammograms obtained at CCE when the concentration AA, UA and DA were simultaneously changed. The calibration curves and correlation coefficients for these biomolecules are shown in inset of Fig. 5B. The detection limit, linear calibration range and sensitivity of AA, UA, DA and adrenaline were

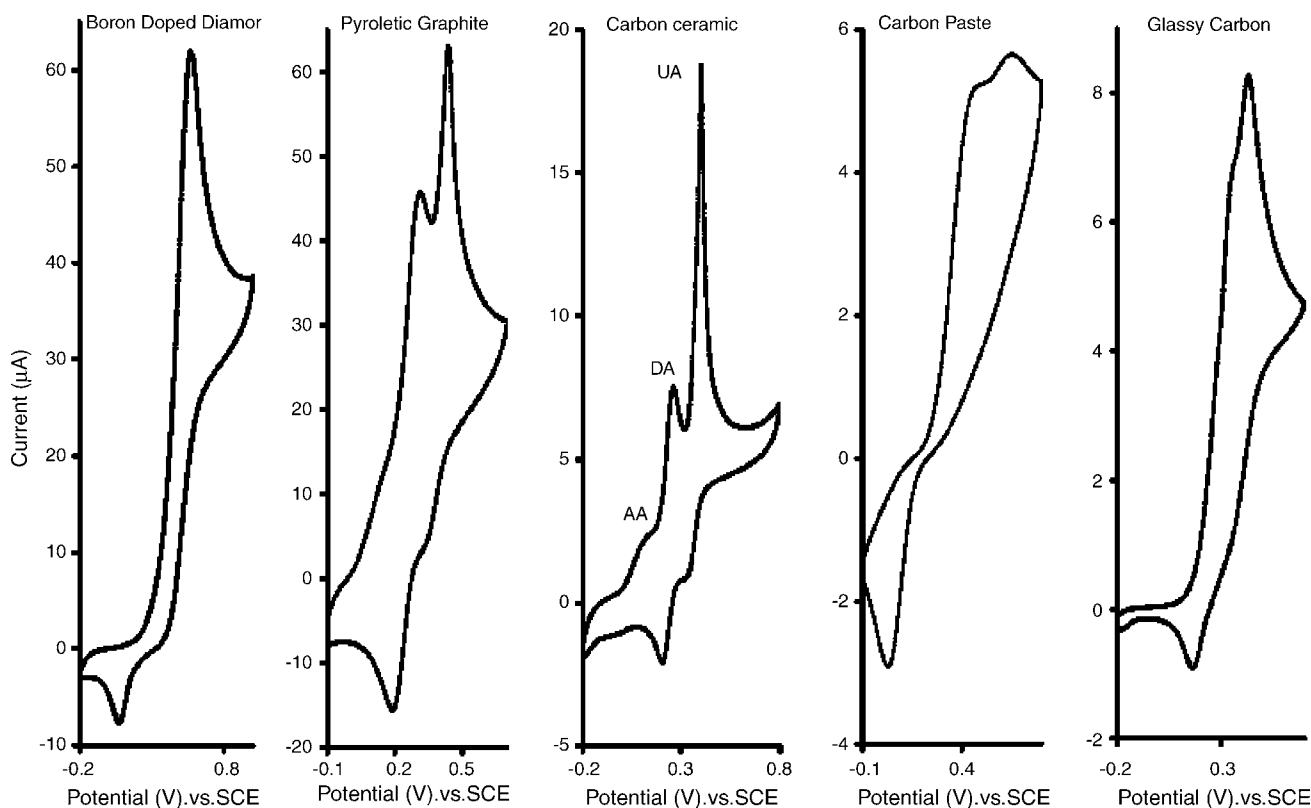


Fig. 4. Cyclic voltammograms a mixture of AA (1 mM), DA (1 mM) and UA (2 mM) at the surface different electrode materials at (pH 5), scan rate 20 mV s^{-1} .

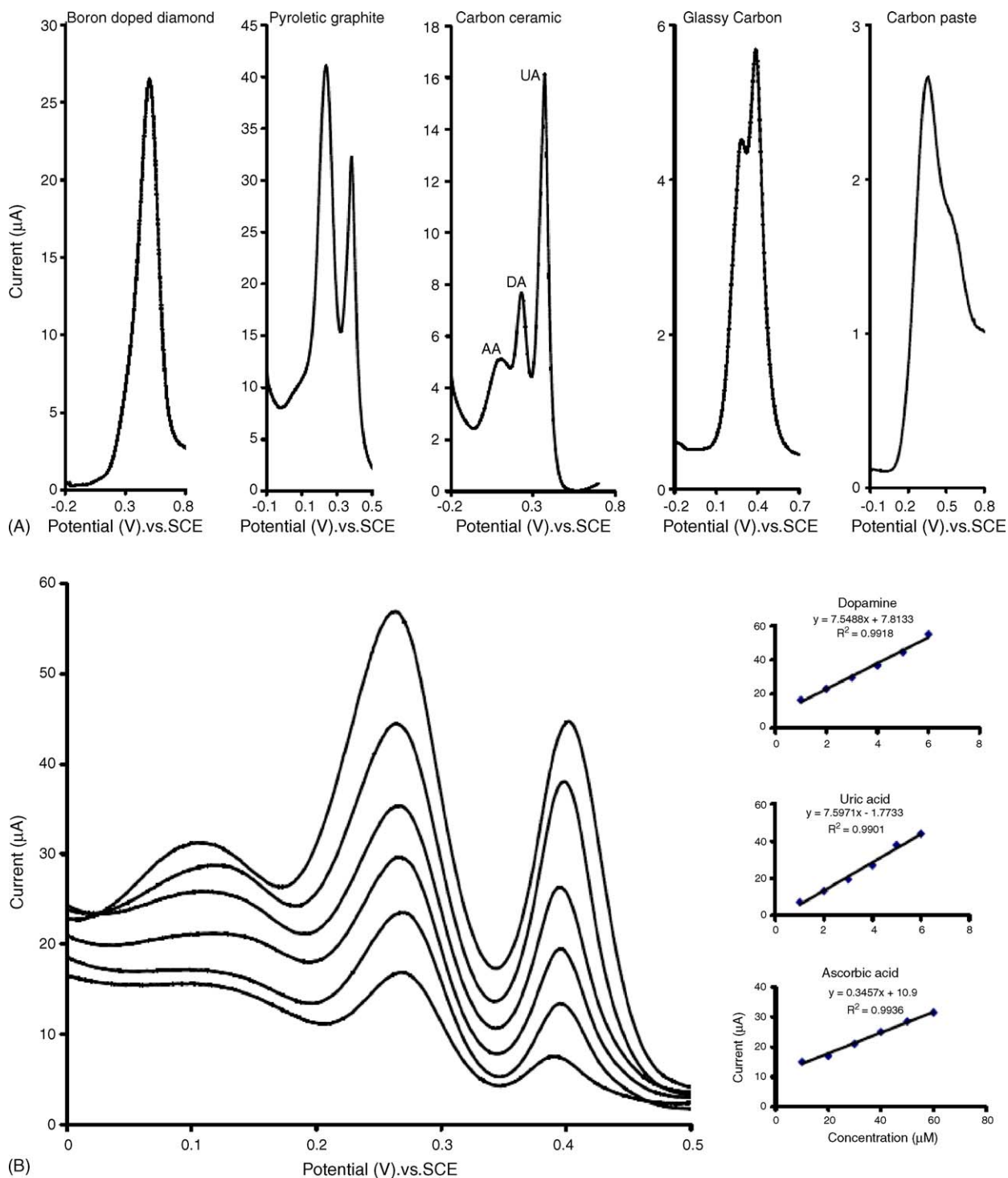


Fig. 5. (A) Square wave voltammograms of 10 μ M of AA, 1 μ M DA, and 2 μ M UA at CCE in 0.1 M phosphate buffer solution (pH 5). (B) as (A) concentration of AA, DA, and UA are simultaneously changed: 10, 20, 30, 40, 50 μ M of AA and 1, 2, 3, 4, 5 and 6 μ M DA and UA. Insets: the variation of peak currents vs. biomolecules concentrations.

reported in Table 1. The analytical parameters are better or comparable with results reported for simultaneous determination of these biomolecules at the surface different modified electrodes (Table 1 results and Refs. [44–50]). The electrode response was very stable and showed excellent antifouling properties against the oxidation products. For evaluation of the ability of CCE to determination UA or catecholamines in the presence of ascorbic

acid, the SQW voltammograms was recorded for the oxidation of UA and dopamine in the presence of AA (Fig. 6). A linear increase in the peak current of neurotransmitters and uric acid were observed while increasing the concentration of dopamine and UA and the value of voltammetric peak current of AA is remained almost unchanged during the addition of UA and neurotransmitters (only the background current is changed). These

Table 1
Analytical parameters for simultaneous detection UA, AA and neurotransmitters at several modified electrodes

Electrode	Method	Analyte	Dynamic range (μM)	Limit of detection (μM)	Sensitivity ($\mu\text{A } \mu\text{M}^{-1}$)	Reference
Sol–gel composite containing copper and MPS ^a	Amperometry	AA	16–5000	8.6	–	[24]
		DA	7.3–2200	5.8	–	
Glassy carbon modified by propionylcholine	DPV ^b	UA	0.07–70	0.02	–	[41]
		AA	–	–	–	
Novel choline and acetylcholine modified glassy carbon	DPV	DA	0.7–5	0.3	–	[14]
		AA	7–90	0.9	–	
		5-HT ^c	1–30	0.5	–	
Sol–gel composite containing silver and MPS	Amperometry	DA in the presence of AA	6.6–1200	1.4	0.026	[25]
Self-assembled lipid bilayer (DTDB-BLM) ^d modified glassy carbon electrode	DPV	DA	0.01–100	0.01	–	[15]
Carbon paste electrode containing iron II phthalocyanin and iron(II) tetrasulfo phthalocyanin GC modified with (BAHHFP) ^g	FIA ^e	In the presence of AA	5×10^{-4} –500	5×10^{-5}	–	[20]
	OSWV ^f	DA	1–15	1	–	
	DPV	5-HT	–	1	–	
Gold modified electrode cationic self-assembled monolayer	SWV ^h	DA	0.05–3	0.005	138	[18]
		UA	0.5–100	0.1	4.67	
		DA	0.5–100	0.5	0.021	
Modified carbon paste electrode by tetrabromo- <i>p</i> -benzoquinone	DPV	AA	0.3–100	0.3	0.036	[19]
		AA	10–600	0.62	0.005	
		DA	10–100	–	0.0074	
Highly oxidized electrode	LSV ⁱ	UA	10–100	–	0.0022	[8]
		AA	100–7000	100	–	
		UA	0.2–60	0.2	–	
Clay modified electrode	SWV	DA	0–6	0.0027	–	[17]
		UA	0.1–10	0.2	17	
Gold electrode self-assembled monolayer of hetroaromatic thiol	OSWV	AA	–	–	6.47	[9]
		UA	1–300	1	14.9	
Oxidized diamond film electrode	Amperometry	UA in the presence of AA	Up to 1	0.015	–	[40]
		AA	–	–	–	
2,4,6-Triphenylpyrylum ion encapsulated into zeolyte Y sol–gel ceramic film incorporating methylene-blue on GC electrode	DPV	DA in the presence of AA	1–250	0.2	0.062	[42]
Sol–gel ceramic film incorporating methylene-blue on GC electrode		UA	–	0.001	–	[43]
		AA	–	0.005	–	
Nafion coated glassy carbon electrode modified with catechin hydrate	Amperometry	DA in the presence of AA	0.1–100	0.011	0.016	[33]
Bppg ^j electrode modified with MWCNTs ^k	Amperometry	Epinephrine in the presence of AA	0.1–100	0.02	0.42	[35]
	Amperometry	DA	0.5–50	0.07	0.0414	
		UA	1–50	0.4	0.0303	
		AA	5–1000	1.7	8.5	
		AD	0.5–100	0.07	0.044	
Sol–gel carbon ceramic composite electrode	SWV	DA	0.5–20	0.1	0.75	This work
		UA	0.5–20	0.1	0.754	
		AA	10–200	5	0.36	
		AD	5–100	1	0.83	

^a (3-Mercaptopropyl)trimethoxysilane.

^b Differential pulse voltammetry.

^c 5-HT serotonin.

^d 5,5-Ditetradecyl-2-(2-trimethyl-ammonioethyl)-1,3-dioxane bromide.

^e Flow injection analysis.

^f Osteryoung square wave voltammetry.

^g 2,2-Bis(3-amino-4-hydroxyphenyl)hexafluoropropane modified GC.

^h Square wave voltammetry.

ⁱ Linear sweep voltammetry.

^j Basal plane pyrolytic graphite.

^k Multi wall carbon nanotube.

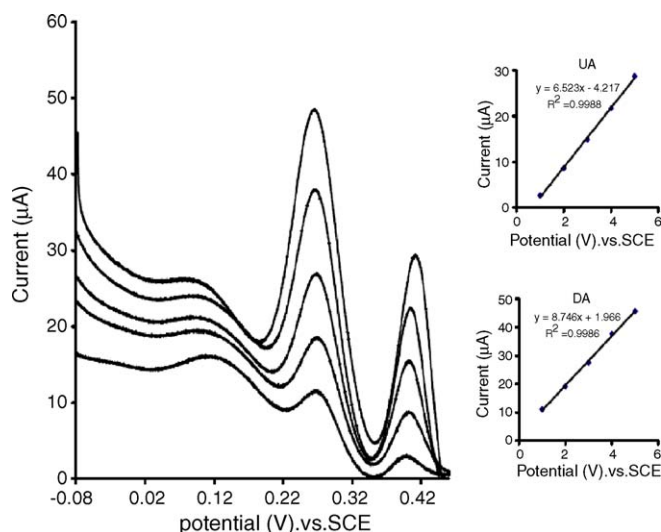


Fig. 6. Square wave voltammograms of simultaneously concentration of DA and UA changed from 1 to 5 μM in 0.1 M phosphate buffer solution (pH 5) in the presence 0.1 mM ascorbic acid.

results indicate that the homogeneous catalytic oxidation has not been found and the voltammetric peak of AA was unaltered by the oxidation of UA and neurotransmitters.

3.4. Amperometric determination of AA, UA, DA and adrenaline

For compounds such as dopamine, adrenaline and other neurotransmitters derivatives, a separation step is needed when mixture of these compounds want to be analysed by using an amperometric detection because of they have the very similar oxidation potentials. The CCE is suitable amperometric detector for catecholamines determination because it has high sensitivity and antifouling properties. Fig. 7 shows amperogram recorded from a rotating disk carbon ceramic electrode. In this experiment the potential of electrode was controlled at the constant value of 0.4 V during the successive addition of 0.5 μM dopamine. A well-defined response is observed for addition of dopamine up to 100 nM. As shown in inset of Fig. 7, the measured currents increase by increasing the dopamine concentration in solution. However, for a high concentration of dopamine the I versus DA concentration curve deviate from linearity. The detection limits, linear calibration range and sensitivity for DA, UA, AA and adrenaline are presented in Table 1. These analytical parameters are comparable or even better than those obtained when using several modified carbon ceramic or other kind modified electrodes (see results of Table 1 and Refs. [44–50]). In addition, small variation (2–4%) in the sensitivity was observed when the concentration of above molecules was changed in the concentration calibration range. Fig. 8 shows the steady-state response of carbon ceramic electrode obtained for the oxidation of DA, AD, UA and ascorbic acid. The steady-state current for oxidation of these biomolecules was examined at constant potential of electrode at 0.5 V in phosphate buffer solution (pH 5) and individually injection of DA, AD, UA and AA into the solution. The current response for adrenaline, UA and

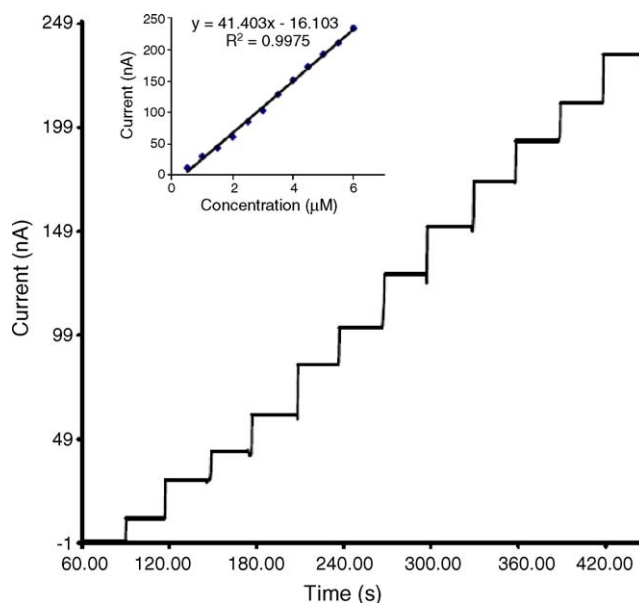


Fig. 7. Chronoamperometric response of the CCE for the successive addition of 0.5 μM dopamine to the stirring 0.1 M phosphate buffer solution; pH 5; stirring rate: 1000 rpm and electrode potential was kept at 0.4 V. Inset: calibration plot.

DA before and after the injection of AA is very similar. This fact confirms the elimination of the above mentioned catalytic oxidation at the CCE, and these results indicate SQW voltammetric measurements. The selective determination of AA, DA and UA can be made by controlling the electrode potential. For instance, by holding the electrode potential at 0.15 V, the selective determination of ascorbic acid can be done. The current responses for AA, DA, UA and adrenaline were very fast (<1 s) and stable upon repeated injection of these biomolecules. Since fabrication procedure of CCE is easy without using any electron transfer or specific reagents, the long-term stability of the electrode is not a big issue. Electrode can be renewed simply by polishing its surface. One of the big advantages of this

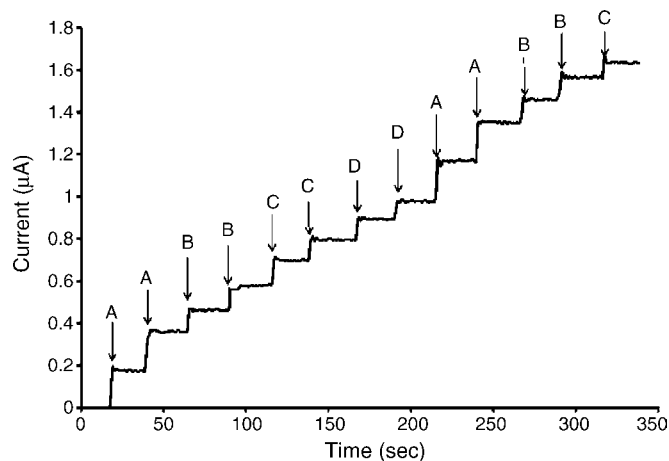


Fig. 8. Steady-state current response obtained for the oxidation of DA, AD, UA and AA at CCE upon the injection of (A), (B), (C) 1 μM of DA, AD, UA and (D) 10 μM of AA, respectively. Electrode potential and rotation rate were 500 mV and 1000 rpm.

Table 2
Determination of UA, AD and DA in real samples with carbon ceramic electrode

Sample	Original (μM)	Added (μM)	Found (μM) ^a	Recovery (%)	Total ^b (mg/l)
Serum	Uric acid, 23.34	10	33 (± 1)	100 (± 3)	41 (± 2)
		20	44 (± 1)	100 (± 3)	
		30	55 (± 1)	103 (± 3)	
Serum	Adrenaline	2	2.1 (± 0.05)	105 (± 2.3)	
		3	3.2 (± 0.1)	106 (± 3)	
		4	4 (± 0.1)	95 (± 4)	
Urine	Uric acid, 39.13	10	50 (± 01)	103 (± 3)	548 (± 3)
		20	58 (± 1)	99 (± 2)	
		30	68 (± 1)	98 (± 2)	
Urine	Dopamine	1	1.02 (± 0.02)	102 (± 1)	
		2	1.85 (± 0.05)	92 (± 2)	
		3	3.3 (± 0.1)	110 (± 3)	

^a The average of three repeated measurements.

^b The total value (average of four measurements) was obtained by multiplying the detected value by the appropriate dilution factor (83 for urine and 10 for serum samples).

CCE for amperometric detection of above biomolecules is its high stability of amperometric response during the long period of time towards AA, DA, UA and adrenaline oxidation. We have recorded the amperometric response of AA, DA, UA and adrenaline for the period 20 min (not shown). It was interestingly observed that oxidation current only decreased less than 5% after 20 min.

3.5. Analytical performance of carbon ceramic electrode for biomolecules determination in real samples

We examined the applicability of our carbon ceramic electrode for determination of uric acid, dopamine and adrenaline in real samples by measuring UA, DA and adrenaline in urine and

serum samples. The urine and serum samples were diluted 83 and 10 times with phosphate buffer solution before the measurements to prevent the matrix effect of real samples. The square wave voltammograms recorded for urine sample were shown in Fig. 9. As can be seen a voltammetric peaks at 0.11 and 0.4 V were observed, corresponding to the oxidation ascorbic and uric acid respectively. However no detectable peaks observed for oxidation DA and adrenaline in potential range of 0.15–0.3 V. The results obtained for UA in serum and urine samples are listed in Table 2. To ascertain the correctness of the results the urine and serum samples were spiked with certain amount of UA, DA and adrenaline in about the same concentration as found in the samples themselves or linear concentration range of method. The recovery rates of the spiked samples were determined and ranged between 97 and 105% for, UA, DA and adrenaline in serum and urine samples. The total value concentration of UA in the serum and urine sample are in close agreement with the value reported by other research groups [16,17,25].

4. Conclusion

In this paper we presented a method for preparation of carbon ceramic electrode by sol–gel technique without using any electron transfer mediator or specific reagents. The results indicated that the CCE facilitates the simultaneous determination of uric acid, ascorbic acid, dopamine or adrenaline with good sensitivity and high selectivity compared to the similar based electrodes. This sensor can be used for square wave voltammetric or amperometric determination of UA, and neurotransmitters as low as 0.5 μM in the presence of 0.1 mM ascorbic acid without interfering and surface fouling. The CCE is sensitive, stable and fast response for biomolecules determination and was shown that it can be successfully used for determination of UA and AA in diluted real samples and neurotransmitters detection in real samples. Simple fabrication procedure, wide linear range, low detection limit, high stability and good reproducibility for repeated determination, suggest this electrode as a good and attractive candidate for practical applications.

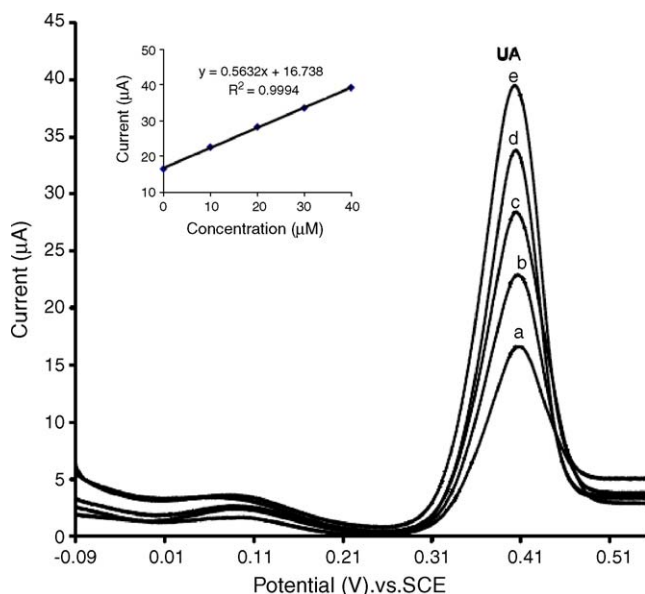


Fig. 9. Square wave voltammogram obtained for the oxidation of real urine sample (83 times diluted with pH 5 phosphate buffer) (a) at CCE surface: serum in the presence different concentration of uric acid, (b) 10 μM , (c) 20 μM , (d) 30 μM and (e) 40 μM .

Acknowledgments

This research was supported by research office of Kurdistan University. The authors are grateful to Dr. Saied Soltanian, Dr. Habibollah Danyali and Dr. Gholam Hossain Karimi Dostan for their valuable discussion and Dr. Mehrdad Parki (Shafa Medical Laboratory of Sanandaj) for serum samples.

References

- [1] T.E. Smith, Textbook of Biochemistry with Clinical Correlations, Wiley/Liss, New York, 1992, p. 929.
- [2] O. Arrigori, C.D. Tullio, Biochim. Biophys. Acta 1569 (2002) 1.
- [3] D.V.S. Gswara, H.A. Mottola, Anal. Chem. 46 (1974) 1777.
- [4] H.A. Harper, Review of Physiological Chemistry, 13th ed., Lange Medical Publications, Los Altos, CA, 1977, p. 406.
- [5] R.D. O'Neill, Analyst 119 (1994) 767.
- [6] J.W. Luo, M. Zhang, D.W. Pang, Sens. Actuators B 106 (2004) 325.
- [7] M. Zhang, K. Gong, H. Zhang, L. Mao, Biosens. Bioelectron. 20 (2004) 1270.
- [8] J. Premkumar, S.B. Khoo, J. Electroanal. Chem. 576 (2005) 105.
- [9] C.R. Raj, T. Ohsaka, J. Electroanal. Chem. 540 (2003) 69.
- [10] P. Ramesh, G.S. Suresh, S. Sampath, J. Electroanal. Chem. 561 (2004) 173.
- [11] T. Selavaraj, R. Ramaraj, Electrochem. Commun. 8 (2003) 667.
- [12] T. Liu, M. Li, Q. Li, Talanta 63 (2004) 1053.
- [13] H. Zhao, Y. Zhang, Z. Yuan, Anal. Chim. Acta 20 (2001) 1270.
- [14] G.P. Jin, X.Q. Lin, J.M. Gong, J. Electroanal. Chem. 569 (2004) 135.
- [15] X. Lin, J. Gong, Anal. Chim. Acta 507 (2004) 255.
- [16] C.R. Raj, K. Tokuda, T. Ohsaka, Biochemistry 53 (2001) 183.
- [17] J.M. Zen, P.J. Chen, Anal. Chem. 69 (1997) 5087.
- [18] G. Milczarek, A. Ciszewski, Electroanalysis 16 (2004) 1977.
- [19] H.R. Zare, N. Nasirzadeh, M. Mazlom Ardakani, J. Electroanal. Chem. 577 (2005) 25.
- [20] J. Oni, T. Nyokong, Anal. Chim. Acta 434 (2001) 9.
- [21] A. Walcarius, Electroanalysis 13 (2001) 701.
- [22] L. Rabinovich, O. Lev, Electroanalysis 13 (2001) 265.
- [23] O. Lev, Z. Wu, S. Bharathi, V. Glezer, A. Modestov, J. Gun, L. Rabinovich, S. Samph, Chem. Mater. 9 (1997) 2354.
- [24] R.D. Shankaran, K. Iimura, T. Kato, Sens. Actuators B 94 (2003) 73.
- [25] R.D. Shankaran, N. Uehara, T. Kato, Anal. Chim. Acta 478 (2003) 321.
- [26] D. Martinez-Perez, M.L. Ferrer, C.R. Mateo, Anal. Biochem. 322 (2003) 238.
- [27] A. Salimi, S. Pourbeyram, M.K. Amini, Analyst 127 (2002) 1649.
- [28] A. Salimi, S. Pourbeyram, Talanta 60 (2003) 205.
- [29] A. Salimi, S. Pourbeyram, H. Hadadzadeh, J. Electroanal. Chem. 542 (2003) 39.
- [30] A. Salimi, K. Abdi, Talanta 63 (2004) 475.
- [31] A. Salimi, K. Abdi, G.R. Khayatian, Electrochim. Acta 49 (2004) 413.
- [32] A. Salimi, K. Abdi, G.R. Khayatian, Microchim. Acta 144 (2004) 161.
- [33] A. Salimi, V. Alizadeh, H. Hadadzadeh, Electroanalysis 16 (2004) 1984.
- [34] A. Salimi, R. Hallaj, Electroanalysis 16 (2004) 1964.
- [35] A. Salimi, C.E. Banks, R.G. Compton, Analyst 129 (2004) 225.
- [36] A. Salimi, R. Hallaj, M.K. Amini, Anal. Chim. Acta 534 (2005) 335.
- [37] M. Tsionsky, G. Gun, V. Glezer, O. Lev, Anal. Chem. 66 (1994) 1747.
- [38] J. Wang, M. Li, Z. Shi, N. Li, Z. Gu, Electroanalysis 14 (2002) 225.
- [39] M. Marazuela, L. Agui, A.G. Cortes, P.Y. Seden, J.M. Pingarron, Electroanalysis 11 (1999) 1333.
- [40] E. Popa, Y. Kubota, D.A. Tryk, A. Fujishima, Anal. Chem. 72 (2000) 1724.
- [41] X.Q. Lin, G.P. Jin, Electrochim. Acta 50 (2005) 3210.
- [42] A. Domenech, H. Garcia, M.T. Domenech-Carbo, M.S. Galletero, Anal. Chem. 74 (2002) 562.
- [43] S.B. Khoo, F. Chen, Anal. Chem. 74 (2002) 5734.
- [44] P. Kalimuthu, S.A. John, Electrochem. Commun. 7 (2005) 1271.
- [45] H.R. Zare, F. Memarzadeh, M.M. Ardakani, M. Namazian, S.M. Golabi, Electrochim. Acta 50 (2005) 3495.
- [46] S.A. John, J. Electroanal. Chem. 579 (2005) 249.
- [47] A.P. dos Reis, C.R.T. Tarley, N. Maniasso, L.T. Kubota, Talanta 67 (2005) 829.
- [48] L. Zhang, X. Jiang, J. Electroanal. Chem. 583 (2005) 292.
- [49] Y. Tang, M. Wu, Talanta 65 (2005) 794.
- [50] Y. Zho, Y. Gao, D. Zhan, H. Liu, Q. Zhao, Y. Kou, Y. Shao, M. Li, Q. Zhuang, Z. Zhu, Talanta 66 (2005) 51.

Short communication

Quantitative and qualitative studies of silica in different rice samples grown in north of Iran using UV–vis, XRD and IR spectroscopy techniques

Abdolraouf Samadi-Maybodi*, Ebrahim Atashbozorg

Department of Chemistry, Faculty of Basic Sciences, Mazandaran University, Babolsar, Iran

Received 22 August 2005; received in revised form 15 January 2006; accepted 3 February 2006

Available online 20 March 2006

Abstract

Silicon is an essential trace element and is found in vegetables, fruits, cereals, water, pasta and rice (*Oryza sativa*). In this work, the silica content of different types of rice grains were measured. Here, we used the heteropoly blue photometric method with a double beam UV–vis spectrophotometer to determine the amount of silicon in rice samples ($n=7$) that were collected in the north of Iran. The samples were digested with wet-ashing method by microwave-assisted heating and then treated with ammonium molybdate to produce a yellow color compound in acidic solution (ca. pH 1.2) and then reduced to give a heteropoly compound with a blue color. Analyses were performed using standard addition method and absorbance values were measured with double beam UV–vis spectrophotometer at $\lambda_{\text{max}}=815$ nm. Results indicated that the silica content was 307–451 mg/kg for the samples. X-ray diffraction patterns and infra-red spectra were obtained from rice samples without any sample treatment.

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Keywords: Silica; Heteropoly blue method; Rice; Microwave digestion; IR; XRD; UV–vis spectrophotometry

1. Introduction

Rice is the main foodstuff for about half of the world's population. For the last decades rice consumption has been expanding beyond the traditional rice-grown areas particularly in Asia and Europe. In most countries surveillance measures are taken regarding the presence of different elements in important foodstuff such as rice to study the structure.

Silicon is one of the most abundant elements on earth after oxygen and is present in nature as silica or silicate. Generally silicon compounds are classified in two classes: (a) water soluble silica (Si-sol) such as silicic acid or low polymerized silicate which is able to react with reagents in solution media and (b) insoluble silica in water (complex polymers) that needs prior treatment to provide a clear solution of silica for analysis by spectroscopic methods. Determination of total silica in natural samples such as aerosols, water, biological and other environmental samples is usually carried out by spectroscopic methods such as UV–vis spectrophotometric method [1,2], direct cur-

rent plasma-atomic emission spectroscopy [3], inductively coupled plasma atomic emission spectrometry [4], flameless atomic absorption spectroscopy [5] and atomic absorption spectroscopy [6]. Frequently determination of reactive silica (Si-sol) in water is considered using colorimetric method based on formation of molybdenum heteropolyacid (MHA) ($[\text{SiMo}_{12}\text{O}_{40}]^{4-}$) or heteropoly anion with blue color ($[\text{SiMo}_{12}\text{O}_{40}]^{5-}$) [7–9]. The yellow molybdenum heteropoly acid (MHA) has a maximum absorption at 410 nm [10,11]. When a reducing agent acts on the yellow MHA an intensive blue color is produced and provides more sensitive measurement at $\lambda_{\text{max}}=815$ nm than at 410 nm for the yellow MHA complex. Several reducing agents have been used such as 1-amino-2-naphthol-4-sulfonic acid (ANS) [1], ascorbic acid [2] and *p*-methylaminophenol sulfate [12].

Analysis of silicon in plant tissue has been accomplished using dissolution techniques. Dissolution techniques are generally classified in two classes: (a) wet-ashing (digestion) and (b) dry-ashing. Wet-ashing methods are usually preferred when organic matrices are decomposed for subsequent determination of trace metals. Dry-ashing is the simplest method for decomposing an organic sample by heating with a flame in an open dish or crucible until all the carbonaceous materials are oxidized by

* Corresponding author. Tel.: +98 112 5242002; fax: +98 112 5242002.
E-mail address: samadi@umz.ac.ir (A. Samadi-Maybodi).

the air. Analysis of the nonvolatile components is carried out after dissolving the residual solid in appropriate solvent.

There are two ways of heating the sample, i.e. with microwave and furnace apparatus. During the past decade microwave digestion methods have become popular because they are more reproducible, more accurate and less time consuming than conventional digestion method on hot plates in open beaker [13]. Microwave heating process use small volume of reagents and allows more samples to be processed in a period of time than conventional digestion methods. Three different ways were developed for the decomposition of biological materials by microwave digestion: open focused microwave systems, closed-pressurized microwave digestion systems and a combination of both above systems [14–24]. Open-vessel focused microwave digestion method significantly increases the efficiency of acid digestion and has overcome several limitations of conventional closed-vessel microwaves. Open-vessel microwave digestions of organic and carbonate samples which produce large volumes of gases during the digestion, can be carried out avoiding the safety concerns of forming high pressure bombs and sample loss. Large organic samples can be digested using the open-vessel microwave which minimizes the time consuming of drying and grinding steps [25]. However, closed-vessel methods are preferred here for low volume of solutions. In low volume of solutions, a large volume of sample solution is rapidly lost because of the rapid and effective microwave heating in a short period of time. Hence, it is better to use closed-vessel containers for sample digestion during microwave heating.

In this work, the rice flour samples were digested by microwave-assisted wet-ashing with solution of $\text{HNO}_3/\text{H}_2\text{O}_2$. In order to determine silica, the solution of the heteropoly blue compound “[$\text{H}_2\text{N}(\text{HO})\text{C}_{10}\text{H}_5\text{SO}_3$] $^+[\text{SiMo}_{12}\text{O}_{40}]^{5-}$ ” was prepared and absorption values were measured by UV–vis spectrophotometry at maximum wavelength of $\lambda_{\text{max}} = 815 \text{ nm}$. Qualitative data were obtained by X-ray patterns and IR spectra of the rice flour without any sample treatment.

2. Experimental

2.1. Apparatus

A Braic 2100 double beam UV–Vis Spectrophotometer was used for recording absorption spectra and an electrical shaker (IKA-VIBRAX-VXR) used for shaking the solutions. To avoid any contamination of silica from glassware, polyethylene volumetric vessels (pipet, beaker and bottles) were used in all experiments. A 1000 W microwave (Panasonic NN-K543WF) and pH meter (Corning-250) was used. The crystallinity of the rice flour samples was tested by using X-ray diffractometer (GBC MMA Instrument) with Be filtered $\text{Cu K}\alpha$ 1.5406 Å radiation operating at 35.4 kV and 28 mA. The framework vibration spectra (IR spectra) were recorded by KBr pellet technique on a FT-IR, Vector 22-Bruker spectrometer. A sample digestion container made of teflon (P.T.F.E) with cap was used for digestion of rice samples in microwave.

2.2. Materials

Nitric acid (65%), hydrochloric acid (37%), hydrogen peroxide (30%), ammonium molybdate, oxalic acid, 1-amino-2-naphthol-4-sulfonic acid, disodium sulfite, silica (SiO_2) and sodium hydrogen sulfite were obtained from Merck. Triply distilled water was used thoroughly. A silica solution was prepared by dissolving silica (Merck) in NaOH (1000 ppm SiO_2) as a stock solution.

2.2.1. Colorimetric agent

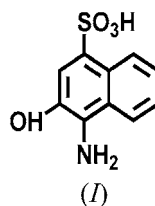
A solution of ammonium molybdate was prepared by dissolving 5 g of ammonium molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$) in 50 ml distilled water and stored in polyethylene bottle.

2.2.2. Masking agent

A solution of oxalic acid was prepared by dissolving 5 g of oxalic acid ($\text{H}_2\text{C}_2\text{O}_4\cdot 2\text{H}_2\text{O}$) in 50 ml distilled water and stored in polyethylene bottle.

2.2.3. Reducing agent

In order to prepare the solution of reducing agent, first 0.0625 g of 1-amino-2-naphthol-4-sulfonic acid (**I**) and 0.1250 g of disodium sulfite were dissolved in 6.25 ml of distilled water (solution A). Then, 3.75 g of sodium hydrogen sulfite was dissolved in 18.75 ml of distilled water (solution B). Solutions A and B were mixed and stored in polyethylene bottle and kept in dark place at low temperature (i.e. $<10^\circ\text{C}$).



(I)

2.3. Sample preparation

Different rice samples were collected from the same area in the north of Iran (Mazandaran). At the first, the rice grains were cleaned with water and finally washed with distilled water. Then were dried (at 50°C for 1 h), grinded and stored in polyethylene bottles at 10°C .

Wet digestion method was performed using microwave-assisted heating. Mixture of $\text{HNO}_3\text{--H}_2\text{O}_2$ –distilled water (10 ml, 4:2:4) was used for digestion of 0.1000 g of rice flour in a closed-vessel teflon container and it was stirred for 10–15 min before placed in microwave. The heating microwave program was performed as shown in Table 1, the mixture was shaken between each step (i.e. resting time). Then the teflon container was cooled down to room temperature and the solution was neutralized with NaOH. The solution was diluted to 50 ml with distilled water and stored in polyethylene bottle at 10°C .

Then the solution of ammonium molybdate, HCl 1:1, oxalic acid and reducing reagent were added to the sample solution. The absorption value was measured by a double beam

Table 1
The heating microwave program for digestion

Step	Time	Microwave power (W)
1	3 min	170
Resting time	5 s	0
2	2 min	340
Resting time	5 s	0
3	2 min	510

Table 2

The results of measuring silica content in the rice samples with standard addition method, the absorbance values were read four times

Sample number	Silica concentration (mg/kg rice)
1	347 (± 0.65)
2	309 (± 0.59)
3	439 (± 0.57)
4	378 (± 0.60)
5	451 (± 0.62)
6	413 (± 0.64)
7	314 (± 0.60)

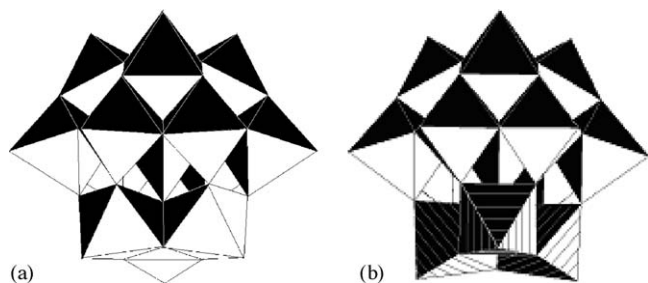
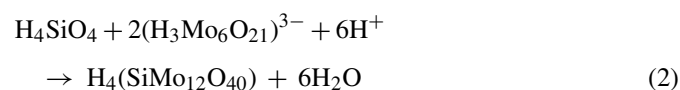
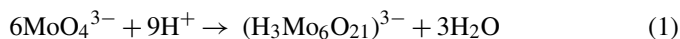


Fig. 1. Structure of: (a) α -isomer and (b) β -isomer of molybdosilicic acid.

UV–vis spectrometer ($\lambda_{\max} = 815$ nm) at ambient temperature (ca. 27 °C).

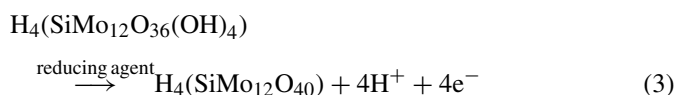
3. Results and discussion

Molybdate ions form hexamolybdenum acid in acidic medium (see Eq. (1)) [26]. Silicic acid reacts with hexamolybdenum acid ion and produces the yellow molybdosilicic acid complex (see Eq. (2)) which exists in two forms depending on pH of solution (see Fig. 1) [27].



The α -isomer (Keggin structure) exists at pH 3.5–4.5 and is very stable, whereas the β -isomer (derived from α -isomer) is formed rapidly at the pH of 0.8–2.5 and is less stable [28–29]. At pH approximately 1.2 only β -[SiMo₁₂O₄₀]⁴⁻ isomer exists but at higher than that both α and β isomers are present. Therefore reduction of β -molybdosilicic acid was carried out in aqueous

acidic solutions at pH of 1.2 and the produced heteropoly blue anion (β -[SiMo₁₂O₄₀]⁵⁻) had a maximum absorption value at 815 nm (see Eq. (3)) [30].



Rice flour is a complex mixture of carbohydrates, proteins, fats, fibers and mineral silicates that requires particular methods to ensure completeness of the decomposition [31]. Here, two different methods were performed for digestion of rice flour, once with HNO₃ and the other with mixture of HNO₃/H₂O₂. Fig. 2 illustrates the absorbance values of digestion with HNO₃ and mixture of HNO₃/H₂O₂ for two different rice samples (0.1000 g rice flour). Results reveal that the latter method is better than that former, this is because of oxidizing characteristic of H₂O₂ which can rapidly react with the organic matrix of sample [32]. Experiments for digestion of the sample were carried out with different ratios of HNO₃/H₂O₂, the optimized ratio for HNO₃/H₂O₂ was 4/1 (by volume).

Phosphate ion (PO₄³⁻) is a serious interference in the determination of silicon because it can also react with molybdate ion and produce a phosphomolybdate complex with the same color (yellow). Addition of oxalic acid avoids reaction with the phosphate and acts as a masking reagent [1]. In series experiments, different amounts of oxalic acid were added to the sample solution. Results indicated that the optimized amount of oxalic acid was 0.4% (w/v). The amount of colorimetric reagents, i.e. ammonium molybdate and reducing reagent were also optimized; the standard addition method was performed for all measurements. The results of standard addition measurements are presented in Table 2 and also illustrated in Fig. 3. As

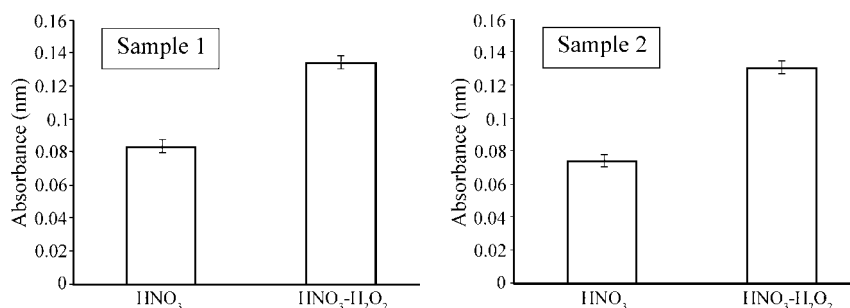


Fig. 2. Results of digestion methods for two different samples with HNO₃ and mixture of HNO₃/H₂O₂.

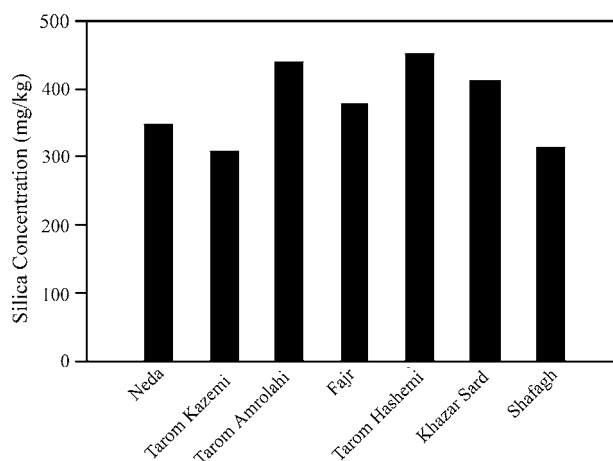


Fig. 3. Silica concentration (mg/kg) in different rice samples.

can be seen the content of silica in the rice samples are different for various samples, which lies between 307 and 451 mg/kg rice. Results indicated that samples No. 3 (Tarom Amrolahi) and samples No. 5 (Tarom Hashemi) contain more silica than the others. It has been proved that the silica content in the rice influences on the rice quality such as rice resistance to lodging and dry matter accumulation in rice [33–35], activity of some enzymes

involved in the photosynthesis in rice [36,37], spikelet fertility [38], improving grain yields of rice cultivars [38], delaying fungal ingress and controlling several diseases of warm-season [39].

The X-ray spectra of the rice flour samples and pure silica (Merck) have been obtained and shown in Fig. 4, no sample treatment has been performed on the samples. The spectrum of pure silica (Fig. 4A) shows a broad peak in the range of $18\text{--}30^\circ$ (2θ), which indicates an amorphous structure. On the other hand, X-ray spectra of the samples consists of narrow peaks indicating that there is a crystalline structure in the rice flour. Since the peaks lie in the same region of the silica, i.e. located between 15° and 30° (2θ), therefore it can be imagined that there is a crystalline structure of silicate in the rice. The X-ray spectra of the samples are almost similar to each other and there are no significant differences between them.

Infra-red spectra of the rice flour samples and pure silica (Merck) were also recorded by FT-IR spectrometer and presented in Fig. 5. Although each spectrum has a number of signals and fully interpretation of them is impossible, but some of signals can be attributed to the specific vibration. For instance, band located at $1114\text{--}1060\text{ cm}^{-1}$ denoted to the Si–O asymmetry stretching, band at $850\text{--}800\text{ cm}^{-1}$ ascribed to the Si–O symmetry stretching, band at $1130\text{--}1000\text{ cm}^{-1}$ belongs to Si–O–Si asymmetric stretching and band centered at 3420 cm^{-1} assign to the H_2O band [40].

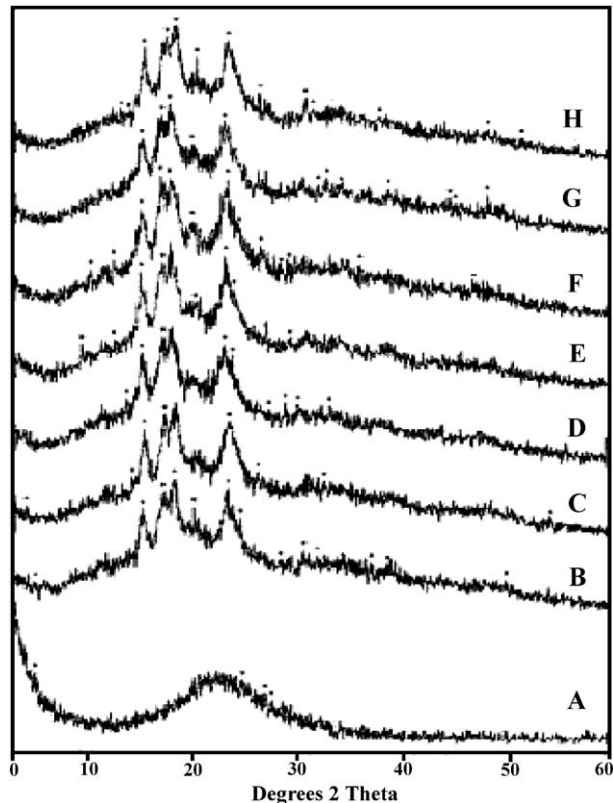


Fig. 4. X-ray diffraction patterns of: pure silica (SiO_2) (A) and rice samples*, Tarom Amrolahi (B), Tarom Hashemi (C), Neda (D), Khazar Sard (E), Tarom Kazemi (F), Fajr (G) and Shafagh (H). *The names of samples are local area names.

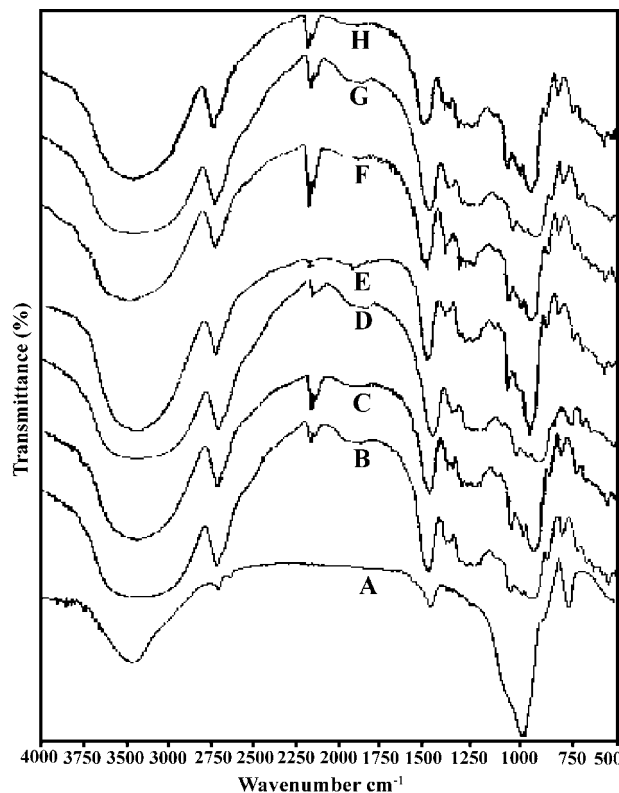


Fig. 5. Infra-red spectra of: pure silica (SiO_2) (A) and rice flour samples, Tarom Amrolahi (B), Tarom Hashemi (C), Neda (D), Khazar Sard (E), Tarom Kazemi (F), Fajr (G) and Shafagh (H).

4. Conclusions

Results obtained from spectrophotometric method based on the formation of heteropoly blue complex for determination of silica in different rice samples (north of Iran) indicated, although they were planted in the same area but the silica content were different in those ones. X-ray diffraction pattern of the rice flour revealed that a crystalline structure of compound can present in that one. IR spectra also give some information about the presence of Si–O and Si–O–Si bands in rice samples.

References

- [1] M.C. Giacomelli, O. Largiuni, G. Piccardi, *Anal. Chim. Acta* 396 (1999) 258–292.
- [2] M.A. Raggi, C. Sabbioni, R. Mandrioli, Q. Zini, G. Varani, *J. Pharm. Biomed. Anal.* 20 (1999) 335–342.
- [3] G.M. Bercow, H. Vo, F. Rieders, *J. Anal. Toxicol.* 18 (1994) 46–48.
- [4] A. Lopez Molinero, L. Martinez, A. Villareal, J.R. Casillo, *Talanta* 45 (1998) 1211–1217.
- [5] Annual Book of ASTM Standards, Water and Environmental Technology, Standard Test Method for Low Level Total Silica in High Purity Water By Flameless Atomic Absorption Spectroscopy, vol. 11.02, Designation D 4517-85, ASTM, Philadelphia, 1990.
- [6] R. Novozamsky, E. Van, V.J.G. Houba, *Commun. Soil Sci. Plant Anal.* 5 (1984) 205–211.
- [7] U. Hase, K. Yoshimura, *Anal. Sci.* 9 (CA 118) (1993) 111–115.
- [8] B. Allen III, P.A. Mayewski, W.B. Lyons, M.J. Spencer, *Ann. Glaciol.* 7 (1985) 1–6.
- [9] Annual Book of ASTM Standards, Water and Environmental Technology, Standard Test Method for Silica in Water, vol. 11.01, Designation D, ASTM, Philadelphia, 1990, pp. 859–888.
- [10] Annual Book of ASTM Standards, Part 3.1, “Water”, Standard D859-68, 1976, p. 401.
- [11] Standard Methods for the Examination of Water and Wastewater, 14th ed., Method 426B, 1975, p. 487.
- [12] K.A. Fanning, M.E.Q. Pilson, *Anal. Chem.* 45 (1973) 135–140.
- [13] H.M. Kingston, L.B. Jassie, *Introduction to Microwave Sample Preparation*, American Chemical Society, Washington, USA, 1998.
- [14] D.A. Instock, W.M. Yeager, P.M. Groshe, A. Gaskill, *Validation of a Method for Determining Elements in Solid Waste by Microwave Digestion*, Report, Research Triangle Park, NC, 1989.
- [15] S.A. Atthes, in: L.B. Jassie, H.M. Kingston (Eds.), *Introduction to Microwave Sample Preparation: Theory and Practice*, ACS, Washington, DC, 1988, p. 33.
- [16] R. Revesz, E. Hasty, *Microwave Digestion of Soils, Sediments, and Waste Water for Analysis of Environmentally Significant Elements*, International Conference on Metals in Soils, Waters, Plants and Animals, 1990, Paper FR.
- [17] V. Ducros, D. Ruffieux, N. Belin, A. Favier, *Analyst* 119 (1994) 1715–1717.
- [18] G.J. DeMenna, G.J. Brown, *Int. Lab.* 24 (1994) 25–27.
- [19] S. Fridlund, S. Littlefield, J.S. Rivers, *Commun. Soil Soc. Plant Anal.* 25 (1994) 933–941.
- [20] L.B. Gilman, W.G. Engelhart, *Spectroscopy* 4 (1989) 14–21.
- [21] W. Lautenschläger, *Spectroscopy* 4 (1989) 16–21.
- [22] J.S. Yaeger, L.L. Smith, *Waste Minimization through High-Pressure Microwave Digestion of Soils for Gross α/β Analyses*, Argonne National Laboratory, ANIJACL-95-3, 1995.
- [23] S. Baldwin, M. Deaker, W. Maher, *Analyst* 119 (1994) 1701–1704.
- [24] B.S. Sheppard, D.T. Heitkemper, C.M. Gaston, *Analyst* 119 (1994) 1683–1686.
- [25] H.L. Zhang, J.A. Hattey, *Commun. Soil Sci. Plant Anal.* 31 (2000) 2959–2967.
- [26] M.T. Pope, *Heteropoly and Isopoly Oxometalates*, Springer-Verlag, Berlin, 1983 (Chapter 2).
- [27] I.A. Weinstock, J.J. Cowan, E.M.G. Barbuzzi, H. Zeng, C.L. Hill, *J. Am. Chem. Soc.* 121 (1999) 4608–4617.
- [28] I.R. Morrison, A.L. Wilson, *Analyst* 88 (1043) (1963) 88–99.
- [29] V.W. Truesdale, C.J. Smith, *Analyst* 100 (1188) (1975) 203–212.
- [30] J.N. Barrow, J.B. Jameson, M.T. Pope, *J. Am. Chem. Soc.* 107 (1985) 1771–1773.
- [31] M.N. St. Paul, in: B.O. Juliano (Ed.), *Rice Chemistry and Technology*, second ed., American Association of Cereal Chemists, 1985 (Chapter 2).
- [32] E. Vassileva, C.R. Quétel, *Anal. Chim. Acta* 519 (2004) 79–86.
- [33] M.H. Adatia, A.T. Besford, *Ann. Bot.* 58 (1986) 343–351.
- [34] E. Epstein, *Proc. Natl. Acad. Sci. U.S.A.* 91 (1991) 11–17.
- [35] Y.C. Liang, Q.R. Shen, Z.G. Shen, T.S. Ma, *J. Plant Nutr.* 19 (1996) 173–183.
- [36] N.K. Savant, G.H. Snyder, L.E. Datnoff, in: D.L. Sparks (Ed.), *Advances in Agronomy*, vol. 58, Academic Press, San Diego, CA, 1997, pp. 151–199.
- [37] R.E. Schmidt, X. Zhang, D.R. Chalmers, *J. Plant Nutr.* 22 (1999) 1763–1773.
- [38] N.K. Savant, L.E. Datnoff, G.H. Snyder, *Commun. Soil Sci. Plant Anal.* 28 (1997) 1245–1252.
- [39] A. Fawe, J.G. Menzies, M. Chérif, R.R. Bélanger, in: L.E. Datnoff, G.H. Snyder, G.H. Korndörfer (Eds.), *Silicon in Agriculture*, Elsevier Science, New York, NY, 2001, pp. 159–169.
- [40] F.L. Galeener, *Phys. Rev. B* 8 (19) (1993) 4292–4297.

Amperometric choline biosensors prepared by layer-by-layer deposition of choline oxidase on the Prussian blue-modified platinum electrode

Haibin Shi^a, Yu Yang^a, Jiadong Huang^a, Zixia Zhao^a,
Xinhua Xu^b, Jun-ichi Anzai^c, Tetsuo Osa^c, Qiang Chen^{a,*}

^a College of Life Science, Nankai University, Tianjin 300071, China

^b School of Materials Science and Engineering, Tianjin University, Tianjin 300072, China

^c Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8578, Japan

Received 8 September 2005; received in revised form 4 February 2006; accepted 7 February 2006

Available online 23 March 2006

Abstract

An amperometric choline biosensor was developed by immobilizing choline oxidase (ChOx) in a layer-by-layer (LBL) multilayer film on a platinum (Pt) electrode modified with Prussian blue (PB). 6-*O*-Ethoxytrimethylammoniochitosan chloride (EACC) was used to prepare the ChOx LBL films. The choline biosensor was used at 0.0 V versus Ag/AgCl to detect choline and exhibited good characteristics such as relative low detection limit (5×10^{-7} M), short response time (within 10 s), high sensitivity ($88.6 \mu\text{A mM}^{-1} \text{cm}^{-2}$) and a good selectivity. The results were explained based on the ultrathin nature of the LBL films and the low operating potential that could be due to the efficient catalytic reduction of H_2O_2 by PB. In addition, the effects of pH, temperature and applied potential on the amperometric response of choline biosensor were evaluated. The apparent Michaelis–Menten constant was found to be $(0.083 \pm 0.001) \times 10^{-3}$ M. The biosensor showed excellent long-term storage stability, which originates from a strong adsorption of ChOx in the EACC multilayer film. When the present choline biosensor was applied to the analysis of phosphatidylcholine in serum samples, the measurement values agreed satisfactorily with those by a hospital method.

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Keywords: Choline oxidase; Amperometric biosensor; Layer-by-layer; Prussian blue; 6-*O*-Ethoxytrimethylammoniochitosan chloride

1. Introduction

The quantitative determination of choline in biological samples such as human bile, serum, amniotic fluid, brain extracts and pharmaceutical products is important in clinical analysis [1–4]. Thus, the development of amperometric choline biosensors utilizing choline oxidase (ChOx) is an active research area because biosensors offer simple, inexpensive, rapid and reliable means for the analysis [5–16].

ChOx has been immobilized onto the electrode surfaces by a variety of techniques, including casting and electropolymerization, cross-linking and self-assembly techniques [6–15]. Recently, ordered protein multilayers can also be produced with a layer-by-layer (LBL) technique based on physical adsorption

driven by an ionic force of attraction [17]. The LBL technique has recently been extended to a variety of materials, including synthetic polymers, nanoparticles, nucleic acids and proteins [5,16,18–25]. As far as proteins are concerned, the major advantage of the LBL technique lies on the possibility of minimizing denaturing because the adsorption process is carried out in aqueous solutions under mild conditions. The LBL film-modified enzyme sensors have been shown to exhibit a good reproducibility, stability and high sensitivity [5,16,18,19,23–25].

Most of amperometric choline biosensors have been used based on the direct oxidation of enzymatically liberated hydrogen peroxide (H_2O_2) or mediator-based determination [5–16]. The direct detection of H_2O_2 is usually accomplished by the oxidation at anodic potentials (greater than +0.6 V versus Ag/AgCl) [5,6,10–13,15–16]. However, electrochemically active interfering species which are usually present in biological fluids, such as ascorbic acid, uric acid and acetaminophen, are easily oxidized at the same potentials and produce an interfering current

* Corresponding author. Tel.: +86 22 23506182; fax: +86 22 23506122.
E-mail address: qiangchen@nankai.edu.cn (Q. Chen).

[18,26]. This results in a difficulty in the quantitative analysis of H_2O_2 and the enzyme substrate.

This drawback can be circumvented by operating the sensor at lower potential range (from -0.2 V to 0.0 V versus Ag/AgCl) where interfering reactions cause only a minor influence on the biosensor response [7–9,12,14]. Up to now, there are only two low-potential transducers for H_2O_2 reduction: peroxidase [9,12] and metal hexacyanoferrate [7,8,14]. Prussian blue (PB) with particular characteristic of catalyzing H_2O_2 reduction at a potential range between -0.2 V and 0.0 V (versus Ag/AgCl), is defined as “artificial peroxidase” and the “range offering the most sensitive and interference-free detection” for amperometric biosensors [7,27–30]. Thus, the effects of the most common interfering species can be avoided or greatly reduced. Additionally, its long-term stability makes it more suitable than horseradish peroxidase (HRP) in assembling modified electrode [7,30]. Recently, papers have appeared on constructing oxidase enzyme-based biosensors for the detection of choline [7,8,14], glucose [24,30–32], glutamate [32–34], lactate [32,35,36], cholesterol [37,38], oxalate [39] and sucrose [40].

We report here an efficient and simple way to prepare choline biosensors using ChOx and 6-*O*-ethoxytrimethylammoniochitosan chloride (EACC) multilayer films deposited on a PB-modified platinum (Pt). According to our knowledge, LBL technique has never been used for the immobilization of ChOx on PB-modified electrode. In addition, no work has concerned the immobilization of enzymes with EACC for biosensor applications. In this paper, EACC is first used instead of chitosan to fabricate layer-by-layer film to improve the characteristics of choline biosensor. EACC films have an excellent film-forming ability, high permeability towards water and small molecules, good adhesion, non-toxicity, high mechanical strength and good biocompatibility [41–43]. All these virtues make it a promising matrix for enzyme immobilization for the practical use of biosensors [42,43]. The experimental conditions related to the preparation and characteristics of the deposition behavior of EACC and ChOx to fabricate the choline biosensor have also been studied in details and discussed. The choline biosensor thus fabricated exhibits excellent performances, such as relative low detection limit, fast response time, high sensitivity and good stability.

2. Experimental

2.1. Reagents

Choline oxidase (ChOx, EC 1.1.3.17, from *Alcaligenes* species, 17 units mg^{-1}) and phospholipase D (PLD, EC 3.1.4.4, from *Streptomyces* species, 150 units mg^{-1}) were obtained from Sigma (USA). 6-*O*-Ethoxytrimethylammoniochitosan chloride (EACC, degree of deacetylation was >85%, MW: 80,000) was gifted from Tianjin University (China). Choline chloride was obtained from Sigma. Serum samples were obtained from the Hospital at Nankai University. All other reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using doubly distilled water.

2.2. Preparation of PB film on the Pt electrode

The Pt electrode (diameter 3 mm) was polished with alumina powder, washed with double distilled water, and finally sonicated thoroughly in double distilled water before use. Then, the Pt electrodes were modified with a PB film according to the reported procedure [24]. The Pt electrode was potentiostatically deposited at a potential of $+0.4\text{ V}$ during 60 s from an aqueous $2 \times 10^{-3}\text{ M K}_3[\text{Fe}(\text{CN})_6] + 2 \times 10^{-3}\text{ M FeCl}_3$ solution in $0.1\text{ M KCl} + 0.01\text{ M HCl}$. After deposition, the modified electrodes were rinsed with double distilled water and immersed into a solution containing $0.1\text{ M KCl} + 0.01\text{ M HCl}$, where the electrode potential was cycled between 0.0 V and 1.0 V at a scan rate of 50 mV s^{-1} , until a stable voltammetric response was obtained [24].

2.3. Preparation of (EACC/ChOx)_n multilayer film-modified choline sensor

ChOx can be used as polyanionic material for preparing LBL film around pH 7.4, because the isoelectric point (*pI*) of ChOx lies around pH 4.5 [16]. The pH of ChOx solution has been set apart from *pI* so that the protein is sufficiently charged. The solution for enzyme immobilization thus used was consisted of 0.2 mg mL^{-1} of ChOx in 0.1 M phosphate buffer solution at pH 7.4.

The multilayer films containing EACC and ChOx were prepared on the PB-modified Pt electrode according to the reported procedure [18]. The PB-modified Pt electrode was immersed in an EACC solution (2 mg mL^{-1} in Dulbecco's buffer solution [18], pH 7.4) for 20 min to deposit the first polycationic layer. After being rinsed in phosphate buffer solution (pH 7.4) for 5 min to remove weakly adsorbed polycations, the Pt electrode was immersed in a ChOx solution for 20 min to immobilize enzyme on the polycation-modified electrode through electrostatic force. Then the Pt electrode was washed with phosphate buffer solution (pH 7.4) for 5 min to remove weakly adsorbed enzymes. Such deposition and rinsing processes were carried out repeatedly to obtain the desired number of layers. The resulting enzyme electrodes were thoroughly washed with phosphate buffer solution (pH 7.4) and stored in phosphate buffer solution (pH 7.4) below 4°C for future use. Fig. 1 shows the chemical structure of EACC and a possible structure of the EACC/ChOx multilayer film.

2.4. Electrochemical measurements

The electrochemical instrument consisted of a Potentiostat–Galvanostat (EG&G PARC Model 283 with a software M270). The electrochemical measurements of the choline biosensor were carried out with a conventional three-electrode system. A PB/(EACC/ChOx)_n film-modified electrode (3 mm diameter) was used as a working electrode, a Pt wire (1 mm diameter) as an auxiliary electrode and an Ag/AgCl as a reference electrode. All the electrochemical measurements were carried out in 20 mL of 0.1 M phosphate buffer solution with a gently stirring. Prior to the experiment, the phosphate buffer solution was deoxygenated by bubbling thoroughly with

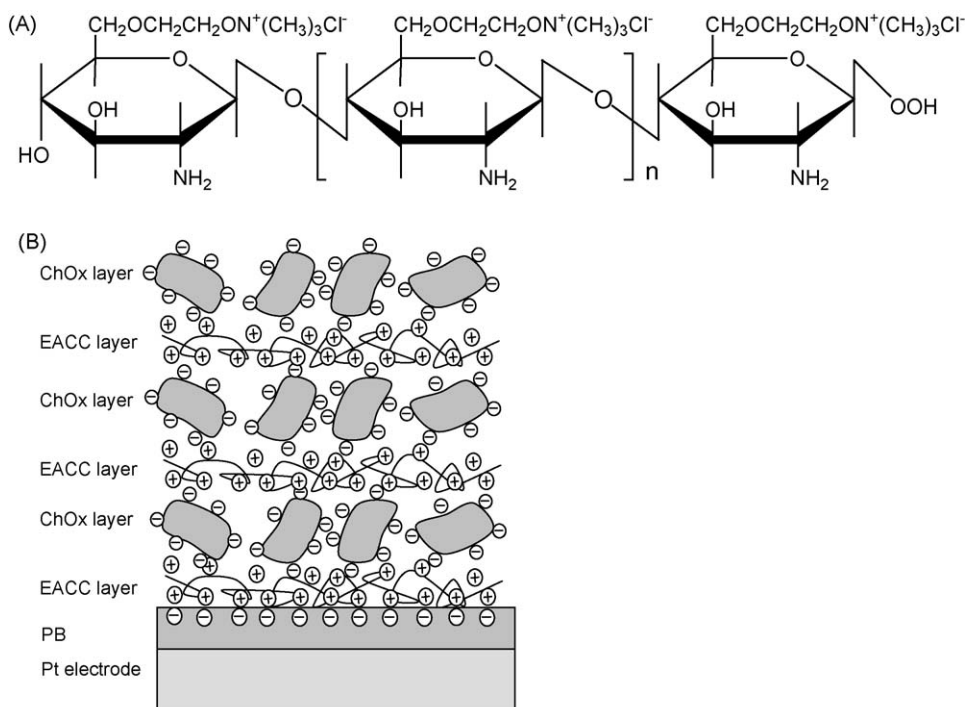


Fig. 1. (A) Chemical structure of 6-*O*-ethoxytrimethylammoniochitosan chloride. (B) Possible structure of Pt/PB/(EACC/ChOx)_{*n*} LBL films.

high-purity nitrogen at least 15 min. Then a stream of nitrogen was blown gently across the surface of the phosphate buffer solution to maintain the solution anaerobic in the experiment. The background current was allowed to decay to a steady value before aliquots of stock choline solution were added.

2.5. Gravimetric measurements

A quartz crystal microbalance (QCM) (QCA 917 Model, EG & G) with an AT-cut quartz resonator (9 MHz) whose surface (effective surface area: 0.2 cm²) is covered with a Pt thin layer was used to monitor the deposition of EACC and ChOx. The deposition process of PB/(EACC/ChOx)_{*n*} is the same as above. In the QCM device used, the adsorption of 1 ng mass induces a −0.91 Hz change in the resonance frequency as discussed in the next section [5].

3. Results and discussion

3.1. QCM Study

Prior to fabricating choline biosensors, a set of QCM measurement was performed to clarify the deposition behavior of the EACC and ChOx. The relationship between the change in resonant frequency (ΔF) resulting from a change in mass (m), is given by Eq. (1) [44]:

$$\Delta F = -2F_0^2(\rho_q\mu_q)^{-1/2}mA^{-1} \quad (1)$$

where ΔF is the measured frequency shift due to the adsorbed mass in Hz, F_0 the fundamental oscillation frequency of the dry crystal (9 × 10⁶ Hz), ρ_q the density of quartz (2.65 g cm^{−3}), μ_q

the shear modulus (2.95 × 10¹¹ dyn cm^{−2}), A the quartz area (0.2 cm²) and m is the deposited mass. For the 9 MHz quartz crystal used in this work, Eq. (1) predicts that an adsorbed mass of 1 ng corresponds to a frequency reduction of 0.91 Hz [5].

The insets of Fig. 2 show the typical adsorption kinetics for two consecutive steps of the EACC/ChOx bilayer assembly. The inset A of Fig. 2 shows frequency changes induced by the adsorption of EACC (curve a) and that during the subsequent washing process (curve b). In curve a, one can see a time course of the frequency decrease due to the adsorption of EACC, where the adsorption of EACC required ca. 20 min to reach equilibrium. Curve b shows the desorption of the small excess parts of the adsorbed EACC by washing with phosphate buffer solution probably due to a weak binding. After washing, the final loading of EACC was ca. 154 ± 10 ng (ca. 140 ± 10 Hz). Frequency changes due to the adsorption of ChOx onto the resulting EACC-covered film are shown in the inset B of Fig. 2, where a similar adsorption and washing behavior was observed. The loading of ChOx was ca. 143 ± 10 ng (ca. 130 ± 10 Hz). The thickness of the each EACC and ChOx layers can be estimated to be 6.4 nm and 5.6 nm, respectively, assuming the density of 1.2 ± 0.1 g cm^{−3} for EACC layer and 1.3 ± 0.1 g cm^{−3} for ChOx layer according to the reported procedure [45]. These results suggest that EACC and ChOx can be assembled successfully into a thin film (ca. 12 nm) by the layer-by-layer deposition technique. Furthermore, EACC is easily soluble in both acidic and basic solutions and is a better candidate for a polycationic material in fabricating biosensors. In fact, the results shows that the assemble effect of water-soluble EACC (6.4 nm) is better than chitosan (4.9 nm) [19]. The reason is that EACC is a stronger polycationic than chitosan, because it has the cationic group of

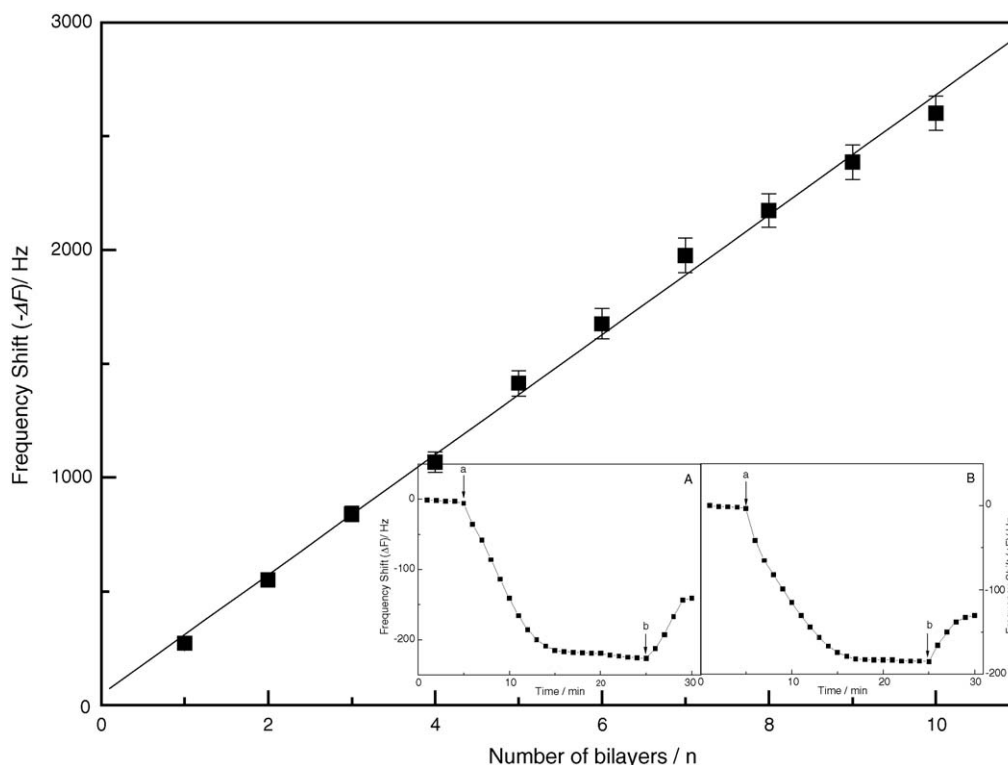


Fig. 2. Relationship between the frequency change and the number of EACC/ChOx bilayers deposited on the quartz resonator. The error bars show the deviations within five measurements. The inserts display the frequency changes of QCM induced by the deposition of EACC (A) and ChOx (B). The quartz resonator was immersed in EACC or ChOx solution at (a) and in phosphate buffer solution at (b).

quaternary ammonium salt in its “O-” site and improves protonation of the amino groups. Therefore EACC is easier to be protonated and stronger base than chitosan, and provides the better properties for fabricating multilayer film with negative enzyme through electrostatic force than chitosan itself.

The multilayer films containing EACC and ChOx were prepared on the quartz resonator to monitor the deposition behavior. Fig. 2 shows the frequency shifts of the resonator during the deposition of the EACC/ChOx bilayer. The resonance frequency changed linearly, confirming a linear growth of the EACC/ChOx multilayer film as a function of the number of bilayers. This results mean that the EACC/ChOx multilayer film is successfully formed through electrostatic force of attraction between the protonated EACC and negatively charged ChOx (pI, 4.5) [16] and that, in each deposition step, the amount of enzyme immobilized is approximately the same.

3.2. Effect of the number of bilayers on the sensitivity of choline sensors

Electrochemical measurements were carried out to evaluate the effects of the number of EACC/ChOx bilayer on the sensitivity of the choline biosensor. The wide concentration range (5×10^{-7} M to 1×10^{-4} M) of choline solutions were used in the present assays. Table 1 shows the sensitivity for choline increases almost linearly with the number of EACC/ChOx bilayers stepwise from $14.7 \mu\text{A mM}^{-1} \text{cm}^{-2}$ for 2 bilayers to $88.6 \mu\text{A mM}^{-1} \text{cm}^{-2}$ for 10 bilayers. The sensitivity of the biosensors obtained from the slope of the initial linear part of

the calibration, with different limits of detection respectively (three times the noise, see Table 1). Such an increase in the sensitivity is attributed to the fact that the larger amount of enzyme was immobilized in the multilayer films when the number of bilayers is increased. These results are promising because the sensitivity could be controlled by changing the number of bilayers. Moreover, the LBL method allows fine control of thickness of the enzyme layer to avoid mass transport problems. As already discussed, the fact that the sensitivity increased well proportionally with the increased EACC/ChOx bilayer means the choline molecular can pass smoothly through the thin EACC/ChOx films and the enzymatic reaction is mainly preceded in diffusion control process. The sensitivity further increased when the number of bilayers was more than 10. However, the response time

Table 1

Effect of the number of bilayers on the sensitivity and limit of detection (LOD) of choline

The number of EACC/ChOx bilayers	Sensitivity ($\mu\text{A mM}^{-1} \text{cm}^{-2}$)	LOD (M)
2	14.7	1×10^{-4}
3	24.3	1×10^{-4}
4	31.7	5×10^{-5}
5	40.9	5×10^{-5}
6	49.3	5×10^{-5}
7	58.4	5×10^{-6}
8	69.4	5×10^{-6}
9	80.2	5×10^{-7}
10	88.6	5×10^{-7}

became slower for the thicker films probably due to the diffusion problem. Based on the above results, we chose 10 bilayers of PB/(EACC/ChOx)₁₀ film-modified electrodes to construct choline biosensors.

3.3. Optimization of experimental parameters

The effects of pH, temperature and applied potential on the behavior of the PB/(EACC/ChOx)₁₀ multilayer film-modified electrode have been investigated.

The effect of the pH value of the sample solution on the current response of the PB/(EACC/ChOx)₁₀ film modified electrodes to 5×10^{-5} M choline was studied at 0.0 V over the pH range 6.0–10.0 in a 0.1 M phosphate buffer solution (data not shown). The currents increased from pH 6.0 to 7.4 and then decreased over pH 7.4. This decrease might be due to the poor enzyme activity. Hence, the optimum pH for the choline biosensor to obtain the maximum response was determined to be 7.4. The result was similar to the literatures data with a maximum of the response current at around pH 7.4 reported at PB modified electrodes [8,14]. The result indicated that the present choline biosensor has not altered the optimum pH of ChOx (pH 7.0–8.0) [46].

The effect of temperature on the current response of PB/(EACC/ChOx)₁₀ film modified electrodes was also studied in 5.0×10^{-5} M choline solutions (pH 7.4) at 0.0 V (data not shown). The current response increased with the increasing temperature and reached the maximum response at ca. 30 °C. Then the current began to decrease as the temperature increased over 30 °C. The current decreased above 30 °C may be due to thermal inactivation of ChOx. Thus, the optimum temperature was ca. 30 °C for the choline biosensor.

The effect of the applied potential on the current response of sensor was also studied. The current response increased from +0.2 V and reached the maximum at 0.0 V (versus Ag/AgCl). Application of the more negative potential did not improve the current response (data not shown). Thus, the choline biosensor was polarized at 0.0 V (versus Ag/AgCl). Based on the results, we used the choline sensors at 0.0 V (versus Ag/AgCl) in this study.

3.4. Effect of interfering materials

The selectivity of the biosensor for choline detection was evaluated in the presence of possible interfering compounds such as ascorbic acid, uric acid and acetaminophen. The effect was tested by adding these substances to the choline solution. As shown in Fig. 3, the electrode was first kept in a stirred phosphate buffer solution (0.1 M, pH 7.4) at 0.0 V and a 0.05 mM choline solution was added. Then, 0.1 mM of ascorbic acid, uric acid and acetaminophen were added sequentially, which were indicated by arrows in Fig. 3. Little changes in current due to the interferences were observed. Following the addition of the interferences, further choline (0.05 mM) was added into the solution. The results confirmed the high selectivity of the choline biosensor, which may be attributed to the low potential (0.0 V versus Ag/AgCl).

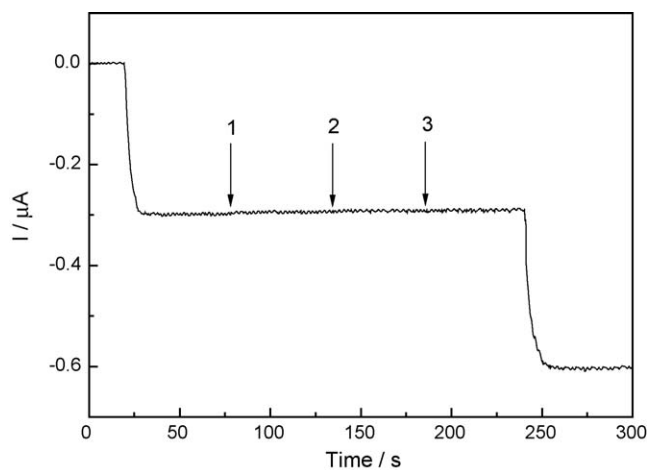


Fig. 3. Effect from the possible interferences in choline sensors. Amperometric response was obtained with 10 bilayers Pt/PB/(EACC/ChOx) biosensor in a 0.1 M phosphate buffer solution (pH 7.4) at 0.0 V vs. Ag/AgCl. The arrows show the each moment at the injection of the interfering compounds of ascorbic acid (1), uric acid (2), acetaminophen and (3) (each injection was controlled to 0.1 mM of interfering compounds). The current plateaus correspond to the injection of choline (the attained concentration is 0.05 mM).

3.5. The properties of choline biosensors

Fig. 4 shows the typical current–time plots for a choline biosensor built with PB/(EACC/ChOx)₁₀ multilayer film-modified Pt electrode upon the addition of successive choline solution in a 0.1 M phosphate buffer solution (pH 7.4) at 0.0 V. Each addition corresponds to a 0.01 mM solution increase of choline concentration. The response time achieved in the 95% value of the steady states current is short (approximately 10 s), probably because the film is ultrathin and the diffusion barrier at the electrode is very low. It is similar to that of choline biosensor prepared with [poly(ethyleneimine)/ChOx]₁₀ film (10 s), and shorter than that of choline biosensor prepared with

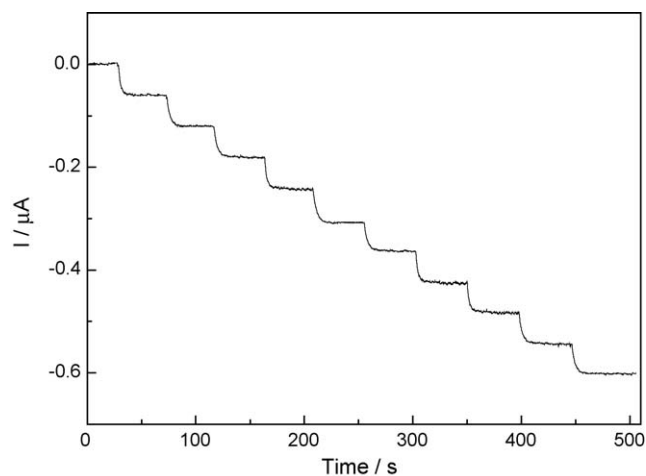


Fig. 4. Amperometric responses obtained upon the successive addition of choline solution at the applied potential 0.0 V in phosphate buffer solution at pH 7.4 with a 10-bilayer LBL film of (EACC/ChOx) deposited on to Pt/PB. Each current step corresponds to an increase of 0.01 mM in choline concentration.

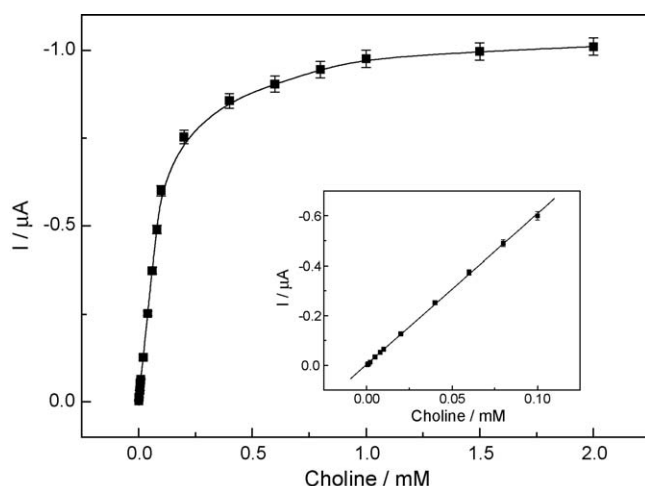


Fig. 5. Calibration curve of the response current of the sensor to choline concentration in phosphate buffer solution at pH 7.4 at the applied potential 0.0 V vs. Ag/AgCl. The error bars show the deviations within five measurements.

[poly(diallyldimethylammomium choride)/ChOx]₈ film (15 s) [16].

A typical calibration curve of the choline biosensor was obtained toward a function of choline concentration at the applied potential of 0.0 V as shown in Fig. 5. According to the Michaelis–Menten equation, the apparent Michaelis–Menten constant K_m is calculated to $(0.083 \pm 0.001) \times 10^{-3}$ M from Fig. 5. As shown in the insert of Fig. 5, the PB/(EACC/ChOx)₁₀ film-coated biosensor indicated a linear calibration graph in the concentration range of 5×10^{-7} M to 1.0×10^{-4} M, with a linear regression equation for I (μA) = $(-0.00316 \pm 0.00177) + (-6.06002 \pm 0.04116) \times c$ (mM) and relative standard deviation $R = -0.9977$. The detection limit of choline biosensor was 5×10^{-7} M, based on three times measurements with the standard deviation of the blank (95% confidence level, $k=3$, $n=5$). This value is same to that of PB-modified enzyme electrode [8] and lower than some of other PB-modified ChOx biosensors [7,14] (see Table 2). The sensitivity of choline biosensor obtained from the slope of the initial linear part of the calibration is $88.6 \mu\text{A mM}^{-1} \text{cm}^{-2}$. This value is also similar to the reported PB-modified enzyme electrode [8] and larger than some of other PB-modified ChOx biosensors [7,14] (see Table 2). These results show that this kind of choline biosensor based on ChOx immobilized in LBL film at PB-modified electrode is actually valuable and sensitive.

Table 2

Prussian blue-based choline biosensors - sensitivity and limit of detection (LOD) in comparison with the literatures

Electrode	Sensitivity ($\mu\text{A mM}^{-1} \text{cm}^{-2}$)	LOD (M)	Reference
LBL Pt/PB/(EACC/ChOx) ₁₀	88.6	5×10^{-7}	This paper
Carbon paste electrode	3.5	2×10^{-5}	[7]
Screen printed electrode	110	5×10^{-7}	[8]
Pt electrode	67	5×10^{-5}	[14]

Table 3

Analysis of choline in serum samples^a

Sample number	Determined by spectrophotometric method (1.0×10^{-4} M)	Determined by choline biosensor (1.0×10^{-4} M)	Recovery (%)
1	0.248 ± 0.005	0.259 ± 0.006	104.4 ± 1.2
2	0.418 ± 0.006	0.442 ± 0.011	105.7 ± 1.8
3	0.674 ± 0.008	0.632 ± 0.028	93.8 ± 3.5
4	0.846 ± 0.010	0.817 ± 0.024	96.6 ± 2.4
5	1.032 ± 0.007	1.096 ± 0.043	106.2 ± 6.1

^a The values are averages of five measurements by the choline biosensor and spectrophotometric method.

We checked a long-term stability of the choline sensor by measuring the current response to 0.05 mM choline once in a day and stored in 0.1 M phosphate buffer solution (pH 7.4) at 4 °C. The choline biosensor retained 85.7% of its initial current response to choline after ca. 2 month. Thereafter, the response deteriorated gradually. The good stability implies that the existence of EACC film is actually helpful for improving the stability of the enzyme electrode. Good stability may be also attributed to the enzyme entrapped strongly in the good biocompatible EACC film which is stable in the detection solutions.

3.6. Analysis of serum samples

Phosphatidylcholine makes up a considerable proportion of the lipid such as brain lipids and the determination of serum phosphatidylcholine is important for the clinical diagnosis of liver diseases. The phosphatidylcholine can be hydrolyzed by phospholipase D (PLD) to phosphatidic acid and free choline, which can become the substrate for ChOx oxidation [47]. In the present work, for the determination of phosphatidylcholine in a serum sample, 1 mL of the sample was first incubated with a stock PLD solution ($100 \mu\text{L}$, 3 mg mL^{-1}) at 37 °C for 20 min to ensure the complete hydrolysis of phosphatidylcholine and then diluted with phosphate buffer solution (pH 7.4). To verify the reliability of the responses of the choline biosensor, the sample was analyzed by the choline biosensor comparing with a standard hospital method (spectrophotometric measurement). The analytical values of choline by the present choline biosensor agreed satisfactorily with those by spectrophotometric method and the recoveries values were in the range of 93.8 ± 3.5 – $106.2 \pm 6.1\%$ (see Table 3).

4. Conclusions

The layer-by-layer (LBL) deposition technique has been exploited to produce the sensitive and stable choline biosensors. The high sensitivity and fast response time may be due not only to the efficacy in the enzyme immobilization but also to the ultrathin nature of the LBL films, in which mass transport problems are minimized. Use of PB layer is useful for electrocatalytic reduction of H_2O_2 at a low potential (0.0 V versus Ag/AgCl), which makes it possible to avoid interference arising

from ascorbic, uric acids and acetaminophen. Another merit is that the choline sensors are stable for approximately 2 months. When the present choline biosensor was applied to the analysis of phosphatidylcholine in serum samples, the obtained values agreed well with those by a hospital method.

Acknowledgements

This work was supported by the National Natural Science Foundation (No. 90209054), the Project 863 (No. 2002AA326060) and the Grant-in-Aid for the High-Tech Cooperation Project of Nankai University and Tianjin University from the Chinese Ministry of Education.

References

- [1] L. Campanella, M. Mascini, G. Palleschi, M. Tomassetti, *Clin. Chim. Acta* 151 (1985) 71.
- [2] L. Campanella, M. Tomassetti, G. De Angelis, M.P. Sammartino, M. Cordatore, *Clin. Chim. Acta* 169 (1987) 175.
- [3] L. Campanella, M. Tomassetti, M.P. Sammartino, *Analyst* 113 (1988) 77.
- [4] L. Campanella, M. Tomassetti, M.P. Sammartino, *Anal. Lett.* 22 (1989) 1389.
- [5] Q. Chen, Y. Kobayashi, H. Takeshita, T. Hoshi, J. Anzai, *Electroanalysis* 10 (1998) 94.
- [6] L. Doretto, D. Ferrara, S. Lora, F. Schiavon, F.M. Veronese, *Enzyme Microb. Technol.* 27 (2000) 279.
- [7] D. Moscone, D. D'Ottavi, D. Compagnone, G. Palleschi, *Anal. Chem.* 73 (2001) 2529.
- [8] F. Ricci, A. Amin, G. Palleschi, D. Moscone, *Biosens. Bioelectron.* 18 (2003) 165.
- [9] S.S. Razola, S. Pochet, K. Grosfils, J.M. Kauffmann, *Biosens. Bioelectron.* 18 (2003) 185.
- [10] H. Gülce, Y.S. Aktas, A. Gülce, A. Yıldız, *Enzyme Microb. Technol.* 32 (2003) 895.
- [11] S. Sen, A. Gülce, H. Gülce, *Biosens. Bioelectron.* 19 (2004) 1261.
- [12] M.A. Rahman, D. Park, Y. Shim, *Biosens. Bioelectron.* 19 (2004) 1565.
- [13] K.M. Mitchell, *Anal. Chem.* 76 (2004) 1098.
- [14] A. Curulli, F. Valentini, S. Orlanduci, M.L. Terranova, G. Palleschi, *Biosens. Bioelectron.* 20 (2004) 1223.
- [15] M.H. Yang, Y.H. Yang, Y. Yang, G.L. Shen, R.Q. Yu, *Anal. Chim. Acta* 530 (2005) 205.
- [16] H.B. Shi, Z. Song, J.D. Huang, Y. Yang, Z.X. Zhao, J. Anzai, T. Osa, Q. Chen, *Sens. Actuator B Chem.* 109 (2005) 341.
- [17] G. Decher, J.D. Hong, J. Schmitt, *Thin Solid Films* 210 (1992) 831.
- [18] T. Hoshi, H. Saiki, S. Kuwazawa, C. Tsuchiya, Q. Chen, J. Anzai, *Anal. Chem.* 73 (2001) 5310.
- [19] Q. Chen, J. Han, H.B. Shi, B.Y. Wu, X.H. Xu, T. Osa, *Sens. Lett.* 2 (2004) 102.
- [20] F. Hua, Y. Lvov, T.H. Cui, *Thin Solid Films* 449 (2004) 222.
- [21] L.P. Lu, S.Q. Wang, X.Q. Lin, *Anal. Chim. Acta* 519 (2004) 161.
- [22] G.E. Pacey, S.D. Puckett, L. Cheng, S. Khatib-Shahidi, J.A. Cox, *Anal. Chim. Acta* 533 (2005) 135.
- [23] S.X. Zhang, W.W. Yang, Y.M. Niu, C.Q. Sun, *Sens. Actuator B Chem.* 101 (2004) 387.
- [24] M. Ferreira, P.A. Fiorito, O.N. Oliveira, S.I. Torresi, *Biosens. Bioelectron.* 19 (2004) 1611.
- [25] L. Coche-Guérente, J. Desbrières, J. Fatissou, P. Labbé, M.C. Rodriguez, G. Rivas, *Electrochim. Acta* 50 (2005) 2865.
- [26] R. Maidan, A. Heller, *Anal. Chem.* 64 (1992) 2889.
- [27] V.D. Neff, *J. Electrochem. Soc.* 125 (1978) 886.
- [28] K. Itaya, H. Akahoshi, S. Toshima, *J. Electrochem. Soc.* 129 (1982) 1498.
- [29] L. Gorton, *Electroanalysis* 7 (1995) 23.
- [30] D. Pan, J.H. Chen, L.H. Nie, W.Y. Tao, S.Z. Yao, *Anal. Biochem.* 324 (2004) 115.
- [31] I.L. De Mattos, M.C. Da Cunha Areias, *Talanta* 66 (2005) 1281.
- [32] S. De Luca, M. Florescu, M.E. Ghica, A. Lupu, G. Palleschi, C.M.A. Brett, D. Compagnone, *Talanta* 68 (2005) 171.
- [33] A.A. Karyakin, E.E. Karyakina, *Sens. Actuator B Chem.* 57 (1999) 268.
- [34] Y. Wang, J. Zhu, R. Zhu, Z. Zhu, Z. Lai, Z. Chen, *Meas. Sci. Technol.* 14 (2003) 831.
- [35] R. Garjonyte, Y. Yigzaw, R. Meskys, A. Malinauskas, L. Gorton, *Sens. Actuator B Chem.* 79 (2001) 33.
- [36] F. Ricci, C. Goncalves, A. Amine, L. Gorton, G. Palleschi, D. Moscone, *Electroanalysis* 15 (2003) 1204.
- [37] J. Li, T. Peng, Y. Peng, *Electroanalysis* 15 (2003) 1031.
- [38] J.C. Vidal, J. Espuelas, E. Garcia-Ruiz, J.R. Castillo, *Talanta* 64 (2004) 655.
- [39] P.A. Fiorito, S.I.C. de Torresi, *Talanta* 62 (2004) 649.
- [40] B. Haghighi, S. Varma, F.M. Alizadeh Sh, Y. Yigzaw, L. Gorton, *Talanta* 64 (2004) 3.
- [41] S.M. Hudson, C. Smith, Polysaccharides—chitin and chitosan: chemistry and technology of their use as structural materials, in: D.L. Kaplan (Ed.), *Biopolymers from Renewable Resources*, Springer, Berlin, 1998, pp. 96–118.
- [42] B. Krajewska, *Enzyme Microb. Technol.* 35 (2004) 126.
- [43] B. Krajewska, *Sep. Purif. Technol.* 41 (2005) 305.
- [44] F. Caruso, E. Rodda, D.N. Furlong, *J. Colloid Interface Sci.* 178 (1996) 104.
- [45] Y. Lvov, K. Ariga, M. Onda, I. Ichinose, T. Kunitake, *Colloid Surf. A* 146 (1999) 337.
- [46] D. Nachmansohn, I.B. Wilson, in: F.F. Nord (Ed.), *Advances in Enzymology*, vol. 12, Interscience Publisher, New York, 1991, p. 259.
- [47] M.H. Yang, Y.H. Yang, Y. Yang, G.L. Shen, R.Q. Yu, *Anal. Biochem.* 334 (2004) 127.

Short communication

^{27}Al NMR and Raman spectroscopic studies of alkaline aluminate solutions with extremely high caustic content – Does the octahedral species $\text{Al}(\text{OH})_6^{3-}$ exist in solution?

 Pál Sipos^{a,b,*}, Glenn Hefter^a, Peter M. May^a
^a Chemistry-DSE, Murdoch University, Murdoch, WA 6150, Australia

^b Department of Inorganic and Analytical Chemistry, University of Szeged, Szeged H-6701, PO Box 440, Hungary

Received 20 September 2005; received in revised form 31 January 2006; accepted 7 February 2006

Available online 13 March 2006

Abstract

^{27}Al NMR and Raman spectra of alkaline aluminate solutions with $0.005\text{ M} \leq [\text{Al(III)}]_T \leq 3\text{ M}$ in various $\text{M}'\text{OH}$ solutions ($\text{M}'^+ = \text{Na}^+$, K^+ and Li^+) were recorded and analysed. Caustic concentrations up to 20 M were used to explore whether higher aluminium hydroxo complexes are formed at extremely high concentrations of hydroxide. A single peak was observed on the ^{27}Al NMR spectrum of each solution. The chemical shift of this peak shifts significantly upfield with increasing $[\text{M}'\text{OH}]_T$ in solutions with $[\text{Al(III)}]_T < 0.8\text{ M}$. This variation shows a strong dependence on the cation of the solution and practically disappears in systems with $[\text{Al(III)}]_T \geq 0.8\text{ M}$. For Raman spectra of solutions with $[\text{Al(III)}]_T = 0.8\text{ M}$ and $[\text{NaOH}]_T \geq 10\text{ M}$, the peak maximum of the symmetric $\nu_1\text{-AlO}_4$ stretching of $\text{Al}(\text{OH})_4^-$ shifted progressively from ~ 620 to $\sim 625\text{ cm}^{-1}$ and decreased in intensity with increasing $[\text{NaOH}]_T$. In parallel, modes centred at ~ 720 and $\sim 555\text{ cm}^{-1}$ (cf. ~ 705 and $\sim 535\text{ cm}^{-1}$ at lower $[\text{NaOH}]_T$), ascribed to a dimeric aluminate species appeared, and their intensities increased with increasing $[\text{NaOH}]_T$. These variations in the ^{27}Al NMR and Raman spectra can be interpreted in terms of contact ion-pairs formed between the cation of the medium and the well-established $\text{Al}(\text{OH})_4^-$ or the dimeric aluminate species. Assumption of higher aluminium hydroxo complex species (e.g., $\text{Al}(\text{OH})_6^{3-}$) is not necessary to explain the spectroscopic effects observed.

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Keywords: ^{27}Al NMR spectroscopy; Raman spectroscopy; Aluminate; Hydroxo complexes; Solution species; Contact ion-pairs; Concentrated electrolyte solutions; Bayer liquors

1. Introduction

Relatively little is known about the thermodynamics and structure of aluminium-containing hydroxo complexes in solutions of $\text{pH} > 13$. Yet such conditions are directly relevant to the industrial Bayer process, which employs highly concentrated hot mixtures of NaOH and $\text{Al}(\text{OH})_3$ (known as Bayer liquors) for the extraction and purification of gibbsite ($\text{Al}(\text{OH})_3(\text{s})$) from bauxitic ores. There is considerable uncertainty as to which chemical species, other than the well-characterized tetrahedral $\text{Al}(\text{OH})_4^-$ ion, exist in Bayer liquors under typical industrial conditions (i.e., $1\text{ M} < [\text{NaOH}]_T \leq 5\text{ M}$, $1\text{ M} < [\text{Al(III)}]_T \leq 3\text{ M}$ and $40^\circ\text{C} \leq T \leq 90^\circ\text{C}$) [1,2]. Since such species may provide a

key to understanding critical aspects of the Bayer process, such as the unexpectedly slow precipitation kinetics, quantification of the chemical speciation in such solutions is of considerable interest.

^{27}Al NMR spectroscopy is a ‘selective’ technique providing direct information of the changes in the environment of aluminium [3]. In alkaline aluminate solutions at industrial concentrations usually only one ^{27}Al NMR line is seen and the chemical shift is always around 80 ppm [3–8] (relative to $\text{Al}(\text{H}_2\text{O})_6^{3+}$ set at 0 ppm); this is typical of four coordinate aluminium [4,5]. The invariance in the chemical shift observed in alkaline aluminate solutions proves that the aluminium is predominantly in a tetrahedral environment. That is, $\text{Al}(\text{OH})_4^-$ [4–8] or closely related coexisting species whose interconversion causes only subtle variations in the observed ^{27}Al NMR chemical shifts [8].

It is well established that Raman spectra of alkaline aluminate solutions at $[\text{Al(III)}]_T \leq 0.5\text{ M}$ show only one significant

* Corresponding author. Tel.: +36 62 544 338; fax: +36 62 420 505.
E-mail address: sipos@chem.u-szeged.hu (P. Sipos).

band, centred at $\sim 620\text{ cm}^{-1}$ assigned to the symmetric ν_1 - AlO_4 stretching of $\text{Al}(\text{OH})_4^-$ [6,9–12]. At $[\text{M}'\text{OH}]_T \leq 10\text{ M}$ (where M' denotes an alkali metal ion) and at $[\text{Al}(\text{III})]_T \geq 0.5\text{ M}$, two new modes appear on both sides of the $\text{Al}(\text{OH})_4^-$ band. These modes, centred on ~ 700 and $\sim 535\text{ cm}^{-1}$, [9–14] are generally considered to arise from the formation of a dimer: $(\text{OH})_3\text{Al}-\text{O}-\text{Al}(\text{OH})_3^{2-}$. This assignment [6] was based on the similarity between the IR and Raman spectra of concentrated alkaline aluminate solutions and the crystalline solid $\text{K}_2[\text{Al}_2\text{O}(\text{OH})_6]$ [15] which, as shown by X-ray diffraction, contains this dimeric anion. At $[\text{M}'\text{OH}]_T \geq 10\text{ M}$ and $[\text{Al}(\text{III})]_T \leq 1.0\text{ M}$ a further band was reported [10] at $\sim 555\text{ cm}^{-1}$, which was attributed to small concentrations of $\text{Al}(\text{OH})_6^{3-}$.

Initial experiments [16] (in accordance with literature data [3,7,8]) indicated that ^{27}Al NMR spectroscopy is of low diagnostic value at typical industrial liquor concentrations (i.e., $2\text{ M} \leq [\text{NaOH}]_T \leq 5\text{ M}$, $1\text{ M} \leq [\text{Al}(\text{III})]_T \leq 3\text{ M}$). On the other hand, detailed analysis of the Raman spectra of such solutions allowed the determination of the formation constant of the dimeric species as a function of water activity [17] indicating that Raman spectra of alkaline aluminate solutions are of high information content. The aim of the current paper is a comparative study of ^{27}Al NMR and Raman spectra of solutions with caustic concentrations outside the industrial ranges (e.g., $1\text{ M} \leq [\text{NaOH}]_T \leq 20\text{ M}$, $0.05\text{ M} \leq [\text{Al}(\text{III})]_T \leq 3\text{ M}$). To explore possible effects associated with the cation of the medium, K^+ and Li^+ aluminate solutions were also investigated.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared from high purity water (Millipore Milli-Q system), which was boiled and purged with nitrogen to minimize carbon dioxide content. Concentrated alkali stock solutions were prepared from analytical grade NaOH (Ajax Chemicals, Australia, “98%” grade), KOH (Ajax, “85% (w/w)” grade with ca. 10–15% (w/w) water) and $\text{LiOH}\cdot\text{H}_2\text{O}$ (Sigma–Aldrich Chemical Company, USA, “99%+” grade). The concentrations of the stock solutions were: $\text{NaOH} \approx 20\text{ M}$, $\text{KOH} \approx 14\text{ M}$ and $\text{LiOH} \approx 4\text{ M}$. Exact concentrations were determined and carbonate contents minimised (to $\leq 0.1\%$ of the total alkalinity) using techniques described elsewhere [18]. Alkaline aluminate solutions were prepared by quantitative dissolution of degreased high purity aluminium wire (Ajax, 99.9%) in the appropriate base solution [19]. The desired concentrations in $[\text{Al}(\text{III})]_T$ and $[\text{M}'\text{OH}]_T$ were then obtained by accurate gravimetric dilution of the aluminate stock solution with a known concentration of $\text{M}'\text{OH}$ and water.

2.2. Apparatus and equipment

^{27}Al NMR spectra of the solutions were measured at 78.204 MHz on a Bruker DPX 300 MHz instrument. The alumi-

nate solutions were held in 4 mm diameter Teflon liners placed in standard 5 mm glass tubes. Spectra were accumulated over 3 min using a single pulse method of 5 μs duration (120 scans), with a repetition delay of 1 s, with an acquisition time of 0.5 s and at a pulse angle of 90° over a sweep-width of 31250 Hz. No exponential filtering was used. Spectra were recorded at ambient temperature ($25 \pm 1^\circ\text{C}$) and unlocked since the field drift was negligible. Spectra were recorded at least twice over 7–10 days to determine the effects of solution ageing. The observed NMR parameters displayed no time dependence except for solutions that precipitated. The chemical shifts of all signals were measured relative to the octahedral hexaquo aluminium(III) ion, $\text{Al}(\text{H}_2\text{O})_6^{3+}$ from a deuterium-locked acidified solution of alum, $\text{KAl}(\text{SO}_4)_2$ (5%, w/v). Five solutions of acidified ($\text{pH} \approx 1$) AlCl_3 in the concentration range $0.2\text{ M} < [\text{Al}(\text{III})]_T < 1.0\text{ M}$ were chosen as calibration standards for purposes of integration of sample peak intensities. This integration was hampered by the presence of a baseline distortion that appeared in the form of a broad hump at ca. 70 ppm, directly in the region of interest. Its origin lies in the aluminium present in the probe alloy [20]. Careful subtraction from all sample spectra of a “blank” sample (e.g., Teflon-lined glass tube filled with distilled water) allowed effective removal of this interference [20]. Errors in measuring the integrals of broad signals were numerically corrected for as suggested by Laszlo [21]. In spite of these precautions, peak integrals (performed usually between 120 and 40 ppm) showed unacceptably large fluctuations, reaching 40% for the broadest signals, and their values depended strongly on the processing parameters. From this we concluded that peak integrals could not be used safely for establishing speciation changes in these solutions.

Line widths of the ^{27}Al NMR spectra were also determined. To account for the viscosity line broadening, densities and viscosities of the solutions were also measured [22] according to the experimental protocol described in [23]. At low $\text{Al}(\text{III})$ -concentrations ($[\text{Al}(\text{III})]_T < 0.2\text{ M}$) the viscosity normalized line widths are narrow ($< 10\text{ Hz/cP}$) and seem to pass through a minimum and then a maximum with increasing $[\text{NaOH}]_T$, while at higher $[\text{Al}(\text{III})]_T$ they are clearly of maximum shape. This non-monotonic behavior suggests that linear viscosity corrections on the ^{27}Al NMR spectra might not be valid for solutions of very high viscosity.

Raman spectra were measured with a Nicolet Magna 850, Series II FT-IR spectrometer equipped with a Magna Raman module using a Nd:YVO_4 laser. For the measurements, 1.5 W laser power, 2 cm^{-1} resolution and 1024 scans were used throughout. Spectra were recorded at $25 \pm 1^\circ\text{C}$ using 5 mm fluorescence-free NMR tubes. The position of the sample tube was optimised using cyclohexane. Thus baseline fluctuations were mainly due to variations in the laser intensity. Spectral intensities varied by not more than $\pm 2\%$. A matrix-matched blank solution (containing $\text{M}'\text{OH}$ in place of $\text{M}'\text{Al}(\text{OH})_4$) was measured for each sample and its spectrum numerically subtracted from the target spectrum. The background-subtracted spectrum was then fitted in the region of interest ($400\text{--}900\text{ cm}^{-1}$) using the Galactic Grams 32.5 software.

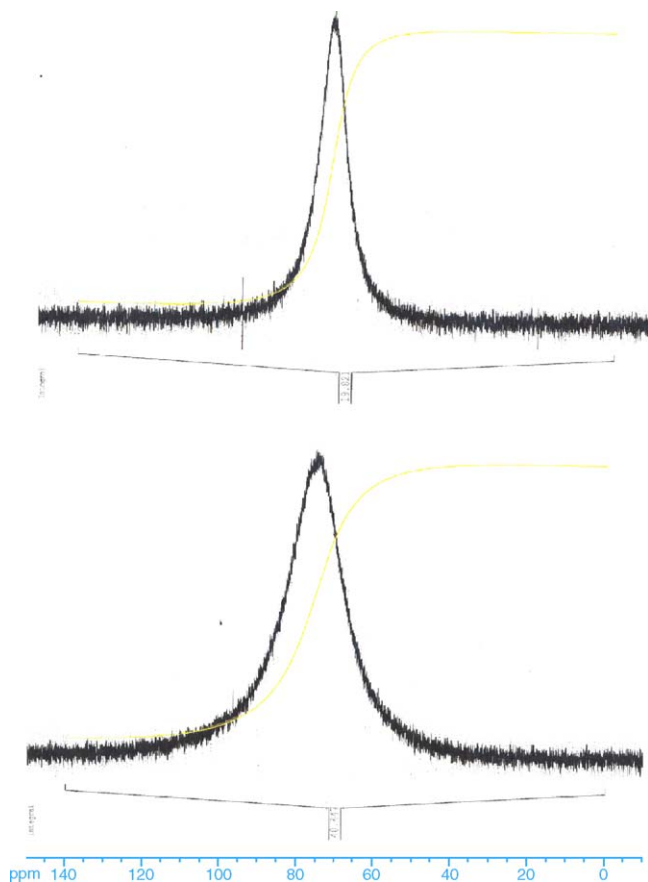


Fig. 1. ^{27}Al NMR spectra of two alkaline aluminate solutions with $[\text{NaOH}]_T = 18.0\text{ M}$. Baseline corrections have been performed as described in Section 2. Upper spectrum: $[\text{Al(III)}]_T = 0.10\text{ M}$, $\delta_{\text{Al}} = 72.8\text{ ppm}$; lower spectrum: $[\text{Al(III)}]_T = 0.80\text{ M}$, $\delta_{\text{Al}} = 76.4\text{ ppm}$.

3. Results and discussion

^{27}Al NMR spectra were recorded for a large number of aluminate solutions over the specified range of $[\text{Al(III)}]_T$ and $[\text{NaOH}]_T$. Only one broad peak was observed for each solution (Fig. 1). Fig. 2 illustrates the variation of chemical shift of this single peak as a function of both $[\text{Al(III)}]_T$ and $[\text{NaOH}]_T$. For solutions with $[\text{NaOH}]_T \leq 5\text{ M}$ (i.e., solutions of industrial

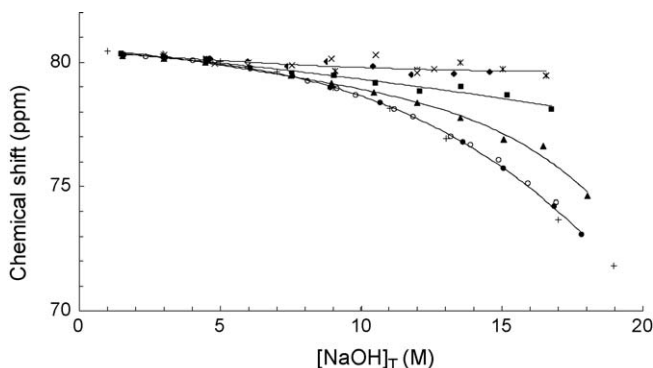


Fig. 2. ^{27}Al NMR chemical shift vs. the total concentration of NaOH (M) at various aluminium concentrations. $[\text{Al(III)}]_T = 0.005\text{ M}$ (+); 0.050 M (○); 0.100 M (●); 0.396 M (▲); 0.800 M (■); 1.20 M (✕); 2.00 M (◆); 3.00 M (x).

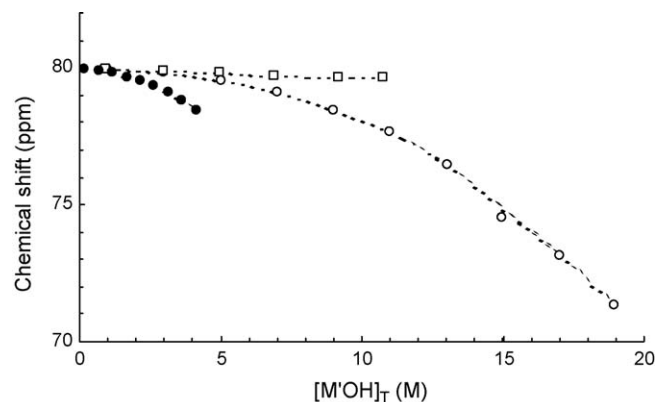


Fig. 3. ^{27}Al NMR chemical shift vs. the total molar concentration of KOH (□), NaOH (○) and LiOH (●) at $[\text{Al(III)}]_T = 0.005\text{ M}$.

composition), the chemical shift is practically independent of $[\text{Al(III)}]_T$ and constant at a value of $80.3 \pm 0.2\text{ ppm}$ in accordance with literature data [3–8]. The single peak is assigned to the tetrahedral Al(OH)_4^- (which is dominant under the prevailing concentration conditions) and/or other possible species with tetrahedral coordination geometry around the Al (cf. NaAl(OH)_4^0 ion-pairs [16,24] or the dimeric aluminate species $(\text{OH})_3\text{Al-O-Al(OH)}_3^{2-}$ [6], etc.) As $[\text{NaOH}]_T$ increases beyond 5 M, however, the chemical shifts show systematic variations both with $[\text{NaOH}]_T$ and $[\text{Al(III)}]_T$. In solutions of lower $[\text{Al(III)}]_T$ the chemical shift moves systematically upfield with the increasing $[\text{NaOH}]_T$ (Fig. 1). For $0.005\text{ M} \leq [\text{Al(III)}]_T \leq 0.1\text{ M}$ the δ_{obs} versus $[\text{NaOH}]_T$ functions are independent of $[\text{Al(III)}]_T$. On the other hand, there are no such variations in δ_{obs} at higher $[\text{Al(III)}]_T$ ($\geq 1.2\text{ M}$).

Measurements were also performed in LiOH medium using $[\text{Al(III)}]_T = 0.005\text{ M}$, due to the very low solubility of Al(III) in LiOH solutions. ^{27}Al NMR spectra of NaOH and KOH-containing systems were also recorded. As seen in Fig. 3, the chemical shift variation depends strongly on the cation of the system and follows the order of $\text{Li}^+ > \text{Na}^+ > \text{K}^+$. Note, that the ^{27}Al NMR peaks of these very low $[\text{Al(III)}]_T$ solutions were found to be hardly distinguishable from the baseline, in particular at the highest $[\text{NaOH}]_T$. Therefore the concentration of 0.005 M can be considered as the detection limit of Al(III) in these highly viscous alkaline aluminate solutions.

Raman spectra of solutions with $[\text{Al(III)}]_T = 0.8\text{ M}$ and at $2\text{ M} \leq [\text{NaOH}]_T \leq 10\text{ M}$ showed only the $\sim 620\text{ cm}^{-1}$ band with unchanged intensity and half width. However, at $[\text{NaOH}]_T \geq 10\text{ M}$, ν_{max} shifted progressively from ~ 620 to $\sim 625\text{ cm}^{-1}$ (Fig. 4) and decreased in intensity. In parallel, concentration-sensitive modes centred at ~ 710 – 720 and ~ 540 – 555 cm^{-1} , i.e., significantly different from the bands seen at ~ 705 and $\sim 535\text{ cm}^{-1}$ at lower $[\text{NaOH}]_T$, appeared. The intensities of both bands increased with increasing $[\text{NaOH}]_T$. The gradual shifts in ν_{max} are similar in magnitude to the shifts observed for CO_3^{2-} and SO_4^{2-} ions in concentrated NaOH and CsOH solutions and therefore are likely to be associated with formation of contact ion-pairs [25].

The variation of ν_{max} from ~ 620 to $\sim 625\text{ cm}^{-1}$ (Fig. 4) and the gradual downfield shift in δ_{obs} with increasing $[\text{NaOH}]_T$

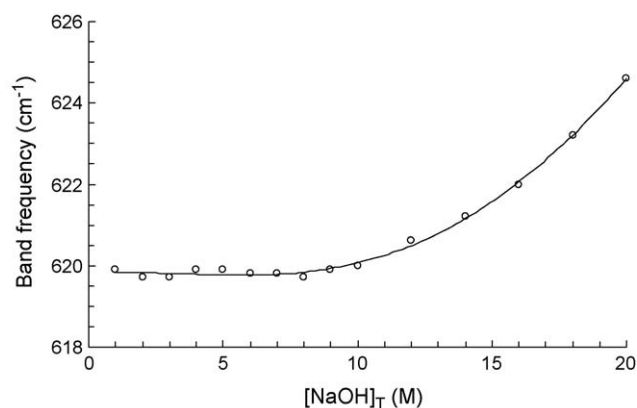


Fig. 4. Position of $\nu_{\max}$ for Raman band at $\sim 620\text{ cm}^{-1}$ as a function of $[\text{NaOH}]_T$ of solutions with $[\text{Al(III)}]_T = 0.800\text{ M}$ (const.).

(Fig. 2) occur in parallel. Hence the origin of these two effects is likely to be the same and thus the variations observed in the ^{27}Al NMR chemical shifts are associated with ion-pairing rather than transformation of aluminium species with four-fold coordination geometry to five- or six-fold ones. This interpretation is further supported by the pronounced cation-dependence of the δ_{obs} versus $[\text{M}'\text{OH}]_T$ functions (Fig. 2) and the observation, that for solutions with $[\text{Al(III)}]_T \geq 1.2\text{ M}$ (e.g., where the formation of contact ion-pairs are expected to be unfavorable due to the high $[\text{Al(III)}]_T/[\text{NaOH}]_T$ δ_{obs} is practically independent of $[\text{NaOH}]_T$ (Fig. 2).

Raman spectra of solutions with $[\text{NaOH}]_T = 18.0\text{ M}$ (const.) and $0.1\text{ M} \leq [\text{Al(III)}]_T \leq 0.8\text{ M}$ are shown in Fig. 5. The combined peak area increases linearly with $[\text{Al(III)}]_T$, however, the intensities of the ~ 720 and $\sim 555\text{ cm}^{-1}$ bands increase at the expense of the $\sim 625\text{ cm}^{-1}$ band in a non-linear manner (Fig. 6). This behavior is analogous to that observed previously for the dimeric aluminate species [17] and strongly suggests that the ~ 720 and $\sim 555\text{ cm}^{-1}$ bands correspond to the same polynuclear complex as the ~ 705 and $\sim 535\text{ cm}^{-1}$ ones observed for aluminate solutions with lower $[\text{NaOH}]_T$ [17]. The band positions are supposedly shifted because of formation of contact ion-pairs. Note, that under $[\text{NaOH}]_T = 18\text{ M}$ (const.) conditions

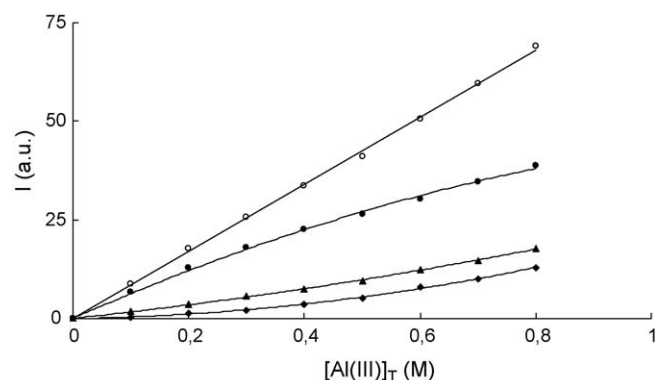


Fig. 6. Integrated intensity of the Raman peaks as a function of $[\text{Al(III)}]_T$ at $[\text{NaOH}]_T = 18.0\text{ M}$ (const.). Peak areas in the range $400\text{--}900\text{ cm}^{-1}$: combined (○); $\sim 625\text{ cm}^{-1}$ band (●); $\sim 555\text{ cm}^{-1}$ band (▲); $\sim 720\text{ cm}^{-1}$ band (◆).

the water activity (a_w), $[\text{Na}^+]_T$ and $[\text{OH}^-]_T$, are practically constant and all these three components are in large excess relative to $[\text{Al(III)}]_T$. If the development of the ~ 720 and $\sim 555\text{ cm}^{-1}$ bands was the result of an interconversion between mononuclear aluminate species, then the Raman intensities would obey the Lambert–Beer law and the individual intensities would increase strictly linearly with $[\text{Al(III)}]_T$.

4. Conclusions

All the variations observed both in the ^{27}Al NMR and Raman spectra can be interpreted in terms of contact ion-pairs formed between the cation of the medium and the well-established Al(OH)_4^- or the dimeric aluminate species. Assumption of a higher aluminium hydroxo complex species, such as Al(OH)_6^{3-} , is not necessary for the explanation of these spectroscopic effects nor is any such species detected by ^{27}Al NMR spectroscopy.

Acknowledgements

This work was funded by the Australian alumina industry through the Australian Mineral Industries Research Association as part of project P380A (Fundamentals of Alumina Precipitation) and by the Australian Government under its Cooperative Research Centres Program. PS also gratefully acknowledges the financial help of the Bolyai fellowship and the Hungarian National Research Fund (OTKA T 043551).

References

- [1] J.R. Glastonbury, Chem. Ind. (London) 5 (1969) 121.
- [2] T.G. Pearson, The Chemical Background of the Aluminium Industry, Royal Institute of Chemistry, London, UK, 1955.
- [3] J.W. Akitt, Prog. NMR Spectrosc. 21 (1989) 1.
- [4] D.E. O'Reilly, J. Chem. Phys. 32 (1960) 1007.
- [5] H. Haraguchi, S. Fujiwara, J. Phys. Chem. 73 (1969) 3467.
- [6] R.J. Moolenaar, J.C. Evans, L.D. McKeever, J. Phys. Chem. 74 (1970) 3629.
- [7] J.W. Akitt, W. Gessner, J. Chem. Soc., Dalton Trans. (1984) 147.
- [8] J.W. Akitt, W. Gessner, M. Weinberger, Magn. Reson. Chem. 26 (1988) 1047.

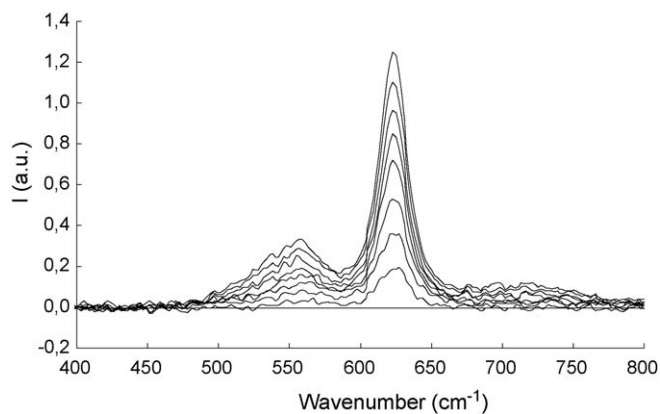


Fig. 5. Raman spectra of sodium-aluminate solutions at room temperature and at $[\text{NaOH}]_T = 18.0\text{ M}$ (const.) with $[\text{Al(III)}]_T = 0\text{--}0.8\text{ M}$ from bottom to top in 0.1 M increments.

- [9] H. Watling, *Appl. Spectrosc.* 52 (1998) 250.
- [10] H.R. Watling, S.D. Fleming, W. van Bronswijk, A. Rohl, *J. Chem. Soc., Dalton Trans.* (1998) 3911.
- [11] C.T. Johnston, S.A. Agnew, J.R. Schoonover, J.W. Kenney III, R. Page, J. Osborne, R. Corbin, *Environ. Sci. Technol.* 36 (2002) 2451.
- [12] N.I. Eremin, Y.A. Volokhov, V.E. Mironov, *Russ. Chem. Rev.* 43 (1974) 92.
- [13] L.P. Ni, E.V. Starshikova, E.A. Kopylova, *Sov. J. Non-Ferrous Met.* 47 (1988) 2632.
- [14] T.P. Porotnikova, V.A. Derevyankin, S.I. Kuznetsov, M.P. Tsvetkova, A.K. Chirkov, *J. Appl. Chem. (USSR)* 46 (1973) 479.
- [15] G. Johansson, *Acta Chem. Scand.* 20 (1966) 505.
- [16] P.M. May, G.T. Hefter, P. Sipos, *Proceedings of the 13th International Conference on the Properties of Water and Steam*, Toronto, Canada, 1999, p. 98.
- [17] P. Sipos, P.M. May, G.T. Hefter, *Dalton Trans.* (2006) 368.
- [18] P. Sipos, P.M. May, G.T. Hefter, *Analyst* 125 (2000) 955.
- [19] P. Sipos, G.T. Hefter, P.M. May, *Aust. J. Chem.* 51 (1998) 445.
- [20] L.-O. Öhman, S. Sjöberg, *Coord. Chem. Rev.* 149 (1996) 33.
- [21] P. Sipos, A. Stanley, S. Bevis, G. Hefter, P.M. May, *J. Chem. Eng. Data* 46 (2001) 657.
- [22] P. Sipos, G. Hefter, P.M. May, *J. Chem. Eng. Data* 45 (2000) 613.
- [23] P. Laszlo, *NMR of Newly Accessible Nuclei*, Academic Press, New York, 1983.
- [24] P. Sipos, I. Bodi, P.M. May, G.T. Hefter, in: G. Ondrejovicz, A. Sirota (Eds.), *Progress in Coordination and Organometallic Chemistry*, Slovak Technical University Press, Bratislava, Slovakia, 1997, p. 303.
- [25] P. Sipos, L. Bolden, G. Hefter, P.M. May, *Aust. J. Chem.* 53 (2000) 88.

Identification and distribution of contamination sources in the Ebro river basin by chemometrics modelling coupled to geographical information systems

Marta Terrado, Damià Barceló, Romà Tauler*

Department of Environmental Chemistry, IIQAB-CSIC, Jordi Girona 18-26, Barcelona 08034, Spain

Received 10 February 2006; received in revised form 3 May 2006; accepted 12 May 2006

Available online 7 July 2006

Abstract

Identification of the main contamination sources of heavy metals, organic compounds and other physicochemical parameters in Ebro river surface waters and description of their temporal and spatial distributions, are analyzed using chemometrics and geostatistical methods. Historical data available from the Confederación Hidrográfica del Ebro (CHE), which is the organization in charge of the management of the Ebro river basin; covering different years since 1992 for water, sediments and fish samples are investigated. Due to the great amount of data in these databases and to their complexity, chemometrics modelling (using principal components analysis, PCA) coupled to geographical information systems (GIS) is proposed to evaluate the environmental quality of the Ebro river basin.

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Keywords: Ebro river; Contamination sources; Chemometrics; PCA; GIS

1. Introduction

Diffuse and point pollution in the Ebro river basin (NE Spain) from agriculture, industry and human sewage, is an issue of great concern since together with changes in climatic conditions and land use practices have produced large scale adverse impacts on water quality and quantity.

The Water Framework Directive 60/2000/CE and the European Directive 76/464/CEE make compulsory the establishment of surveillance stations for the control of contamination in the aquatic environment (water, sediments and biota) by dangerous compounds in principal emission focus downstream. In waters, quality objectives have been fixed whereas in sediments and biota the main objective is to avoid a significant rise in time. The Confederación Hidrográfica del Ebro (CHE) [1] has been controlling the concentration of some specific compounds in the whole basin with the establishment of a control network: the Red de Control de Sustancias Peligrosas (RCSP).

Through the environmental monitoring program performed at different sampling sites and environmental compartments of the network, a large amount of concentration values of chemicals spread into the Ebro river basin are obtained. In order to derive useful environmental information from the data, the application of modern chemometric methods based in new multivariate factor analysis [2] tools is proposed. The basic assumption of these methods when they are applied to environmental data tables is that each value of a measured variable in a particular sample is due to the sum of contributions from individual independent sources of different origin. Each one of these sources is characterized by a particular chemical composition profile and distributed among samples in a different way. As a result of the application of chemometric methods, main point and diffuse sources of contamination in the environment and their origin may be identified and their distribution profiles among samples (geographical, temporal, among environmental compartments) are characterized. Distribution of contamination sources and their impact over the territory can be assessed by the use of geographical information systems [3,4] by means of cartographic techniques of symbols and pollution prediction maps. Geostatistical methods [5–7] based on mathematical and statistical functions are used, which allow the estimation of continuous

* Corresponding author. Tel.: +34 93 4006140; fax: +34 93 2045904.
E-mail address: rtaqam@iiqab.csic.es (R. Tauler).

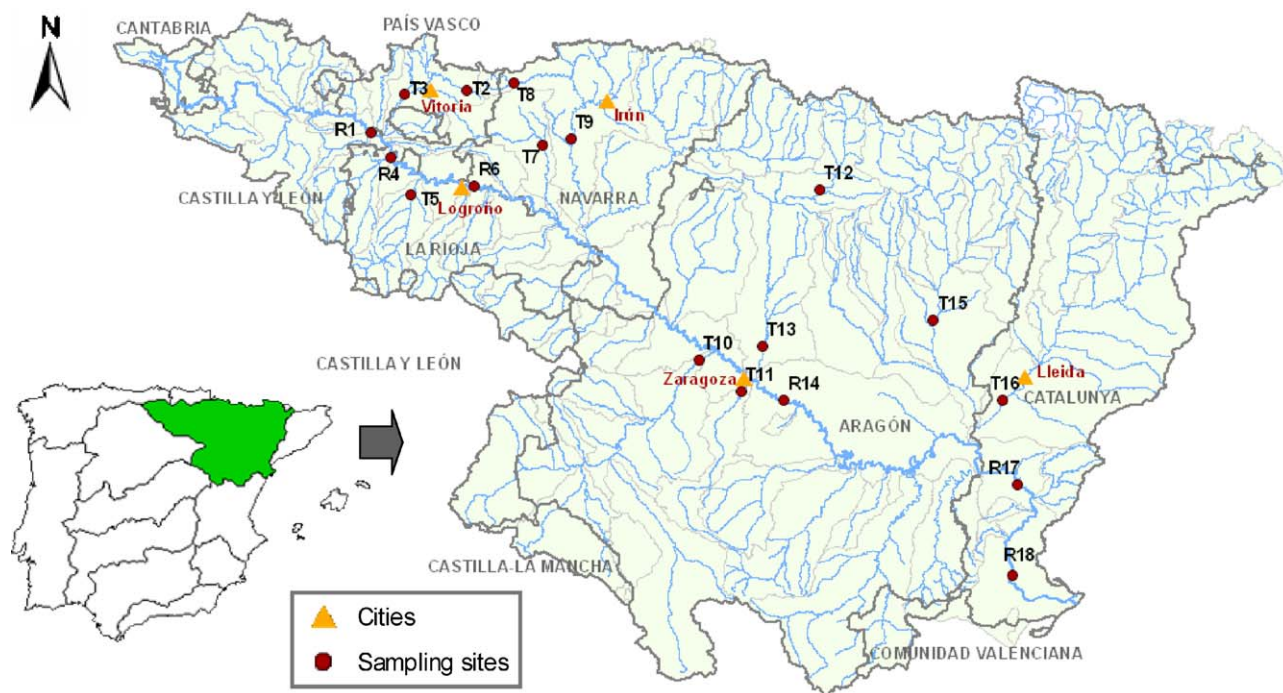


Fig. 1. Identification and location of Red de Control de Sustancias Peligrosas (RCSP) sampling points. Sampling sites identification: R1, Ebro in Miranda de Ebro (Burgos); T2, Zadorra in Salvatierra (Álava); T3, Zadorra in Vitoria-Trespuestas (Álava); R4, Ebro in Conchas de Haro (La Rioja); T5, Najerilla in Nájera downstream (La Rioja); R6, Ebro in Logroño downstream (La Rioja); T7, Ega in Arinzano (Navarra); T8, Araquil in Alsasua (Navarra); T9, Arga in Puente la Reina (Navarra); T10, Jalón in Grisén (Zaragoza); T11, Huerva in Fuente de la Junquera (Zaragoza); T12, Gállego in Jabarrella (Huesca); T13, Gállego in Villanueva de Gállego (Zaragoza); R14, Ebro in Presa de Pina (Zaragoza); T15, Cinca in Monzón downstream (Huesca); T16, Segre in Torres de Segre (Lleida); R17, Ebro in Ascó (Tarragona); and R18, Ebro in Tortosa (Tarragona).

surfaces using the measured variables to predict unknown values by interpolation and, at the same time, give an estimation of the errors associated to these predictions.

In order to perform an investigation about the causes of the environmental impact over Ebro river's quality, the following aspects have been studied in detail in this work:

- (1) Historical data arrangement of the CHE [1] data base has been performed to get the available information in a proper way to do the analysis (original information had a remarkable uniformity lack for variables in samples at different sampling campaigns).
- (2) Chemometric analysis of data using principal components analysis (PCA) [8] has been performed to get useful environmental information about contamination sources in the Ebro river basin.
- (3) Geographical display of the results has been obtained using geographical information systems (GIS) tools and, where possible, geostatistical interpolation techniques.

2. Study area

The region under study is the whole Ebro river basin (NE Spain), a geographical area defined by UTM coordinates (International 1924) projected in the 30 N zone: 385,775.28 ($x_{\min}$), 924,410.62 ($x_{\max}$), 4,781,293.49 ($y_{\max}$) and 4,468,155.50 ($y_{\min}$). The approximate drainage area of the basin is 85,000 km², discharging into the Mediterranean Sea and originating a delta of

more than 30,000 ha. This area comprises territories of nine Autonomous Communities, from Cantabria to Catalonia (see Fig. 1). The average population density of the basin is 38 people/km², although population is distributed in a very heterogeneous way. There are five cities with a population greater than 100,000 people: Irún (Pamplona), Lleida, Logroño, Vitoria and Zaragoza [9]. These cities concentrate around the 45% of the population of the basin. It is the most important irrigated land in Spain and agriculture varies with relief and particular climate. Cereal crops (wheat, maize and barley) dominate much of the agricultural production of the basin. In the Southern part, vines, olives and industrial crops such rape and sunflowers are important [10]. Fruit trees are also grown all over the basin, and the Ebro delta is a major centre of rice production. Pig and sheep farming have also proliferated in urban areas as an alternative economic activity, which causes contamination problems in water too [11]. The intensification of agriculture in Spain in recent decades has resulted in a dramatic increase in productivity but at a cost of diffuse pollution in the Ebro basin from the historic and ongoing use of pesticides. The Ebro river basin is also dominated by industrial activities concentrated close to the cities of Zaragoza, Vitoria, Pamplona, Logroño, Huesca, Lleida and Tarragona (Flix), which have caused contamination problems to the basin. A historical Hg pollution from chemical plants manufacturing solvents and chlorinated pesticides is well known [12]. Besides, automobile, textile, food and wood industry as well as mining activities in the northern part of the river are important. Some authors in previous studies remark the effects of agricul-

tural and industrial contamination on the Ebro delta wildlife [13], noting the existence of a contamination source from industrial and agricultural wastes, which ends up on the delta or into the sea, and another one due to a large annual input of pesticides in the delta crops to fight agricultural pests. A detrimental effect of the use of herbicides is the destruction of the habitat needed by animal communities to develop.

Many of the chemical and physicochemical characteristics studied in the Ebro river basin are physically and statistically correlated and in many cases they show spatial dependence. This fact along with the spatial distribution of experimental data makes GIS a suitable tool for such analysis, especially for information management and helping in the evaluation and decision making process.

Basic cartography of the study zone is necessary to locate the interest areas, and additional cartographic data are also useful since they give a richer knowledge of the environment and help to find out the reasons for high pollution levels in specific areas according to territorial aspects and uses [14–16].

3. Description of data

Monitoring data have been obtained from the RCSP of CHE. This network consists on 18 sampling points where different compounds are analyzed once a year (see Fig. 1 for the situation of the sampling points). These points are numbered from 1 to 18 from north to south and “R” indicates “Ebro river” whereas “T” holds for “Tributary”. All studied sample areas include risk sites either for their industrial activities, urban impact or finally agricultural practices.

Data have been divided into six different groups according to the physical compartment (water, sediments and biota) and the analyzed variables (metals, organic compounds and physicochemical parameters). Analyzed parameters and sampling sites have not been the same for all compartments and years because of the availability of data (see Table 1 for variables identification). Sediment and water sample data covering years from 1996 to 2003 have been selected, because before 1996 the data set is too much incomplete. Time interval for biota has been reduced, covering only years from 2000 to 2003 for metals and from 1999 to 2002 for organic compounds.

Spatial data layers used in this study have been obtained from the CHE web site: (a) Ebro river basin limits (1:50,000); (b) sub basins; (c) autonomous communities limits; (d) hydrology (Ebro river and tributaries); (e) RCSP sampling points; (f) urban areas; (g) land use; (h) areas affected by nitrates contamination; and (i) digital terrain model (DTM).

(a) Metals in sediments

Concentrations of metals in sediments have been arranged in a data matrix of size 9×8 (rows $\times$ columns). Rows correspond to sampling sites and columns to concentrations of the analyzed metals in mg/kg of sediment. Metals analyzed have been As, Cd, Cr, Cu, Hg, Ni, Pb and Zn. Samples have been arranged according to sampling years from 1996 to 2003 (1999 excluded).

Table 1
Variables identification

Labels	Variables
HCHs	Total hexachlorocyclohexanes
HCB	Hexachlorobenzene
HCBu	Hexachlorobutadiene
TCBs	Total trichlorobenzenes
DDTs	Total dichlorodiphenyltrichloroethanes
NAP	Naphthalene
FLU	Fluoranthene
BaPY	Benzo(a)pyrene
BbF	Benzo(b)fluoranthene
BghiPE	Benzo(g,h,i)perylene
BkF	Benzo(k)fluoranthene
I123-cdPY	Indeno(1,2,3-cd)pyrene
Length	Length
op-DDD	<i>ortho, para</i> -Dichlorodiphenyldichloroethane
op-DDE	<i>ortho, para</i> -Dichlorodiphenyldichloroethylene
op-DDT	<i>ortho, para</i> -Dichlorodiphenyltrichloroethane
pp-DDD	<i>para, para</i> -Dichlorodiphenyldichloroethane
pp-DDE	<i>para, para</i> -Dichlorodiphenyldichloroethylene
pp-DDT	<i>para, para</i> -Dichlorodiphenyltrichloroethane
ALCALI	Alkalinity
CL ⁻	Chlorides
CN ⁻	Cyanides
COL_TO	Total coliforms
pH	pH
BOD ₅	Biological oxygen demand
COD _{UV}	Chemical oxygen demand
FLUOR	Fluorides
SM	Suspended matter
NH ₄ ⁺	Total ammonium
NO ₃ ⁻	Nitrates
O ₂	Dissolved oxygen
COND20	Conductivity at 20 °C
PO ₄ ³⁺	Phosphates
SO ₄ ⁼	Sulphates
TEM_AG	Water temperature
TEM_AI	Air temperature

(b) Metals in fish

Concentrations of metals in fish have been arranged in a data matrix of size 9×8 (rows $\times$ columns). Rows correspond to sampling sites and columns to concentrations of the analyzed metals and one physical parameter. Analyzed parameters have been Cd, Cr, Cu, Hg, Pb, Se, Zn and fish length. Metal concentration units are mg/kg of biota whereas cm is the unit for the length of the fish. Data have been arranged according to sampling years from 2000 to 2003.

(c) Metals in surface water

Concentrations of metals in waters have been arranged in a data matrix of size 10×13 (rows $\times$ columns). Rows correspond to sampling sites (not all years have the same number of them) and columns to concentrations of the analyzed metals in mg/L of water. Analyzed parameters have been As, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn. Samples have been arranged according to sampling years from 1996 to 2003.

(d) Organic compounds in sediments

Concentrations of organic compounds in sediments have been arranged in a data matrix of size 9×12 (rows $\times$ columns). Rows correspond to sampling sites and

columns to concentrations of the analyzed organic compounds in $\mu\text{g/kg}$ of sediment. Organic compounds have been HCHs, DDTs, HCB (pesticides); HCBu, TCBs (volatile organo-halogenous compounds); Naphthalene, Fluoranthene, Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(g,h,i)perylene, Benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene (polycyclic aromatic hydrocarbons, PAHs). Samples have been arranged according to sampling years from 1996 to 2003 (1999 excluded).

(e) Organic compounds in fish

Concentrations of organic compounds in fish have been arranged in a data matrix of size 9×12 (rows $\times$ columns). Rows correspond to sampling sites and columns to concentrations of the analyzed compounds and one physical parameter. Analyzed parameters have been HCB, HCHs, o,p-DDD, o,p-DDE, o,p-DDT, p,p-DDD, p,p-DDE, p,p-DDT, DDTs (pesticides); TCBs, HCBu (volatile organo-halogenous) and length. Organic compounds concentration units are $\mu\text{g/kg}$ of biota whereas cm is the unit for the length of the fish data have been arranged according to sampling years from 1999 to 2002.

(f) Physicochemical parameters in surface water

Values of physicochemical parameters in waters have been arranged in a data matrix of size 10×17 (rows $\times$ columns). Rows correspond to sampling sites (not all years have the same number of them) and columns to concentrations of the analyzed parameters, each one with their own units. Analyzed parameters have been alkalinity (mg/L of CaCO_3), chlorides (mg/L of Cl^-), cyanides (mg/L of CN^-), total coliforms (MPN/100 mL), conductivity at 20°C ($\mu\text{S/cm}$), BOD_5 (biological oxygen demand, in mg/L of O_2), COD_{UV} (chemical oxygen demand, in mg/L of O_2), fluorides (mg/L of F), MES (suspended matter, in mg/L), NH_4^+ (mg/L), NO_3^- (mg/L), O_2 (mg/L), pH, PO_4^{3-} (mg/L), SO_4^{2-} (mg/L), water temperature ($^\circ\text{C}$) and air temperature ($^\circ\text{C}$). Samples have been arranged according to sampling years from 1996 to 2003.

4. Methodology

Fig. 2 shows a flowchart of the methodology used in this work. From the CHE database [1] historical data have been obtained and arranged in data sets according to different compartments and variable groups. A preliminary data description has been performed using EXCEL software (2002, Microsoft Corporation, Redmond, WA, USA) and data representation of single variables by GIS has been done with the ArcGis software (v.9.0, 2005, ESRI, Redlands, CA, USA). After this, data sets have been transferred to MATLAB software (v.7.0, 2004, The Mathworks, MA, USA) to perform data analysis. PCA results have been exported to a GIS environment, where prediction maps have been obtained using geostatistics [5–7].

4.1. Data pretreatment

Data were initially organized in different data matrices, each one corresponding to one of the different data groups (see Sec-

tion 3) and to one sampling campaign. $\mathbf{D}$ is the notation used to indicate the matrix or table of data, which has a specific number of rows (NR), corresponding to geographical sampling sites and a number of columns (NC) corresponding to the measured variables (concentration of chemical contaminants or other environmental parameters). Variables having very few values above the detection limit should be removed before multivariate analysis is applied. When a particular compound has not been detected, its concentration value has been set equal to half of its detection limit, if it is known. If not, half the value of the lowest measured value of the specific variable has been used. For missing values, imputation methods have been applied whenever they are a small fraction of the values, using appropriate PCA imputation functions in PLS toolbox (v.3.5, Eigenvector Research, Mason, WA, USA) under MATLAB computer environment. Selected data pretreatment method has been autoscaling, which is a combination of data mean centering and standardization. Mean centering removes average and constant background contributions, which are considered of no interest for data variance interpretation. Some kind of scaling is mandatory when variables are of different type and their values are at different scales and units. Standardization is a scaling obtained dividing each value of the variables by their norm or standard deviation.

4.2. Chemometric analysis

Chemometric data analysis methods provide powerful tools for the analysis and interpretation of large environmental multivariate data sets generated within environmental monitoring programs. To investigate multivariate correlations between data, identify contamination sources and deduce their geographical and temporal distributions, principal components analysis [8] is proposed. The use of multivariate statistical analysis methods to evaluate water quality is reported in other examples [15,17].

PCA is a chemometric method, which assumes a bilinear model to explain the observed data variance using a reduced number of components. Using the linear combination of the original variables (loadings), a new group of non-correlated orthogonal variables is deduced, which makes possible the calculation of new axes (orthogonal) of data representation (scores), in which the data variance is explained in a decreasing order.

The product of scores and loadings gives the original matrix of data for a specific number of components $\mathbf{D} \rightarrow \mathbf{D}^* = \mathbf{U}\mathbf{V}^T$, where $\mathbf{D}$ is the experimental data array arranged in a data matrix. $\mathbf{V}^T$ (loadings matrix), identifies the nature of the main sources of data variance, $\mathbf{U}$ (scores matrix) provides sample scores for these contamination patterns, describing the distribution of contamination between samples (geographical, temporal or between environmental compartments), and $\mathbf{D}^*$ is the calculated data matrix using a particular number of principal components. Determination of the number of principal components is performed as a compromise among model simplicity (few components), maximum variance explained by the model (more components) and model interpretability.

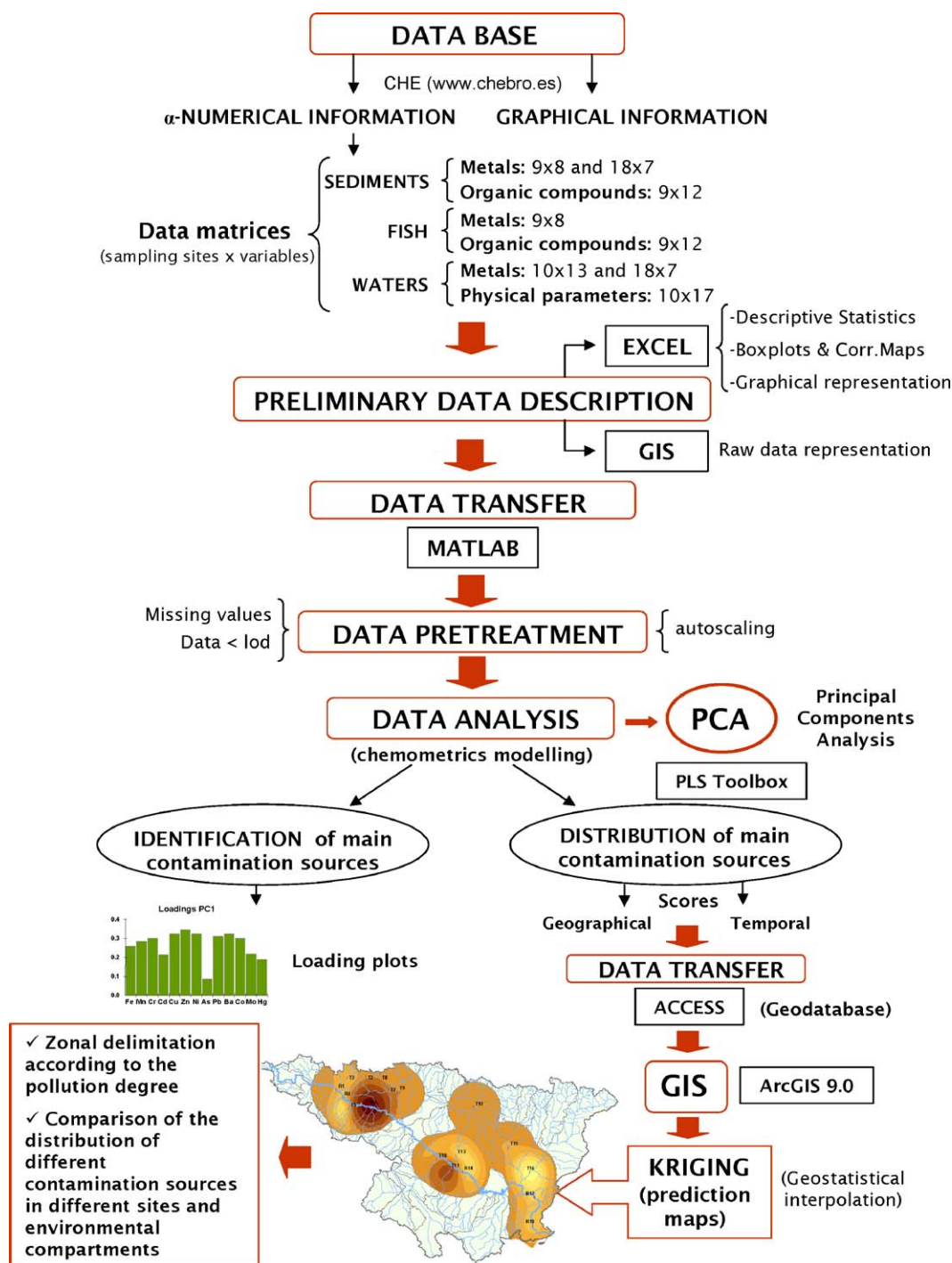


Fig. 2. Flowchart of the methodology followed in this work (see Section 3).

Each data matrix corresponds to 1-year sampling campaign (d_{year} in Fig. 3) and contains a specific number of samples (rows) with various variables measured in each sample (columns). There are as many data matrices as years in the study. To perform PCA, matrices are concatenated one on top of the other (D in Fig. 3) giving a new augmented data matrix. Results also have a temporal dimension. However, since not the same samples were acquired and reported during all the investigated years, a more systematic study of annual changes has not been possible.

4.3. Geographical information systems

PCA results (scores) are transferred to an ACCESS (2002, Microsoft Corporation, Redmond, WA, USA) geodatabase where, by using the Geostatistical Analyst extension (ArcGIS software), the elaboration of prediction maps [18] is possible. Geostatistical Analyst uses sample points taken at different locations in a landscape and creates (interpolates) a continuous surface. It provides two different groups of interpolation techniques: deterministic and geostatistical. All methods rely

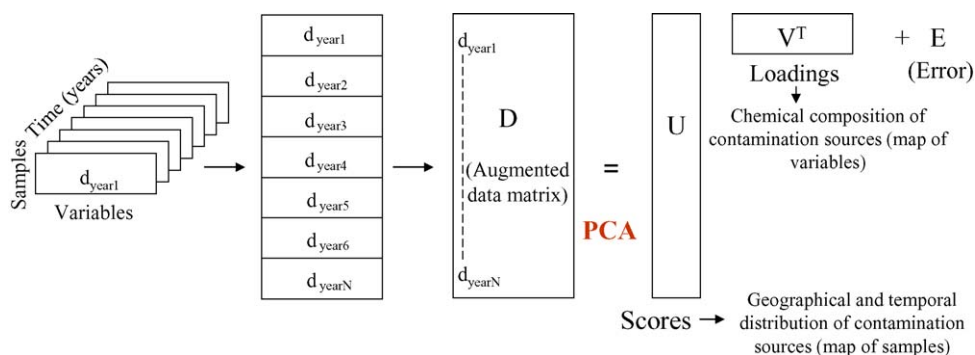


Fig. 3. Matrix augmentation PCA modelling (see chemometric analysis in Section 4.2).

on the similarity of nearby sample points to create the surface. However, while deterministic techniques only use mathematical functions for interpolation, geostatistics relies on both statistical and mathematical methods [5–7]. Geostatistical techniques have been selected in this work, because they produce not only prediction surfaces but also error or uncertainty surfaces, giving an indication of how good the predictions are. Ordinary Kriging has been applied as geostatistical interpolator which, during the calculation process, follows the following steps: (a) calculation of the empirical semivariogram built on the basis that closer objects are more alike than those farther away; (b) fitting a model defining the mathematical function that provides the best fit through the points; (c) creation of weight matrices determining the kriging weights assigned to each measured value from the spatial autocorrelation among the measured sample locations and the predicted ones; and (d) prediction of unknown values. The important feature of the semivariogram is that, unlike the

variance and covariance values used to describe spatial variation patterns, it measures the average dissimilarity between data according to their separation distance. The spatial structure in the data distribution described by the semivariogram is a prerequisite for the application of Geostatistics [7,19]. Although interpolation techniques are based in proximity relationships, to make a correct prediction, map sites have to be interconnected, that is, the analysis should be performed at a sub-basin level. In this work, this has not been possible due to the lack of enough number of sampling sites and, for this reason, interpolation has only been applied in the main Ebro river stream (using data from all the sites building the net), which is considered to be affected downstream by tributary influences.

Cross-validation was used to indicate whether the predicted model and parameters are reasonable. It uses all the available data to estimate the autocorrelation model. Then it removes each data location, one at a time, and predicts the associated data

Table 2
Descriptive statistics of metals in sediments, fish and water

Metals in sediments (mg/kg)	Cd	Cr	Cu	Pb	Zn	Hg	As	Ni					
Minimum	0.05	4.00	6.25	7.16	30.00	0.02	0.01	2.30					
Maximum	23.42	464.20	359.02	123.00	4036.76	5.60	18.67	170.00					
Mean	2.41	56.25	43.10	40.68	292.58	0.56	5.51	31.18					
Median	0.39	34.00	22.40	27.05	94.35	0.24	5.00	23.00					
Standard deviation	4.75	80.59	57.59	30.67	646.39	0.97	4.19	29.11					
Kurtosis	8.41	12.13	15.41	0.57	19.27	16.23	1.13	9.44					
Asymmetry coefficient	2.85	3.31	3.60	1.23	4.10	3.83	1.02	2.86					
Metals in fish (mg/kg)	Cd	Cr	Cu	Pb	Zn	Hg	Se	Long (cm)					
Minimum	0.01	0.08	0.47	0.04	18.52	0.02	0.12	13.33					
Maximum	0.29	3.24	3.58	0.43	92.40	1.64	1.23	40.38					
Mean	0.04	0.48	1.24	0.14	39.94	0.24	0.49	25.24					
Median	0.02	0.32	1.03	0.09	36.82	0.08	0.40	24.78					
Standard deviation	0.07	0.57	0.69	0.11	15.68	0.35	0.27	6.57					
Kurtosis	5.48	15.95	3.20	0.75	3.11	6.70	0.35	0.05					
Asymmetry coefficient	2.45	3.62	1.73	1.30	1.56	2.48	0.87	0.48					
Metals in water (mg/L)	Cd	Cr	Cu	Pb	Zn	As	Ni	Ca	Fe	K	Mg	Mn	Na
Minimum	0.000	0.000	0.000	0.000	0.000	0.000	0.000	56.650	0.030	0.000	4.900	0.007	19.300
Maximum	0.001	0.060	0.007	0.006	0.304	0.004	0.019	216.000	1.650	10.450	97.333	0.094	261.000
Mean	0.000	0.006	0.002	0.002	0.052	0.001	0.005	103.365	0.136	3.129	21.006	0.026	79.258
Median	0.000	0.003	0.002	0.001	0.042	0.001	0.003	96.700	0.085	3.076	21.073	0.020	76.192
Standard deviation	0.000	0.008	0.001	0.002	0.040	0.001	0.005	33.743	0.210	2.178	15.034	0.019	52.571
Kurtosis	3.619	30.305	2.785	−0.810	21.277	6.820	2.477	0.741	39.326	2.584	8.506	3.379	1.671
Asymmetry coefficient	2.110	4.988	0.722	0.791	3.900	2.058	1.818	0.928	5.932	1.151	2.265	1.834	1.207

5. Results and discussion

5.1. Preliminary description of data

Heavy metals are mostly originated by industry, mining activities, agriculture or urban sewage but some of them can also have a natural origin depending on the soil nature. In this work, Zn, Cu and Cr are the metals presenting the highest concentration values whereas Hg and Cd have the lowest ones.

They are mostly accumulated in sediments (see Table 2). Zn levels in sediments have presented concentrations from 30 to 4036 mg/kg, while natural levels usually found for this element, range from 60 to 90 mg/kg [20]. Half of the sediment samples in the study have exceeded the considered natural level for Zn. For Cu, Cr and Pb, maximum measured levels in sediments have been 359 mg/kg, 464 mg/kg and 123 mg/kg, respectively. Cu average levels of uncontaminated sediments are around 14 mg/kg [21]; 34–50 mg/kg in the case of Cr and 17–30 mg/kg for Pb [20]. All these metals have been frequently detected at high concentrations at sampling site T3, Zadorra in Vitoria Trespuentes,

Table 3
Descriptive statistics of organic compounds in sediments and fish

Organic compounds in sediments (µg/kg)	HCHs	HCB	HCBu	TCBs	DDTs	NAP	FLU	BaPY	BbF	BghiPE	BkF	I123-cdPY
Minimum	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Maximum	9.10	4532	8.71	272	79287	131	4461	941	994	412	1762	335
Mean	0.77	94.8	0.61	12.3	1610	20.8	187	63.2	98.4	39.3	74.7	34.6
Median	0.25	1.00	0.25	0.25	5.40	13.0	30.0	10.0	20.0	10.0	9.76	3.00
Standard deviation	1.62	576	1.41	38.6	10104	26.4	576	149	190	76.4	238	73.1
Kurtosis	13.9	59.3	21.3	33.8	58.9	4.13	50.7	20.2	9.86	11.5	41.8	7.72
Asymmetry coefficient	3.63	7.62	4.49	5.36	7.59	1.77	6.83	4.11	2.99	3.21	6.11	2.86
Organic compounds in fish (µg/kg)	HCHs	HCB	HCBu	TCBs	DDTs	op-DDD	op-DDE	pp-DDT	pp-DDD	pp-DDE	pp-DDT	Length (cm)
Minimum	0.96	0.25	0.27	3.00	3.21	0.25	0.25	0.25	0.25	2.76	0.71	13.3
Maximum	24.5	446	8.00	16.0	3964	92.6	520	70.1	312	3865	147	38.9
Mean	5.96	45.6	1.31	4.33	536	16.2	48.3	12.3	27.3	482	14.2	23.8
Median	4.25	6.02	1.00	3.00	63.1	2.97	2.27	1.44	3.96	50.7	4.15	23.8
Standard deviation	5.78	94.5	1.81	2.92	1009	26.5	121	21.3	59.6	954	27.0	6.85
Kurtosis	2.85	9.44	10.1	9.72	4.86	2.38	8.06	2.05	14.9	5.70	17.4	-0.43
Asymmetry coefficient	1.56	2.95	3.25	2.95	2.35	1.90	2.95	1.83	3.59	2.50	3.90	0.38

Table 4
Descriptive statistics of physicochemical parameters in surface water

Physicochemical parameters in water (mg/L)	ALCALI	CL [−]	CN [−]	COL_TO (MPN/100 mL)	pH	BOD ₅	COD _{uv}	FLUOR	SM
Minimum	123	23.8	<LOD	816	7.59	1.48	5.44	0.08	0.75
Maximum	271	375	0.03	270000	8.24	20.1	21.8	0.58	857
Mean	176	112	0.006	22359	8.00	6.25	13.1	0.20	46.1
Median	166	107	0.005	12669	8.00	5.60	12.8	0.19	14.4
Standard deviation	31.1	82.2	0.004	41953	0.16	3.60	4.19	0.10	117
Kurtosis	1.32	1.73	27.9	21.6	−0.41	3.16	−1.17	4.25	34.5
Asymmetry coefficient	1.14	1.27	4.41	4.45	−0.51	1.52	0.17	1.77	5.58
	NH ₄ ⁺	NO ₃ [−]	O ₂	COND20 (μS/cm)	PO ₄ ^{3−}	SO ₄ ⁼	TEM_AG (°C)	TEM_AI (°C)	
Minimum	0.03	2.80	6.50	433	0.02	35.9	11.1	11.1	
Maximum	8.52	27.8	10.7	2200	1.32	598	19.4	24.7	
Mean	0.76	11.8	9.10	916	0.24	175	15.4	16.7	
Median	0.12	11.0	9.38	855	0.17	180	15.6	15.9	
Standard deviation	1.80	4.88	0.99	386	0.23	112	1.63	3.24	
Kurtosis	9.78	1.82	0.06	0.59	7.90	1.50	−0.24	−0.63	
Asymmetry coefficient	3.26	0.94	−0.82	0.88	2.58	0.94	0.008	0.51	

nearly every year. Cd has been found at concentrations ranging from 0.05 to 23.4 mg/kg, while natural levels of this element are around 0.4 mg/kg [21]. R1, Ebro in Miranda de Ebro and T3, are the sites where higher Cd concentrations have been found.

Hg, Cu and Zn are the metals that can most affect fish. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), the current standard of Hg used to control the consumption of adulterated tissue in fish is 0.5 mg/kg (it assumes that all the mercury in fish is methylated). The average value of mercury measured in fish samples in this work is lower (0.24 ppm) but there are various samples with Hg concentrations higher than 0.5 ppm in some specific sites. Year 2002 is the one in which the concentration of Hg in fish was higher. Sampling sites with these high values are R17, Ebro in Ascó (1.64 ppm); T12, Gállego in Jabarrella (1.13 ppm) and T15, Cinca in Monzón downstream (0.8 ppm). It is important to remark that these are average values among different samples from the same year.

For waters, measured heavy metal concentrations have not exceeded legal values established by the Spanish Real Decreto 140/2003, for human water consumption quality.

Organic compounds investigated in this work have always an anthropogenic origin. These are hydrophobic compounds which can hardly be measured in surface waters (concentrations are usually below their detection limits). The abundance of organic compounds in sediments has followed the sequence: DDTs > PAHs (specially fluoranthene, followed by benzo(b)fluoranthene and benzo(k)fluoranthene) > HCB > the rest of the analyzed compounds (see Table 3). These results differ from those obtained in another study performed in the same area [13] only for year 2003, where PAHs concentration was higher than DDTs concentration. In years previous to 2003, DDTs presence in sediments was more important, especially for sampling sites T15, Cinca in Monzón (2081 μg/kg in 1997); R17, Ebro in Ascó (79287 μg/kg in 1998) and R18, Ebro in Tortosa (1774 μg/kg in 1997). Fluoranthene has been the hydrocarbon most frequently detected in sediments, especially in the

upper part of the Ebro river. In both studies, HCB has presented its highest levels at R17 and R18, close to the end of the river. Mean concentration value of HCB measured in sediments has been twice the mean value measured in fish (see Table 3).

In fish, abundance of organic compounds has followed the sequence: DDTs (specially the DDE isomer) > HCB > HCHs > the rest of analyzed compounds. DDTs have been detected in fish at levels from 3.2 to 3964.5 μg/kg, with p,p-DDE levels from 2.8 to 3865 μg/kg. In a previous study for year 2003 [12], bioaccumulation of DDT isomers in fish was also important but at lower levels. The sampling site with larger DDTs concentrations in fish has been T15 for all studied years, followed by R17 and R18. In the Ebro river basin, DDT and DDT metabolites are probably derived from the historic use of DDT in this area. It is interesting to remark that HCB, DDTs, PAHs and TCBs have presented higher values in sediment samples while HCHs and HCBu have shown their highest concentrations in fish samples (see Table 3).

Physicochemical parameters (see Table 4) show different aspects of surface water quality. Variables presenting more skewed distributions were SO₄⁼, NH₄⁺ and MES. The variable with the lowest measured concentrations in waters were cyanides; however, they are known to have a very toxic effect. According to the Spanish Real Decreto 140/2003, cyanides, fluorides and nitrates in this study are always below the maximum established levels. For ammonium, it is important to remark that for years 1996–2002, levels of this species surpassed the legal drinking water threshold of 0.5 mg/L at sampling site T3, Zadorra in Vitoria Trespuentes, reaching a concentration of 8.56 mg/L of ammonium in 2002. Levels in 2003 have decreased to 0.65 mg/L. In the case of chlorides, legal level is established at 250 mg/L, and only at sampling site T13, Huerva in Fuente de la Junquera, their concentration has reached the level of 375 mg/L. For sulphates, the maximum legal level for drinking waters is established at 250 mg/L. Sampling sites around Zaragoza (in the central part of the Ebro river) have presented the highest concentration levels, reaching 414.5 mg/kg at T10, Jalón in Grisén

and 598 mg/kg at T13. Levels of COD and BOD are similar to those obtained in other studies of water quality [22], while phosphates concentrations are slightly higher. For suspended matter, measured levels present a mean value of 46.1 mg/L in contrast to 17.7 mg/L, measured in the previous study [22]. Sampling sites presenting higher values of suspended matter are those in the central part of the Ebro river. Finally, for total coliform levels, 0 CFU/100 mL (Colony-forming units per 100 mL of water) are established in the Spanish Real Decreto 140/2003 for drinking water. Levels for total coliforms observed elsewhere [23] ranged from 0 to 100 CFU/100 mL, which indicated a quite good water quality (only at one sample site 600 CFU/100 mL was measured). In the Ebro river, estimated coliform-group number of organisms (MPN, most probable number of coliform-group organisms per unit volume of sample water) range from 816 to 270000 MPN/100 mL, the latter indicating sampling sites where water quality is rather bad.

General trends in temporal tendencies have not been observed in the analyzed historical data. A temporal increase in Cu concentration has been detected in the upper part of the Ebro river, especially at sampling sites R1 and T3. Some Hg concentration peaks have also been measured at T9 (in the upper part of the river) and at R17 and R18 (in the mouth of the river).

5.2. PCA results

PCA results are summarized in Fig. 4, which shows the loading plots characterizing the main contamination patterns in every analyzed data set and their explained variance. High positive and negative loadings indicate that these variables have an important presence in the considered principal component. Whenever possible, interpretation of principal components is done in terms of contamination sources operating over the region under study.

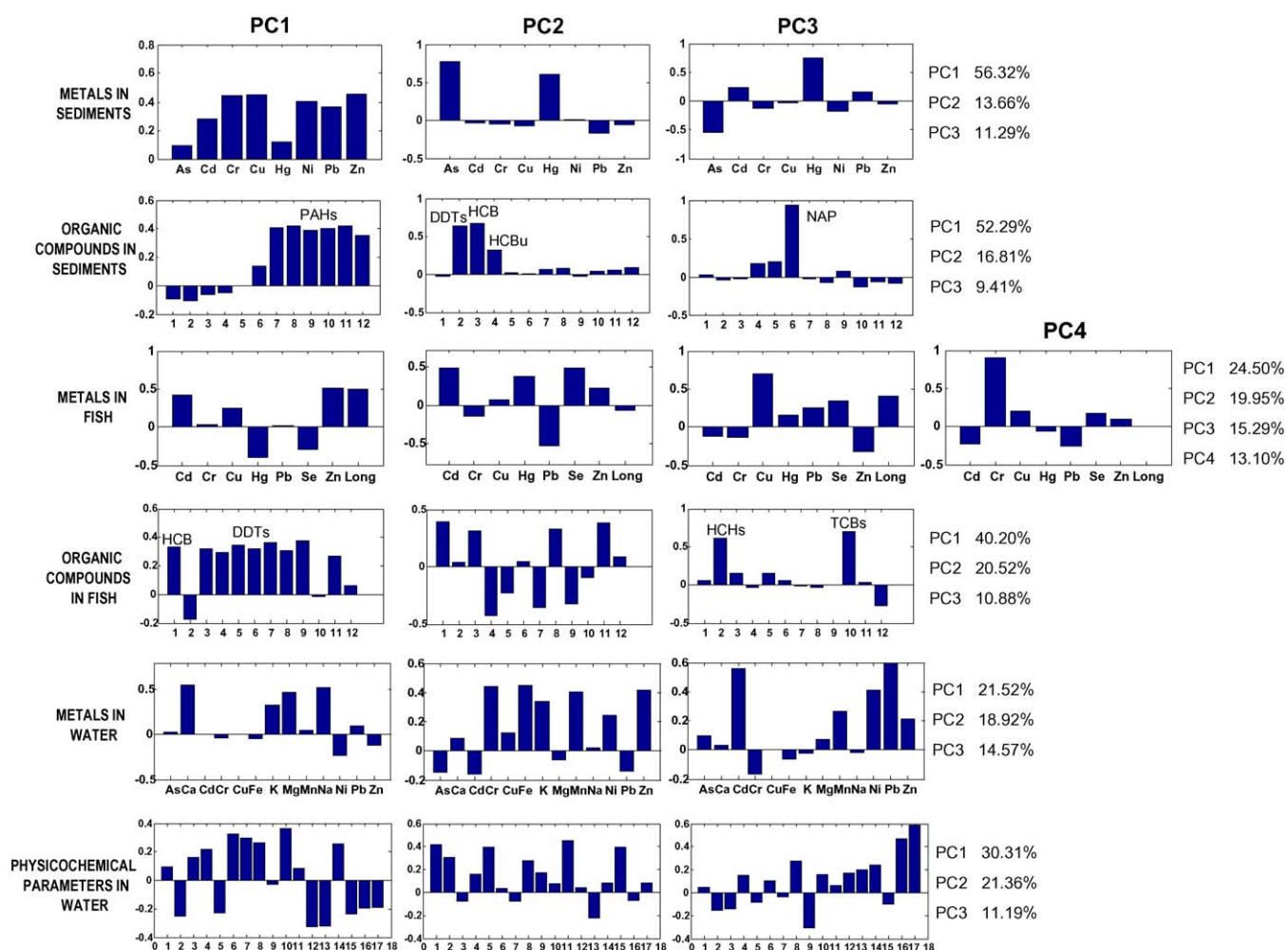


Fig. 4. Main contamination sources identified by PCA for sediments, fish and surface waters in the Ebro river basin and explained variances for each PC. Variables identification. Organic compounds in sediments: 1, summatory of hexachlorocyclohexanes (HCHs); 2, summatory of DDTs (DDTs); 3, hexachlorobenzene (HCB); 4, hexachlorobutadiene (HCBu); 5, summatory of trichlorobenzenes (TCBs); 6, Naphthalene; 7, fluoranthene; 8, benzo(a)pyrene; 9, benzo(b)fluoranthene; 10, benzo(g,h,i)perylene; 11, benzo(k)fluoranthene; 12, indene(1,2,3-cd)pyrene. Organic compounds in fish: 1, hexachlorobenzene (HCB); 2, summatory of hexachlorocyclohexanes (HCHs); 3, o,p-DDD; 4, o,p-DDE; 5, o,p-DDT; 6, p,p-DDD; 7, p,p-DDE; 8, p,p-DDT; 9, summatory of DDTs (DDTs); 10, summatory of trichlorobenzenes (TCBs); 11, hexachlorobutadiene (HCBu); 12, fish length. Physicochemical parameters in water: 1, alkalinity; 2, chlorides; 3, cyanides; 4, total coliforms; 5, conductivity at 20 °C; 6, biological oxygen demand; 7, chemical oxygen demand; 8, fluorides; 9, suspended matter; 10, total ammonium; 11, nitrates; 12, dissolved oxygen; 13, phosphates; 14, sulphates; 15, water temperature; 16, air temperature.

(a) Metals in sediments

For sediments, PC1 (56.32% of the data variance) describes a general metal contamination pattern, mostly characterized by the simultaneous presence of Zn, Cu, Cr, Ni and Pb. This contribution is especially relevant at sampling site T3, in Zadorra river in Vitoria Trespuentes (Álava, País Vasco). It is an irrigated land, next to the city of Vitoria, which has an important industrial activity. Industry could possibly be the cause for this heavy metal contribution [24], but care has to be taken in its interpretation, because in some cases, these metals can be attributed to a natural origin. PC2 (13.66% of the data variance) is loaded by a high presence of As and Hg. In this source, the two metals covariate positively. R17, at Ebro river in Ascó (Tarragona, Catalonia) is the sampling site with a higher influence of this pollution pattern. It is in an irrigated land, where possible contaminants from agriculture are released to the river and which also receives a very strong industrial pressure. As is a metal frequently used in different types of fertilizers. Hg has generally an industrial origin. It is used as a catalyser in the chlor-alkali industry as well as a pigment in paintings and in paper elaboration, as an active element in batteries, thermometers, some medicines and also in some pesticide formulations [12]. It is important to consider the existence of the nearby electrochemical industry Erkimia, located at Flix, among other industries, which since 1949 produces soda by electrolysis, a process that uses Hg and produces chlorine. PC3 (11.29% of the data variance) is also loaded by As and Hg but, in this case, they covariate negatively (in some samples there is a high content of one element but the level of the other element is lower and vice versa). For instance, R18, Ebro in Tortosa (Tarragona, Catalonia) keeps the high Hg content measured in the previous sampling point (R17), although it has a lower concentration of As.

(b) Metals in fish

Evaluation of geographical distribution of river contamination on fish samples is more difficult than in the case of sediments, probably because of their mobility, which in some cases can give confusing results. PC1 in fish defines the contrast between two inversely correlated contamination profiles. Positive loadings show a contribution defined mostly by Zn and Cd whereas negative loadings define a contribution characterized by Hg and Se content. Together, these two tendencies describe a 24.5% (Fig. 4) of the total data variance. Cd and Zn can have both an industrial and agricultural origin. They present high concentrations in fish samples taken at R18 sampling site, Ebro in Tortosa-Campredó (years 2001 and 2003); T9, Arga in Puente la Reina (2003) and R17, Ebro in Ascó (2001). Hg covariates with Se at sampling sites T12, Gállego river in Jabarrella (Huesca); T15, Cinca river in Monzón downstream (Huesca); and T3, Zadorra river in Vitoria-Trespuestas (Álava). While Se is possible that has a natural origin, Hg appears in the environment only by anthropogenic means. The existence of a relatively large textile industry in Monzón, could be a cause of Hg presence in the Cinca river. Besides, Gállego river (T12) drains a significant extension of traditional irrigation areas, being very

much affected by the sewage of two large paper mills with insufficient wastewater treatment facilities close to its mouth [11]. PC2 (19.95% of the data variance) describes also the contrast between two contamination patterns. Positive loadings describe a Cd, Se and Hg contribution while negative loadings describe a more specific contribution of Pb. Positive loadings in second PC have their major influence in the Ebro mouth, at sampling sites R17 and R18 (where Hg concentrations are higher than the average concentration of this metal in fish and sediments). Pb has its highest levels at R14, Ebro in Presa de Pina (Aragón) and T9, Arga river in Puente la Reina (Navarra). Pb is a metal with a predominant anthropogenic origin and a high toxicity. PC3 (15.29% of the data variance) is characterized by Cu and Zn inversely correlated. Cu presence is important at R1, Ebro in Miranda de Ebro (Burgos, Castilla y León) in years 2002 and 2003, and at T15, Cinca in Monzón (Huesca) in 2003. R1 is located in an urban area and T15 is close to an industrial emplacement, where industry could be responsible for this contamination. Zn contamination pattern is important at R14 during year 2001 and at sampling sites R17 and R18 during years 2000 and 2001. PC4 (13.10% of the data variance) is mostly defined by Cr contribution, which has important applications in industry (galvanotechnics, paintings, textile dyes, etc.). R14, in Zaragoza, is the sampling site where a higher input of this contamination pattern has been found.

(c) Metals in surface water

A first source for metals in waters is defined by PC1 (21.52% of the data variance), with high loadings for Ca, Na, Mg and K. This cannot be regarded as a contamination source, since these metals have no toxic effect and only reflect the salinity content of waters. Three different geographical areas with different inputs of this metal source are established: area 1, grouping sampling sites in the upper part of the Ebro river (R1, T3 and R4); area 2, grouping sampling sites in the middle part of the river around Zaragoza (T10, T13 and R14) and finally area 3, with sampling sites closer to the mouth of the river (T15, T16, R17 and R18). Area 2 has the richest content in nutrients and salinization because of the nature of parental rocks and the intensive irrigation agriculture developed. PC2 (18.92% of the data variance) is characterized by the presence of Cr, Fe, Mn and Zn. PC3 (14.57% of the data variance) is loaded by Cd, Pb and Ni. The most polluted sampling sites by PC2 and PC3 are T3, Zadorra river in Vitoria-Trespuestas (País Vasco), and R17 and R18, at the lowest part of the river (last years).

(d) Organic compounds in sediments

For sediments, PC1 (52.29% of the data variance) describes a contamination pattern of polycyclic aromatic hydrocarbons, except for naphthalene. This compound is the most volatile compound of this group and has a slightly different chemical behaviour. This PAHs source is highly present in the upper part of the Ebro river, mainly at sampling site T9, Arga river in Puente la Reina (Navarra), followed by R1 and T3, Ebro river in Miranda de Ebro (Burgos, Castilla y León) and Zadorra river in Villodas (Álava, País Vasco), respectively. All these sampling sites are located very close

to Vitoria and Pamplona, cities with an important industrial activity. PC2 (16.81% of the data variance) describes a contamination pattern mostly loaded by pesticides: DDTs and HCB (and it has also some contribution of HCBu). It mostly affects the lower part of the Ebro river, with highest inputs at sampling sites R17, Ebro in Ascó (Tarragona, Catalonia) and R18, Ebro in Tortosa-Campredó (Tarragona, Catalonia). At T15, Cinca in Monzón downstream (Huesca, Aragón) a relatively high input of this contamination pattern was detected in 1997. Three main industries are located in this area: the electrochemical industry Erkimia in Flix, the paper factory Celulosas de Levante in Tortosa and a dying factory in Móra d'Ebre. Besides, organochlorinated compounds have been intensively used in the Ebro river downstream to prevent agricultural plagues and mosquitoes. The Ebro delta is one of the most polluted humid areas in Spain by organochlorinated compounds because, although the amount of industry in this area is relatively low in comparison to other areas in Catalonia, it is the zone where more typical rice pesticides (molinate, bentazone, etc.) and other industrial products have been released directly to the river flow with very little or none depuration processes for years. PC3 (9.41% of the data variance) is a rather specific contamination pattern for naphthalene, which is also generated from industrial activities. Sites where this source is especially present are T3 and R1 (in the País Vasco). Other punctual concentration peaks of naphthalene have been observed at T12, Gállego river in Jabarrella (Aragón) in year 2001; R14, Ebro in Presa de Pina (Aragón) in year 2002 and R17, Ebro in Ascó (Catalonia) in year 1996. A more detailed study of organic compounds in sediment samples of the Ebro river basin is being reported elsewhere [25].

(e) Organic compounds in fish

Aromatic hydrocarbons have not been included in the case of fish evaluation because they were not available for some years or there were too many values below their detection limit. PC1 in fish (40.20% of the data variance) describes the same contamination pattern as PC2 in sediments (by DDTs, HCB and HCBu) whose higher inputs are detected at T15 and R17, where a tendency to decrease year after year has been observed. PC2 (20.52% of the data variance) defines a contrast between two inversely correlated groups of compounds. Compounds with positive loadings are HCB, HCBu, o,p-DDD and p,p-DDT, whereas other DDTs (o,p-DDE, p,p-DDE and o,p-DDT) have negative loadings. Therefore, this PC shows a distinction between the behaviour and distribution of different DDTs. Whereas R17 and R18 are affected by those compounds with positive loadings, at T15, compounds with negative loadings are the ones which are more important. PC3 (10.88% of the data variance) is characterized by a high contribution of HCHs and TCBs. Its presence was only detected in year 2001 at T3, Zadorra in Vitoria-Trespuestas (where a high presence of PAHs and naphthalene has been found for sediments); R17, Ebro in Ascó (also affected by DDTs and HCB pollution); T9, Arga in Puente la Reina (PAHs contamination too); and T12, Gállego in Jabarrella (also characterized by naphthalene presence). These sites

do not have any geographical relation, since they belong to different tributaries, but a similar industrial pollution effects are observed on them.

(f) Physicochemical parameters in surface water

Three principal contamination patterns have been derived for physicochemical parameters in waters, explaining as much as 62.86% (Fig. 4) of the total data variance. PC1 (30.31% of the data variance) describes two different contributions that covariate inversely. The first one is defined by variables with positive loadings: NH_4^+ , BOD_5 , COD_{uv} , PO_4^{3-} and fluorides mainly. These characteristics correspond to waters with a high content of organic matter and, therefore, with a low content of oxygen and subsequent eutrophication problems. Sampling sites more affected by these contributions are located at the upper part of the river. These are T3, Zadorra in Vitoria Trespuestas (País Vasco) and R4, Ebro in Conchas de Haro (La Rioja), and both are situated after the river flow through urban areas receiving important effluents from industrial complexes and urban sewage with high concentrations of these compounds. A second type of profile is defined by PC1 negative loadings and has a high contribution of pH, oxygen, chlorides, $\text{SO}_4^{=}$ and conductivity variables. There is not an specific contribution of this profile in any sampling site, but at R17, Ebro in Ascó and R18, Ebro in Tortosa, both close to the mouth of the river. PC2 (21.36% of the data variance) is defined by high NO_3^- , alkalinity, conductivity and $\text{SO}_4^{=}$ values. Nitrates can come from animal wastes and agriculture, but it is important to remark that in this study their concentrations have not exceeded EC drinking water limit of 50 ppm in any sample. PC2 mostly affects the central part of the Ebro river, in Zaragoza. Salinization is especially evident in this part due to the high salt content of soils and underlying geology. Sampling sites with higher values are T10, Jalón river in Grisén, T13 Gállego river in Villanueva de Gállego and R14, Ebro river in Presa de Pina. PC3 (11.19% of the data variance) describes a contamination pattern with positive and negative loadings. Positive loadings show a contribution defined by high temperatures in water and in air and also by a relatively high fluoride concentration. It is especially present at sampling sites near the mouth of the river: R17, Ebro in Ascó; R18, Ebro in Tortosa, as well as T16, Segre in Torres de Segre (Lleida, Catalonia) and R14, Ebro in Presa de Pina (Zaragoza). T3, Zadorra in Vitoria Trespuestas, has also some influence of this pattern. The area at the final part of the river is a region whose climate is characterized by high temperatures (Mediterranean zone). Anthropogenic interventions also have important effects on water temperature in the lower course of the Ebro river. The nuclear power station in Ascó, at 4 Km downstream from Flix, is the responsible of the 3 °C increase in river temperature during the year [26,27]. Besides, it is also very important the effect of reservoirs located at the lower part of the river: Mequinensa, Riba-Roja and Flix. Fluorides are used in various industrial processes in chemical synthesis, like in phosphate production. Negative loadings in PC3 define a contribution characterized by a high presence of suspended matter, which is mainly found

at T13, Gállego river in Villanueva de Gállego (Aragón) and sampling sites R1 and R4, at the upper part of the river. Suspended matter reduces the amount of light passing through the water, which can cause photosynthesis to slow down and reduce dissolved oxygen in water.

5.3. GIS results

In this section, two different data matrices have been created to perform a good geostatistical interpolation and prediction with as many sampling sites as possible. Data matrices with fewer variables (7 metals) but with more samples (up to 18 sampling sites) for metals in sediments and surface waters have been arranged for years from 2000 to 2003. In the first data matrix, analyzed metals for sediments (concentrations in mg/kg of sediment) have been As, Cr, Cu, Ni, Pb, Se and Zn (Hg, analyzed in Section 5.2, was removed because it was only measured at few sampling sites). In the second data matrix, analyzed metals in surface water (concentrations in mg/L of water) have been As, Cd, Cr, Cu, Pb, Se and Zn (some of the metals from Section 5.2 have not been included here because their concentrations have not been available for all sampling sites). In both cases, score values obtained by PCA have been used to perform the kriging geostatistical operation.

In sediments as well as in surface waters, a river display of each described metal contamination pattern has been obtained (see Figs. 5–7). Each single map contains two different ways of presenting the contamination distribution over the territory. On the one side, graphic bars are displayed at each sampling site representing the scores value for the four consecutive studied years, from 2000 to 2003 (each bar corresponds to one year). At the right side of the map, there is a loadings plot which describes the

contribution of variables to the considered contamination pattern. On the other side, a river display using colour graduation has been obtained according to the presence of the described metal contamination pattern. In this way, not only the punctual information is given but also the main patterns of contamination distribution according to river zonation resulting from geostatistical predictions. White river areas correspond to parts where values could not be predicted.

For metals in sediments, the first general pattern identified (see Fig. 5) has contributions of all analyzed metals (77.53% of the data variance). From the graphic bars showing annual score values for each sampling point, T3, Zadorra river in Vitoria-Trespuentes (País Vasco) appears to be the most affected site. It is important to remark however, that this high contribution has not been found at the other sampling sites following this point and located in the main river (Ebro). Probably, this contribution has been diluted in its way through the tributary to the main river. For this reason, when colour graduation is displayed in the Ebro river, the upper part of the river does not show high values of this contamination pattern in the map (light colour), because prediction values are based on proximity. The highest presence of this metal pollution pattern (dark colour) has been predicted in the central part of the Ebro river, in the community of Aragón, around the city of Zaragoza.

For metals in surface waters, two contamination patterns have been identified. First PC (43.63% of the data variance) describes a general heavy metal pollution mostly characterized by Pb, Se, Cu, Zn and As (see Fig. 6), which is especially high at sampling site T11, Huerva river in Fuente de La Junquera (Aragón), (the tallest graphic bars, specially for year 2001). Ebro river downstream from this point presents the highest levels of metal contamination according to the prediction map displayed using

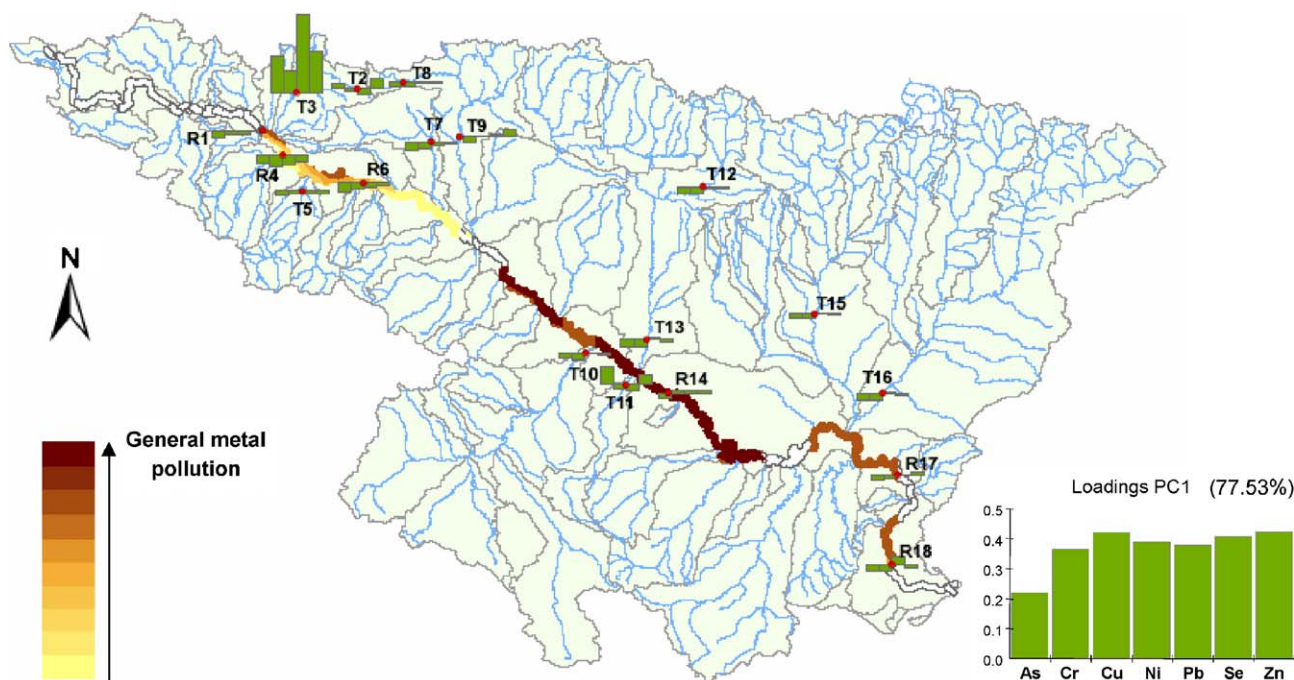


Fig. 5. Geostatistical kriging interpolation of contamination source 1 for metals in sediments of the Ebro river. Graphical scores representation for each sampling site from years 2000 to 2003.

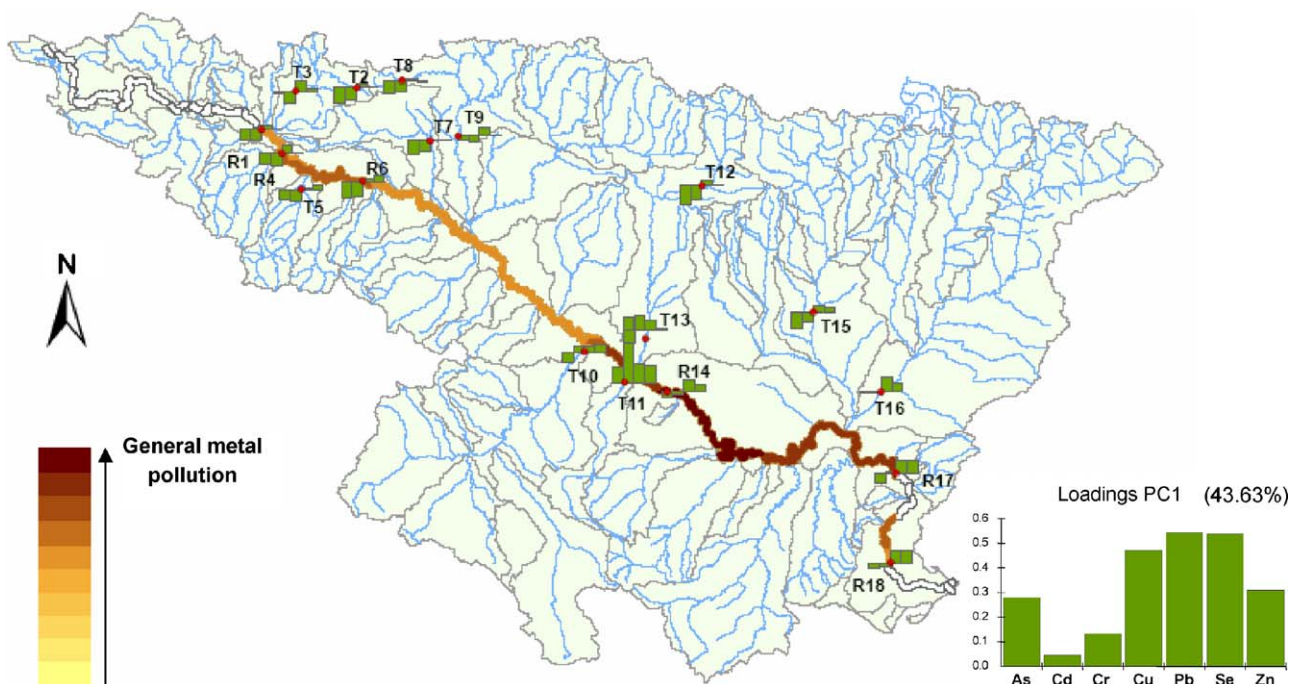


Fig. 6. Geostatistical kriging interpolation of contamination source 1 for metals in surface waters of the Ebro river. Graphical scores representation for each sampling site from years 2000 to 2003.

colour graduation. However, near the mouth of the river, heavy metal presence seems to be lower. It might be attributed to a dilution factor or to their precipitation due to pH increase in this part of the river. Second PC (23.41% of the data variance) describes two inversely correlated contributions (see Fig. 7). Positive loadings describe an important presence of Cd and As whereas negative loadings are mainly characterized by Cr pres-

ence. According to the graphic bars displayed at each sampling site, Cd and As pollution is especially high at T11, Huerva river in Fuente La Junquera, and, for years 2000 and 2001, at R6, Ebro river in Logroño (La Rioja). Central and upper parts of the Ebro river, especially the central part in Aragón, have a higher As and Cd concentration and they are displayed in the prediction map in darker colours. Cr pollution is mainly present in the mouth

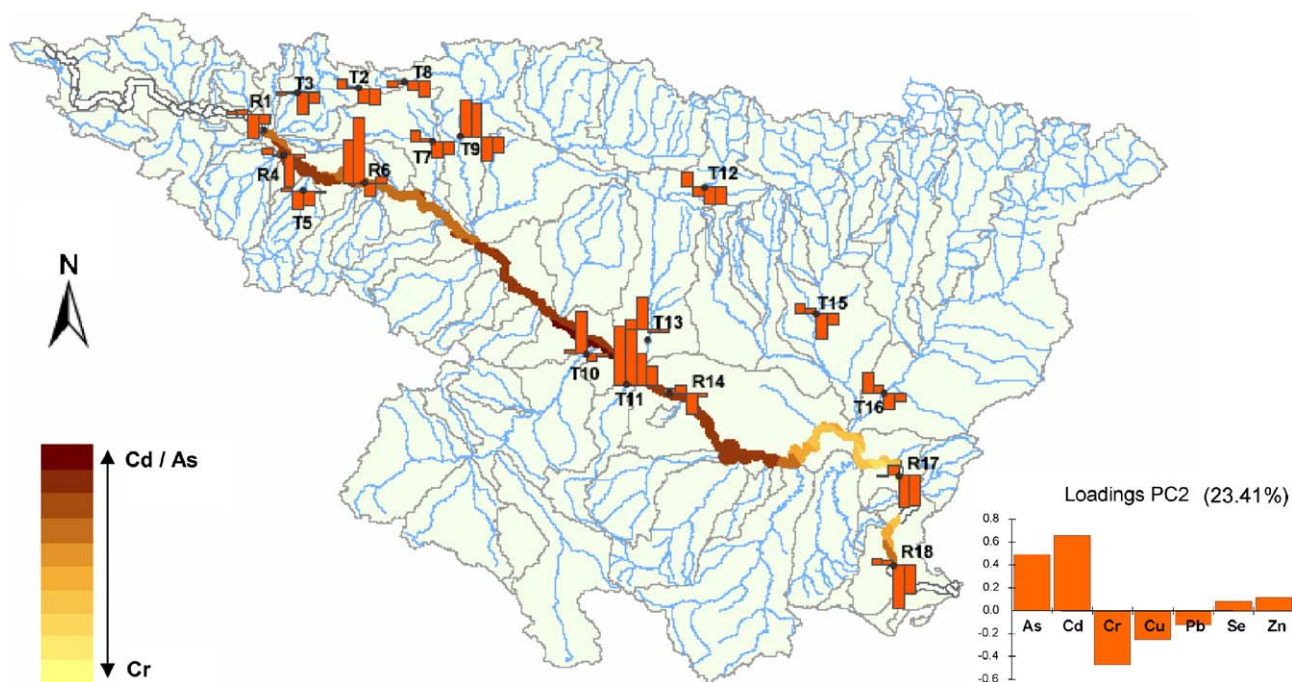


Fig. 7. Geostatistical kriging interpolation of contamination source 2 for metals in surface waters of the Ebro river. Graphical scores representation for each sampling site from years 2000 to 2003.

of the river, at sampling sites R18, Ebro in Tortosa (Catalonia) and R17, Ebro in Ascó (Catalonia) in years 2002–2003, which show the highest negative scores in the graphic bars. The more affected geographical zone by Cr pollution in the Ebro river is shown in light colours in the prediction map.

6. Conclusions

Environmental quality assessment of the Ebro river basin from 1996 to 2003 has been performed looking for different contamination sources and trends. Three geographical areas showing different tendencies have been roughly delimited according to the distribution of the identified contamination patterns. A correlation between land uses and characteristics of the river water, sediments and biota has been attempted. Area 1, in the upper part of the river (points R1, T3, R4, T9) is characterized by high inputs of contamination sources from industry and urbanisation: heavy metals (Zn, Cu, Cr, Pb, Cd and Hg), PAHs, HCHs, TCBs and the existence of eutrophic conditions (high content of NH_4^+ , MES, organic matter, PO_4^{3-} , etc.). Area 2 covers the central part of the Ebro river (points T10, T11, T13, R14), in Aragón. High accumulation of nutrients in water like Ca, Na, Mg and K, as well as the high values of alkalinity, conductivity, NO_3^- , SO_4^{2-} , MES, etc. shows an important salinization effect in this geographical region, with lower inputs from contamination sources derived in Area 1. Soils and underlying geology in this area have a high salt content which together with intensive irrigation techniques from agricultural activities have made it worse. Area 3 is located in the Ebro river downstream, from T15 in Monzón to the mouth of the river (points T15, T16, R17, R18). In this area, organic and heavy metal contamination sources come mainly from industrial activities, although agriculture (rice production) is also responsible for the encountered sources of high releases of pesticides into the Ebro basin. Metals such Hg, Cd, Zn and As have a high influence in this lower part of the river. DDTs, HCB and HCBu are the organic pollutants with the highest concentrations and there are also some punctual influences of HCHs and TCBs.

Acknowledgements

This work is supported by the European Union and the Spanish Ministry of Education and Science and reflects the author's view and the EU is not liable for any use that may be made of the information contained there in "Integrate modelling of the river-sediments-soil-groundwater system; advanced tools for the

management of catchment areas and river basins in the context of global change" [AQUATERRA, GOCE 505428] and [CTM2005-25168-E].

References

- [1] CHE: www.chebro.es and www.opf.chebro.es.
- [2] A. Smilde, R. Bro, P. Geladi, Multi-way Analysis with Applications in the Chemical Sciences, John Wiley & Sons Ltd., 2004.
- [3] D. Comas, E. Ruiz, Fundamentos de los Sistemas de Información Geográfica, Ariel Geografía, Barcelona, 1993.
- [4] J. Gutiérrez-Puebla, M. Gould, Sistemas de Información Geográfica, Síntesis, Madrid, 1994.
- [5] F. Calvete, J. Carrera, Geoestadística: Aplicaciones a la hidrología subterránea, Centro Internacional de Métodos Numéricos en Ingeniería, UPC, Barcelona, 1990.
- [6] N. Cressie, Statistics for Spatial Data, John Wiley & Sons Inc., USA, 1993.
- [7] P. Goovaerts, Geostatistics for Natural Resources Evaluation, Oxford University Press, 1997.
- [8] I. Jolliffe, Principal Component Analysis, Springer, New York, 1986.
- [9] World Resources Institute: www.earthtrends.wri.org, 2005.
- [10] J. Moradillo, J. Ramos, IENICA (Interactive European Network for Industrial Crops and their Applications), Report from the Kingdom of Spain (www.ienica.net), 2004.
- [11] N. Torrecilla, J. Galve, L. Zaera, J. Retamar, A. Alvarez, J. Hydrol. 304 (2005) 166–182.
- [12] S. Lacorte, D. Raldúa, E. Martínez, A. Navarro, S. Diez, J. Bayona, D. Barceló, Environ. Pollut. (2005).
- [13] S. Mañosa, R. Mateo, R. Guitart, Environ. Monitoring Assessment 71 (2001) 187–205.
- [14] R. Aspinall, D. Pearson, J. Environ. Manage. 59 (2000) 299–319.
- [15] C.S. Lee, X.D. Li, W.Z. Shi, S.C. Cheung, I. Thornton, Sci. Total Environ. 356 (2006) 45–61.
- [16] T. Oguchi, H.P. Jarvie, C. Neal, Sci. Total Environ. 251 (2000) 9–26.
- [17] P. Simeonova, V. Simeonov, G. Andreev, Central Eur. J. Chem. 1 (2003) 121–136.
- [18] K. Johnston, J. Ver Hoef, K. Krivoruchko, N. Lucas, Using ArcGis Geostatistical Analyst, Environmental Systems Research Institute, USA, 2001.
- [19] P. Goovaerts, Geoderma 89 (1999) 1–45.
- [20] A. Palanques, J.A. Sanchez-Cabeza, P. Masque, L. Leon, Mar. Chem. 63 (1998) 183 (vol. 61, p. 209, 1998).
- [21] A. Palanques, J.I. Diaz, Mar. Environ. Res. 38 (1994) 17–31.
- [22] V. Simeonov, J. Stratis, C. Samara, G. Zachariadis, D. Voutsas, A. Anthemidis, M. Sofoniou, T. Kouimtzis, Water Res. 37 (2003) 4119–4124.
- [23] P. Karanis, I. Chronis, G. Zakas, C. Kourenti, I. Sotiriadou, C. Papadopoulou, Acta Hydrochim. Hydrobiol. 33 (2005) 346–354.
- [24] J. Moore, S. Ramamoorthy, Heavy Metals in Natural Waters, Applied Monitoring and Impact Assessment, Springer-Verlag, New York, 1984.
- [25] A. Navarro, R. Tauler, S. Lacorte, D. Barceló, Anal. Bioanal. Chem. (2006), in press.
- [26] J. Prats, R. Val, J. Armengol, J. Dolz, Claves para la gestión sostenible del agua (2004) 1–6.
- [27] J. Prats, R. Val, J. Armengol, J. Dolz, Aquatics Habitats: Anal. Restoration (2004) 872–879.

Highly collinear three-way photoinduced spectrofluorimetric data arrays modelled with bilinear least-squares: Determination of tetracyclines in surface water samples

Rosario Santiago Valverde^a, M. Dolores Gil García^a,
M. Martínez Galera^{a,*}, Héctor C. Goicoechea^{b,**}

^a Departamento de Hidrogeología y Química Analítica, Facultad de Ciencias Experimentales, Universidad de Almería, la Cañada de San Urbano, 04120 Almería, Spain

^b Laboratorio de Control de Calidad de Medicamentos, Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, S3000ZAA Santa Fe, Argentina

Received 24 October 2005; received in revised form 24 January 2006; accepted 24 January 2006

Available online 3 March 2006

Abstract

A new sensitive method for the determination of three tetracyclines in surface water samples (tetracycline, chlortetracycline and oxytetracycline) using highly collinear data corresponding to photochemical-induced fluorescence excitation emission matrices, modelled with three-way data arrays chemometric tools has been developed. The method involves irradiation of aqueous basic solution of antibiotics with UV light and fluorescence determination of the generated photoproducts after solid phase preconcentration (SPE) with Oasis hydrophilic lipophilic balance (HLB) cartridges. The chemometric models were built by using an eight samples calibration set and samples corresponding to both a validation set and a test set. The latter set containing unsuspected interferents. Considering this latter samples, recoveries of 101.5, 97.9 and 102.2 were obtained for the three analytes. Evaluation of constant and proportional bias and precision were made by using the elliptical joint confidence region (EJCR) test for the estimated intercept and slope.

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Keywords: Tetracyclines; Water; Photochemically induced fluorescence; PARAFAC; NPLS; BLS/RBL

1. Introduction

Currently, the availability of high-order instrumental data and the proliferation of chemometric algorithms for data processing [1] gave raise to the regular use of multi-way analysis for solving complex analytical problems [2]. A predominantly attractive property of multi-way data is the second-order advantage [3], a term coined to describe the analysis in the presence of unsuspected interferents. Second-order, i.e., matrix data for a given sample can be produced in a variety of ways among which the simpler one is an excitation–emission fluorescence matrix (EEM). When a sample produces a data matrix (a second-

order tensor), such as an EEM, the corresponding set obtained by ‘stacking’ the training matrices is a three-way array. A variety of chemometric methods have been applied to analyze these arrays, specifically generalized rank annihilation (GRAM) [4], parallel factor modelling (PARAFAC) [5,6], N-way partial least-squares (NPLS) [5], multivariate curve resolution coupled to alternating least-squares (MCR-ALS) [7] and the recently presented bilinear least-squares (BLS) [8,9].

In this report, we focus our attention to the photochemically induced fluorescence EEM (PIF-EEM) based analysis of tetracycline antibiotics in surface water. Tetracyclines are an important group of antibiotics that are extensively used to the treatment of human infections and in veterinary medicine. Also, livestock farms often apply antibiotics as a preventive measure or as growth promoters, thus being added to the feed. Recently, there has been a growing interest in the presence of pharmaceutical substances in the aquatic environment due to the possible enhancement of resistance formation in bacteria.

* Corresponding author. Fax: +34 950015483.

** Corresponding author. Tel.: +54 342 4575205; fax: +54 342 4575205.

E-mail addresses: mmartine@ual.es (M.M. Galera),
hgoico@fcb.unl.edu.ar (H.C. Goicoechea).

Some previous studies stated that the total amount of antibiotics used for human and veterinary medication is in the same range as the applied amount of some pesticides, which are subjected to extensive studies in the aquatic environment for many years. Generally, after intake drugs are subjected to metabolic reactions, but a significant amount of the original substance will leave the organism unmetabolized, via urine or feces. Hirsch et al. reported that tetracycline, chlortetracycline and oxitetracycline are excreted unchanged in percentages higher than 70, 80–90 and 80%, respectively [10]. In addition, their elimination in sewage treatment plants is often incomplete [11]. Hence one can expect to find antibiotics drugs in surface waters.

The determination of tetracyclines in water samples has traditionally been performed using fluorescence detection based on their metallic complexes with Mg^{2+} , Ca^{2+} or Eu^{2+} under neutral or alkaline conditions and with Al^{3+} or Zr^{4+} in acidic solutions [12], by using batch [13–16] or continuous methods, i.e., liquid-chromatography (LC) with fluorescence detection [17,18] or pulsed amperometric detection [19] and capillary electrophoresis (CE) using diode array (DAD) [20] and laser-induced fluorescence detection [12]. In addition, LC with UV detection [21–23] and flow injection analysis (FIA) with chemiluminescence detection [24,25] have been employed, even though, today the technique of choice for analysis of this group of substances seems to be liquid-chromatography coupled to mass spectrometry (LC–MS) [26,27] and tandem mass spectrometry [28–30]. The above-mentioned conventional techniques lack of enough selectivity, requiring of time consuming clean up steps, and in some cases, strategies for solving matrix effect have been necessary. In addition, mass instruments are still quite expensive being not readily available for chemists in testing laboratories and, in some cases, matrix component caused ion suppression/enhancement effects during the MS detection [31].

The present work tries to demonstrate that modelling three-way data arrays is a convenient way of simultaneously measuring the concentration of three tetracyclines with strongly overlapping spectra, in the presence of uncalibrated interferents. The employment of an adequate chemometric methodology for data analysis in the presence of strong colinearities produces results crucial to the success of the analytical determination. As shown in the present report, NPLS and PARAFAC are not able to handle these extreme collinearities, especially when unsuspected interferents are present. Consequently, we have explored the alternative use of a recently presented BLS methodology.

2. Theory

2.1. PARAFAC

When a sample produces a $J \times K$ data matrix, such as the PIF-EEM landscape (J is the number of emission wavelengths, K the number of excitation wavelengths), the corresponding set obtained by ‘stacking’ the training matrices $X_{c,i}$ and the unknown one X_u is a three-dimensional array, of dimen-

sions $(I+1) \times J \times K$ (I is the number of training samples). In the PARAFAC model, a generic element X_{ijk} of the three-dimensional array X can be written as follows:

$$X_{ijk} = \sum_{n=1}^N a_{ni} b_{nj} c_{nk} + E_{ijk} \quad (1)$$

where N is the total number of responsive components, E_{ijk} is an element of a residual error array E of the same dimensions as X , a_{ni} , b_{nj} and c_{nk} are the elements of the column vectors a_n , b_n and c_n , which are the relative concentration $[(I+1) \times 1]$, emission $(J \times 1)$ and excitation $(K \times 1)$ profiles for component n , respectively. The column vectors a_n , b_n and c_n are collected into the three loading matrices A , B and C (b_n and c_n are usually normalized to unit length). Decomposition of the three-way array provides the profiles B and C , corresponding to the emission and excitation wavelengths and the relative concentrations (A) of individual components in the $(I+1)$ mixtures, whether they are chemically known or not. This constitutes the basis of the so-called second-order advantage. Theoretically, this property should allow the analyst to obtain the concentration values of calibrated constituents in the presence of any number of uncalibrated components.

Several different methods can be used to estimate the number of responsive components (N). Among them, the consideration of the PARAFAC internal parameter known as core consistency [32] and the study of the regression error of the pseudounivariate calibration line [33] are useful techniques. In the present case, the number of components is equal to the number of different species in the sample. For ternary mixture standards, this number is four, on account of the presence of the three analytes plus the background signal originated by the pre-treatment sample. For real samples the number is likely to be larger (presence of uncalibrated components).

Identification of the chemical constituent under investigation is done with the aid of the profiles B and C , as extracted by PARAFAC, and compared them with those for a standard solution of the analyte of interest. On the other hand, absolute analyte concentrations are obtained after proper calibration, since only relative values (A) are provided by decomposing the three-way data array. Experimentally, this is done by using the information for the set of standards of known composition. The calibration procedure involves decomposing an array formed by joining the matrices for the I training samples with that for the unknown. Resorting to Eq. (2), once the component profiles have been obtained, the scores for the standard samples associated with a particular component are linearly related to the nominal concentrations of the analyte:

$$[a_{n1}|a_{n2}|\cdots|a_{nI}] = k_{\text{PARAFAC}} y \quad (2)$$

where n identifies a PARAFAC component, and y is an $I \times 1$ vector holding the nominal analyte concentrations in the I calibration standards. It should be noticed that employment of this joined calibration mode implies that the array decomposition should be repeated for each newly analyzed sample.

2.2. N-PLS

Multiway regression methods such as *N*-PLS extend the traditional PLS algorithm to higher orders, using the multidimensional structure of the data for model building and prediction [34]. In the case of three-way data, the model is given by the following equation:

$$x_{ijk} = \sum_{f=1}^F t_{tf} w_{jf}^J w_{kf}^K + e_{ijk} \quad (3)$$

where x_{ijk} is the fluorescence intensity measured for sample i at wavelength emission j and wavelength excitation k , F is the number of components, t_{tf} is an element of the score matrix $\mathbf{T}$, w_{jf}^J and w_{kf}^K are elements of two $\mathbf{W}$ loading matrices and e_{ijk} is a residue not fitted by the model. The model finds the scores yielding maximum covariance with analyte concentrations as the dependent variable, in a three-dimensional sense. The advantage of using *N*-PLS over bidimensional regression is a stabilization of the decomposition involved in Eq. (3), which potentially gives increased interpretability and better predictions. This methodology, however, does not exploit the second-order advantage, and in principle, it should lead to results of a lower quality as compared to PARAFAC or BLS in samples of complex composition.

2.3. BLS

BLS employs concentration information into the calibration step, without including data for the unknown sample. This is done in order to estimate pure-analyte matrices at unit concentration ($\mathbf{S}_n$) for specific calibrated analytes ($\mathbf{n}$).

For this purpose, the I calibration data matrices $\mathbf{X}_{c,i}$ (each of size $J \times K$) are first vectorized and grouped into a $JK \times I$ matrix $\mathbf{V}_X$ [35]:

$$\mathbf{V}_X = [\text{vec}(\mathbf{X}_{c,1}) | \text{vec}(\mathbf{X}_{c,2}) | \cdots | \text{vec}(\mathbf{X}_{c,I})] \quad (4)$$

where ‘vec’ implies the unfolding operation. Then a direct least-squares (LS) procedure is employed to obtain the pure-analyte information, analogous to first-order classical least-squares multivariate calibration [35]:

$$\mathbf{V}_S = \mathbf{V}_X \mathbf{Y}^T \quad (5)$$

where $\mathbf{Y}$ is an $I \times N_c$ matrix collecting the nominal concentrations of the calibrated analytes, N_c is the number of calibrated analytes, and $\mathbf{V}_S$ (size $JK \times N_c$) contains the required $\mathbf{S}_n$ matrices in vectorized form:

$$\mathbf{V}_S = [\text{vec}(\mathbf{S}_1) | \text{vec}(\mathbf{S}_2) | \cdots | \text{vec}(\mathbf{S}_{N_c})] \quad (6)$$

To obtain the profiles in both dimensions which are present in the $J \times K \mathbf{S}_n$ matrices, two procedures have been discussed in the literature [8,9]: the least-squares profile estimator and the singular value decomposition (SVD) profile estimator, the most reliable and simple seems to be the latter one. In this case, the component profiles are obtained by single-component singular

value decomposition (SVD_1) of each of the $\mathbf{S}_n$ matrices, obtained after suitable reshaping of the unfolded $\text{vec}(\mathbf{S}_n)$:

$$(g_n, \mathbf{b}_n, \mathbf{c}_n) = \text{SVD}_1(\mathbf{S}_n) \quad (7)$$

where g_n is the first singular value, and $\mathbf{b}_n$ and $\mathbf{c}_n$ are the $J \times 1$ and $K \times 1$ left and right eigenvectors of $\mathbf{S}_n$, respectively. This completes the calibration process.

After calibration, concentrations are estimated in test samples by direct least-squares, provided no interferences occur in the unknown sample [35]:

$$\mathbf{y}_u = \mathbf{S}_{\text{cal}}^+ \text{vec}(\mathbf{X}_u) \quad (8)$$

where $\mathbf{X}_u$ is the $J \times K$ matrix data for the test sample, $\mathbf{y}_u$ is an $N_c \times 1$ vector holding the estimated concentrations of the N_c analytes in the sample, and $\mathbf{S}_{\text{cal}}$ is a calibration $JK \times N_c$ matrix given by:

$$\mathbf{S}_{\text{cal}} = [g_1(\mathbf{c}_1 \otimes \mathbf{b}_1) | g_2(\mathbf{c}_2 \otimes \mathbf{b}_2) | \cdots | g_{N_c}(\mathbf{c}_{N_c} \otimes \mathbf{b}_{N_c})] \quad (9)$$

where $\otimes$ implies the well-known Kronecker product.

The occurrence of uncalibrated compounds in a test sample is investigated after the calibration step is completed. If an interferent occurs, the situation is handled by a separate residual bilinearization (RBL) [8,9], in which profiles for the interferent are found, and incorporated into an expanded version of $\mathbf{S}_{\text{cal}}$:

$$\mathbf{S}_{\text{exp}} = [\mathbf{S}_{\text{cal}} | g_{\text{int}}(\mathbf{c}_{\text{int}} \otimes \mathbf{b}_{\text{int}})] \quad (10)$$

where the profiles $\mathbf{b}_{\text{int}}$ and $\mathbf{c}_{\text{int}}$ are obtained by a procedure involving the minimization of the residual matrix $\mathbf{E}_u$, computed while fitting the sample data to the sum of the various component contributions:

$$\mathbf{X}_u = \sum_{n'=1}^{N_c} g_{n'} \mathbf{b}_{n'} (\mathbf{c}_{n'})^T \mathbf{y}_{n',u} + g_{\text{int}} \mathbf{b}_{\text{int}} (\mathbf{c}_{\text{int}})^T + \mathbf{E}_u \quad (11)$$

During the minimization, the interferent profiles are estimated by SVD of a residual matrix $\mathbf{E}_c$, obtained by subtracting the analyte contributions to the total signal $\mathbf{X}_u$:

$$\mathbf{E}_c = \mathbf{X}_u - \sum_{n'=1}^{N_c} \mathbf{S}_{n'} \mathbf{y}_{n',u} \quad (12)$$

$$(g_{\text{int}} \mathbf{b}_{\text{int}} \mathbf{c}_{\text{int}}) = \text{SVD}_1(\mathbf{E}_c) \quad (13)$$

We used a Gauss–Newton minimization procedure of $\|\mathbf{E}_u\|$ in Eq. (11), implemented under MATLAB, during which g_{int} , $\mathbf{b}_{\text{int}}$, and $\mathbf{c}_{\text{int}}$ are found by singular value decomposition of $\mathbf{E}_c$ [36]. The procedure renders the value of $y_{ns,u}$ required to minimize $\mathbf{E}_u$ in Eq. (11).

BLS is a two-step calibration-prediction mode where concentration prediction is guided by a least-squares minimization, while the second-order advantage is left for a subsequent stage, in which the matrix residuals are analyzed in order to estimate the interferent profiles. This second step serves to expand the loadings and to correctly estimate the analyte concentrations even in the presence of unexpected constituents. Notice that no initialization or constraining procedures are required taken less computation time than the other algorithms.

3. Experimental

3.1. Instrumentation

An Oriel 50-500 W model 66901 Xenon lamp with an Oriel model 8500 power supply was used for the photolysis reactions. The photochemical reactor included a light box consisting of a fan, a Xenon lamp and a convenient quartz lens to concentrate the radiation on a standard Hellman 10 mm quartz cell containing the sample. For the photolysis reactions, the solutions of tetracyclines, were placed on an optical bench at 30 cm from the Xenon lamp and stirred magnetically.

The spectral fluorescence measurements were performed on an Aminco Bowman Series 2 Luminescence Spectrometer controlled by a PC Novo Pentium microcomputer and an AB2 software version 2.0 running under OS/2 version 2.0. A vacuum system Büchi Vac V-500 and a vacuum controller V-800 (Switzerland) connected to an extraction manifold Waters (Milford, USA) was used as pre-concentration water system.

3.2. Software

All calculations were done using MATLAB 6.0 [37]. Routines for *N*-PLS and PARAFAC are available on the Internet [38] although a useful MATLAB graphical interface was developed for easy data manipulation and graphics presentation [33] now including the BLS code [39].

3.3. Reagents and standards

Analytical standards of tetracycline hydrochloride (TC), chlortetracycline hydrochloride (CTC) and oxytetracycline (OTC) were obtained from Riedel-de Hën (Seelze, Germany). Analytical-reagent grade solvents, methanol (MeOH), sodium carbonate and sodium hydrogen carbonate were obtained from Merck (Darmstadt, Germany). Ethylenediaminetetracetic acid disodium salt 2-hydrate (EDTA- Na_2) for analysis, chlorhydric acid and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Ultrapure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The solid phase preconcentration (SPE) of water samples was carried out using Oasis hydrophilic lipophilic balance (HLB) 6cc cartridges containing 0.2 g of stationary phase from Waters (Milford, Massachusetts, USA).

Individual standard solutions of 200 mg L^{-1} of each tetracycline were prepared by exactly weighing and dissolving the corresponding compounds in methanol. These standard solutions are stable for a maximum of 1 month. The working standard solutions were prepared daily by diluting these stock standard solutions in methanol. All solutions were protected against light and stored in a refrigerator at 4°C .

3.4. Photochemically induced procedure for the determination of tetracyclines

Different quantities of standard solutions in MeOH were added to a flask of 5.00 mL and diluted to the total volume

with buffer carbonate–bicarbonate 0.02 M (pH 10). The solutions were placed in a quartz cell and stirred magnetically during 8 min without UV irradiation. Finally, these solutions were irradiated with UV light for 3 min with agitation.

The excitation and emission spectra were recorded between 270 and 370 nm ($\Delta\lambda = 1 \text{ nm}$) and between 370 and 500 nm ($\Delta\lambda = 3 \text{ nm}$), respectively. Thus, the dimension of the ($J \times K$) data matrices was (131×23).

3.5. Procedure to determine tetracyclines in surface water

Tetracyclines were extracted from water samples using 500 mg Oasis HLB cartridges. Aqueous samples were prepared for extraction by adding 2 mL of 5% Na_2EDTA to a flask containing 500 mL of water, and pH of the sample was adjusted to 2.8 with HCl immediately prior to extraction.

The cartridges were preconditioned with 20 mL of MeOH and 10 mL of Milli-Q water. Then 500 mL of water sample, treated as described above, were passed through the cartridge at a flow-rate of 8 mL min^{-1} . The analytes retained on the SPE cartridge were eluted with 5 mL of MeOH and the extracts were concentrated to dryness under a stream of N_2 and re-dissolved with $125 \mu\text{L}$ of MeOH. Before the analysis, carbonate/bicarbonate buffer 0.02 M (pH 10) was added until a final volume of 5 mL. Then, the solutions were placed in a quartz path and treated according to the previous procedure.

3.6. Calibration and validation sets and real spiked samples

An 8-samples calibration set was randomly built for NPLS, PARAFAC and BLS models, including three pure standard of each analyte. The concentrations are displayed in Table 1. On the other hand, a 9-sample set was also randomly built to be used for validating the models (see Tables 2–4). Both, the calibration and validation sets were prepared in MQ-water.

Additionally, nine samples with the same analytes concentrations than those for the validation set were prepared by spiking real surface water samples. This test set was used to evaluate the ability of the models when uncalibrated components were present.

3.7. Data processing

PARAFAC was applied separately for each sample on three-way data arrays formed by PIF-EEMs corresponding to eight

Table 1
Calibration design

Calibration samples	Actual (mg L^{-1})		
	OTC	TC	CTC
C-1	0.60	0.00	0.00
C-2	0.00	0.50	0.00
C-3	0.00	0.00	0.40
C-4	0.30	0.60	0.60
C-5	0.60	0.50	0.40
C-6	1.00	0.40	0.20
C-7	0.70	0.30	0.50
C-8	0.40	1.00	0.80

Table 2

Results obtained in the analysis of OTC in synthetic samples prepared in MQ water

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
S-1	0.60	0.57 (3.5)	95.0	0.54 (3.4)	90.0	0.61 (3.3)	101.7
S-2	0.30	0.30 (3.2)	100.0	0.47 (3.3)	156.7	0.31 (3.3)	103.3
S-3	0.80	0.75 (3.4)	93.8	0.77 (3.4)	96.3	0.76 (3.5)	95.0
S-4	1.00	0.95 (3.0)	95.0	0.86 (3.1)	86.0	0.97 (3.0)	97.0
S-5	0.50	0.44 (3.1)	88.0	0.46 (3.1)	92.0	0.46 (3.2)	92.0
S-6	0.36	0.32 (3.1)	88.9	0.47 (3.2)	130.6	0.33 (3.1)	91.7
S-7	0.50	0.35 (3.1)	70.0	0.38 (3.0)	76.0	0.40 (3.1)	80.0
S-8	0.00	-0.03	—	0.08	—	-0.01	—
S-9	0.70	0.59 (3.2)	84.3	0.53 (3.1)	75.7	0.67 (3.1)	95.7
Mean recovery (%)			89.4		100.4		94.5

^a Between parentheses the CV% corresponding to three replicates.

Table 3

Results obtained in the analysis of TC in synthetic samples prepared in MQ water

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
S-1	0.40	0.39 (2.9)	97.5	0.50 (3.0)	125.0	0.37 (3.1)	92.5
S-2	0.50	0.58 (3.1)	116.0	0.65 (3.0)	130.0	0.55 (2.9)	110.0
S-3	0.30	0.29 (3.0)	96.7	0.44 (3.1)	146.7	0.20 (2.9)	66.7
S-4	0.40	0.30 (2.9)	75.0	0.41 (3.2)	102.5	0.34 (3.0)	85.0
S-5	0.80	0.76 (3.5)	95.0	1.02 (3.3)	127.5	0.76 (3.2)	95.0
S-6	0.70	0.69 (3.3)	98.6	0.63 (3.2)	90.0	0.73 (3.2)	104.3
S-7	0.70	0.51 (3.2)	72.9	0.47 (2.8)	67.1	0.65 (3.0)	92.9
S-8	0.50	0.63 (3.3)	126.0	0.69 (3.5)	138.0	0.55 (3.1)	110.0
S-9	0.00	0.18 (2.5)	—	0.13 (2.5)	—	0.05	—
Mean recovery (%)			97.2		115.9		95.5

^a Between parentheses the CV% corresponding to three replicates.

calibration samples together with a water sample. The interface used (see Section 3.2) provides a simple mean of loading the data matrices into the MATLAB working space before running PARAFAC. The **B** and **C** profiles provided by the latter are separately plotted in order to allow users to identify the analyte

of interest. The pseudo-univariate calibration graph [40] corresponding to this particular component is then displayed, along with the regression error, which can be subsequently employed for estimating the correct number of components (see Section 2). Once this is done, the results are conveniently shown

Table 4

Results obtained in the analysis of CTC in synthetic samples prepared in MQ water

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
S-1	0.50	0.49 (3.5)	98.0	0.55 (3.0)	110.0	0.51 (3.0)	102.0
S-2	0.60	0.56 (3.1)	93.3	0.59 (3.0)	98.3	0.57 (2.9)	95.0
S-3	0.40	0.42 (3.2)	105.0	0.45 (2.9)	112.5	0.45 (2.9)	112.5
S-4	0.30	0.33 (2.9)	110.0	0.39 (2.9)	130.0	0.32 (3.1)	106.7
S-5	0.70	0.71 (3.0)	101.4	0.73 (2.9)	104.3	0.70 (3.3)	100.0
S-6	0.50	0.48 (3.1)	96.0	0.47 (3.1)	94.0	0.45 (3.1)	90.0
S-7	0.00	0.03	—	0.00	—	-0.01	—
S-8	0.70	0.66 (3.1)	94.3	0.68 (3.2)	97.1	0.68 (3.3)	97.1
S-9	0.50	0.42 (3.1)	84.0	0.51 (3.2)	102.0	0.48 (2.9)	96.0
Mean recovery (%)			97.8		106.0		99.9

^a Between parentheses the CV% corresponding to three replicates.

in terms of predicted concentration and analytical figures of merit.

On the other hand, the best NPLS model was applied to both the validation and the test set.

Finally, for application of the BLS algorithm to each test sample, the total PIF-EEMs corresponding to the calibration set were first vectorized in order to apply the mathematical expressions described above. Residual bilinearization was then applied to all samples requiring the second-order advantage. The number of interferents was assessed by increasing a trial number of components until the BLS prediction residuals stabilize at a value compatible with the instrumental noise level. The latter was estimated by suitable blank replication measurements.

4. Results and discussion

In a previous paper we found that TC, OTC and CTC are converted into strongly fluorescent photoproducts when they are irradiated with UV light in carbonate/bicarbonate buffer (pH 10; $C_T = 0.02$ M) 2.5% MeOH, after stirring for 6–8 min in this medium [41]. Samples were stirred before irradiation to eliminate fluctuations which appear in measurements when solutions are irradiated immediately after pH adjustment. In order to determine the three tetracyclines at the concentration levels expected in surface water samples, a SPE procedure using Oasis HLB cartridges was performed [42]. Recoveries obtained, however, were rather low due to the fact that the signal is attenuated by the cartridge background and, therefore a PDS calibration transfer model was developed in order to solve this drawback. In addition, the signal was also influenced by the source of the surface water and, definitively, the problem was overcome by using river water from different sources to build the transfer set in the PDS transference model.

Fig. 1A and B show the normalized emission and excitation spectra of the three tetracyclines together with both the background signal originated in the pre-treatment procedure and the signal corresponding to a real water sample tetracyclines free, before the antibiotics were spiked. As can be seen, both the high spectral overlapping and the colinearity are evident. In addition, real water samples present interferences that may be variable from sample to sample. These facts become the present studied problem in a challenge for multi-way models, especially those that take into account the second-order advantage.

The number of latent variables was established in NPLS by resorting to the well-known cross-validation leave-one-sample-out procedure. This number was four for all the three calibration models. The quality of the fitting can be judged by computing the root-mean-square error of cross-validation, $RMSECV = [(1/I) \sum_1^I (c_{act} - c_{pred})^2]^{1/2}$, where I is the number of calibration samples and c_{act} and c_{pred} the actual and predicted concentration by the cross-validation procedure, respectively. The RMSECV obtained are the following: 0.046 mg L^{-1} for OTC, 0.033 mg L^{-1} for TC and 0.022 mg L^{-1} for CTC.

The core consistency analysis was applied for selecting the number of spectral components in PARAFAC [32]. This latter test allowed us to find the optimum number of factors to be used when predicting the analytes concentration in both validation

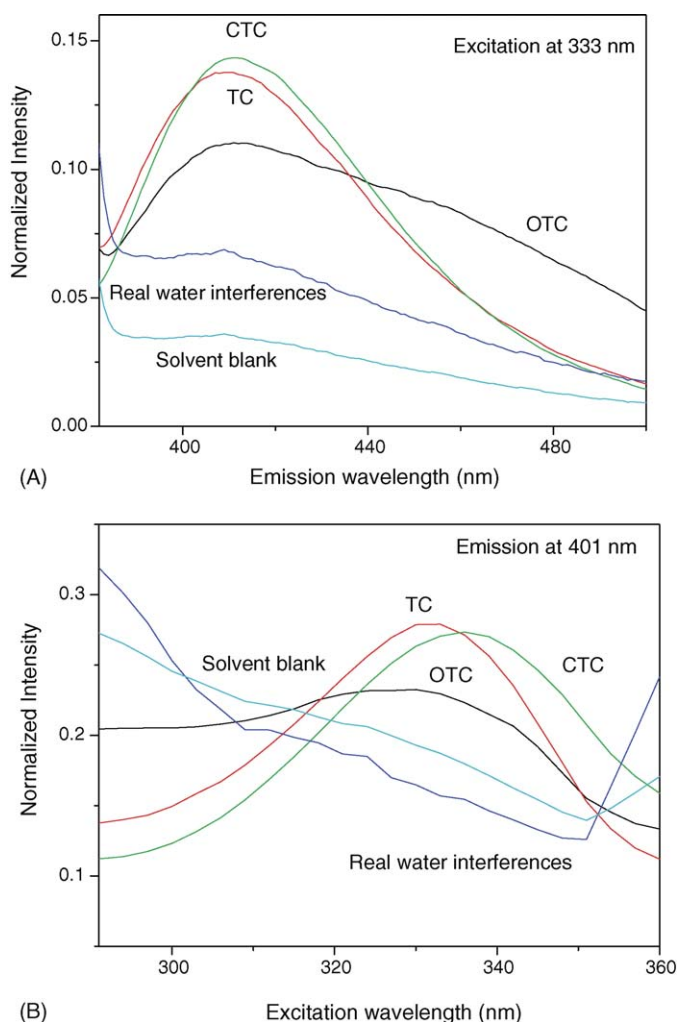


Fig. 1. Normalized pure emission (A) and excitation (B) spectra of the three tetracyclines together with both the background signal originated in the pre-treatment procedure and the signal corresponding to a real water sample tetracyclines free, before the antibiotics were spiked.

and test samples. Interestingly, for the validation set, which was prepared by spiking Milli-Q water, the number of factors was four, while this number reached five for those samples prepared by spiking real water. Analogous considerations can be made for the selection of the number of factors in the BLS analyses. Four components were necessary to decrease the BLS prediction residuals for each validation sample, until it stabilizes at a value compatible with the instrumental noise level. Fig. 2 shows that when the analysed sample is one corresponding to the test set (R-1, uncalibrated component presence), the necessary number of factors reaches five. As can be seen, the model with five components shows a prediction residual comparable to the instrumental noise level (ca. 0.07 fluorescence units).

The application of the multivariate models to both validation and test samples yielded analyte profiles and estimated concentrations. All spectral profiles agree well with those corresponding to standards. As an example, Fig. 3A and B show the five components, as suggested by the consideration of BLS prediction residuals for a typical real sample. Prediction with this BLS model furnishes the spectral profiles for each component.

Table 5

Results obtained in the analysis of OTC in real water samples

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
R-1	0.60	0.72 (3.1)	120.0	0.74 (3.0)	123.3	0.60 (3.2)	100.0
R-2	0.30	0.69 (3.0)	230.0	0.63 (3.0)	210.0	0.31 (3.1)	103.3
R-3	0.80	1.07 (2.9)	133.8	1.33 (3.0)	166.3	0.68 (3.0)	85.0
R-4	1.00	1.20 (3.8)	120.0	1.27 (3.2)	127.0	1.05 (3.5)	105.0
R-5	0.50	0.82 (3.5)	164.0	1.11 (3.4)	222.0	0.48 (3.4)	96.0
R-6	0.36	0.55 (3.0)	152.8	0.67 (2.9)	186.1	0.39 (2.8)	108.3
R-7	0.50	0.68 (3.0)	136.0	0.53 (3.1)	106.0	0.52 (3.0)	104.0
R-8	0.00	0.30 (2.9)	–	0.41 (3.1)	–	0.10 (3.0)	–
R-9	0.70	0.74 (3.5)	105.7	0.74 (3.3)	105.7	0.77 (3.2)	110.0
Mean recovery (%)			145.3		155.8		101.5

^a Between parentheses the CV% corresponding to three replicates.

Table 6

Results obtained in the analysis of TC in real water samples

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
R-1	0.40	−0.03	−7.5	0.41 (3.2)	102.5	0.39 (3.1)	97.5
R-2	0.50	0.02	4.0	0.45 (3.5)	90.0	0.40 (3.1)	80.0
R-3	0.30	−0.16	−53.3	0.40 (3.4)	133.3	0.30 (3.0)	100.0
R-4	0.40	−0.01	−2.5	0.25 (2.9)	62.5	0.50 (3.1)	125.0
R-5	0.80	0.22	27.5	0.74 (3.5)	92.5	0.76 (3.0)	95.0
R-6	0.70	0.36	51.4	0.52 (3.0)	74.3	0.69 (3.2)	98.6
R-7	0.70	0.35	50.0	0.17 (3.0)	24.3	0.72 (3.4)	102.9
R-8	0.50	0.33	66.0	0.41 (3.1)	82.0	0.42 (3.3)	84.0
R-9	0.00	−0.10	−7.5	0.34 (3.1)	102.5	0.00	97.5
Mean recovery (%)			16.9		82.7		97.9

^a Between parentheses the CV% corresponding to three replicates.

In comparison with Fig. 1, the analyte spectra can be readily matched with those of standards. In the case of the interferent spectral profiles, they consist of principal components of the matrix of prediction residuals, and are thus linear combination

of real spectra. In this sense, they are not strictly comparable to those shown for a blank sample in Fig. 1. BLLS, nevertheless, is successful in obtaining the correct analyte profiles to be employed for concentration prediction in complex real samples.

Table 7

Results obtained in the analysis of CTC in real water samples

Sample	Actual concentration (mg L ⁻¹)	Method					
		NPLS		PARAFAC		BLLS	
		Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)	Predicted ^a (mg L ⁻¹)	Recovery (%)
R-1	0.50	0.49 (2.8)	98.0	0.55 (2.9)	110.0	0.48 (2.9)	96.0
R-2	0.60	0.54 (2.9)	90.0	0.63 (2.8)	105.0	0.63 (3.0)	105.0
R-3	0.40	0.44 (3.0)	110.0	0.40 (2.9)	100.0	0.44 (3.0)	110.0
R-4	0.30	0.27 (2.7)	90.0	0.36 (3.1)	120.0	0.38 (3.0)	126.7
R-5	0.70	0.67 (3.4)	95.7	0.69 (3.1)	98.6	0.55 (3.1)	78.6
R-6	0.50	0.47 (3.2)	94.0	0.46 (3.3)	92.0	0.53 (3.1)	106.0
R-7	0.00	0.05	–	0.01	–	0.05	–
R-8	0.70	0.60 (3.2)	85.7	0.60 (3.1)	85.7	0.60 (3.3)	85.7
R-9	0.50	0.45 (3.0)	90.0	0.55 (3.1)	110.0	0.55 (3.1)	110.0
Mean recovery (%)			94.2		102.7		102.2

^a Between parentheses the CV% corresponding to three replicates.

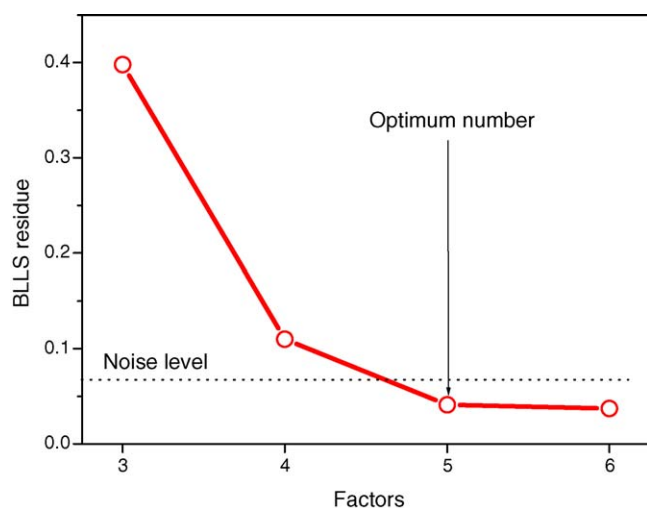
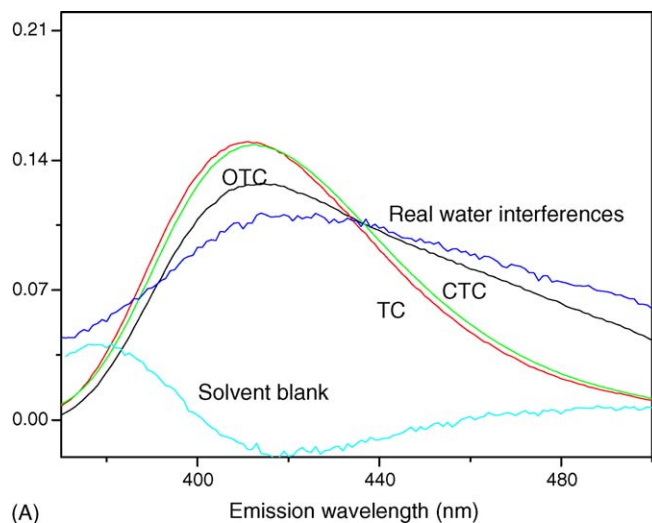
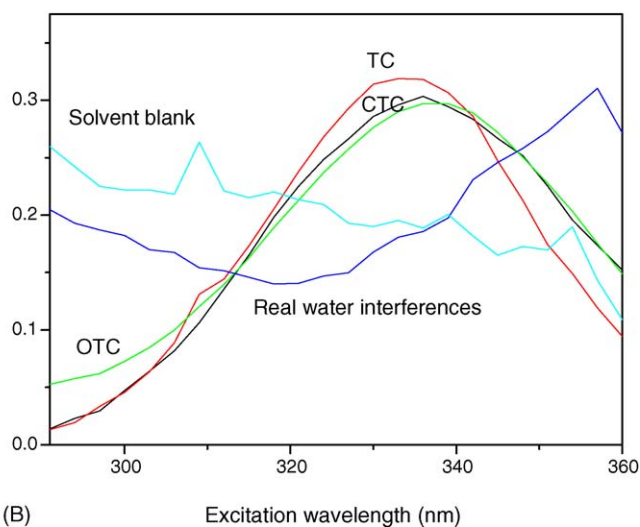


Fig. 2. Plot of the prediction residuals as a function of a trial number of factors for a test sets sample (sample R-1). The noise level in this system is ca. 0.07 FU (fluorescence intensity units).

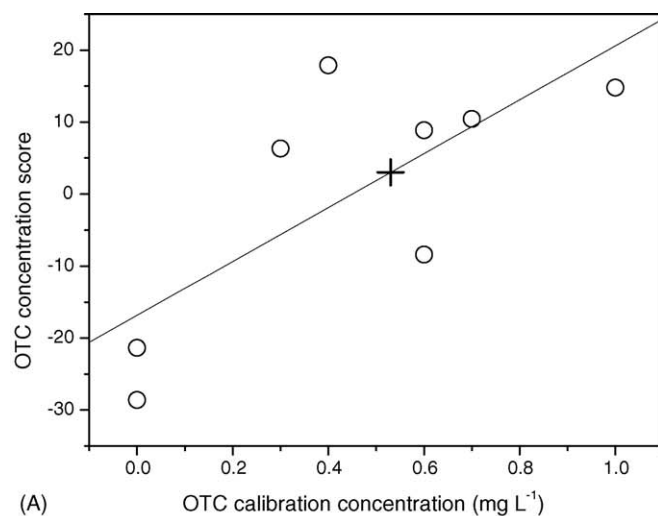


(A)

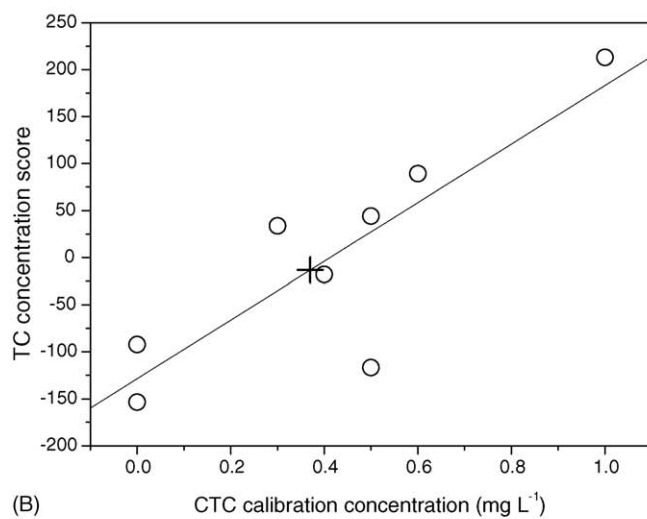


(B)

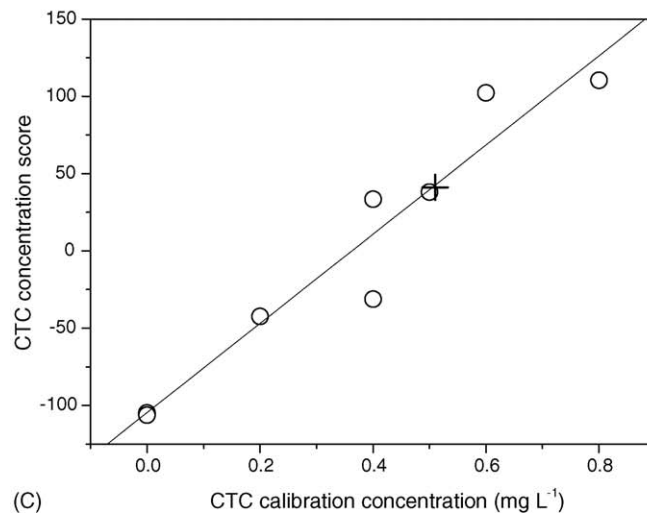
Fig. 3. Emission (A) and excitation (B) profiles for the five factors present in sample R-1, as retrieved by BLLS analysis. In both cases the profiles are normalized to unit length.



(A)



(B)



(C)

Fig. 4. Pseudo univariate calibration graphs corresponding to the PARAFAC models: (A) OTC model; (B) TC model; and (C) CTC model.

As regard the pseudo univariate calibration graphs corresponding to the PARAFAC models, they are displayed in Fig. 4A–C. Interestingly, better fitting is obtained for CTC than for the other two components. This fact agrees well with that observed when applying NPLS, and is probably due to the more significant spectral differences for this component, especially if the excitation mode is analyzed (see Fig. 1). Predictions corresponding to the validation set are displayed in Tables 2–4. These tables show a better performance for the latter mentioned compound agreeing well with the previously mentioned observations. In addition, predictions for the test set are showed in Tables 5–7. In this latter case, a lack of accuracy is observed for NPLS and PARAFAC, although results for CTC can be considered acceptable.

Based on the results presented in Tables 2–7, predicted versus actual concentration regression plots were built for all the three analytes together as recommended by Martínez et al. for multianalyte determination methods [43]. The estimated intercept and slope ($\hat{a}$ and $\hat{b}$, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test, in this case by using an ordinary least-squares fit-

ting (OLS) [44]. Fig. 5A shows the ellipses corresponding to the validation samples predictions (Tables 2–4), that are seen to contain the theoretical ($a=0, b=1$) point. This fact is indicative that constant and proportional bias is absent for all the chemometric methods, although better precision can be observed by the BLS predictions. On the other hand, when comparing the predictions obtained on the real spiked samples (i.e., the test set) (Tables 5–7), the better results furnished by BLS application are remarkable (see Fig. 5B). This fact is indicative of the ability of the recently presented multi-way model for taking into account not only unsuspected components but also high colinearities in the data. In addition, recoveries found in real water samples by applying the latter approach are in the same order that those reported by other authors [20,26].

5. Conclusions

A new sensitive method for the determination of three tetracyclines in water samples using photochemical-induced fluorescence excitation emission matrices modelled with three-way data arrays chemometric tools has been developed. The method involves irradiation of aqueous basic solution of antibiotics with UV light and fluorescence determination of the generated photoproducts. The main advantage of using derivatization with UV light is that naturally photodegraded tetracyclines in surface water are taken into account in the determination procedure.

The presently analyzed example challenges the most advanced second-order multivariate methodologies with data showing strong spectral colinearities. Bilinear least-squares emerges as a promising technique for processing these kind of data, yielding analyte profiles and concentrations in samples where a strong both overlapping and background occur.

Acknowledgements

The authors are grateful to Ministerio de Ciencia y Tecnología (Project BQU2003-07697) for financial support. H.C.G. thanks to CONICET and Universidad Nacional del Litoral.

References

- [1] N.M. Faber, R. Bro, P.K. Hopke, *Chemom. Intell. Lab. Syst.* 65 (2003) 119.
- [2] P.K. Hopke, *Anal. Chim. Acta* 500 (2003) 365.
- [3] K.S. Booksh, B.R. Kowalski, *Anal. Chem.* 66 (1994) 782A.
- [4] E. Sanchez, B.R. Kowalski, *Anal. Chem.* 58 (1986) 496.
- [5] R. Bro, *Chemom. Intell. Lab. Syst.* 38 (1997) 149.
- [6] C.M. Andersen, R. Bro, *J. Chemom.* 17 (2003) 200.
- [7] R. Tauler, *Chemom. Intell. Lab. Syst.* 30 (1995) 133.
- [8] M. Linder, R. Sundberg, *Chemom. Intell. Lab. Syst.* 42 (1998) 159.
- [9] M. Linder, R. Sundberg, *J. Chemom.* 16 (2002) 12.
- [10] R. Hirsch, T. Ternes, K. Haberer, K.-L. Kratz, *Sci. Total Environ.* 225 (1999) 109.
- [11] T.A. Ternes, *Water Res.* 32 (1998) 3245.
- [12] J. Tjørnelund, S.H. Hansen, *J. Chromatogr. A* 779 (1997) 235.
- [13] X. Liu, Y. Li, Y. Ci, *Anal. Chim. Acta* 345 (1997) 213.
- [14] J. Simal-Gándara, *Anal. Chim. Acta* 455 (2002) 143.
- [15] R.C. Rodríguez-Díaz, M.P. Aguilar-Caballero, A. Gómez-Hens, *Anal. Chim. Acta* 494 (2003) 55.
- [16] M.C. Mahedero, D. Bohoyo, F. Salinas, T. Ardila, D. Airado, B. Roldán, *J. Pharm. Biomed. Anal.* 37 (2005) 1101.

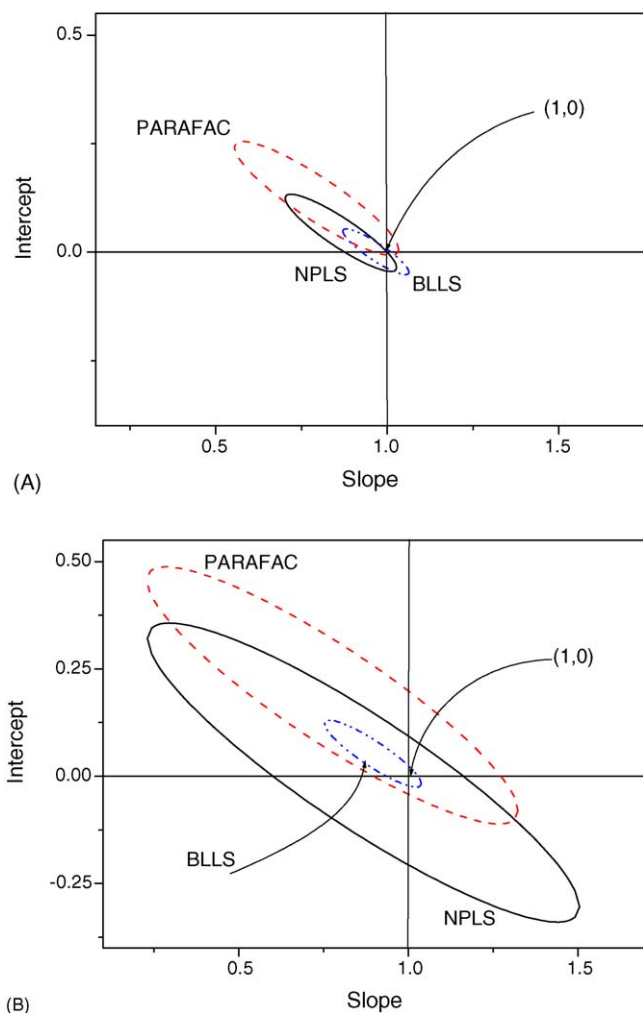


Fig. 5. Elliptical joint confidence regions (EJCR) for the multianalyte determination: (A) predictions on validation set and (B) predictions on test set.

- [17] D.S. Vienneau, C.G. Kindberg, *J. Pharm. Biomed. Anal.* 16 (1997) 111.
- [18] A. Pena, C. Carmona, A. Barbosa, C. Lino, I. Silveira, B. Castillo, *J. Pharm. Biomed. Anal.* 18 (1998) 839.
- [19] T. Charoenraks, S. Chuanuwatanakul, K. Honda, Y. Yamaguchi, O. Chailapakul, *Anal. Sci.* 21 (2005) 241.
- [20] L. Nozal, L. Arce, B.M. Simonet, A. Rios, M. Valcárcel, *Anal. Chim. Acta* 517 (2004) 89.
- [21] N. Furusawa, *Talanta* 59 (2003) 155.
- [22] M.A. Rudek, C.L. March, K.S. Bauer Jr., J.M. Pluda, W.D. Figg, *J. Pharm. Biomed. Anal.* 22 (2000) 1003.
- [23] A.L. Cinquina, F. Longo, G. Anastasi, L. Giannetti, R. Cozzani, *J. Chromatogr. A* 987 (2003) 227.
- [24] X. Zheng, Y. Hei, Z. Zhang, *Anal. Chim. Acta* 440 (2001) 143.
- [25] Y. Fuster Mester, L. Lahuerta Zamora, J. Martínez Calatayud, *Luminescence* 16 (2001) 213.
- [26] S. Reverté, F. Borrell, E. Pocurull, R.M. Marcé, *J. Chromatogr. A* 1010 (2003) 225.
- [27] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K.-L. Kratz, *J. Chromatogr. A* 815 (1998) 213.
- [28] M. Cherlet, M. Schelkens, S. Croubels, P. De Backer, *Anal. Chim. Acta* 492 (2003) 199.
- [29] A.M. Jacobsen, B. Halling-Sørensen, F. Ingerslev, S.H. Hansen, *J. Chromatogr. A* 1038 (2004) 157.
- [30] S. Yang, J. Cha, K. Carlson, *J. Chromatogr. A* 1097 (2005) 40.
- [31] R. Lindberg, P.-A. Jarnheimer, B. Olsen, M. Johanson, M. Tysklind, *Chemosphere* 57 (2004) 1479.
- [32] R. Bro, H.A.L. Kiers, *J. Chemom.* 17 (2003) 274.
- [33] A. Muñoz de la Peña, A. Espinosa Mansilla, D. González Gómez, A.C. Olivieri, H.C. Goicoechea, *Anal. Chem.* 75 (2003) 2640.
- [34] R. Bro, *J. Chemom.* 10 (1996) 47.
- [35] N.M. Faber, J. Ferré, R. Boqué, J.H. Kalivas, *Chemom. Intell. Lab. Syst.* 63 (2002) 107.
- [36] P.C. Damiani, A.J. Nepote, M. Bearzotti, A.O. Olivieri, *Anal. Chem.* 76 (2004) 2798.
- [37] MATLAB 6.3, The Math Works Inc., Natick, Massachusetts, USA, 2000.
- [38] <http://www.models.kvl.dk/source/>.
- [39] H.C. Goicoechea, A.C. Olivieri, *Appl. Spectrosc.* 59 (2005) 67.
- [40] N.M. Faber, *Chemom. Intell. Lab. Syst.* 50 (2000) 107.
- [41] R. Santiago Valverde, M.D. Gil García, M. Martínez Galera, H.C. Goicoechea, *Anal. Chim. Acta*, in press.
- [42] S.M. Croubels, K.E.I. Vanoosthuyze, C.H. Van Peterghem, *J. Chromatogr. B* 690 (1997) 173.
- [43] A. Martínez, J. Riu, O. Busto, J. Guasch, F.X. Rius, *Anal. Chim. Acta* 406 (2000) 257.
- [44] A.G. González, M.A. Herrador, A.G. Asuero, *Talanta* 48 (1999) 729.

Attenuated total reflectance infrared determination of sodium nitrilotriacetate in alkaline liquid detergents

Josep F. Ventura-Gayete, Miguel de la Guardia, Salvador Garrigues*

Department of Analytical Chemistry, Universitat de València, Edifici Jeroni Muñoz, 50 Doctor Moliner St., 46100 Burjassot, València, Spain

Received 22 September 2005; received in revised form 20 January 2006; accepted 7 February 2006

Available online 23 March 2006

Abstract

An attenuated total reflectance infrared (ATR–FTIR) method has been developed for the direct determination of the chelating agent sodium nitrilotriacetate (NTA) in high pH cleaning formulations. Aqueous samples and standards were placed directly on the diamond ATR cell without any pre-treatment and FTIR spectra were recorded between 4000 and 600 cm^{-1} . Measurement conditions were evaluated. Results obtained for five different commercial formulations containing from 6.22 to 9.25% (w/w) of NTA were in good agreement with the manufacturer's declared content (differences between 3.2 and -3.7%). Recovery studies evidenced the accuracy of the developed method, having found values between 98.4 and 102.4%. The developed procedure provides an excellent throughput of 50 independent determinations per hour, and each measurement required only less than 0.2 ml of sample or standard.

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Keywords: Diamond ATR–FTIR; Detergents; Sodium nitrilotriacetate; Quantitative analysis

1. Introduction

Quality control of industrial products requires faster, simpler and cheaper methods day by day. Frequently, the objective of the analysis is the verification of a correct manufacturing process, concerning the proportions of the chemicals which constitute the product or information about its homogeneity. So, it is necessary to develop robust analytical techniques applicable for a big number of samples of different types, from aqueous to organic nature and working from the mildest conditions to the most corrosive ones.

Detergents commercialized for hand use are normally neutral or slightly acid in order to protect human skin. However, a lot of household and industrial cleaning formulations have a high pH, being highly corrosive for skin and for laboratory instrumentation [1].

Classical attenuated total reflectance (ATR) cells coupled with Fourier transform infrared (FTIR) spectrometers allow to obtain the infrared spectra of aqueous samples. However, these cells usually employ ZnSe, ZnS or KRS-5 crystals, which were

attacked by strong acid, alkaline solutions or oxidizing agents. Ge is also a typical material for ATR crystals more resistant than those previously indicated but it has high reflection losses due its refractive index. Diamond ATR cells have a thin polished diamond mounted on the surface of a ZnSe crystal (or other similar materials), protecting it from scratching and from corrosion by chemicals. So, with this material it is possible to obtain ATR–FTIR spectra of strong acid, alkaline or oxidizing solutions or abrasive liquids without damaging the ATR cell [2]. In recent years, ATR–FTIR applications with diamond cells have been expanded, with applications such as the on-line determination of glucose and poly (beta-hydroxybutyric acid) in *Escherichia coli* [3], pesticide screening in aqueous samples [4], bacteria spectroscopic discrimination [5], determination of alkaloids in poppy products [6], glucose determination in dried-down nanolitre samples [7], study of metabolic intermediates [8], and the determination of carbohydrates, alcohols and organic acids in wine, coupled with high performance liquid chromatography (HPLC) [9], but none of these applications involves the use of aggressive media and thus the main reason for the use of diamond ATR is absent.

Sodium nitrilotriacetate (NTA) is a chelating agent widely used in detergent formulations for avoiding the precipitation of salts during the washing process [10]. It is highly nocive

* Corresponding author. Tel.: +34 96 354 31 58; fax: +34 96 354 48 38.
E-mail address: salvador.garrigues@uv.es (S. Garrigues).

for the aquatic environment and it has been suggested as a possible carcinogen in mammals [11]. Methods for its determination as a total chelons concentration include polarography [12] or capillary electrophoresis [13]. For its direct determination differential-pulse anodic stripping voltammetry [14,15] and HPLC [16] have been proposed. All that methods are time-consuming and laborious, difficult to be coupled on-line and not very appropriate for quality control purposes when a rapid response is required.

The objective of this work is to develop a simple and fast ATR–FTIR method for the direct quantification of NTA in high pH commercial liquid detergents, showing the usefulness of ATR–FTIR measurements in the field of quality control and to evaluate the usefulness of its hypothetic automation in a flow procedure using diamond probe fibers.

2. Experimental

2.1. Apparatus

A Bruker (Bremen, Germany) FT-IR spectrometer, model Tensor 27, equipped with a MIR source, a Ge/KBr beamsplitter and a temperature-stabilized lanthanum doped deuterated triglycine sulfate (DLATGS) detector was employed for obtaining FTIR spectra of aqueous standards and samples between 4000 and 600 cm^{-1} , using a three reflection DuraSamplIR™ Diamond ATR system from Smiths Detection (Warrington, UK).

The OPUS (Version 4.2) software from Bruker was used for controlling spectrometer and for the acquisition of data.

Omnic (Version 6.1) software, used for processing individual spectra, and TQ Analyst software, used for carrying out validation studies, are both from Nicolet (Madison, WI, USA).

A vortex mixer from Velp Scientifica (Milano, Italy) was used for homogenization of samples and standards.

2.2. Reagents and samples

NTA technical grade (92%) was supplied from BASF (Ludwigshafen, Germany) and aqueous standards from 2.56 to 19.60% (w/v) were prepared by dilution with nanopure water, obtained from a Milli-Q water purifier system (Millipore, Bedford, MA).

Samples of five different commercial liquid detergent formulations were obtained from Sanmarino Consorcio (Quart, Valencia, Spain). Table 1 summarizes the main ingredients and pH of those formulations. A series of laboratory samples were prepared at reduced scale, in the same way than commercial ones to be used as reference samples.

2.3. Proposed ATR–FTIR procedure

A drop of sample or standard was placed onto the surface of the diamond-ATR cell of the FTIR spectrometer. ATR–FTIR spectra were recorded between 4000 and 600 cm^{-1} with a nominal resolution of 8 cm^{-1} and accumulating 50 scans per spectrum. In these conditions an acquisition time of 23.5 s was required for each measurement. The surface of the diamond cell

Table 1

Chemical composition of the commercial liquid detergent formulations analysed in this work

Formulation	pH	Major components
F1	11.4	NTA, methyl ammonium alkyl sulfate, tri phosphate
F2	11.8	NTA, alpha-olefin sulfonate, tri phosphate, hydroxide
F3	11.5	NTA, tri phosphate, tripolyphosphate
F4	11.6	NTA, alpha-olefin sulfonate, alkyl sulfonate, polyglycoether, metasilicate
F5	11.0	NTA, polyglycoether, alpha-olefin sulfonate, tri phosphate

Ingredients are listed by relative percentage (w/w) respect to the corresponding technical raw material. The main ingredient for all formulations was distilled water.

was cleaned with water and dried with soft paper between measurement of different samples or standards. The measurement of the water reference spectrum was acquired in the same conditions.

As measurement criterion it was selected the peak area values between 1604.5 and 1546.6 cm^{-1} , corrected with a two-point baseline established between 1743.3 and 1365.4 cm^{-1} .

NTA concentration was obtained by interpolating peak area values of samples in a calibration line established from six standard aqueous solutions containing between 0.00 and 19.60% (w/v) and measured in the same experimental conditions.

3. Results and discussion

3.1. ATR–FTIR spectra of NTA and samples

Fig. 1 shows the ATR–FTIR spectra of NTA standards ranging from 2.56 to 19.60% (w/v) in front of the water blank. As can be seen, the main NTA bands appeared in the fingerprint region. The strongest bands of the NTA at 1576 and at 1405.1 cm^{-1} correspond to the asymmetric and symmetric stretching of the carboxylate group, respectively. A series of small bands appeared at 1332.2 cm^{-1} , 1265 cm^{-1} (C–N stretching) and 1143.9 cm^{-1} (C–O stretching) [17]. High background values are evident at 2000 cm^{-1} , due to the diamond absorption. However, in the fin-

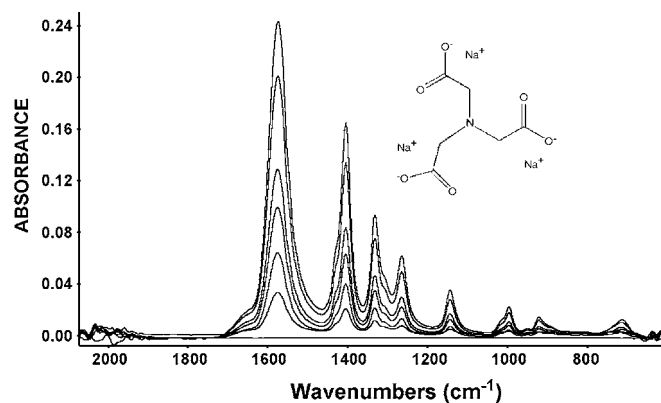


Fig. 1. ATR–FTIR spectra of NTA standards in water, ranging from 2.57 to 19.60% (w/v). Measurement conditions: 8 cm^{-1} of nominal resolution, 50 accumulated scans per spectrum, water background spectrum was used.

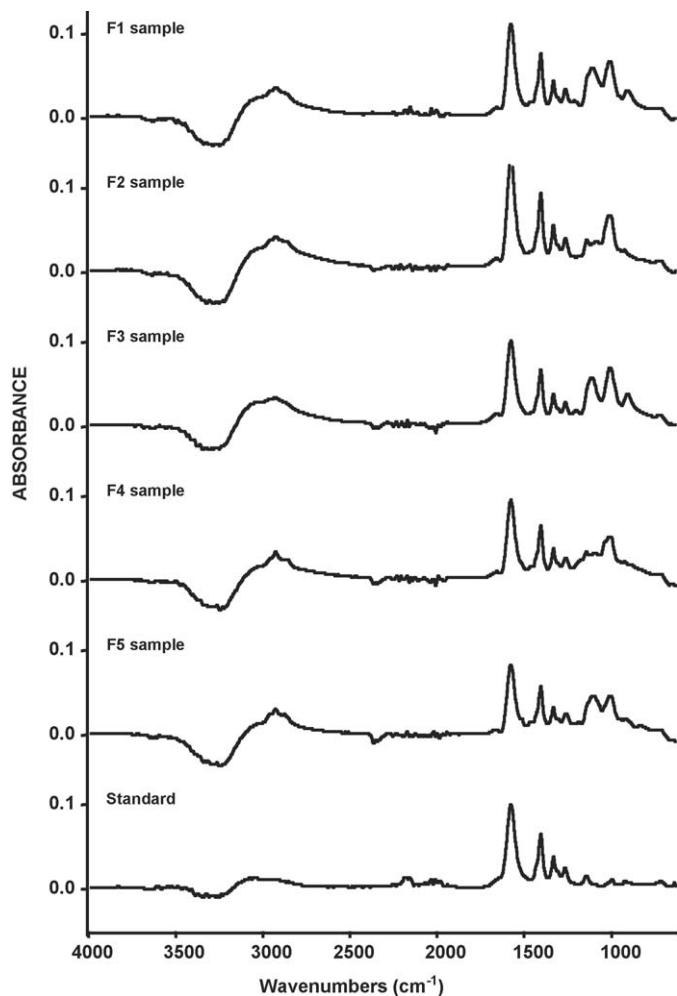


Fig. 2. ATR-FTIR spectra of a 7.81% (w/v) NTA standard and different commercial liquid detergent formulations. Measurement conditions: 8 cm⁻¹ of nominal resolution, 50 accumulated scans per spectrum, background from water.

gerprint region, the water baseline is stable, allowing to use this zone for NTA determination.

Fig. 2 shows the spectra of the five commercial formulations investigated in this work compared with a 7.81% (w/v) NTA standard. All formulations have clear differences with NTA spectrum, except for the most intense bands at 1576 and 1405.1 cm⁻¹, which seems not be affected by other ingredients present in the products. So, it was reasonable the use of these two bands for the quantification of NTA in liquid detergent formulations. The ATR-FTIR absorbance spectra of the principal ingredients of each formulation were recorded (spectra not shown) for evaluating the possible spectral interferences in the NTA determination. Finally, the two carboxylate stretching bands were selected for carrying out future studies due to the absence of significant band overlapping.

3.2. Study and selection of the experimental measurement conditions

The effect of both, the number of scans and the nominal resolution, on the absorbance values of samples and standards were

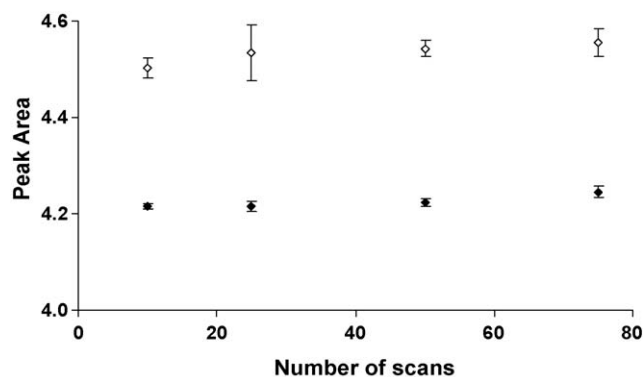


Fig. 3. Effect of the number of accumulated scans per spectrum on the absorbance peak area between 1604.5 and 1546.6 cm⁻¹ (corrected with a baseline established between 1743.3 and 1365.4 cm⁻¹), for a 7.81% (w/v) NTA standard (●) and for the F1 reference sample (○). Results are expressed as mean value $\pm$ standard deviation for three independent replicates. Nominal resolution was 8 cm⁻¹.

studied in terms of sensitivity and precision. Fig. 3 shows the effect of accumulated scans on peak area values obtained for sample F1 and a 7.81% (w/v) NTA standard for a fixed nominal resolution of 8 cm⁻¹. As can be seen, the mean absorbance values were not affected by the number of accumulated scans but this parameter affects the repeatability, especially for sample measurement. A value of 50 scans was selected, that provide a good signal-to-noise ratio, a precision better than 0.4% and an acquisition time of 23.5 s.

The nominal resolution had a clear and critical influence on the peak average area and precision, as can be seen in Fig. 4, specially for low resolution values. The use of a spectral resolution of 2 cm⁻¹ provides the highest peak area signal but the spectral noise found in these conditions provides a loss of spectral repeatability. Absolute signal values obtained for 8 and 16 cm⁻¹ are similar but the use of the last one had a lack of spectral information. Sampling throughput also is affected by the nominal resolution and, in this sense, as a compromise between sensitivity, precision and measurement frequency a nominal resolution of 8 cm⁻¹ was selected for subsequent studies.

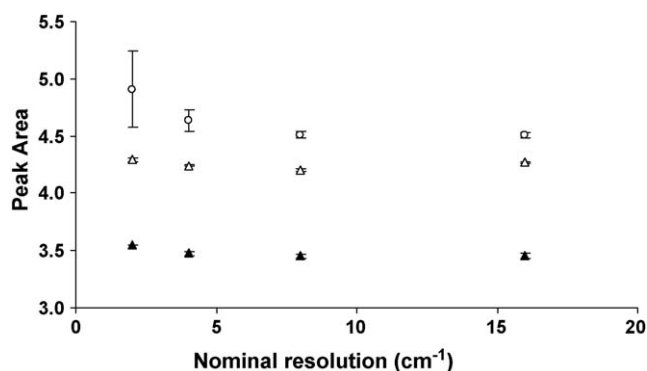


Fig. 4. Effect of the nominal resolution on the peak area, for a 7.81% (w/v) NTA standard (Δ), reference sample F5 (▲) and for sample F1 (○). Results are expressed as mean value $\pm$ standard deviation obtained from three independent replicates. Number of scans 50 and peak area values calculated as indicated in Fig. 3.

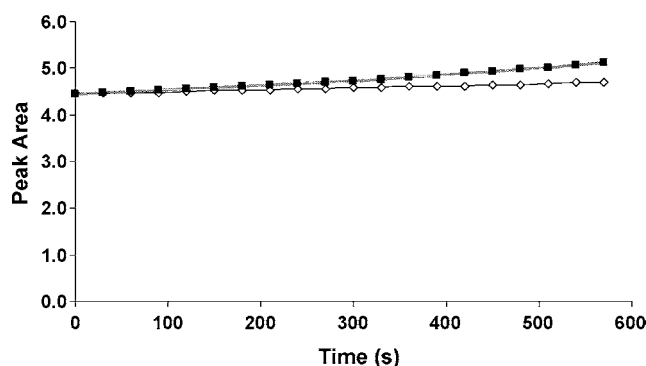


Fig. 5. Effect of the residence time of the liquid sample in the diamond ATR cell on the peak area values, for sample F1, using metallic ring ($\diamond$) and without it ($\blacksquare$). Each measurement was obtained accumulating 25 scans with a nominal resolution of 8 cm^{-1} . Peak area measurement conditions as indicated in Fig. 3.

One of the most important limitations in ATR–FTIR spectrometry is the difficulty of cleaning the ZnSe crystal surface, for ensuring the absence of cross contamination. Diamond-ATR cells allow us to make easier this process. In this sense, scratching of cell surface is avoided due to the diamond hardness.

On the other hand, as the cell surface is plain and the liquid to analyse has a reduced surface tension, it spreads over it, becoming a liquid microfilm, with the subsequent increased evaporation of solvent and changes in the analyte concentration, which is the case of working with liquid detergents usually. Diamond-ATR accessories include a metallic ring to minimize this effect. However, its use complicates the cleaning process. So, it was studied the variations of the peak area as a function of the sample residence time, comparing both approaches (with ring and without ring) for standards and samples. Fig. 5 shows the results of this study. The increase of absorbance, due to the loss of water from the cell surface, is small when using the metallic ring, whereas the increase is notable if the ring is not used due to the evaporation of the solvent. For a measuring time smaller than 100 s, there are no significant differences between both approaches. So, as 50 scans were accumulated in less than 50 s for a nominal resolution of 8 cm^{-1} , it was decided not to use the ring in order to reduce sampling time and facilitate the cleaning step between measurements.

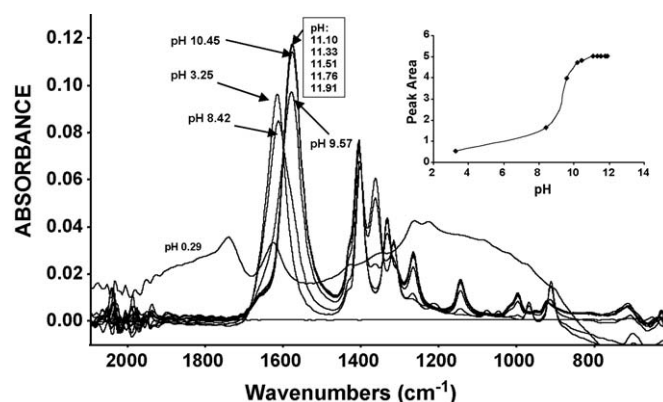


Fig. 6. Effect of pH on peak area values for a 10% (w/v) NTA standard. Each measurement was obtained accumulating 50 scans with a nominal resolution of 8 cm^{-1} . (Inset) Plot of the peak area values between 1604.5 and 1546.6 cm^{-1} (corrected with a baseline established between 1743.3 and 1365.4 cm^{-1}) vs. pH.

3.3. Selection of measurement criteria

Different measurement criteria (peak area, peak height and baseline correction) were evaluated for the direct determination of NTA in detergent formulations using an external calibration. Table 2 shows the main analytical features obtained for different measurement criteria. As can be seen, it has been obtained an excellent correlation for all NTA calibrations ($R^2 \geq 0.9997$), using both, peak height or peak area, corrected with different baselines. The best sensitivity was obtained for the peak area between 1643.3 and 1491.3 cm^{-1} corrected with a baseline established between 1805.3 and 1365.6 cm^{-1} . In all cases the repeatability was better than 0.5% and limits of detection between 0.015 and 0.037% (w/v) were obtained, which are adequate for the determination of NTA in this type of samples. However, it was necessary to carry out validation studies with reference samples, in order to select the adequate criterion which avoids interferences from the presence of other ingredients in the liquid detergent formulations.

It was selected the peak area between 1604.5 and 1546.6 cm^{-1} , corrected with a baseline established between 1743.3 and 1365.3 cm^{-1} , as the measurement condition that provides the lowest errors in the analysis of reference samples.

On the other hand, it must be noticed that the protonation of the carboxylate groups present in NTA has a dramatic effect on the peak area, as can be seen in Fig. 6. It is due to the shift

Table 2

Analytical features of the ATR–FTIR determination of NTA using different bands and measurement modes

Measurement mode	Wavenumber (cm^{-1})	Baseline correction	Calibration curve $y = a + bC_{\text{NTA}}\%$ (w/v)			R.S.D. ^a (%)	LOD ^b % (w/v)
			$a \pm s_a$	$b \pm s_b$	R^2 ($n = 25$)		
Height	1406	1743.3–1365.6	-0.0008 ± 0.0002	0.00721 ± 0.00002	0.9997	0.42	0.020
Height	1574	1743.3–1365.6	0.0010 ± 0.0004	0.01171 ± 0.00004	0.9998	0.32	0.017
Area	1604.5–1546.6	1743.3–1465.8	0.044 ± 0.017	0.5186 ± 0.0017	0.9997	0.45	0.032
Area	1438.8–1377.1	1743.3–1365.4	-0.021 ± 0.006	0.2278 ± 0.0006	0.9998	0.24	0.023
Area	1643.3–1491.3	1805.3–1365.6	0.06 ± 0.03	0.713 ± 0.003	0.9997	0.26	0.037
Area	1604.5–1546.6	1743.3–1365.3	0.044 ± 0.017	0.5250 ± 0.0017	0.9997	0.33	0.015

^a Relative standard deviation for a standard solution containing 7.81% (w/v) ($n = 3$).

^b Limit of detection established from the standard deviation of 10 blank measurements ($k = 3$, a probability level of 99.6%).

of carboxylate bands and that of baseline points. However, in the 11.0–12.0 pH value, that corresponding with the studied samples, the peak area value is constant. So it is unnecessary to do any sample treatment nor to match the pH of standards which is approximately 11.1 for the concentration range assayed.

3.4. Analysis of samples by external calibration and recovery studies

Table 3 summarizes the results obtained for the determination of NTA in commercial liquid detergent formulations, also including reference samples prepared at laboratory scale, to verify the absence of errors. Accuracy error was under 1% for the formulations F1, F2 and F4 as compared with the manufacturer data. For formulation F3 it was found a 1.84% error. However, the determination of NTA in formulation F5 had a relative error of –3.6%. As quality control required a 5% tolerance for this product, it was concluded that the developed method was suitable for our purpose. Moreover, it is observed that there was a small variation coefficient (CV) between samples of the same formulation.

The precision obtained, expressed as the variation coefficient was between 0.5 and 2%.

The accuracy of the ATR–FTIR determination of NTA was also evaluated through recovery studies. Table 4 summarizes the results obtained for all reference samples spiked at 70–200 mg level. For this study, in order to avoid the formation of instable

Table 4

Recovery studies for samples diluted and spiked with different amounts of NTA

Sample	NTA mass added (mg)	Individual recovery (% $\pm$ s)	Mean recovery (% $\pm$ s)
F1 laboratory	72.4	101.8 $\pm$ 0.1	101.8 $\pm$ 0.6
	136.5	102.4 $\pm$ 0.6	
	183.4	101.2 $\pm$ 0.2	
F2 laboratory	114.3	100.2 $\pm$ 0.2	99.7 $\pm$ 0.4
	169.2	99.5 $\pm$ 0.2	
	223.1	99.5 $\pm$ 0.3	
F3 laboratory	89.1	101.1 $\pm$ 0.4	101.4 $\pm$ 0.7
	125.7	102.2 $\pm$ 0.1	
	173.6	101.0 $\pm$ 0.4	
F4 laboratory	87.4	102.04 $\pm$ 0.06	102.0 $\pm$ 1.0
	123.1	102.03 $\pm$ 0.08	
	157.7	100.4 $\pm$ 0.2	
F5 laboratory	71.2	101.8 $\pm$ 0.3	99.7 $\pm$ 1.8
	120.6	98.8 $\pm$ 0.2	
	162.9	98.4 $\pm$ 0.3	

Results are expressed as mean value $\pm$ standard deviation for three independent replicates. Measurement conditions: as indicated in Table 3.

emulsions or the precipitation of some ingredients, samples were diluted before the addition of NTA. As can be seen, all recoveries were near to 100%, showing the accuracy of the ATR–FTIR developed method.

In addition, there were prepared some different spiked samples from commercial formulations (F1, F2) by adding very small amounts of analyte being obtained recoveries around 100% (results not shown).

3.5. Analysis of samples by standard addition

The standard addition method was tested in order to investigate possible matrix effects, specially for formulation F5 which has a Youden blank clearly different to 0. In this study, we added different quantities of NTA to a previously diluted aliquot of sample and recorded the FTIR spectra in the selected conditions.

From peak area values, we established a relation between the corrected signal and the added NTA, taking into consideration the density of sample and standard added. For sample F5 the equation obtained for the standard addition was corrected signal = $(0.0339 \pm 0.0005) + (0.511 \pm 0.005)C$, being C the NTA added, with an $R^2 = 0.9989$. The content obtained by this standard addition approach was $6.34 \pm 0.14\%$ (w/w) (reference value of 6.22 (w/w)), being the relative error of 1.9% instead –3.6% obtained for external calibration.

In conclusion, for the analysis of sample F5 could be recommended the use of the standard addition method instead the external calibration in order to avoid the matrix effects and to obtain accurate results.

4. Conclusion

The developed method, based on the direct measurement of alkaline samples by using diamond ATR, provides accurate

Table 3

Analysis of commercial liquid detergent formulations by ATR–FTIR using external calibration

Sample ^a	% NTA (w/w) ^b		Error (%)
	Reference value	ATR–FTIR	
F1 batch 1	7.95	7.90 $\pm$ 0.03	–0.7
F1 batch 2	7.95	8.12 $\pm$ 0.01	2.2
F1 laboratory	7.95	7.95 $\pm$ 0.03	0.0
F2 batch 1	9.25	9.37 $\pm$ 0.03	1.3
F2 batch 2	9.25	9.38 $\pm$ 0.05	1.4
F2 batch 3	9.25	9.45 $\pm$ 0.03	2.1
F2 laboratory	9.25	9.33 $\pm$ 0.01	0.9
F3 batch 1	7.07	7.24 $\pm$ 0.03	2.4
F3 batch 2	7.07	7.30 $\pm$ 0.02	3.2
F3 laboratory	7.07	7.20 $\pm$ 0.05	1.8
F4 batch 1	6.80	6.99 $\pm$ 0.08	2.8
F4 batch 2	6.80	6.80 $\pm$ 0.004	0.0
F4 laboratory	6.80	6.87 $\pm$ 0.02	1.0
F5 batch 1	6.22	6.09 $\pm$ 0.02	–2.1
F5 batch 2	6.22	5.99 $\pm$ 0.02	–3.7
F5 batch 3	6.22	6.08 $\pm$ 0.03	–2.1
F5 laboratory	6.22	5.99 $\pm$ 0.01	–3.6

Measurement conditions: peak area between 1604.5 and 1546.6 cm^{-1} (baseline 1743.3–1365.3 cm^{-1}).

^a Reference samples were prepared at laboratory scale, in front of commercial samples which were prepared at industrial scale.

^b Percentage in (w/w) was obtained from the concentration in terms of (w/v) obtained by interpolating sample peak area in the calibration line also considering the sample density. Results are expressed as mean value $\pm$ standard deviation of three independent replicates.

and precise results in the analysis of commercial cleaning formulations using an external calibration and has an excellent throughput of 50 replicates h^{-1} , with a sample consumption of less than 0.2 mL per three independent replicates. These features are in good agreement with the quality control analysis exigencies, being remarkably the low LOD achieved of 0.015% (w/w), adequate for its use in the analysis of cleaning formulations. Automation of the developed method would be possible through the use of diamond-ATR probes, inserted directly in the product.

Acknowledgements

The authors acknowledge the financial support of the Direcció General d'Universitats i Investigació de la Generalitat Valenciana (Project GV04B/247 and Grupos 03-118) and Universitat of Valencia (Project UV-AE-20050203).

References

- [1] U. Zoller, G. Broze (Eds.), *Handbook of Detergents: Properties*, Marcel Dekker, Inc., New York, 1999.
- [2] J. Coates, J. Sanders, *Spectrosc. Eu.* 12 (2000) 12.
- [3] G. Jarute, A. Kainz, G. Schroll, J.R. Baena, B. Lendl, *Anal. Chem.* 76 (2004) 6353.
- [4] A. Colume, J. Diewok, B. Lendl, *Int. J. Environ. Anal. Chem.* 84 (2004) 835.
- [5] C.L. Winder, R. Goodacre, *Analyst* 129 (2004) 1118.
- [6] H. Schulz, M. Baranska, R. Quilitzsch, W. Schuetze, *Analyst* 129 (2004) 917.
- [7] E. Diessel, S. Willmann, P. Kamphaus, R. Kurte, U. Damm, H.M. Heise, *Appl. Spectrosc.* 58 (2004) 442.
- [8] K. Nakanishi, A. Hashimoto, T. Pan, M. Kanou, T. Kameoka, *Appl. Spectrosc.* 57 (2003) 1510.
- [9] A. Edelmann, J. Diewok, J. Rodríguez-Baena, B. Lendl, *Anal. Bioanal. Chem.* 376 (2003) 92.
- [10] T.P. Knepper, *Trends Anal. Chem.* 22 (2003) 708.
- [11] <http://www.cdc.gov/niosh/ipcsneng/neng1239.html>.
- [12] S. Esser, B.W. Wenclawiak, H. Gabelmann, *Fresen. J. Anal. Chem.* 368 (2000) 250.
- [13] G. Owens, V.K. Ferguson, M.J. McLaughlin, I. Singleton, R.J. Reid, F.A. Smith, *Environ. Sci. Technol.* 34 (2000) 885.
- [14] A. Voulgaropoulos, N. Tzivanakis, *Electroanalysis* 4 (1992) 647.
- [15] P. Schoen, K.H. Bauer, V. Wiskamp, *Fresen. J. Anal. Chem.* 358 (1997) 699.
- [16] R. Geshke, M. Zehringer, *Fresen. J. Anal. Chem.* 357 (1997) 773.
- [17] D. Lin-Vien, N.B. Colthup, W.G. Fateley, J.G. Grasselli, *Infrared and Raman Characteristic Frequencies of Organic Molecules*, Academic Press, London, 1991.

Simple procedure for the determination of Cd, Pb, As and Se in biological samples by electrothermal atomic absorption spectrometry using colloidal Pd modifier

Anu Viitak^a, Anatoly B. Volynsky^{b,*}

^a Institute of Chemistry, Tallinn Technical University, Ehitajate Tee 5, 19086 Tallinn, Estonia

^b Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, Kosygin St. 19, 119991 Moscow, Russia

Received 3 November 2005; received in revised form 18 January 2006; accepted 7 February 2006

Available online 24 March 2006

Abstract

Electrothermal atomic absorption spectrometry was used for the determination of Cd, Pb, As and Se in the whole blood, serum, hair reference standard materials and the samples of algae collected at the coastal Estonian regions of Baltic sea. Instead of tedious and time-consuming experimental comparison of various chemical modifiers, theoretical consideration of the problem was applied for choosing the most prospective one (colloidal Pd) for solution of the given task. The experimental data obtained proved correctness of the choice. Pure standard solutions in diluted nitric acid were used for construction of the calibration graphs. The same experimental conditions were applied for each analyte for calibration solutions and all samples studied. In spite of very limited optimization procedures used, all the values obtained agree well with the corresponding reference values. Accuracy of the analysis of the algae samples was checked by recoveries of the spikes that were in the region 91–109%. Detection limits reached are 0.021, 1.2, 0.62 and 1.1 ng ml⁻¹ for Cd, Pb, As and Se, respectively, in digests of biological samples.

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Keywords: Electrothermal atomic absorption spectrometry; Whole blood; Serum; Hair; Algae; Cadmium; Lead; Arsenic; Selenium; Colloidal palladium

1. Introduction

Atomic absorption spectrometry (AAS) is relatively simple, fast and powerful method of analytical chemistry [1]. That is why it has been widespread and developed very intensively. Good understanding of the phenomena dealing with the method resulted in disappearance of the articles devoted to fundamentals of flame AAS about two decades ago. Although some important topics in electrothermal AAS (ETAAS) are still unclear (such as mechanisms of the action of some chemical modifiers [2,3]), the number of the articles devoted to development of the method permanently decreases.

The fortunes of these two most widespread “branches” of AAS are not the same. Flame AAS is already for decades “a working horse” of most analytical laboratories. Possibilities and limitations of the method are very well known. Optimization of the method is practically limited to choosing the

flame type (C₂H₂–air or C₂H₂–N₂O) and addition of the modifier (usually La salts), if necessary. It is hardly surprising that flame AAS may be effectively used by a non-experienced technician.

As for ETAAS, the number of variables determining its efficiency became significantly less after wide introduction of the STPF-concept [4] to the analytical practice. However, the number of these parameters is still much higher compared to flame AAS. First of all, this is a time–temperature program of electrothermal atomizer–ETA (with temperatures, heating rates and hold times that may be critical at least for pyrolysis and atomization stages) and a chemical modifier. In addition to this, even today some analysts do not apply completely the potential of the STPF-concept and waste their time for comparison of peak height and peak area data or do not use a graphite platform. That is why sometimes analysis of even relatively simple samples by ETAAS becomes a problem.

This work is devoted to simple optimization, interference-free analysis of various biological samples for some toxic metals (Pb, Cd) and semi-metals (As, Se) by ETAAS using colloidal Pd modifier.

* Corresponding author. Tel.: +7 495 939 7035; fax: +7 495 938 2054.
E-mail address: ras@a-volynsky.msk.ru (A.B. Volynsky).

2. Experimental

2.1. Instrumentation

A SpectraAA 220Z (Varian, Mulgrave, Australia) atomic absorption spectrometer equipped with end-heated GTA-110Z graphite atomizer, Zeeman-effect background correction and integrated autosampler was used. Graphite tubes with a pyrolytic graphite coating and platforms made of a pyrolytic graphite were used throughout the work. Argon of 99.998% purity (AGA, Helsinki, Finland) was used as the purge gas.

A microwave oven Anton Paar Multiwave 3000 (Graz, Austria) with Teflon vessels was used for the microwave-assisted acid digestion.

2.2. Reagents and samples

Nitric acid 66% “suprapur” grade (Merck, Darmstadt, Germany), hydrochloric acid 36–38% “for trace metal analysis” (Baker, Phillipsburg, USA) and hydrogen peroxide 35% “puriss” (Riedel-de Haën, Seelze, Germany) were used. Polyvinylpyrrolidone (PVP) K-90 ($M_r \approx 360\,000$) “for molecular biology” and palladium(II) chloride anhydrous were purchased from Fluka (Buchs, Switzerland). Methyl carbinol 96.8° (Moe joogid, Tallinn, Estonia) and high-purity de-ionized water obtained by a MilliQ water purification system (Millipore, Bedford, USA) were used.

The stock atomic spectroscopy standard solutions (1000 mg l^{-1}) for Pb, Cd, As and Se (Fluka) and Multielement Quality Control Standard 26 (High-Purity Standards, Charleston, USA) were gradually diluted with 4% HNO_3 before use.

SeronormTM Trace Elements Whole Blood Level 2 (MR9067), Whole Blood Level 3 (OK0337), Serum Level 1 (MI0181) from Sero AS (Billingstad, Norway) and Hair NCS DC73347 (China National Analysis Center for Iron and Steel, Beijing, China) standard reference materials were used.

The red algae *Fucus vesiculosus* and the green algae *Furcellaria lumbricalis* were collected on the coastal regions of Estonia. The fresh samples were washed by MilliQ water and dried firstly at room temperature and then at 105°C till the constant weight. After drying the samples of algae were homogenized using agate mortar.

2.3. Procedure

Two millilitres of blood, serum or approximately 0.1–0.2 g hair or algae samples were mineralized by 3 ml of concentrated HNO_3 and 1 ml of concentrated H_2O_2 in Teflon bombs in microwave oven at temperatures up to 180°C for 30 min. After cooling down the bombs the solutions were transferred to the volumetric flasks (25 ml) with MilliQ water. Per sample, two weights ($N=2$) and two blanks were performed at least.

Colloidal Pd modifier was synthesized according to the procedure described in [5]. Concentration of the modifier solution was 1 mg ml^{-1} .

Instrumental parameters and temperature programs for atomic absorption measurements used are given in Tables 1

Table 1

Instrumental parameters used for the determination of Cd, Pb, As and Se by ETAAS (peak area quantitation)

Element	Pb	Cd	As	Se
Wavelength (nm)	283.3	228.8	193.7	196.0
Bandwidth (nm)	0.5	0.5	0.2	1.0

Table 2

Temperature programs used for the determination of Cd, Pb, As and Se by ETAAS

Step	Temperature ($^\circ\text{C}$)	Time (s)	Ar flow rate (l min^{-1})
1. Drying	110	5.0	3.0
2. Drying	110	20.0	3.0
3. Drying	130	10.0	3.0
4. Pyrolysis	700 (Cd, Pb); 1300 (As); 1100 (Se)	10.0	3.0
5. Pyrolysis	700 (Cd, Pb); 1300 (As); 1100 (Se)	25.0	3.0
6. Pyrolysis	700 (Cd, Pb); 1300 (As); 1100 (Se)	6.2	0
7. Atomization	1800 (Cd); 2000 (Pb); 2600 (As); 2100 (Se)	0.7	0
8. Atomization	1800 (Cd); 2000 (Pb); 2600 (As); 2100 (Se)	3.0	0
9. Clean	2200 (Cd); 2300 (Pb, Se); 2600 (As)	2.0	3.0

and 2, respectively. The total volume of the solution pipetted into the atomizer was always $20\text{ }\mu\text{l}$ ($10\text{ }\mu\text{l}$ of sample solution plus $10\text{ }\mu\text{l}$ of the modifier solution). At least two replicates ($n=2$) were measured for each solution. As a rule, the reproducibility of the measurements was better than 10%.

3. Results and discussion

3.1. Choice of a chemical modifier

Choice of the best chemical modifier for solution of a concrete task seems to be the most critical point of modern ETAAS. The huge number of the modifiers described just complicates the problem, as the corresponding recommendations often contradict each other. That is why in reality analysts usually try to apply universal $\text{Pd} + \text{Mg}(\text{NO}_3)_2$ modifier [6] under the recommended determination conditions and stop further trials in the case of the failure. Generally, choice of optimum modifiers on the basis of chemical and physical properties of analytes, sample matrix and modifiers themselves is still impossible. However, numerous researches of mechanisms of the action of the modifiers and huge experimental materials on their comparative efficiency collected may provide valuable information to a qualified researcher. Let us try to evaluate the performance of several forms of the most universal PGM modifiers on the basis of information available in the literature.

3.1.1. $\text{Pd} + \text{Mg}(\text{NO}_3)_2$ modifier

This modifier was proposed by Schlemmer and Welz [6] 20 years ago. Main modes of the action of this modifier are:

- (1) thermal stabilization of the analytes [7];

- (2) elimination of interfering chloride ions out of ETA during the pyrolysis stage [8].

This modifier is very effective for a variety of samples, as chloride ions are the most widespread strong interferents [1], and for numerous analytes [9], as Pd interacts with most analytes forming solid solutions and/or intermetallic compounds [10].

The main disadvantage of Pd + Mg(NO₃)₂ modifier seems to be the instability of Pd(NO₃)₂ in the presence of sample matrices. As is known, complex Pd chlorides are easily formed in aqueous solutions and the corresponding solid salts are quite thermally stable (for references, see [2]). Unfortunately, chloride derivatives of palladium are not effective thermal stabilizers [2]. Careful optimization of the experimental conditions (time–temperature program, ratio Pd:Mg and their concentrations) enhances the modifier efficiency. However, without these time-consuming procedures the efficiency of Pd + Mg(NO₃)₂ modifier may be similar to that of Pd(NO₃)₂ alone or of its mixture with ascorbic acid [11].

Numerous efforts to discover more effective complex Pd-based modifiers (e.g., [12,13]) have been not very successful because of the same reasons.

3.1.2. Stable form Pd (and other PGMs) modifiers

If Pd modifier exists in its active form (elemental Pd and/or PdO [2]) already during the drying stage (to prevent losses of very volatile analyte compounds, such as organoelement ones), it has to be more robust. The experimental experience collected supports this viewpoint.

3.1.2.1. Pre-reduced PGM modifiers. The first modifier of such a type (pre-reduced Pd) was proposed in 1984 by Grobowski et al. [14]. In this case Pd solution is introduced into the atomizer before the solution to be analysed, then it is dried and subjected to the pyrolysis step. After cool-down the atomizer, sample solution is introduced. The elemental Pd formed, since it is a noble metal does not react with most acids, organic and inorganic matrices at drying and pyrolysis temperatures. That is why even high concentrations of complex matrices do not suppress its modifying ability (e.g., [15,16]). However, the pre-reduction stage increases analysis time and may not be feasible with some autosamplers.

3.1.2.2. Permanent PGM modifiers. The other analogous approach is application of large amounts (100–500 µg) of low-volatile PGMs (Rh, Ru or Ir) as permanent modifiers [17]. This approach reduces the analysis time, decreases blank values and often increases lifetime of graphite tubes. Most systematically application of permanent PGM modifiers was studied by Lima et al. (e.g., [18,19]). In most of their works they used combinations of 200 µg of Rh, Ru or Ir with 250 µg W as the modifiers. They received very good results for the determination of As, Cd, Pb, Se, Sb and Cu in variety of biological and environmental samples, including some slurry samples. Significant advantage of the approach developed is a unified time–temperature program for each analyte independently on the sample nature. Only for pure analyte solutions used for the construction of the calibration

graphs, pyrolysis temperature and time may slightly differ [19].

Alas, this quite attractive approach possesses some disadvantages as well [20]. Most important of them seems to be the overstabilization of some analytes and matrix components by huge amounts of PGM modifiers. For example, Slaveykova and Hoenig [21] noted that the atomization temperature had to be increased above 2600–2700 °C for effective release of lead atoms in the presence of more than 10 µg of iridium modifier. Volynsky and Wennrich [22] observed overstabilization of As in the presence of as few as several micrograms of Rh modifier. References to some other analogous observations may be found in [23]. These experimental data make necessary to be cautious about the approach discussed.

3.1.2.3. Colloidal Pd modifier. We proposed use of solution of colloidal Pd as a modifier [5]. Tiny particles of elemental Pd (3–2000 nm in diameter) also are not susceptible to the negative influence of most matrices. Solution of colloidal Pd that may be easily synthesized in any chemical laboratory is stable under room temperature for at least 10 years without losing its modification ability. Colloidal palladium allows determining Se in a sodium chloride media, Sn and Cd in the presence of high amounts of sodium sulphate, and Te in a glucose media [24]. This modifier was used for the determination of As, Sb and Pb spikes in undiluted sea water sample [25].

From theoretical viewpoint, colloidal Pd modifier does not possess any disadvantages. That is why we chose namely this modifier for the determination of Cd, Pb, As and Se in a variety of biological samples.

3.2. Optimization study

An analytical method may be evaluated as a reliable one if an analyst may analyse an unknown sample for several days. In the other case the sample may be analysed by another method (or passed to another laboratory). That is why nowadays the works with careful optimization of the ETAAS experimental conditions including comparison of various chemical modifiers seem to be outdated. The works like these were acceptable only for early ETAAS, when owing to outstanding sensitivity of the method its only competitor was slow and expensive neutron activation analysis. With widespread of ICP-MS techniques the situation changed. Therefore, the ETAAS works with tedious optimization of the experimental conditions are still appropriate only for the analysis of really complex samples, such as solid samples (e.g., [26]).

As for relatively simple and well-studied digests of biological materials, in the ideal situation an analyst has to dissolve the sample, choose the definitely effective modifier on the basis of the literature data and his personal experience, shortly optimize the standard experimental conditions towards analysis of the concrete samples and to analyse them for a couple of days all together. Recovery of the spikes and/or analysis of the standard reference materials may be used to check accuracy of the method. Our work was generally conducted in accordance with this scheme.

Fixed amount of the modifier ($10\ \mu\text{g}$ Pd) was used throughout the whole work. In our previous work [24] 6 and $15\ \mu\text{g}$ of colloidal Pd was used for pure standard solutions and for the analysed solutions, respectively. This might be one of the reasons why the corresponding time–temperature programs slightly differed. Our preliminary experiments showed that application of $10\ \mu\text{g}$ Pd modifier allows receiving good recoveries for the analytes studied in biological samples and resulting to unification of the time–temperature programs for standard and analysed solutions.

Durations of all stages of the time–temperature program and the corresponding heating rates were the same for all samples and all analytes. These parameters were mostly taken from our previous works [24,25]. Only duration of the pyrolysis stage was decreased from 60 s for sea water sample [25] to 35 s for the digests of biological samples with their less concentrated inorganic matrix. The pyrolysis stage 6 (see Table 2) operating under “gas-stop” conditions was introduced to the program according to the recommendations of the producer for the equipment used [27]. Increase in duration of this stage up to 6.2 s versus 2 s indicated in the standard determination conditions [27] was recommended to enhance reliability of the method [28]. Under the specified experimental conditions, no significant background absorption that might influence the data obtained was registered.

In the beginning of the work, detailed pyrolysis (see Figs. 1–4) and atomization curves for the blood sample and pure standards were obtained for all analytes. For subsequent samples, only several points in the neighbourhood of optimum pyrolysis temperature value were checked (more developed curves 3 in Figs. 1–4 were obtained especially for the publication purpose). Other parameters were not checked at all. This procedure needed only half an hour for each analyte. Then the measurements of the samples were conducted.

3.3. Analysis of the samples

One may see that the data obtained (Table 3) correspond well to the reference data for all four SRMs and for all analytes stud-

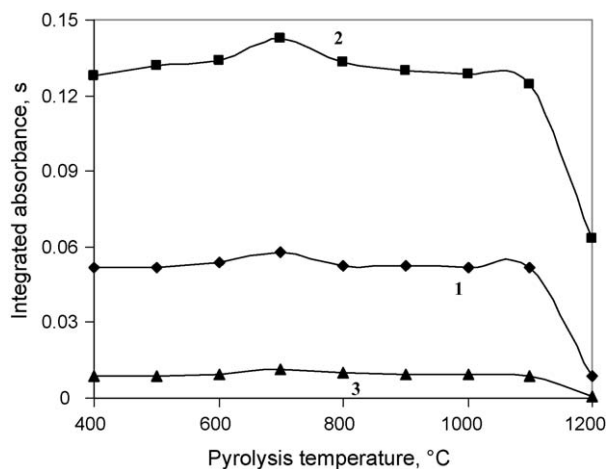


Fig. 1. Pyrolysis curves for Pb in the presence of $10\ \mu\text{g}$ of colloidal Pd modifier: (1) pure standard solution ($20\ \text{ng ml}^{-1}$); (2) SRM Whole Blood Level 3 ($34.6\ \text{ng ml}^{-1}$); (3) algae *Fucus vesiculosus* (Suurupi) ($3.35\ \text{ng ml}^{-1}$).

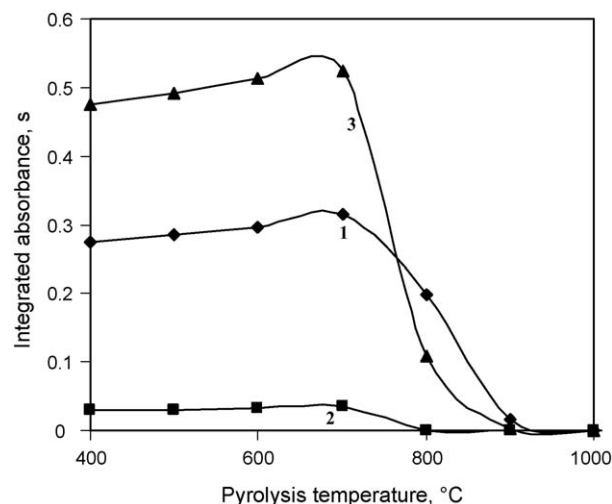


Fig. 2. Pyrolysis curves for Cd in the presence of $10\ \mu\text{g}$ of colloidal Pd modifier: (1) pure standard solution ($6\ \text{ng ml}^{-1}$); (2) SRM Whole Blood Level 3 ($0.91\ \text{ng ml}^{-1}$); (3) algae *Fucus vesiculosus* (Suurupi) ($12.3\ \text{ng ml}^{-1}$).

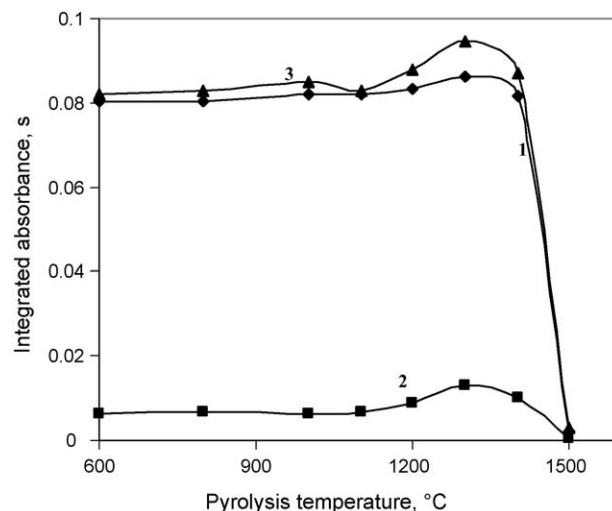


Fig. 3. Pyrolysis curves for As in the presence of $10\ \mu\text{g}$ of colloidal Pd modifier: (1) pure standard solution ($50\ \text{ng ml}^{-1}$); (2) SRM Whole Blood Level 3 ($2.32\ \text{ng ml}^{-1}$); (3) algae *Fucus vesiculosus* (Suurupi) ($52.4\ \text{ng ml}^{-1}$).

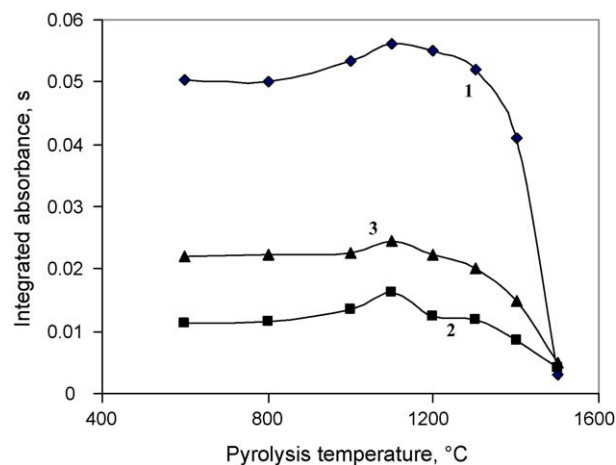


Fig. 4. Pyrolysis curves for Se in the presence of $10\ \mu\text{g}$ of colloidal Pd modifier: (1) pure standard solution ($50\ \text{ng ml}^{-1}$); (2) SRM Whole Blood Level 3 ($13.0\ \text{ng ml}^{-1}$); (3) algae *Fucus vesiculosus* (Suurupi) ($21.4\ \text{ng ml}^{-1}$).

Table 3

Results of the determination of Cd, Pb, As and Se in biological samples ($P=0.95$; $N=6$; $n=2-4$)

Sample	Cd	Pb	As	Se
Whole Blood Level 2 ($\mu\text{g ml}^{-1}$)	7.0 ± 0.2 (5.0–7.6) ^a	433 ± 5 (349–448) ^a	11.0 ± 0.6 (10.6–11.8) ^a	123 ± 4 (108–126) ^a
Whole Blood Level 3 ($\mu\text{g ml}^{-1}$)	11.3 ± 0.3 (11.0–12.6) ^a	614 ± 7 (606–676) ^a	27.1 ± 0.8 (24–29) ^a	162 ± 1 (151–169) ^a
Serum Level 1 ($\mu\text{g ml}^{-1}$)	– ^b	– ^b	– ^b	80 ± 2 (78–84) ^a
Hair NCS DC73347 ($\mu\text{g g}^{-1}$)	0.102 ± 0.004 (0.11 $\pm$ 0.02) ^a	9.8 ± 0.6 (8.8 $\pm$ 0.9) ^a	0.31 ± 0.02 (0.28 $\pm$ 0.04) ^a	0.7 ± 0.1 (0.60 $\pm$ 0.03) ^a
<i>Furcellaria lumbricalis</i> /Viimsi 2003 ($\mu\text{g g}^{-1}$)	0.62 ± 0.04	0.90 ± 0.03	4.57 ± 0.09	0.22 ± 0.01
<i>Furcellaria lumbricalis</i> /Viimsi 2005 ($\mu\text{g g}^{-1}$)	0.20 ± 0.01	1.07 ± 0.01	6.2 ± 0.1	0.42 ± 0.01
<i>Fucus vesiculosus</i> /Suurupi 2005 ($\mu\text{g g}^{-1}$)	0.54 ± 0.03^c	0.120 ± 0.008^c	10.73 ± 0.03^c	0.43 ± 0.02^c
<i>Fucus vesiculosus</i> /Kakumäe 2005 ($\mu\text{g g}^{-1}$)	0.78 ± 0.06^c	0.24 ± 0.01^c	14.2 ± 0.1^c	0.064 ± 0.004^c
<i>Fucus vesiculosus</i> /Viimsi 2005 ($\mu\text{g g}^{-1}$)	0.65 ± 0.06^c	0.43 ± 0.02^c	13.7 ± 0.2^c	0.014 ± 0.003^c

For algae samples the corresponding coastal Estonian region and year of collection are indicated.

^a Reference data.^b Not attested.^c $N=2$.

Table 4

Recoveries of the spikes for two algae samples ($P=0.95$; $n=4$)

Analyte	Found in solution of the original sample ($\mu\text{g ml}^{-1}$)	Spike added ($\mu\text{g ml}^{-1}$)	Total concentration found ($\mu\text{g ml}^{-1}$)	Spike found ($\mu\text{g ml}^{-1}$)	Recovery (%)
<i>Furcellaria lumbricalis</i> /Viimsi 2003					
Cd	4.48 ± 0.04	5	9.44 ± 0.09	4.96	99
Pb	9.1 ± 0.3	10	19.0 ± 0.1	9.9	99
As	21.2 ± 0.1	10	31.6 ± 0.7	10.4	104
Se	3.0 ± 0.1	20	22.9 ± 0.2	19.9	99.5
<i>Furcellaria lumbricalis</i> /Viimsi 2005					
Cd	1.03 ± 0.03	5	5.57 ± 0.06	4.54	91
Pb	3.79 ± 0.04	10	13.2 ± 0.5	9.41	94
As	21.7 ± 0.3	10	31 ± 1	9.3	93
Se	2.25 ± 0.09	20	24 ± 1	21.75	109

Table 5

Metrological parameters for the ETAAS method developed (for samples dissolved)

	Analyte			
	Pb	Cd	As	Se
Detection limit (3σ) ($\mu\text{g l}^{-1}$)	1.2	0.021	0.62	1.1
Quantitation limit (10σ) ($\mu\text{g l}^{-1}$)	2.8	0.057	1.15	2.6

ied. The accuracy of the data obtained for the algae samples was checked using recovery of spikes. For this purpose, spikes of the analytes were added to the digests of biological materials before the measurements. Recoveries for the spikes for all analytes studied were in the range 91–109% (see Table 4). Detection limits and quantitation limits for the method developed are given in Table 5.

4. Conclusion

The data obtained proved the high potential of modern ETAAS towards fast and accurate determination of some toxic elements in biological samples. Application of full potential of the STPF-concept and colloidal Pd modifier allows analysing “new” (for a given laboratory) biological samples after about half an hour optimization of the ETAAS procedure. Although the method discussed was developed for Cd, Pb, As and Se, it

probably may be also applied for other analytes determined by ETAAS using Pd modifiers (Te, Sb, Bi, Sn, etc.).

As is known, biological materials usually contain similar concentrations of main matrix components (Na, Cl, Ca, Mg, S and P). That is why the method developed may be applied for the analysis of a wide range of the corresponding materials in proposal of transferring 0.1–0.2 g of a solid sample (2 ml of a liquid sample) to 25 ml of solution. For some samples the weights dissolved may be probably increased without decreasing the accuracy of the method, but this point needs additional check up for every sample type.

Moreover, the approach developed may be applied for some samples of other types. We used it for the successful determination of Cd and As in River Sediment LGC6187 certified reference material (LGC, Teddington, UK) after its microwave decomposition. The values obtained were $(2.6 \pm 0.3) \mu\text{g g}^{-1}$ and $(26 \pm 2) \mu\text{g g}^{-1}$ for Cd and As, respectively (the corresponding certified values are $(2.7 \pm 0.3) \mu\text{g g}^{-1}$ and $(24 \pm 3) \mu\text{g g}^{-1}$).

Acknowledgements

The authors thank Prof. Kalle Truus and Dr. Merike Vaher for the algae samples, and Adrien Delrue for technical assistance. The authors are also grateful to the Estonian Science Foundation (grant no. 5618) for partial financial support of this work.

References

- [1] B. Welz, M. Sperling, *Atomic Absorption Spectrometry*, third ed., Wiley-VCH, Weinheim, 1999.
- [2] A.B. Volynsky, *Spectrochim. Acta Part B* 55 (2000) 103–150.
- [3] H.M. Ortner, E. Bulska, U. Rohr, G. Schlemmer, S. Weinbruch, B. Welz, *Spectrochim. Acta Part B* 57 (2002) 1835–1853.
- [4] W. Slavin, D.C. Manning, G.R. Carnrick, *At. Spectrosc.* 2 (1981) 137–145.
- [5] A.B. Volynsky, V. Krivan, *J. Anal. At. Spectrom.* 11 (1996) 159–164.
- [6] G. Schlemmer, B. Welz, *Spectrochim. Acta Part B* 41 (1986) 1157–1165.
- [7] R.D. Ediger, *At. Absorpt. Newslett.* 14 (1975) 127–130.
- [8] Z.-M. Ni, X.-Q. Shan, *Spectrochim. Acta Part B* 42 (1987) 937–949.
- [9] B. Welz, G. Schlemmer, J.R. Mudakavi, *J. Anal. At. Spectrom.* 7 (1992) 1257–1271.
- [10] Landolt-Börnstein, in: O. Madelung (Ed.), *New Series, Group IV, Phase Equilibria, Crystallographic and Thermodynamic Data of Binary Alloys*, vol. 5, Springer-Verlag, Berlin, 1991.
- [11] X.-Q. Shan, B. Wen, *J. Anal. At. Spectrom.* 10 (1995) 791–798.
- [12] O. Acar, Z. Kilic, A.R. Türker, *Anal. Chim. Acta* 382 (1999) 329–338.
- [13] O. Acar, *Talanta* 65 (2005) 672–677.
- [14] Z. Grobowski, W. Erler, U. Voellkopf, *At. Spectrosc.* 6 (1985) 91–93.
- [15] A.B. Volynsky, V. Krivan, *J. Anal. At. Spectrom.* 12 (1997) 333–340.
- [16] A.B. Volynsky, R. Wennrich, *J. Anal. At. Spectrom.* 16 (2001) 179–187.
- [17] I.L. Shuttler, M. Feuerstein, G. Schlemmer, *J. Anal. At. Spectrom.* 7 (1992) 1299–1301.
- [18] E.C. Lima, R.V. Barbosa, J.L. Brasil, A.H.D.P. Santos, *J. Anal. At. Spectrom.* 17 (2002) 1523–1529.
- [19] E.C. Lima, J.L. Brasil, A.H.D.P. Santos, *Anal. Chim. Acta* 484 (2003) 233–242.
- [20] D.L. Tsalev, V.I. Slaveykova, L. Lampugnani, A. D’Ulivo, R. Georgieva, *Spectrochim. Acta Part B* 55 (2000) 473–490.
- [21] V.I. Slaveykova, M. Hoenig, *Analyst* 122 (1997) 337–343.
- [22] A.B. Volynsky, R. Wennrich, *Spectrochim. Acta Part B* 57 (2002) 1301–1316.
- [23] A.B. Volynsky, *Spectrochim. Acta Part B* 59 (2004) 1799–1821.
- [24] A.B. Volynsky, V. Krivan, *Spectrochim. Acta Part B* 52 (1997) 1293–1304.
- [25] A.B. Volynsky, S. Akman, C.E. Dogan, U. Koklu, *Spectrochim. Acta Part B* 56 (2001) 2361–2369.
- [26] M. Resano, M. Aramendia, A.B. Volynsky, M.A. Belarra, *Spectrochim. Acta Part B* 59 (2004) 523–531.
- [27] E. Rothery (Ed.), *Analytical Methods for Graphite Tube Atomizers*, Varian, Mulgrave, Australia, 1988.
- [28] M. Smolka, Personal communication, 2004.

Coulometric determination of total sulfur and reduced inorganic sulfur fractions in environmental samples

Richard T. Wilkin^{*}, Kelly J. Bischoff¹

*U.S. Environmental Protection Agency, National Risk Management Research Laboratory,
Ground Water and Ecosystems Restoration Division, 919 Kerr Research Drive, Ada, OK 74820, USA*

Received 1 November 2005; received in revised form 23 January 2006; accepted 23 January 2006
Available online 28 February 2006

Abstract

Evaluation of the solid-phase partitioning of sulfur is frequently an important analytical component of risk assessments at hazardous waste sites because minerals containing reduced-sulfur can significantly affect the transport and fate of organic and inorganic contaminants in natural environments. We applied selected methods for the determination of total sulfur, acid-volatile sulfide (AVS), chromium-reducible sulfur (CRS), and extractable-sulfate in standard reference materials and sediment samples from a contaminated site. A coulometric titration method is presented and evaluated for total sulfur, AVS, and CRS. This method is especially advantageous for AVS and CRS determinations because hydrogen sulfide gas evolved during chemical extraction is detected and quantitated in-line; consequently, measurement endpoints can be precisely determined without need for setting arbitrary time limits. The coulometric method allows for improved data quality and increased laboratory throughput of samples. Data on sulfur partitioning are presented for four standard reference materials (NIST 1646a, NIST 2780, CCRMP LKSD-1, CCRMP RTS-3) for the purpose of supporting quality control in environmental studies involving the geochemical and biochemical cycling of sulfur. Published by Elsevier B.V.

Keywords: Sulfur; Coulometry; Site characterization; Acid-volatile sulfide; Chromium-reducible sulfur

1. Introduction

In environmental materials, solid-phase sulfur is associated with organic compounds, sulfate minerals, sulfide minerals, and/or elemental sulfur. The specific form or mixture of sulfur compounds encountered in a given sample is primarily dictated by pH, oxidation–reduction conditions, and the nature of biogeochemical processes important during sulfur mineralization. Evaluation of solid-phase associations of sulfur, particularly reduced inorganic forms, is frequently an important factor in contaminant transport and fate assessments at hazardous waste sites. For example, measurements of the quantity of sulfur present as metallic sulfides contained in mine tailings are used to predict the acid generating capacity of waste deposits at active and abandoned mine sites [1]. On the other hand, laboratory and field investigations indicate that iron sulfide minerals

contribute a positive role in attenuating halogenated aliphatic compounds in anaerobic groundwater systems through surface-mediated electron transfer reactions [2–4]. In addition, metal sulfides are known to sequester and immobilize metal and metalloid contaminants via adsorption and/or precipitation reactions [5–7]. Not surprisingly, therefore, some anaerobic remediation technologies for the treatment of contaminated groundwater take advantage of, or attempt to promote, sulfate-reducing conditions and precipitation of iron sulfides through addition of organic carbon substrates to the subsurface to support microbial respiration [8–12]. Accurate methods for determining solid-phase sulfur partitioning are needed to improve predictions of contaminant attenuation by sulfur mineral phases and to evaluate the contribution of abiotic attenuation pathways in environmental restoration efforts.

Several approaches have been developed for the determination of total sulfur and sulfur partitioning in environmental solids. Analysis methods for total sulfur include digestion with concentrated acids followed by emission spectroscopy [13], high temperature combustion followed by gas detection [14,15], and X-ray fluorescence spectrometry [16]. Partitioning of reduced

^{*} Corresponding author. Tel.: +1 580 436 8874; fax: +1 580 436 8703.

E-mail address: wilkin.rick@epa.gov (R.T. Wilkin).

¹ Present address: School of Geology and Geophysics, University of Oklahoma, Norman, OK, USA.

sulfur in environmental samples is most often assessed by using chemical extraction methods, such as digestion in dilute hydrochloric acid [17,18], digestion in acidic reductants such as chromous chloride [19–22], and digestion in oxidizing solutions such as hydrogen peroxide [23]. These partial extraction methods are useful for distinguishing reactive pools of sulfur in environmental samples; however, chemical extraction tests are by nature operationally defined and they do not necessarily target specific mineral forms [24].

As noted by Atkin and Somerfield [14], one of the problems in determining sulfur partitioning in geological samples is the lack of characterized reference materials needed to support quality control of solid-phase measurements. In this paper, we present a new coulometric method for determining total sulfur and reduced sulfur partitioning in environmental samples. Selected standard reference materials are characterized using this method with the goal of providing sulfur-partitioning information on widely available materials used for quality assurance in environmental studies. In addition, the sulfur partitioning method is applied to sediment samples collected from a hazardous waste site.

2. Experimental

2.1. Materials

Sulfur-partitioning measurements were carried out on a selection of standard reference materials (SRMs) and applied to the characterization of contaminated sediments collected from a Superfund Site. SRMs were chosen to match mineralogical compositions frequently encountered in environmental materials and to test the reliability of coulometric methods for the determination of total sulfur and reduced-sulfur partitioning. The following SRMs were used in this study: Lake Sediment (Canadian Certified Reference Materials Project LKSD-1), Estuary Sediment (National Institute of Standards and Technology 1646a), Hard Rock Mine Waste (National Institute of Standards and Technology 2780), and Sulphide Ore Mill Tailings (Canadian Certified Reference Materials Project RTS-3). Other materials used for total sulfur measurements included elemental sulfur (Aldrich, 99.9%), barium sulfate (Aldrich, 99.999%), and sulfanilamide (Aldrich, 99+%).

Sediment samples were collected from a small pond situated adjacent to a Superfund Site located approximately 16 km northwest of downtown Boston, MA (USA). The pond receives discharge of groundwater with elevated concentrations of arsenic, petroleum hydrocarbons, ferrous iron, and sulfate. Sediment core NC01 was collected from a water depth of 2.2 m using a 5-cm diameter piston-coring device. Note that preservation of sample redox status is a critical component of any solid-phase investigation relating to sulfur. The core was sealed immediately after recovery and kept upright before and during freezing. The frozen sediment core was sub-sampled by slicing off 2- to 5-cm thick sections. The sections were immediately bagged and kept frozen for subsequent solid-phase analyses. Core subsections were thawed and dried at room temperature in a Coy anaerobic glove box (96:4 v/v N₂–H₂ gas mixture). Dried sediments were

homogenized with an agate mortar and pestle and retained in the anaerobic glove box until analysis.

For total sulfur measurements, ultra-high purity oxygen gas and granular vanadium pentoxide (V₂O₅, Aldrich, 98+%) were used as oxidizing agents. Blank measurements indicated detectable concentrations of sulfur in V₂O₅ (<7 µgS per g of V₂O₅). Thus, blank corrections are on the order of 10 µgS for a standard V₂O₅ dosing of 1.5 g and are significant only for samples with very low total sulfur contents (<50 µg g⁻¹). Ultra-high purity nitrogen gas was used as the carrier gas for all coulometer measurements, including total sulfur measurements by combustion and reduced sulfur measurements by chemical extraction and gas purging.

AVS measurements were made with 1 M HCl prepared by diluting concentrated HCl with distilled water. The AVS extraction procedure is intended to digest labile sulfide compounds, including iron monosulfides such as mackinawite (FeS). The chromous chloride solution used for CRS measurements was prepared by passing 1 M CrCl₃·6H₂O (Aldrich, 98+%; Fluka, 98+%) dissolved in 0.5 M HCl through a standard Jones reductor [19]. The resulting Cr(II) chloride solution was observed to have an optimum level of effectiveness when stored in an anaerobic glove box; otherwise, the solution had a maximum shelf life of approximately 2 weeks if stored in a glass bottle with headspace purged by nitrogen gas, presumably due to the oxidation of Cr(II) back to Cr(III). When used in a sequential extraction scheme following AVS measurements, the CRS extraction procedure is intended to digest refractory sulfur-bearing compounds, such as pyrite/marcasite (FeS₂) and elemental sulfur (S⁰).

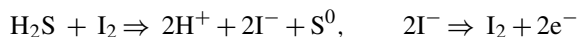
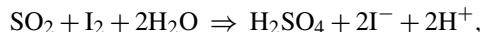
2.2. Coulometer instrumentation and analysis

A UIC Inc. sulfur coulometer (model number CM5014S) was used for the determination of total sulfur, AVS, and CRS. The sulfur coulometer provides accurate and absolute quantification of sulfur in SO₂- and H₂S-containing gas streams, with sulfur detection in the range of 0.01 µg to 10 mg. The direct coulometric method is especially advantageous for wet chemical extractions because the rate of gas evolution (e.g., H₂S(g)) can be monitored as a function of reaction time and because the detection and quantitation of sulfide is performed in-line. In this method, therefore, extraction endpoints can be precisely determined, for example, an extraction endpoint is reached when the cumulative sulfur total increases ≤0.5% after 1 min of reaction. This approach contrasts with other conventional AVS and CRS methods, such as trapping evolved H₂S in base followed by sulfide detection using ion-specific electrodes, iodometric titration, or colorimetric techniques. When using these indirect or off-line sulfide detection methods, the extraction endpoint is arbitrarily selected by the analyst based upon experience with the sample type [25].

The coulometer titration cell contains an anode and cathode compartment. The anode compartment contains platinum-detector and -generating electrodes. The cathode compartment contains a single platinum cathode. The anode compartment is filled with a solution containing methanol, pyridine, water, and tetrabutylammonium iodide; the cathode cell compartment

contains a phosphoric acid solution. Inert carrier gas, containing sulfur as SO_2 or H_2S released from a sample, is delivered to the anode cell compartment. Free iodine is electrochemically produced in the anode cell and reacts with the absorbed sulfur gas, as illustrated in the following reactions:

Anode reactions :



Cathode reaction : $2\text{H}_2\text{O} + 2\text{e}^- \Rightarrow \text{H}_2\uparrow + 2\text{OH}^-$

The decrease in free iodine proportionally decreases the sulfur detector current, automatically activating the titration current and generating I_2 stoichiometrically. Hydrogen gas is produced in the cathode cell at a rate equivalent to that of iodine generation. After the majority of the analyte is titrated, an increase in the detector current and free I_2 occurs, ultimately stopping the titration when the initial iodine concentration is reached. The titration current is continuously monitored, integrated, and used to calculate the quantity of sulfur delivered to the anode cell. The significant advantage associated with the coulometric titration of sulfur is that the current is the titrant and as a result there is no need for generating and applying standard calibration curves [14]. Most sample analyses for total sulfur can be completed within 10 min. Longer analysis times result if the sample contains more than about 3000 μg of sulfur or if sulfur-bearing compounds in the sample resist oxidation. Analysis times for AVS and CRS measurements are highly dependent on the sample type and reactivity of reduced sulfur compounds present in the sample.

The analysis of total sulfur was accomplished by combustion using two furnaces aligned in sequence (UIC model number CM3220). Samples were weighed into ceramic combustion boats and covered with V_2O_5 . The boat was pushed inside the first furnace where sample ignition occurred at 1050 °C and sulfur was converted to SO_2 and SO . These gas products were carried through the second furnace, set at 850 °C, where they reacted with a mixture of granular copper oxide and reduced copper filings to ensure that all sulfur was converted to SO_2 . The SO_2 was purged into the sulfur coulometer cell, where it was absorbed and titrated.

AVS and CRS were measured using the coulometer by wet chemical extraction methods as illustrated in Fig. 1. AVS is determined by digestion of samples in boiling 1 M hydrochloric acid and quantifying the amount of H_2S gas evolved. The same procedure is followed for CRS measurements with the exception of Cr(II) solution in place of hydrochloric acid. Samples were weighed into 50 mL glass flasks in an anaerobic glove box. Next the flasks were capped, removed from the glove box, and connected to the distillation apparatus (Fig. 1). Approximately 10 mL of 1 M HCl was pumped into the flask to initiate the release of acid-volatile sulfide and the sample plus acid solution was then heated to the boiling point. Nitrogen carrier gas was bubbled into the flask at a rate of 50–100 $\text{cm}^3 \text{min}^{-1}$. The gas stream was sent to the coulometer titration cell (Fig. 1). For most samples, hydrogen sulfide evolution was typically complete after approximately 10 min and next 10 mL of Cr(II) solution was added to the glass reaction flask to initiate a second release of hydrogen sulfide (CRS). The AVS and CRS procedures can be performed sequentially or in single step extractions on fresh samples. When performed separately, CRS measurements include both AVS and CRS.

2.3. Sulfate–sulfur analysis

Dry anoxic sediment was added to a 30-mL plastic bottle and extracted in 20 mL of sodium bicarbonate/sodium carbonate solution (2.4 mM NaHCO_3 plus 2.6 mM Na_2CO_3 ; Baker Reagent) for the determination of soluble sulfates [18]. Bicarbonate/carbonate extractions (pH 9.5) were conducted in an anaerobic glove box to prevent the oxidation of sulfides and production of soluble sulfate. The extracting solution was flushed with N_2 gas to remove dissolved O_2 . The supernatant solution was collected after 24 h of extraction, filtered with a 0.22 μm membrane syringe filter, and analyzed for sulfate and chloride by capillary electrophoresis with indirect UV detection.

2.4. Microscopy

Particle morphology and elemental composition was examined using a JEOL JSM-6360 scanning electron microscope (SEM) equipped with an Oxford Instruments energy dispersive

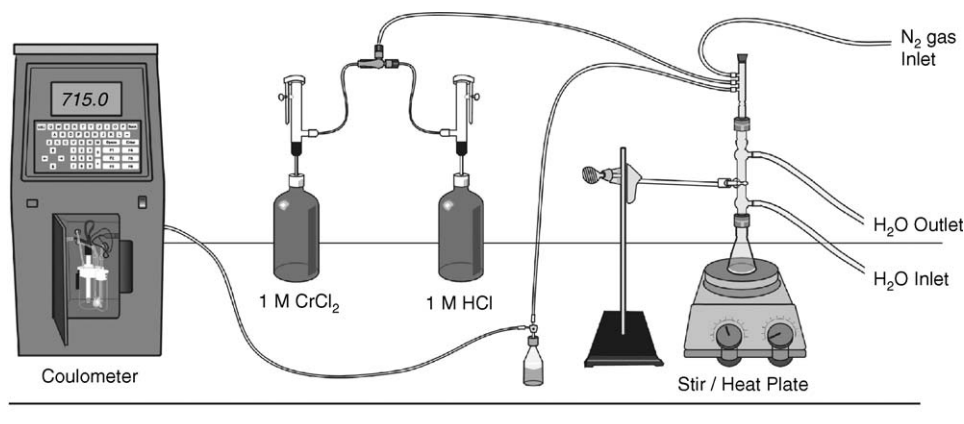


Fig. 1. Schematic of in-line detection system for hydrogen sulfide gas released during AVS and CRS extractions.

X-ray spectrometer (EDX). Imaging and elemental analysis was conducted using an electron accelerating voltage of 20 kV. A backscattered electron detector was used for imaging and particles with bright backscattered response were selected for spot- or area-mode EDX analysis.

3. Results and discussion

3.1. Total sulfur

The concentration of total sulfur in sediments, aquifer materials, and mine wastes is important for understanding the factors that control the geochemical cycling of sulfur, especially in studies where solid-phase partitioning of sulfur is evaluated. Accurate total sulfur values are necessary in order to determine the fractional abundance of any operationally defined form of sulfur (e.g., AVS, CRS), to evaluate sulfur mass balance, and to compare with total concentrations of other redox-sensitive elements (e.g., carbon, iron, manganese). Results of total sulfur analyses in SRMs using coulometry are in excellent agreement with certified values (Table 1); a paired *t*-test showed no difference between the measured and certified values at the 95% confidence level. In Fig. 2, linear regression trends demonstrate

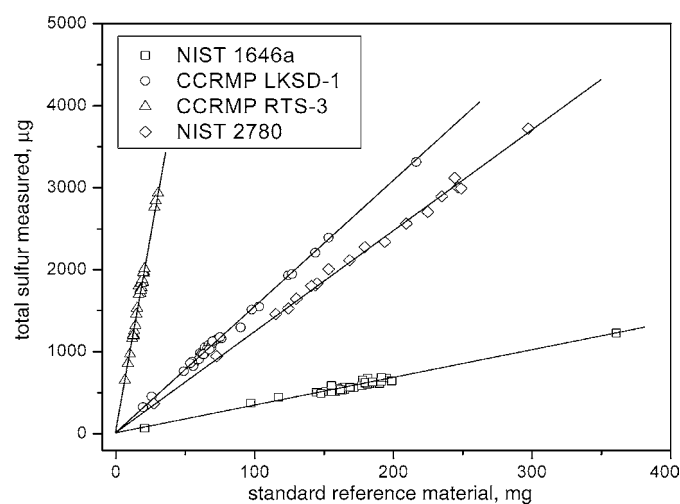


Fig. 2. Linearity of total sulfur determinations on selected standard reference materials.

Table 1
Coulometric determinations of total sulfur in standard reference materials and selected chemicals

Sample	Determined value, wt. %	R.S.D., %	Certified value, wt. %
Lake Sediment (CCRMP LKSD-1)	1.56	3.47 (<i>n</i> = 42)	1.57 ± 0.13
Estuary Sediment (NIST 1646a)	0.341	5.04 (<i>n</i> = 36)	0.352 ± 0.004
Hard Rock Mine Waste (NIST 2780)	1.26	3.07 (<i>n</i> = 20)	1.26 ± 0.042
Ore Mill Tailings (CCRMP RTS-3)	9.86	3.42 (<i>n</i> = 30)	9.98 ± 0.26
Sample	Determined value, wt. %	R.S.D., %	Expected value, wt. %
Barium sulfate	13.76	2.16 (<i>n</i> = 7)	13.8
Sulfanilamide	18.64	5.76 (<i>n</i> = 7)	18.6
Elemental sulfur	101.6	7.4 (<i>n</i> = 9)	100

Note: Certified values for total sulfur were determined by multi-acid digestion followed by isotope dilution thermal ionization mass spectrometry (NIST 1646a and NIST 2780), Leco combustion apparatus with infrared sulfur detection (CCRMP RTS-3), and X-ray fluorescence spectrometry (CCRMP LKSD-1).

that the sulfur values obtained were reproducible over a range of sample masses and total sulfur concentrations that span about two orders of magnitude. The linear range of sulfur determination by combustion extends from <100 to 5000 µgS. Data presented in Table 1 were collected over a 4-year period and highlight the reproducibility of the method. The R.S.D. values varied between 3.1% and 5.0% (Table 1). The detection limit of the method is determined to be 15 µgS g⁻¹ (0.0015 wt.%) for a 1-g sample (the value of the blank plus three times the standard deviation). The maximum titration rate of the coulometer cell, 240 mA (2400 µgS min⁻¹), permitted completion of most analyses in 7–10 min.

Measured total sulfur values for barium sulfate, elemental sulfur, and sulfanilamide are in good agreement with expected values. Analytical results presented in Table 1 suggest that coulometric determination of total sulfur is accurate and precise for a wide range of sulfur forms encountered in geological materials, including sulfides, sulfates, elemental sulfur, and organic sulfur.

3.2. Sulfur partitioning in SRMs

The AVS and CRS methods are conventionally applied either as single-step or sequential extraction tests for the evaluation of solid-phase sulfur partitioning. These chemical extraction methods were developed and are applied to samples for different purposes. AVS measurements are generally considered to capture hydrogen sulfide released during the acid dissolution of iron monosulfides. AVS can also include sulfide associated with other acid-soluble sulfide minerals (e.g., poorly crystalline ZnS), residual pore-water sulfide [26], sulfide adsorbed to mineral or organic surfaces released at low pH, or sulfide associated with metal sulfide nanoparticles or clusters [24]. In addition, the determination of AVS and simultaneously extracted metals (SEM) in sediments by means of digestion in dilute hydrochloric acid is used as an assessment tool to predict the potential for metal toxicity to sediment-dwelling organisms [27]. On the other hand, CRS measurements are commonly used to determine the pyrite-sulfur content of sediments. The CRS method was first introduced by Zhambina and Volkov [19] and later developed for geochemical applications [17,20–22]. The CRS method is frequently carried out in sediment diagenesis studies to assess sulfur partitioning and sulfide mineral transformations

Table 2
Sulfur partitioning results for selected standard reference materials

Sample	CRS, wt. %	AVS, $\mu\text{g g}^{-1}$	Sulfate-S, wt. %	% of Total S
Lake Sediment (CCRMP LKSD-1)	0.954 ± 0.118	6 ± 1	0.116 ± 0.004	68.6
Estuary Sediment (NIST 1646a)	0.242 ± 0.026	6 ± 1	0.043 ± 0.001	83.8
Hard Rock Mine Waste (NIST 2780)	0.594 ± 0.165	Not detected	0.074 ± 0.001	53.0
Ore Mill Tailings (CCRMP RTS-3)	6.55 ± 0.46	115 ± 25	1.315 ± 0.054	80.0

Note: % of Total S = [(CRS + AVS + Sulfate-S)/Total S] $\times$ 100.

in anoxic systems. It is generally accepted that reaction of materials with acidic chromous chloride solution releases sulfur from fine-grained pyrite, marcasite, elemental sulfur, and from acid-volatile sulfides [22].

Due to the non-uniformity of AVS and CRS measurement methods in environmental studies, there is a need for the characterization of widely available SRMs that can be used to assess laboratory determinations of AVS and CRS. Concentrations of AVS in the standard reference materials are reported in Table 2. The AVS detection limit of the coulometric method is determined to be $5 \mu\text{g S g}^{-1}$ for a 1-g sample. Not surprisingly AVS concentrations in the SRMs examined here are low (<0.02 wt. %) and variable. Compounds that contribute to AVS are generally unstable in air and would be expected to oxidize in SRMs, primarily because no special handling procedures are typically followed to preserve the redox-sensitive components of these materials. The detection of AVS in NIST 1646a and CCRMP LKSD-1 indicates that these sediments contain an oxidation-resistant, labile sulfide component. Although AVS concentrations in the SRMs are near the analytical detection limit, the presence of AVS is consistent with the findings of Brumbaugh and Arms [28]. They also reported low but stable levels of AVS in river sediment (NIST 1645).

To the best of our knowledge, CRS data presented in Table 2 are the first such values reported for SRMs. CRS values in the selected SRMs range from 0.240 ± 0.026 wt. % in NIST 1646a to 6.55 ± 0.46 wt. % in CCRMP RTS-3. As a percent of the total sulfur content in the lake and estuarine sediments, CRS comprises from 61 to 71% in CCRMP LKSD-1 and NIST 1646a, respectively. Results from our laboratory over a 2-year period indicate that the results of CRS determinations on SRMs are more variable compared to total sulfur measurements. The R.S.D. values range from 7 to 25%. The reasons for this analytical variability are uncertain but may be due to the apparent diminishing quality of Cr(II) solutions as a function of time after their preparation and/or due to the more complex nature of chemical extraction/purging measurements compared to high temperature combustion measurements.

Fig. 3 shows the cumulative total of sulfur extracted by HCl and Cr(II) as a function of time for three different masses of CCRMP LKSD-1 and NIST 1646a. Neither of these materials contains any significant amount of AVS. With smaller sample weights, shorter reaction times are needed to reach the CRS extraction endpoint. However, increased analytical significance can be achieved with larger sample weights. Results in Fig. 3 indicate that 25 min were sufficient to reach the extraction endpoint for NIST 1646a; whereas, up to 40 min of reaction time

were needed for CCRMP LKSD-1. These findings are broadly consistent with the conclusion of Canfield et al. [20]. They reported an optimum reaction time for CRS measurements of ~ 1 h for modern sediments. As previously noted, however, the advantage of the coulometric method for sulfur detection is that the endpoint of the chemical extraction can be precisely determined for each sample type without need for selecting arbitrary time limits, thereby increasing laboratory efficiency, sample throughput and data reliability. It is also possible that detailed analysis of sulfide release trends as presented in Fig. 3 could shed additional insight into the nature of reduced sulfur compounds in sample materials, e.g., regarding particle reactivity and grain size.

Mylon et al. [29] have recently suggested that sulfate can be reduced by Cr(II). This possibility introduces a potentially significant interference to CRS measurements, especially in systems with molar Cr/SO₄ ratios in excess of 10^4 . In the sediment extractions carried out in this study, molar Cr/SO₄ ratios were

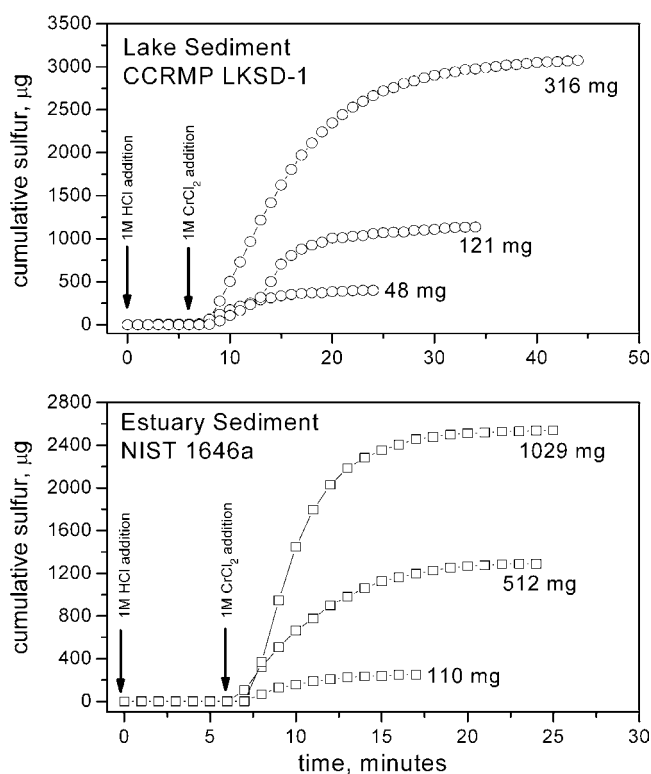


Fig. 3. Cumulative total of sulfur released with time during AVS and CRS extractions from CCRMP LKSD-1 and NIST 1646a. For each SRM, data are shown for three sample masses.

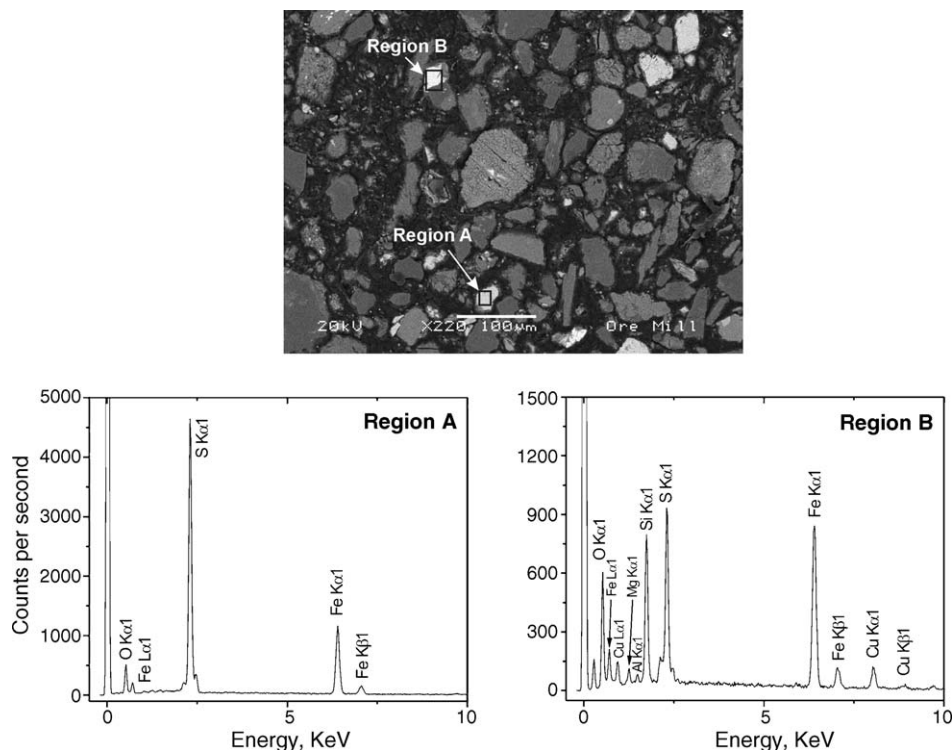


Fig. 4. Backscattered electron photomicrograph of grains from CCRMP RTS-3. Emission spectra are shown for regions A and B. Region A contains Fe and S in a ratio consistent with FeS_2 . Region B includes an area from a locked polymineralic grain including a Cu–Fe–S phase (bright) and a Mg–Al–Si–O phase (dark).

below $10^{3.7}$. Results presented in Ref. [29] suggest that at this Cr/ SO_4 ratio <1% of sulfur from sulfate should be recovered as sulfide. This potential interference should not impact CRS determinations for most sediment or aquifer samples; nevertheless, the possibility of sulfate reduction by Cr(II) needs to be considered in CRS extraction tests. We note, however, that we were unsuccessful in reducing Na_2SO_4 using Cr(II) solutions prepared from $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$.

Extractable sulfate represents soluble sulfate compounds and is likely dominated by precipitates that form as residual pore water dries or is the product of metal sulfide oxidation. Extractable sulfate values for the selected SRMs are reported in Table 2. Extractable sulfate comprises from 6 to 13% of total sulfur in NIST 2780 and CCRMP RTS-3, respectively. The recommended value of sulfate–sulfur in CCRMP RTS-3 is 1.54 ± 0.20 wt.%, which is in reasonable agreement with the value reported in Table 2, 1.32 ± 0.05 wt.%. The lower value reported here is likely due to the anaerobic extraction procedure, which is expected to decrease the sulfate-soluble component by minimizing or eliminating sulfide mineral oxidation during wet chemical extraction.

In sediment samples NIST 1646a and CCRMP LKSD-1, the sum of sulfur fractions AVS, CRS, and sulfate–sulfur accounts for 84 and 69% of the total sulfur concentration, respectively (Table 2). The remaining quantity of sulfur in these SRMs may be reasonably operationally defined as the organic sulfur fraction [30]. In mine waste samples NIST 2780 and CCRMP RTS-3, smaller percentages total sulfur are accounted for by the AVS, CRS, and sulfate–sulfur fractions (Table 2). In these samples with low total organic carbon contents it is unlikely that organic

sulfur is a significant component. Microscopic inspection of CCRMP RTS-3 revealed the presence of sulfide grains partially locked by gangue phases (Fig. 4). Textural features indicated that not all sulfide grains, potentially reactive with Cr(II), are indeed available for reaction during wet chemical extractions with Cr(II) solutions. In addition, energy dispersive X-ray analysis showed that particles of iron–copper sulfide (chalcopyrite) are present. It is unknown whether chalcopyrite or other copper sulfides are reactive in Cr(II) solution, so that the presence of mixed-metal sulfides could result in reduced CRS extraction efficiency. More work is needed to better understand the reactivity of Cr(II) solutions with reduced sulfur phases other than iron monosulfides, iron disulfides, and elemental sulfur.

3.3. Sulfur partitioning in sediments

Total sulfur measurements and chemical extraction tests for AVS, CRS, and sulfate–sulfur were carried out on sediment cores collected from a hazardous waste site. Sulfur partitioning results for the core materials are presented in Fig. 5. At core depths <10 cm, the sediments are rich in reduced sulfur compounds. This is significant because mineral sulfides play a role in attenuating the mobility of metals out of the sediments into pore water and the overlying water column. The abrupt transition at about 10 cm depth in sediment composition from high-sulfur content to low-sulfur content corresponds with a decrease in organic carbon concentrations, from >10 to <1 wt.%. Organic carbon along with dissolved sulfate drives microbially mediated sulfate reduction and the build-up of solid-phase sulfides [31]. Total sulfur values are highly correlated with the sum of AVS, CRS, and

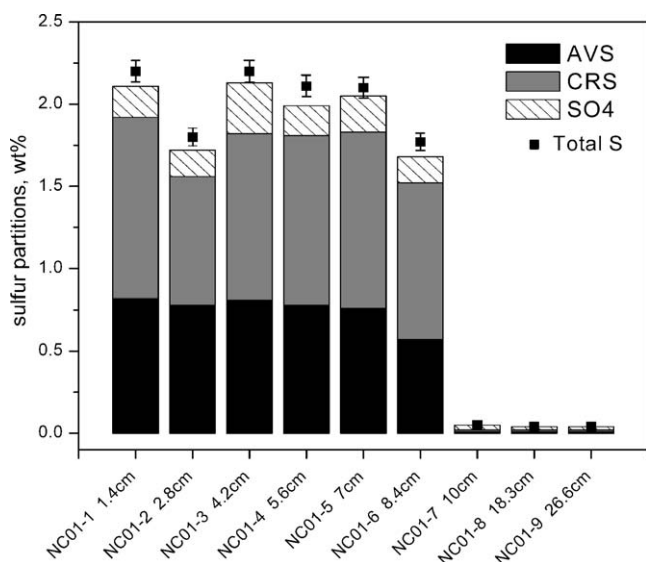
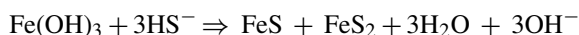


Fig. 5. Sulfur partitions and total sulfur (wt.%) in nine sediment core samples collected from a contaminated site.

sulfate–sulfur ($R = 0.9998$; $n = 9$). However, total sulfur values are consistently in excess of the sum of the individually determined partitions. Considering the abundance of TOC, the excess sulfur at depths <10 cm is likely associated with organic compounds. Assigning the un-partitioned fraction of total sulfur as organic sulfur yields organic sulfur to organic carbon ratios of 0.0038–0.0082. For comparison, the organic sulfur to organic carbon ratio of aquatic biota typically falls in the range from 0.005 to 0.03 [30].

In sediments above a depth of 10 cm below the sediment–water interface, the molar FeS/FeS_2 ratio is 1.54 ± 0.26 , assuming that all AVS is partitioned into FeS and all CRS is partitioned into FeS_2 . Equivalent molar amounts of FeS and FeS_2 are expected to form as ferric hydroxide progressively reacts with bisulfide, e.g., following the reaction:



If no oxidant other than $\text{Fe}(\text{OH})_3$ is present, equal quantities of mackinawite and pyrite should precipitate as a consequence of sulfide production by microbes and mineral transformation processes [32]. This pattern appears to be generally followed in core NC01; however, it is noted that other non-sulfidic iron minerals could be present in the sediments. The molar excess of FeS over FeS_2 might be due to the fact that some AVS is from a metal sulfide phase other than FeS or AVS is present in labile compounds other than iron monosulfide. These data illustrate the applicability of the coulometric methods for determining solid-phase sulfur partitioning in environmental materials.

4. Conclusions

The presented methods enable rapid and reliable determination of total sulfur, reduced sulfur forms, and water-soluble sulfate present in environmental solids. The coulometric method is especially advantageous for AVS and CRS determinations because the evolution of hydrogen sulfide gas is detected and

quantitated in-line. In this way, chemical extraction endpoints can be precisely determined without need for setting arbitrary time limits as in existing methods for measuring AVS and CRS. The quantification limit of total sulfur and reduced sulfur forms using the methods presented here are 15 and $5 \mu\text{g g}^{-1}$, respectively, for a 1-g sample. Data are presented on the solid-phase partitioning of four SRMs to aid in developing laboratory quality control protocols. The methods documented here can be used to evaluate the concentration distribution of sulfur and reduced sulfur compounds in environmental samples, including sediments, soils, aquifer solids, and waste products. Further studies are needed to better understand the solid-phase components that release hydrogen sulfide during AVS and CRS extractions.

Acknowledgements

The U.S. Environmental Protection Agency through its Office of Research and Development funded the research described here. It has not been subjected to Agency review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. We gratefully acknowledge the analytical support provided by Shaw Environmental (Contract #68-C-03-097), T. Lee for the electron microscopy, and F. Beck and P. Clark for field assistance.

References

- [1] U.S. Environmental Protection Agency (USEPA), Acid Mine Drainage Prediction, EPA-530/R-94-036, 1994.
- [2] M.R. Kriegman-King, M. Reinhard, *Environ. Sci. Technol.* 28 (1994) 692.
- [3] C. Butler, K.F. Hayes, *Environ. Sci. Technol.* 33 (1999) 2021.
- [4] W. Lee, B. Batchelor, *Environ. Sci. Technol.* 36 (2002) 5147.
- [5] J.W. Morse, *Mar. Chem.* 46 (1994) 1.
- [6] B.-G. Lee, J.-S. Lee, S.N. Luoma, H.J. Choi, C.-H. Koh, *Environ. Sci. Technol.* 34 (2000) 4517.
- [7] T. Jong, D.L. Parry, *J. Environ. Monit.* 6 (2004) 278.
- [8] S.G. Benner, D.W. Blowes, C.J. Ptacek, K.U. Mayer, *Appl. Geochem.* 17 (2002) 301.
- [9] L.G. Kennedy, J.W. Everett, K.J. Ware, R. Parsons, V. Green, *Bioremediation J.* 2 (1998) 259.
- [10] L.G. Kennedy, J.W. Everett, T. Dewers, W. Pickins, D. Edwards, *J. Environ. Eng.* 125 (1999) 47.
- [11] J.A. Saunders, M.-K. Lee, L.W. Wolf, C.M. Morton, Y. Feng, I. Thompson, S. Park, *Bioremediation J.* 9 (2005) 33.
- [12] O. Gibert, J. de Pablo, J.L. Cortina, C. Ayora, *J. Chem. Technol. Biotechnol.* 78 (2003) 489.
- [13] M.A. Tabatabai, *Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties*, Soil Science Society of America, Madison, WI, 1982, pp. 501–538 (Chapter 28).
- [14] B.P. Atkin, C. Somerfield, *Chem. Geol.* 111 (1994) 131.
- [15] J.M.M. Leitão, F.S. Costa, F.M.G. Tack, *Intern. J. Environ. Anal. Chem.* 80 (2000) 219.
- [16] M. Andersson, A. Olin, *Talanta* 37 (1990) 185.
- [17] M.L. Tuttle, M.B. Goldhaber, D.L. Williamson, *Talanta* 33 (1986) 953.
- [18] F.M. Tack, F. Lapauw, M.G. Verloo, *Talanta* 44 (1997) 2185.
- [19] N.N. Zhambina, I.I. Volkov, in: W.E. Krumbein (Ed.), *Environmental Biogeochemistry and Geomicrobiology*, Ann Arbor Science Publishers, Ann Arbor, MI, 1978, pp. 735–746.
- [20] D.E. Canfield, R. Raiswell, J.T. Westrich, C.M. Reaves, R.A. Berner, *Chem. Geol.* 54 (1986) 149.

- [21] J.W. Morse, J.C. Cornwell, *Mar. Chem.* 22 (1987) 55.
- [22] R.E. Allen, R.J. Parkes, in: M.A. Vairavamurthy, M.A.A. Schoonen (Eds.), *Geochemical Transformations of Sedimentary Sulfur*, American Chemical Society, Washington, DC, 1998, pp. 243–257 (Chapter 13).
- [23] L.A. Sullivan, R.T. Bush, D. McConchie, G. Lancaster, P.G. Haskins, M.W. Clark, *Aust. J. Soil Res.* 37 (1999) 255.
- [24] D. Rickard, J.W. Morse, *Mar. Chem.* 97 (2005) 141.
- [25] L.A. Sullivan, R.T. Bush, D.M. McConchie, *Aust. J. Soil Res.* 38 (2000) 729.
- [26] J.W. Morse, D. Rickard, *Environ. Sci. Technol.* 38 (2004) 131A.
- [27] D.M. DiToro, J.D. Mahony, D.J. Hansen, K.J. Scott, M.B. Hicks, S.M. Mayer, M.S. Redmond, *Environ. Toxicol. Chem.* 9 (1990) 1487.
- [28] W.G. Brumbaugh, J.W. Arms, *Environ. Toxicol. Chem.* 15 (1996) 282.
- [29] S.E. Mylon, H. Hu, G. Benoit, *Anal. Chem.* 74 (2002) 661.
- [30] J. Werne, D.J. Hollander, T.W. Lyons, J.S. Sinninghe Damste, in: J.P. Amend, K.J. Edwards, T.W. Lyons (Eds.), *Sulfur Biogeochemistry, Past and Present*, Geological Society of America, Denver, CO, 2004, pp. 135–150.
- [31] R.A. Berner, *Geochim. Cosmochim. Acta* 48 (1984) 605.
- [32] C. Boesen, D. Postma, *Am. J. Sci.* 288 (1988) 575.

Preparation and application of functionalized nanoparticles of CdSe capped with 11-mercaptoundecanoic acid as a fluorescence probe

Ping Zhong^a, Ying Yu^{a,*}, Jianzhong Wu^a, Yan Lai^a, Bo Chen^a,
Zhaoyang Long^b, Chunsui Liang^b

^a School of Chemistry and Environment, South China Normal University, Guangzhou 510006, PR China

^b Center for Disease Control and Prevention of Guangdong Province, Guangzhou 510300, PR China

Received 17 October 2005; received in revised form 19 February 2006; accepted 3 March 2006

Available online 18 April 2006

Abstract

A new fluorescence probe, CdSe nanocrystal has been prepared and modified with 11-mercaptoundecanoic acid [HS-(CH₂)₁₀-COOH]. The functionalized nanoparticles were characterized using transmission electron microscopy (TEM), ultraviolet–visible (UV–vis) spectroscopy and photoluminescence (PL) spectroscopy. The results demonstrate that CdSe is dispersed homogeneously in aqueous solution and well protected from the environmental oxygen. They can be used as a new fluorescence probe for lysozyme, which was simple, rapid and specific. Under the optimum condition, the response is linearly proportional to the amount of lysozyme from 0.20 to 26.0 µg ml⁻¹, and the limit of detection is 0.115 µg ml⁻¹. The proposed method has been applied to the determination of lysozyme in egg white, with the recovery of 96–105%.

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Keywords: Nanoparticles; Nanocrystals; 11-Mercaptoundecanoic acid; Fluorescence probe; Lysozyme

1. Introduction

Using fluorescence probes to label biological molecules is a common and very useful practice in fields of chemistry, biology and medical science. Organic dyes are the most used [1]. However, they have significant limitations, including photobleaching and low signal intensities. Furthermore, most of them have narrow excitation spectra, and often exhibit broad emission bands with red tailing, which makes it difficult for simultaneous quantitative evaluation of relative amounts of two or more sensors, presenting in the same sample because of spectral overlap [2]. It is a challenge to find proper fluorescence probes for tracing biomolecules.

Colloidal semiconductor nanocrystals (NCs), often referred as quantum dots (QDs), have novel properties such as improved brightness, resistance against photobleaching, and readily tunable spectra [2–6], so they are emerging as a new class of biological labels with properties that are not available among tra-

ditional organic dyes and fluorescent proteins. There are numerous methods for synthesis of semiconductor NCs [7–10], but most luminescent NCs are prepared in organic solvents, which are costly, requiring extreme reaction conditions, and may result in low output of NC product, especially not suitable for biological application. In recent years, NCs have attracted considerable attention as novel luminescence probes [2,3,11]. Gattás-Asfura and Leblanc [12] synthesized the peptide-coated CdS NCs for optical detection of Cu(II) and Ag(I). Wang and Wang [13–15] have reported a class of nanometer-sized luminescent particles that allow ultrasensitive detection of nucleic acids and proteins. However, these CdS NCs showed broad emission peaks, indicating that these particles contain a larger number of surface defects. The molecular chains of these stabilizing agents were too short to prevent the attack of water molecules, and could not reserve the NCs for a long time. Goldman et al. [16,17] synthesized CdSe–ZnS NCs conjugated to antibodies in fluoroimmunoassays to detect protein and small molecules. Although the NCs were water-soluble, the synthetic route was so complicated that could not be used widely.

Therefore, we have optimized water-phase synthesis procedure and developed a simple and low-cost method to produce very stable (>7 months) CdSe semiconductor nanocrystals by

* Corresponding author. Tel.: +86 20 39315993; fax: +86 20 85213368.

E-mail addresses: pingzhong119@163.com (P. Zhong),
yuyhs@163.com (Y. Yu).

using 11-mercaptoundecanoic acid as stabilizing agent to cap CdSe NCs for the first time. These CdSe NCs allow ultrasensitive quantitative detection of lysozyme. As lysozyme has the property of dissolving some bacteria [18], it can be applied in medical treatment, biological engineering, and especially in food antisepsis to replace chemically synthesized ones. Hence, lysozyme was selected as a representative protein.

2. Experiment

2.1. Apparatus

UV–vis absorption spectra were recorded with a UV–vis 8500 spectrophotometer (Techcomp, Shanghai, China). Fluorescence measurements were performed using a F-2500 spectrofluorometer (Hitachi, Japan). The fluorescence spectra were recorded at $\lambda_{\text{ex}} = 365$ nm. pH values were measured with a model PHS-3C pH meter (Leici Analytical Instrument Factory, Shanghai, China). High-resolution transmission electron micrograph (HRTEM) was performed on a Hitachi HR 2000 microscope operating at 200 kV accelerating voltage. Clusters of closely spaced particles were obtained by sample spreading and dried on the 5 mm thick carbon-coated copper grids at room temperature, with the excess solution being wiped away.

2.2. Reagents

All chemicals used were of analytical grade or of the highest purity available. All solutions were prepared with double-distilled water.

11-Mercaptoundecanoic acid was purchased from Sigma–Aldrich (USA). Lysozyme, Se powder, NaBH_4 , NaOH and $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ were acquired from Shanghai Reagent Company (China) and used as received. Britton–Robinson (B–R) buffer solution was prepared by mixing $0.04 \text{ mol l}^{-1} \text{ H}_3\text{PO}_4$, $0.40 \text{ mol l}^{-1} \text{ CH}_3\text{COOH}$, $0.04 \text{ mol l}^{-1} \text{ H}_3\text{BO}_3$ and $0.20 \text{ mol l}^{-1} \text{ NaOH}$ solution to required pH value. Na_2HPO_4 – NaCl – KH_2PO_4 (PBS) buffer solution was prepared by mixing $0.07 \text{ mol l}^{-1} \text{ Na}_2\text{HPO}_4$, $0.07 \text{ mol l}^{-1} \text{ KH}_2\text{PO}_4$ and $0.05 \text{ mol l}^{-1} \text{ NaCl}$ solution to required pH value.

2.3. Synthesis of CdSe nanocrystals

CdSe nanocrystals were prepared, following the method reported by previous literatures [19–21]. 11-Mercaptoundecanoic acid was used as the capping agent to synthesize CdSe NCs because its hydrosulfide group (HS^-) can directly interact with the cadmium ions and its carboxyl group (COO^-) can act on biomolecules like lysozyme. 0.0228 g of $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ was dissolved in 125 ml of water, while 0.0524 g of 11-mercaptoundecanoic acid, dissolved with NaOH solution, was added under stirring and then adjusted to pH 11 by dropwise addition of 1.0 mol l^{-1} solution of NaOH. The solution was placed in a three-necked flask fitted with a septum and valves, deaerated by N_2 bubbling for about 30 min. Under stirring, $100 \mu\text{l}$ of freshly prepared oxygen-free NaHSe solution (0.50 mol l^{-1}) was added to the solution quickly. NaHSe was generated by reaction of Se powder with NaBH_4 . The molar ratio of $\text{Cd}^{2+}:\text{HSe}^-:11\text{-mercaptoundecanoic acid}$ was 1:0.5:2.4. The resulting mixture refluxed at 96°C for 2 h was adopted in this assay to promote growth of NCs. The final concentration of NCs was ca. $2 \times 10^{-4} \text{ mol l}^{-1}$ according to the Se^{2-} concentration.

2.4. Determination of lysozyme with CdSe NCs

1.5 ml of $2 \times 10^{-4} \text{ mol l}^{-1}$ NCs solution mentioned above, 0.5 ml B–R buffer (pH 7.24) and different amounts of lysozyme were successively placed into a 10-ml calibrated test tube, then diluted to the mark with water and mixed thoroughly. The fluorescence intensity of the solution was recorded at 542 nm with the excitation wavelength set at 365 nm. The slit widths of both excitation and emission are 5.0 nm.

3. Results and discussion

3.1. Characterization of NCs

The particle size and degree of dispersion were determined by HRTEM. Fig. 1 shows the TEM images of the CdSe nanocrystals. From Fig. 1a, it is observed that the NCs

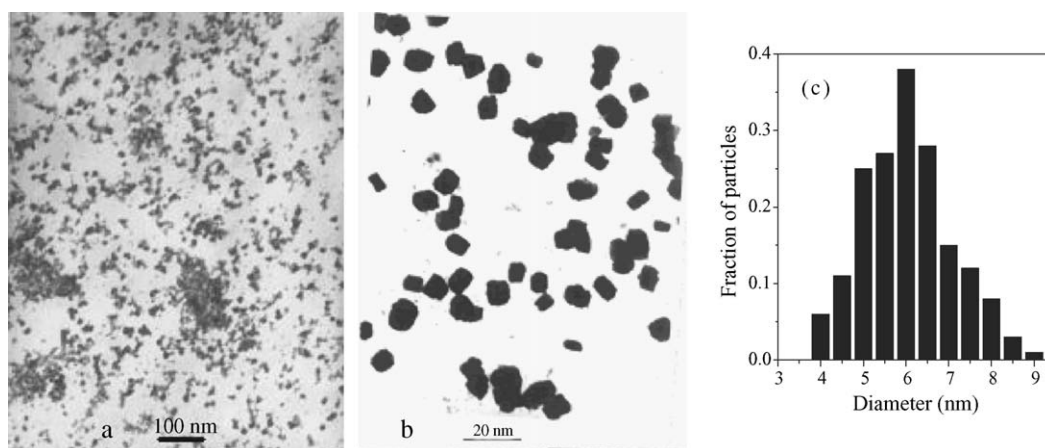


Fig. 1. Low-magnification (a) and higher-magnification (b) TEM images of CdSe NCs and particle size distribution diagram based on TEM images (c).

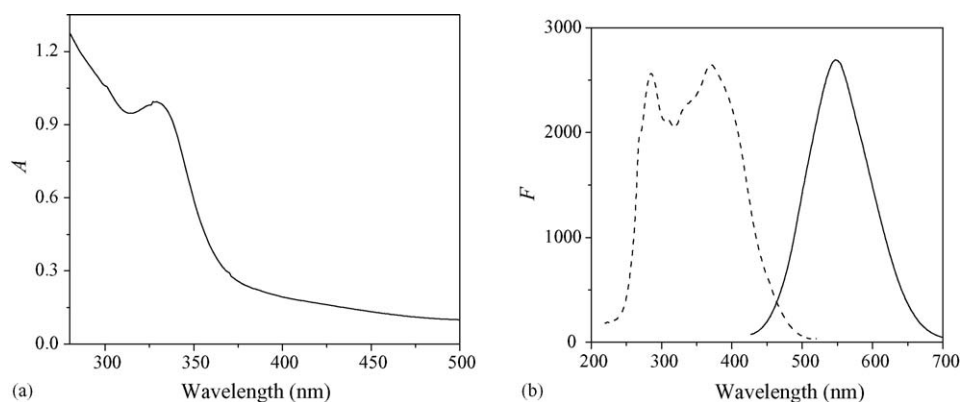


Fig. 2. Absorption (a), excitation (b dashed) and emission (b solid) spectra of CdSe NCs.

are almost monodispersed. And the TEM image of higher magnification (Fig. 1b) indicates an average particle diameter of ca. 6.0 nm. The histogram of cluster size obtained is shown in Fig. 1c, also indicating a size of ca. 6.0 nm of the nanocrystals.

Fig. 2a shows UV–vis absorption spectrum of the CdSe nanocrystals, with a sharp peak centered around 327 nm. It means that the particle size distribution is relatively narrow and particle size is almost homogeneous [22], which is consistent with the conclusion drawn from TEM studies. Emission and excitation spectra of the CdSe NCs are shown in Fig. 2b. The excitation spectrum is broad, while the emission spectrum is narrow and symmetric, which provides a possibility to develop multiplexed detection schemes, allowing excitation of multiple fluorophores with a single light source. Thus it is advisable to use CdSe NCs in bio-detection.

3.2. Stability of CdSe NCs

As observed in Fig. 3, exposure of the NCs solution to the room light results in enhanced luminescence intensity in the first 100 days, and then decreased. This phenomenon may be attributed to the photochemical etching of low quantity of NCs as well as Se atoms with dangling bonds on the NC surface [23,24]. Due to the large surface/volume ratio and lots of vacant electron sites on NCs surface, NCs become reactive upon illumina-

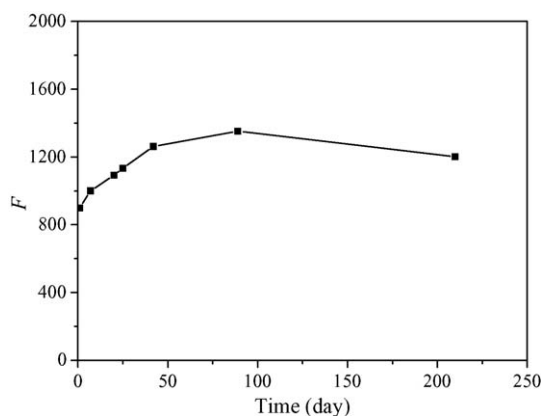


Fig. 3. The stability of CdSe nanocrystals.

tion. Up to now, photoluminescence enhancement effects by illumination were generally explained by photochemical degradations of nanoparticles themselves [25–28].

3.3. Effects of lysozyme concentration

Fig. 4 shows the effects of lysozyme on fluorescence intensity of CdSe NCs. The intrinsic spectral peak wavelength of NCs–Lys was unchanged in comparison with that of free NCs. But the fluorescence intensity was enhanced with the increasing concentration of lysozyme. On this basis, a novel quantitative assay of lysozyme was developed.

3.4. Effects of reaction time and mixing sequence

According to the fluorescence spectra in Fig. 5, the reaction between NCs and lysozyme reaches equilibrium very quickly, and the fluorescence signals are stable for at least 3 days. The good stability is possibly attributed to the long chain molecule of 11-mercaptopundecanoic acid. The polarization of H₂O molecules to CdSe can be prevented by its long chain [29]. And it can also provide a long conjunctive arm between semiconductor nanocrystals and biological materials, making

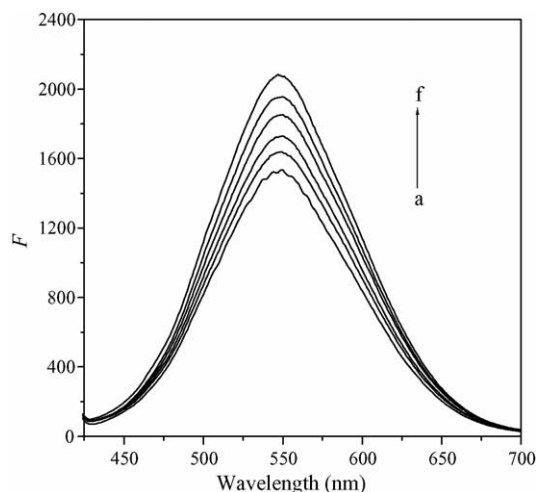


Fig. 4. Fluorescence emission spectra of NCs–Lys solution system. $C_{\text{NCs}} = 2 \times 10^{-4} \text{ mol l}^{-1}$; C_{Lys} ($\mu\text{g ml}^{-1}$): (a) 0; (b) 0.4; (c) 0.8; (d) 1.2; (e) 1.6; (f) 2.0.

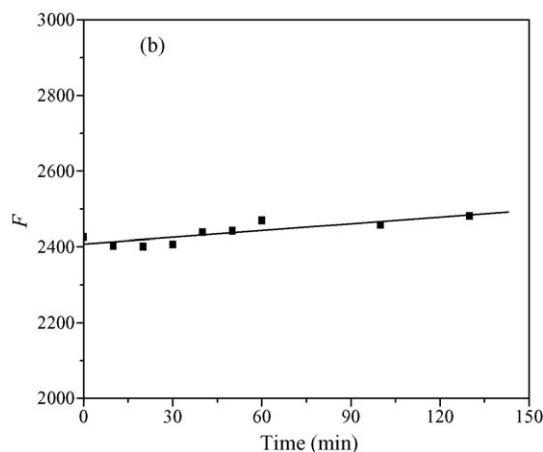


Fig. 5. Effects of reaction time on the fluorescence intensity of NCs–Lys solution system ($C_{\text{CdSe}} = 2 \times 10^{-4} \text{ mol l}^{-1}$; $C_{\text{Lys}} = 2.0 \mu\text{g ml}^{-1}$; pH 7.24).

them combine more easily. Furthermore, the biomolecules could still maintain their activity [29]. This is the unique merit of the NCs, which is lacked among traditional organic dyes. The fluorescence signals were recorded after the system had reacted for 20 min. After investigating several addition sequences, it is found that there is little influence on fluorescence intensity, so NCs and buffer was mixed first, and then lysozyme.

3.5. Effects of pH and buffer

The influence of B–R buffer and PBS buffer on fluorescence intensity was investigated (Fig. 6). It was found that the optimum range of pH was 6.80–7.60 for B–R buffer. If pH value were out of the range, the relative fluorescence intensity would be significantly lower. The reason may be as follows: in acid medium, the fluorescence decreases as a result of the decomposition of the annulus of the Cd^{2+} –11-mercaptoundecanoic acid complexes due to the protonation of the surface-binding thiolates [30]. When pH is too high, lysozyme may lose its activity. In this experiment, 0.5 ml of B–R buffer with pH 7.24 was chosen to run the assay. The pH value is close to human's physiological acidity.

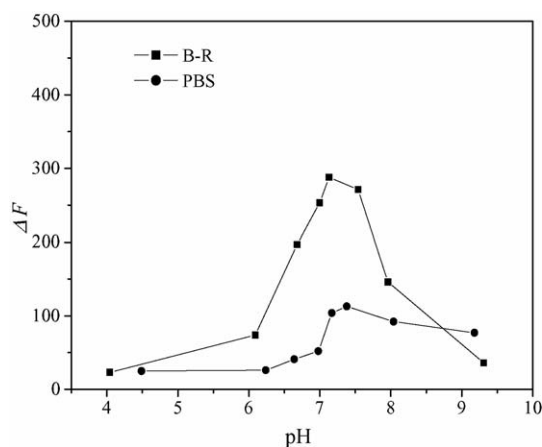


Fig. 6. Effects of buffer solution on the relative fluorescence intensity of system (CdSe : $1.5 \text{ ml } 2 \times 10^{-4} \text{ mol l}^{-1}$; C_{Lys} : $2.0 \mu\text{g ml}^{-1}$).

3.6. Effects of the concentration of NCs

NCs concentration also had effect on fluorescence intensity. If the concentration is too low, the limited NCs molecules cannot occupy all non-specific binding sites of lysozyme coexisting in the system. When the concentration of fluorescence materials is too high, the fluorescence intensity may decrease because of self-quenching effect [31,32]. Considering these factors, 1.5 ml NCs concentration of $2 \times 10^{-4} \text{ mol l}^{-1}$ was applied.

3.7. Effects of diverse substances

To investigate the possibility of practical application, the interference from diverse substances was investigated under chosen condition. For this study, the criterion for interference was fixed at $\pm 10\%$ variation of the average fluorescence intensity calculated for established level of sample. The results show that, Ca(II) , 22.0; Mg(II) , 8.40; Zn(II) , 4.48; Fe(III) , 2.00; Cu(II) , 1.66; Pb(II) , 0.26; Hg(II) , 0.26; Ag(I) , 6.50; glucose, 40.0; fish sperm DNA, 35.0; bovine serum albumin, 32.0; phenylalanine, 12.0; L-tryptophan, 14.8; L-tyrosine, 6.50. Most of the diverse substances have no obvious interference in determination. Although some cations like Hg^{2+} and Pb^{2+} interfered in determination, but their amount is still larger than that in organism.

3.8. Linearity, sensitivity and precision

Under the optimal condition mentioned above, there are good linear relationships between relative fluorescence intensity ($\Delta F = F_0 - F$) and the concentration of lysozyme in the range of 0.200 – $26.0 \mu\text{g ml}^{-1}$, with a correlation coefficient (r) of 0.9994. The calibration curve established the following equation, $\Delta F = 4.89 + 51.83C$ (C , $\mu\text{g ml}^{-1}$). The detection limit ($3S_0/K$) was $0.115 \mu\text{g ml}^{-1}$, where S_0 is the standard deviation of blank measurements ($n = 10$) and K is the slope of calibration line. The relative standard deviation (R.S.D.) is 1.72% obtained from a series of 10 standards, each containing $2.00 \mu\text{g ml}^{-1}$ of lysozyme.

3.9. Analytical applications

To test the applicability of the proposed spectrofluorometric method, the CdSe NCs fluorescence probe was used to determine lysozyme in eggs.

The method of extracting lysozyme from eggs followed previous literatures [18,33]. Determination of the extracted lysozyme is described in Section 2.4. The recovery (R) is defined by equation $R = [(C_T - C_1)/C_2] \times 100\%$, where C_1 is the amount of extracted lysozyme from eggs, and C_2 is the amount of standard solution of lysozyme. C_T is obtained from the linear equation $\Delta F = 4.89 + 51.83C$. The results are shown in Table 1. It can be seen that the recovery is between 96% and 105%, which meets the requirement of microanalysis. The R.S.D. is lower than 5%, showing a good precision of this method.

Table 1

Analytical results of the samples ($P=95\%$, $n=5$)

Sample	Extracted Lys (C_1 , $\mu\text{g ml}^{-1}$)	Added Lys (C_2 , $\mu\text{g ml}^{-1}$)	Found ^a (C_T , $\mu\text{g ml}^{-1}$)	Average recovery (%)	R.S.D. (%)
1	13.6 ± 0.1	4.0	17.6 ± 0.4	100	1.7
2	18.0 ± 0.5	2.0	20.1 ± 0.8	105	3.5
3	9.0 ± 0.3	5.0	13.8 ± 0.5	96	2.7

^a Found value together with confidence interval.

4. Conclusions

A novel kind of CdSe nanocrystals as a sensitive and photostable fluorescence probe in biological assays was developed. The advantage of this method is outstanding high selectivity for lysozyme. This feasibility of conjugation is due to electrostatic interactions between negatively charged 11-macaptoundecanoic-acid-capped CdSe NCs and positively charged lysozyme. When the buffered solutions are at pH 7.24, it would be positively charged as the isoelectric pH value of lysozyme is 11.2. While a large sum of other protein is lower than 7, it would be negatively charged. That is why the NCs so specific for lysozyme.

The synthesis of these NCs is easily carried out at low cost. They can be used as direct sensors in real samples for their optical features of tunability. The approach for assaying lysozyme proposed in this article can be a general one, not restricted to a particular type of biomolecule as selected in this study. The nanocrystals would attract more attention and be applied widely in biological assays.

Acknowledgement

The present work was supported by the Natural Science Foundation of Guangdong Province (No. 04010369).

References

- [1] E. Schröck, S. du Manoir, T. Veldman, B. Schoell, J. Wienberg, M.A. Ferguson-Smith, Y. Ning, D.H. Ledbetter, I. Bar-Am, D. Soenksen, Y. Garini, T. Ried, *Science* 273 (1996) 494.
- [2] M.B. Jr, M. Moronne, P. Gin, S. Weiss, A.P. Alivisatos, *Science* 281 (1998) 2013.
- [3] W.C.W. Chan, S.M. Nie, *Science* 281 (1998) 2016.
- [4] F.X. Wu, J.W. Lewis, D.S. Kliger, J.Z. Zhang, *J. Chem. Phys.* 118 (2003) 12.
- [5] X.H. Zhong, Y.Y. Feng, W. Knoll, M.Y. Han, *J. Am. Chem. Soc.* 125 (2003) 13559.
- [6] M. Shingyoji, D. Gerion, D. Pinkel, J.W. Gray, F.Q. Chen, *Talanta* 67 (2005) 472.
- [7] K. Richard, C.L.T.M. laudia, K.M. Rajesh, *J. Colloid Interface Sci.* 227 (2000) 561.
- [8] C.P. Collier, S. Henrichs, J.J. Shiang, R.J. Saykally, J.R. Heath, *Science* 277 (1997) 1978.
- [9] N. Gomez, J.O. Winter, F. Shieh, A.E. Saunders, B.A. Korgel, C.E. Schmidt, *Talanta* 67 (2005) 462.
- [10] Z.Q. Ye, M.Q. Tan, G.L. Wang, J.L. Yuan, *Talanta* 65 (2005) 206.
- [11] M.Y. Han, X.H. Gao, J.Z. Su, S.M. Nie, *Nat. Biotechnol.* 19 (2001) 631.
- [12] K.M. Gattás-Asfura, R.M. Leblanc, *Chem. Commun.* 21 (2003) 2684.
- [13] L.Y. Wang, L. Wang, *Analyst* 127 (2002) 1531.
- [14] L.Y. Wang, L. Wang, *Analyst* 127 (2002) 977.
- [15] L. Wang, L.Y. Wang, *Anal. Chem. Acta* 468 (2002) 35.
- [16] E.R. Goldman, G.P. Anderson, P.T. Tran, H. Mattoussi, P.T. Charles, J.M. Mauro, *Anal. Chem.* 74 (2002) 841.
- [17] B.M. Lingerfelt, H. Mattoussi, E.R. Goldman, J.M. Mauro, G.P. Anderson, *Anal. Chem.* 75 (2003) 4043.
- [18] L. Pellegrino, A. Tirelli, *Int. Dairy J.* 10 (2000) 435.
- [19] M. Gao, A.L. Rogach, A. Kornowski, S. Kirstein, A. Eychmüller, H. Mohwald, H. Weller, *J. Phys. Chem. B.* 102 (1998) 8360.
- [20] E. Hao, H. Zhang, B. Yang, H. Ren, J.C. Shen, *J. Colloid Interface Sci.* 238 (2001) 285.
- [21] H. Zhang, Z. Zhou, B. Yang, *J. Phys. Chem. B* 107 (2003) 8.
- [22] Y. Zhang, Q. Peng, X. Wang, Y.D. Li, *Chem. Lett.* 33 (2004) 1320.
- [23] D.V. Talapin, A.L. Rogach, E.V. Shevchenko, A. Kornowski, M. Haase, H. Weller, *J. Am. Chem. Soc.* 124 (2002) 5782.
- [24] N. Gaponik, D.V. Talapin, A.L. Rogach, K. Hoppe, E.V. Shevchenko, A. Kornowski, H. Weller, *J. Phys. Chem. B* 106 (2002) 7177.
- [25] S.R. Cordero, P.J. Carson, R.A. Estabrook, G.F. Strouse, S.K. Buratto, *J. Phys. Chem. B* 104 (2000) 12137.
- [26] D.E. Dunstan, A. Hagfeldt, M. Almgren, H.O.G. Siegbahn, E. Mukhtar, *J. Phys. Chem.* 94 (1990) 6797.
- [27] H. Asami, Y. Abe, T. Ohtsu, I. Kamiya, M. Hara, *J. Phys. Chem. B* 107 (2003) 12566.
- [28] A. Shavel, N. Gaponik, A. Eychmüller, *J. Phys. Chem. B* 108 (2004) 5905.
- [29] H. Mattoussi, J.M. Mauro, E.R. Goldman, G.P. Anderson, V.C. Sundar, F.V. Mikulec, M.G. Bawendi, *J. Am. Chem. Soc.* 122 (2000) 12142.
- [30] M. Gao, S. Kirstein, H. Mhwal, A.L. Rogach, A. Kornowski, A. Eychmüller, H. Weller, *J. Phys. Chem. B* 102 (1998) 8360.
- [31] K.K. Rohatgi, *Indian J. Chem.* 7 (1970) 1020.
- [32] G.Z. Chen, X.Z. Huang, J.G. Xu, *Fluorimetry [M]*, Science Publication, Beijing, 1990, pp. 27–28.
- [33] X.Q. Qi, *Appl. Sci. Technol. (China)* 7 (1999) 6.