

Talanta

The International Journal of Pure and Applied Analytical Chemistry

Editors-in-Chief

Professor G.D. Christian, University of Washington, Department of Chemistry, 36 Bagely Hall, P.O. Box 351700, Seattle, WA 98195-1700, U.S.A.

Professor J.-M. Kauffmann, Université Libre de Bruxelles, Institut de Pharmacie, Campus de la Plaine, C.P. 205/6, Boulevard du Triomphe, B-1050 Bruxelles, Belgium

Associate Editors

Professor J.-H. Wang, Research Center for Analytical Sciences, Northeastern University, Box 332, Shenyang 110004, China

Professor J.L. Burguera, Los Andes University, IVAQUIM, Faculty of Sciences, P.O. Box 542, 5101-A Mérida, Venezuela.

Assistant Editors

Dr R.E. Synovec, Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195-1700, U.S.A.

Professor J.-C. Vire, Université Libre de Bruxelles, Institut de Pharmacie, Campus de la Plaine, C.P. 205/6, Boulevard du Triomphe, B-1050 Bruxelles, Belgium

Talanta

R. Apak (Istanbul, Turkey)
E. Bakker (Auburn, AL, U.S.A.)
D. Barceló (Barcelona, Spain)
B. Birch (Luton, UK)
K. S. Booksh (Tempe, AZ, U.S.A.)
J.-L. Capelo-Martinez (Caparica, Portugal)
Z. Cai (Kowloon, Hong Kong)
S. Cosnier (Grenoble, France)
D. Diamond (Dublin, Ireland)
W. Frenzel (Berlin, Germany)
A.G. Gonzales (Seville, Spain)
E.H. Hansen (Lyngby, Denmark)
P. de B. Harrington (OH, U.S.A.)

A. Ho (Hsin-chu, Taiwan)
J. Kalivas (Pocatella, ID, U.S.A.)
B. Karlberg (Stockholm, Sweden)
J.-M. Lin (Beijing, China)
Y. Lin (Richland, WA, U.S.A.)
M.D. Luque de Caastro (Cordoba, Spain)
I.D. McKelvie (Victoria, Australia)
S. Motomizu (Okayama, Japan)
D. Nacapricha (Bangkok, Thailand)
J.-M. Pingarron (Madrid, Spain)
E. Pretsch (Zürich, Switzerland)
W. Schuhmann (Bochum, Germany)
M. Shamsipur (Kermanshah, Iran)

M. Silva (Porto Alegre, Brazil)
P. Solich (Hradec Králové, Czech Republic)
K. Suzuki (Yokohama, Japan)
D.G. Themelis (Thessaloniki, Greece)
D.L. Tsalev (Sofia, Bulgaria)
Y. van der Heyden (Belgium)
B. Walczak (Katowice, Poland)
J. Wang (Tempe, AZ, U.S.A.)
J.D. Winefordner (Gainesville, U.S.A.)
Xiu-Ping Yan (Tianjin, China)
E.A.G. Zagatto (Piracicaba, SP, Brazil)

Copyright © 2007 Elsevier B.V. All rights reserved

Publication information: *Talanta* (ISSN 0039-9140). For 2007, volumes 71–73 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.

Orders, claims, and journal enquiries: please contact the Customer Service Department at the Regional Sales Office nearest you:

Orlando: Elsevier, Customer Service Department, 6277 Sea Harbor Drive, Orlando, FL 32887-480 USA; phone: (+1) (877) 8397126 [toll free number for US customers], or (+1) (407) 3454020 [customers outside US]; fax: (+1) (407) 3631354; e-mail: usjcs@elsevier.com

Amsterdam: Elsevier, Customer Service Department, PO Box 211, 1000 AE Amsterdam, The Netherlands; phone: (+31) (20) 4853757; fax: (+31) (20) 4853432; e-mail: nlinfo-f@elsevier.com

Tokyo: Elsevier, Customer Service Department, 4F Higashi-Azabu, 1-Chome Bldg, 1-9-15 Higashi-Azabu, Minato-ku, Tokyo 106-0044, Japan; phone: (+81) (3) 5561 5037; fax: (+81) (3) 5561 5047; e-mail: jp.info@elsevier.com

Singapore: Elsevier, Customer Service Department, 3 Killiney Road, #08-01 Winsland House I, Singapore 239519; phone: (+65) 63490222; fax: (+65) 67331510; e-mail: asiainfo@elsevier.com

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\$ 3,818 (valid in North, Central and South America), including air speed delivery. Application to mail at periodical postage rate is paid at Rathway, NJ and additional mailing offices.

USA POSTMASTER: Send address changes to *Talanta*, Publications Expediting Inc., 200 Meacham Avenue, Elmont, NY 11003.

AIRFREIGHT AND MAILING in the USA by Publications Expediting Inc., 200 Meacham Avenue, Elmont, NY 11003.

Separation and determination of alkylglycosides by liquid chromatography with electrospray mass spectrometric detection

Miriam Beneito-Cambra, Virginia Bernabé-Zafón,
José Manuel Herrero-Martínez, Guillermo Ramis-Ramos *

Departament de Química Analítica, Universitat de València, Dr. Moliner 50, E-46100 Burjassot, València, Spain

Received 26 February 2007; received in revised form 10 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

The separation of alkylpolyglycosides by liquid chromatography with electrospray mass spectrometric detection, using either an alkylamide or a cyanopropyl column, and acetonitrile/water mixtures as mobile phases, was developed. Using the alkylamide column and isocratic elution, the α - and β -epimers and ring isomers (pyranosides and furanosides) of the alkylmonoglycosides were resolved. The ring isomers were also resolved in a much shorter time using the cyanopropyl column with gradient elution. Using these columns, the isomers of the alkyldiglycosides and alkyltriglycosides were also partially resolved. The equilibration time was much shorter with the cyanopropyl column, which was selected to perform quantitation studies. The response factors increased more than an order of magnitude with the length of the alkyl chain, from the methyl to the decylmonoglycoside, and decrease largely for the dodecyl and tetradecylmonoglycoside. The limits of detection were of ca. 25 μ M from the hexyl up to the dodecylmonoglycoside. The procedures were applied to the characterisation and determination of alkylmonoglycosides in toiletries. © 2007 Elsevier B.V. All rights reserved.

Keywords: Alkylpolyglycosides; Alkylglycofuranosides; Alkylglycopyranosides; Electrospray ionisation mass spectrometry; Ion-trap mass spectrometry

1. Introduction

The alkylpolyglycosides (APGs) are non-ionic surfactants produced from renewable materials (glucose and fatty alcohols) [1–6]. Owing to their favourable properties in terms of foaming performance, synergism with other surfactants, and environmental and skin compatibility, they are widely used in cleaning and personal care products [2,4,6–8], and in the oil and gas industry [9]. APGs are industrially obtained as complex mixtures of alkyloligoglycosides. The oligomers are distinguished by the alkyl chain length, the number of glucose units and the isomerism (Fig. 1). Three types of isomerism are present: stereoisomerism with α - and β -epimers, ring isomerism (pyranoside and furanoside forms), and positional isomers, with predominant 1,4- and 1,6-interglycosidic linkages between glucose units.

The total amount of APGs can be determined by hydrolysis followed by derivatisation with anthrone and colorimetry [10,11], potentiometric titration after sulfonation with the

SO₃–DMF complex [12], near-infrared spectrometry [13] and enzymatic hydrolysis [14]. APGs can be determined in environmental samples after hydrolysis, followed by distillation of the alcohols and fluorogenic derivatisation [15]. The separation by thin layer chromatography on C8 and C18 phases has been described [11,12,16–18]. Underivatised alkylmonoglycosides can be separated with isomer resolution using high-temperature GC [4,17]. APGs have been also separated by GC after silylation [12,17,19]. The pyranoside and furanoside forms can be distinguished by the different fragments obtained by GC–MS [19]. Micellar electrokinetic chromatography with electrochemical detection has been applied to the determination of APGs in shampoo [20]. To implement the photometric detection of APGs after HPLC separation, several post-column chromogenic derivatisation procedures have been described [21]. Alternatively, refractive index detection [18], evaporative light scattering (ELSD) [11,12,22–24] or MS detection [23,25–27], can be used. The chromatographic behaviour of alkylglycopyranoside standards using C8, C18, phenyl and polyvinylalcohol columns has been described [22], and the separation of APGs with silica, C18 and polyvinylalcohol columns have been compared [23]. Eichhorn and Knepper [25] have used HPLC–MS

* Corresponding author. Tel.: +34 963 543 003; fax: +34 963 544 436.
E-mail address: ramis@uv.es (G. Ramis-Ramos).

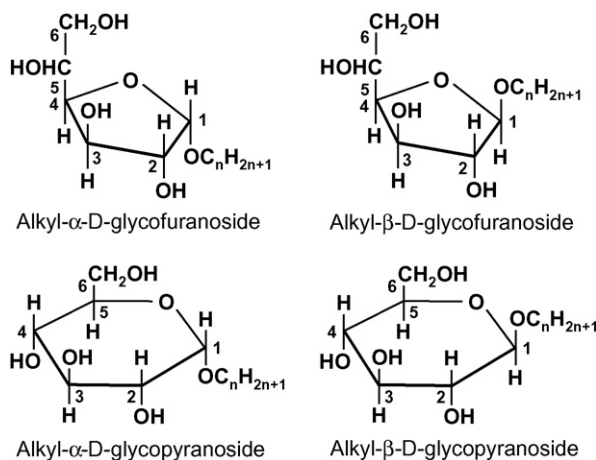


Fig. 1. Chemical structures of the epimers and ring isomers of alkylmonoglycosides.

with an electrospray ion source (ESI) to determine APGs in spiked river and waste waters. Using a C18 column and gradient elution with acetonitrile/water, the α - and β -epimers and the ring isomers were resolved. Alkylglycopyranosides can be further distinguished by their higher affinity to form $[M + \text{NH}_4]^+$ adducts with respect to alkylglycofuranosides. Kuhn and Neubert [27] have used HPLC–ESI–QTOF–MS with a C18 column, a methanol/water mobile phase and gradient elution, to characterise industrial mixtures of APGs; however, isomers were not resolved.

In this work, the HPLC separation of APG mixtures using either an alkylamide or a cyanopropyl column, with acetonitrile/water mixtures as mobile phases, was studied. The alkylamide column in isocratic conditions was adequate to separate alkylmonoglycosides with full resolution between epimers and ring isomers. Retention was lower on the cyanopropyl column, but equilibration time was much shorter. Using the cyanopropyl column with gradient elution the ring isomers were also resolved. The influence of the alkyl chain length on the response factors was studied. The procedures were applied to the characterisation and determination of APGs in toiletries.

2. Experimental

2.1. Reagents and acronyms

Reagent grade methyl- α - (>99%), methyl- β - (>99%), hexyl- β - (>98%), octyl- α - (>98%), octyl- β - (>99%), decyl- β - (>99%) and dodecyl- β -D-glycopyranosides (>99%, Fluka, Buchs, Switzerland), were used as standards. Nonyl- β -D-glycopyranoside (>99%, Glycon Bioch. GmbH, Biotechnol., Luckenwalde, Germany) was used as internal standard. Glucopone 215 CS UP (technical grade mixtures of APGs, Fluka), Plantacare 818 UP (kindly donated by Dr. U. Gollman, Cognis Deutschland, Düsseldorf, Germany), acetonitrile, methanol, acetic acid, HCl (Scharlab, Barcelona, Spain), sodium acetate (Sigma–Aldrich, Steinheim, Germany) and deionised water (Barnstead deionizer, Sybron, Boston, MA) were also used. Acronyms of the form C_nG_m , where n is the number of car-

bon atoms in the alkyl chain, and m the number of glucose units, were used, and when necessary, a “p” or an “f” was added to distinguish between pyranosides and furanosides (e.g. C8G1p = octylmonoglycopyranoside).

2.2. Instrumentation and procedures

An 1100 Series VL ITMS system, provided with an ESI source (Agilent Technologies, Waldbronn, Germany) was used. For continuous infusion in the MS, a syringe pump (kdScientific, Holliston, MA, USA) set at a constant flow of 0.3 mL h^{-1} ($5 \mu\text{L min}^{-1}$), was used. Continuous infusion experiments were used both to examine the spectra of the standards and industrial mixtures, and to optimise the MS detection conditions. For HPLC–MS, an alkylamide column ($100 \text{ mm} \times 2.1 \text{ mm}$, ID $3 \mu\text{m}$, Supelco, Bellefonte, USA) and a cyanopropyl column ($75 \text{ mm} \times 3 \text{ mm}$, ID $3 \mu\text{m}$, Supelco) were used. Acetonitrile/water mixtures, containing 20 mM acetic acid and 20 mM sodium acetate (pH 4.7 buffer), at a flow rate of 0.2 mL min^{-1} , were used as mobile phases; however, a flow rate of 0.4 mL min^{-1} was also used to speed up isocratic elution with mobile phases containing 20% acetonitrile. The scan range was m/z 50–800, the capillary voltage was 4 kV, skimmer 2 was set at 6 V, and the voltage of skimmer 1 was automatically selected as a function of the target mass. The m/z value of the most abundant ion was selected as target mass. In all cases, the maximum loading of the ion trap was 3×10^4 counts with a maximum accumulation time of 300 ms. With the continuous infusion experiments, the spectra were averaged during 2 min. Nitrogen (Gaslab NGLC MS 20 generator, Equcien, Madrid, Spain) was used as the spray (25 psi) and dry gas (8 L min^{-1} at 300°C), and He (C-50, Carbueros Metálicos, Aranjuez, Spain) was the collision gas. The Agilent LC/MSD v. 4.2 software was used for data analysis.

Stock solutions of the standards and technical grade mixtures containing $1000 \mu\text{g mL}^{-1}$ were prepared in 50:50 (v/v) MeOH/water; however, owing to its limited solubility, the stock solution of C12G1 was $500 \mu\text{g mL}^{-1}$. For continuous infusion in the MS, these solutions were further diluted with a 50:50 (v/v) MeOH/water mixture containing 20 mM sodium acetate. For HPLC, $2000 \mu\text{g mL}^{-1}$ Glucopone and Plantacare solutions in 50:50 (v/v) MeOH/water were injected. A hand cream and a baby shampoo were 1:100 diluted with 50:50 (v/v) MeOH/water and injected. All injected solutions were filtered through a $0.45 \mu\text{m}$ pore-size nylon membrane (Albet, Germany). The HPLC injection volume was $20 \mu\text{L}$.

3. Results and discussion

3.1. Optimisation of working conditions using continuous infusion MS

Using continuous infusion MS in the negative-ion mode, peaks of the $[M - \text{H}]^-$ ions of the standards and the components of Glucopone and Plantacare were obtained. In the positive-ion mode, the corresponding $[M + \text{H}]^+$ and $[M + \text{Na}]^+$ peaks were observed. The $[M + \text{Na}]^+$ peaks largely predominated

when sodium acetate was added to the infused solution. Maximal intensity was obtained with 20 mM sodium acetate. The positive-ion mode, which gave peak intensities ca. 20 times larger than those obtained with the negative-ion mode, was selected. Glucopone and Plantacare showed several groups of $[M + Na]^+$ peaks, each of them corresponding to the oligomers with a given number of glucose units, up to $m = 6$. Within each group, two large peaks corresponding to the C8Gm and C10Gm oligomers, and a small one due to the C12Gm oligomer, were observed. In agreement with the declared composition, the intensity decreased as m increased. Using infusion of the Glucopone solution, the dry gas flow, spray temperature and spray pressure were optimised.

3.2. Separation of APGs by HPLC–MS in isocratic conditions

The alkylamide column and a mixture containing 20:80 acetonitrile/water in the presence of the acetic acid/sodium acetate buffer were first used. The extracted-ion chromatograms (EICs) at the m/z values observed in the infusion spectra were examined. The alkylmonoglycopyranoside standards gave single chromatographic peaks, and complex chromatograms were obtained for the technical grade mixtures. As shown in Fig. 2A for Glucopone, a complete separation of the four C8G1 isomers (α - and β -epimers, and ring isomers, on the EIC at m/z 315) was achieved. The epimers and ring isomers of C10G1 and C12G1 appeared also resolved at longer retention times (not shown). Also, a partial separation of the isomers of the alkyldiglycosides (C8G2) was evidenced (EIC at m/z 477). The identification of α - and β -C8G1p was made by injecting solutions of the corresponding standards. As far as we know, standards of alkylpolyglycofuranosides are not available; however, the two peaks of low intensity which appeared at longer retention times than the α - and β -C8G1p peaks on the m/z 315 trace, were attributed to the corresponding α - and β -C8G1f isomers. Retention should be higher for these isomers as a consequence of the longer $-\text{CH}(\text{OH})\text{CH}_2\text{OH}$ chain bound to the alkylglycofuranoside ring in comparison with the $-\text{CH}_2\text{OH}$ chain of the alkylglycopyranosides.

Next, to reduce the analysis time of the C10Gn and C12Gn isomers, the acetonitrile concentration in the mobile phase was increased. With 30% acetonitrile (Fig. 2B), the α - and β -epimer pairs of C8G1 were not resolved, but the four isomers of C10G1 were resolved within 20 min. As also shown in Fig. 2B, the presence of alkyldiglycosides (C8G2 and C10G2) and alkyltriglycosides (C8G3) with partial isomer resolution was evidenced. The chromatograms of Plantacare were similar to those of Glucopone, but with higher proportions of the C10Gm and C12Gm oligomers with respect to those of C8Gm (not shown). A chromatogram of a baby shampoo, eluted with 50% acetonitrile, is shown in Fig. 2C. The ring isomers of the alkylmonoglycosides of C8G1, C10G1, C12G1 and C14G1 were resolved. The α - and β -epimers of C12G1f, C14G1p and C14G1f were also resolved. The isomers of the alkyldiglycosides C8G2, C10G2 and C12G2 were partially resolved.

Retention was much weaker with the cyanopropyl column; for instance, using 20% acetonitrile, the C8G1 isomers were

eluted within 6 min (not shown). Using 95 and 100% water in the mobile phase, the C8G1 isomers appeared within 30 and 40 min, respectively. In all cases, the ring isomers were well resolved, but the epimer pairs were poorly resolved with this column.

3.3. Separation of APGs by HPLC–MS with gradient elution

The alkylamide column and mobile phases containing 60:40 (A) or 90:10 (B) acetonitrile/water (v/v) in the presence of the acetic/acetate buffer were used. Mobile phase A was first pumped until stabilisation of the pressure, then the Plantacare solution was injected and the composition of the mobile phase was linearly varied from A to B in 20 min. The alkylmonoglycosides were separated with partial resolution between the epimers, and with excellent resolution between the ring isomers, within 12 min (not shown). However, the re-equilibration of this column was observed to be rather slow. Pumping of mobile phase A during several hours was required to achieve reproducibility between successive injections.

Using the cyanopropyl column, mobile phases containing 25:75 (A) and 90:10 (B) acetonitrile/water (v/v) in presence of the acetic acid/sodium acetate buffer, were used. The mobile phase was linearly varied from A to B in 15 min. As shown in Fig. 3A for Plantacare, all the alkylmonoglycosides, from C8G1 up to C16G1, were separated within 14 min. The epimer pairs were not resolved, but a good resolution between the ring isomers was achieved. Successive injections of the sample were performed after pressure stabilisation (15 min). Excellent reproducibility of the chromatograms was obtained. The chromatogram of a hand cream, which has a rather complex matrix, showed the resolved peaks of the ring isomers of C12G1 and C14G1 (Fig. 3B).

3.4. Quantitation studies

The calibration curves were obtained using the cyanopropyl column in the optimised gradient elution conditions. Solutions containing a mixture of the standards (β -pyranosides C1G1, C6G1, C8G1, C10G1 and C12G1), in the presence of $50 \mu\text{g mL}^{-1}$ β -pyranoside C9G1 as internal standard, were injected. Mixtures of the α -pyranosides of C1G1 and C8G1 were also injected. The concentration of the standards was varied from 10 to $120 \mu\text{g mL}^{-1}$ (from ca. 60–600 μM for C1G1 to 30–360 μM for C12G1). In all cases, the calibration curves gave a good linearity, with $r^2 > 0.99$ (6 points per curve).

As shown in Fig. 4, the relative sensitivities of the β -epimers (or response factors, calculated by using α -C1G1p as reference) increased almost linearly from C1G1 to C8G1, reached a maximum at C10G1 and decreased for C12G1. Relative rather than absolute sensitivities were plotted in this figure to enhance the differences between the standards, independently from detector sensitivity and some working conditions. A range of relative sensitivities for C14G1 was also plotted on Fig. 4. This range was obtained by injecting Plantacare, and using the sum of the areas of the C14G1p and C14G1f peaks, and the composition given

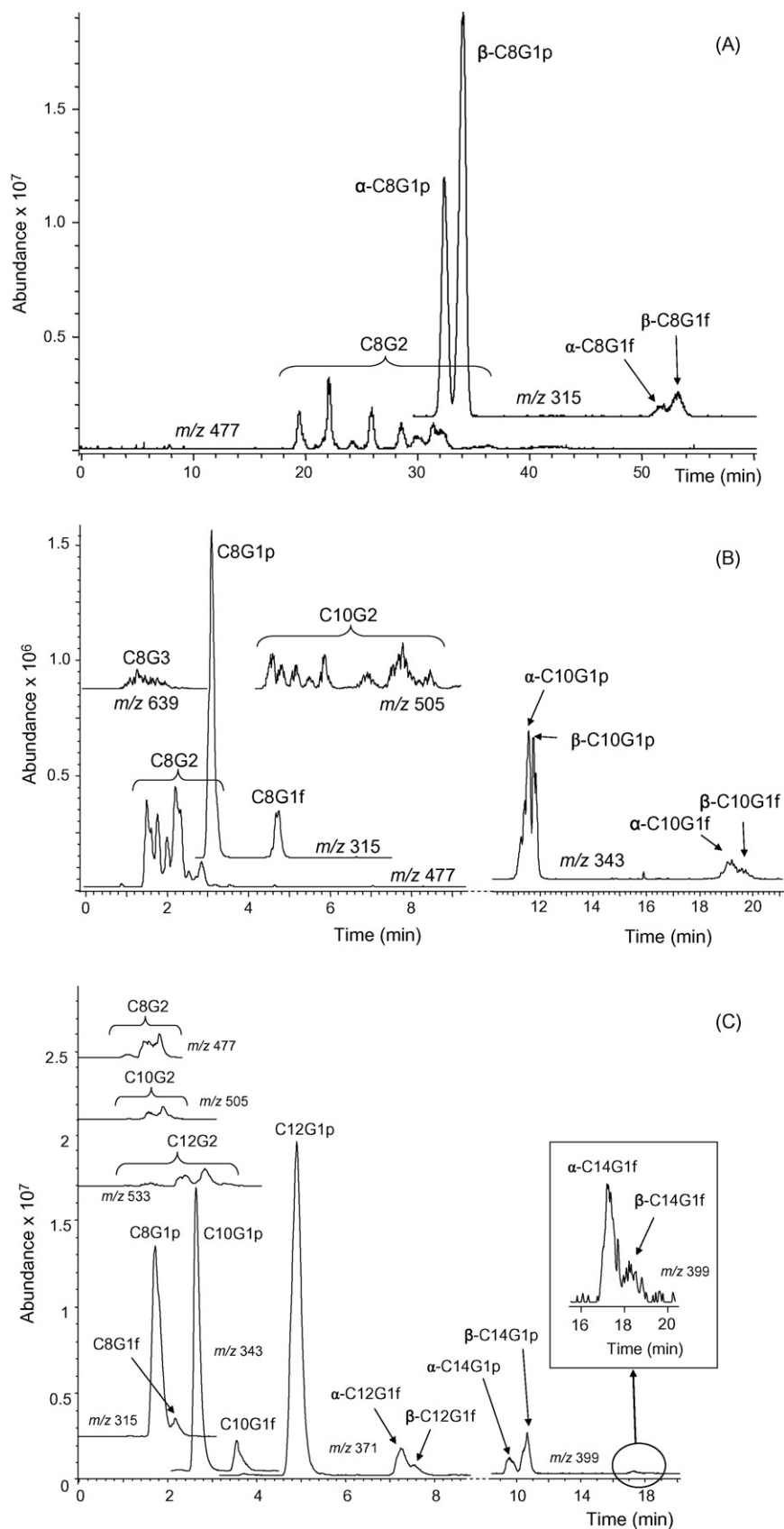


Fig. 2. EICs of Glucopone (A and B) and a baby shampoo (C). The alkylamide column was used. The mobile phase contained water with 20% (A), 30% (B) and 50% (C) acetonitrile in the presence of 40 mM acetic/acetate buffer of pH 4.7. The flow rate was 0.4 mL min^{-1} (A) and 0.2 mL min^{-1} (B and C). In (C), the inset shows the m/z 399 trace with the intensity axis multiplied by 50.

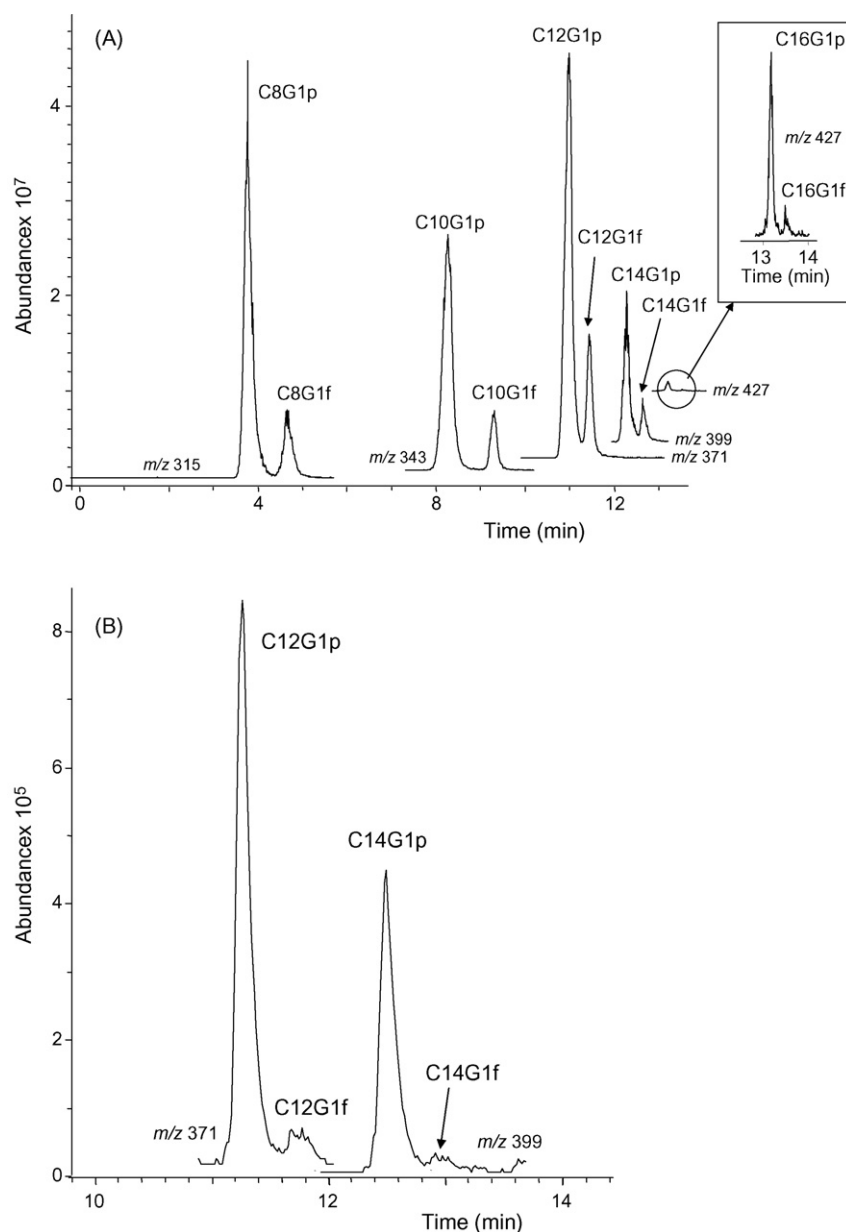


Fig. 3. EICs of Plantacare (A) and a hand cream (B). The cyanopropyl column was used. The composition of the acetonitrile/water mobile phase (also containing 40 mM of the acetic/acetate buffer of pH 4.7) was linearly varied from 25 to 90% acetonitrile in 15 min. The flow rate was 0.2 mL min⁻¹. In (A), the inset shows the *m/z* 427 trace with the intensity axis multiplied by 20.

by the manufacturer (6.1–9.5% total C14G1 for a 51–53% pure product), to calculate the relative sensitivity range. As deduced from Fig. 4, this range indicated a further decrease of the relative sensitivity for alkyl chains longer than 12 carbon atoms. Since a range of concentrations was not declared (Table 1), the relative sensitivity for C16G1 could not be calculated.

The influence of the mobile phase composition on the relative sensitivities was also studied. For this purpose, a 20:80 (A') or a 75:25 (B') acetonitrile/water (v/v) mixtures, containing 20 mM sodium acetate and 20 mM acetic acid, were used. Dilutions of the stock solutions of the standards were prepared using both A' and B'. The dilution factor was 1:20, but 1:10 was used for C12G1. All the diluted solutions also contained 50 µg mL⁻¹ of the internal standard, β-C9G1p. The autosampler

Table 1
Declared and found concentrations of alkylmonoglucosides in Plantacare^a

Compound	Declared (%)	Found (%)
C6G1	Max. 0.26	0.06
C8G1	12.2–15.9	13.0
C10G1	7.6–11.7	9.7
C12G1	18.9–22.3	18.9
C14G1	6.1–9.5	–
C16G1	Max. 2.12	–

^a Range from minimal up to maximal declared concentrations multiplied by minimal (51%) and maximal (53%) declared purity, respectively.

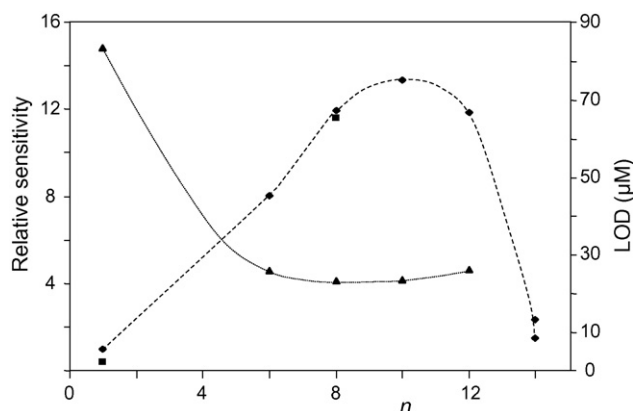


Fig. 4. Relative sensitivities (left axis and dashed line) of α - (■) and β -alkylglycopyranosides (◆) with respect to β -C1G1, and LODs (▲, right axis and dotted line) vs. the number of carbon atoms in the alkyl chain, n (β -C9G1p used as internal standard). All values were obtained from pure standards, but for C14G1 the two points delimit the range of relative sensitivity, which was estimated from its concentrations in Plantacare (6.1–9.5% C14G1 for a 51–53% pure product, as declared by the manufacturer).

was directly connected with the ESI source with a PEEK tube in the absence of the separation column, and the solutions were injected successively using A' and B' as mobile phases. No significant differences between the peak areas obtained with mobile phases A' and B' were observed. Thus, the differences among the response factors observed in Fig. 4 should be attributed to the length of the alkyl chains rather than to the increase of the acetonitrile concentration along the elution gradient.

Taking into account that Na^+ is bound to the same oxygen as the alkyl chain [26], the increase of the relative sensitivity from C1G1 up to C10G1 can be due to stabilisation of the adduct by the higher electronic density provided by the longer alkyl chain. The smaller volatility, the formation of ion-pairs or micellization are possible explanations for the sensitivity decrease when the alkyl chain has 12 and 14 carbon atoms. Thus, the response factor can be approximately predicted by linear interpolation within the C1G1–C10G1 range, but not when $n > 10$. Owing to the large differences, large systematic errors should be expected if calibration is not performed by using all the appropriate $\text{C}_n\text{G1}$ standards.

The relative sensitivities of the α -epimers of C1G1p and C8G1p with respect to β -C1G1p (used as a reference compound) were also plotted on Fig. 4. The α -epimer/ β -epimer sensitivity ratio was 0.995 for C8G1, but 0.43 for C1G1. Thus, the response factor of the α -epimer was less than half that of the β -epimer for C1G1, approaching to the sensitivity of the corresponding β -epimer for longer alkyl chains. This can be explained by a lower ability of the α -epimer of C1G1 to bind Na^+ with respect to the β -epimer. Further, the higher electronic density on the oxygen atom bound to the chain could explain both the sensitivity increase when the alkyl chain becomes longer, and the small sensitivity differences between the C8G1 epimers. Small or negligible systematic errors should be expected by using the calibration curve of a $\text{C}_n\text{G1}$ epimer ($n \geq 6$) to predict concentrations of the other epimer, or the sum of the concentrations of the two epimers.

The limits of detection (LODs) were obtained as the concentrations giving a peak area equal to three times the standard deviation of the calibration points with respect to the regression straight-line (standard deviation of the residuals). As observed in Fig. 4, the LODs were 85 μM for C1G1 and of ca. 25 μM from C6G1 up to C12G1, which agrees with the higher response factors of the latter. Also, from the standard deviation of the residuals, the limits of quantitation (as the concentrations giving a 10% precision in the determination of the analytes) were 280 μM for C1G1 and 80 μM from C6G1 up to C12G1.

When the industrial products were analysed, significant differences between the peak area ratios of the respective furanose/pyranose forms were observed. Thus, integration of the areas for the C10G1 and C12G1 isomer pairs showed that this ratio was 0.12–0.13 for Glucopone and the baby shampoo, 0.26–0.30 for Plantacare and ca. 0.04 for the hand cream. Thus, the furanose/pyranose concentration ratio can be used to characterise industrial samples. However, owing to the lack of standards, only the total concentration of each $\text{C}_n\text{G1}$ group of isomers was obtained. For this purpose, the sum of the peak areas of the pyranose and furanose forms, and the calibration curve of the corresponding β -pyranose standard, were used. For Plantacare, the calibration curves predicted concentrations within the ranges declared by the manufacturer (Table 1). The analysis of a baby shampoo gave 0.5% C8G1, 0.2% C10G1, and 0.6% C12G1. The peaks of the C14G1 isomers were also observed in the chromatograms of this sample.

4. Conclusions

The mass spectra of the APGs in the positive-ion mode were constituted by the single peaks of the $[\text{M} + \text{Na}]^+$ ions. Using an alkylamide column, isocratic elution and MS detection, the alkylmonoglycosides can be separated with resolution between the α - and β -epimers and ring isomers. The isomers of the alkyldiglycosides and alkyltriglycosides are also partially resolved. The ring isomers can be also resolved in a shorter time using a cyanopropyl column with gradient elution. Also in comparison with the alkylamide column, the cyanopropyl column requires a much shorter equilibration time. The APGs present in industrial mixtures and products (toiletries) can be quickly identified and quantified by any of these two methods. Using ESI–MS, the response factors of alkylmonoglycosides are independent from the acetonitrile contents of the mobile phase, but increase more than one order of magnitude from C1G1 up to C10G1, and decrease again for longer alkyl chains; thus, standards of all the oligomers are required to avoid systematic error in the determination of the alkylpolyglycosides of most industrial interest (C10G1–C16G1).

Acknowledgements

Work supported by projects CTQ2004-06302/BQU (MEC of Spain and FEDER funds), ACOMP/2007/168 (Conselleria d'Empresa, Universitat i Ciència, Generalitat Valenciana) and Químicas Oro S.A. (Sant Antoni de Benaixeve, València, Spain).

JMH-M thanks the MEC of Spain and the University of Valencia for a “Ramón y Cajal” research contract.

References

- [1] H. Koch, R. Beck, H. Roper, *Starch-Starke* 45 (1993) 2.
- [2] D. Balzer, *Tenside Surfact. Deterg.* 33 (1996) 102.
- [3] K. Hill, W. Rybinski, G. Stoll, *Alkyl Polyglycosides: Technology, Properties and Applications*, VCH, Weinheim, Germany, 1997.
- [4] W. Rybinski, K. Hill, *Angew. Int. Ed.* 37 (1998) 1328.
- [5] D. Balzer, H. Lüders, *Nonionic Surfactants: Alkyl Polyglycosides*, Marcel Dekker, New York, USA, 2000.
- [6] M. Biermann, K. Schmid, P. Schulz, *Starch-Starke* 45 (1993) 281.
- [7] M.T. García, I. Ribosa, E. Campos, J.S. Leal, *Chemosphere* 35 (1997) 545.
- [8] L. Uppgard, M. Sjöström, S. Wold, *Tenside Surfact. Deterg.* 37 (2000) 131.
- [9] W.M. McGregor, D. Fornara, T. Pellizzon, *Tenside Surfact. Deterg.* 41 (2004) 220.
- [10] N. Buschmann, S. Wodarczak, *Tenside Surfact. Deterg.* 32 (1995) 336.
- [11] N. Buschmann, A. Kruse, S. Wodarczak, *Agrofood Ind. Hi-Tech.* 7 (1996) 6.
- [12] N. Buchmann, F. Hülskötter, A. Kruse, S. Wodarczak, *Fett/Lipid* 98 (1996) 399.
- [13] J.Y. Kim, Y.A. Woo, H.J. Kim, J.D. Kim, *J. Pharm. Biomed. Anal.* 26 (2001) 73.
- [14] L.W. Kroh, T. Neubert, E. Raabe, H. Waldhoff, *Tenside Surfact. Deterg.* 36 (1999) 19.
- [15] C. Meissner, H. Engelhardt, *Cromatographia* 49 (1999) 12.
- [16] N. Buschmann, L. Merschel, S. Wodarczak, *Tenside Surfact. Deterg.* 33 (1996) 16.
- [17] R. Spilker, B. Menzebach, U. Schneider, I. Venn, *Tenside Surfact. Deterg.* 33 (1996) 21.
- [18] S. Klaffke, T. Neubert, L.W. Kroh, *Tenside Surfact. Deterg.* 35 (1998) 108.
- [19] P. Billian, H.-J. Stan, *Tenside Surfact. Deterg.* 35 (1998) 181.
- [20] J. Hübner, A. Nguyen, F. Turcu, D. Melchior, H.W. Kling, S. Gäb, O.J. Schmitz, *Anal. Bioanal. Chem.* 384 (2006) 259.
- [21] M. Kramer, H. Engelhardt, *J. High Resol. Chromatogr.* 15 (1992) 24.
- [22] M. Lafosse, P. Marinier, B. Joseph, M. Dreux, *J. Chromatogr.* 623 (1992) 277.
- [23] G. Czichocki, F. Harald, K. Haage, H. Much, S. Weidner, *J. Chromatogr. A* 943 (2002) 241.
- [24] J.V. Armari, Z. Lian, T. Lowden, U. deAlwis, P. Levesque, *J. Chromatogr. Sci.* 41 (2003) 234.
- [25] P. Eichhorn, T.P. Knepper, *J. Chromatogr. A* 854 (1999) 221.
- [26] S. Klaffke, T. Neubert, L.W. Kroh, *Tenside Surfact. Deterg.* 36 (1999) 178.
- [27] A.V. Kühn, R.H.H. Neubert, *Pharm. Res.* 21 (2004) 2347.

Chromium determination in pharmaceutical grade barium sulfate by solid sampling electrothermal atomic absorption spectrometry with Zeeman-effect background correction

Rodrigo Cordeiro Bolzan^a, Luis Frederico Rodrigues^b, Júlio Cezar Paz de Mattos^b,
Valderi Luiz Dressler^b, Érico Marlon de Moraes Flores^{b,*}

^a Departamento de Ciências da Saúde, Universidade Regional Integrada do Alto Uruguai e das Missões, Campus de Frederico Westphalen, 98400-000, Frederico Westphalen, RS, Brazil

^b Departamento de Química, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil

Received 17 February 2007; received in revised form 19 May 2007; accepted 21 May 2007

Available online 26 May 2007

Abstract

A procedure for chromium (Cr) determination in pharmaceutical grade barium sulfate by direct solid sampling electrothermal atomic absorption spectrometry (DSS-ET AAS) with Zeeman-effect background correction was developed. Operational conditions for the proposed procedure and the use of citric acid, ammonium phosphate, palladium and magnesium nitrate as chemical modifiers were evaluated. Pyrolysis and atomization temperatures were set at 1500 and 2400 °C, respectively and the use of matrix modifiers did not improve these conditions. Graphite platform presented high degradation rate, but minima changes were observed in the sensitivity or signal profile. Samples (0.3–1 mg) were weighted and introduced into the furnace using a manual solid sampling system. The linear concentration range of the calibration curve was from 100 to 1800 pg ($R^2 > 0.995$). The characteristic mass was 7.7 pg and the limit of detection was 2.4 pg. Chromium concentration in commercial samples ranged from 0.45 to 1.06 $\mu\text{g g}^{-1}$ and these results were confirmed by standard addition method. The mean reproducibility was 12% ($n = 20$ in a 3-day period) and repeatability was less than 9%. Results obtained using inductively coupled plasma optical emission spectrometry and conventional electrothermal atomic absorption spectrometry after extraction with HNO_3 were around 20% lower than those obtained by the proposed procedure. It was assumed that the low results were due to incomplete extraction even using hard conditions related to temperature and pressure. The proposed procedure by DSS-ET AAS provided some advantages related to recommended pharmacopoeias methodology, as lower risks of contamination and analyte losses, higher specificity, accuracy and sensitivity, no toxic or unstable reagents are required, and calibration with aqueous standards was feasible.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Chromium determination; Solid sampling; Barium sulfate; Electrothermal atomic absorption spectrometry

1. Introduction

Determination of trace inorganic impurities is a critical aspect in the quality control of pharmaceutical products. Several trace elements have toxic effects, besides some of them could cause problems in drug effectiveness or could decrease drug stability even at low concentrations [1]. Barium sulfate is a raw material widely used in pharmaceutical industry for production of suspensions for radiographic templates. In view of the presence

of some contaminants in barium sulfate, specially those classified as heavy metals by official pharmacopoeias, their control in pharmaceutical industry routine is very important [2,3]. Among the elements that need to be currently determined in pharmaceutical products, chromium (Cr) is important in view of some health risks as e.g., chromosomal aberration, mutations, carcinogenicity, transformation in cultured mammalian cells and a variety of DNA lesions [4,5]. Therefore, regarding to the potential health risk, Cr was included in routine analysis of limit test for heavy metals in the United States and European Pharmacopoeias. In these pharmacopoeias the maximum heavy metals content was set as 10 $\mu\text{g g}^{-1}$ for pharmaceutical grade barium sulfate. However, the analysis of this matrix is prone to some

* Corresponding author. Fax: +55 55 3220 9445.

E-mail address: flores@quimica.ufsm.br (É.M.d.M. Flores).

drawbacks, in part because this substance is practically insoluble in water and acids. This fact makes the sample preparation step based on wet digestion more difficult. According to the recommendations of some pharmacopoeias, the limit test for heavy metals should be performed by extraction in open vessels with diluted acid solution and subsequent reaction with thioacetamide or hydrogen sulphide [2,3]. In spite of this procedure to be recommended in official pharmacopoeias, it is obviously prone to several drawbacks as (i) contamination, (ii) limit test is not specific for Cr, but gives only general information based on color appearance for a group of elements, (iii) lack of accuracy and sensitivity, (iv) time consuming, and (v) use of toxic and unstable reagents. Thus, there is a need for development of reliable analytical techniques allowing the determination of trace metals in pharmaceutical substances, not only to meet the established specifications, but also to ensure the safety and efficacy of drugs for human consumption. On the other hand, there is a trend to change the general sulphide-assay by specific tests based on spectroscopic techniques [6–8].

Electrothermal atomic absorption spectrometry (ET AAS) is a well established technique for Cr determination in several kinds of samples [9]. However, its conventional application involves sample digestion steps, usually ashing or acid digestion which are time consuming and prone to contamination and analyte losses [10]. On the other hand, direct solid sampling using electrothermal atomic absorption spectrometry (DSS-ET AAS) has been proposed as a good alternative to the conventional methods of analysis where problems related to low limits of detection (LOD) or difficulties of sample digestion are present [11]. In addition, this technique has been applied for trace element determination at low levels in a wide variety of samples [12–14], in view of some features as (i) reduced sample pre-treatment and hence, increasing the throughput; (ii) low contamination risk, an essential requirement when trace levels of metals must be determined; (iii) lower risks of analyte losses during the sample pretreatment; and (iv) the use of corrosive and hazardous chemicals is avoided [15,16]. The use of DSS-ET AAS for Cr determination at low levels in BaSO₄ could present advantages over the conventional techniques based on wet analysis, taking into account the very low solubility of BaSO₄, and the necessity of high throughput for quality control in pharmaceutical industry. Moreover, the relatively chemical inertness of BaSO₄ is a drawback for conventional digestion procedures.

Although conventional ET AAS has been used successfully for the determination of low concentrations of several elements, it has limitations for the determination of carbide-forming metals. The possible formation of these compounds, as Cr and Ba carbides, could be the reason for the poor sensitivity attained during the determination of these analytes by ET AAS [17,18]. Analysis by X-ray diffraction has indicated the formation of chromium carbide (Cr₃C₂) at thermal pretreatment temperatures higher than 1030 °C [19]. Using ⁵¹Cr radiotracer techniques, it was found that Cr was irreversibly retained on the graphite tube, depending on the composition of the matrix, temperature control of the furnace and surface condition of the graphite tube [20].

In literature it was found only one study related to trace elements determination in a similar matrix used in the present work

[21]. Authors demonstrated the feasibility of DSS-ET AAS for analysis of barytes (a mineral composed basically by BaSO₄) and that concentrated nitric acid could be effective as chemical modifier for Cr determination. However, in spite of good results found for other elements, no information was given for Cr related to the graphite platform lifetime. Moreover, calibration must be performed with solid reference materials having different matrix composition (soil or sediments). Data for calibration using reference solutions was not supplied and, unfortunately, no information was given about the accuracy or LOD for Cr in the analyzed samples.

Based on these considerations, in this work is proposed a procedure for Cr determination in pharmaceutical grade BaSO₄ by DSS-ET AAS. Operational parameters were evaluated in order to consider the possibility of calibration using standard calibration technique with aqueous solutions. The use of citric acid, ammonium phosphate, palladium and magnesium nitrate as chemical modifiers was evaluated. As there is no available certified reference material with composition based on BaSO₄, the obtained results were compared to those obtained by inductively coupled plasma optical emission spectrometry (ICP OES) and ET AAS after acid extraction using microwave oven in closed vessels. In addition, comparative tests were also performed using the conditions described in reference [21].

2. Experimental

2.1. Instrumentation and operating conditions

Chromium determinations were carried out using a Model AAS ZEEnit 60 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite atomizer Zeeman-effect background correction system and a hollow cathode lamp for chromium operated at 4 mA (wavelength of 357.9 nm, spectral bandpass of 0.8 nm). This equipment was used for conventional ET AAS and DSS-ET AAS analysis. Spectrometer was also equipped with a special device for solid introduction into the graphite furnace (manual solid sampling system, Model SSA-5, Analytik Jena). Pyrolytic coated graphite tubes (Analytik Jena, Part No. 407–152.023) were used throughout. Integrated absorbance (peak area) was used for signal evaluation with an integration time of 12 s. A Model M2P microbalance (Sartorius, Göttingen, Germany) with a resolution of 1 µg was used for weighing the samples.

Sample acid digestion (extraction) was performed in closed quartz vessels using a high pressure Model Multiwave 3000 microwave oven (Anton Paar, Graz, Austria, maximum temperature of 280 °C and maximum pressure of 80 bar). Extracts were analyzed by ICPOES and ET AAS. Determinations by ICPOES were performed using a Model Optima 4300 DV axial viewing inductively coupled plasma optical emission spectrometer (Perkin Elmer, Shelton, USA), equipped with a spray Scott-type nebulization chamber. A pneumatic nebulizer GemCone-type coupled to an alumina injector tube was used for Cr determination. The operating conditions were: radiofrequency power of 1400 W, principal plasma gas flow of 15 L min⁻¹, nebulizer gas flow of 0.85 L min⁻¹ and auxiliary gas flow of 0.2 L min⁻¹.

Selected spectral line was 205.560 nm and peak area was used for signal acquisition, using three points per peak and two points for background (BG) correction. For analysis by conventional ET AAS the conditions recommended by the manufacturer were used throughout (Analytik Jena AG, Win AAS V 3.13.0 eng, 1998–2004, Jena, Germany).

2.2. Samples and reagents

Powdered pharmaceutical grade BaSO₄ samples were purchased from pharmaceutical industries (samples “A” to “D”) and they were previously dried in a conventional oven at 105 °C × 2 h. The particle size distribution of BaSO₄ samples were checked by optical microscopy. Sample “A” was used for initial tests and evaluation of the proposed procedure. Distilled and deionized water was further purified using a Milli-Q system (Millipore Corp., Bedford, USA). All reagents, including chemical modifiers (palladium, citric acid, magnesium nitrate, ammonium phosphate) were of analytical grade or better (Merck, Darmstadt, Germany) and their respective solutions were prepared by sequential dilution in water. Reference solutions were daily prepared by serial dilution of stock Cr solutions (1 g L⁻¹ Cr in 2% HNO₃). Concentrated nitric acid used for sample digestion/extraction was doubly distilled in a Milestone sub-boiling system (Model duoPUR 2.01 E, Bergamo, Italy).

2.3. Procedures

For the proposed procedure, test samples of barium sulfate were weighted (between 0.1 and 1.5 mg) directly on the graphite platform and transferred to the graphite tube using a manual device for solid introduction. Blanks were evaluated by simulating the same individual steps (weighing, transport, and modifier addition) using an empty platform. Pyrolysis and atomization curves were established with the correspondent temperatures ranging from 800 to 1700 °C and from 1900 to 2600 °C, respectively. The effectiveness of palladium, citric acid, magnesium nitrate and ammonium phosphate as chemical modifiers was evaluated using aqueous solutions correspondent to 5, 25, 50, and 50 µg, respectively. Each modifier solution was added directly on the solid sample after the weighing in the graphite platform. The correspondent chemical modifier masses were chosen based on the conditions described in the literature [22] with the exception of citric acid. For this modifier, no references were found related to its use for Cr determination and the citric acid mass of 25 µg was chosen based on the successfully conditions used for Pb determination in sulfate matrix by ET AAS [23]. Platform mass losses were evaluated by performing successive weighing steps just after each heating cycle of atomizer. In addition, a study related to the mass interval useful for solid analysis was performed according to reference [24].

For Cr determinations by ICP OES and ET AAS, samples were previously extracted using a microwave-assisted system in quartz closed vessels. About 1 g of sample was transferred to the vessels and 6 mL of concentrated HNO₃ was added. Vessels were closed and the following heating program of the microwave oven was applied: 600 W for 10 min, 1400 W for 20 min, and 20 min

for cooling. After cooling, digests (extracts) were diluted with water up to 50 mL.

3. Results and discussion

3.1. Pyrolysis and atomization profile

Initially, pyrolysis and atomization curves for chromium in barium sulfate using sample “A” were performed (Fig. 1). Pyrolysis curve shows that chromium is thermally stable in BaSO₄ at temperatures ranging from 800 to 1600 °C. The maximum temperature is in agreement to literature data for other kinds of samples without using chemical modifiers [25,26]. On the other hand, atomization curve shows that chromium signals are practically the same in the range between 1900 and 2400 °C. At temperatures higher than 2400 °C, in general those considered better for Cr atomization [25–27], a decrease of absorbance was observed and the BG signal was strongly increased. Therefore, in the presence of barium sulfate matrix it was impossible to use the normal recommended atomization temperatures for Cr and 2400 °C was chosen for further studies. Background could be related to the co-volatilization of analyte and matrix with possible formation of matrix derived species, as S₂, S₃₋₈ and CS or CS₂ compounds in the vapor phase. The absorption due to these compounds could not be completely compensated using conventional BG correctors [28].

In general, the proposed mechanism of Cr atomization is the thermal dissociation of its solid carbides, with subsequent transfer of Cr to the gas phase by thermal desorption in a first-order process [29]. At pyrolysis temperatures around 1300 °C, the formation of Cr₃C₂ is possible, and therefore the atomization mechanism could include (i) the thermal decomposition of Cr₃C₂ in gaseous Cr and, (ii) the thermal desorption of adsorbed chromium [30]. In this work it was observed that about 64% of the original sample mass remains in the graphite platform even after heating at 1500 °C during 15 s, even using nitric acid as chemical modifier (pyrolysis step, Table 1). The residual mass has a black appearance that is an indicative of carbide formation. No attempts were made to determine the exact residue composition. However, this residue was completely decomposed

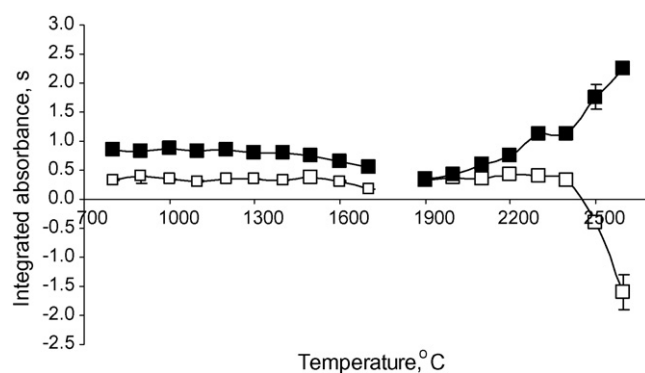


Fig. 1. Pyrolysis and atomization □ analytical and ■ BG signals curves for chromium in barium sulfate (pyrolysis set at 1100 °C and atomization at 2200 °C). Vertical bars are the correspondent standard deviations ($n = 3$). Barium sulfate mass was 0.467 ± 0.058 mg, $E_{\max} = 0.8$ T.

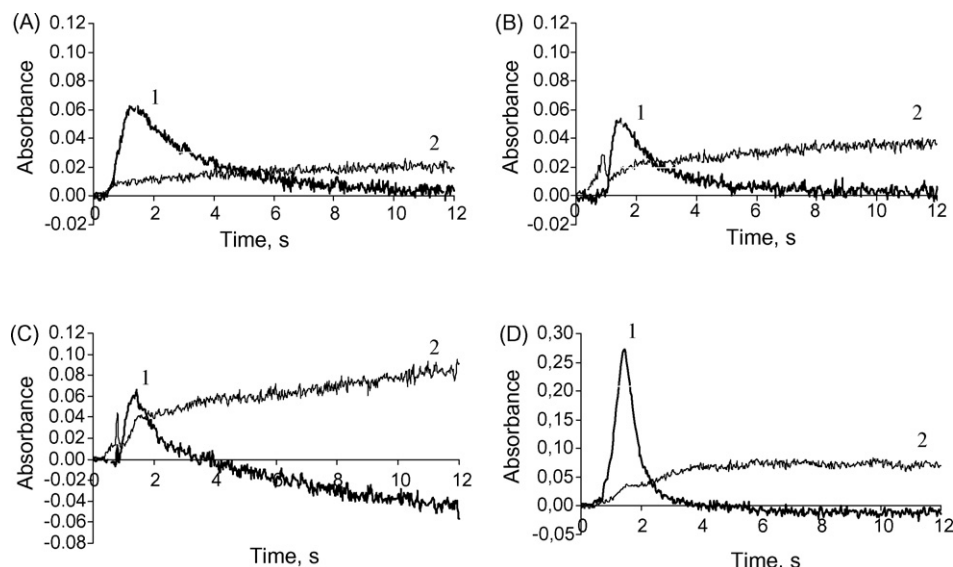


Fig. 2. Analytical (1) and BG (2) signals for chromium in barium sulfate by DSS GF-AAS, pyrolysis at 1500 °C and atomization at 2400 °C. A: 100 pg Cr aqueous reference solution; B: 0.431 mg barium sulfate; C: 0.388 mg barium sulfate, atomization 2500 °C; D: 0.742 mg barium sulfate + 5 μL pure HNO₃, atomization 2400 °C. $E_{\max} = 0.8 T$.

after the cleanout step and this fact is in agreement to literature data that report the BaC₂ decomposition at high temperatures [31].

Typical analytical and BG signals are shown in Fig. 2 A for reference Cr solutions. When performing barium sulfate analysis, Cr signal starts at 2 s and decreases until around 8 s (Fig. 2 B). Moreover, it can be seen a large BG peak starting also around 1 s, possibly promoted by matrix vaporization, that may be responsible for a small overcorrection effect before 1 s. However, when performing barium sulfate analysis with atomization at 2500 °C, an overcorrection effect occurred after 4 s causing a strong signal suppression resulting in negative values (Fig. 2-C). Despite the BG signal being less than 0.1 Zeeman-effect corrector was not able to compensate it. In this work it was supposed that the reason for these results could be due to the concomitant vaporization of matrix during Cr atomization causing uncorrected results. As barium sulfate is practically insoluble in HNO₃, the content of sulfate in solution after digestion is low. Then, as expected, the shape of signal for Cr reference aqueous solution added to digests obtained after extraction with 6 mL of HNO₃ was practically the same when compared to that for conventional Cr aqueous reference solution (Fig. 2-A).

Nitric acid was reported as chemical modifier for Cr determination in barytes samples [21]. In that case, authors reported very

low BG with use of 2350 °C as atomization temperature. In addition, a heating ramp of 2150 °C s⁻¹ and pyrolysis temperature of 1000 °C was used. However, in the present study, no differences between signal shape or sensitivity was found between the reported conditions and selected temperatures with heating ramp of 3000 °C s⁻¹, with or without nitric acid. Therefore, the use of HNO₃ as matrix modifier was considered ineffective. Although analytical peak became more symmetric an overcorrection effect was still observed (Fig. 2-D). Then, atomization temperature of 2400 °C and pyrolysis at 1500 °C were chosen for subsequent studies.

3.2. Barium sulfate effect on the graphite platform degradation

In this study, the graphite platform mass losses were evaluated without use of chemical modifiers. Just after each heating cycle the platform mass was determined and losses were observed as shown in Fig. 3. When solid barium sulfate was analyzed the loss

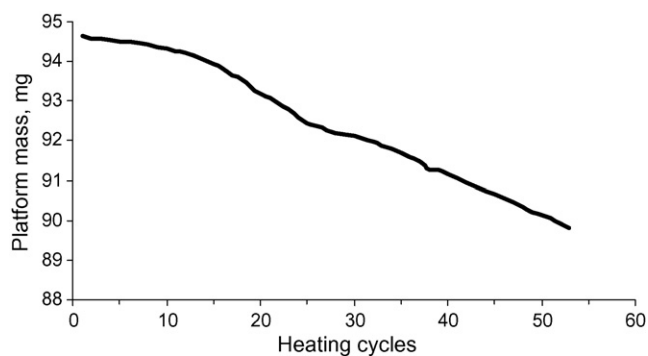


Fig. 3. Platform mass variation during chromium determination in barium sulfate by DSS-ET AAS. Pyrolysis temperatures ranged from 800 °C to 1700 °C and atomization from 1900 to 2600 °C.

Table 1
Graphite furnace heating program for chromium determination in barium sulfate by DSS-ET AAS

	Temperature (°C)	Ramp (°C s ⁻¹)	Time (s)	Inert gas
Drying #1	110	15	30	Max
Drying#2	120	15	10	Max
Pyrolysis	1500	400	15	Max
Auto zero	1500	0	6	Stop
Atomization	2400	3000	12	Stop
Clean out	2600	3000	4	Max

of mass was less than 0.5% after 13 heating cycles up to start the damages of the pyrolytic layer. Damages were detected by visual inspection of platform just after each heating cycle. However, when solid barium sulfate was placed on the platform after the destruction of pyrolytic layer the mass losses were higher (about 5%) with only 54 firings. In this case, the loss of mass was 0.46 ± 0.23 mg lost/heating cycle. In this condition the maximum lifetime was only 120 firings. These data show that barium sulfate causes significant mass losses from the graphite platform, probably through the formation of Ba carbides by reaction with the graphite layer that are volatilized during the clean out step [32].

The reductant properties of active graphite sites, which provide a relatively large surface area in contact with the sample, seem to have an important role in the decomposition processes of metal sulfates and some of their by-products [33]. If carbon takes contact with sulfur compounds in vapor phase most of sulfur containing compounds can generate molecules of the type C_nS_m (with $n > m$) in vapor phase at high temperatures [28]. In this situation, graphite surfaces could reduce the diffusion of the analyte through the graphite wall and prevent the formation of carbides [9] that, in the case of chromium, has low thermal stability [29]. Despite the degradation of graphite platform could change the atomization profile, no differences were observed in this study between normalized analytical and BG signals after and before platform pyrolytic surface rupture. In a previous study [34] it was tried to obtain better results for chromium determination in pieces of titanium by DSS-ET AAS by recovering the graphite platform with carbon powder. However, accuracy and precision of the results were rather poor. In the case of determining Cr in $BaSO_4$, recovering the graphite platform also did not provide any advantage, probably because Cr could form carbides easier since there was higher surface area for reacting chromium atoms with carbon. A tentative to minimize the degradation effect was investigated in this work by using iridium (Ir) to covering the graphite platform. The procedure for Ir deposition was performed according to reference [35]. Using Ir, no damages were observed, but this condition was unsuitable in this study in view of losses of Ir during the heating cycle. In addition, using Ir the correlation between sample mass and absorbance was unsuitable ($R = 0.72$) and relative standard deviation (R.S.D) was higher than 25%.

3.3. Sample mass

The maximum sample mass that could be introduced in the graphite tube under optimized conditions (without chemical modifier) was investigated and the chosen procedure was performed similarly to procedure described in reference 24. Barium sulfate masses between 0.1 and 1.5 mg were weighed in the graphite platform. Small sample masses (from 0.1 to 0.3 mg) lead to overestimated values, while relatively large amounts (above 1 mg) give rise to underestimated results as can be seen in Fig. 4. It was observed a good correlation between integrated absorbance and $BaSO_4$ masses from 0.3 to 1 mg. Therefore, the interval correspondent to sample masses from 0.3 to 1 mg was chosen for further studies.

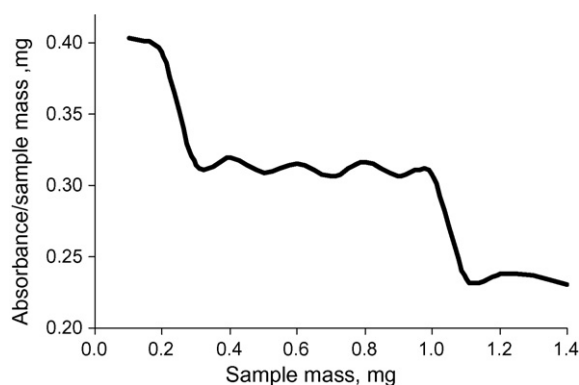


Fig. 4. Influence of sample ($BaSO_4$) mass on analytical results for Cr determination by the proposed DSS-ET AAS, pyrolysis set at 1500°C and atomization at 2400°C , $E_{\text{max}} = 0.8$ T.

3.4. Chemical modifiers

Many attempts have been made to improve the performance of the analytical methods for Cr determination in several samples based on ET AAS, with use of chemical modifiers. In this work, citric acid, ammonium phosphate, palladium and magnesium nitrate were evaluated to increase the atomization temperature and Cr peak shape.

Citric acid is an organic matrix modifier that can improve metal vaporization [36], and has been already used for determination of metals in complex matrices [23]. In view of the co-vaporization of matrix with Cr and consequently high BG signal, citric acid was tested as chemical modifier in an attempt to use the recommended atomization temperature of 2500°C [25–27] in order to separate matrix and analyte volatilizations in the atomization step. Citric acid was not effective and no changes in absorbance profile were observed during $BaSO_4$ analysis. Ammonium phosphate, palladium and magnesium nitrate were also tested as chemical modifiers, but again, no analytical advantages were obtained. Results for ammonium phosphate as matrix modifier are in agreement to literature data which do not report advantages in using this matrix modifier for Cr [22]. In spite of palladium and magnesium nitrate have been recommended as modifiers for other samples [22,37,38] their use for $BaSO_4$ did not present advantages by comparing with condition without modifier addition proposed in this work.

3.5. Figures of merit for the proposed procedure

Optimized conditions for chromium determination in barium sulfate by DSS-ET AAS are shown in Table 1. Calibration was performed using aqueous reference standard solutions. The linear concentration range of the calibration curve was from 100 to 1800 pg, with a determination coefficient, R^2 , better than 0.995. Limit of detection was 2.4 pg (3σ , $n = 10$) and characteristic mass was 7.7 pg. The characteristic mass found in this work is in agreement with literature data that describes values between 3.5 and 11.2 pg for chromium determination in slurries of environmental samples [27] and complex matrices [39].

The chromium concentration in pharmaceutical grade barium sulfate samples ranged from 0.45 to $1.06 \mu\text{g g}^{-1}$ using the

Table 2

Results for the determination of chromium in barium sulfate ($\mu\text{g g}^{-1}$) by the proposed DSS-ET AAS procedure and by ICP OES and conventional ET AAS after extraction step ($n \geq 3$)

Samples	DSS-ET AAS	ET AAS	ICP OES
A	0.45 ± 0.04	0.32 ± 0.04	0.35 ± 0.05
B	1.02 ± 0.05	0.77 ± 0.06	0.73 ± 0.05
C	0.95 ± 0.06	0.72 ± 0.08	0.65 ± 0.07
D	1.06 ± 0.01	0.81 ± 0.08	0.87 ± 0.09

proposed procedure (Table 2). These results did not agree with those obtained using ICP OES and conventional ET AAS after extraction with 6 mL of HNO_3 in high pressure microwave oven (t -test, $p < 0.05$). Results for the proposed procedure by DSS-ET AAS were more than 20% higher than those using ICP OES and ET AAS after extraction. As no certified reference BaSO_4 was available, recovery tests were performed for the proposed procedure and chromium reference solutions were added to the solid BaSO_4 (correspondent to $0.48 \mu\text{g g}^{-1}$) after weighing. Recoveries were between 98 and 103%. It is important to point out that Cr extraction from this kind of matrix, even using an aggressive sample treatment step, is currently unsatisfactory. Incomplete extraction of Cr by acid digestion has been observed by some authors and has been attributed to the presence of certain chromium-containing minerals (e.g., chromite), which are not dissolved by several acid mixtures [40–42].

It is well known that the particle size and the homogenous distribution of the analyte in a solid sample can influence the accuracy and precision in analysis by DSS-ET AAS [21]. In the present work, more than 95% of particle size was smaller than $20 \mu\text{m}$ for all samples. The mean reproducibility obtained in 20 measurements in three different days with BaSO_4 sample “A” was 12%, which indicates suitable intra-laboratory variability. In addition, the repeatability of the proposed procedure was less than 9% which was considered appropriated by using DSS procedure.

4. Conclusion

A procedure for Cr determination in pharmaceutical grade BaSO_4 by DSS-ET AAS has been developed. This method presented good accuracy and precision. Moreover, using optimized temperature parameters it was possible to minimize the matrix effects for chromium determination and hence no need of chemical modifiers addition was found. The use of ICP OES and conventional ET AAS requires previous sample preparation step that involves the use of corrosive and hazardous chemicals that is a time consuming procedure and a possible source of contamination. These undesirable steps were minimized using the proposed procedure.

Acknowledgements

The authors thank to FAPERGS, CNPq and Agência Nacional de Vigilância Sanitária (ANVISA) for supporting this study.

References

- [1] A. Kelkó-Lévai, I. Varga, K. Zih-Perényi, A. Lásztity, *Spectrochim. Acta Part B* 54 (1999) 827.
- [2] United States Pharmacopoeia, 29th ed., USP Convention, Rockville, MD, USA, 2006.
- [3] European Pharmacopoeia, fifth ed., Council of Europe, Strasbourg, France, 2005.
- [4] E.T. Snow, *Pharmacol. & Therapeutics* 53 (1992) 31.
- [5] T.J. O'Brien, S. Ceryak, S.R. Patierno, *Mutat. Res.: Fundam. Mol. Mech. Mutagen.* 533 (2003) 3.
- [6] R.N. Rao, M.V.N.K. Talluri, *J. Pharmaceut. Biomed. Anal.* 43 (2007) 1.
- [7] N. Lewen, S. Mathew, M. Schenkenberger, T. Raglione, *J. Pharmaceut. Biomed. Anal.* 35 (2004) 739.
- [8] S. Görög, *Trends Anal. Chem.* 25 (2006) 755.
- [9] K. Pyrzynska, *Spectrochim. Acta Part B* 50 (1995) 1595.
- [10] B. Docekal, V. Krivan, *J. Anal. At. Spectrom.* 8 (1993) 637.
- [11] H.M. Dong, V. Krivan, B. Welz, G. Schlemmer, *Spectrochim. Acta Part B* 52 (1997) 1747.
- [12] H.M. Dong, V. Krivan, *Spectrochim. Acta Part B* 56 (2001) 1645.
- [13] B. Welz, *Spectrochim. Acta Part B* 54 (1999) 2081.
- [14] M. Resano, J. Briceño, M. Aramendía, M.A. Belarra, *Anal. Chim. Acta* 582 (2007) 214.
- [15] M.A. Belarra, M. Resano, F. Vanhaecke, L. Moens, *Trends Anal. Chem.* 21 (2002) 828.
- [16] M.G.R. Vale, N. Oleszczuk, *Appl. Spectrosc. Rev.* 41 (2006) 377.
- [17] R.M. Camero, J. Alvarado, *Spectrochim. Acta Part B* 55 (2000) 875.
- [18] F. Barbosa Jr., S.S. Souza, D. Santos Jr., F.J. Krug, *Microchem. J.* 78 (2004) 7.
- [19] W. Wendl, G. Mtiller-Vogt, *Spectrochim. Acta Part B* 39 (1984) 237.
- [20] C. Veillon, B.E. Guthrie, W.R. Wolf, *Anal. Chem.* 52 (1980) 457.
- [21] R. Nowka, I.L. Marr, T.M. Ansari, H. Müller, *Fresenius J. Anal. Chem.* 364 (1999) 533.
- [22] P. Bermejo-Barrera, J. Moreda-Pineiro, A. Moreda-Pineiro, A. Bermejo-Barrera, *J. Anal. At. Spectrom.* 13 (1998) 777.
- [23] J.C.P. Mattos, A.M. Nunes, A.F. Martins, V.L. Dressler, E.M.M. Flores, *Spectrochim. Acta Part B* 60 (2005) 687.
- [24] M.A. Belarra, C. Crespo, M.P. Martínez-Garbayo, J.R. Castillo, *Spectrochim. Acta Part B* 52 (1997) 1855.
- [25] J. Mierza, Y.C. Sun, M.H. Yang, *Spectrochim. Acta Part B* 53 (1998) 63.
- [26] M.J. Marquez, A. Salvador, A. Morales-Rubio, M. Guardia, *Microchem. J.* 62 (1999) 363.
- [27] P. Pazos-Capeans, M.C. Barciela-Alonso, P. Bermejo-Barrera, *Anal. Chim. Acta* 524 (2004) 121.
- [28] M. Lemme, D.A. Katskov, P. Tittarelli, *Spectrochim. Acta Part B* 59 (2004) 101.
- [29] A.B. Volynsky, *Spectrochim. Acta Part B* 53 (1998) 1607.
- [30] N.S. Thomaidis, E.A. Piperaki, *Spectrochim. Acta Part B* 55 (2000) 611.
- [31] D.R. Lide (Ed.), *Physical Constants of Inorganic Compounds*, in *CRC Handbook of Chemistry and Physics*, 85th ed., CRC Press, Boca Raton, FL, 2005.
- [32] M. Suzuki, K. Ohta, *Talanta* 28 (1981) 177.
- [33] M.A. Castro, K. Faulds, W.E. Smith, A.J. Aller, D. Littlejohn, *Spectrochim. Acta Part B* 59 (2004) 827.
- [34] V. Krivan, H.M. Dong, *Anal. Chem.* 70 (1998) 5312.
- [35] M.M. Silva, I.C.F. Damin, M.G.R. Vale, B. Welz, *Talanta* 71 (2007) 1877.
- [36] N. Zhe-ming, S. Xiao-Quan, *Spectrochim. Acta Part B* 42 (1987) 937.
- [37] M.I.C. Monteiro, A.K. Ávila, R. Neumann, *Anal. Chim. Acta* 428 (2001) 265.
- [38] J.L. Burguera, M. Burguera, C. Rondon, L. Rodriguez, P. Carrero, Y.P. de Pena, E. Burguera, *J. Anal. At. Spectrom.* 14 (1999) 821.
- [39] M. Felipe-Sotelo, A. Carlosena, J.M. Andrade, M.J. Cal-Prieto, E. Fernandez, D. Prada, *Microchem. J.* 81 (2005) 217.
- [40] R. Villares, X. Puente, A. Carballeira, *Environ. Monit. Assess.* 83 (2003) 129.
- [41] P. Pazos-Capeans, M.C. Barciela-Alonso, A. Bermejo-Barrera, P. Bermejo-Barreira, *Anal. Chim. Acta* 524 (2004) 121.
- [42] R.A. Nadkarni, *Anal. Chem.* 56 (1984) 2233.

Determination of lamotrigine by adsorptive stripping voltammetry using silver nanoparticle-modified carbon screen-printed electrodes

M. Encarnación Burgoa Calvo, Olga Domínguez Renedo, M. Julia Arcos Martínez*

*Departamento de Química, Área de Química Analítica, Facultad de Ciencias, Universidad de Burgos,
Plaza Misael Bañuelos s/n, E-09001 Burgos, Spain*

Received 22 February 2007; received in revised form 9 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

Carbon screen-printed electrodes (CSPE) modified with silver nanoparticles present an interesting alternative in the determination of lamotrigine (LTG) using differential pulse adsorptive stripping voltammetry.

Metallic silver nanoparticle deposits have been obtained by electrochemical deposition. Scanning electron microscopy measurements show that the electrochemically synthesized silver nanoparticles are deposited in aggregated form.

The detection limit for this analytical procedure was 3.72×10^{-7} M. In terms of reproducibility, the precision of the above mentioned method in %R.S.D. values was calculated at 2.58%.

The method was applied satisfactorily to the determination of LTG in pharmaceutical preparations.

© 2007 Published by Elsevier B.V.

Keywords: Silver nanoparticles; Screen-printed electrodes; Lamotrigine; Adsorptive stripping voltammetry

1. Introduction

Lamotrigine (LTG), 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (Fig. 1) is a new-generation antiepileptic drug registered for treatment of patients with refractory partial seizures with or without secondary generalization [1,2]. Lamotrigine is thought to act at voltage-sensitive neuronal membranes and inhibit the release of excitatory amino acid neurotransmitters, in particular glutamate and aspartate, which play an important role in the generation and spread of epileptic seizures.

HPLC [3–7] and capillary electrophoresis [8] are among the different techniques generally used for the measurement of LTG concentrations in pharmaceutical products and biological fluids. Electrochemical techniques applied to the determination of LTG provide an interesting alternative to the chromatographic methods that are widely used at present. Together with the recognized advantage of the relatively low cost of electrochemical

instrumentation, one should bear in mind the high sensitivity of methods such as stripping voltammetry, based on the adsorption exhibited by numerous organic compounds on some electrodes. Despite the presence of redox groups in this molecule, only one work can be found in the literature describing the electrochemical analysis of LTG by means of adsorptive stripping voltammetry using a HMDE electrode [9].

Screen-printed electrodes are planar devices with plastic substrates that are coated with layers of electroconductive and insulating inks at controlled thickness. The advent of screen-printed (thick-film) technology has made it possible to mass-produce inexpensive disposable electrodes for use with electrochemical instruments [10–14]. Their use in potentiometric, amperometric and voltammetric devices have been reported for the detection of different compounds [15–27] although the bibliography shows very few examples of determination of drugs such as lamotrigine [28,29].

The great versatility of screen-printed electrodes resides in their wide range of possible modifications. In fact, the composition of the inks used in the printing process can be modified by the addition of substances of a very different nature, such as metals, enzymes, polymers, complexing agents, etc. Moreover, the possibility also exists of modifying the electrodes once they

* Corresponding author.

E-mail addresses: meburgoa@beca.ubu.es (M.E. Burgoa Calvo),
olgado@ubu.es (O. Domínguez Renedo), jarcos@ubu.es
(M.J. Arcos Martínez).

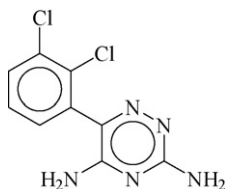


Fig. 1. Chemical structure of lamotrigine.

have been printed through the deposition of films containing those substances.

The design of new nanoscale materials has acquired ever-greater importance in recent years due to their wide-ranging applications in various fields. Among these materials, metallic nanoparticles are of great interest due to their important properties and their numerous possible applications [30,31]. The bibliography lists numerous methods describing the synthesis of metallic silver nanoparticles in solution as well as by deposition on solid surfaces. These include chemical synthesis by means of reduction with different reagents [32], UV light or electron-beam irradiation [33] and electrochemical methods [34–39]. The latter provides an easy and rapid alternative for the preparation of metallic nanoparticle electrodes in a short period of time.

The aim of this work is to determine LTG by differential pulse adsorptive stripping voltammetry (DPAdSV) using screen-printed electrodes modified with silver nanoparticles and, to the best of the author's knowledge, presents the first ever electrochemical detection of this molecule with these types of electrodes.

2. Experimental

2.1. Reagents

All solutions were prepared with deionized water obtained with a Barnstead NANO Pure II system.

Electrodag PF-407 A (carbon ink), Electrodag 418 SS (silver ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) were supplied by Achenson Colloiden (Scheemda, The Netherlands).

Britton-Robinson solutions were used as buffers. A 0.04 M Britton-Robinson buffer solution for the o-boric, o-phosphoric and acetic acids was prepared using Merck analytical grade reagents. Solutions of different pH values were prepared from this by the addition of 0.2 M sodium hydroxide (analytical-reagent grade, Merck, Darmstadt, Germany).

Silver nitrate was from VWR International and hydrochloric acid was purchased from Merck (analytical-reagent grade, Merck, Darmstadt, Germany).

Commercial capsules of LAMICTAL[®] were obtained from GlaxoSmithKline. The excipients of the tablet are: blackcurrant flavour, calcium carbonate, low-substituted hydroxypropylcellulose, magnesium aluminium silicate, magnesium stearate, povidone, saccharin sodium and starch glycolate.

2.2. Apparatus

Screen-printed electrodes were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

Voltammetric measurements were taken using a μ Autolab electrochemical system with GPES software (Eco Chemie, Utrecht, The Netherlands).

Centrifugation of samples was carried out in an Angular 6 centrifuge (Selecta, Barcelona, Spain).

Scanning electron microscopy (SEM) images were obtained using a JEOL JSM-6460LV with an INCA elemental X-ray analysis system.

2.3. Screen printed electrodes preparation

Hand-made screen-printed electrodes were used in the determination of LTG. For the construction of the screen-printed electrodes successive layers of different inks were printed onto a PVC strip substrate (30 mm $\times$ 15 mm, 0.5 mm thick) using four different screens with appropriate stencils to transfer the required design (Fig. 2). The printing procedure was as follows:

- (1) Firstly, three parallel conducting base-patterns were screen-printed on the PVC strip substrate with commercial silver ink, giving them an effective conductive surface, and they were then cured for 15 min at 90 °C. The base-pattern at the left was used as the counter electrode.
- (2) A silver/silver chloride reference electrode was screen-printed using silver/silver chloride ink on the silver base-pattern at the right, as can be seen in Fig. 2, and then cured for 15 min at 90 °C.
- (3) The working electrode was formed by screen printing a graphite layer over the silver base pattern at the centre using commercial graphite ink and was then cured for 15 min at 90 °C.

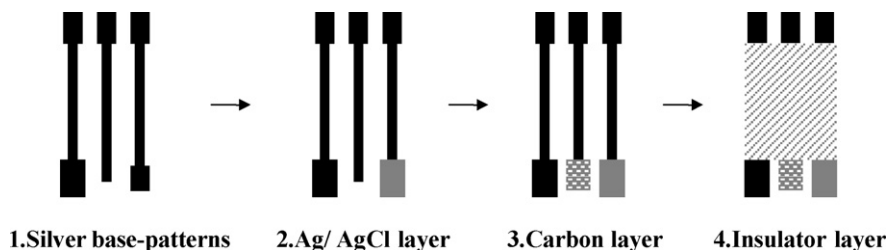


Fig. 2. Schematic diagram of the sensor preparation procedure.

- (4) An insulator layer was finally printed over all of the sensor strip except for the three electrode surfaces and the electrical connection at the reverse end of the sensor strip, which was subsequently cured under UV radiation.

2.4. Preparation of nanoparticle-modified electrodes

Despite the potential advantages of using silver nanoparticle-modified carbon screen-printed electrodes as working electrodes in electrochemical techniques, no works have been found in the reference literature on the applications of such electrodes for drug analysis.

In comparison to other electrochemical methods described for the generation of silver nanoparticles [38,39], this work describes a new and easy procedure to obtain silver nanoparticle-modified CSPE. The method consists in the electrochemical deposition of silver on the working-electrode surface, using a solution of AgNO_3 in Britton-Robinson (pH 2). The deposition was performed by applying an accumulation potential during a time under stirring.

3. Results and discussion

3.1. Optimization of the experimental parameters for silver nanoparticle-modified CSPE formation

No analytical signal for LTG was observed when Ag solid electrodes were used. However, modification of the graphite electrode by the deposition of silver nanoparticles produced signals of a sufficiently high quality for analytical purposes, as may be observed from Fig. 3. This figure shows a peak due to

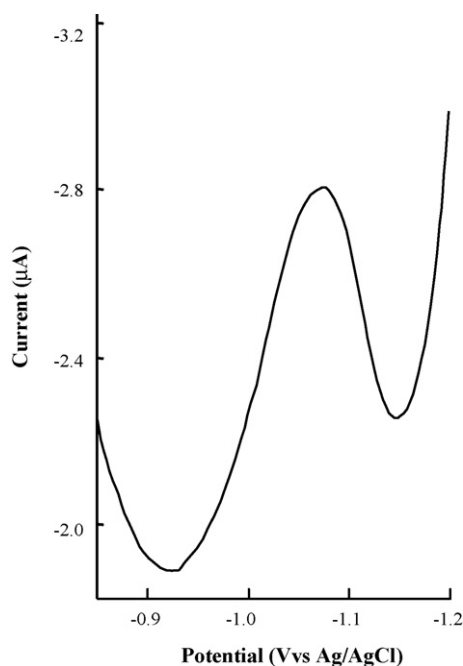


Fig. 3. Differential pulse voltammograms obtained for LTG in Britton-Robinson buffer, using silver nanoparticle-modified CSPE. pH 5.5, $t_{\text{dep}} = 200$ s, $E_{\text{dep}} = -0.9$ V and $[\text{LTG}] = 1.56 \times 10^{-5}$ M. Optimum silver concentration was used for the preparation of the electrode.

reduction of LTG at a potential about -1.06 V, which is notably influenced by the features of the silver coating.

Previous experiments showed that very acid pH values are needed to deposit silver in the carbon surface of the electrode. For this reason pH 2 Britton-Robinson solution was used in the deposition stage. In the same way, to deposit silver in the carbon surface of the electrodes, long deposition times or very negative potentials were needed so, we decided to use -1.20 V and 120 s as conditions for the deposition.

Finally, silver concentration was optimized by addition of different volumes of 5.0×10^{-3} M AgNO_3 solution into a 10 mL cell containing Britton-Robinson pH 2. The silver nanoparticle modified CSPE was introduced into a solution containing 1.56×10^{-5} M of LTG and a DPAdSV scan was carried out under the following conditions, pH 5.5, $E_{\text{dep}} = -0.90$ V and $t_{\text{dep}} = 200$ s.

As shown in Fig. 4 the highest peak intensity was reached for a concentration of AgNO_3 of 0.83 mM.

It has been observed that a poor response for LTG is obtained for high concentrations of AgNO_3 . This behaviour can be explained by the formation of a partial silver film on the CSPE electrode as can be seen in Fig. 5, which shows the SEM images of the silver nanoparticles electrodeposited onto the CSPE under different operating conditions.

3.2. Characterization of silver deposition

SEM analysis shows the formation of silver nanoparticles on the CSPE surface which are deposited in aggregated form. The effect of the silver concentration in the solution is shown in Fig. 5, which depicts different images taken, as well as the image of a clean CSPE. It can be observed that the higher silver concentration, the greater the nanoparticles size obtained (Fig. 5b–d).

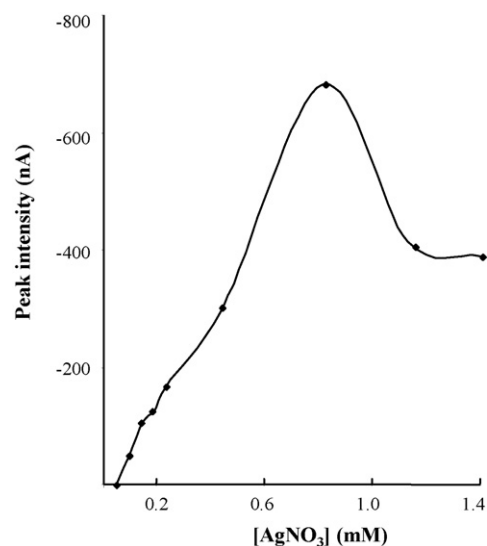


Fig. 4. Effect of silver concentration in the LTG reduction peak using a silver nanoparticle-modified CSPE. pH 5.5, $t_{\text{dep}} = 200$ s, $E_{\text{dep}} = -0.9$ V and $[\text{LTG}] = 1.56 \times 10^{-5}$ M.

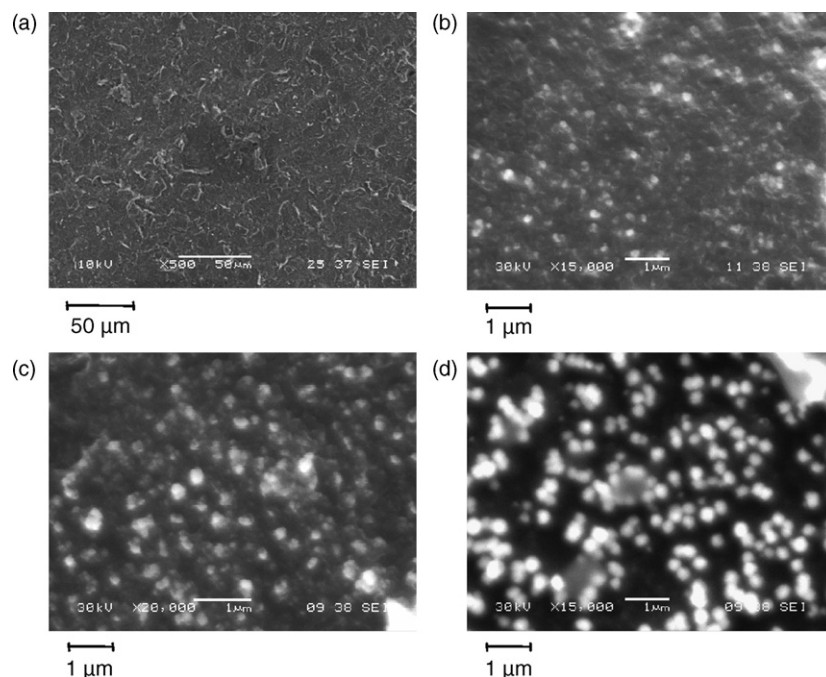


Fig. 5. SEM images of no modified CSPE and silver nanoparticle-modified CSPE: (a) no modified CSPE; (b–d) silver nanoparticle-modified CSPE prepared by electrodeposition at a potential of -1.2 V during 120 s from a pH 2 Britton-Robinson solution containing various concentrations of AgNO_3 ; (b) 0.45 mM AgNO_3 ; (c) 0.83 mM AgNO_3 ; (d) 1.15 mM AgNO_3 .

Moreover, energy-dispersive X-ray analysis (EDX) of CSPE confirmed the presence of deposited silver.

3.3. Optimization of adsorptive stripping parameters for LTG determination

Once silver coated has been optimized the next step is to optimize the parameters which influence on DPAdSV.

It is well known that deposition potential (E_{dep}), deposition time (t_{dep}) and pH value have a great influence in DPAdSV. Previous experiments have shown that there is no analytical signal for pH values very different of 5.5. For this reason a Britton-Robinson buffer solution of pH 5.5 was chosen as the supporting electrolyte. In order to obtain a sensitive analytical signal a 2^2 central composite design was carried out with the remaining factors. The values which correspond to the high (+) and low (−) levels and to the central point (0) for each factor are as follows:

$$\begin{array}{lll} E_{\text{dep}}(+)= -0.70 \text{ V} & E_{\text{dep}}(-)= -1.10 \text{ V} & E_{\text{dep}}(0)= -0.90 \text{ V} \\ t_{\text{dep}}(+)= 250 \text{ s} & t_{\text{dep}}(-)= 50 \text{ s} & t_{\text{dep}}(0)= 150 \text{ s} \end{array}$$

The response to be optimized was the intensity ($-i_p$), at a potential of -1.06 V, of a LTG sample at a concentration of 2.0×10^{-6} M.

From analysis of the variance (ANOVA) in Table 1, it can be seen that a second order function is adequate to model the data because the lack of fit is not significant at the 95% confidence level. A maximum can be observed in Fig. 6 which corresponds to a potential of -0.90 V and an accumulation time of 147 s.

3.4. Calibration and detection limit

Once the optimal experimental conditions were found for the analysis of LTG by means of silver nanoparticle-modified CSPE, a calibration was performed using a least-median-squares regression (LMS) to detect the existence of anomalous points [40], which might have led to incorrect adjustments altering the sensitivity and the detection limit.

The calibration equation obtained by DPAdSV for standard solutions containing LTG concentrations of between 3.30×10^{-7} and 1.50×10^{-6} M was $I = -33.98 + 1.25 \times 10^8 C$ ($R^2 = 0.99$, $s_{yx} = 4.99$).

Table 1

ANOVA of the data obtained with the 2^2 central composite design for optimization of experimental variables in LTG determination with silver nanoparticle-modified CSPE

Effect	SS	d.f.	MS	F_{ratio}	P_{level}
A: E_{dep}	888.06	1	888.06	1.03	0.418
B: t_{dep}	173.83	1	173.83	0.20	0.698
AA	52050.70	1	52050.70	60.16	0.016 ^a
AB	190.44	1	190.44	0.22	0.685
BB	32833.70	1	32833.70	37.95	0.025
Lack of fit	3493.91	3	1165.64	1.35	0.453
Pure error	1730.34	2	865.17		
Total	72781.70	10			
$R^2 = 0.933$					

[LTG] = 2.0×10^{-6} M. SS, sum of squares; d.f., degrees of freedom; MS, mean squares; F_{ratio} : $\text{MS}_{\text{factor}}/\text{MS}_{\text{error}}$. P_{level} , probability level.

^a Significant factor at $\alpha = 0.05$.

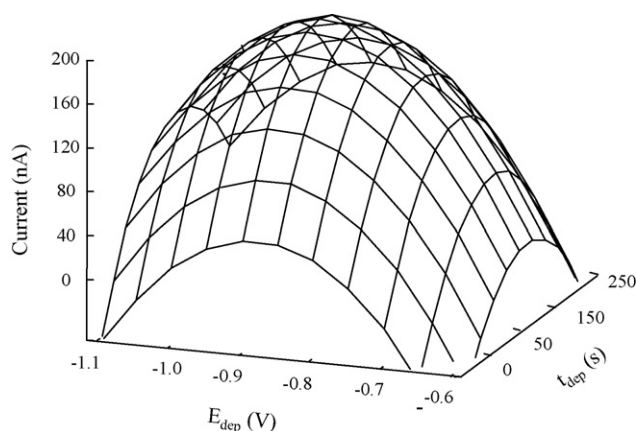


Fig. 6. Response surface for the 2^2 central composite design for optimization of experimental variables in LTG determination by DPAdSV.

A key feature of any analytical method is its detection limit; the smallest concentration of the analyte that can be detected to a specified degree of certainty. Calculation of the detection limit, based on the variability of ten samples with a very low analyte concentration, was calculated according to [41] and ISO 11843–2 [42]. At the chosen probability level of 5% ($\alpha = \beta = 0.05$), the detection limit was 3.72×10^{-7} M.

This limit of detection value is one order less than the values obtained when LTG was measured with carbon and mercury-film carbon screen-printed electrodes. Moreover, as can be seen in Fig. 7, the analytical signal is higher when LTG is analyzed with nanoparticle-modified electrodes.

3.5. Precision

This parameter was calculated in terms of reproducibility. A series of measurements of three different samples containing 2.0×10^{-7} M of LTG were carried out obtaining a %R.S.D. value of 2.58%.

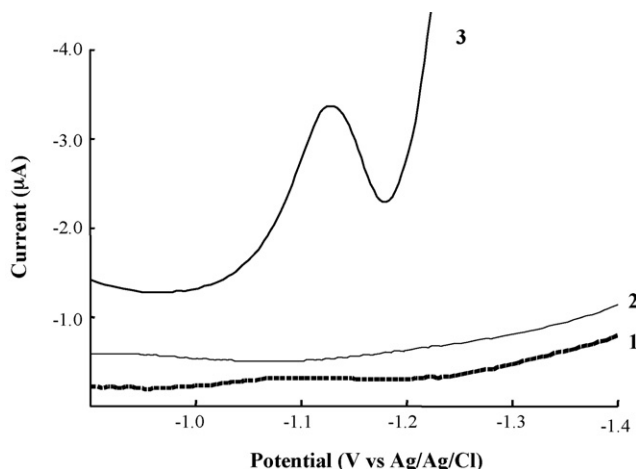


Fig. 7. DPAdSV voltammograms of 5×10^{-6} M of LTG in: (1) Hg-film-CSPE (pH 5.53, $t_{\text{dep}} = 105$ s and $E_{\text{dep}} = 0.40$ V); (2) CSPE (pH 5.50, $t_{\text{dep}} = 28$ s and $E_{\text{dep}} = 0.05$ V); (3) silver nanoparticle-modified CSPE (pH 5.5, $t_{\text{dep}} = 147$ s and $E_{\text{dep}} = -0.90$ V). Measurements were carried out in the optimal conditions for each electrode.

3.6. Analytical application

The concentration of LTG in commercial capsules of LAMICTAL® (GlaxoSmithKline) with a known concentration of analyte, was determined using the DPAdSV method, so as to evaluate the accuracy of the proposed method.

In order to determine the concentration of LTG in LAMICTAL® 10 different capsules of the pharmaceutical preparation were analyzed. The following procedure was carried out for each capsule:

- (1) The tablet was pulverized with a pestle and dissolve in HCl 0.1 M.
- (2) The resulting solution was centrifuged at 5000 rpm for 1 h.
- (3) After centrifugation the supernatant was collected and used as a stock solution of the sample.

Finally, the determination of the LTG content was made in an aliquot of the stock solution described above by means of the AdSV using the silver nanoparticle-modified CSPE developed in this work.

Good agreement was obtained between the amount of LTG found by the standard addition 26 ± 1 mg with $n = 10$ and $\alpha = 0.05$ and the value supplied by the manufacturer 25 ± 1 mg. These results were also checked using the HPLC method described in Ref. [4] as a reference technique obtaining 25 ± 1 mg ($n = 3$, $\alpha = 0.05$).

4. Conclusions

The silver nanoparticle-modified carbon screen printed electrodes developed in this work present an environmentally-friendly method for the analysis of LTG. Their most important advantage is the high sensitivity in the determination of LTG in real samples. In fact, the detection limit obtained was less than the values found when LTG was measured with carbon and mercury-film carbon screen-printed electrodes. For this reason we can assure that this is the best way, known at the present time, to analyze LTG using screen printed electrodes.

Acknowledgements

The financial support made available by the Junta de Castilla y León (Burgos 08/04) and the Ministerio de Ciencia y Tecnología (BQU2001-1126) is gratefully acknowledged. The authors also thank Dr. Álvaro Colina from Universidad de Burgos for the help with SEM images. M. Encarnación Burgoa Calvo also thanks Ministerio de Educación y Ciencia for the F.P.U. grant.

References

- [1] M. Bialer, S.I. Johannessen, H.J. Kupferberg, *Epilepsy Res.* 34 (1999) 1.
- [2] S. Shorvon, H. Stefan, *Epilepsia* 38 (1997) S45.
- [3] D. Londero, P. LoGreco, *J. Chromatogr. B* 691 (1997) 139.
- [4] K.M. Matar, P.J. Nicholis, S.A. Bawazir, M.I. Al-Hassan, A. Tekle, *J. Pharm. Biomed. Anal.* 17 (1998) 525.

- [5] P. Angelis-Stoforidis, D.J. Morgan, T.J. O'Brien, F.J.E. Vajda, J. Chromatogr. B 727 (1999) 113.
- [6] E. Vidal, C. Pascual, L. Pou, J. Chromatogr. B 736 (1999) 295.
- [7] N.R. Barbosa, A.F. Midio, J. Chromatogr. B 741 (2000) 289.
- [8] J. Zheng, M.W. Jann, Y.Y. Hon, S.A. Shamsi, Electrophoresis 25 (2004) 2033.
- [9] M.E. Burgoa, O. Domínguez, M.J. Arcos, Anal. Chim. Acta 549 (2005) 74.
- [10] J. Wang, J. Lu, B. Tian, C. Yarnitzky, J. Electroanal. Chem. 361 (1993) 77.
- [11] C. Yarnitzky, J. Wang, B. Tian, Talanta 51 (2000) 333.
- [12] P. Ugo, L.M. Moretto, P. Bertoncello, J. Wang, Electroanalysis 10 (1998) 1017.
- [13] D. Desmond, B. Lane, J. Alderman, M. Hill, D.W.M. Arrigan, J.D. Glennon, Sens. Actuators B 48 (1998) 409.
- [14] M. Jasinski, P. Gründler, G.U. Flechsig, J. Wang, Electroanalysis 13 (2001) 34.
- [15] V. Beni, V.I. Ogurtsov, N.V. Bakunin, D.W.M. Arrigan, M. Hill, Anal. Chim. Acta 552 (2005) 190.
- [16] I. Palchetti, S. Laschi, M. Mascini, Anal. Chim. Acta 530 (2005) 61.
- [17] B.B. Rodriguez, J.A. Bolbot, I.E. Tothill, Anal. Bioanal. Chem. 380 (2004) 284.
- [18] K.C. Honeychurch, D.M. Hawkins, J.P. Hart, D.C. Cowell, Talanta 57 (2002) 565.
- [19] R.O. Kadara, I.E. Tothill, Talanta 66 (2005) 1089.
- [20] I. Palchetti, S. Majid, A. Kicela, G. Marrazza, M. Mascini, Int. J. Environ. Anal. Chem. 83 (2003) 701.
- [21] J.M. Zen, C.C. Yang, A.S. Kumar, Anal. Chim. Acta 464 (2002) 229.
- [22] J.Y. Choi, K. Seo, S.R. Cho, J.R. Oh, S.H. Kahng, J. Park, Anal. Chim. Acta 443 (2001) 241.
- [23] O. Domínguez Renedo, M.A. Alonso-Lomillo, M.J. Arcos Martínez, Talanta, in press.
- [24] V.V. Shumyantseva, S. Carrara, V. Bavastrello, D.J. Riley, T.V. Bulko, K.G. Skryabin, A.I. Archakov, C. Nicolini, Biosens. Bioelectron. 21 (2005) 217.
- [25] J. Razumiene, A. Vilkanauskite, V. Gureviciene, J. Barkauskas, R. Meskys, V. Laurinavicius, Electrochim. Acta 51 (2006) 5150.
- [26] L. Micheli, A. Radoi, R. Guarrina, R. Massaud, C. Bala, D. Moscone, G. Palleschi, Biosens. Bioelectron. 20 (2004) 190.
- [27] B. Bucur, D. Fournier, A. Danet, J.L. Marty, Anal. Chim. Acta 562 (2006) 115.
- [28] G. Bagni, D. Osella, E. Sturchio, M. Mascini, Anal. Chim. Acta 573 (2006) 81.
- [29] Y. Shih, J.M. Zen, H.H. Yang, J. Pharm. Biomed. Anal. 29 (2002) 827.
- [30] M. Starowicz, B. Stypula, J. Banas, Electrochem. Commun. 8 (2006) 227.
- [31] C.M. Welch, R.G. Compton, Anal. Bioanal. Chem. 384 (2006) 601.
- [32] L. Sun, Z. Zhang, H. Dang, Mater. Lett. 57 (2003) 3874.
- [33] M. Fukushima, H. Yanagi, S. Hayashi, N. Suganuma, Y. Taniguchi, Thin Solid Films 438 (2003) 39.
- [34] E. Majid, S. Hrapovic, Y. Liu, K.B. Male, J.H.T. Luong, Anal. Chem. 78 (2006) 762.
- [35] X. Dai, O. Nekrasova, M.E. Hyde, R.G. Compton, Anal. Chem. 76 (2004) 5924.
- [36] M.S. El-Deab, T. Okajima, T. Ohsaka, J. Electrochem. Soc. 150 (2003) A851.
- [37] C.M. Welch, C.E. Banks, A.O. Simm, R.G. Compton, Anal. Bioanal. Chem. 382 (2005) 12.
- [38] B. Yin, H. Ma, S. Wang, S. Chen, J. Phys. Chem. B 107 (2003) 8898.
- [39] Z. Tang, S. Liu, S. Dong, E. Wang, J. Electroanal. Chem. 502 (2001) 146.
- [40] P.J. Rousseeuw, A.M. Leroy, Robust Regression and Outlier Detection, John Wiley and Sons, New York, 1989.
- [41] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S.P. De Jong, J. Levi, S. Verdeke, Handbook of Chemometrics and Qualimetrics, Part A, Elsevier, Amsterdam, 1997.
- [42] ISO 11843-2, Capability of Detection, Geneva, Switzerland, 2000.

Comparison of two extraction methods for evaluation of volatile constituents patterns in commercial whiskeys Elucidation of the main odour-active compounds

M. Caldeira, F. Rodrigues, R. Perestrelo, J.C. Marques, J.S. Câmara*

Centro de Química da Madeira, Departamento de Química da Universidade da Madeira, 9000-390 Funchal, Portugal

Received 8 March 2007; received in revised form 15 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

An analytical procedure based on manual dynamic headspace solid-phase microextraction (HS-SPME) method and the conventional extraction method by liquid–liquid extraction (LLE), were compared for their effectiveness in the extraction and quantification of volatile compounds from commercial whiskey samples. Seven extraction solvents covering a wide range of polarities and two SPME fibres coatings, has been evaluated. The highest amounts extracted, were achieved using dichloromethane (CH_2Cl_2) by LLE method ($\text{LLE}_{\text{CH}_2\text{Cl}_2}$) and using a CAR/PDMS fibre ($\text{SPME}_{\text{CAR/PDMS}}$) in HS-SPME. Each method was used to determine the responses of 25 analytes from whiskeys and calibration standards, in order to provide sensitivity comparisons between the two methods. Calibration curves were established in a synthetic whiskey and linear correlation coefficient (r) were greater than 0.9929 for $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ and 0.9935 for $\text{SPME}_{\text{CAR/PDMS}}$, for all target compounds. Recoveries greater than 80% were achieved. For most compounds, precision (expressed by relative standard deviation, R.S.D.) are very good, with R.S.D. values lower than 14.78% for HS-SPME method and than 19.42% for LLE method. The detection limits ranged from 0.13 to 19.03 $\mu\text{g L}^{-1}$ for SPME procedure and from 0.50 to 12.48 $\mu\text{g L}^{-1}$ for LLE.

A tentative study to estimate the contribution of a specific compound to the aroma of a whiskey, on the basis of their odour activity values (OAV) was made. Ethyl octanoate followed by isoamyl acetate and isobutyl alcohol, were found the most potent odour-active compounds.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Whiskey; Volatile organic compounds; Liquid–liquid extraction; Solid-phase microextraction; Odour activity values

1. Introduction

Analysis of flavour/aroma compounds is one of the most important steps in the evaluation of whiskey quality. Being a combination of taste and olfaction senses, these characteristics are crucial factors in consumer acceptance of drinks and foods.

It has been reported [1–3] that whiskeys have several volatile compounds, which belong to a great variety of families such as: *ethyl esters, higher alcohols, fatty acids, higher alcohols acetates, carbonyl compounds* such as *aldehydes and ketones, sulphur compounds, furanic compounds, lactones, volatile phenols*, among others. This great diversity of compounds are produced through metabolic pathways and their genesis depends on many factors related to raw materials and the subsequent pro-

cesses of mashing, fermentation, distillation and ageing, others are oak derived, while others depend on the type of technological treatment. Many of these compounds are common to different whiskeys but differ analytically in terms of the relative amount. The qualitative and quantitative study with some whiskeys available commercially, is an important database for ensuring process continuity and product authenticity [4,5].

Ethyl esters are an essential family among the aroma components of whiskeys. Even present in small amounts, they have very intense odour characterized by pleasant aromas, such as *fruity* and *floral* aromas that generally contribute positively to the global quality of whiskeys [5]. The higher alcohols are also an important family, quantitatively and qualitatively speaking, being characterized by their *strong* and *pungent* smell and taste, causing a positive contribution to the overall sensory properties when present at levels lower than 300 mg L^{-1} . Higher alcohol acetates are correlated with *freshness* and *fruitiness* character, while fatty acids can contribute with *fruity*, *cheese*, *fatty* and

* Corresponding author. Tel.: +351 291705112; fax: +351 291705149.
E-mail address: jsc@uma.pt (J.S. Câmara).

rancid notes to the whiskeys sensory. However, not all compounds contribute to the same extent to whiskey aroma. The particular importance of each compound to the final aroma is related to its odour perception threshold, which is defined as the lowest concentration that can be detected by smelling. In fact, if the *concentration/olfactory* threshold ratio of each compound, known as the odour activity value (OAV) is ≥ 1 , this allows to estimate the contribution of a specific compound to aroma of a whiskey. To our knowledge no scientific studies on the most potent aroma compounds of commercial whiskey samples, has been previously published.

In order to achieve a practical and reliable method for the analysis of volatiles in complex matrices such as whiskey samples, several extraction methods have been developed and used, including steam distillation, liquid–liquid microextraction [6–8], simultaneous distillation–solvent extraction [9], solid-phase extraction [6], supercritical fluid extraction [10], microwaves extraction [11] and ultrasound extraction [12], among others. Although offering specific advantages under certain circumstances, these analytical methods have some drawbacks such as the possibility of contamination with solvents, and later solvent concentration, generation of artifacts and the length of time analysis. In spite of these inconvenient, LLE methods continue to be the reference technique for determination of volatile constituents from several beverages. An advantage of this method is that all volatile compounds (low, medium and high volatility) can be analysed in one extraction step, but the method may require solvent evaporation, which, in some cases, results in the loss or degradation of some compounds.

Pawliszyn and co-workers in 1990 [13–15] develop a new variation of adsorption techniques called solid-phase microextraction (SPME). Compared to conventional techniques this new technique offers many advantages such as high sensitivity and reproducibility, does not require solvent and combines extraction and pre-concentration in a single step without pre-treatment of samples. Moreover, it is fast, inexpensive and requires small sample volumes. This technique, based on absorption and/or adsorption mechanism, depending on the fibre coating, can be successfully applied for polar and non-polar compounds in gaseous, liquid and solid samples and can be easily coupled with various analytical instruments such as GC, GC–MS, HPLC and LC–MS [16–23]. It has been used routinely in combination with gas chromatography (GC) and gas chromatography coupled to mass spectrometry with ion trap detection (GC–ITDMS), and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from environmental [24], biological [25] and food [26–31] samples. More recently Deng et al. [32] developed a SPME methodology for investigation of lung cancer volatile biomarkers. The same authors applied HS-SPME with on-fibre derivatization for the determination of hexanal and heptanal in normal blood and lung cancer blood [33].

Lately 1990s, a new technique, namely stir bar sorptive extraction (SBSE) has also been developed by Baltussen et al. [34] and applied to the extraction of volatile constituents [35]. Zalacain et al. [36] used this technique to the analysis of wine

primary aroma compounds, while Demyttenaere et al. [37] used the same methodology to extract whiskey volatiles. The characterization of the main chemical composition and the aroma profile of Madeira wine by sorptive extraction techniques were made by Alves et al. [38].

The present work was carried out to evaluate the performance of two extraction methods (LLE and dynamic HS-SPME) in the determination of volatile compounds from commercial Scotch whiskeys. Seven different extraction solvents [CH_2Cl_2 , $\text{H}(n\text{-hexane})$, $\text{CH}_2\text{Cl}_2\text{-DE}$ (diethyl ether) (3:1 and 1:3), $\text{CH}_2\text{Cl}_2\text{-H}$ (3:1 and 1:3) and DE-H (1:3)] and two kinds of commercially available fibres: carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm), apolar, and carbowax/divinylbenzene (CW/DVB, 65 μm), polar, were used to test the extraction efficiency of volatile compounds. The best solvent and SPME fibre were applied by employing GC with flame ionisation detector (FID) to the determination of absolute content of whiskey volatiles and GC–MS to the volatile identification. The selectivity of each method for specific classes of flavour compounds was evaluated. Linearity, detection and quantification limits, and precision of the whole analytical procedure have also been calculated for the 25 target compounds selected to study the methods performance. Given the lack of information about the whiskeys odour-active compounds, the levels of recognized odorants were used to establish exactly the flavour differences between the whiskeys, and to calculate the odour activity values in order to elucidate the most potent aroma compounds of the studied samples. The results obtained applying the two analytical procedures are shown and discussed.

2. Experimental

2.1. Chemicals and materials

All reagents used were of analytical quality. *n*-Hexane (99%) and diethyl ether (99.5%) were supplied by Lab-Scan (Dublin, Ireland); ethanol absolute ($\geq 99.5\%$), solid anhydrous sodium sulphate (99%) and analytical-grade hydrochloric acid ($\approx 37\%$) used to adjust the standards pH, were purchased from Panreac Quimica SA (Barcelona, Spain). Dichloromethane ($\geq 99.8\%$), sodium hydroxide ($>99\%$), sodium chloride (99.5%) that was used to obtain the adequate ionic strength (decrease the solubility of the aroma molecules which then partition more readily into the headspace improving the adsorption of analytes in SPME analysis), were supplied by Merck (Darmstadt, Germany). Methanol of Chromasolv quality ($\geq 99.9\%$) and the standards of aroma compounds, which ranged from 98% to 99.5% purity, were obtained from Sigma–Aldrich (Steinheim, Germany). The *n*-alkane mixture, consisting of C_8 to C_{20} straight-chain alkanes, was purchased from Fluka. Solvents did not require additional distillation, were used as received. Water was purified through a Milli-Q purification system (Millipore).

Seventy-five micrometers CAR/PDMS and 65 μm of CW/DVB SPME fibres, purchased from Supelco (Bellefonte, PA, USA) were conditioned under helium at a flow-rate of 1.0 mL min^{-1} in the hot injection port of a gas chromatograph at 300°C for 2 h for CAR/PDMS and 220°C for 0.5 h for

CW/DVB. Fiber blanks, taken prior to analysis, confirm the quality of conditioning.

Stock solution of target compounds to be determined was prepared in methanol. A mixed standard solution for the preparation of spiked samples was obtained diluting the stocks. All the standards solution was stored at 4 °C in the darkness, until use.

Synthetic whiskey samples were prepared by an ethanol solution at 12% (v/v) to which hydrochloride acid 1 M was added to adjust solution pH.

2.2. Whiskey samples

A total of 24 commercial Scotch whiskey samples (40% (v/v), alcohol), Famous Grouse (FG), Dewar's (DW), Red Label (RL), Black Label (BL), Grant's (GRA), Long John (LJ), Ballantines (BAL) and Highland Clan (HC), three samples from each whiskey, were purchased from a local store (Funchal, Madeira Island) and frozen at –28 °C until their analysis. Before extraction of the volatile compounds, the samples were unfrozen at 3–4 °C. Commercial BL whiskey sample was used to select the best solvent and the fiber with higher extraction efficiency for whiskey volatile constituents.

The high ethanol concentration of the whiskeys required dilution to 12% (v/v) alcohol for both LLE and SPME methods. This dilution minimized emulsion formation during LLE method and loss of sensitivity for most volatiles determined by SPME.

2.3. Extraction methodologies

2.3.1. LLE method

Quantitative analysis and identification of volatile compounds were carried out by the procedure described by Perestrelo et al. [39]. In accordance with this method, 50 mL of whiskey sample, to which 250 μ L of octan-3-ol and 1 mL of 4-methylpentan-2-ol, in hydro alcoholic solution (1:1, v/v) at 422 mg L^{–1} as internal standards were added, was poured into a 100 mL flask. Five grams of sodium sulphate was added. The samples were then extracted twice with 5 mL of CH₂Cl₂. The solution was stirred during 15 min at 400 rpm. Both organic phases obtained were blended and dried over anhydrous sodium sulphate and concentrated to 500 μ L under a gentle stream of pure nitrogen (N45, Air Liquide, Portugal). Injection of the concentrated extract was made in split mode (split ratio, 1:10; sample size, 1 μ L) in the GC-FID, for quantification and onto GC-MS for volatile identification.

2.3.2. Solid-phase microextraction procedure

After adjust to 12% (v/v) alcohol by dilution with distilled water, whiskey samples were adjusted to pH 3.3 and the ionic strength was increased, using NaCl (30%), to improve the extraction efficiency. A 60 mL vial containing 35 mL of sample, spiked with octan-3-ol (Sigma–Aldrich) and 4-methylpentan-2-ol, which were used as internal standards (50 μ L of alcoholic solution at 422 mg L^{–1}), was placed in a thermostatic block on a stirrer. The fibre was then exposed to the gaseous phase during 60 min at temperature of 40 $\pm$ 1 °C. As stirring usually improves

the extraction, because the static layer resistant to mass transfer is destroyed (facilitate mass transport between the bulk of the aqueous sample and the fibre), all the experiments were performed under constant stirring velocity (1250 rpm). After extraction, the SPME fibre was withdrawn into the needle, removed from the vial and inserted into the hot injector port (260 °C) of the GC–MS system for 6 min where the extracted chemicals were desorbed thermally and transferred directly to the analytical column.

2.4. Instrumental analysis

2.4.1. Gas chromatography (GC) analysis

A Hewlett-Packard HP 5890 series II gas chromatograph equipped with a split/splitless capillary injection port and flame ionization detector (FID) was used. Separation was performed on a fused silica capillary column DB-Waxetr: 30 m $\times$ 0.25 mm I.D. $\times$ 0.5 μ m film thickness from JW Scientific (USA). The column was maintained at 40 °C for 1 min after desorption and then ramped at 1 °C min^{–1} to 120 °C (2 min) followed by 1.7 °C min^{–1} to 180 °C where it was held for 1 min and finally to 220 °C at 25 °C min^{–1} and held isothermally for 10 min. The FID temperature was 300 °C and the injector temperature 260 °C. The carrier gas was helium (99.99995%) at a flow-rate of 1 mL min^{–1}. Hydrogen and air at 30 and 300 mL min^{–1}, respectively, were used in FID, with nitrogen (35 mL min^{–1}) as a make-up gas. A Star Chromatography workstation version 4.0 was used for acquiring and processing the data. Measured retention times and peak areas represented an average from three injections.

The quantification was based on the calibration curves of respective standards in the synthetic whiskeys, with octan-3-ol and 4-methylpentan-2-ol as internal standards. Since the repeatability of the chromatographic method was very good (with coefficients of variation ranging from 2.0% to 12% in average), only tree injection of each CH₂Cl₂ extract was carried out. *n*-Alkane standards (Fluka) were run under the same chromatographic conditions as the samples, to calculate the Kovats retention indices of the compounds.

2.4.2. Gas chromatography–ion trap mass spectrometry (GC–ITDMS) analysis

The identification and confirmation of analytes identity in real samples was performed by a GC–ITDMS, Varian Star 3400 Cx Series II gas chromatograph equipped with Varian Saturn III mass selective detector and a Saturn GC–MS workstation software, in the electron impact mode (EI). Chromatographic conditions were similar to the described in the GC-FID analysis. The ion trap detector was set as follows: transfer line temperature 220 °C; manifold and trap temperatures 180 °C. The mass range was m/z = 30–300 amu, the emission current 15 μ A and the electron multiplier was set in the relative mode to the auto tune procedures. All mass spectra were acquired in the electron impact (EI) mode (E_i = 70 eV, source temperature, 180 °C).

The identification of the volatile compounds was achieved by comparing their mass spectra with those stored in the US Government National Institute of Standards and Technology

Table 1

Performance characteristics of liquid–liquid extraction method (LLE_{CH₂Cl₂}): concentration range, linearity, detection (LOD) and quantification (LOQ) limits, recoveries and yield extraction data

Compound	Range concentration (mg L ⁻¹)	<i>r</i>	Slope	Intercept	LOD (µg L ⁻¹)	LOQ (µg L ⁻¹)	Recovery (%)	Yield (%)	R.S.D. (%)	IS
Higher alcohols										
Propan-1-ol	0.40–28.91	1.0000	0.21	0.22	1.65	5.50	84.67	106.94	5.57	3-OCT
2-Methylpropan-1-ol	0.26–20.12	0.9989	1.41	−0.01	0.50	1.66	69.05	84.00	3.19	4M2P
Butan-1-ol	0.74–29.16	0.9995	0.24	0.04	1.21	4.04	85.76	123.04	7.06	3-OCT
3-Methylbutan-1-ol	0.74–29.12	0.9989	0.22	0.05	0.95	3.15	85.16	102.43	6.98	4M2P
Hexan-1-ol	3.80–29.48	0.9995	0.13	0.04	0.97	3.23	101.24	101.94	7.82	4M2P
Methionol	0.54–42.12	0.9995	0.05	0.03	1.39	4.62	100.77	109.18	7.81	4M2P
β-Phenylethanol	0.47–36.83	0.9985	0.14	0.14	2.79	9.29	101.18	83.27	9.36	3-OCT
Benzyl alcohol	0.48–37.62	0.9989	0.12	0.09	2.38	7.95	105.46	102.87	8.48	4M2P
2-Phenoxyethanol	0.51–39.67	0.9929	0.12	0.14	4.90	16.34	114.28	113.30	7.81	4M2P
Acetate										
Isoamyl acetate	4.00–31.54	1.0000	0.11	0.03	0.92	3.08	101.12	107.49	8.93	4M2P
Ethyl esters										
Ethyl butanoate	0.37–31.61	1.0000	0.10	0.09	0.76	2.54	104.39	106.31	14.88	4M2P
Ethyl hexanoate	0.80–31.43	0.9995	0.13	0.08	1.22	4.06	100.97	103.52	10.05	4M2P
Ethyl lactate	0.96–37.51	0.9995	0.04	0.01	1.41	4.72	96.60	125.26	6.92	4M2P
Ethyl octanoate	0.80–31.61	0.9995	0.15	0.07	1.42	4.74	98.90	106.01	9.73	4M2P
Ethyl decanoate	0.80–31.03	1.0000	0.21	−0.04	0.70	2.33	100.45	92.34	9.16	4M2P
Diethyl succinate	0.48–37.69	0.9995	0.11	0.01	4.43	14.77	106.18	95.89	9.16	4M2P
Ethyl dodecanoate	5.60–124.84	0.9959	0.44	0.46	12.48	41.59	92.49	115.18	11.37	3-OCT
Fatty acids										
Hexanoic acid	0.43–33.72	0.9969	0.08	−0.13	3.18	10.60	88.93	89.57	16.25	4M2P
Octanoic acid	0.42–32.79	0.9959	0.08	−0.13	3.73	12.44	93.62	88.71	19.42	4M2P
Decanoic acid	0.72–55.99	0.9965	0.13	−0.07	8.90	29.68	82.69	96.42	13.11	3-OCT
Carbonyl compounds										
Acetaldehyde	3.60–28.37	0.9995	0.35	0.24	3.92	13.07	71.03	83.68	4.64	3-OCT
Syngaldehyde	3.50–54.86	0.9939	0.05	−0.01	7.65	25.49	90.13	99.77	13.67	4M2P
Furanic compounds										
Furfural	0.96–37.77	0.9995	0.08	−0.01	1.29	4.31	91.70	99.42	4.42	4M2P
5-Methyl-2-furfural	1.20–39.85	0.9995	0.09	0.05	1.86	6.21	95.61	101.66	8.10	4M2P
Volatile phenol										
Guaiacol	0.46–36.18	0.9985	0.13	0.12	2.40	8.01	102.74	123.13	8.62	4M2P

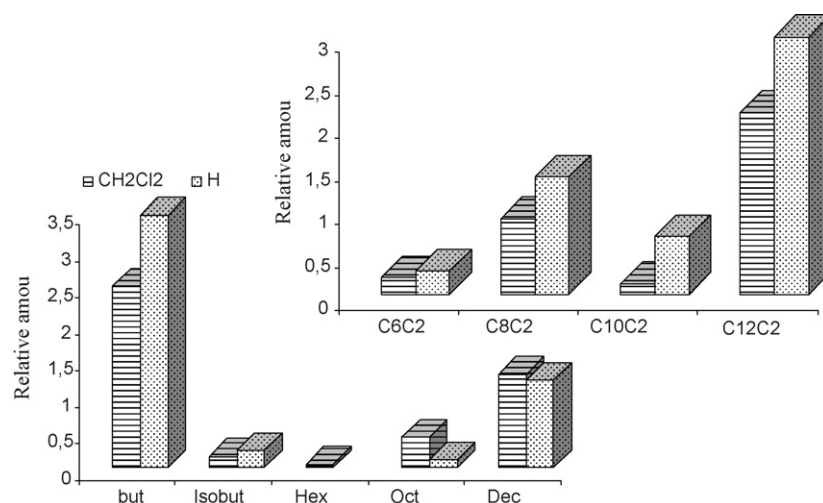


Fig. 1. Comparison of the extraction efficiency for different chemical families by LLE method using CH₂Cl₂ and H (But: butanoic acid; Isobut: 3-methylbutanoic acid; Hex: hexanoic acid; Oct: octanoic acid; Dec: decanoic acid; C6C2: ethyl hexanoate; C8C2: ethyl octanoate; C10C2: Ethyl decanoate; C12C2: ethyl dodecanoate).

(NIST) library and by the Kovats retention indices calculated for each peak with reference to the normal alkanes C₈–C₂₀ series according to the following equation [40]:

$$RI = 100z + 100 \left[\frac{RT_i - RT_z}{RT_{z+1} - RT_z} \right]$$

where RI is the retention index of the unknown peak, RT_i the retention time for the unknown peak, RT_z and RT_{z+1} are the retention times for the *n*-alkanes that bracket the unknown peak, *z* is the number of carbon atoms in the *n*-alkane standard that elute just before the unknown peak. All experiments were carried out in triplicate.

2.5. Analytical methods validation

Twenty-five volatile target compounds (Table 1) were selected to study the methods performance. Calibrations have to be carried out for each compound and by the two methods

(LLE_{CH₂Cl₂} and HS-SPME_{PDMS}) in order to achieve accurate quantitative results.

For linearity study, calibration graphs were established with five standard solutions in synthetic whiskey. Duplicate calibration graphs, were drawn by the least-squares linear regression method using the relative peak area as response versus concentration. Regression slope and origin intercept were calculated by linear least-squares regression. Repeatability (precision) was evaluated by the relative standard deviation of six independent assays performed under the same analytical conditions in the shortest period of time. For each assay the mean values, standard deviation and coefficients of variation for all compounds were calculated.

The recovery rates of volatile compounds were evaluated by addition of known amounts of the target compounds to the BL whiskey sample. Samples were submitted to six successive extractions with CH₂Cl₂ and with a CAR/PDMS fibre. For each volatile compound the recovery rate was calculated by the ratio

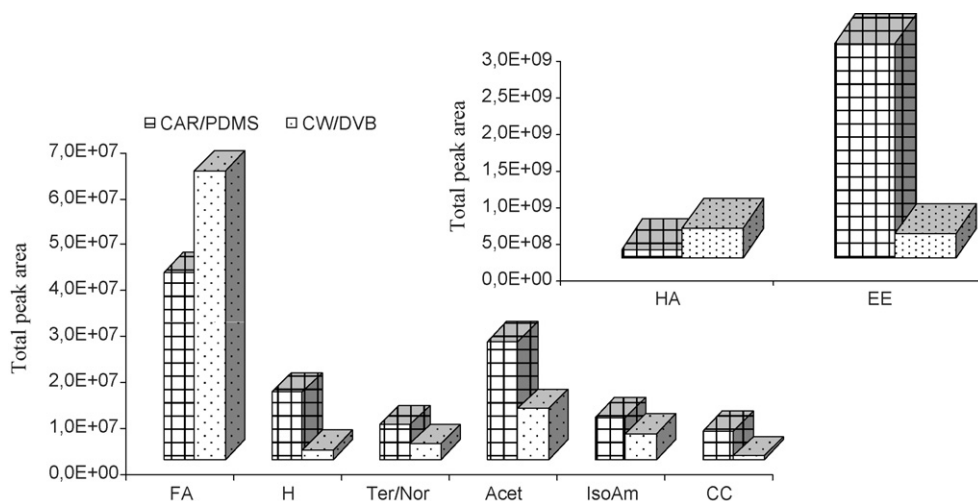


Fig. 2. Influence of the type of HS-SPME fibre coatings on the GC–MS peak area for the total fraction of the volatile compounds in BL whiskey sample (extraction temperature: 40 °C; extraction time: 60 min) (HA: higher alcohols; EE: ethyl esters; FA: fatty acids; H: hydrocarbons; Ter/Nor: monoterpenes and C₁₃-norisoprenoids; Acet: higher alcohol acetates; Isoam: isoamyl esters; CC: carbonyl compounds).

$((C_1 - C_0)/C_2) \times 100$, where C_0 is the concentration of the analyte in the whiskey before addition, C_1 the concentration of the analyte in spiked whiskey sample and C_2 is the amount of the analyte added to whiskey sample.

The limits of detection (LOD) were estimated as the concentration of the analyte that produce a *signal-to-noise* ratio of three times the standard deviation of the y -residuals of the calibration graph, which is $3s_{y/x}/b$, where $s_{y/x}$ is the blank standard deviation and b is the slope of the line regression. The linear range experiments provide the necessary information to calculate the limits of detection, by extrapolating from the lowest concentration point on the linear calibration curve. The limit of quantification (LOQ) can also be estimated as the concentration of analyte producing a signal 10 times that of the noise.

Extraction yield were calculated using the internal standard method. Standard solutions containing the volatiles and the internal standard at known concentrations were injected into the chromatograph system. The response factor (K) of each compound was calculated using the following equation: $K = A_c \times C_{IS}/A_{IS} \times C_c$, where A_c is the area of the compound, A_{IS} area of the internal standard, C_c concentration of the compound and C_{IS} is the concentration of the internal standard. Then 50 mL of synthetic whiskey containing the volatile compounds at known concentrations was subjected to the LLE

procedure described above. The concentration of each compound can be calculated applying the response factor equation (K). Taking into account the organic phase volume, the amount of the extracted analyte was calculated and the yield expressed as: $\% = C_e/C_0 \times 100$, where C_e is the concentration of the extracted analyte and C_0 is the initial concentration. A similar procedure, to the described in Section 2.3.2, was made for HS-SPME_{CAR/PDMS}.

2.6. Whiskey samples differentiation

In this study, eight different commercial whiskeys were analyzed in triplicate following the methodology proposed. Whiskey sample differentiation was performed with SPSS program Version 13.0 statistical package for Windows (SPSS, Chicago, IL).

3. Results and discussion

First, the commercial whiskeys were analysed by GC–MS to identify the volatile fraction constituents. Many compounds were identified, but only 25 (Table 1) including higher alcohols, ethyl esters, fatty acids and furanic compounds, were selected to study the figures of merit of the methods.

Table 2

Performance parameters of the solid-phase microextraction method (HS-SPME_{CAR/PDMS}): concentration range, linearity, detection (LOD) and quantification (LOQ) limits

Compounds	Range concentration (mg L ⁻¹)	<i>r</i>	Slope	Intercept	LOD (μg L ⁻¹)	LOQ (μg L ⁻¹)	R.S.D. (%)	IS
Higher alcohols								
2-Methylbutan-1-ol	3.90–60.0	0.9985	18.37	−6.48	5.44	18.12	7.40	4M2P
Butan-1-ol	20.0–200.0	0.9955	15.10	2.80	10.26	34.20	5.17	4M2P
3-Methylbutan-1-ol	12.75–176.05	0.9969	0.96	−4.68	19.03	63.43	4.25	4M2P
Hexan-1-ol	10.14–219.77	0.9969	0.94	−2.81	8.03	26.77	12.78	3-OCT
Methionol	0.46–11.26	0.9989	12.96	−1.94	0.67	2.22	9.64	3-OCT
β-Phenylethanol	7.70–228.33	0.9995	2.72	−1.00	8.55	9.43	6.31	3-OCT
Benzyl alcohol	0.93–87.64	0.9989	0.82	0.25	1.59	5.32	11.22	4M2P
2-Phenoxyethanol	17.95–151.78	0.9935	0.15	6.05	13.43	44.76	9.16	3-OCT
Acetate								
Isoamyl acetate	0.39–24.78	0.9989	2.10	0.84	1.44	4.81	4.97	4M2P
Ethyl esters								
Ethyl butanoate	0.91–122.03	0.9989	55.73	4.39	2.37	7.91	13.75	3-OCT
Ethyl hexanoate	0.91–24.92	0.9995	67.71	6.23	1.06	3.53	3.97	4M2P
Ethyl lactate	4.10–29.53	0.9969	0.02	0.01	3.06	10.19	7.83	4M2P
Ethyl octanoate	0.40–4.90	1.0000	459.48	−15.55	0.13	0.42	4.98	3-OCT
Ethyl decanoate	0.40–4.81	0.9995	117.40	−4.14	0.24	0.79	10.54	3-OCT
Diethyl succinate	1.49–10.52	0.9955	0.74	0.42	1.34	4.48	6.81	4M2P
Ethyl dodecanoate	0.40–4.37	0.9995	75.19	−29.15	0.20	0.66	13.05	3-OCT
Fatty acids								
Hexanoic acid	1.60–92.77	0.9995	0.408	0.10	3.02	10.08	8.89	4M2P
Octanoic acid	3.20–90.87	1.0000	1.081	0.91	2.50	8.32	14.78	4M2P
Decanoic acid	7.90–58.77	0.9959	1.921	2.46	6.91	23.03	2.71	4M2P
Furanic compounds								
Furfural	2.9–14.26	0.9985	9.00	−1.24	1.07	3.56	10.11	3-OCT
5-Methyl-2-furfural	2.5–11.77	0.9975	12.53	−2.39	1.09	3.64	12.04	3-OCT
Volatile phenol								
Guaiacol	0.90–10.79	0.9995	7.22	0.54	0.54	1.79	7.52	3-OCT

Table 3
Comparison of liquid–liquid extraction using dichloromethane as extraction solvent (LLE_{CH₂Cl₂}) and solid-phase microextraction using two different coatings (HS-SPME_{CAR/PDMS} and HS-SPME_{CW/DVB}) analysis of chemical composition of the main volatile compounds in BL whiskey

RT (min)	KI ^a	Compound	Identity ^b	%RPA ± %R.S.D.		
				^c LLE _{CH₂Cl₂}	^c HS-SPME _{CAR/PDMS}	^c HS-SPME _{CW/DVB}
2.09	907	Ethyl acetate	A, B	ND ^d	0.14 ± 1.21	0.56 ± 18.29
2.96	984	Ethanol	A, B	ND	16.44 ± 1.58	42.57 ± 13.37
4.13	1033	2-Methylpropan-1-ol	A, B	3.71 ± 9.72	ND	ND
5.52	1121	Butan-1-ol	A, B	0.061 ± 12.14	0.26 ± 6.89	ND
6.18	1144	Isoamyl acetate	A, B	0.056 ± 8.76	0.13 ± 1.45	0.36 ± 5.07
6.44	1152	1,2-Dimethylbenzene	B	ND	0.04 ± 8.72	ND
8.17	1202	1,3-Dimethylbenzene	B	ND	0.02 ± 8.48	ND
9.48	1211	1,2-Dihydro-3,6,8-trimethylnaftalene	B	ND	0.06 ± 6.90	0.085 ± 12.94
10.26	1255	3-Methylbutan-1-ol	A, B	46.39 ± 13.64	2.57 ± 13.00	4.85 ± 9.86
10.37	1257	Ethyl hexanoate	A, B	0.056 ± 16.31	1.81 ± 0.65	0.26 ± 8.59
12.03	1292	Styrene	B	0.032 ± 8.97	2.32 ± 19.11	ND
12.48	1310	Hexyl acetate	A, B	0.036 ± 7.43	ND	ND
14.04	1333	2-Methyl furane	B	ND	ND	0.022 ± 2.80
15.32	1358	1,2,4-Trimethylbenzene	B	ND	ND	0.046 ± 2.59
17.27	1391	4-Ethyl-1,2-dimethylbenzene	B	ND	ND	0.0037 ± 18.98
18.41	1413	Hexan-1-ol	A, B	ND	0.02 ± 8.66	0.044 ± 7.51
18.52	1418	Ethyl lactate	A, B	1.14 ± 5.61	ND	ND
19.13	1426	(Z)-3-Hexen-1-ol	A, B	ND	0.04 ± 4.46	ND
20.22	1447	2-Methyl undecanal	B	ND	0.09 ± 2.86	ND
22.34	1467	TTN ^e	B	ND	0.0021 ± 8.59	0.0051 ± 13.90
23.07	1496	Ethyl octanoate	A, B	3.39 ± 9.74	14.16 ± 13.16	13.38 ± 4.22
24.58	1509	Isoamyl hexanoate	B	ND	0.03 ± 18.65	0.0092 ± 6.15
24.91	1520	1-Octen-3-ol	A, B	0.0091 ± 18.50	0.0057 ± 3.74	0.011 ± 12.41
25.16	1529	Acetal	B	0.025 ± 13.26	ND	ND
25.21	1538	2-Ethylhexan-1-ol	B	ND	0.03 ± 7.30	0.0096 ± 17.99
26.43	1556	Acetic acid	A, B	2.09 ± 6.71	ND	0.086 ± 7.07
27.09	1567	4-Ethyl- <i>m</i> -xylene	B	ND	0.0067 ± 5.37	0.021 ± 5.09
27.24	1570	Furfural	A, B	0.53 ± 7.09	0.90 ± 0.23	0.034 ± 14.49
28.08	1584	1,10-Decanediol	B	0.014 ± 12.42	0.04 ± 0.22	ND
28.33	1588	Vitispyrane (I)	B, C	ND	0.016 ± 17.16	0.0070 ± 2.56
28.48	1590	Vitispyrane (II)	B, C	ND	0.015 ± 12.55	0.032 ± 11.42
29.25	1703	Propyl octanoate	B	ND	0.07 ± 14.90	0.13 ± 12.43
30.26	1719	2-Hydroxy-1-phenylethanone	B	0.034 ± 9.34	ND	0.012 ± 4.40
31.34	1741	Ethyl nonanoate	B	0.035 ± 14.04	ND	ND
32.04	1756	Butyl caprilate	B	ND	0.036 ± 21.50	0.024 ± 23.45
32.12	1758	Linalool	A, B	ND	ND	0.013 ± 1.27
33.05	1773	Propionic acid	A, B	0.067 ± 5.44	ND	ND
34.03	1789	2-Methylpropanoic acid	A, B	0.0064 ± 7.39	ND	ND
34.52	1800	Decahydronaphthalene	B	ND	0.05 ± 1.98	ND
35.11	1812	Methyl decanoate	B	ND	0.11 ± 7.30	ND
35.52	1848	5-Methyl furfural	A, B	0.024 ± 11.72	ND	ND
37.57	1860	Ethyl decanoate	A, B	6.67 ± 10.16	34.06 ± 2.54	1.27 ± 3.49
38.41	1881	3-Methylbutanoic acid	A, B	0.20 ± 9.81	ND	ND
40.05	1906	Isoamyl octanoate	B	ND	0.89 ± 14.23	0.17 ± 3.38
40.50	1915	Ethyl benzoate	A, B	ND	0.59 ± 4.44	0.060 ± 16.36
41.39	1933	Ciclohexanemethanol	B	ND	0.24 ± 5.83	0.21 ± 3.25
41.84	1941	Diethyl succinate	A, B	0.49 ± 5.56	ND	ND
42.22	1949	Ethyl 9-decanoate	B	ND	3.45 ± 6.15	0.77 ± 4.27
44.46	2001	TDN	A, B	ND	0.036 ± 11.47	0.19 ± 1.51
45.33	2009	Propyl decanoate	B	0.013 ± 9.15	0.04 ± 4.11	0.0090 ± 10.40
45.56	2022	Azulene	B	0.11 ± 6.71	0.28 ± 1.82	0.034 ± 14.02
46.36	2030	Butyl decanoate	B	ND	0.051 ± 3.25	0.092 ± 0.41
48.01	2062	Dodecan-1-ol	B	ND	0.40 ± 16.38	0.13 ± 11.59
51.26	2129	β-Damascenone	A, B	ND	0.21 ± 3.94	0.032 ± 8.57
51.45	2128	2-Phenylethyl acetate	A, B	0.74 ± 8.64	0.43 ± 3.02	0.38 ± 26.86
52.49	2156	Ethyl dodecanoate	A, B	4.44 ± 6.41	3.74 ± 9.76	21.95 ± 7.62
54.01	2189	Isoamyl decanoate	B	0.012 ± 13.76	0.70 ± 9.70	0.46 ± 2.08
54.10	2192	Hexanoic acid	A, B	0.32 ± 4.76	ND	ND
54.32	2197	1,4-Bis(1-methylethenyl)benzene	B	ND	0.05 ± 3.66	0.063 ± 0.95
55.33	2218	(Z)-11-Hexadecan-1-ol	B	ND	0.07 ± 12.72	0.039 ± 8.63
56.10	2235	Ethyl benzenepropanoate	B	ND	0.08 ± 4.36	0.016 ± 11.24

Table 3 (Continued)

RT (min)	KI ^a	Compound	Identity ^b	%RPA $\pm$ %R.S.D.		
				^c LLECH ₂ Cl ₂	^c HS-SPME _{CAR/PDMS}	^c HS-SPME _{CW/DVB}
57.31	2257	Benzeneacetaldehyde	B	0.31 $\pm$ 10.14	ND	ND
57.81	2272	α -Ionol	A, B	ND	0.35 $\pm$ 8.70	0.087 $\pm$ 2.74
58.50	2287	2-Phenylethanol	A, B	5.52 $\pm$ 12.40	1.00 $\pm$ 5.99	0.24 $\pm$ 17.52
61.11	2301	Whiskey lactone	B	0.17 $\pm$ 7.34	ND	ND
61.23	2314	1,8-Dimethyl naphthalene	B	ND	ND	0.0067 $\pm$ 6.78
61.35	2346	1,14-Tetradecanediol	B	ND	0.31 $\pm$ 6.88	0.19 $\pm$ 4.55
62.31	2365	Biphenyl	B	ND	0.01 $\pm$ 2.07	0.012 $\pm$ 3.41
65.19	2423	2-Methoxyphenol	A, B	0.026 $\pm$ 14.61	ND	ND
65.74	2431	2-Methyl phenol	A, B	0.026 $\pm$ 15.06	0.023 $\pm$ 1.93	0.033 $\pm$ 14.97
66.02	2440	Ethyl tetradecanoate	A, B	0.604 $\pm$ 9.57	0.41 $\pm$ 0.54	0.97 $\pm$ 8.15
66.24	2447	Ethyl laurate	A, B	ND	0.0023 $\pm$ 8.47	0.039 $\pm$ 14.97
66.69	2456	Nerolidol	A, B	0.062 $\pm$ 12.18	0.17 $\pm$ 0.29	0.16 $\pm$ 13.53
67.45	2468	Octanoic acid	A, B	4.01 $\pm$ 8.15	2.22 $\pm$ 0.83	1.37 $\pm$ 19.76
68.18	2495	Ethyl 3-hydroxyhexanoate	B	ND	0.0067 $\pm$ 16.11	0.095 $\pm$ 5.63
70.09	2512	4-Methyl phenol	B	0.085 $\pm$ 11.61	ND	ND
70.52	2523	2-Ethyl phenol	B	0.014 $\pm$ 16.23	ND	ND
74.04	2554	Cyclododecanemethanol	B	0.72 $\pm$ 11.47	0.054 $\pm$ 13.61	0.75 $\pm$ 5.83
75.31	2591	4-Ethyl phenol	B	0.045 $\pm$ 10.34	ND	ND
77.32	2667	Ethyl hexadecanoate	A, B	1.06 $\pm$ 7.76	0.05 $\pm$ 27.07	0.34 $\pm$ 21.16
78.10	2683	Ethyl 9-hexadecenoate	B	1.20 $\pm$ 4.12	0.03 $\pm$ 8.48	0.74 $\pm$ 7.75
79.14	2704	Decanoic acid	A, B	9.01 $\pm$ 11.07	4.08 $\pm$ 0.70	4.53 $\pm$ 10.94
82.29	2772	Oleyl alcohol	B	0.79 $\pm$ 5.73	0.03 $\pm$ 1.03	0.19 $\pm$ 16.30
85.30	2828	Benzoic acid	A, B	ND	0.04 $\pm$ 19.15	ND
86.20	2846	Dodecanoic acid	A, B	4.08 $\pm$ 7.91	0.93 $\pm$ 2.12	1.51 $\pm$ 9.85
87.54	2865	5-Hydroxymethyl furfural	A, B	0.53 $\pm$ 8.53	ND	ND
88.51	2894	Vanillin	A, B	0.46 $\pm$ 12.41	ND	ND

^a Experimentally determined Kovats indices on the DB-Waxetr column, relative to C₈–C₂₀ hydrocarbons.

^b A: components identified on the basis of the retention time and EI mass spectra of pure standard; B: components identified on the basis of their EI mass spectra only; C: mass spectra agreed with literature data [42].

^c Averages of three independent extractions.

^d Not detected.

^e 1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene.

3.1. Solvent and fibre selection

The choice of the extraction solvent depends on the type of food and the information needed. It is of great importance to recover an aromatic extract that as representative as possible of the product. Initially, some preliminary experiments were performed in order to establish the most suitable extraction solvent. Two solvents, CH₂Cl₂, H, and five mixtures CH₂Cl₂–DE (3:1 and 1:3), CH₂Cl₂–H (3:1 and 1:3) and DE–H (1:3), and two SPME fibres (CAR/PDMS and CW/DVB) were investigated and evaluated. More than 50 compounds were detected and identified in concentration above 0.05% in the volatile fractions obtained by CH₂Cl₂. The total composition of volatile fractions isolated by CH₂Cl₂–DE (3:1 and 1:3), and CH₂Cl₂ differed significantly ($p < 0.05$) from H, CH₂Cl₂–H (1:3) and DE–H (1:3). Highest extraction efficiency of whiskey volatile compounds was obtained when CH₂Cl₂ was used as solvent extraction. H extracted the lowest volatile fraction (about 24% of CH₂Cl₂). Similar amounts of volatiles were extracted when using either CH₂Cl₂–DE (3:1 and 1:3) (94 and 87% of CH₂Cl₂, respectively) or CH₂Cl₂–H (3:1) (82% of CH₂Cl₂). CH₂Cl₂ is a very good extraction solvent, because all the target compounds were extracted and the yields were bigger in most cases, hence, it was the solvent chosen for extraction/isolation the

target compounds from whiskeys by LLE. Fig. 1 compares the main fatty acids and ethyl esters found in CH₂Cl₂ extract of BL whiskey with those obtained by using H as extraction solvent.

The main volatile compounds found in CH₂Cl₂ extracts of BL whiskey were 3-methylbutan-1-ol with a relative peak area (RPA) of 46.4, decanoic acid (RPA = 9.0), ethyl decanoate (RPA = 6.7), β -phenylethanol (RPA = 5.5), and in lower extent dodecanoic (RPA = 4.1) and octanoic (RPA = 4.0) acids. 3-Methylbutan-1-ol (RPA = 7.3), butanoic acid (RPA = 3.5), ethyl dodecanoate (RPA = 2.9) and ethyl octanoate (RPA = 1.4), were the main compounds found in *n*-hexane extract.

The relative amount of the higher alcohols, dropped considerably from 88.44% (CH₂Cl₂–DE, 3:1) to 44.14% (H), whilst the ethyl esters fraction rose from 3.92% (CH₂Cl₂) to 24.20% (H).

To investigate the extraction yields of the whiskey volatile components by HS-SPME, two fibre coatings, CAR/PDMS and CW/DVB, were used. Some experimental factors which influence the HS-SPME extraction yield, namely time required for the target analytes to reach equilibrium and extraction temperature, were previously evaluated by Câmara et al. [41]. The equilibration temperature was investigated by performing the analysis at four different temperatures (25, 40, 50 and 60 °C) at

fixed equilibration time of 60 min. To select an optimal equilibration exposure time of the SPME fibre, extractions were performed at 5, 15, 30, 45 and 60 min [41]. In view of the obtained results, a temperature of 40 °C and an extraction time of 60 min were chosen as the optimized conditions to carrying the analysis of all the whiskey samples under investigation. For reasons of comparability all tests were carried out with the same whiskey sample (BL). The peak areas of the total free fraction present in the BL whiskey were used for the evaluation of the optimal fibre. With the HS-SPME method, more than 60 volatile compounds were identified by using the CAR/PDMS and CW/DVB coatings. The results were shown in Fig. 2, indicating that the semi-polar CAR/PDMS fibre was more efficient than the CW/DVB fibre for extracting total volatiles in whiskeys. Therefore, this fibre was chosen for the remaining studies including validation. CAR/PDMS coating is less polar than CW/DVB, thus it is widely used for the extraction of non-polar compounds. For volatile low-molecular-mass and polar compounds like ketones and alcohols polar coatings like CW/DVB work better. From Fig. 2, it can be observed that the two fibres show different selectivity to different groups of compounds. The ethyl esters from fatty acids (EE) have a larger affinity for CAR/PDMS fibre. This coating has better sensitivity for hydrocarbons (H), monoterpenes and C_{13} norisoprenoids (Ter/Nor), higher alcohol acetates (Acet), isoamyl esters (IsoAm) and carbonyl compounds (CC). Higher alcohols (HA) and fatty acids (FA) were more effectively extracted by using the CW/DVB coating.

The results presented above suggest that there is a great variability in the aroma compounds obtained, depending on the solvent and the fibre coating employed. Some characteristic compounds of whiskey aroma were isolated by the both methods, such as 3-methylbutan-1-ol, β -phenylethanol, ethyl esters: hexanoate, octanoate, decanoate, dodecanoate, tetradecanoate and hexadecanoate, and fatty acids: octanoic, decanoic and dodecanoic.

3.2. LLE and headspace SPME: performance characteristics

Once the extraction solvent (for LLE method) and the fibre (for SPME method) were selected, the performance characteristics of the methods in terms of linearity, precision and accuracy, were evaluated. The results are shown in Table 1 (LLE $_{CH_2Cl_2}$) and Table 2 (SPME $_{CAR/PDMS}$). Other analytical parameters that have been studied include the limits of detection (LOD) and quantification (LOQ), the percentage of recovery and the extraction yield.

The linearity of the method was evaluated through the construction of two calibration curves for each analyte (five levels of concentration were tested), using the same analytical conditions of the sample. For each compound the dynamic concentration ranges together with the response factor for each of the analytes are shown in Table 1 (LLE $_{CH_2Cl_2}$) and Table 2 (SPME $_{CAR/PDMS}$). In general, the volatile compounds have shown a good linearity in the range of concentrations studied, as regression coefficients (r) vary between 0.9929 (β -phenoxyethanol) and

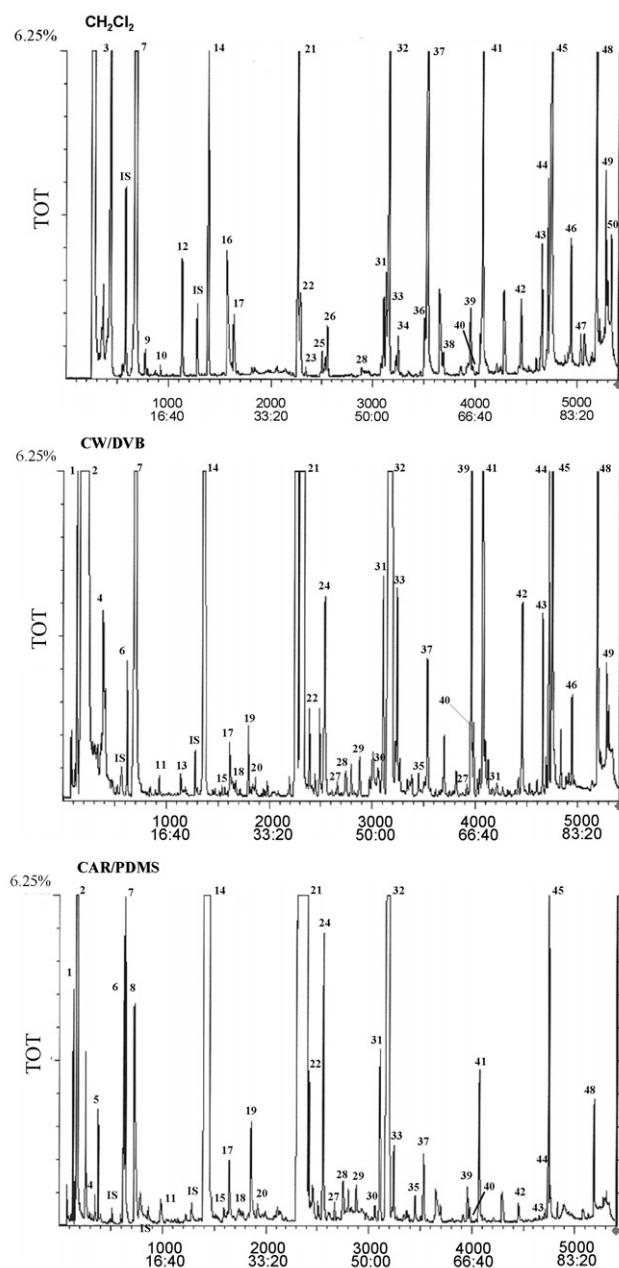


Fig. 3. Comparison of TIC chromatograms of the volatile fraction extracted/isolated from BL whiskey sample obtained by LLE method using CH_2Cl_2 as extraction solvent (LLE $_{CH_2Cl_2}$) and by dynamic HS-SPME using a 75 μ m CAR/PDMS fibre coating (HS-SPME $_{CAR/PDMS}$). Peak Identification: 1: ethyl acetate; 2: ethanol; 3: 2-methylpropan-1-ol; 4: butan-1-ol; 5: isoamyl acetate; IS: 4-methylpentan-2-ol; 6: ethyl hexanoate; 7: 3-methylbutan-1-ol; 8: styrene; 9: acetal; 10: hexyl acetate; 11: ethyl heptanoate; IS: octan-3-ol; 12: ethyl lactate; 13: hexan-1-ol; 14: ethyl octanoate; 15: linalool; 16: acetic acid; 17: furfural; 18: vitispirane (isomer I + II); 19: propyl octanoate; 20: butyl caprylate; 21: ethyl decanoate; 22: isoamyl octanoate; 23: butanoic acid; 24: *trans*-ethyl 2-decenoate; 25: 3-methylbutanoic acid; 26: diethyl succinate; 27: azulene; 28: butyl decanoate; 29: dodecan-1-ol; 30: β -damascenone; 31: 2-phenylethanol acetate; 32: ethyl dodecanoate; 33: isoamyl decanoate; 34: hexanoic acid; 35: α -ionol; 36: benzeneacetaldehyde; 37: β -phenylethanol; 38: *cis*-whiskey lactone; 39: ethyl tetradecanoate; 40: nerolidol; 41: octanoic acid; 42: 1,12 dodecanediol; 43: ethyl hexadecanoate; 44: ethyl 9-hexadecanoate; 45: decanoic acid; 46: cyclododecanemethanol; 47: ethyl succinate; 48: dodecanoic acid; 49: 5-(hydroxymethyl) furfural; 50: vanillin.

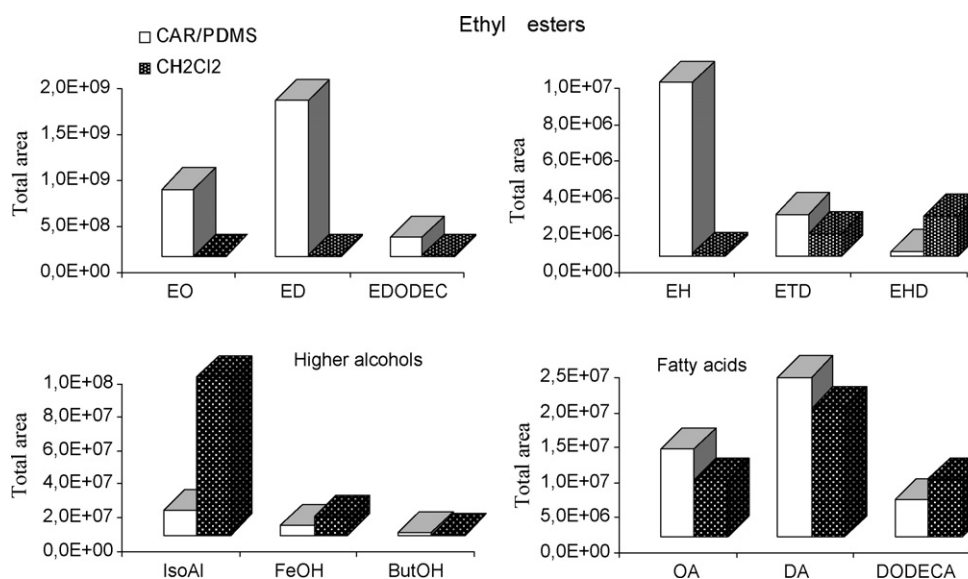


Fig. 4. Proportion of the chromatographic profile between $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ and $\text{SPME}_{\text{CAR/PDMS}}$ methods for analysis of different chemical classes and specific compounds determined in BL whiskey (DCM: CH_2Cl_2 ; EO: ethyl octanoate; ED: ethyl decanoate; EDODEC: ethyl dodecanoate; EH: ethyl hexanoate; ETD: ethyl tetradecanoate; EHD: ethyl hexadecanoate; OA: octanoic acid; DA: decanoic acid; DODECA: dodecanoic acid; IsoAl: 3-methylbutan-1-ol; FeOH: β -phenylethanol; ButOH: butan-1-ol).

1.000 (propan-1-ol, isoamyl acetate, ethyl butanoate and ethyl decanoate) for $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ (Table 1) and between 0.9935 (β -phenoxyethanol) and 1.000 (ethyl octanoate, octanoic acid) for $\text{HS-SPME}_{\text{CAR/PDMS}}$ (Table 2).

The precision was studied as reproducibility and expressed as relative standard deviation, (R.S.D.) for BL whiskey which was extracted six times and injected in triplicate. Percent R.S.D. values range from 3.19% (2-methylpropan-1-ol) to 19.42% (decanoic acid) for the $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ (Table 1) and between 3.97% (ethyl hexanoate) and 14.78% (octanoic acid), for $\text{HS-SPME}_{\text{PDMS}}$ method (Table 2).

The accuracy of both the analytical methods was evaluated from the determination of the recovery, obtained by the addition of known quantities of the target compounds in a commercial BL whiskey. No significant differences were found between them at a significance level of 5%. Recoveries near 100% were obtained for all the volatile compounds under study.

The values obtained for detection and quantification limits (Tables 1 and 2), are in general, low enough to permit the determination of these compounds in whiskey samples. The values obtained by $\text{SPME}_{\text{CAR/PDMS}}$ were much lower than the values determined by $\text{LLE}_{\text{CH}_2\text{Cl}_2}$.

Table 4
Odour description, odour threshold and odour activity values of the main odour-active compounds of commercial whiskeys

Compound	Odour description	Odour threshold ($\mu\text{g L}^{-1}$)	OAV ^a							
			HC	FG	DW	RL	BL	GRA	JL	BAL
Acetaldehyde	Green, nutty,	500 ^b	6.02	7.75	9.44	7.59	12.49	4.09	6.04	8.17
Butan-1-ol	Pungent, harsh	150000 ^c	0.62	1.30	0.87	0.66	3.01	0.12	< 0.1	0.45
2-Methylpropan-1-ol	Wine, solvent	40000 ^b	55.53	64.10	66.42	57.76	75.26	65.32	60.46	57.65
Isoamyl acetate	Banana	30 ^b	150.35	125.43	121.35	92.72	100.21	190.51	128.27	127.61
3-Methylbutan-1-ol	Sweet, fusel	30000 ^b	3.25	3.81	3.89	3.90	5.57	4.49	2.60	3.81
Ethyl hexanoate	Green apple, anise	14 ^c	< 0.1	7.12	27.63	< 0.1	22.42	21.13	< 0.1	5.44
Ethyl octanoate	Sweet, fruity, fresh	5 ^c	423.47	787.35	915.87	603.01	1011.35	864.43	255.70	638.17
Furfural	Fruity, floral	20000 ^d	0.27	1.08	0.66	0.72	2.66	0.79	0.06	0.71
Ethyl decanoate	Floral, soap	200 ^c	26.56	50.13	42.15	39.14	45.36	49.61	20.09	37.53
β -Phenylethanol	Floral, rose, honey	14000 ^c	0.19	0.16	0.14	0.18	0.20	0.32	< 0.1	0.20
Ethyl dodecanoate	fruity, floral	1200 ^c	3.47	6.73	2.97	4.86	4.71	4.37	3.39	4.69
Hexanoic acid	Sweet, cheesy	420 ^c	4.83	5.31	6.60	6.12	7.40	6.42	4.21	6.17
Octanoic acid	Cheese	500 ^c	8.93	13.85	19.56	17.22	23.37	19.99	6.48	18.11
Decanoic acid	Fatty	1000 ^c	11.74	6.30	12.78	17.55	7.57	8.85	9.62	11.77

^a Odour activity value calculated by dividing concentration by odour threshold value of the compound. In bold compounds with OAV < 1.

^b Guth [43]. The matrix was a 10% water/ethanol solution.

^c Ferreira et al. [44]. The matrix was a 11 % water/ethanol solution containing 7 g L⁻¹ of glycerol and 5 g L⁻¹ of tartaric acid, with the pH adjusted to 3.4 with 1 M NaOH.

^d Boidron et al. [45]. The matrix was a synthetic wine containing 12% ethanol, 8 g L⁻¹ of glycerol and different salts.

3.3. Volatile compounds in commercial whiskeys

The R.S.D. values for triplicate samples were <10%. 3-Methylbutan-1-ol is the main component in all whiskeys volatile fractions, followed by decanoic acid and ethyl decanoate. It was observed that the total average concentration of volatile constituents in BL whiskeys are of 262.65 mg L^{-1} while for the other commercial whiskeys, the average concentration are significantly lower ($p < 0.05$, 95%). The higher alcohols are the chemical family that contributes for this significant difference, from which 3-methylbutan-1-ol and 2-methylpentan-1-ol are highlighted. Our results, using both $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ and $\text{SPME}_{\text{CAR/PDMS}}$ methods, indicate that BL has significantly elevated levels of higher alcohols and furanic compounds as compared to JL and HC whiskeys. Levels of higher alcohol acetates and fatty acids are significantly lower in RL and FG whiskeys, respectively.

3.4. Comparison of the two analytical methods

The conventional LLE method and the new HS-SPME methodology were applied to the analysis of nine commercial whiskey samples in order to determine the volatile profile and to compare the different samples. The instrumental analytical conditions chosen allow the separation of the all compounds studied. Relative peak area counts (RPA) and relative standard deviation (R.S.D.) of volatile compounds detected in whiskey using LLE and SPME, together with their calculated Kováts indices are shown in Table 3 in order of elution. As can be seen from the data, 65 volatile organic compounds were tentatively identified in whiskey extracts using the $\text{SPME}_{\text{CAR/PDMS}}$ technique and 61 by $\text{SPME}_{\text{CW/DVB}}$, whereas 55 compounds were positively identified by $\text{LLE}_{\text{CH}_2\text{Cl}_2}$.

Basically, volatile flavour compounds comprised: ethyl esters (17 SPME and 14 CH_2Cl_2), higher alcohols (16 SPME and 8 CH_2Cl_2), higher alcohol acetate (3 SPME and 2 CH_2Cl_2), isoamyl acetates (4 SPME and 1 CH_2Cl_2), fatty acids (4 SPME

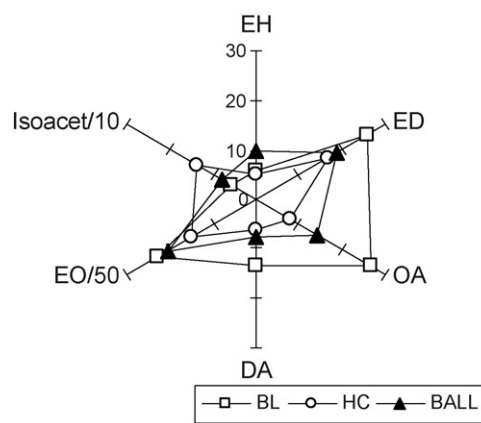


Fig. 5. Main odorants found in BL, HC and RL whiskeys determined by HS- $\text{SPME}_{\text{CAR/PDMS}}$ methodology (EH: ethyl hexanoate; ED: ethyl decanoate; OA: octanoic acid; DA: decanoic acid; EO/50: ethyl octanoate concentration divided by 50; Isoacet: 3-methylbutan-1-ol acetate concentration divided by 10).

and 9 CH_2Cl_2), carbonyl compounds (3 SPME and 7 CH_2Cl_2), hydrocarbons (9 SPME and 4 CH_2Cl_2), monoterpenes and C_{13} -norisoprenoids (8 SPME and 2 CH_2Cl_2) and volatile phenols (4 SPME and 6 CH_2Cl_2). Typical GC-ITDMS chromatographic profile of the volatile fraction isolated from BL whiskey by $\text{LLE}_{\text{CH}_2\text{Cl}_2}$, HS- $\text{SPME}_{\text{CAR/PDMS}}$ and HS- $\text{SPME}_{\text{CW/DVB}}$ methods, are shown in Fig. 3. Table 3 displays the semi-quantitative results of the volatile organic components derived from triplicate extractions.

Major compounds in the TIC/HS- $\text{SPME}_{\text{CAR/PDMS}}$ profile were ethyl esters ($\text{C}_{10} \gg \text{C}_8 > \text{C}_{12}$, and in lower content $\text{C}_{14} > \text{C}_{16}$), higher alcohols (3-methylbutan-1-ol > β -phenylethanol) and fatty acids ($\text{C}_{10} > \text{C}_8 > \text{C}_{12}$), while in the TIC/ $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ profile, higher alcohols (3-methylbutan-1-ol > β -phenylethanol), ethyl esters ($\text{C}_{10} > \text{C}_{12} > \text{C}_8$), 5-hydroxymethylfurfural and vanillin are the major ones. This suggests that SPME is generally a more sensitive technique than LLE for the extraction of aroma compounds from whiskey, although in practice the two methods may complement each other.

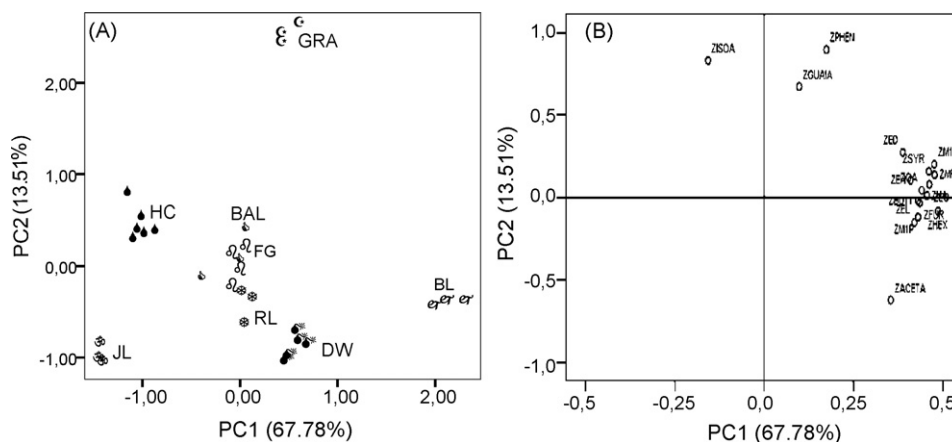


Fig. 6. PC1 vs. PC2 scatter plot of the main sources of variability between different whiskeys: (A) distinction between the samples (scores) and (B) relation between the 22 volatile compounds (loading). Identification of the variables: M1P: 2-Methylpropan-1-ol; BUT: butan-1-ol; M1B: 3-methylbutan-1-ol; HEX: hexan-1-ol; PHEN: 2-phenylethanol; ISOA: isoamyl acetate; EH: ethyl hexanoate; EL: ethyl lactate; EO: ethyl octanoate; ED: ethyl decanoate; HA: hexanoic acid; OA: octanoic acid; ACET: acetaldehyde; SYR: syringaldehyde; FUR: 2-furfural; MF: 5-methyl-2-furfural; GUA: guaiacol.

Differences in the selectivity between each extraction method were observed. In general, $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ extracted higher alcohols more efficiently than $\text{HS-SPME}_{\text{CAR/PDMS}}$. However, this methodology was more selective for ethyl esters and fatty acids than was $\text{LLE}_{\text{CH}_2\text{Cl}_2}$. The major compounds appeared to be the same as those in CH_2Cl_2 extracts. As can be seen, the values obtained for these compounds by $\text{SPME}_{\text{CAR/PDMS}}$ differed to those obtained by $\text{LLE}_{\text{CH}_2\text{Cl}_2}$. Fig. 4 shows the proportion of some of volatile compounds identified in whiskey samples by both $\text{SPME}_{\text{CAR/PDMS}}$ and $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ methods.

3.5. Identification of the main odorants

To estimate the sensory contribution of the volatiles to the overall flavour of whiskeys the OAV values were calculated. Aroma-active compounds detected in whiskeys and their aroma properties are given in Table 4. However, the ranking of the OAV values were different because of differences in concentration levels. From all volatile quantified compounds, those present at concentrations higher than their odour threshold are mainly considered as aroma contributing substances. The results showed that, 14 out of 25 components (56%) quantified in the studied commercial whiskeys were found at concentrations higher than their corresponding threshold values ($\text{OAVs} \geq 1$). According to the OAV values, ethyl octanoate, a compound associated with *sweet, fruity* and *fresh* notes, was the most intense odorant in the studied samples (Fig. 5). Isoamyl acetate was the next most significant compound with an OAV values that ranges from 92.72 (RL) to 190.52 (GRA). This compound contributed for the total aroma of the whiskeys, with a characteristic *banana-like* odour. With regard to alcohols, 3-methylbutan-1-ol (*fruity*) and 2-methylpropan-1-ol (*bitter, harsh*), were the only ones found contributing to whiskey aroma. Butan-1-ol and β -phenylethanol, although exhibiting OAV values lower than 1 also could contribute to the *floral* character of studied whiskeys, due to the synergic effects in the matrix. Ethyl hexanoate showing a *green apple* and *anise* aroma, was identified as another important aroma-active compounds in $\text{DW} > \text{BL} > \text{GRA} > \text{FG} > \text{BAL}$ whiskeys. In HC, RL and LJ samples, this compound present OAV values lower than 0.1, and do not contribute to the aroma of these whiskeys. Hexanoic, octanoic and decanoic acids exhibit an aroma index very high, indicating that these compounds probably can contribute individually to the studied whiskeys aroma.

There is a similarity in the OAV values between the different commercial whiskey samples. The most significant differences were observed for BL and GRA (Fig. 5). The highest OAV values were gotten for by-products of the fermentative metabolism, such as: ethyl octanoate, isoamyl acetate, ethyl decanoate and 2-methylpropan-1-ol. The volatile compounds that contribute less for the aromatic profile are butan-1-ol, furfural and β -phenylethanol.

3.6. Whiskey differentiation

The proposed $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ method was applied to nine different commercial whiskeys. Evidently, the different concentrations of the volatile constituents determined in these samples

allow their differentiation. The obtained results were subjected to principal components analysis (PCA) which revealed that GRA, HC, LJ, and BL whiskeys were clearly separated in a two-dimensional projection. The PCA results show that 81.29% for the obtained results can be represented in a subspace formed by two coordinates (principal components) constructed from linear combinations of the various concentrations that describe each volatile fraction. Fig. 6 shows how GRA whiskey is clearly separated from the others. From the latest, LJ and BL are the most differentiated ones, followed by HC and DW. The BAL, FG and RL whiskeys were too closed that confirm that they have very similar compositions. The compounds which contributed most to the differentiation were hexan-1-ol, 3-methylbutanoic acid and β -phenylethanol.

Factor 1 which explains 67.78% of the information is constructed mainly from contribution of hexan-1-ol (0.97), 3-methylbutanoic acid (0.97), 3-methylbutan-1-ol acid (0.96) and 5-methylfurfural (0.96). Factor 2 correspond to 13.5% of the information and consist mainly of β -phenylethanol (0.96) and isoamyl acetate (0.90).

4. Conclusions

For a complete and quantitative study of volatile composition in food beverages, two or more sample preparation techniques are recommended. Volatile compounds of whiskeys extracted using the two methods LLE and SPME were identified by GC–MS. Twenty-five of them were quantified by GC–FID, and their odour-active compounds identified. $\text{HS-SPME}_{\text{CAR/PDMS}}$ provides a more sensitive technique to the traditional method of LLE. Sixty-five volatile organic compounds were identified using $\text{HS-SPME}_{\text{CAR/PDMS}}$ applied to the extraction of BL whiskey, whereas only 55 aroma compounds were identified using traditional LLE. The main advantages of LLE compared to other conventional methods are that is simple, rapid and no specific instrumentation was required. The $\text{LLE}_{\text{CH}_2\text{Cl}_2}$ –GC–FID analysis revealed that volatile fractions of the studied whiskeys were very rich in ethyl esters followed by higher alcohols and fatty acids.

HS-SPME method using CAR/PDMS coating fibre followed by GC and MS detector is a good procedure for the analysis of several whiskey flavour compounds. The study has revealed potent odorants that are responsible for the overall flavour of the investigated whiskeys. Ethyl octanoate followed by isoamyl acetate, 2-methylpropan-1-ol, ethyl decanoate and decanoic acid, were identified as the main odour-active compounds, on the basis of their high OAV values.

The identification of whiskey aroma compounds and the determination of analytical ratio combined with statistics provide efficient tools like: differentiation of whiskies, establishing criteria for genuineness, improvement of quality, prevention of frauds and guaranteeing their origin.

Acknowledgments

The authors are grateful to the Famous Grouse Company (Scotland) and Prime Drinks, Lda. (Portugal) for supply of the samples used in this study.

References

- [1] G. Fitzgerald, K.J. James, K. MacNamara, M.A. Stack, J. Chromatogr. A 896 (2000) 351.
- [2] W. Wardencki, P. Sowinski, J. Curylo, J. Chromatogr. A 984 (2003) 89.
- [3] S.E. Ebeler, M.B. Terrien, C.E. Butzke, J. Sci. Food Agric. 80 (2000) 625.
- [4] D. González-Arajona, V. Gozález-Gallero, F. Pablos, A.G. González, Anal. Chim. Acta 381 (1999) 257.
- [5] K.Y. Mónica Lee, A. Paterson, J.R. Piggett, G.D. Richardson, Food Qual. Prefer. 12 (2001) 109.
- [6] R. López, M. Aznar, J. Cacho, V. Ferreira, J. Chromatogr. A 966 (2002) 166.
- [7] V. Ferreira, R. López, J. Cacho, J. Sci. Food Agric. 80 (2000) 1659.
- [8] V. Ferreira, N. Ortin, A. Escudero, R. Lopez, J. Cacho, J. Agri. Food Chem. 50 (2002) 4048.
- [9] J.M. Nuñez, H. Bemelmans, J. Chromatogr. A 294 (1984) 361.
- [10] G.P. Blanch, G. Reglero, M. Herraiz, J. Agric. Food Chem. 43 (1995) 1251.
- [11] A. Razungles, H. Tarhi, R. Baumes, C. Günata, C. Tapiero, C. Bayonno, Sci. Aliment. 14 (1993) 59.
- [12] C. Cocito, G. Gaetano, C. Delfini, Food Chem. 52 (1995) 311.
- [13] C. Arthur, J. Pawlizin, Anal. Chem. 62 (1990) 2145.
- [14] H.L. Lord, J. Pawlizin, Anal. Chem. 69 (1997) 3899.
- [15] C. Arthur, L. Killam, K. Buchholz, J. Pawlizin, J. Berg, Anal. Chem. 64 (1992) 1960.
- [16] J.S. Câmara, P. Herbert, J.C. Marques, M.A. Alves, Anal. Chim. Acta 513 (2004) 203.
- [17] S.M. Rocha, L. Maeztu, A. Barros, C. Cid, M.A. Coimbra, J. Sci. Food Agric. 84 (2003) 43.
- [18] J.J. Rodríguez-Bencomo, J.E. Conde, M.A. Rodríguez-Delgado, F. García-Montelongo, J.P. Pérez-trujillo, J. Chromatogr. A 963 (2002) 213.
- [19] J.S. Câmara, M.A. Alves, J.C. Marques, Anal. Chim. Acta 555 (2006) 191.
- [20] S.M. Rocha, P. Coutinho, A. Barros, I. Delgadillo, M.A. Coimbra, J. Chromatogr. A 1114 (2006) 188.
- [21] E. Coelho, S.M. Rocha, I. Delgadillo, M.A. Coimbra, Anal. Chim. Acta 563 (2006) 204.
- [22] J.J. Rodríguez-Bencomo, J.E. Conde, F. García-Montelongo, J.P. Pérez-Trujillo, J. Chromatogr. A 991 (2003) 13.
- [23] S.M. Rocha, F. Rodrigues, P. Coutinho, I. Delgadillo, M.A. Coimbra, Anal. Chim. Acta 513 (2001) 257.
- [24] T.A. Ternes, Water Res. 32 (1998) 3245.
- [25] M. Moeder, S. Schrader, M. Winkler, P. Popp, J. Chromatogr. A 873 (2000) 95.
- [26] H. Kataoka, H.L. Lord, J. Pawlizin, J. Chromatogr. A 880 (2000) 35.
- [27] J.S. Câmara, M.A. Alves, J.C. Marques, Talanta 68 (2006) 1512.
- [28] M.A. Pozo-Bayón, E. Pueyo, P.J. Martín-Álvarez, M.C. Polo, J. Chromatogr. A 922 (2001) 267.
- [29] L.M.T.V. Freire, A.M.C. Freitas, A.M. Relva, J. Microcol. Separ. 13 (2001) 236.
- [30] L. Mondello, R. Costa, P.Q. Tranchida, P. Dugo, M. Lo Presti, S. Festa, A. Fazio, G. Dugo, J. Separ. Sci. 28 (2005) 1101.
- [31] L. Lecanu, V. Ducruet, C. Jouquand, J.J. Gratadoux, A. Feigenbaun, J. Agric. Food Chem. 50 (2002) 3810.
- [32] C. Deng, N. Li, X. Zhang, J. Chromatogr. B 808 (2004) 269.
- [33] C. Deng, X. Zhang, N. Li, J. Chromatogr. B 813 (2004) 47.
- [34] E. Baltussen, P. Sandra, F. David, C. Cramers, J. Microcol. Separ. 11 (1999) 737.
- [35] D.J.C. Quantrill, A.J. Buglass, J. Chromatogr. A 1117 (2006) 121.
- [36] A. Zalacain, J. Marín, G.L. Alonso, M.R. Salinas, Talanta 71 (2007) 1610.
- [37] J.C.R. Demyttenaere, J.I.S. Martinez, R. Verhé, P. Sandra, N. De Kimpe, J. Chromatogr. A 985 (2003) 221.
- [38] R.F. Alves, A. Nascimento, J.M. Nogueira, Anal. Chim. Acta 546 (2005) 11.
- [39] R. Perestrelo, A. Fernandes, F.F. Albuquerque, J.C. Marques, J.S. Câmara, Anal. Chim. Acta 563 (2006) 154.
- [40] H. van den Dool, P.D. Kratz, J. Chromatogr. 11 (1963) 463.
- [41] J.S. Câmara, J.C. Marques, R.M. Perestrelo, F. Rodrigues, L. Oliveira, P. Andrade, M. Caldeira, J. Chromatogr. A 1150 (2007) 198.
- [42] Eight Peak Index of Mass Spectra, 2nd ed., The Mass Spectra Data Centre, Nottighan, UK, 1974.
- [43] H. Guth, J. Agric. Food Chem. 45 (1997) 3027.
- [44] V. Ferreira, R. López, J.F. Cacho, J. Sci. Food Agric. 80 (2000) 1659.
- [45] J.N. Boidron, P. Chatonnet, M. Pons, Connaissance Vigne Vin 22 (1988) 275.

Low power electrocorrosion for sample preparation: Identification of metals in alloys

Kimberly Charleton^{a,b}, Tyler Buffie^c, Douglas M. Goltz^{a,*}

^a C-SCAPE, Centre for Scientific and Curatorial Analysis of Painting Elements, Department of Chemistry,
University of Winnipeg, 515 Portage Avenue, Winnipeg, Man., Canada R3B 2E9

^b Department of Chemistry, University of Manitoba, Winnipeg, Man., Canada R3T 2N2

^c Department of Chemistry, University of Winnipeg, Winnipeg, Man., Canada R3B 2E9

Received 13 April 2007; received in revised form 16 May 2007; accepted 17 May 2007

Available online 26 May 2007

Abstract

Rapid surface oxidation of a metal was carried out using a low voltage (9–18 V dc) power supply to examine the feasibility of low power electrocorrosion as an alternative to current metal sampling techniques such as acid digestion. Potential was applied between a metal alloy and a cellulose electrode that was made conductive using an aqueous solution (NH_4NO_3 , KCl). Metal ions diffuse into the cellulose as rapid surface oxidation of the metal occurs. The metal ions can then be extracted and analyzed using atomic spectroscopy (e.g. GFAAS). Steel (316L, SRM 663), brass and aluminum alloys were electro-corroded using constant potential. At short intervals (<2 min) the mass of corroded metal increased linearly with time. Corrosion rates for Cr, Ni, Cu and Mn ranged from 870 to 34 pg s^{-1} . The mass of metal corroded increased as applied potential/current increased and depends on the surface area of the cellulose–metal contact. Experiments showed that preferential metal corrosion does not occur in steel samples. SEM images show that there is a relatively large area ($\sim 1 \text{ mm}^2$) of homogenous corrosion and that the most damage occurs closest to the edge of filter paper/metal contact. Low power electrocorrosion was used to identify metals in a fork of unknown composition. Multiple techniques (GFAAS, FAAS and SN-ICP-MS) were used for analysis and it was found that Ag and Cu were the primary metals in the alloy, in a ratio of 3:1. Trace amounts of other metals (<1%) were found but not quantified.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Electrocorrosion; Atomic spectroscopy; Sample preparation; Dissolution; Alloy; GFAAS; ICP-MS

1. Introduction

Metal analyses can be carried out using spectroscopic methods such as flame atomic absorption spectroscopy (FAAS), graphite furnace atomic absorption spectroscopy (GFAAS) and inductively coupled plasma atomic emission spectroscopy/mass spectrometry (ICP-AES/MS), which typically require the target analyte to be dissolved in a dilute aqueous solution. Therefore, sample dissolution, usually in acid, may be required prior to analysis. Apart from traditional acid digestion, many sample preparation methods are useful for sample dissolution including: acid digestion [1,2], microwave digestion [1,3–7], pressure ashing [3,8,9], ultrasonication [10,11], high and low power spark ablation [2,12–15], and high power electrocorrosion [16]. The

major drawback of all of these methods is that they will damage the object under investigation and may not be suitable for valuable samples, such as cultural objects, where the integrity of the object must be maintained.

Several analytical techniques are available to the analyst that minimize sample damage such as: proton-induced X-ray emission (PIXE) [17–23], neutron activation analysis (NAA) [24,25] and X-ray fluorescence (XRF) [17,26–29]. External beam PIXE is minimally destructive, is capable of multi-element analysis and applicable to many types of materials [17–23]. One drawback of using PIXE analysis for cultural objects is that it has low sensitivity (ppm) and the size, shape or nature of the object may not be compatible with the vacuum and the small sample enclosures of the instrument [17]. Neutron activation analysis (NAA) has also been used to characterize cultural objects [24,25]. Neutron activation offers very good sensitivity and accuracy and is especially useful for the determination of some trace elements (e.g. Pb and Fe). It is a multi-element technique that can

* Corresponding author. Fax: +1 204 775 2114.

E-mail address: d.goltz@uwinnipeg.ca (D.M. Goltz).

be used with or without sample preparation, however, routine applications of NAA are limited by the availability of a reactor [25].

X-ray fluorescence is one of the most common methods for analysis of cultural objects; it does not require sample preparation and portable instruments are available. In general, XRF can have low measurement sensitivity for lighter elements (i.e. Al) [26]. Quantification errors also occur when XRF is applied on curved metal surfaces and the use of conventional XRF instruments (large beam diameter) for quantitative analysis is limited. Micro-XRF offers similar capabilities as external beam PIXE but with better lateral resolution. Small beams allow sampling on locations that resemble flat surfaces [17]. Surface sampling techniques (<100 μm depth), such as XRF, may also be affected by the presence of surface corrosion and contamination because the depth of analysis is usually less than the patina thickness [19,28]. For example, over time silver can develop a patina with a thickness ranging from a few micrometers to hundreds of micrometers. This can be a result of artificial enrichment with silver after the minting process, or due to naturally selective corrosion phenomena [29]. The presence of a patina makes it very difficult to achieve quantitative results through direct, non-destructive surface analysis because it is not always possible to remove the patina due to curatorial concerns. However, in many cases exhaustive quantitative analysis is not required in order to achieve material identification (brass, bronze, silver alloy or tin) [28].

Low power electrocorrosion can be an alternative approach for rapid metal dissolution for the purpose of identifying surface metals in alloys. Several groups have used anodic dissolution for rapid metal analysis [16,30–32]. In anodic dissolution the conductive metal sample acts as the anode. The metal sample is immersed in an electrolytic solution, usually 0.5–1.5 M HNO_3 , with the cathode and typically 2–5 A (current density 1.6–5 A cm^{-2}) is applied. Sampling intervals are short, usually being less than 10 s. After electrolysis the electrodisolved sample can be determined using either voltammetry or spectroscopy.

The objective of this study was to investigate some of the properties and to examine the feasibility of using low power (3–18 V) electrocorrosion for identifying metals in alloys. A low voltage potential (9 V dc), is applied between the metal sample and a cellulose electrode, which has been made conductive by an aqueous solution. Rapid oxidation of the alloy surface occurs and the metal ions diffuse into the cellulose, prior to analysis by GFAAS or ICP-MS.

2. Experimental

2.1. Chemicals

All solutions were prepared using de-ionized water (18 $\text{M}\Omega^{-1}\text{cm}$ Milli-Q). Standard metal solutions were prepared by appropriate dilution from 1000 $\mu\text{g mL}^{-1}$ standards (SCP Science). High purity AR Select[®] Plus HNO_3 (Mallinckrodt) was used for acid dissolution and solution matrix matching; KCl (Aldrich) and NH_4NO_3 (Aldrich), were reagent grade and used without purification. All glassware and Petri dishes were pre-

soaked in 50% HNO_3 for at least 24 h and rinsed with de-ionized water prior to use.

For the cellulose electrode, filter paper (VWR 5.5 cm) was sectioned into approximately 1 cm $\times$ 2 cm pieces using tweezers wrapped in Parafilm[®] wax to minimize metal contamination. Filter paper was chosen because it is flexible and was found to be relatively free of transition metals. Flexibility was required to sample curved surfaces and small spaces. Metals were extracted in disposable 1.5 mL polystyrene cups, cups were not pre-treated prior to use. Several 9–1.5 V (AA) batteries were used in series to generate 3–27 V of applied potential. Alloys that were used for studying corrosion properties included two stainless steels (304L and 316L), aluminum (6061), brass and the standard reference material SRM 663 stainless steel.

2.2. Methods

The filter paper (anode) was saturated with aqueous solution and then placed on the bottom of a Petri dish and the metal (cathode) was contacted to the paper, typically for 10–120 s. Metals were extracted from the cellulose with concentrated HNO_3 and diluted to 2 mL prior to analysis (GFAAS or ICP-MS). Hydrochloric acid was avoided as the presence of large amounts of Cl^- in the sample can cause matrix effects for some elements [33] with GFAAS and with ICP-MS analysis, it can also cause a spectral interference $^{40}\text{Ar}^{35}\text{Cl}^+$ with $^{75}\text{As}^+$.

2.3. Instrumentation

Absorption spectroscopy was carried out using a Perkin-Elmer 3100 flame atomic absorption spectrophotometer (FAAS) and a Perkin-Elmer AAnalyst 800 graphite furnace atomic absorption spectrophotometer (GFAAS) equipped with an AS80 autosampler. Recommended optical and lamp conditions were used for both the FAAS and GFAAS. For GFAAS, Zeeman effect background correction was used for all measurements with the recommended temperature program for each element. A Pd modifier (500 ppm $\text{Pd}(\text{NO}_3)_2$) was also used for the determination of Mn. External calibrations and method of standard addition were used for quantitative analysis.

A Perkin-Elmer Elan DRC II inductively coupled plasma mass spectrometer (ICP-MS) with solution nebulization was also used for the analysis of the fork. Experimental parameters for the ICP-MS are indicated in Table 1. A Cambridge

Table 1
SN-ICP-MS instrumental operating and data acquisition parameters

ICP mass spectrometer	
RF power	1100 W
Coolant Ar flow	15.0 L min^{-1}
Auxiliary Ar flow	1.4 L min^{-1}
Carrier Ar flow	1.0 L min^{-1}
ICP-MS data acquisition	
Dwell time	50 ms
Scan mode	Peak hopping
Signal measurement	Average counts
Resolution	0.7 amu at 10% peak height

Stereoscan 120 Scanning electron microscope equipped with a four-quadrant semi-conductor back scattered electron detector and a Kevex 7000 energy dispersive spectrometer was used to collect SEM images and ED-XRF data.

3. Results and discussion

A number of different alloys were used as test materials in this project including brass, steel, aluminum and silver. The electrocorrosion process for oxidizing and removing a small amount of metal sample appeared to work equally well for all of the alloys tested. Fig. 1 shows the corroded masses of Cr, Cu, Ni and Mn from SRM 663 stainless steel after specific time intervals from 60 to 480 s. The electrocorrosion was achieved using 18 V of applied potential between the steel sample and the cellulose electrode that was soaked in 0.6 M NH_4NO_3 and 0.125 M HNO_3 . For short sampling times (<2 min) the mass of metal corroded appeared to increase linearly (Fig. 1a) and the relative masses of metal removed were consistent with the composition of the steel alloy, Mn (1.50%), Cr (1.31%), Ni (0.32%) and Cu (0.098%). This experiment illustrates for metals in high enough concentrations, the possibility of obtaining a representative sample and performing semi-quantitative analysis after only 60 s.

The rate of metal corrosion for SRM 663 was plotted for each time interval and the results are shown in Fig. 1b. This figure shows that the corrosion rates for each metal in the alloy decrease as soon as potential is applied. The decrease in rate of electrocorrosion eventually levels off after longer time intervals (>2 min). A logical explanation for the decrease in rate would likely be physical (evaporation) and chemical changes (oxidation). The maximum corrosion rate for each metal decreased from 870 to 125 pg s^{-1} for Mn and from 34 to 5 pg s^{-1} for Cu. Depending on the composition of the alloy under investigation, optimal sampling times for most metals range from 10 to 120 s; collecting samples for longer periods did not significantly increase the amount of metal removed.

A number of experimental parameters affect the mass of metal that is corroded from the surface of an alloy including: sampling time, applied potential and ionic strength. The effect of increasing applied potential on the mass corroded at specific time intervals was examined. Fig. 2 shows the relationship between applied voltage (1.5–27 V) and mass corroded for both

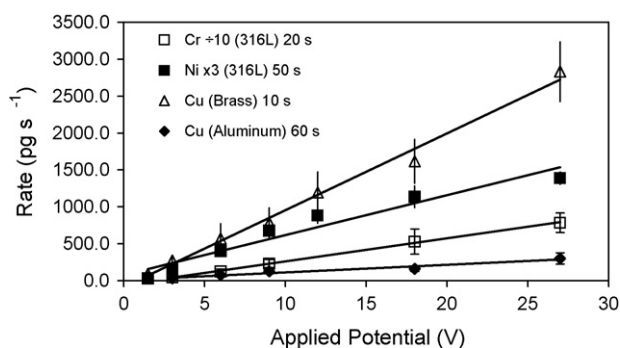


Fig. 2. Rate of Cr and Ni removal from steel 316L, Cu from brass and Cu from aluminum 6061 as a function of applied potential ($n=6$).

ferrous and non-ferrous alloys. In this experiment the applied voltage was increased by using additional batteries in series. For consistent electrocorrosion and reproducible sampling of the metals, exact duplication of experimental conditions was required. This is important because resistance of the filter paper increases significantly with distance from the electrical contact and highest concentrations of corroded metal are at the metal–electrode interface. The rate of metal corrosion in both ferrous and non-ferrous metals increased linearly with applied potential. In this experiment the Cu in aluminum was much lower (4–6%), compared to Cu in brass (30–60%) and therefore, a longer electrocorrosion time was used for the aluminum alloy. Unlike Cr in this alloy, the mass and therefore, rate of electrocorrosion of Ni did not increase linearly with applied potential. One possibility is that saturation of the cellulose electrode with Ni is occurring, however this would likely be ruled out since it was not observed with Cr. For steel, a black film was visible on the surface after electrocorrosion which indicates that an oxide species, possibly NiO may be forming [34]. Formation of a stable oxide layer could change the conductivity which likely affects the rate of corrosion of some metals on the sample surface. Furthermore, some metal oxides may be quite stable and difficult to oxidize.

Prior to electrocorrosion of a sample, the cellulose electrode was soaked in an aqueous salt solution in order to facilitate the movement of charge and hence oxidation of the metal surface. Since the concentration of the salt solution affects the rate of corrosion, experiments were carried out to observe the effect

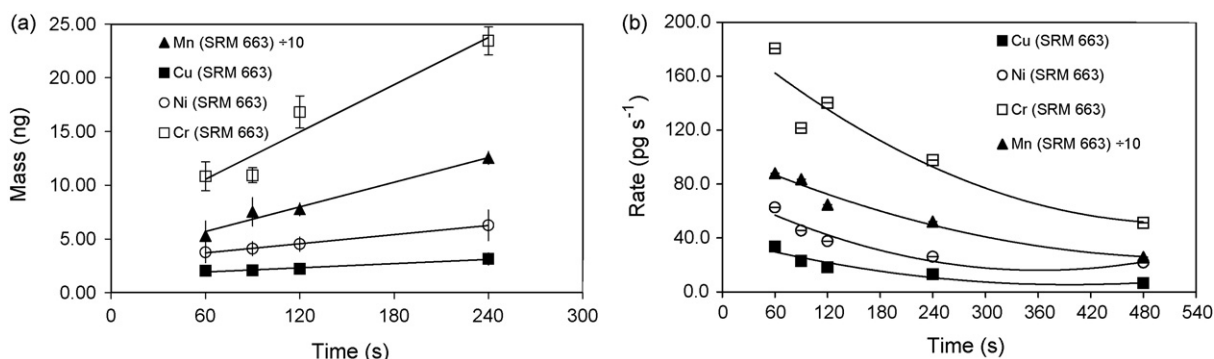


Fig. 1. (a) Mass of Cu, Cr, Mn and Ni removed from SRM 663 steel at various sampling intervals ($n=6$). (b) Rate of metal removal as a function of sampling time ($n=6$).

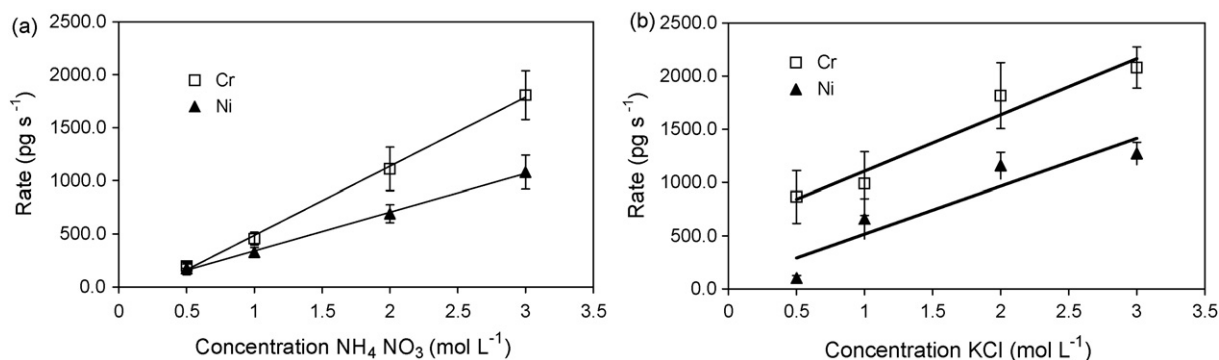


Fig. 3. (a) Rate of Cr and Ni removal from 316L steel as a function of concentration of NH_4NO_3 ($n=6$). (b) Rate of Cr and Ni removal from steel 316L as a function of concentration of KCl ($n=6$).

of ionic strength of the aqueous solution on the rate of metal corroded. Experiments were performed using either NH_4NO_3 or KCl in concentrations ranging from 0.3 to 2.4 M and acidified with 0.125 M HNO_3 . This is similar to work done in previous studies [30–32], where higher concentrations of HNO_3 were added to the solution as a supporting electrolyte prior to analysis. Fig. 3 shows the rates of corrosion of Cr and Ni from SRM 663 with various concentrations of NH_4NO_3 (Fig. 3a) and KCl (Fig. 3b) when 18 V was applied for 10 s. As expected the rate of Cr and Ni corrosion increased as the ionic strength increased when the electrode was soaked in either NH_4NO_3 or KCl. The rates of corrosion of either Ni or Cr were not affected by the type of electrolyte significantly when a high ionic strength solution was used (3 M). Since the ratio of Cr to Ni is 2:1 in this alloy, preferential corrosion of Ni appears to be occurring when low ionic strength solutions of NH_4NO_3 are used. This does not appear to be the case when KCl is used.

Fig. 4 shows a plot of the mol% of corroded Cr with respect to Ni in a sample of 316L steel. For this experiment the metal was corroded at 10, 20, 30, and 60 s using an electrode soaked with 2.4 M NaCl. The slope of the curve is in agreement with the expected ratio (2:1) of Cr to Ni in this steel (16–18% Cr, 10–14% Ni). The high degree of linearity ($r^2 = 0.999$) suggests that neither Cr nor Ni is removed preferentially under these conditions. To verify that preferential corrosion was not occurring, several certified metals in SRM 663 steel were determined to observe if the ratio of each metal pair agrees with certified values. The steel

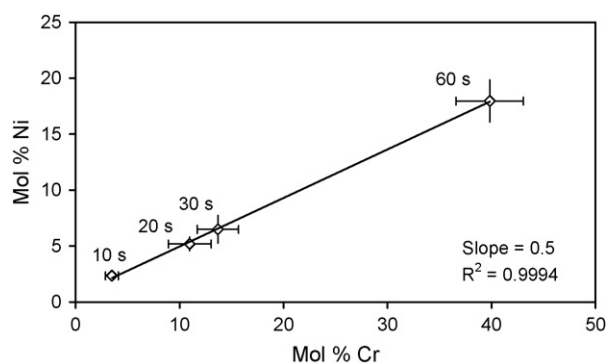


Fig. 4. Mol% Ni plotted against the mol% Cr removed during low power electrocorrosion of 316L stainless steel.

was sampled with 9 V applied potential and 3 M NH_4NO_3 or KCl in 0.125 M HNO_3 . The alloy was sampled six times each at 40, 60 and 90 s. Fig. 5 shows the certified ratio and experimentally determined ratios of Cu, Cr, Mn and Ni that were found after 90 s of electrocorrosion for SRM 663 steel, using electrodes that were soaked in either NH_4NO_3 (Fig. 5a) or KCl (Fig. 5b). The ratio of each metal does not change significantly from 40 to 90 s. For this experiment shorter time intervals were avoided because of sampling uncertainty due to the small sample size. After 90 s the amounts of metals removed from the surface of SRM 663 were: 305 ± 25.3 ng for Mn, 180 ± 27.0 ng for Cr, 48.0 ± 0.94 ng for Ni and 21.6 ± 1.93 ng for Cu when NH_4NO_3 was used. Given the magnitude of the sampling uncertainty ($\pm 10\%$ R.S.D.) the expected ratios of metals were generally within one standard deviation of the experimentally determined ratios. When KCl was used the amounts of metals removed from the surface of SRM 663 after 90 s were: 757 ± 148 ng for Mn, 612 ± 154 ng for Cr, 366 ± 37.0 ng for Ni and 54.8 ± 15.4 ng for Cu. Unlike NH_4NO_3 , when KCl was used it appeared that relative to other metals, more Ni was corroded than expected. This may indicate preferential corrosion of Ni, however, it should also be noted that the precision was considerably worse for all of the metals with KCl. This is partly due to the matrix effect of Cl^- in the graphite furnace and partly due to sampling. As such, the magnitude of the expected ratios of all the metals was still within one standard deviation of the experimentally determined ratios. In general, preferential corrosion should not occur for most metals (e.g. steel, brass, aluminum) provided that a sufficient ionic strength solution, sampling time and potential are used. This result is significant as it validates this technique for determining approximate elemental ratios for characterizing and identifying alloys.

Prior to using this approach on valuable samples, examination of the corroded surface of a test samples was performed using microscopy. Since optical microscopy did not provide satisfactory detail of the corroded surface, scanning electron microscopy (SEM) was used. Fig. 6 shows images of aluminum 6061 after low power electrocorrosion was applied to the surface. The alloy was corroded for 1 s with a 9 V battery and a cellulose electrode soaked with 3 M NH_4NO_3 in 0.125 M HNO_3 (Fig. 6a). The same spot was then corroded for another 30 s and the sample was imaged again (Fig. 6b). At $40\times$ magnification some pitting is observed and is represented by the lighter regions on the left

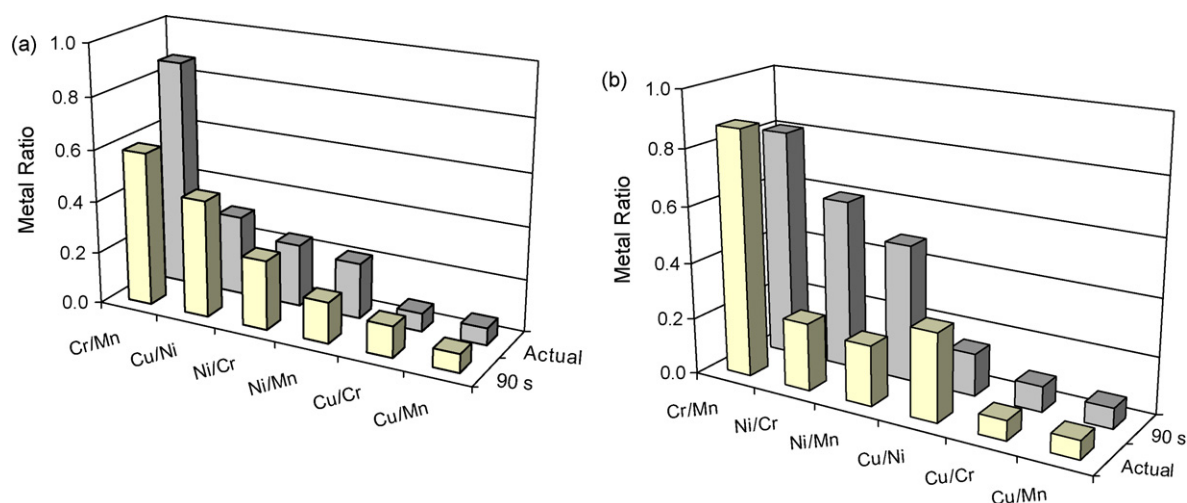


Fig. 5. (a) Corrosion of Cu, Cr, Mn and Ni from SRM 663 steel ($n=8$) electrode soaked with NH_4NO_3 . (b) Corrosion of Cu, Cr, Mn and Ni from SRM 663 steel ($n=8$) electrode soaked with KCl.

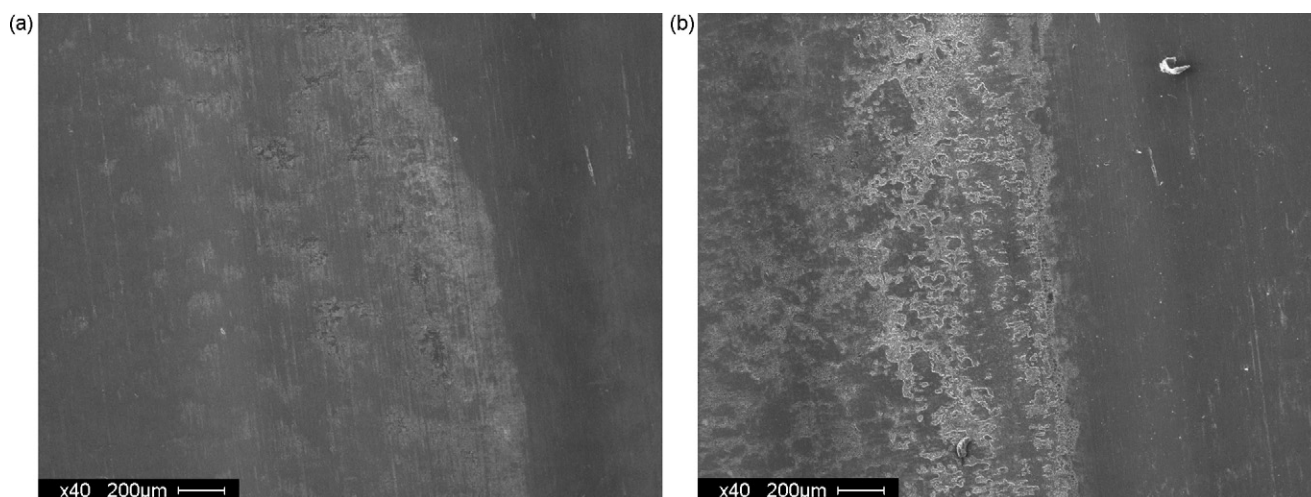


Fig. 6. (a) SEM image of aluminum 6061 40 $\times$ magnification after low power electrocorrosion (1 s). (b) SEM image of aluminum 6061 40 $\times$ magnification after low power electrocorrosion (30 s).

side of the image. At higher magnification (Fig. 7), it becomes apparent that the majority of the corrosion occurs at the point of closest contact between the power supply and the metal sample. This area is located on the light side of the boundary in this image and corresponds to the area of greatest current density in the cellulose that makes contact with the sample. Measurements with a voltmeter indicated that this area with the greatest corrosion corresponded to lowest resistance between the metal sample and the cellulose. Resistance between the cellulose and the metal sample increased significantly with distance from the point of contact of the cellulose. This coincides with areas on the metal surface with decreasing corrosion. Another interesting feature is that in addition to pitting, there also appears to be individual grains and clumps of particles breaking apart from the surface.

For comparison, an identical experiment was also performed using stainless steel 304L. This alloy was chosen because, in contrast to the aluminum, it is a harder alloy that is expected to be less susceptible to corrosion under similar experimental conditions. For shorter time intervals (1 s) very little corrosion

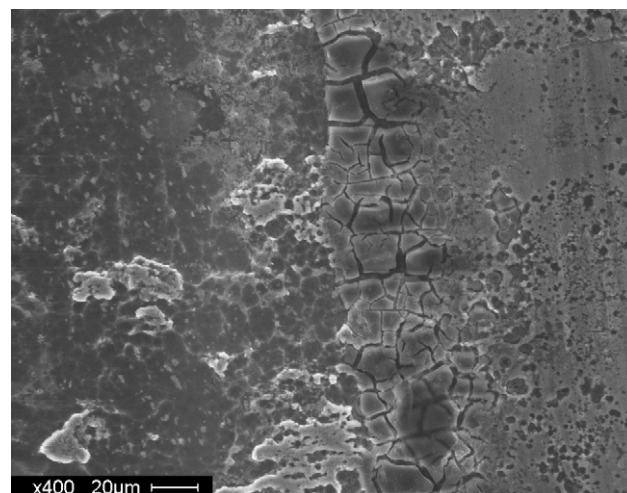


Fig. 7. SEM of aluminum 6061 400 $\times$ magnification after low power electrocorrosion (30 s).

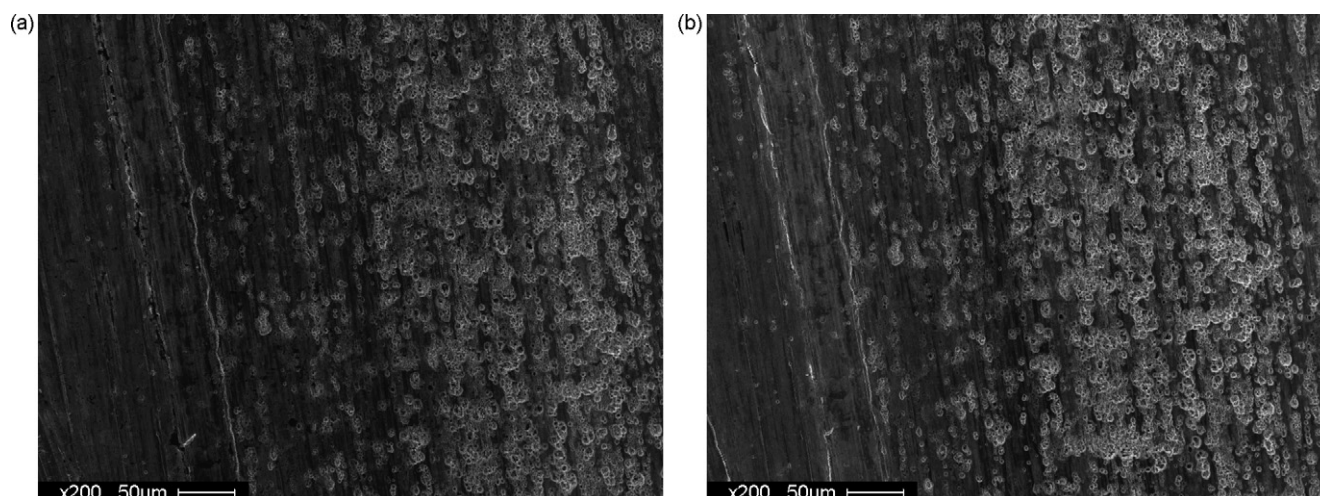


Fig. 8. (a) SEM of steel 304L 200 $\times$ magnification after low power electrocorrosion (20 s). (b) SEM of steel 304L 200 $\times$ magnification after low power electrocorrosion (40 s).

Table 2
Analysis of a silver alloy using electrocorrosion and acid digestion

	Ag (ng)	Cu (ng)	Ratio Ag:Cu
Electrocorrosion (GFAAS)	10 ± 1	2.0 ± 0.5	5.1 ± 1.4
Acid digestion (FAAS)	$20 \times 10^5 \pm 4 \times 10^5$	$40 \times 10^4 \pm 8 \times 10^4$	4.3 ± 1.2

$n = 8$.

was observed on the surface with the unaided eye. At longer time intervals (20 s) some corrosion could be observed, however, the effects of corrosion were much less damaging when compared to aluminum 6061. Fig. 8 which shows the pitting in the stainless steel 304L after 20 s (Fig. 8a) and 40 s (Fig. 8b) confirms this observation. In these images the cellulose electrode contact was on the right side and pitting is represented by the lighter regions. Apart from the minimal damage to the surface, the amount of pitting was only marginally increased after 40 s. Pitting of the surface should create a larger surface area, which should also cause the corrosion rate to increase with respect to time. In reality, the rate of corrosion tends to decrease with time which suggests that, formation of stable oxides, evaporation of the aqueous solution and saturation of the cellulose likely have greater effects on the rate of corrosion.

During this study our research group received a metal fork from a local conservator in Manitoba (Parks, Canada). The fork had a missing tine and the composition of the alloy was required in order to repair it. As well as collecting images with the SEM, energy dispersive (ED) X-ray fluorescence (XRF) was used for non-destructive analysis of the fork. Analysis of the fork by XRF indicated it was a Ag–Cu alloy. The irregular shape and lack of standards eliminated the possibility of quantitative analysis. Therefore, electrocorrosion was used for sample preparation prior to analysis using FAAS, ICP-MS and GFAAS. For this experiment a 9 V potential was applied between the fork and the cellulose electrode, which was soaked with 3 M NH_4NO_3 and 0.125 M HNO_3 .

To establish that representative sampling could be achieved with electrocorrosion, a silver alloy that was similar in composition was also analyzed. The alloy was purchased locally and was

not a reference material, but its composition was determined by obtaining a much larger size sample using acid digestion with concentrated HNO_3 . The same Ag alloy was then analyzed by FAAS using an external calibration curve and matrix matched standards. This Ag alloy was then analyzed using electrocorrosion for sample preparation and GFAAS to compare the two approaches. The results of this analysis, presented in Table 2, show the similarity of the ratios of Ag to Cu with either acid digestion or electrocorrosion. A t -test at the 95% confidence level also confirmed this result statistically.

Table 3 shows the ratio of Ag to Cu in the fork when electrocorrosion was used for sample preparation. For FAAS an external calibration curve was used and the Ag to Cu ratio was found to be 3.6 ± 0.2 . Longer sampling intervals were used which gave us a representative sample and this is reflected in the precision of the data. While FAAS gave satisfactory data for Ag and Cu, it lacked the sensitivity required for trace analysis. Therefore, solution nebulization (SN) inductively coupled plasma mass spectrometry (ICP-MS) was used to identify trace metals. The ratio of Ag to Cu was found to be 3.8 ± 0.2 , which is similar to the results obtained using FAAS.

The most important advantage of using ICP-MS is that the elemental abundance for most of the elements can be obtained

Table 3
Analysis of fork of unknown composition using electrocorrosion

	Ag (ng)	Cu (ng)	Ratio Ag:Cu
FAAS	399 ± 17	111 ± 6	3.6 ± 0.2
SN-ICP-MS	377 ± 12	97 ± 4	3.8 ± 0.2
GFAAS	33 ± 1	13 ± 2	2.6 ± 0.9

$n = 8$.

by performing a mass scan. The mass scan did not reveal any other major elements; however, traces of ^{51}V , ^{208}Pb , ^{27}Al and ^{66}Zn were detected but not quantified. One of the limitations of ICP-MS is isobaric interferences. For example, elements such as $^{56}\text{Fe}^+$, $^{52}\text{Cr}^+$ and $^{75}\text{As}^+$ overlap with $^{40}\text{Ar}^{16}\text{O}^+$, $^{40}\text{Ar}^{12}\text{C}^+$ and $^{40}\text{Ar}^{35}\text{Cl}^+$, respectively. The large sample size requirements for FAAS and ICP-MS are also a disadvantage because of potential damage to the surface. For this reason, the fork was also analyzed using GFAAS, because of the small sample size requirement ($<20\ \mu\text{L}$) and high sensitivity of this technique. It was also possible to quantify the elements that are problematic with ICP-MS such as As which has been found as an impurity in antique silver. The GFAAS analysis of the fork (Table 3), gave a ratio of 2.6 ± 0.9 for Ag to Cu. Although this result is lower than that obtained with ICP-MS and FAAS the standard deviation from the GFAAS analysis is quite large. The main reason for the poor precision is likely caused by the small sample sizes. The standard deviations of the analyses using ICP-MS or FAAS and GFAAS overlap indicating that the results can be considered similar. Other metals, such as Al and Pb, were also detected, but not quantified as they were found to be present in trace amounts ($<0.1\%$).

4. Conclusion

Portable ED-XRF instruments, either benchtop or handheld models, are commercially available. The handheld model requires little or no sample preparation and can provide very quick sample turnaround time for the analyst. In the absence of a portable ED-XRF, low power electrocorrosion with GFAAS can be a useful alternative to identify metals and alloys. Damage to the surface is minimal when the corrosion is applied for 10–30 s. The filter paper was an easily manoeuvred, flexible, electrode that can be used for irregularly shaped or large objects. Solution nebulization ICP-MS provided a mass scan with greater sensitivity than SEM for identifying alloy metals that were in low concentration. The drawback of SN is that it requires a large sample volume and some elements can have spectral interferences. Graphite furnace AAS offers the possibility of determining small quantities of material, which makes it a very powerful technique for detecting trace levels of metals. Successive measurements of the same spot did not give increasing amounts of Cu indicating that a Ag enriched patina was not found.

Multiple techniques were used in this study to obtain both quantitative and qualitative information about the unknown composition of the fork. It was only possible to use FAAS for this object because a large quantity of material could be removed. Flame AAS and SN-ICP-MS may not be a feasible approach for more valuable objects because of their large sample size requirements and low sensitivity. Although the small sample size requirement for GFAAS solves this problem, a small sample size may not be representative of the sample which can limit the accuracy of a quantitative analysis.

Acknowledgements

Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada and

Canadian Foundation for Innovation. The authors gratefully acknowledge Eno Busse from Parks Canada for access to material and Sergio Mejia from the University of Manitoba for SEM measurements. K. Charleton is a graduate student at the University of Manitoba.

References

- [1] G. Knapp, *Mikrochim. Acta* 11 (1991) 445.
- [2] D.M. Goltz, M. Hinds, J. Coombs, A. Jagdeo, D. Craig, *J. Anal. At. Spectrom.* 17 (2002) 395.
- [3] M.Y. Perez-Jordan, A. Salvador, M. delaGuardia, *Anal. Lett.* 31 (1998) 867.
- [4] R. Chakraborty, A.K. Das, M.L. Cervera, M. delaGuardia, *Anal. Lett.* 30 (1997) 283.
- [5] X.H. Wen, L.Z. Wu, Y. Zhang, Y. Chu, *Fresenius J. Anal. Chem.* 357 (1997) 1111.
- [6] J.G. SenGupta, N.B. Bertrand, *Talanta* 45 (1995) 1947.
- [7] S. Fadda, A. Rivoldini, I. Cau, *Geostand. Newslett.* 19 (1995) 41.
- [8] M. Somenath, *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons Inc., New Jersey, 2003.
- [9] P. Halmos, J. Borszeki, E. Gegus, *Magy. Kem. Foly.* 99 (1993) 420.
- [10] K. Ashley, *Electroanalysis* 7 (1995) 1.
- [11] S. Mamba, B. Kratochvil, *Int. J. Environ. Anal. Chem.* 60 (1995) 295.
- [12] B.V. L'vov, A.V. Novichikhin, *Atom. Spec.* 11 (1990) 1.
- [13] H.P. Longerich, D. Gunther, S.E. Jackson, *Spectrochim. Acta B* 54 (1999) 381.
- [14] Z. Zhou, K. Zhou, X. Hou, H. Luo, *Appl. Spec. Rev.* 40 (2005) 165.
- [15] D.M. Goltz, G. Kostic, G. Reinfelds, *Talanta* 52 (2002) 1131.
- [16] M. Boileau, D. Goltz, M. Hinds, *Can. J. Anal. Sci. Spec.* 49 (2004) 185.
- [17] K. Janssens, G. Vittiglio, I. Deraedt, A. Aerts, B. Vekemans, L. Vincze, F. Wei, I. Deryck, O. Schalm, F. Adams, A. Rindby, A. Knochel, A. Simionovici, A. Snigirev, *X-ray Spectrom.* 29 (2000) 73.
- [18] R. Linke, M. Schreiner, G. Demortier, M. Alram, *X-ray Spectrom.* 32 (2003) 373.
- [19] M.H. Abraham, G.W. Grime, J.P. Northover, C.W. Smith, *Nucl. Instrum. Methods B* 150 (1999) 651.
- [20] M. Hajivaliei, M.L. Garg, D.K. Handa, K.L. Govil, T. Kakavand, V. Vijayan, K.P. Singh, I.M. Govil, *Nucl. Instrum. Methods B* 150 (1999) 645.
- [21] I. Gyodi, I. Demeter, K. Hollos-Nagy, I. Kovacs, Z. Szokefalvi-Nagy, *Nucl. Instrum. Methods B* 150 (1999) 605.
- [22] G. Demortier, F. Fernandez-Gomez, M.A. Ontalba Salamanca, P. Coquay, *Nucl. Instrum. Methods B* 158 (1999) 275.
- [23] G. Demortier, Y. Morciaux, D. Dozot, *Nucl. Instrum. Methods B* 150 (1999) 640.
- [24] E. Panczyk, M. Ligeza, L. Walis, *J. Radioanal. Nucl. Chem.* 244 (2000) 543.
- [25] J.H. Zaidi, S. Waheed, S. Ahmed, *J. Radioanal. Nucl. Chem.* 242 (1999) 259.
- [26] G. Vittiglio, K. Janssens, B. Vekemans, F. Adams, A. Oost, *Spectrochim. Acta B* 541 (1999) 1697.
- [27] C.L. Wei, Q.M. Fan, Y.W. Liu, D.L. Li, *X-ray Spectrom.* 25 (1990) 138.
- [28] C.P. Swann, S. Caspi, J. Carlson, *Nucl. Instrum. Methods B* 150 (1999) 571.
- [29] M. Milazzo, C. Cicardi, *X-ray Spectrom.* 26 (1997) 211.
- [30] J.B.B. da Silva, M.B.O. Giacomelli, I.G. de Souza, A.J. Curtius, *Talanta* 47 (1998) 1191.
- [31] T. Tanaka, K. Shitan, H. Kondo, *Buns. Kag.* 50 (2001) 855.
- [32] A.P. Packer, A.P.G. Gervasio, C.E.S. Miranda, B.F. Reis, A.A. Menegario, M.F. Gine, *Anal. Chim. Acta* 485 (2003) 145.
- [33] J.P. Byrne, M.M. Lamoureux, C.L. Chakrabarti, T. Ly, D.C. Gregoire, *J. Anal. Atom. Spectrom.* 8 (1993) 599.
- [34] E.E. Abd El Aal, *Corr. Sci.* 45 (2003) 641.

Determination of andrographolide and dehydroandrographolide in rabbit plasma by on-line solid phase extraction of high-performance liquid chromatography

Ligang Chen^a, Aimin Yu^a, Xiaodong Zhuang^b, Kui Zhang^b,
Xiupin Wang^a, Lan Ding^{a,*}, Hanqi Zhang^a

^a College of Chemistry, Jilin University, 2699 Qianjin Street, Changchun 130012, PR China

^b College of Life Sciences, Northeast Normal University, 5268 Renmin Street, Changchun 130024, PR China

Received 12 March 2007; received in revised form 22 May 2007; accepted 22 May 2007

Available online 31 May 2007

Abstract

The high-performance liquid chromatography (HPLC) coupled with on-line solid phase extraction (SPE) and ultraviolet (UV) detection was developed for determining andrographolide and dehydroandrographolide in rabbit plasma. Plasma samples (100 μL) were injected directly into a C18 SPE column and the biological matrix was washed out for 6 min using 15% aqueous methanol. By rotation of the switching valve, andrographolide and dehydroandrographolide were eluted in the back-flush mode and transferred to the analytical column by the chromatographic mobile phase consisted of methanol:acetonitrile (ACN):water (50:10:40; v/v). The UV detection was performed at 225 nm. The calibration curves showed excellent linear relationship ($R \geq 0.9993$) over the concentration range of 0.05–5.0 $\mu\text{g mL}^{-1}$. The within- and between-day precisions (R.S.D.) of two analytes were in the range of 1.2–6.5% and the accuracies were between 92.0% and 102.1%. Their recoveries were all greater than 94%. The limits of detection were 0.019 $\mu\text{g mL}^{-1}$ for andrographolide and 0.022 $\mu\text{g mL}^{-1}$ for dehydroandrographolide. This method was successfully applied to the plasma concentration–time curve study after oral administration of *Andrographis paniculata* Nees extract in rabbit. © 2007 Elsevier B.V. All rights reserved.

Keywords: On-line solid phase extraction; HPLC; Plasma; Andrographolide; Dehydroandrographolide

1. Introduction

Andrographis paniculata Nees (*A. paniculata*) grows widely in the tropical area of Asia, and belongs to the acanthaceae family [1]. The herb is commonly used in combating the common cold and respiratory inflammations. It is reported to remove latent heat, counteract hepatotoxicity and induce hypoglycemia [2–5]. The primary medicinal constituents of *A. paniculata* are andrographolide (Fig. 1a) and dehydroandrographolide (Fig. 1b) [5].

Several methods have been reported for the determination of two lactones in *A. paniculata*, including thin layer chromatography (TLC), HPLC, capillary electrophoresis (CE), micellar electrokinetic capillary chromatography (MECC) and micel-

lar liquid chromatography (MLC) [5–12]. Some papers have studied their pharmacokinetics by HPLC with different sample pretreatment methods. The most routine approach is precipitation of plasma proteins using an organic reagent followed by centrifugation, furthermore, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were used to extract two lactones from biological fluids [13–15]. Those methods require large sample volumes, and are time consuming and laborious.

On-line SPE using column switching technique is an interesting alternative to liquid–liquid extraction or off-line solid-phase extraction for the preparation of biological matrices [16–23]. This automated technique involves the use of two columns connected by a switching valve and is particularly useful when a large number of assays have to be performed. The role of the first column (SPE column) is to retain selectively the analytes. After rotation of the switching valve, the analytes are eluted and transferred to the analytical column and individually quantitated. Although the technique has been developed widely,

* Corresponding author. Tel.: +86 431 85168399; fax: +86 431 85112355.
E-mail address: analchem@mail.jlu.edu.cn (L. Ding).

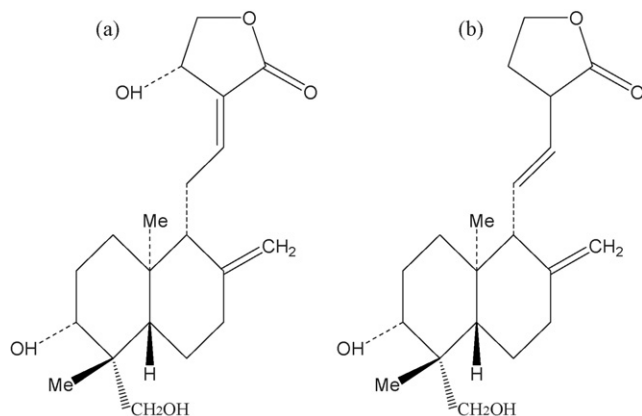


Fig. 1. The structures of andrographolide (a) and dehydroandrographolide (b).

the analysis objective realm is localized at western medicines. Assaying complex herb medicine plasma samples using this technique was still scarce. In this paper, the on-line solid phase extraction of high-performance liquid chromatography was applied to simultaneous determination of andrographolide and dehydroandrographolide in rabbit plasma.

2. Experimental

2.1. Chemicals and materials

The standard of andrographolide and dehydroandrographolide were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Three male and three female white rabbits were purchased from the Center of Experimental Animals, Jilin University, China. Rabbit plasma was obtained by centrifugation of rabbit blood (6000 rpm, 5 min) after anti-coagulation treatment with heparin. The raw *A. paniculata* material was obtained from the Jilin Great Medicine Store (Changchun, China). The material was powdered using a cyclone mill and passed through a 40 mesh sieve. HPLC grade methanol and ACN were obtained from Fisher Corporation, USA. Pure water was obtained by the Milli-Q water purification system (Millipore Corporation, USA).

2.2. Apparatus

The on-line SPE-HPLC system is represented in Fig. 2. In this system, an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) was used, which was equipped with a 7725i injection valve (V 1), an automatic ten-port switching valve (V 2), a quaternary pump (Pump B), a heated column compartment, a UV detector, a LC workstation and a Pinnacle 11 C18 column (250 mm × 4.6 mm i.d., 5 μm) used as analytical column (Restek, Bellefonte, PA, USA). A guard column packed with C18 sorbent (4.6 mm × 12.5 mm i.d., 5 μm) was used as SPE column for concentration of the analytes. A P230 high-pressure pump (Pump 2, Elite Analytical Instruments Co., Dalian, China) was used for completing the extraction of analytes.

2.3. Calibration standards and quality control samples

Andrographolide and dehydroandrographolide were dissolved in methanol (1.0 mg mL⁻¹) as a stock solution, and then they were diluted with methanol for further concentration levels. Calibration work solutions were prepared by addition of the diluted stock solutions into blank rabbit plasma giving final concentration series of 0.05–5 μg mL⁻¹. Quality control (QC) samples containing low (0.05 μg mL⁻¹), middle (0.5 μg mL⁻¹) and high (5.0 μg mL⁻¹) concentrations of standards were prepared by the same procedure as the calibration standards. Calibration work solutions and QC samples were stored at -18 °C.

2.4. Preparation of *A. paniculata* extract

The raw *A. paniculata* powder 10 g was weighted and then put in the distilling flask, fitted with a water-cooling condenser, into which 250 mL of 80% aqueous ethanol was added. After being heated for 1 h, the extract was filtrated. The sample was extracted twice again. All the filtrates were combined, ethanol was removed under reduced pressure by rotary evaporation, and the residue was recovered in distilled water [24]. It was determined by HPLC that the dose of 2.0 mL extract was equivalent to 35.2 mg andrographolide and 20.7 mg dehydroandrographolide.

2.5. On-line solid phase extraction high-performance liquid chromatography

Step 1 (Fig. 2a), pump A and pump B were activated. The SPE column was conditioned in sequence with 5.0 mL methanol and 5.0 mL 15% aqueous methanol at a flow rate of 2.5 mL min⁻¹. At the same time, 100 μl plasma samples were injected into the sampling loop with syringe. The mobile phase was introduced into the analytical column.

Step 2 (Fig. 2b), pump A and pump B were activated. The plasma sample extract collected in the sample loop was introduced into the SPE column for analyte concentration by 15% aqueous methanol at a flow rate of 1.0 mL min⁻¹. The baseline of the detector was established by the chromatographic mobile phase (methanol:ACN:water, 50:10:40).

Step 3 (Fig. 2c), pump A was stopped and pump B was activated. The analytes trapped on the SPE column were eluted into the analytical column in the back-flush mode with mobile phase at a flow rate of 1.0 mL min⁻¹ and the eluate was monitored at 225 nm.

When the analytes were transferred from the SPE column to the analytical column completely, the valves 1 and 2 were switched (Fig. 3a). The SPE column was conditioned again for the next assay during the previous chromatographic separation. The overall analysis time can be shortened in this way.

2.6. Validation of the method

2.6.1. Specificity

The specificity of the method was investigated by comparing the chromatograms of blank plasma sample with that of blank

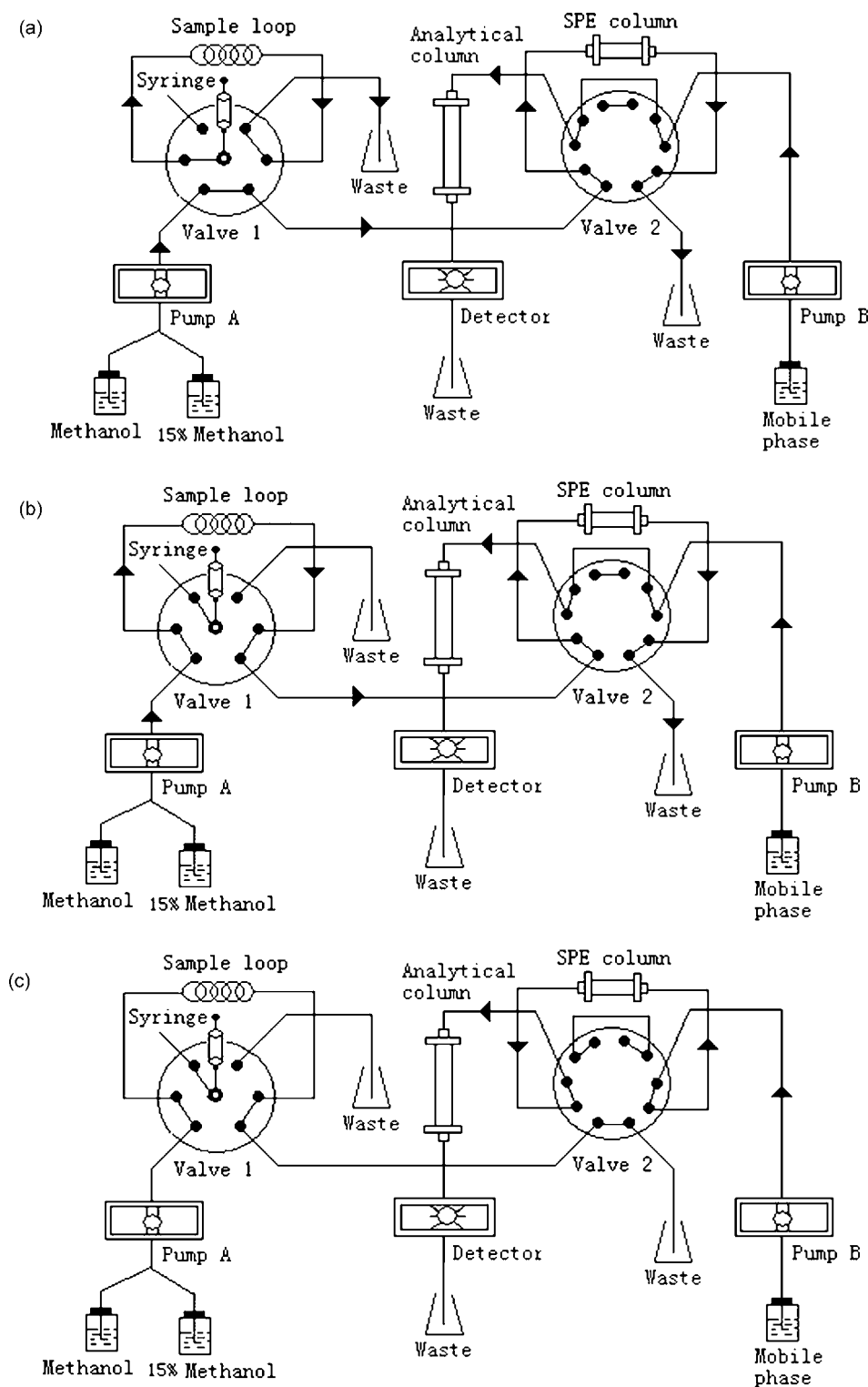


Fig. 2. The manifold diagram of on-line SPE-HPLC system.

plasma spiked with standard solutions of andrographolide and dehydroandrographolide.

2.6.2. Calibration curve

The chromatographic identification of the analytes andrographolide and dehydroandrographolide were realized by their relative retention time. Calibration curves were obtained by plot-

ting the peak-area of the studied analytes versus the theoretical concentration of the analytes added into drug-free rabbit plasma. The curves were constructed from replicate measurements of six concentrations over a range of $0.05\text{--}5\text{ }\mu\text{g mL}^{-1}$. The data were subjected to least squares regression analysis, to provided information on linearity of the method. Six standard curves were prepared on six separate days.

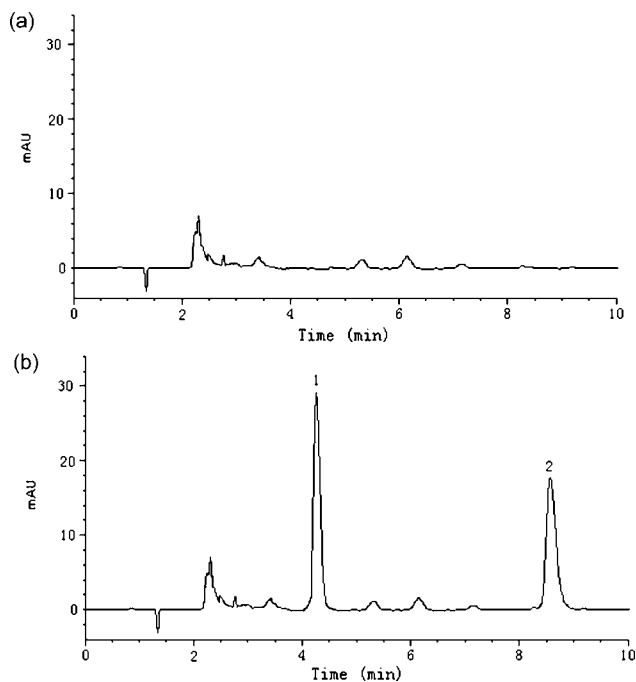


Fig. 3. HPLC chromatograms of blank rabbit plasma (a), rabbit plasma spiked with andrographolide and dehydroandrographolide ($2.0 \mu\text{g mL}^{-1}$) (b). 1, andrographolide, $R_t = 4.3$ min; 2, dehydroandrographolide, $R_t = 8.7$ min.

2.6.3. Precision and accuracy

For the within-day assay precision and accuracy, six replicates of quality control samples were analyzed for the same day. For the between-day assay precision and accuracy, control samples were analyzed once a day for six sequence days.

2.6.4. Sensitivity

The limit of detection (LOD) in plasma was defined as the concentration providing a signal-to-noise ratio of three. The limit of quantification (LOQ) is the minimum injected amount that demonstrated precise measurements (R.S.D. was less than 15% and accuracy was more than 85%) [25].

2.6.5. Extraction recovery

The extraction recovery was determined by comparing peak areas obtained with methanol solutions containing analyte by direct injecting into the HPLC and those with plasma standards containing same analyte concentrations, submitted to the on-line SPE-HPLC system.

2.6.6. Stability

The short-term stability of andrographolide and dehydroandrographolide in rabbit plasma was studied by placing quality control samples at 18°C for 24 h.

The freeze-thaw stability of andrographolide and dehydroandrographolide was also assessed by analyzing quality control samples undergoing three freeze (-18°C)–thaw (room temperature) cycles.

The long-term stability of andrographolide and dehydroandrographolide in rabbit plasma was assessed by placing quality control samples at -18°C for 1 month.

2.7. Application of the proposed method

Three male and three female white rabbits were fasted overnight prior to the experiment of oral administration of *A. paniculata* extract with a dose of 2 mL kg^{-1} . No other food was allowed until after collection of the 12 h sample while water intake was free. Blood samples (1.0 mL) were collected into heparinized tubes from the marginal vein of the ear at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 h following oral administration of *A. paniculata* extract. Plasma was separated by centrifugation (6000 rpm, 5 min) and stored at -18°C until analysis.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The HPLC method proposed provides a simple procedure for the determination of andrographolide and dehydroandrographolide in biological samples.

The maximum absorption wavelength of andrographolide is 225 nm. Dehydroandrographolide had two maximum absorption wavelengths at 225 and 252 nm. A value of 225 nm was used to detect two analytes with a good sensitivity.

In order to optimize the separation and get the maximal signal of peaks, different concentrations of methanol and ACN were used as the mobile phase. As the concentration of organic solvent increased, the signal of the two analytes, especially the dehydroandrographolide, was increased. When the organic solvent concentration was too high, the analytes and other compounds were not separated fully. In this work, the solvent (methanol:ACN:water, 50:10:40; v/v) was selected as mobile phase.

The influence of the mobile phase flow rate in the range of 0.5 – 1.5 mL min^{-1} was studied, and the best separation was obtained at the flow rate of 1.0 mL min^{-1} . As can be seen from Fig. 3b, the separation process can be completed in 10 min.

3.2. Method development for sample clean-up

3.2.1. The optimization of washing solvent

Some methanol was added in the water as washing solvent in order to obtain an efficient washing of the sample matrix. The flow rate was set at 1.0 mL min^{-1} . The washing time was set at 6 min. As can be seen from Fig. 4, when the methanol concentration was equal to or lower than 20%, the recovery values ranging from 93.2% to 98.1% were obtained. However, more co-extracted matter retained on the SPE column when lower methanol concentration was used as washing solvent. When methanol concentration was 25%, the recovery of andrographolide decreased. In this work, 15% aqueous methanol was selected as washing solvent.

3.2.2. The optimization of washing time

It was necessary to measure the time needed for a complete elution of the proteins present in plasma samples from the SPE column and retaining the analytes on it. Such experiments

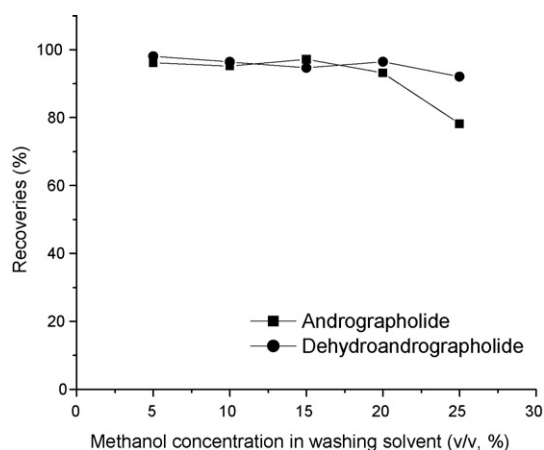


Fig. 4. The effect of methanol concentration in washing solvent on the recoveries of andrographolide and dehydroandrographolide from rabbit plasma.

were achieved by directly connecting the SPE column to the UV detector set at 225 nm and injecting blank plasma samples (100 μ L). The flow rate was set at 1.0 mL min⁻¹. A complete elution of proteins was achieved within 5 min with a washing solvent of 15% aqueous methanol. The washing time was therefore set at 6 min. This time was enough for completing elution of the proteins and was still lower than the time corresponding to the beginning of the elution of andrographolide (16 min) and dehydroandrographolide (29 min).

3.2.3. The optimization of transfer time of analytes from the SPE column to the analytical column

When the concentration was completed, the transfer of the analytes from the SPE column to the analytical column is a critical step in column switching. If the time is too short, poor recovery of the analytes is observed. If the time is too long, many endogenous compounds can be transferred to the analytical column, which will affect the long-term performance of the assay. A transfer time of 2 min in the back-flush mode was found to be ideal for the assay.

3.3. Method validation

3.3.1. Specificity

Fig. 3 shows the typical chromatograms of a blank plasma sample (Fig. 3a) and a blank plasma sample spiked with

andrographolide and dehydroandrographolide (Fig. 3b). No significant interferences of endogenous substances from the blank rabbit plasma with andrographolide or dehydroandrographolide were detected. Therefore, the proposed method was selective for the determination of andrographolide and dehydroandrographolide in rabbit plasma.

3.3.2. Calibration curve

The calibration curves in six different days demonstrated linearity between the response and corresponding concentrations of andrographolide or dehydroandrographolide over the range of 0.05–5 μ g mL⁻¹.

The mean regression equation and correlation coefficients (*R*) are:

Andrographolide: $A = (272.13 \pm 11.28) C + (13.25 \pm 4.37)$, $R = 0.9994 \pm 0.0003$

Dehydroandrographolide: $A = (195.52 \pm 8.52) C + (2.51 \pm 4.84)$, $R = 0.9993 \pm 0.0002$

where *A* represents the peak areas and *C* represents the concentration of andrographolide and dehydroandrographolide in μ g mL⁻¹.

3.3.3. Precision and accuracy

The within- and between-day precisions and accuracies of the assay are summarized in Table 1. The R.S.D. of two analytes was in the range of 1.2–6.5% and the accuracies were between 92.0% and 102.1%.

3.3.4. Sensitivity

The LODs were 0.019 μ g mL⁻¹ for andrographolide and 0.022 μ g mL⁻¹ for dehydroandrographolide. From Table 1, we find that at concentration of 0.050 μ g mL⁻¹ for andrographolide and dehydroandrographolide, the R.S.D. was less than 15% and accuracy was more than 85%. Lower concentration than 0.050 μ g mL⁻¹ added into plasma sample was also test in the work, but the R.S.D. was increased and accuracy was less than 85%. Thus, 0.050 μ g mL⁻¹ for andrographolide and dehydroandrographolide was defined as the LOQ.

Table 1
Within- and between-day precisions and accuracies of the assays (*n* = 6)

Analytes	Theoretical concentration (μ g mL ⁻¹)	Within-day			Between-day		
		Concentration found (mean $\pm$ S.D.) (μ g mL ⁻¹)	R.S.D. (%)	Accuracy (%)	Concentration found (mean $\pm$ S.D.) (μ g mL ⁻¹)	R.S.D. (%)	Accuracy (%)
Andrographolide	0.050	0.048 $\pm$ 0.001	2.1	96.0	0.051 $\pm$ 0.001	2.0	102.0
	0.500	0.505 $\pm$ 0.012	2.4	101.0	0.487 $\pm$ 0.031	6.4	97.4
	5.000	4.731 $\pm$ 0.205	4.3	94.6	4.756 $\pm$ 0.112	2.4	95.1
Dehydroandrographolide	0.050	0.046 $\pm$ 0.003	6.5	92.0	0.048 $\pm$ 0.002	4.2	96.0
	0.500	0.470 $\pm$ 0.012	2.6	94.0	0.507 $\pm$ 0.024	4.7	101.4
	5.000	5.104 $\pm$ 0.224	4.4	102.1	4.892 $\pm$ 0.057	1.2	97.8

Table 2

The extraction recovery of andrographolide and dehydroandrographolide from rabbit plasma

Analytes	Concentration spiked ($\mu\text{g mL}^{-1}$)	Extraction recovery (% , mean $\pm$ S.D.)	R.S.D. (%)
Andrographolide	0.050	94.1 $\pm$ 2.8	3.0
	0.500	98.2 $\pm$ 0.9	0.9
	5.000	97.1 $\pm$ 4.2	4.3
Dehydroandrographolide	0.050	94.5 $\pm$ 3.5	3.7
	0.500	95.2 $\pm$ 3.7	3.9
	5.000	95.5 $\pm$ 2.4	2.5

3.3.5. Extraction recovery

The recoveries of all compounds from methanol and plasma standard were evaluated to test the efficiency of the extraction procedure. The recovery ranged from 94.1% to 98.2% for andrographolide and 94.5% to 95.5% for dehydroandrographolide (Table 2).

3.3.6. Stability

No significant difference of concentrations for andrographolide and dehydroandrographolide in rabbit plasma was observed during the 24-h period of assay, the R.S.D. between the initial concentrations and the concentrations of andrographolide and dehydroandrographolide stored at 18 °C for 24 h was less than 4.8%.

Freezing and thawing did not reveal any detrimental effect on the absolute concentrations of analyte spiked to rabbit plasma. After completion of three freezing and thawing cycles, the recoveries of andrographolide and dehydroandrographolide still ranged between 93.4% and 98.7%.

Without any obvious trend of concentration change of andrographolide and dehydroandrographolide was observed over 1 month. The spiked samples after stored at -18°C for 1 month, the recoveries of andrographolide and dehydroandrographolide still ranged between 94.2% and 97.1%.

3.4. Method comparison with existing reports

There are several reports on assaying andrographolide and dehydroandrographolide in plasma. Han et al. measured dehydroandrographolide in rat plasma [13]. The intra- and inter-day precisions (R.S.D.) were 1.06–2.32% and 2.91–6.14% in a 0.509–10.175 $\mu\text{g mL}^{-1}$ range. The recoveries were 94.10–104.23%. Cai et al. reported pharmacokinetic study of andrographolide in rabbit plasma [14]. The intra- and inter-day precisions (R.S.D.) were 0.81–3.92% and 1.11–4.09% in a 0.204–8.250 $\mu\text{g mL}^{-1}$ range. The recoveries were 85.7–96.8%. Considering the results above, the proposed method can be used as a simpler procedure with similar precision and accuracy but all the reported method mentioned above had to go through a time-consuming and arduous pretreatment step. Moreover, sample used in the proposed method (100 μL) is very little. It makes possible that multiple constituents of herb medicine in biological fluid can be simultaneously determined by automatization.

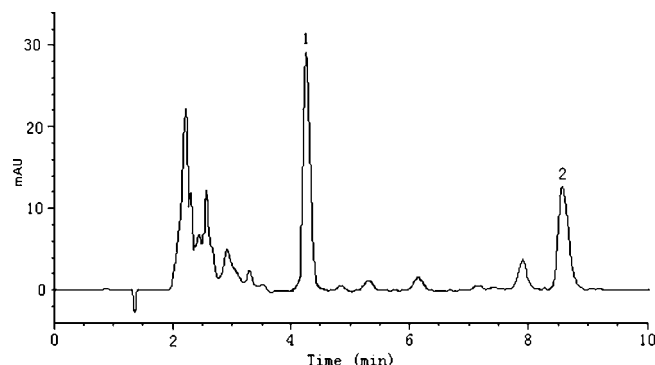


Fig. 5. HPLC chromatograms from rabbit plasma sample, obtained after oral administration of *A. paniculata* extract at 0.75 h. 1, andrographolide; 2, dehydroandrographolide.

3.5. Application of the proposed method

The proposed method was applied to determination of andrographolide and dehydroandrographolide in plasma after oral administration of *A. paniculata* extract at a dose of 2 mL kg^{-1} in rabbits. HPLC chromatograms from rabbit plasma sample, obtained after oral administration of *A. paniculata* extract at 0.75 h are shown in Fig. 5. Mean plasma concentration–time curves of two analytes are shown in Fig. 6. The C_{max} (mean value) of andrographolide was 2.28 $\mu\text{g mL}^{-1}$ appearing at 1.0 h. The C_{max} (mean value) of dehydroandrographolide was 1.33 $\mu\text{g mL}^{-1}$ appearing at 0.75 h.

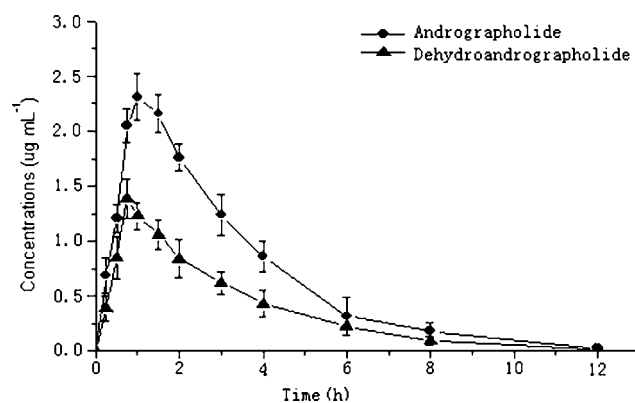


Fig. 6. Plasma concentration–time curves of andrographolide and dehydroandrographolide after oral administration of *A. paniculata* extract ($n=6$; error bars = S.D.).

4. Conclusions

An on-line SPE-HPLC method was developed for determination of andrographolide and dehydroandrographolide in rabbit plasma. A comparatively clean chromatogram was obtained though a complex herb medicine plasma sample was injected. The method showed excellent precision, accuracy, stability and high recovery. The SPE column can be used repeatedly. Under the proposed experimental conditions, the SPE column was shown to obtain perfect recovery for over 200 injections of 100 μ l of plasma, although a little increase in back-pressure of the system was observed after a total of 150 samples. The suitability of the method was proved in the plasma concentration–time curve study of andrographolide and dehydroandrographolide after oral administration of *A. paniculata* extract in rabbits.

Acknowledgement

This work was supported by the Development Program of the Ministry of Science and Technology of Jilin Province, China (grant number 20050560).

References

- [1] R. Wongkittipong, L. Prat, S. Damronglerd, C. Gourdon, Sep. Purif. Technol. 40 (2004) 147.
- [2] G.A. Akowuah, I. Zhari, I. Norhayati, A. Mariam, J. Food Compos. Anal. 19 (2006) 118.
- [3] J.X. Zhao, G.L. Yang, H.X. Liu, D.X. Wang, X.R. Song, Y. Chen, Phytochem. Anal. 13 (2002) 222.
- [4] W. Ruengsitagoon, K. Anuntakarun, C. Aromdee, Talanta 69 (2006) 900.
- [5] The Pharmacopoeia Commission of the People's Republic of China, in: Pharmacopoeia of PRC, Chemical Industry Press, Beijing, 2005, p. 189.
- [6] S. Saxena, D.C. Jain, M.M. Gupta, Phytochem. Anal. 11 (2000) 34.
- [7] A. Srivastava, H. Misra, R.K. Verma, Phytochem. Anal. 15 (2004) 280.
- [8] D.C. Jain, M.M. Gupta, S. Saxena, S. Kumar, J. Pharm. Biomed. Anal. 22 (2000) 705.
- [9] W.K. Li, J.F. Fitzloff, J. Liq. Chromatogr. Rel. Technol. 25 (2002) 1335.
- [10] W.K. Li, J.F. Fitzloff, J. Liq. Chromatogr. Rel. Technol. 27 (2004) 2407.
- [11] N. Pholphana, N. Rangkadilok, S. Thongnest, Phytochem. Anal. 15 (2004) 365.
- [12] Q. Du, G. Jerz, P. Winterhalter, J. Chromatogr. A 984 (2003) 147.
- [13] F.M. Han, W.T. Cai, Q.S. Xia, Y. Chen, J. Chin. Pharm. 20 (2005) 206.
- [14] W.T. Cai, F.M. Han, Y. Chen, J. Hubei Univ. (Nat. Sci.), China 27 (2005) 399.
- [15] Z.Y. Xu, Q.D. Liu, Y.Q. Zhang, J. Guangzhou Univ. TCM 22 (2005) 401.
- [16] N.F.C. Visser, H. Lingeman, H. Irth, J. Pharm. Biomed. Anal. 32 (2003) 295.
- [17] M. Yritia, P. Parraa, E. Iglesiasa, J.M. Barbanojb, J. Chromatogr. A 870 (2000) 115.
- [18] M. Li, Y. Alnouti, R. Leverence, H. Bi, A.I. Gusev, J. Chromatogr. B 825 (2005) 152.
- [19] Y. Alnouti, K. Srinivasan, D. Waddell, H. Bi, O. Kavetskaia, A.I. Gusev, J. Chromatogr. A 1080 (2005) 99.
- [20] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. B 801 (2004) 141.
- [21] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.T. Brinkman, J. Pharm. Biomed. Anal. 17 (1998) 1161.
- [22] C. Kousoulos, Y. Dotsikas, Y.L. Loukas, Talanta 72 (2007) 360.
- [23] M.R. Fuh, S.W. Lin, L.L. Chen, T.Y. Lin, Talanta 72 (2007) 1329.
- [24] C.C. Zhu, J.X. Mo, S.H. Gui, X.J. Zhang, X.J. Zhao, X.P. Lai, Chin. Pharm. Clin. 18 (2002) 9.
- [25] H.A. Nguyen, J. Grellet, B.B. Ba, C. Quentin, M.C. Saux, J. Chromatogr. B 810 (2004) 77.

The analysis of volatile siloxanes in waste activated sludge

Raf Dewil^{a,*}, Lise Appels^b, Jan Baeyens^c, Anna Buczynska^d, Luc Van Vaeck^d

^a Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

^b Department of Chemical Engineering, Catholic University of Leuven, W. De Croylaan 48, B-3001 Heverlee, Belgium

^c Department of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

^d Department of Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

Received 19 June 2006; received in revised form 13 May 2007; accepted 18 May 2007

Available online 26 May 2007

Abstract

The increasing presence of siloxanes in waste activated sludge (WAS) considerably hampers the energy use of the biogas obtained during the anaerobic digestion of the sludge when concentrations exceed critical limits. To prevent the occurrence of unacceptable operating conditions, it is hence necessary to have a reliable analysis method for determining the siloxane content of the sludge. This paper describes and validates such a method, consisting of the extraction of the siloxanes using *n*-hexane and a subsequent analysis of the extract using GC-FID. The validation procedure confirms the excellent recovery and repeatability of the proposed method.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Waste activated sludge; Volatile siloxanes; D4; D5; Extraction; Gas chromatography

1. Introduction

Anaerobic digestion is widely applied in the treatment of waste activated sludge, commonly called biosolids, since it offers several advantages, e.g. a significant reduction of the sludge quantity (by 25–30%), a partial removal of pathogens and a stabilisation of the sludge [1]. Moreover, during the digestion process, an energy-rich biogas is formed, which can be valorised energetically (for the production of heat or electricity). The energy produced in this way is moreover recognised as a form of renewable energy. The presence of increasing amounts of siloxanes in the sludge, however, considerably hampers the energy use of the biogas [2].

Siloxanes are a subgroup of silicones, containing Si–O bonds with organic radicals (mostly methyl-groups) bound to Si. They are widely used in various industrial processes and consumer products such as detergents, shampoos, cosmetics, paper-coatings and textile because of several beneficial properties and are moreover feedstock chemicals for the production of silicones where they are released as a residue [2]. Most of the

used siloxanes are very volatile and disperse into the atmosphere where they are decomposed (mostly hydroxyl radical initiated). Some of them however end up in the wastewater. Siloxanes are not decomposed in the waste activated sludge process, have a significantly lower solubility in water and are more adsorptive than many organic compounds. They do therefore not accumulate in the water phase, but are preferentially adsorbed onto the extracellular polymeric substances (EPS) of the sludge flocs (as is also the case for other micro-pollutants like heavy metals) [3], thus significantly increasing the content of siloxanes in the sludge.

During the anaerobic digestion, most siloxanes are released from the sludge flocs due to the breakdown of organic matter and the increased temperature (up to 37 °C for mesophilic digestion). They then end up in the biogas, especially the cyclic siloxanes octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) which are detected in significant amounts in the biogas produced during sludge digestion [4]. Smaller molecules such as D3 (hexamethylcyclotrisiloxane) volatilise rapidly and are only present in wastewater in small amounts. Larger molecules, such as D6 (dodecamethylcyclohexasiloxane) do not volatilise during sludge digestion due to their low vapour pressure and remain in the WAS. This is also true for the non-volatile polydimethylcyclsiloxanes $[-(\text{Me}_2\text{SiO})_x-]$. Some physicochemical properties of siloxanes are presented in Table 1.

* Corresponding author. Tel.: +32 3 2653569; fax: +32 3 2653225.
E-mail address: raf.dewil@skynet.be (R. Dewil).

Table 1
Some selected properties of the targeted siloxanes

Name	Representation	Abbreviation	Boiling point (°C)	Vapour pressure at 25 °C (Pa)	Water solubility 25 °C (mg/l)	Log K_{ow}	Henry cte. H_c
Hexamethyldisiloxane	HxMe-diSX	L2	106.9	5626.2	0.93	4.2	397
Hexamethylcyclotrisiloxane	HxMe-cyTrSX	D3	135.2	1146.6	1.56	3.85	–
Octamethylcyclotetrasiloxane	OcMe-cyPeSX	D4	175.7	131.99	0.056	4.45	259
Decamethylcyclopentasiloxane	DeMe-cyPeSX	D5	211.2	23.198	0.017	5.2	185
Dodecamethylcyclohexasiloxane	DoMe-cyHxSX	D6	245.1	3.999	0.005	5.86	104

For a more extensive description the reader is referred to Palczewska-Tulinska and Oracz [5].

The presence of siloxanes (and silicon-containing compounds in general) in biogas can seriously damage the biogas beneficiation equipment [6]. During combustion, abrasive microcrystalline silica is formed which leads to serious fouling of all exposed surfaces. Its hardness leads to abrasion of moving motor parts and as thermal insulator it contributes to overheating of sensitive motor parts and abrasion of gas motor surfaces [2]. Schweigkofler and Niessner [4] found siloxane-concentrations up to 24 mg/m³ at a wastewater treatment plant in Munich (Germany).

Bearing all previous problems in mind, it is important to be able to measure the siloxane-concentration in WAS by a reliable analytical technique.

The analysis of volatile siloxanes in biogas has been extensively described in literature. Most sources describe the use of a gas chromatography (GC) method [7–9]. Although the analysis itself is relatively simple, the sampling is not since siloxanes adsorb readily to the surfaces of the sampling container. Therefore, several sampling methods were suggested. Hagmann et al. [7] used GC–MS (gas chromatography coupled with a mass spectrometer) for the analysis and proposed several sampling methods, including adsorption, absorption and the use of gas bags. These methods are listed in Table 2. Huppmann et al. [8] used gas chromatography on a fused silica column and flame ionisation detection (GC–FID) for siloxane-analysis in biogas. To extract the siloxanes from the biogas, XAD resins, activated carbon, polyurethane foam and tetradecane were tested. Adsorption from the gas was successfully accomplished with XAD resins, activated carbon and tetradecane, but quantitative recoveries from the adsorbents were achieved only using XAD and ultrasound treatment. Schweigkofler and Niessner [9] used air-evacuated stainless steel canisters for sampling siloxanes.

Literature references for the analysis of siloxanes in fluid and solid matrices are however scarce. Prior to the chemical analysis, the siloxanes should be extracted from the solid matrix. For the analysis of siloxanes in sludge, Huppmann et al. [8] describe a method which consists of stripping the siloxanes from the samples by helium. Subsequently, this gas mixture was sent through a XAD-resin for adsorbing the siloxanes, followed by a desorption and an analysis by GC–FID (as described above).

Some authors have described analysis methods for siloxanes in biological matrices like animal tissue. Varaprath et al. [10] describe a method for measuring D4 in animal tissue, blood and urine. They carried out extractions of D4 and its metabolites with various organic solvents, followed by an HPLC or GC–MS analysis. Flassbeck et al. [11] also used an extraction with *n*-hexane and a subsequent analysis with GC–MS for the determination of low molecular weight cyclic siloxanes (D3, D4, D5, D6) in plasma and blood with an extraction efficiency of 90% for plasma and 80% for blood. These authors used the same technique for determining siloxanes in human breast tissue in a later study [12]. Varaprath and Cao [13] propose a solid phase extraction for measuring the siloxane content of aqueous matrices.

It is clear that the cited methods are tedious and neither tested nor validated for the analysis of siloxanes in a sludge matrix. Therefore, this paper is dedicated to the description and validation of a combined extraction-analysis method for determining the volatile siloxanes D4 and D5 in WAS.

2. Experimental set-up and procedures

2.1. Sludge samples

The waste activated sludge samples used in the experiments were obtained from the full-scale wastewater treatment plant (WWTP) of Deurne-Schijnpoot, located in Flanders (Bel-

Table 2
Sampling techniques for biogas

Sampling techniques	Materials used	Disadvantages	Advantages
Adsorption on activated coal	Activated coal, solvent for desorption (CS ₂ , benzyl alcohol, CH ₂ Cl ₂)	Poor recoveries	–
Adsorption on ion exchange resins	XAD 2, XAD 4, solvent for desorption (hexane)	Poor recoveries	–
Gas collection bags	Linde gas bags	Gas bags are sealed with silicon septa and adhesives; blank results are too high	–
Glass collection containers	Glass containers	Condensation and adsorption effects of siloxanes on glass walls	Easy to apply
Liquid adsorption	Various liquids (methanol, hexane, diesel); gas washing bottles	Methanol and hexane must be cooled; diesel oil is not suitable for GC/MS	All siloxanes detectable quantitatively

gium). The samples were taken from the secondary clarifier. The dry solids content of the sludge was determined according to standard analytical methods [14]. The sludge was spiked with known amounts of D4 and D5 for the experiments. The D4 had a purity of 98% and was obtained from Acros Organics (Belgium), the D5 had a purity of 97% and was obtained from Merck KGaA (Darmstadt, Germany). The sludge–siloxane mixture was gently stirred for 10 min. Thereafter it was stored at 4 °C for 24 h in order to achieve a complete adsorption of the siloxanes to the sludge flocs.

The amounts of siloxanes added were chosen in the same order of magnitude as frequently measured in heavily siloxane-loaded sludge, e.g. at the WWTP of Tricatti and Minworth, where siloxane concentrations up to 0.03 g/g DS are measured in secondary sludge [15].

2.2. Extraction procedure

An extraction procedure was applied to the sludge samples. The solvent should be chosen according to some essential properties. Firstly, it needs to be immiscible with water, since the sludge samples contain up to 99.5% of water. Moreover its density should be lower than the density of water: the solvent layer will otherwise be situated between the water layer (on top) and the thickened sludge layer (consisting of particulate matter) on the bottom of the tube, making it very difficult to remove the intermediate solvent layer containing the extract for the analysis without contamination with water. Obviously the solvent should not be aggressive to the chromatograph column. One of the most common GC solvents, *n*-hexane was therefore chosen. This solvent meets all pre-defined properties and was already suggested by Flassbeck et al. [11], who moreover detected traces of D3–D6 in other solvents such as THF and ethyl acetate.

After homogenising the sludge samples, 50 ml of sludge was introduced in a calibrated flask and 10 ml of *n*-hexane (obtained from Merck, Darmstadt, Germany) was added for extraction. The sludge–hexane mixture was vortex-mixed at high speed for 10 min. The extracts were subsequently centrifuged at 4400 rpm (approx. $8900 \times g$) for 5 min. Since *n*-hexane and water are immiscible, a phase separation is obtained during centrifugation. The top phase consisting of *n*-hexane containing the extracted siloxane is removed by suction pipette.

2.3. Analysis

The analysis of the siloxane extracts was performed using a Varian 3400 gas chromatograph with an 8200 autosampler, coupled with a FID detector. The separation was carried out in a Varian FactorFour VF-1MS capillary column. The injector port temperature was set at 125 °C. The initial oven temperature was 60 °C. This temperature was kept for 4 min, after which the temperature was linearly increased to 250 °C at a rate of 8 °C/min. This temperature was held for another 15 min. The detector temperature was set at 250 °C.

The high end-temperature and the extended elution time per sample were applied to guarantee that all extracted compounds left the column before introducing the next sample.

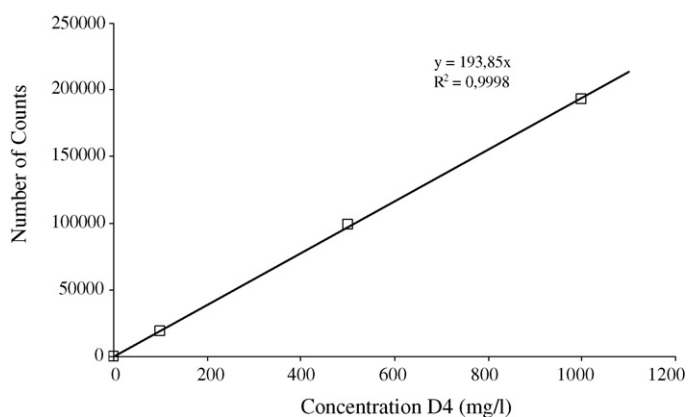


Fig. 1. Calibration curve for D4 in hexane.

3. Results and discussion

3.1. Calibration curve

A calibration curve was obtained by subjecting D4, resp. D5 solutions of known concentration in *n*-hexane to the same separation and measurement process. The calibration curves for D4 and D5 are presented in Figs. 1 and 2, respectively. The high value of the correlation coefficients (R^2) illustrates the linearity of the detector within the considered concentration range. A typical chromatogram for D4 and D5 in *n*-hexane is shown in Fig. 3. The peak with a retention time of 5.539 min corresponds with D4 (being the more volatile) and the peak with a retention time of 9.034 min with D5.

Hereafter the spiked sludge samples were analysed. The chromatogram of a sludge sample spiked with D4 is presented in Fig. 4 as an example. Peak identification by GC–MS was carried out on both a D4 and D5 spiked sludge sample. The mass spectra are presented in Figs. 5 and 6. The spectra were identified by comparison of the obtained mass spectra with NIST 98 MS library spectra. The spectrum of the first peak was indeed identified as D4 with a correlation of 98.7% and the second peak as D5 with a correlation of 98.2%. These results confirm the suitability of using these peaks for determining the D4 and D5 concentrations in sludge samples.

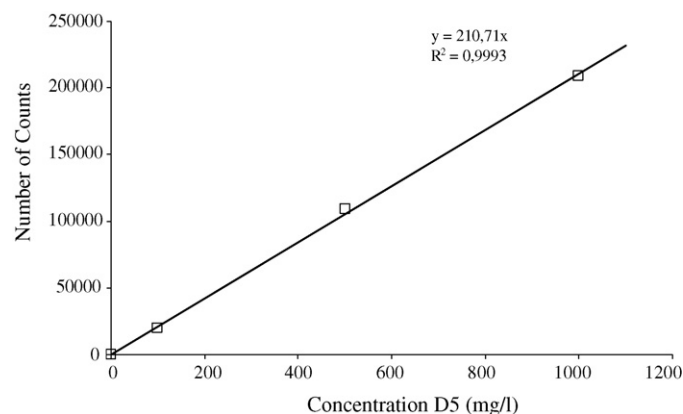


Fig. 2. Calibration curve for D5 in hexane.

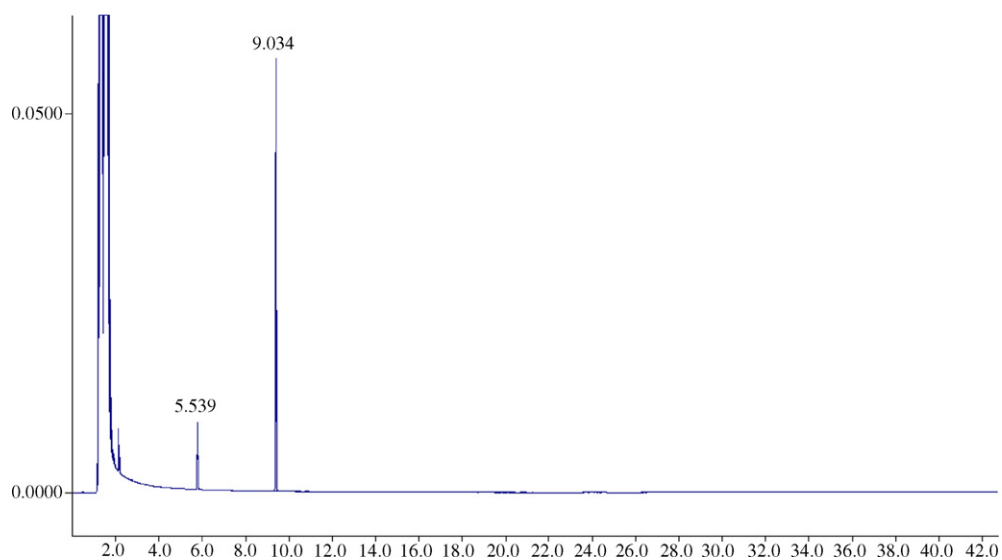


Fig. 3. GC-FID chromatogram of D4 and D5 in hexane (500 mg/l).

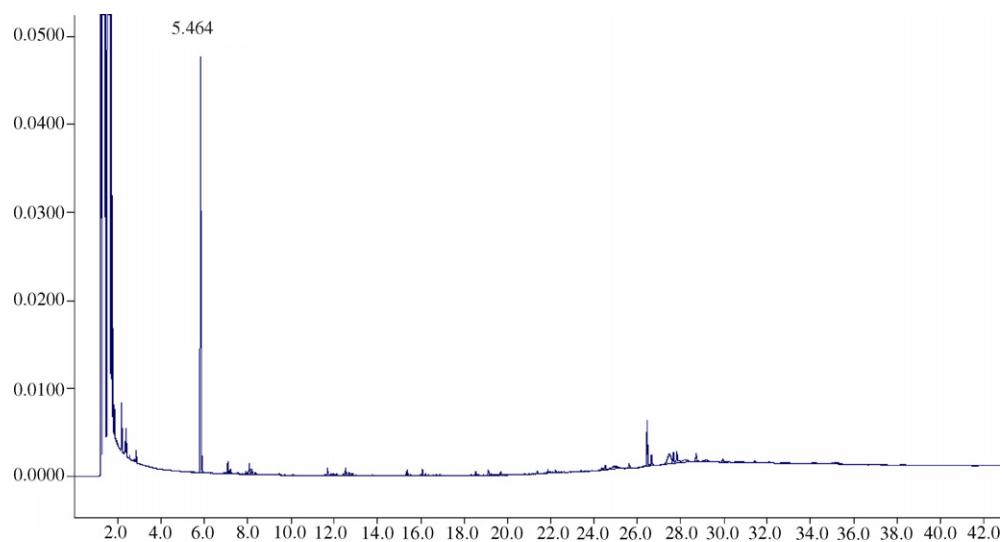


Fig. 4. GC-FID chromatogram of a sludge sample spiked with D4.

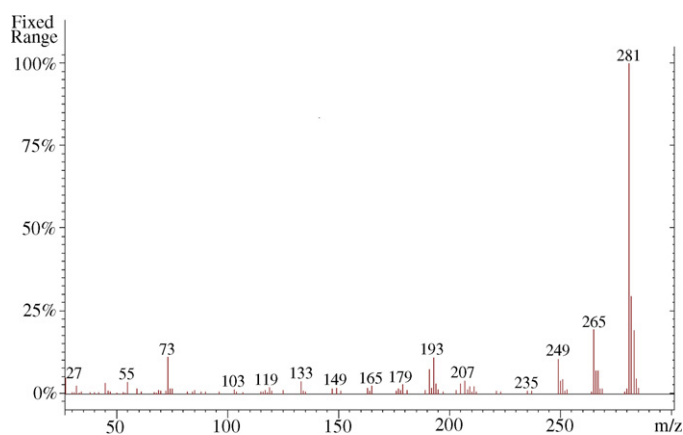


Fig. 5. Mass spectrum of D4 peak in spiked sludge sample.

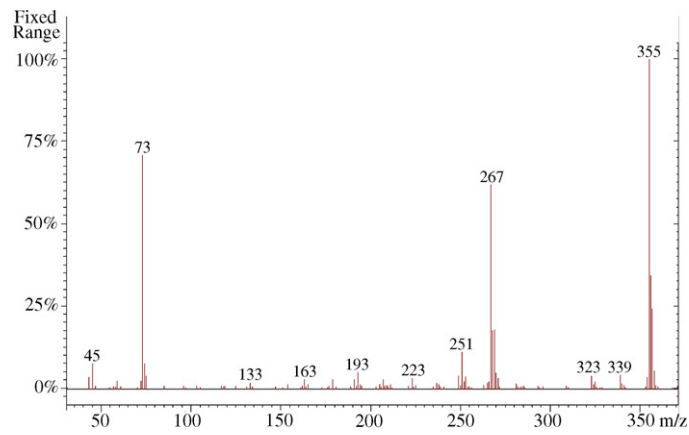


Fig. 6. Mass spectrum of D5 peak in spiked sludge sample.

Table 3
Validation of analysis procedure for D4

D4 added (g/g DS)	Total D4 measured (g/g DS)					Average measured value (g/g DS)	Standard deviation (g/g DS)	Recovery (%)
	1	2	3	4	5			
0	0.00091	0.00090	0.00091	0.00078	0.00085	0.0009	0.000056	–
0.0083	0.0096	0.0054	0.0065	0.0071	0.0065	0.0070	0.0016	73.7
0.0417	0.0515	0.0350	0.0326	0.0395	0.0350	0.0387	0.0076	90.8
0.0833	0.0948	0.0703	0.0777	0.0696	0.0818	0.0788	0.010	93.6
0.1667	0.1864	0.1581	0.1470	0.1517	0.1512	0.1589	0.016	94.8

Table 4
Validation of analysis procedure for D5

D5 added (g/g DS)	Total D5 measured (g/g DS)					Average measured value (g/g DS)	Standard deviation (g/g DS)	Recovery (%)
	1	2	3	4	5			
0	0.000567	0.000569	0.000582	0.000573	0.000585	0.000575	7.95×10^{-6}	–
0.0118	0.0125	0.0128	0.0129	0.0127	0.0112	0.0124	0.00069	100.8
0.0588	0.0581	0.0580	0.0605	0.0570	0.0596	0.0586	0.0014	98.7
0.1176	0.1061	0.1193	0.1149	0.1286	0.1181	0.1174	0.0081	99.3
0.2353	0.2363	0.2274	0.2346	0.2420	0.2364	0.2353	0.0052	99.8

For determining the repeatability of the method, sludge sample spiked with varying concentrations of D4 and D5, respectively, as well as a blank (reference, non-spiked) sample were analysed in five-fold. Subsequently the recovery and the standard deviation of the results were calculated for each sample. The reproducibility (which is defined as the standard deviation for a set of measurements carried out at different times) of the analysis can however not be determined: the samples cannot be conserved since the analysis must be directly performed on fresh sludge samples, due to the microbial instability of the sludge (even kept at 4 °C).

The average results for the analysis of D4 are presented in Table 3. The standard deviation of the analysis was 5.47×10^{-5} g/g DS, confirming the excellent repeatability of

the analysis procedure (6.3% of the mean value). The values of the biases were also very low, except for the lowest addition of D4. This can be explained by the partial evaporation of D4 during the analysis procedure.

The same procedure was followed for D5, and results are presented in Table 4. The standard deviation on the measurements was 7.95×10^{-6} g/g DS, representing 1.36% of the average value. The bias is almost zero. These results are even more promising than the D4-analysis. The lower bias-values are explained by the difference in volatility between D4 and D5. During the hexane-extraction, when the sludge/hexane mixture is heavily stirred, the more volatile siloxanes can be released in the air. This effect was also observed by Schweigkofler and Niessner [4] during siloxane absorption on full scale plants. The

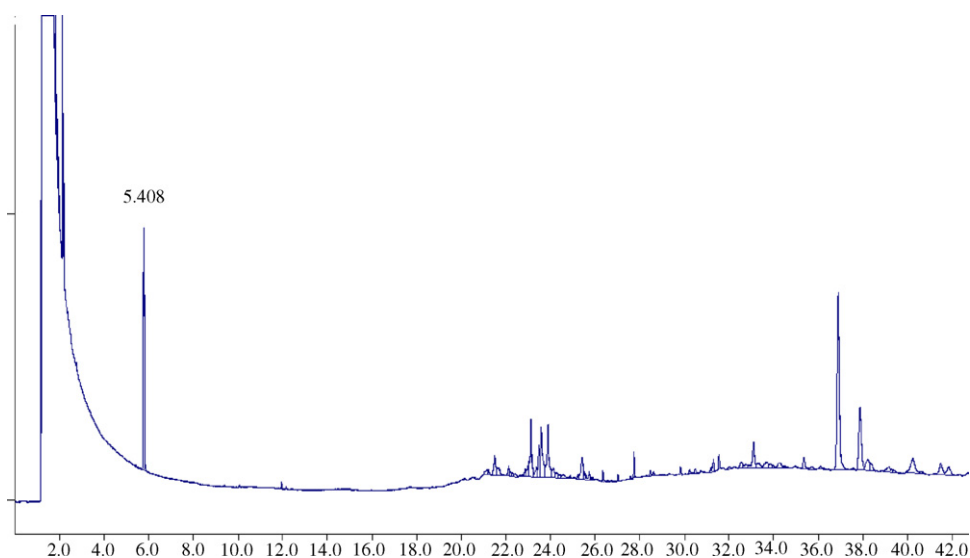


Fig. 7. GC-FID chromatogram of a real sludge sample.

lower volatility of D5 versus D4 explains why D5 is only released to a minor extent.

The proposed method was furthermore tested for sludge samples taken directly from the WWTP. By way of example, the chromatogram of a such a sludge sample, taken at the WWTP of Fenham (Coventry, UK) is shown in Fig. 7, which reveals especially a high concentration of D4 being present. Although the chromatogram is more complex, the D4 peak remains clearly separated from other peaks.

4. Conclusions

The increasing presence of siloxanes in WAS considerably hampers the energy use of the biogas obtained during the anaerobic digestion of the sludge. It is necessary to be capable to accurately analyse the siloxane content of the sludge. This paper described and validated such a method, consisting of the extraction of the siloxanes using *n*-hexane and a subsequent analysis of the extract using GC-FID. The validation procedure confirms the excellent recovery and repeatability of the proposed method. This method is hence suitable for determining the siloxane content of WAS.

References

- [1] Metcalf and Eddy Inc., Wastewater Engineering: Treatment and Reuse, 4th ed., McGraw-Hill, New York, 2003, p. 1819.
- [2] R. Dewil, L. Appels, J. Baeyens, Energy Conv. Manag. 47 (2006) 1711–1722.
- [3] E. Neyens, J. Baeyens, R. Dewil, B. De heyder, J. Hazard. Mater. 106 (2004) 83–92.
- [4] M. Schweigkofler, R. Niessner, J. Hazard. Mater. 83 (2001) 183–196.
- [5] M. Palczewska-Tulinska, P. Oracz, J. Chem. Eng. Data 50 (2005) 1711–1719.
- [6] UK Environment Agency, Guidance on Gas Treatment Technologies for Landfill Gas Engines, Bristol, UK, 2002.
- [7] M. Hagmann, E. Heimbrand, P. Hentschel, Proceedings Sardinia 99, Seventh International Waste Management and Landfill Symposium, Cagliari, Italy, 4–8 October, 1999.
- [8] R. Huppmann, H.W. Lohoff, H.F. Schröder, Fresenius J. Anal. Chem. 354 (1996) 66–71.
- [9] M. Schweigkofler, R. Niessner, Environ. Sci. Technol. 33 (1999) 3680–3685.
- [10] S. Varaprath, K.L. Salyers, K.P. Plotzke, S. Nanavati, Anal. Biochem. 256 (1998) 14–22.
- [11] D. Flassbeck, B. Pfeleiderer, R. Grumping, A.V. Hirner, Anal. Chem. 73 (2001) 606–611.
- [12] D. Flassbeck, B. Pfeleiderer, P. Klemens, K.G. Heumann, E. Eltze, A.V. Hirner, Anal. Bioanal. Chem. 375 (2003) 356–362.
- [13] S. Varaprath, L. Cao, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 1539–1553.
- [14] Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA, 1998.
- [15] P. Griffin, Siloxanes in Wastewater and Biogas, Seminar, Severn Trent Water, U.K., 2004.

Solid-phase extraction *versus* matrix solid-phase dispersion: Application to white grapes

M.S. Dopico-García, P. Valentão, A. Jagodzińska, J. Klepczyńska,
L. Guerra, P.B. Andrade, R.M. Seabra *

*REQUIMTE-Serviço de Farmacognosia, Faculdade de Farmácia, Universidade do Porto,
R. Aníbal Cunha 164, 4050-047 Porto, Portugal*

Received 15 November 2006; received in revised form 11 May 2007; accepted 18 May 2007
Available online 24 May 2007

Abstract

The use of matrix solid-phase dispersion (MSPD) was tested to, separately, extract phenolic compounds and organic acids from white grapes. This method was compared with a more conventional analytical method previously developed that combines solid liquid extraction (SL) to simultaneously extract phenolic compounds and organic acids followed by a solid-phase extraction (SPE) to separate the two types of compounds. Although the results were qualitatively similar for both techniques, the levels of extracted compounds were in general quite lower on using MSPD, especially for organic acids. Therefore, SL-SPE method was preferred to analyse white “*Vinho Verde*” grapes. Twenty samples of 10 different varieties (Alvarinho, Avesso, Asal-Branco, Batoca, Douradinha, Esganoso de Castelo Paiva, Loureiro, Pedernã, Rabigato and Trajadura) from four different locations in Minho (Portugal) were analysed in order to study the effects of variety and origin on the profile of the above mentioned compounds. Principal component analysis (PCA) was applied separately to establish the main sources of variability present in the data sets for phenolic compounds, organic acids and for the global data. PCA of phenolic compounds accounted for the highest variability (77.9%) with two PCs, enabling characterization of the varieties of samples according to their higher content in flavonol derivatives or epicatechin. Additionally, a strong effect of sample origin was observed. Stepwise linear discriminant analysis (SLDA) was used for differentiation of grapes according to the origin and variety, resulting in a correct classification of 100 and 70%, respectively.

© 2007 Elsevier B.V. All rights reserved.

Keywords: MSPD; Multivariate analysis; Organic acids; Phenolics; “*Vinho Verde*”; White grapes

1. Introduction

Matrix solid-phase dispersion (MSPD) has been widely used in the last years for the isolation of a wide range of drugs, pesticides, naturally occurring constituents and other compounds from different complex plant and animal tissues providing, in many cases, equivalent or superior results to older official methods conducted by more classical extraction and/or SPE techniques (see reviews [1–5]).

Usually, for the analysis by conventional techniques of solid, semi-solid and/or highly viscous biological samples, several steps are necessary for their preparation, extraction and fractionation. However, MSPD enables to combine all these steps in

just one, because the entire sample is homogeneously dispersed in a solid support, usually a C18 or C8-bonded silica, creating a unique chromatographic phase that is used as the stationary phase of a column. The extraction of the analytes and clean-up are carried out simultaneously with, generally, good recoveries and precision [3–4].

MSPD enables complete sample disruption and dispersal into particles of very small size, providing an enhanced surface area for subsequent extraction of the compounds [3], whereas in SL/SPE sample disruption must be conducted separately and many of the sample components must be discarded before an extractive solution is ready to be added to an SPE column. In SPE the extracted compounds are usually absorbed onto the top of the column packing material, not throughout the column as in MSPD. The physical and chemical interactions among the components of the system are greater in MSPD and different in many aspects from those that take

* Corresponding author.

E-mail address: rseabra@ff.up.pt (R.M. Seabra).

place in classical SPE or other forms of liquid chromatography.

Although MSPD has been found to be a technique generally simpler, faster and requiring much less solvent than classical methods and has been widely used for the analysis of different analytes in plants (see review Barker [3]), only in a few studies phenolic compounds, as phenolic acids [6] or isoflavonoids, [7–8] have been analysed by this technique. However, these aforementioned studies do not show clearly that their complete extraction can be easily obtained by MSPD. Xiao et al. [7] compared the efficiency of MSPD with ultrasonic and Soxhlet methods to extract isoflavonoids from *Radix astragali*, the dried root of a medicinal Chinese plant. Four main isoflavonoids were identified, two aglycones and two glycosides, but while the amounts of the two aglycones were higher when using MSPD, the efficiency of the extraction was better for the glycosides if the conventional techniques, ultrasonic or Soxhlet (specially this last one) were used. De Rijke et al. [8] employed MSPD to extract and isolate isoflavone glucoside malonates from leaves of leguminous plants but, compared with solid liquid extraction, the efficiency for extracting the glucosides was found to be lower [4]. In the work of Ziaiová et al. [6] that tried the extraction of phenolic acids by MSPD, from a medicinal plant, the results obtained were not quantitatively compared with any other conventional techniques.

Grapes are complex matrices and the complexity of its study is increased by the numerous varieties of *Vitis vinifera* used over the various producing areas. Several studies have been carried out in order to try to correlate the variety with the chemical composition of the grapes and wines obtained from them. Among the metabolites used for such purpose, we can mention the phenolics, coloured or non-coloured [9–14], since, besides working as marker compounds, these substances are known to possess several interesting properties related with health [15–17]. In addition, these constituents contribute strongly to the organoleptic characteristics of grapes, and therefore of the wine obtained from them [18].

“Vinho Verde” is considered a QWPSR (Quality Wine Produced in a Specified Region) and is confined to the north-west region of Portugal. A search over the published literature showed that only few studies were carried out on the chemical composition of these wines [19–22] and even less on their producing grapes [23]. As far as we know, their content of phenolic compounds and organic acids has not been determined yet.

In a previous work [24] a conventional analytical method was developed to determine phenolic compounds and organic acids from white grapes. This method needed two steps, one for the simultaneous extraction of both types of compounds by solid liquid extraction (SL), and another for the separation and clean-up of organic acids and phenolic compounds by solid-phase extraction (SPE).

In this paper, the first objective was to test the ability of MSPD to, separately, extract phenolic compounds and organic acids from white grapes in just one step, simplifying the SL-SPE analytical procedure. The target compounds were determined using high performance liquid chromatography (HPLC) coupled to a

diode array detector (DAD) for phenolic compounds or to an UV detector for organic acids.

Once the advantages of the SL-SPE analytical method for the studied matrix were checked, samples of all the white grape varieties of the “Vinho Verde” recommended by “Comissão de viticultura da região dos Vinhos Verdes” [25] (i.e. Alvarinho, Avesso, Asal Branco, Batoca, Loureiro, Pedernã and Trajadura) and a few white grape varieties authorized (Douradinha, Esganoso Castelo Paiva and Rabigato), were analysed. Afterwards, multivariate techniques of data analysis, principal components analysis (PCA) and linear discriminant analysis (LDA) were employed in order to establish differentiation criteria as a function of the types of grapes. Although other factors such as polymorphism [26] can influence chemical composition of the grapes and, therefore, be important for the differentiation among them, in this study only the variety and origin of the samples have been considered for statistical analysis.

2. Experimental

2.1. Reagents and solvents

Methanol (MeOH), ethanol and formic acid were obtained from Merck (Darmstadt, Germany), hydrochloric acid from Pronalab (Lisboa, Portugal) and sulphuric acid from Fluka Sigma–Aldrich (Seetze, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). The ultrasonic bath was from Bandelin (Berlin, Germany).

The phenolic compounds and the organic acids used as references were obtained from the following sources: oxalic, citric, fumaric, L(–)malic, (–)shikimic and DL-tartaric acids, quercetin and quercetin-3-*O*-glucoside from Sigma–Aldrich (Steinheim, Germany); (–)-epicatechin, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutoside and quercetin-3-*O*-rutoside from Extrasynthèse (Genay, France).

2.2. Procedures

2.2.1. Grape samples

Samples of different varieties of white “Vinho Verde” grapes were collected in different locations and vineyards: Famalicão (one vineyard), Felgueiras (one vineyard), Monção (three vineyards: Quinta de Menanços, Quinta de Moreira and Quinta de Ceivães) and Ponte de Lima (two vineyards: Quinta de Barreiros and Quinta da Facha) in September of 2005. The varieties under study were: Alvarinho, Asal Branco, Avesso, Batoca, Douradinha, Esganoso Castelo Paiva, Loureiro, Pedernã, Rabigato and Trajadura (Table 1). After harvest, the entire grapes were stored at –20 °C and freeze-dried in a Labconco 4.5 apparatus (Kansas City, MO).

2.2.2. Extraction and solid-phase extraction (SPE)

The solid-phase extraction columns used were Chromabond C18 non-encapped (NEC) columns (50 µm particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) from Macherey-Nagel (Düren, Germany).

Table 1
Variety and origin of the “Vinho Verde” white grape samples studied

Observation	Identification	Variety	Geographical origin ^a
1	AlvCei	Alvarinho	Monção (Ceivães)
2	AlvFel	Alvarinho	Felgueiras
3	AlvMen	Alvarinho	Monção (Menanços)
4	AlvMor	Alvarinho	Monção (Moreira)
5	AsFel	Asal Branco	Felgueiras
6	AvFel	Avesso	Felgueiras
7	BatFel	Batoca	Felgueiras
8	DouFel	Douradinha	Felgueiras
9	EsgFel	Esganoso Castelo de Paiva	Felgueiras
10	LouQBa	Loureiro	Ponte da Lima (Quinta Barreiros)
11	LouQFa	Loureiro	Ponte da Lima (Quinta Facha)
12	PedFam	Pedernã	Famalicao
13	PedFel	Pedernã	Felgueiras
14	PedQBa	Pedernã	Ponte da Lima (Quinta Barreiros)
15	PedQFa	Pedernã	Ponte da Lima (Quinta Facha)
16	RabFel	Rabigato	Felgueiras
17	TraFam	Trajadura	Famalicao
18	TraFel	Trajadura	Felgueiras
19	TraQBa	Trajadura	Ponte da Lima (Quinta Barreiros)
20	TraQFa	Trajadura	Ponte da Lima (Quinta Facha)

^a When grapes come from different locations in the same geographical origin, it is noted in brackets.

The experimental procedure SL-SPE was described in a previous work [24]. Each sample (1 g) was accurately weighed and mixed with aqueous HCl (pH 2) containing 5% MeOH (5 × 50 mL) at 40 °C, in an ultrasonic bath during 20 min for each solvent fraction. The extracts were filtered under vacuum and combined. The aqueous solution was then passed through a Chromabond C18 (NEC) column, previously conditioned with 30 mL of MeOH and 70 mL of aqueous HCl (pH 2). The aqueous extract, containing the organic acids, was evaporated until dryness under reduced pressure (30 °C) and redissolved in 0.01N sulphuric acid (1 mL). Twenty microlitres of the redissolved extract were analysed by HPLC-UV.

After the elution of organic acids and other polar compounds with the aqueous solvent, the retained phenolic fraction was eluted with 150 mL of ethanol. The extracts were taken to dryness under reduced pressure (30 °C) and redissolved in MeOH (1 mL). This methanolic solution (20 µL) was analysed by HPLC-DAD.

2.2.3. Alkaline hydrolysis of the methanolic extract obtained by SL-SPE

Four millilitres of 2N NaOH were added to 0.4 mL of methanolic extract obtained as previously mentioned. The solution was kept in the dark for 4 h, acidified with HCl and passed through a C18 Bond Elut cartridge, preconditioned with MeOH and 2N HCl. The phenolic compounds were eluted with MeOH. This solution was taken to dryness under reduced pressure (30 °C), dissolved in 1 mL of MeOH, and 20 µL were analysed by HPLC.

2.2.4. Matrix solid-phase dispersion (MSPD)

The MSPD grade Isolute sorbents, MSPD C18 non-endcapped and MSPD C18 endcapped (EC) (mean particle size: 40–70 µm, average pore size: 60 Å) were from International Sorbent Technology Ltd. (Hengoed Mid Glam, UK). C18 EC

sorbents present a structure of C18 silane and a trimethyl silyl group, covalently bonded to the surface of the silica particle, which reduce the polar secondary interactions associated with surface silanol groups. These columns exhibit non-polar retention mechanism. C18 NEC sorbents have a structure of only C18 silane covalently bonded to the surface of the silica particle, which provide additional polar interactions associated with surface silanol groups. These columns exhibit non-polar, polar and cation exchange retention mechanisms.

1.0 g portions of sample were weighted, mixed and blended carefully with 2.0 g of C18 sorbent in a mortar in order to obtain a mixed stationary phase. Then, the mixture was introduced into a 15 mL syringe body and packed using a syringe plunger.

Aqueous HCl (pH 2) or aqueous HCl (pH 2) containing 5% MeOH was passed through the mixture (at vacuum) to recover the organic acid fraction. This aqueous extract was evaporated until dryness under reduced pressure (30 °C), and the residue was redissolved in 1 mL of 0.01N sulphuric acid and analysed by HPLC-UV (20 µL).

After the elution of organic acids and other polar compounds with the aqueous solvent, the retained phenolic fraction was eluted with ethanol. This extract was taken to dryness under reduced pressure (30 °C), redissolved in MeOH (1 mL), and 20 µL were analysed by HPLC-DAD.

2.2.5. HPLC-UV analysis of organic acids

The chromatographic experiments were performed on an analytical HPLC unit (Gilson) as previously reported in Ref. [27]. The compounds were separated using an ion exclusion Nucleogel Ion 300 OA (300 mm × 7.7 mm) column, in conjunction with a column heating device at 30 °C. Elution was carried out at a solvent flow rate of 0.2 mL min⁻¹, isocratically, with 0.01N sulphuric acid as the mobile phase. Detection was performed with a UV detector set at 214 nm. Each compound was identified comparing its retention time with the corresponding peak in

a standard solution. Organic acids quantification was achieved using a calibration plot of external standard at 214 nm for all compounds.

2.2.6. HPLC-DAD analysis of phenolic compounds

The extracts were analysed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column (25.0 cm × 0.46 cm; 5 µm particle size Waters, Milford, MA, USA) [27]. The solvent system used was a gradient of water/formic acid (19:1) (A) and MeOH (B), starting with 5% MeOH, followed by gradient steps of 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min and 100% B at 60 min, at a solvent flow rate of 0.9 mL min⁻¹. Detection was done using a Gilson diode array detector. The compounds quantified in each sample were identified comparing their retention times and UV–vis spectra in the 200–400 nm range with individual standards. Phenolic compounds quantification was achieved using a calibration plot of external standard at 350 nm for all compounds, except epicatechin, for which the calibration plot was at 280 nm. Quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside were quantified together as quercetin-3-*O*-glucoside. Kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside were quantified together as kaempferol-3-*O*-rutinoside.

2.3. Statistical analysis

2.3.1. One-way analysis of variance (ANOVA)

Significant differences among the 10 white “Vinho Verde” grape varieties for each of the compounds were determined by ANOVA using a SPSS Program (version 14.0). Levene’s test for homogeneity of variance was used to assess the validity of the ANOVA analysis. When variance homogeneity was not acceptable, the Welch test, a one-way robust test of equality of means, was employed instead of ANOVA.

2.3.2. Principal component analysis (PCA) and linear discriminant analysis (LDA)

PCA and LDA were performed by Statgraphics. PCA is a tool for data exploration which allows the reduction of the dimensionality of data facilitating the analysis of intersample relationships. New variables, so called principal components, are obtained to explain the greater part of total variance with a minimum of information loss [28,29]. LDA is employed as a classification tool to discriminate between two or more groups of samples.

PCA was performed separately for each chemical parameter studied (phenolic and organic acid profiles) and also for the global data. LDA was performed for the global data.

3. Results and discussion

3.1. Identification of compounds

White “Vinho Verde” grapes showed a phenolic profile composed by 10 identified phenolic compounds: caftaric acid,

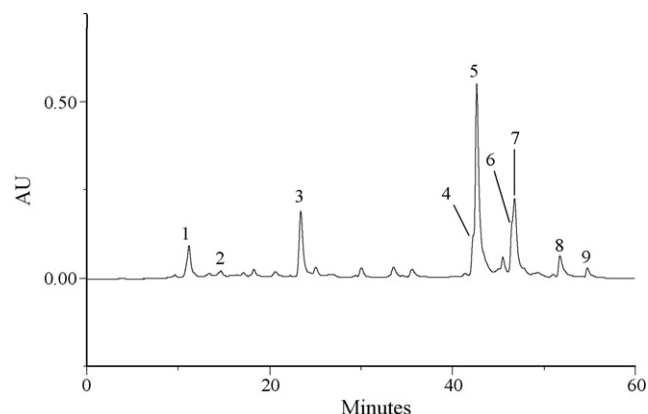


Fig. 1. HPLC chromatogram of phenolic compounds of a sample of white “Vinho Verde” grapes (AlvMen) at 350 nm. Peak identities: (1) caftaric acid; (2) coutaric acid; (3) fertaric acid; (4) quercetin-3-*O*-glucoside; (5) quercetin-3-*O*-rutinoside; (6) kaempferol-3-*O*-glucoside; (7) kaempferol-3-*O*-rutinoside; (8) quercetin; (9) kaempferol.

coutaric acid, fertaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, quercetin, kaempferol and epicatechin. The HPLC chromatogram obtained at 350 nm is shown in Fig. 1.

Quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin, kaempferol and epicatechin were identified by comparing their retention times and UV–vis spectra in the 200–400 nm range with individual standards. Other HPLC conditions were tried for the separation of coeluting compounds (pairs 4/5 and 6/7), namely different gradients and columns, but we decided for those described in Section 2, which allowed a better separation and quantification of the aglycons. Nevertheless, the compounds of each pair are structurally related: compounds 4 and 5 are both glycosidic derivatives of quercetin, while compounds 6 and 7 are kaempferol glycosides. In addition, the aglycone is the molecule’s portion responsible for the UV–vis absorption spectrum of each compound. So, the quantification of the pair as a whole does not introduce great alterations in the quantitative information of the class of compounds in each sample.

Caftaric (Rt 11.2 min), coutaric (Rt 14.7 min) and fertaric (Rt 23.4 min) acids were identified after a chromatographic analysis under the conditions previously used in our laboratory [22] for an extract of white “Vinho Verde” grapes obtained by SL-SPE. Alkaline hydrolysis of this extract showed the absence of the chromatographic peaks corresponding to coutaric, caftaric and fertaric acids and the presence of *p*-coumaric, caffeic and ferulic acids, which confirmed the identity of these compounds. However, these compounds could not be quantified due to their high instability in the extracts.

The identified organic acids were oxalic, citric, tartaric, malic, shikimic and fumaric acids. The chromatographic peak between tartaric and malic acids was identified as fructose. The HPLC chromatogram obtained for organic acids is shown in Fig. 2.

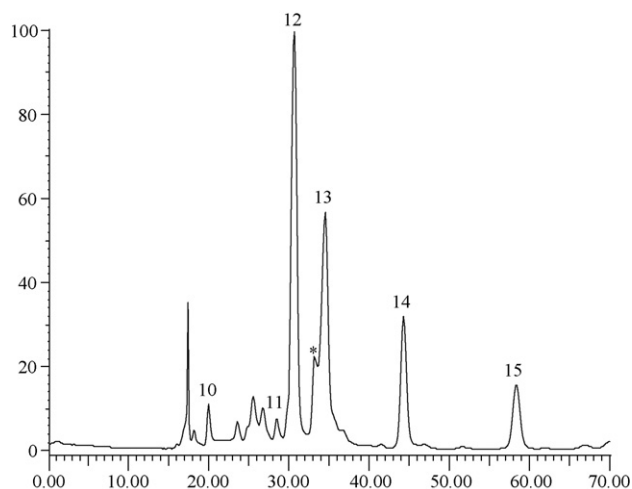


Fig. 2. HPLC chromatogram of the organic acids of a sample of white “Vinho Verde” grapes (BatFel) at 214 nm. Peak identities: (10) oxalic acid; (11) citric acid; (12) tartaric acid; (13) malic acid; (14) shikimic acid; (15) fumaric acid; (*) fructose.

3.2. MSPD versus SL-SPE for white grapes

The SL-SPE method previously developed [24] to extract phenolic compounds and organic acids from white grapes, consisted of a first simultaneous extraction of both types of compounds using aqueous HCl (pH 2) with 5% MeOH in ultrasonic bath and a second step of clean-up, where the organic acids and phenolic compounds were separated using a C18 NEC column. When the acidic aqueous extract was passed through the column, organic acids were eluted while phenolic compounds were retained on the C18 NEC sorbent and eluted later with ethanol. Finally, both fractions were analysed separately.

In this work, the potential use of MSPD to separately extract organic acids and phenolic compounds from white grapes was tested. Since in MSPD the entire sample is blended into the column, it is theoretically possible to perform the sequential elution of the sample to isolate several classes of compounds from it [1].

Some assays were planned based on the results previously obtained during the development of the SL-SPE method. The type of solvent and sorbent, have shown to be two of the most influential variables for the extraction and the separation of the analytes, respectively. C18 sorbent was chosen because of its wide use for MSPD [3,5] and, specifically, to extract phenolic compounds from plants [6–8]. Although C18 non-encapped and encapped did not show to influence the extraction of phenolic compounds from medicinal plants [6], both types of sorbents, or their combination, were tested because in our previous work using SL-SPE, they showed a different effect in the extraction of organic acids and phenolic compounds.

The solvents chosen to sequentially elute the analytes were those that have shown to be efficient to separate the compounds with the C18 SPE column: aqueous HCl (pH 2) with 5% MeOH was tested as first eluent to recover the organic acids and ethanol as second eluent to recover the phenolic compounds. In the development of the SL-SPE procedure [24], MeOH was added

Table 2
Experimental conditions employed for MSPD and SL-SPE

Type of sorbent	1-MSPD	2-MSPD	3-MSPD	4-MSPD	5-MSPD	6-MSPD	SL-SPE
First eluent ^a	NEC 150 mL aqueous HCl (pH 2) with 5% MeOH	EC 150 mL aqueous HCl (pH 2) with 5% MeOH	NEC 150 mL aqueous HCl (pH 2)	EC 150 mL aqueous HCl (pH 2)	50% EC/50% NEC 150 mL aqueous HCl (pH 2)	NEC 150 mL aqueous HCl (pH 2) with 5% MeOH	NEC 150 mL aqueous HCl (pH 2) with 5% MeOH
Second eluent	70 mL ethanol	70 mL ethanol	70 mL ethanol	70 mL ethanol	70 mL ethanol	150 mL ethanol	150 mL ethanol

NEC: non-encapped; EC: encapped; HCl: hydrochloric acid; MeOH: methanol.
^a pH was modified with HCl.

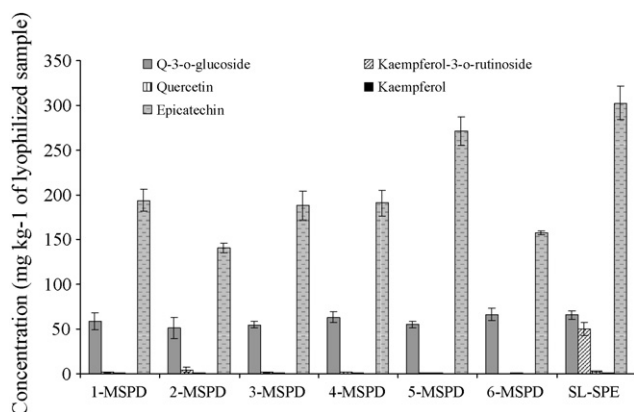


Fig. 3. Comparison between MSPD and SL-SPE for the extraction of phenolic compounds from a sample of white grapes. Experimental conditions of each assay according to Table 2.

to aqueous HCl (pH 2) to improve the recovery of the analytes. Therefore, the addition of MeOH to the acid water was also tested.

In literature, sample/sorbent ratios typically range from 1:1 to 1:4 [5]; therefore a ratio of 1 g sample:2 g of sorbent was chosen.

For these preliminary assays, large volumes of solvents (150 mL of aqueous HCl (pH 2) and 70 mL of ethanol) were employed to guarantee the complete elution of the analytes. This precaution was taken because although small volumes are usually reported to be sufficient for MSPD procedures, the study carried out by Ziaková et al. [6] showed that 20–25 mL of some eluents were necessary to get quantitative recoveries of some phenolic acids.

Considering all these variables, five different assays (1-MSPD to 5-MSPD) were carried out. One more assay (6-MSPD) was planned using the same sorbent, volume and type of eluents as in the SL-SPE method, although these volumes are much higher than the ones usually employed for MSPD. Their experimental conditions are shown in Table 2.

Finally, the grapes were also analysed by the SL-SPE method (Table 2) to compare the results obtained with both techniques. The obtained results as mean of three determinations are shown in Figs. 3 and 4 for phenolic compounds and organic acids,

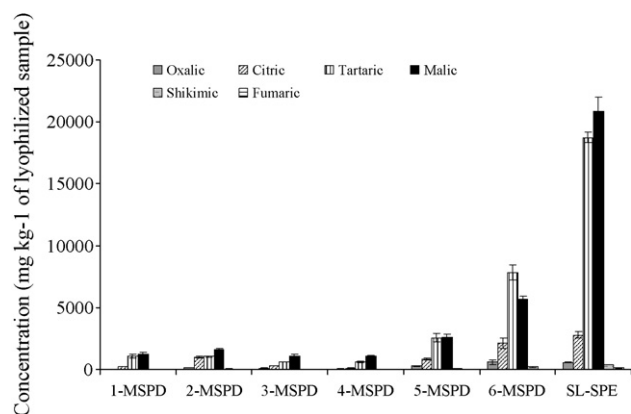


Fig. 4. Comparison between MSPD and SL-SPE for the extraction of organic acids from a sample of white grapes. Experimental conditions of each assay according to Table 2.

respectively. Results are given as concentration of each compound in the lyophilized sample.

3.2.1. Phenolic compounds

As can be seen in Fig. 3, recoveries obtained for phenolic compounds were, in general, lower for MSPD than for SL-SPE. Only the recoveries obtained for the pair quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside and for epicatechin were similar, in some assays, to those obtained using SL-SPE. So, for the pair quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside, the signal obtained with the assay 6-MSPD was similar to the one obtained with SL-SPE (101%), but a very high volume of solvents (150 mL of ethanol), not usual in MSPD, was necessary. The best results for epicatechin, on using MSPD, were obtained with experiment 5-MSPD when a sorbent obtained mixing 50% NEC C18 and 50% EC C18 was employed. The amount of epicatechin obtained was 90% in relation to SL-SPE.

For the rest of the phenolic compounds the recoveries obtained for MSPD were very poor in comparison to those obtained for SL-SPE. The pair kaempferol-3-*O*-glucoside/kaempferol-3-*O*-rutinoside was not recovered at all in experiment 6-MSPD, even using the highest elution volumes. This indicates that probably these compounds were eluted with the first solvent (aqueous HCl (pH 2)) instead of ethanol. Another assay using 45 mL of aqueous HCl (pH 2) and 70 mL of ethanol was carried out to check this hypothesis (data not shown). As was expected, the amount of kaempferol-3-*O*-glucoside/kaempferol-3-*O*-rutinoside increased, although only 52% of the amount recovered with SL-SPE method was obtained while the recoveries of the other compounds were negatively affected.

Recoveries of quercetin and kaempferol, in all the assays carried out by MSPD, were always much lower than by SL-SPE, with the best recoveries being around only 30%.

3.2.2. Organic acids

As can be seen in Fig. 4, recoveries obtained for organic acids were, in general, much lower for MSPD than for SL-SPE, and much poorer than those obtained for phenolic compounds. The best results with MSPD were obtained with assay 6-MSPD that uses the highest volumes of eluents. In these conditions, the best recoveries were obtained for oxalic and citric acids, for which the obtained area was 103 and 77%, respectively, of the signal obtained with SL-SPE. For the other acids the obtained signal was much lower on using MSPD: 55% for shikimic acid, 42% for tartaric acid, 27% for malic acid and 6% for fumaric acid.

Addition of MeOH to the aqueous HCl (pH 2), or the use of C18 NEC or EC did not show to be very influential, although better results were obtained for some compounds (epicatechin and organic acids) when both sorbents were combined 1:1 (5-MSPD). The best results for MSPD were obtained using the highest volume of eluent, which is considerably higher than usually employed with this technique.

In conclusion, MSPD can be useful for the qualitative analysis of phenolic compounds and organic acids from white grapes but, for the quantitative analysis, SL-SPE has shown to have higher extractive capacity, especially for organic acids. This much lower extractive capacity of MSPD for organic acids

was probably related to the high content in sugars of the white grapes that coelute with them making their complete extraction difficult. On the other hand, results obtained for phenolic compounds agree with those obtained by Xiao et al. [7] who got lower extraction levels of glycoside phenolic compounds with MSPD than with other conventional techniques (Soxhlet, ultrasonic).

In a more specific point of view, with the exception of oxalic acid, the recuperation of organic acids was very small. For phenolic compounds, only quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and epicatechin presented similar recuperation values for both methods, but for the remaining ones it was very low and kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside even disappeared with MSPD.

Considering these results, the previously developed SL-SPE was chosen to analyse white “Vinho Verde” grapes. This analytical method was validated in a previous work [24]. The analytical method was precise since relative standard deviation (R.S.D.) was 2.2–9.1% for organic acids and 2.8–15% for phenolic compounds. Limits of detection were between 1.5 and 86 mg kg⁻¹ for organic acids and 0.55 and 3.5 mg kg⁻¹ for phenolic compounds while limits of quantification ranged between 5.1 and 286, and 1.8 and 12 mg kg⁻¹ for organic acids and phenolic compounds, respectively. Recovery values of the method were between 72 and 95% for phenolic compounds and between 72 and 103% for organic acids which demonstrates the effectiveness of the extraction.

Under the described assay conditions, a linear relationship between the concentration of each compound and their UV absorbance was obtained, at 214 nm for oxalic, citric, L-tartaric, L-malic, shikimic and fumaric acids, at 280 nm

for epicatechin and at 350 nm for quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin and kaempferol. The correlation coefficient for the standard curves invariably exceeded 0.99 for all studied compounds. The regression equations for oxalic, citric, L-tartaric, L-malic, shikimic and fumaric acids were $y = 1.5 \times 10^3 x + 5.5 \times 10^4$, $y = 2.4 \times 10^2 x + 7.0 \times 10^3$, $y = 8.6 \times 10^2 x + 7.8 \times 10^3$, $y = 2.5 \times 10^2 x - 1.5 \times 10^4$, $y = 1.3 \times 10^4 x + 2.6 \times 10^4$, $y = 2.6 \times 10^4 x + 1.6 \times 10^4$, respectively. The regression equations for epicatechin, quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin and kaempferol were $y = 3.3 \times 10^5 x - 8.1 \times 10^4$, $y = 1.0 \times 10^6 x - 3.6 \times 10^5$, $y = 8.4 \times 10^5 x - 7.4 \times 10^5$, $y = 1.2 \times 10^6 x - 4.8 \times 10^5$, $y = 1.5 \times 10^6 x + 1.1 \times 10^5$.

The repeatability and reproducibility of the chromatographic method was evaluated by measuring the peak chromatographic area of each compound six times on the same standard solution in the same day and in different days, respectively. The chromatographic method is precise: the repeatability study showed relative standard deviation (R.S.D.) between 1.6 and 4.4% for organic acids, and between 3.5 and 4.7% for phenolic compounds. The reproducibility study showed that R.S.D. ranged from 6.7 to 9.9% for organic acids and from 3.8 to 4.9 for phenolic compounds.

3.3. Analysis of white “Vinho Verde” grapes

3.3.1. Phenolic compounds

Seven phenolic compounds were quantified in white “Vinho Verde” grapes: quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, quercetin, kaempferol and epicatechin (Table 3).

Table 3
Phenolic composition of white “Vinho Verde” grape samples (mg kg⁻¹ of lyophilized sample)

		Q3gluc (Rt 42.2) + Q3rutin (Rt 42.7)		Kaemp3rutin (Rt 46.5) + Kaemp3gluc (Rt 46.8)		Quercetin (Rt 51.8)		Kaempferol (Rt 54.8)		Epicatechin (Rt 18.6)		Total
		Mean	S	Mean	S	Mean	S	Mean	S	Mean	S	
1	AlvCei	247	2.4	65	0.86	6.8	0.023	1.8	0.017	91	4.7	411
2	AlvFel	228	3.4	308	5.2	16	1.3	3.5	0.43	350	6.3	906
3	AlvMen	281	13	138	9.4	29	3.8	5.4	0.87	106	8.3	559
4	AlvMor	278	5.8	209	2.2	28	2.1	5.9	0.42	175	5.6	695
5	AsFel	234	3.3	76	6.3	4.9	0.55	nq		97	6.8	412
6	AvFel	92	1.2	79	2.1	4.9	0.14	nq		281	1.8	457
7	BatFel	44	0.26	52	2.0	nq		nq		331	9.8	426
8	DouFel	439	9.1	427	29	11	1.3	nq		174	9.3	1051
9	EsgFel	79	4.8	58	3.0	nd		nd		152	26	290
10	LouQBa	69	6.9	18	0.88	nq		nd		644	59	731
11	LouQFa	171	7.0	124	1.3	10	0.61	2.0	0.039	158	0.086	465
12	PedFam	191	13	76	2.9	nq		nq		174	4.6	441
13	PedFel	100	7.3	45	6.5	10	0.92	1.8	0.11	116	24	273
14	PedQBa	87	6.6	12	1.2	nd		nd		450	0.90	549
15	PedQFa	288	8.0	145	22	9.5	0.85	nq		289	18	732
16	RabFel	395	22	159	17	16	2.4	2.2	0.25	nd		573
17	TraFam	121	3.2	17	1.3	nd		nd		274	5.8	412
18	TraFel	127	11	52	2.5	9.3	0.16	nq		258	16	446
19	TraQBa	74	1.7	6.5	0.92	5.5	0.19	nq		263	7.7	349
20	TraQFa	149	10	58	2.6	50	2.2	4.0	0.42	179	2.2	440

Q3gluc: quercetin-3-*O*-glucoside; Q3rutin: quercetin-3-*O*-rutinoside; Kaemp3rutin: Kaempferol-3-*O*-rutinoside; Kaemp3gluc: Kaempferol-3-*O*-glucoside. Results are expressed as mean of three determinations and standard deviation. nd = not detected (concentration sample < detection limit). nq = not quantified (detection limit < concentration sample < quantification limit).

Table 4

Eigenvalues, percentage of variance and cumulative percentage explained by the most important principal components for each chemical group under study

Group	Principal component	Eigenvalue	Percent of variance (%)	Cumulative percentage (%)
Phenolic compounds	1	2.75	55.1	55.1
	2	1.14	22.8	77.9
Organic acids	1	2.12	35.3	35.3
	2	1.58	26.4	61.6
	3	1.06	17.7	79.4
Global data	1	3.46	31.4	31.4
	2	2.19	19.9	51.4
	3	1.64	15.0	66.4
	4	1.14	10.3	76.7

The most abundant phenolic compounds found in all the studied grapes were epicatechin (39–82%) or the pair quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside (37–69%).

Epicatechin was the most abundant phenolic compound for the grapes of the varieties Avesso, Batoca, Esganoso, Castelo de Paiva and Trajadura while quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside was the most abundant for Alvarinho (except those from Felgueiras), Asal Branco, Douradinha and Rabigato. For the varieties Loureiro and Pedernã the amounts of epicatechin and quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside were similar, except for grapes from Quinta de Barreiros, which content of epicatechin was considerably higher.

Kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside appeared in all samples in lower proportion (1.9–41%). Quercetin and, in particular, kaempferol were found in such low amounts, that they could not be quantified in most samples.

This phenolic profile with high content of glycosylflavonols or epicatechin and low levels of quercetin and kaempferol agrees with results obtained for other grapes. Quercetin-3-*O*-glucoside was the most abundant non-coloured phenolic compound in different red and white varieties of grapes studied by Cantos et al. [9] (who also quantified it together with quercetin-3-*O*-rutinoside), and Amico et al. [11] and it was the second most abundant in skin of white Weisser Riesling grapes (where quercetin-3-*O*-glucuronide was the most abundant) [12]. On the other hand, epicatechin was the most abundant compound in seeds of muscadine grapes [10] and in some red grapes [30]. Kaempferol-3-*O*-glucoside was detected in low quantity in white grapes [9] and red [11,13] and in higher concentration in the skin of Weisser Riesling grapes [12].

Quercetin or kaempferol were not detected in other grape samples such as in Weisser Riesling [12], or were found in only low quantities in muscadine grapes [10].

ANOVA showed significant differences between the phenolic profiles among samples of different varieties, in terms of kaempferol-3-*O*-rutinoside/kaempferol-3-*O*-glucoside ($p < 0.05$). Similarly, the Welch test showed differences in terms of quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside ($p < 0.05$). ANOVA also showed the influence of geographical origin on kaempferol ($p < 0.05$).

3.3.1.1. Principal component analysis. The differences among the samples, according to the variety and origin, were

emphasized by the PCA. For phenolic compounds, PCA yields two principal components explaining 77.9% of the total variance in the data (Table 4). Fig. 5a shows the corresponding loading plots that establish the relative importance of each variable and it is therefore useful for the study of relations among the variables and grapes. The first PC, which explains 55.1% of the variance, correlates positively with epicatechin and negatively with the rest of the phenolic compounds. The second PC correlates positively

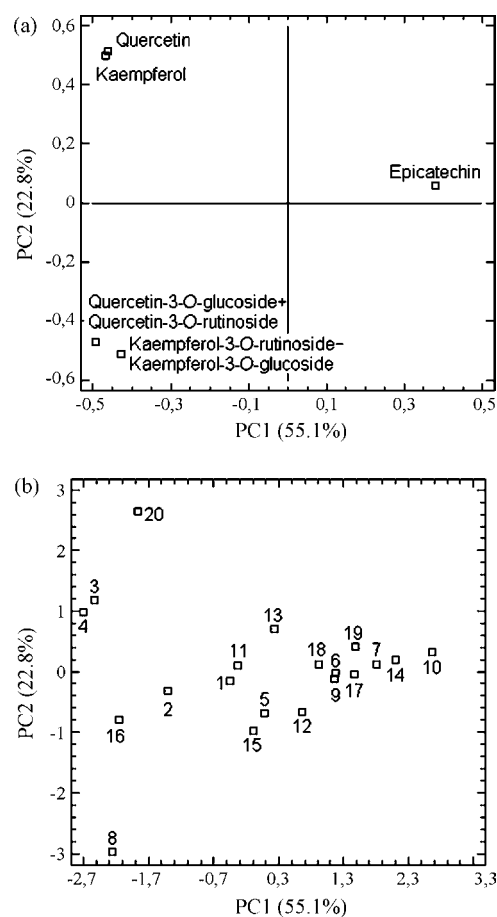


Fig. 5. PC1 vs. PC2 scatter plot of the main sources of variability between the “Vinho Verde” white grapes (a) relation between the phenolic compounds (loadings); (b) distinction between the samples (scores). Observations identities are listed in Table 1.

with epicatechin, kaempferol and quercetin, and negatively with kaempferol-3-*O*-rutinoside/kaempferol-3-*O*-glucoside and quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside.

As can be seen in Fig. 5b phenolic composition was strongly related to both origin and variety of the grapes: the grapes of the varieties Douradinha, Rabigato and Alvarinho (observations 1, 2, 3, 4, 8 and 16) appeared in the third/fourth quadrants, separated from the rest of the samples, due to their higher content of glycosyl flavonols and flavonols. The scores of the rest of the samples fall along the second PC. These last grapes belonged to the varieties Loureiro, Asal Branco, Pedernã, Esganoso Castelo de Paiva, Trajadura, Avesso and Batoca. In general, these grapes show higher content in epicatechin and lower content in flavonols.

However, differences among the varieties were observed according to their different geographical origin. In this way, for the first group, grapes from Felgueiras (observations 2, 8 and 16) showed the highest content in the glycosyl flavonols while the rest of samples (1, 3 and 4, all Alvarinho) showed lower levels of these compounds. Even the Alvarinho grapes from Monção appeared separated in two subgroups, one with grapes with higher content of quercetin and kaempferol (Menanços and Moreira) and another with lower content (Ceivães) of those compounds. This may be explained because Menanços and Moreira are geographically closer.

In the second group, if only the variety is considered, the samples are strongly mixed. However, it could be observed that samples of different varieties with the same origin appeared quite close in the PC plot, like Trajadura and Pedernã from Famalicão (observations 12 and 17), Avesso, Batoca, Esganoso, Pedernã and Trajadura from Felgueiras (6, 7, 9, 13 and 18) and Trajadura, Loureiro and Pedernã from Quinta de Barreiros (10, 14 and 19),

showing that both origin and variety influenced the phenolic composition of the studied grapes. It could be noticed again that samples of the same variety (Loureiro and Pedernã) and the same geographical origin (Ponte de Lima) but from different vineyards (observations 11 and 15, 10 and 14) showed differences in their phenolic profiles.

3.3.1.2. Phenolic profile of varieties with the same origin. If only the data of grapes from Felgueiras are considered (Table 3), the phenolic profile of grapes of nine different varieties with the same origin can be compared. Varieties Douradinha and Alvarinho showed the higher total content in phenolic compounds, followed by Trajadura, Asal Branco, Avesso and Batoca with a similar content, while the lowest levels were found in Esganoso Castelo Paiva and Pedernã. Quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside was the most abundant compound for Asal Branco (57%), Douradinha (42%) and Rabigato (69%), whereas epicatechin was the most abundant for Alvarinho (39%), Avesso (62%), Batoca (78%), Esganoso (52%), Pedernã (43%) and Trajadura (58%), the sum of glycosylflavonols was more abundant for Rabigato (97%) and Douradinha (82%) followed by Asal Branco (75%), while Alvarinho, Avesso, Esganoso, Pedernã, and Trajadura showed lower levels, between 37 and 59%, and Batoca the lowest content (22%). All the varieties showed very low contents in quercetin, with a slightly higher content for Pedernã and Rabigato. Kaempferol was only quantified in Pedernã. If the ratios between the compounds are considered to design the phenolic fingerprint, which can be easily observed drawing the graphs, it can be observed that Rabigato, Douradinha, Alvarinho, Esganoso and Batoca have unique and distinct profiles while those of Pedernã, Trajadura and Avesso show more similar profiles.

Table 5
Organic acids composition of white “Vinho Verde” grape samples (mg kg⁻¹ of lyophilized sample)

		Oxalic (Rt 19.8)		Citric (Rt 28.3)		Tartaric (Rt 30.5)		Malic (Rt 34.4)		Shikimic (Rt 44.2)		Fumaric (Rt 58.2)		Total
		Mean	S	Mean	S	Mean	S	Mean	S	Mean	S	Mean	S	
1	AlvCei	733	10	459	48	3602	30	3288	245	17	2.3	nq		8100
2	AlvFel	1371	64	1167	17	3990	135	5822	345	43	4.9	nd		12392
3	AlvMen	721	83	527	35	3999	215	5505	106	19	1.6	nq.		10771
4	AlvMor	694	42	593	27	2738	397	6061	465	18	0.036	9.1	1.3	10114
5	AsFel	630	63	646	38	3025	12	4960	463	45	0.78	nq		9305
6	AvFel	409	13	507	21	3542	151	4314	537	51	2.0	nq		8822
7	BatFel	363	44	588	50	3395	75	5845	425	101	9.0	7.1	1.0	10299
8	DouFel	543	2.5	650	97	4139	147	4850	72	147	5.2	nq		10329
9	EsgFel	606	13	1110	89	5172	445	6731	556	193	5.7	nq		13811
10	LouQBa	731	31	2014	21	4827	659	11269	161	57	4.8	9.2	0.1	18907
11	LouQFa	417	10	529	99	4433	92	4335	186	18	2.1	8.2	0.16	9740
12	PedFam	465	69	nq		3105	82	3273	258	58	2.2	11	1.3	6911
13	PedFel	228	14	710	80	4017	307	5154	750	99	6.8	nq		10208
14	PedQBa	434	4.1	731	19	3543	27	5340	626	126	4.8	nq		10172
15	PedQFa	422	28	nq		4733	105	1857	105	37	1.7	nq		7050
16	RabFel	231	24	692	106	3015	222	5223	350	158	9.3	nq		9319
17	TraFam	544	28	1515	133	3754	623	4394	791	192	24	9.4	1.0	10410
18	TraFel	500	39	505	146	3158	93	4069	212	118	5.9	nq		8349
19	TraQBa	300	30	711	135	3008	83	8537	1305	188	4.8	12	2.9	12757
20	TraQFa	445	47	977	124	3414	395	3920	371	183	8.3	7.4	0.66	8947

Results are expressed as mean of three determinations and standard deviation. nd = not detected (concentration sample < detection limit). nq = not quantified (detection limit < concentration sample < quantification limit).

3.3.2. Organic acids

The identified organic acids were oxalic, citric, tartaric, malic, shikimic and fumaric acids (Table 5). ANOVA showed significant differences between the organic acids profile among samples of different varieties, in terms of shikimic acid ($p < 0.05$). Welch test showed the influence of geographical origin on shikimic and fumaric acids ($p < 0.05$).

Malic acid was the most abundant compound in most of the samples, followed by tartaric acid. The sum of these two compounds constituted 79–92% of the total amount of the organic acids analysed. The highest percentage of oxalic or citric acids was 11% and shikimic acid was found in the range 0.18–2.0%. The fumaric acid concentration was so low that it could not be quantified in most samples.

3.3.2.1. Principal component analysis. PCA for organic acids yields three principal components with eigenvalues greater than 1 accounting for 79.4% of the total variance in the data (Table 4). Considering that the first two components already account for 61.6% of the total variance, the third one was not considered to simplify the analysis of the results. The first PC, which account for 35.3% of the variance, was correlated positively with all of the compounds, and the second one, which accounts for 26.4%, was correlated positively with malic, shikimic and fumaric acids and negatively with citric, oxalic and tartaric acids (Fig. 6a).

The scores of most of the samples fall along the second PC (Fig. 6b), due to their similar content in malic and tartaric acids. Some samples appeared more separated, for example, Alvarinho grapes appeared in the negative quadrant (observations 1, 2 and 3) due to a higher content in oxalic acid, while Trajadura grapes appeared in the positive quadrant (observations 17, 19 and 20) due to their higher content in citric, fumaric and shikimic acids. A strong mix of the samples from different varieties could also be observed due to the influence of the origin. So, Asal, Avesso, Batoca, Douradinha and Esganoso grapes (observations 5, 6, 7, 8 and 9) all of them from Felgueiras, appeared very close in the plot.

3.3.2.2. Organic acids profile of varieties with the same origin.

If only the data of grapes from Felgueiras are considered, the organic acid profiles of grapes of nine different varieties with the same origin can be compared. The highest content in organic acids was found in the varieties Esganoso de Castelo Paiva and Alvarinho, followed by Batoca, Douradinha and Pedernã, while the lowest content was found in Rabigato, Asal Branco, Aveso and Trajadura. Malic acid was the most abundant compound in all the varieties (47–57%), with the sum of tartaric and malic acids being very similar for all of them (79–90%). Fumaric acid was the organic acid found in lowest amount and could only be quantified in Batoca grapes.

The highest percentage of oxalic acid was found in Alvarinho (11%), followed by Asal branco (6.8%) and Trajadura (6%) while Avesso, Batoca, Douradinha and Esganoso Castelo Paiva, Pedernã and Rabigato showed levels between 2.2 and 5.3%. The highest level of citric acid was found in Alvarinho (9.4%) and Esganoso Castelo Paiva (8%) while the rest of the samples showed levels around 5.7–7.4%.

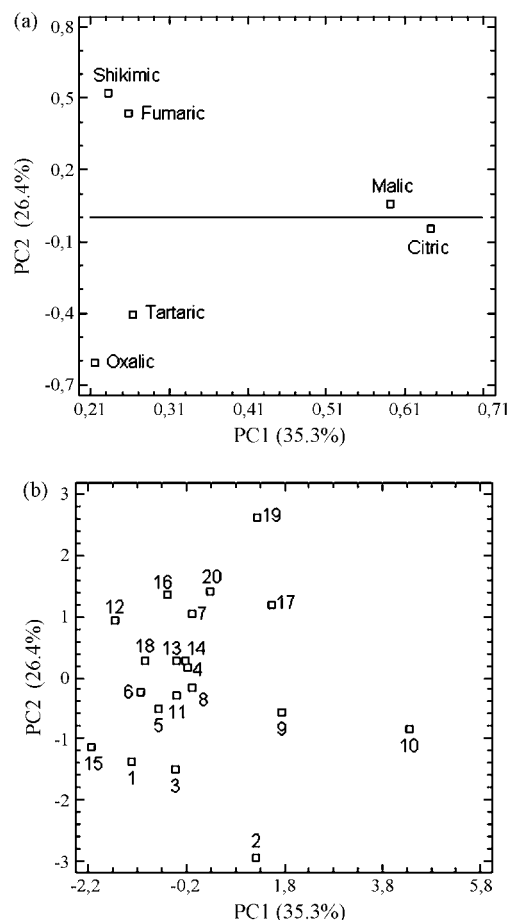


Fig. 6. PC1 vs. PC2 scatter plot of the main sources of variability between the “Vinho Verde” white grapes (a) relation between the organic acids (loadings); (b) distinction between the samples (scores). Observations identities are listed in Table 1.

The highest level of shikimic acid was found in Rabi-gato (1.7%), followed by Douradinha, Esganoso and Trajadura (1.4%) while the rest of the varieties showed levels equal or lower than 1%.

3.3.3. Global analysis

3.3.3.1. Principal component analysis. Fig. 7 presents the PCs of grape composition (phenolic compounds and organic acids). Although the analysis yields four PCs with eigenvalues greater than 1, the scree plot suggested involving two PCs into the model. So, only two PCs were retained to simplify the analysis of the results [31]. The two retained PCs account for 51.4% of the total variance: PC1 31.4% and PC2 19.9%.

PC1 was correlated positively with epicatechin and all of the organic acids except oxalic, and negatively with oxalic acid and flavonols. PC2 was correlated positively with all the compounds, except shikimic acid (Fig. 7a).

Grapes appeared again strongly mixed due to the influence of both origin and variety (Fig. 7b). Samples belonging to the same variety appear similar (Alvarinho, observations 1, 2, 3 and 4; Pedernã, observations 12, 13, 14 and 15; Trajadura, observations 17, 18, 19 and 20) but show important variations due to the dif-

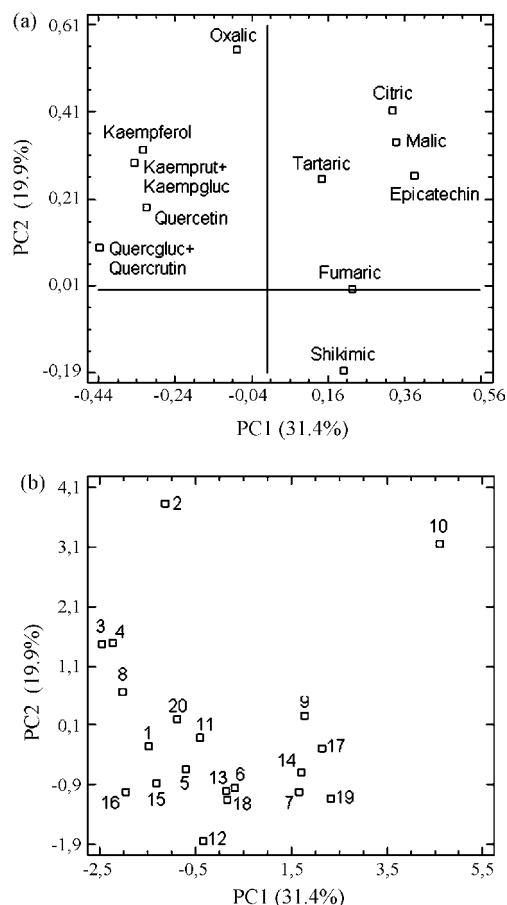


Fig. 7. PC1 vs. PC2 scatter plot of the main sources of variability between the “Vinho Verde” white grapes (a) relation between the phenolic and organic acids (loadings); (b) distinction between the samples (scores). Observations identities are listed in Table 1.

ferent origin. Samples of different variety but of same origin also look similar (different varieties from Felgueiras, observations 5, 6, 7, 8, 9, 13, 16 and 18).

3.3.3.2. Linear discriminant analysis. After PCA, a stepwise linear discriminant analysis was applied in order to obtain the most useful variables in the differentiation between white “Vinho Verde” grapes using the same data set as for PCA. Forward selection method was employed using F to enter and to remove 4 (default value used in the Statgraphics software). A 5% significance level was predefined. Those variables which surpassed the predefined significance limit were excluded.

For differentiation of grapes according the variety, two variables had the highest discrimination power at the 5% significance level: quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside and shikimic acid. The percentage of correctly classified grapes with these variables was 70% (Table 6). Samples of the Alvarinho, Pedernã, Loureiro and Trajadura varieties were incorrectly classified by LDA as being of the Avesso, Asal Branco or Esganoso varieties. As only one sample of each of the Avesso, Asal Branco or Esganoso varieties was studied, this clearly shows that more samples need to be studied to improve the classification.

Table 6

Stepwise discriminant analysis of phenolics and organic acids-classification^a according to variety

Observed group	Number of samples	Correct classification (%)
Alvarinho	4	75
Asal Branco	1	100
Avesso	1	100
Batoca	1	100
Douradinha	1	100
Esganoso Castelo de Paiva	1	100
Loureiro	2	50
Pedernã	4	75
Rabigato	1	100
Trajadura	4	25
Total	20	70

^a Variables: Quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside and shikimic acid.

Table 7

Stepwise discriminant analysis of phenolics and organic acids-classification^a according to geographical origin

	Number of samples	Correct classification (%)
Famalicão	2	100
Felgueiras	9	78
Monção	3	67
Ponte da Lima	6	17
Total	20	60

^a Variables: kaempferol and fumaric acid.

For differentiation of grapes according to geographic origin (Famalicão, Felgueiras, Monção, Ponte da Lima) two variables had the highest discrimination power at the 5% significance level: kaempferol and fumaric acid. The percentage of correctly classified grapes with these variables was 60% (Table 7). But when a more detailed origin was introduced, Menanços, Moreira, Ceivães for the samples from Monção and Quinta de Barreiros and Quinta da Facha for the samples from Ponte da Lima, the correctly classified grapes were 100%. This result shows the importance of the origin in the composition of the grapes.

4. Conclusions

In summary, comparing MSPD and SL-SPE for extracting organic acids and phenolic compounds from white grapes, MSPD showed to be a simpler and faster technique but a complete extraction of all the compounds studied could not be achieved, especially for organic acids. So, although MSPD could be a useful tool to identify the phenolic and organic acids composition of white grapes, SL-SPE was preferred to get quantitative recoveries of these compounds.

Phenolic compounds and organic acids profiles were obtained for white “Vinho Verde” grapes of 10 different varieties grown in four different geographical locations. The most abundant compounds found were quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside and epicatechin, and tartaric and malic acids.

Analysis of the results by using multivariate techniques showed the high influence of both variety and origin in their composition. PCA of phenolic compounds accounted for the highest variability (77.9%) with two PCs, enabling characterization of the varieties of samples according their higher content in epicatechin or flavonols. One hundred and 70% of recognition ability was obtained by the application of step-wise linear discriminant analysis (SLDA) using origin or variety as classification variable, respectively. Considering the strong effect of origin on the results, another study with a higher number of samples of each variety and origin would be interesting to improve the differentiation of grapes according to their variety.

The study of phenolic compounds and organic acids profiles using PCA accounted for the percentages of variance in the data set similar to those obtained by other authors that compared grapes employing variables such as physicochemical measurements (70.1%) [32] or carotenoids (85%) [33], or wines, employing variables such as amines and organic acids (77–84%) [34], volatile compounds (67–91%) [35] and free amino acids and biogenic amines (77%) [36]. Therefore, phenolic compounds and organic acids have been shown to be useful variables to characterize white “Vinho Verde” grapes.

Acknowledgements

The authors would like to thank Eng. José Manuel Afonso, Eng. Óscar Pereira and Eng. Leão from the Direcção Regional de Agricultura de Entre-Douro-e-Minho (Portugal), for supplying the samples.

M.S. Dopico-García is indebted to the Fundação para a Ciência e a Tecnologia (SFRH/BPD/21757/2005) for her grant. A. Jagodzińska and J. Klepczyńska are grateful to European Union Erasmus/Sócrates for their grants.

References

- [1] S.A. Barker, J. Chromatogr. A 880 (2000) 63–68.
- [2] S.A. Barker, J. Chromatogr. A 885 (2000) 115–127.
- [3] S.A. Barker, J. Biochem. Biophys. Methods 70 (2007) 151–162.
- [4] E. de Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A 1112 (2006) 31–63.
- [5] E.M. Kristenson, L. Ramos, U.A.Th. Brinkman, TrAC Trends Anal. Chem. 25 (2006) 96–111.
- [6] A. Ziaiová, E. Brandsteterová, E. Blahová, J. Chromatogr. A 983 (2003) 271–275.
- [7] H.B. Xiao, M. Krucker, K. Albert, X.M. Liang, J. Chromatogr. A 1032 (2004) 117–124.
- [8] E. de Rijke, F. de Kanter, F. Ariese, U.A.Th. Brinkman, C. Gooijer, J. Sep. Sci. 27 (2004) 1061–1070.
- [9] E. Cantos, J.C. Espín, F.A. Tomás-Barberán, J. Agric. Food Chem. 50 (2002) 5691–5696.
- [10] E. Pastrana-Bonilla, C.C. Akoh, S. Sellappan, G. Krewer, J. Agric. Food Chem. 51 (2003) 5497–5503.
- [11] V. Amico, E.M. Napoli, A. Renda, G. Ruberto, C. Spatafora, C. Tringali, Food Chem. 88 (2004) 599–607.
- [12] D. Kammerer, A. Claus, R. Carle, A. Schieber, J. Agric. Food Chem. 52 (2004) 4360–4367.
- [13] R. Rodríguez Montealegre, R. Romero Peces, J.L. Chacón Vozmediano, J. Martínez Gascuña, E. García Romero, J. Food Comp. Anal. 19 (2006) 687–693.
- [14] P.B. Andrade, R.M. Seabra, M.A. Ferreira, F. Ferreres, C. Garcia-Viguera, Z. Lebensm. Unters. Forsh A 206 (1998) 161–164.
- [15] B. Halliwell, in: C. Rice-Evans (Ed.), Wake up to Flavonoids, International Congress and Symposium Series 226, Royal Society of Medicine Press, UK, 2000, pp. 13–23.
- [16] A. Van de Wiel, P.H.M. Van Golde, H.Ch. Hart, Eur. J. Int. Med. 12 (2001) 484–489.
- [17] P. Zafrilla, J. Morillas, J. Mulero, J.M. Cayuela, A. Martínez-Cachá, F. Pardo, J.M. López Nicolás, J. Agric. Food Chem. 51 (2003) 4694–4700.
- [18] R.S. Jackson, Wine Science—Principles, Practice, Perception, second ed., Academic Press, New York, 1994, pp. 544–577.
- [19] J.M. Oliveira, M. Faria, F. Sá, F. Barros, I.M. Araújo, Anal. Chim. Acta 563 (2006) 300–309.
- [20] N. Moreira, F. Mendes, O. Pereira, P. Guedes de Pinho, T. Hogg, I. Vasconcelos, Anal. Chim. Acta 458 (2002) 157–167.
- [21] J.J. Castillo-Sánchez, J.C. Mejuto, J. Garrido, S. García-Falcón, Food Chem. 97 (2006) 130–136.
- [22] P.B. Andrade, B.M. Oliveira, R.M. Seabra, M.A. Ferreira, F. Ferreres, C. Garcia-Viguera, Electrophoresis 22 (2001) 1568–1572.
- [23] J.M. Oliveira, I.M. Araújo, O.M. Pereira, J.S. Maia, A.J. Amaral, M.O. Maia, Anal. Chim. Acta 513 (2004) 269–275.
- [24] M.S. Dopico-García, P. Valentão, L. Guerra, P.B. Andrade, R.M. Seabra, Anal. Chim. Acta 583 (2007) 15–22.
- [25] Comissão de viticultura da região dos Vinhos Verdes, <http://www.vinhoverde.pt/>.
- [26] M.D. Loureiro, M.C. Martinez, J.M. Boursiquot, P. This, J. Am. Soc. Hort. Sci. 123 (1998) 842–848.
- [27] P. Valentão, R.M. Seabra, G. Lopes, L.R. Silva, V. Martins, M.E. Trujillo, E. Velázquez, P.B. Andrade, Food Chem. 100 (2007) 64–70.
- [28] J.M. Millar, J.C. Miller, Estadística y Quimiometría para Química Analítica, Prentice Hall, Pearson Educación, Madrid, 2002, pp. 220–245.
- [29] M. Milan, J. Militky, M. Fauna, Chemometrics for Analytical Chemistry, vol. 1, Ellis Horwood, New York, 1992.
- [30] M. Monagas, C. Gómez-Cordovés, B. Bartolomé, O. Laureano, J.M.J. Ricardo da Silva, Agric. Food Chem. 51 (2003) 6475–6481.
- [31] G. Ramis Ramos, M.C. García Álvarez-Coque, Quimiometría para Química Analítica, Síntesis, Madrid, 2001, pp. 157–197.
- [32] G.E. Pereira, J.-P. Gaudillere, C. Van Leeuwen, G. Hilbert, O. Lavielle, M. Maucourt, C. Deborde, A. Moing, D. Rolin, J. Agric. Food Chem. 53 (2005) 6382–6389.
- [33] C. Oliveira, A. Ferreira, P. Costa, J. Guerra, P. Guedes de Pinho, J. Agric. Food Chem. 52 (2004) 4178–4184.
- [34] J. Kiss, A. Sass-Kiss, J. Agric. Food Chem. 53 (2005) 10042–10050.
- [35] J.S. Cámara, M.A. Alves, J.C. Marques, Talanta 68 (2006) 1512–1521.
- [36] K. Héberger, E. Csomós, L. Simon-Sarkadi, J. Agric. Food Chem. 51 (2003) 8055–8060.

Isolation of flavonoids from aspen knotwood by pressurized hot water extraction and comparison with other extraction techniques

Kari Hartonen^{a,*}, Jevgeni Parshintsev^a, Kati Sandberg^a, Eija Bergelin^b,
Linda Nisula^b, Marja-Liisa Riekkola^a

^a Laboratory of Analytical Chemistry, Department of Chemistry, P.O. Box 55, FI-00014, University of Helsinki, Finland

^b Laboratory of Wood and Paper Chemistry, Åbo Akademi University, Porthansgatan 3, FI-20500, Turku, Finland

Received 7 February 2007; received in revised form 30 April 2007; accepted 18 May 2007

Available online 26 May 2007

Abstract

Pressurized hot water extraction (PHWE) conditions (time, temperature, pressure) were optimized for the extraction of naringenin and other major flavonoids (dihydrokaempferol, naringin) from knotwood of aspen. Extracts were analysed by GC–FID, GC–MS, HPLC–UV and HPLC–MS. The results were compared with those obtained by Soxhlet, ultrasonic extraction and reflux in methanol. Flavonoids were most efficiently extracted with PHWE at 150 °C and 220 bar with 35 min extraction time. Soxhlet with methanol gave slightly higher recoveries, but an extraction time of 48 h was required. Naringenin concentration was highest in knotwood (1.15% dry weight) and much lower in the sapwood. PHWE proved to be cheap, fast and effective for the isolation of biofunctional flavonoids from aspen knotwood, producing higher recoveries than 24 h Soxhlet extraction, sonication or 24 h reflux.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Pressurized hot water extraction; Soxhlet; Aspen (*Populus tremula*); Flavonoids; Naringenin; Dihydrokaempferol; Liquid chromatography; Knotwood; Isolation

1. Introduction

Naringenin (4',5,7-trihydroxyflavanone) is a plant flavonoid (phenolic antioxidant), which is found in large amounts in citrus fruits and tomato. It exhibits anti-estrogenic activity [1], which may be responsible for the lower incidence of breast cancer in women consuming large amounts of phytoestrogens [2], and it could exert cholesterol-lowering properties by inhibiting cholesteryl ester synthesis [3]. Naringenin also seems to affect various oxidative processes associated with chronic degenerative diseases. It partially deactivates the Fenton reaction [4], restores glutathione-dependent protection against lipid peroxidation in α -tocopherol-deficient liver microsomes [5], and inhibits malonaldehyde production induced by ascorbic acid in rat brain mitochondria [6] or by autoxidation in rat brain homogenates [7]. Naringenin also may modulate cytochrome P450-dependent monooxygenase, the primary enzyme involved

in the metabolism of drugs, carcinogens, environmental pollutants and other xenobiotics [8]. Furthermore, naringenin is reported to have inhibitory effects on microorganisms, in contrast to the corresponding glycoside (naringin), which appears to be inactive [9].

Heartwood, foliage, bark and cork of several species of trees have been identified as sources of natural phenolic antioxidants [10,11]. The extract yields obtained from these materials are low, however, and the extract usually contains a wide variety of phenolic and nonphenolic compounds, both as glycosides and as free aglycones. The degree of glycosylation affects the antioxidant properties of phenolic compounds. The major hydrophilic compounds in softwood knots are free aglycones of lignans, oligolignans, stilbenes and flavonoids which could be expected to have antioxidant properties [12–14]. The amount of extractable phenolic compounds, including flavonoids, tends to be much greater in knotwood than in other parts of the tree and in the best cases may account for up to 30% of the dry weight. The average is about 15% (w/w) [15].

Usually flavonoids are extracted from wood with a polar solvent, such as methanol, by Soxhlet-, ultrasonic- or accelerated

* Corresponding author. Tel.: +358 9 191 50 265; fax: +358 9 191 50 253.
E-mail address: kari.hartonen@helsinki.fi (K. Hartonen).

solvent extraction [16]. Supercritical fluid extraction (CO₂) can be applied, but a modifier such as methanol is then required [17]. The use of pure water as an extraction solvent for phenolic compounds in wood material has been proposed by Holmbom et al. [18]. We have not, however, found any reports of the application of pressurized hot water extraction (PHWE) to knotwood.

The use of large amounts of organic hazardous solvents in sample preparation and the increasing cost of solvent waste disposal have created a growing demand for better and more environment-friendly extraction methods for analytical laboratories. Water is an interesting alternative to the usual solvents, particularly in view of its low cost, polarity and non-toxic character. The dissolving power (polarity) of water can be modified merely by adjusting the temperature. The dielectric constant is the key parameter involved in solvent–solute interactions and may be related to polarity. The dielectric constant of water is high at room temperature (78.5), but it decreases as the temperature increases. At the critical point (374 °C and 221 bar), dielectric constants and densities are the same for gaseous and liquid water. Exceedingly low values for the dielectric constant can be obtained for supercritical water, for example 1.5 at 500 °C and 225 bar. Although supercritical water is an excellent solvent for all kinds of organic compounds, its high critical temperature and corrosive nature hinder its wider use as an extraction medium. Fortunately, the solubility of many low polarity compounds in water is sufficient to allow their extraction at temperatures much below the critical temperature.

Recently, both liquid water and steam at temperatures from 200 to 300 °C have been used to efficiently extract organics of different polarity from solid sample matrices. Satisfactory recoveries (>90%) have been reported, for example, for polar persistent organic pollutants such as phenols. Temperatures of about 200 °C have been used for quantitative extraction of more unpolar compounds such as pesticides and low-molecular-mass polycyclic aromatic hydrocarbons (PAHs) [19–21]. Temperatures of 250–300 °C were required for the extraction of polychlorinated biphenyls (PCBs) and high-molecular-mass PAHs from soil and sediments [22], and *n*-alkanes were extracted only at temperatures higher than 300 °C [19,22,23]. Steam conditions were necessary for the quantitative extraction of other highly hydrophobic pollutants such as polychlorinated dibenzofurans (PCDFs) and polychlorinated naphthalenes (PCNs) from naturally contaminated soils [23]. Isoflavones have been successfully extracted from defatted soybean flakes, and catechins with proanthocyanidins from winery by-products, at temperatures up to 150 °C and pressures up to 60 bar [24,25].

The primary aim of this investigation was to optimize pressurized hot water extraction conditions for the isolation of flavonoids and other phenolic compounds from aspen (*Populus tremula*) knotwood, and to compare the results with those obtained by other techniques (Soxhlet, reflux and ultrasonic extraction in organic solvent). A further aim was to efficiently isolate naringenin and dihydrokaempferol from the knotwood by PHWE and purify the extracts by solid phase extraction (SPE).

2. Experimental

2.1. Chemicals

(±)-Naringenin (95% purity) was purchased from Sigma–Aldrich Chemie GmbH. (Steinheim, Germany), dihydrokaempferol (>95%) was from ArboNova (Turku, Finland), and taxifolin (≥85%) and naringin (≥95%) were from Fluka Chemie (Buchs, Switzerland). Silylation reagents *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) for GC analysis were purchased from Sigma (Steinheim, Germany) and Acros (Geel, Belgium), while internal standards heneicosanic acid, cholesterol, cholesteryl heptadecanoate and triglyceride standard (1,3-dipalmitoyl-2-oleyl-glycerol) were from Sigma. Milli-Q water (18 MΩ; Millipore, USA) was used for all solutions and PHWE. Methanol for standards and eluents was from VWR (Prolabo, West Chester, USA). Glacial acetic acid for eluents was 99–100% pure (J.T. Baker, Deventer, Holland).

2.2. Wood material

The wood material was aspen (*P. tremula*) from Närpes in Ostrobothnia, Finland. The tree was healthy and estimated to be 25 years old. A wood disc containing stemwood was cut out 1.5 m above the ground. Two other discs containing knotwood, one with a living branch and one with a dead branch, were sawn out from the same tree and transported directly to the laboratory. The outer branch was cut away and the knotwood discs were air dried before sampling of knotwood. The knotwood and ordinary wood were separated according to the fiber direction change in the boundary. Fibers perpendicular to fibers in the stemwood were considered as knotwood fibers. All reaction wood was removed and the samples did not contain any bark. The samples were splintered, freeze-dried, ground to 20 mesh size and then freeze-dried again.

2.3. Extraction of knotwood with PHWE

The instrument for pressurized hot water extraction is described in detail elsewhere [23,26]. The system consists of a Jasco PU-980 HPLC pump to pressurize the water and a Fractovap Series 2150 oven (Carlo Erba, Milan, Italy) to heat the laboratory-made, stainless steel 3 ml extraction vessel. The vessel has been described earlier [27]. A 30-15HF4-HT high temperature three-way valve (High Pressure Equipment, Erie, PA, USA), a manually adjustable pressure restrictor (model CC-A16A21APK, Tescom, Elk River, MN, USA) and a 10 or 5 ml measuring flask for the collection were employed. On/off valves were type 15-11AF1 from High Pressure Equipment. Approximately 3 m of 1/16 in. stainless steel tubing (i.d. 0.02 in.) was used for the preheating coil and about 1 m was used for the cooling coil. The same tubing was also used for all other connections.

Approximately 0.2 g of granular knotwood sample was placed in the extraction vessel. A sealing ring made of copper was inserted between the vessel body and the cover and the

vessel was tightened with four screws. Pumping of the water (1 ml/min, RT) was started and the oven was switched on. The temperature was set to the desired level and the pressure in the system was adjusted to 220–240 bar or 150 bar (for 150 °C only) with a restrictor. The extraction time count was started when the oven temperature and system pressure were stable. Samples were collected in measuring flasks that were changed after 10 min (sample 1) and then every 5 min for a further 25 min (samples 2–6). The extraction temperatures were 50, 100, 150, 200 and 250 °C. Extractions were made in triplicate. Samples were also extracted with 1% NaOH solution at 150 °C to check for hydrolysis.

Samples for GC–FID/MS analysis were prepared as follows: after pressure equilibrium, the temperature was raised in 50 °C steps, and 10 ml of extract was collected in each step from 50 to 350 °C. The purpose of this experiment was to determine the most suitable temperature for each compound.

2.4. Extraction of knotwood with Soxhlet

Soxhlet extraction was performed using a 30 ml Soxhlet extractor with a cellulose extraction thimble (Whatman International Ltd.). The extraction times were 24 and 48 h with 0.5–1.0 g of the knotwood sample, and 50 ml methanol was used as an extraction solvent. The time for one extraction cycle was ca. 5 min. When the extraction was completed, 1 ml of the extract was filtered through a 0.45 µm syringe filter and 10 µl was injected directly to the HPLC apparatus.

2.5. Preparation of knotwood extracts by ultrasonic extraction

Knotwood samples (0.5 g) for ultrasonic extraction were placed in a 50 ml Erlenmeyer flask and approximately 40 ml methanol was added. The extraction was performed in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corp., USA) for 3 h and the extract was kept over night in a hood. The extracts were filtered before HPLC analysis, as described above for the Soxhlet extracts.

2.6. Extraction of knotwood with reflux

Knotwood (0.5 g) was extracted by refluxing for 24 h in 50 ml of methanol containing 1% (w/w) of NaOH. The extracts were filtered through a 0.45 µm syringe filter and 10 µl was injected directly to the HPLC apparatus.

2.7. GC–FID and GC–MS analysis of PHWE extracts

For GC–FID and GC–MS analysis, the PHWE sample was made up 10 ml with water, and an aliquot of 4 ml was taken for liquid–liquid extraction with methyl-*tert*-butyl ether (MTBE) at pH 3 [28]. After evaporation the extracts were silylated with 80 µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 40 µl trimethylchlorosilane (TMCS). Samples were held in a 70 °C oven for 45 min before analysis by GC–FID and GC–MS. Heneicosanic acid, cholesterol, cholesteryl heptadecanoate and

a triglyceride standard (1,3-dipalmitoyl-2-oleyl-glycerol) were used as internal standards.

Individual extracted compounds were analysed with a Perkin-Elmer Autosystem XL (Wellesley, MA, USA) GC equipped with an HP-1 column (25 m, 0.20 mm i.d. and film thickness 0.11 µm) and flame ionization detector. The initial temperature was 120 °C (1 min), the temperature gradient 6 °C/min. The final temperature was 300 °C (10 min). The injection temperature was 175 °C and the detector temperature 290 °C. Split injection was used with ratio about 1:20; the injection volume was 1 µm. The carrier gas was H₂ with constant flow at 14 psi. Fatty acids and alcohols were quantified against heneicosanic acid standard and unsaponifiable components against cholesterol. No response factors were used. Steryl esters and triglycerides were determined with a Varian 3400 GC on a short column (DB-1, 5 m, 0.53 mm i.d. and film thickness 0.15 µm) where the initial temperature was 100 °C (1.5 min) and the temperature gradient was 12 °C/min with final temperature of 340 °C (5 min). The injection was on column with the injection volume 0.4 µm, and the injector temperature was programmed to 80 °C (0.5 min) and then 200 °C/min to 340 °C (18 min). The detection temperature was 340 °C. The carrier gas was H₂ with constant flow determined at 100 °C as 18 ml/min.

GC–MS analysis was done with an HP 6890GC-5973MSD instrument (Hewlett-Packard, Palo Alto, CA) with column parameters HP-1, 25 m, 0.20 mm i.d., 0.11 µm film thickness. The carrier gas was helium flowing at a constant 0.8 ml/min and with split flow of 15 ml/min. The injector temperature was 280 °C and the oven temperature was programmed from 80 °C (0.25 min) to 300 °C, increases at 8 °C/min. The injection was 1 µl split injection (split ratio 1:20). The MS transfer line was held at 300 °C and the MS ionization mode was EI with 70 eV electron energy. The temperature of the MS ion source and quadrupole were 230 and 150 °C, respectively. The scan range was from 35 to 800 amu. Identifications were based on a comparison of the retention times and EI spectra with those in our own database as well as in NIST98 and WILEY275.

2.8. HPLC–UV and HPLC–MS analysis of flavonoids

The quantitative analyses of flavonoids were performed on an HP 1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA) system with UV–vis detector. Separation was achieved on a Phenomenex Gemini RP-18 column (5 µm, 110 Å, 150 mm × 2.00 mm i.d., Phenomenex, Torrance, CA, USA) with precolumn (RP-18, Phenomenex, USA). Eluent A was 2% acetic acid/8% methanol/90% H₂O (pH 2.7) and eluent B 2% acetic acid/8% H₂O/90% methanol (pH 3.3). A gradient program 10% B–100% B in 40 min was used. The flow rate was 0.35 ml/min. The detector was set to 280 nm, which is a typical wavelength for phenolic compounds. The flavonoids were quantified with use of an external standard method (calibration curve obtained with identical standards).

The column and gradient used in HPLC–MS analysis were the same as above. A Bruker Esquire 3000 plus ion-trap mass spectrometer (Bruker Daltonics Inc., USA) was used in negative ion mode with electrospray ionization (*m/z* from 25 to 500;

capillary exit –109,8 V; skim –40 V; trap drive 44.3; nebulizer 10.0 psi; dry gas 6.0 l/min; dry temperature 300 °C). Identification in the LC–MS analysis were made with ions: taxifolin ($m/z = 302.9$), naringin ($m/z = 270.9$, detected as aglycon), dihydrokaempferol ($m/z = 286.9$) and naringenin ($m/z = 270.9$). All ions were of $[M-1]^-$ type.

2.9. Purification of flavonoids in PHWE extracts

Speedisk C18 (J.T. Baker, Mallinckrodt Baker Inc., USA) solid phase extraction material was used for the purification of flavonoids. The same eluents (A and B) were used as in the HPLC analysis. Taxifolin, dihydrokaempferol and naringenin were eluted from the SPE material with 39% B + 61% A, 48% B + 52% A and 61.3% B + 38.7% A, respectively.

3. Results and discussion

All extracts of the knotwood of *P. tremula* contained principally flavonoid aglycones with dihydrokaempferol (aromadendrin) as the major component. Naringenin was the second most prominent compound in the extracts, and naringin and taxifolin were found in good amounts. Identification of the four flavonoids was done by GC–MS and HPLC–MS with the help of standards. Other compounds were identified from their MS-fragmentation spectra and retention times but they were not quantified. These included syringaldehyde, hydroquinone, vanillin, levoglucosan, 2',4',6',4-tetrahydroxychalcone and its glycoside, 3,4-dihydroxybenzaldehyde, stigmasta-3,5-diene, 4,4'-dihydroxy-3,3'-dimethoxystilbene, sitostadiene-7-one, 3-methoxy-4-hydroxyacetophenone, 3-methoxy-5-hydroxycinnamaldehyde, 3,5-dimethoxy-4-hydroxycinnamaldehyde, syringaresinol, medioresinol, pinioresinol, sitosterol, tocopherol, isolariciresinol, monomethyldihydroxykaempferol, coniferyl alcohol, pyrogallol, syringol, catechol, 4-methylsyringol and many carboxylic acids. Natural extracts often contain hundreds of chemically different compounds, so identification

Table 1

PHWE of flavonoids in aspen knotwood (150 °C, 220 bar, 35 min) quantified by HPLC–UV

Flavonoid	Retention time, min (S.D.)	Amount, mg/g (S.D.)
Taxifolin	11.02 (0.30)	1.16 (0.10)
Naringin	15.15 (0.34)	10.61 (1.71)
Dihydrokaempferol	15.51 (0.35)	17.25 (0.25)
Naringenin	20.37 (0.32)	9.80 (2.10)
Total		4% (w/w) of dry sample

and quantitation of all of them would be both difficult and unreasonable. Quantitation of the four flavonoids was done by HPLC–UV because derivatization was not then required. The HPLC separation was very good, with no optimization needed. As has been noticed previously, acidic eluents are an excellent choice for the reliable separation of phenolic compounds in the reverse phase column. The GC–MS technique is suitable for identification but, owing the need for derivatization, not good for routine use or quantification. The knotwood extract of aspen is rich in bioactive compounds, which have been studied intensively in the past few years. As shown in Table 1, the four flavonoids studied here made up 4% of the dry weight, and if we add to these all other phenolic species that were found, the total amount of extracted bioactive compounds is in agreement with amounts reported in previous studies [15]. A typical HPLC chromatogram for the PHWE extract of aspen knotwood is shown in Fig. 1.

The PHWE results for naringenin at different temperatures are summarized in Table 2 and Fig. 2. The lower extraction efficiency at 200 °C than at 150 °C can be explained by the weaker solubility of naringenin at this temperature, where water is less polar, and probable degradation. The PHWE efficiency for other qualitatively analysed compounds is presented in Table 3. As can be seen, these compounds were identified in the knotwood extract by varying the temperature, but mostly the amounts were considerably smaller than for the four flavonoids.

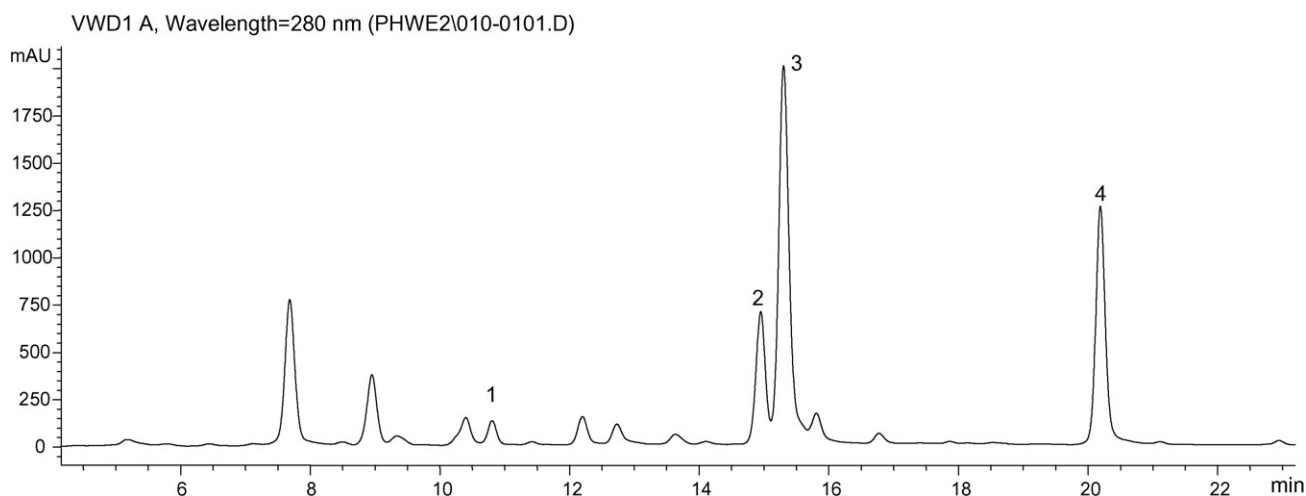


Fig. 1. Typical HPLC chromatogram of PHWE extract. Extraction conditions: 150 °C, 220 bar, 10 min. Ten microliter of filtered undiluted sample. Peaks are 1 = taxifolin, 2 = naringin, 3 = dihydrokaempferol and 4 = naringenin. HPLC conditions explained in Section 2.8.

Table 2
Comparison of extraction techniques for naringenin in aspen knotwood

Extraction technique	Conditions	Amount, mg/g (R.S.D., %)	Extraction time, h
PHWE	100 °C, 220 bar	8.1 (21.4)	0.58
PHWE	150 °C, 220 bar	9.8 (21.4)	0.58
PHWE	200 °C, 220 bar	8.1 (21.4)	0.58
PHWE	1% NaOH, 150 °C	5.1	0.58
PHWE	150 bar, 150 °C	9.1	0.58
Soxhlet (MeOH)	–	8.8	24
Soxhlet (MeOH)	–	11.5 (4.17)	48
Reflux (MeOH)	–	3.0	24
Ultrasonic (MeOH)	–	5.0 (4.41)	3

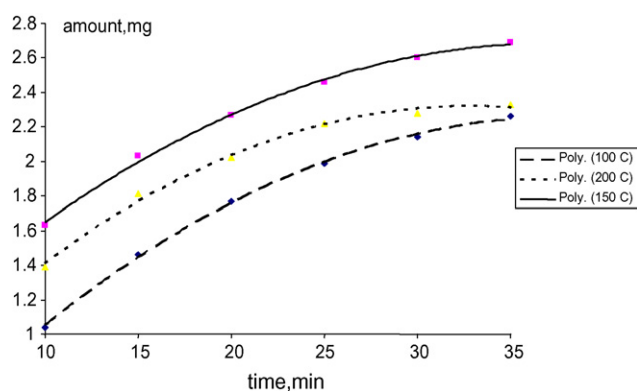


Fig. 2. Influence of temperature on the rate of extraction of naringenin by PHWE. Sample size 0.2 g.

The best recovery of naringenin was obtained at 150 °C and 220 bar with an extraction time of 35 min. Higher temperatures were not effective because the compounds began to decompose and undesirable products were obtained. Also, the knotwood began to dissolve too much and the filter in the extraction vessel became blocked. The extraction process was slower at lower pressure (150 bar), but the same recoveries could be achieved by increasing the extraction time. Nevertheless, the standard deviations of the naringenin and naringin concentrations in the PHWE extraction were relatively high (compared with the other compounds, see Table 1). This may be a consequence of the decomposition of naringin (naringenin glycoside) to naringenin, which is not quantitative or repeatable in such a short time under the conditions employed. The relatively large deviations of the naringin and naringenin concentrations in extracts (Table 1) support this explanation.

Table 3
Compounds extracted by PHWE and identified by GC–MS

Compound	50 °C	50–100 °C	100–150 °C	150–200 °C	200–250 °C	250–300 °C	300–350 °C
Syringaldehyde				+	+++		
Hydroquinone						+	+
Vanillin				+	++	++	+
Levogluconan						+	
2',4',6',4-Tetrahydroxychalcone	+++	+	+				
2',4',6',4-Tetrahydroxychalcone glycoside	+++	+	+				
3,4-Dihydroxybenzaldehyde						+	+
Stigmasta-3,5-diene						+	++
4,4'-Dihydroxy-3,3'-dimethoxystilbene						+	
Sitostadiene-7-one						+	++
3-Methoxy-4-hydroxyacetophenone						+	+
3-Methoxy-5-hydroxycinnamaldehyde					+	++	+
3,5-Dimethoxy-4-hydroxycinnamaldehyde					+	++	+
Syringaresinol				+	++	+	
Medioresinol				++	+	+	
Pinoresinol	+	+	+				
Sitosterol						+	++
Tocopherol							+
Isolariciresinol				+	++		
Monomethyldihydroxykaempferol	+	+++	++				
Coniferyl alcohol				++	++		
Pyrogallol						+	
Syringol						+	+
Catechol						+	+
4-Methylsyrinol							+

+++ Indicates the temperature at which compound was extracted most efficiently, while ++ and + indicate temperatures at which extraction was moderate and observable.

Table 4
Amount of naringenin in different sources

Source	Amount
Orange juice, Valio	41 mg/l
Grapefruit juice, Valio	349 mg/l
Pera orange, pulp	170 mg/kg FW
Pera orange, peel	374 mg/kg FW
Lima orange, pulp	286 mg/kg FW
Lima orange, peel	251 mg/kg FW
<i>Populus tremula</i> knots ^a	9800 mg/kg SW

FW, fresh weight, SW, sample weight [29,30].

^a Results obtained from this research.

Tests were made of different pH conditions in PHWE. Addition of a diluted acid would have caused corrosion, but addition of base to the extracting water could be performed more safely and could be expected to increase the recoveries of flavonoids through the hydrolysis of glycosides. Addition of 1% (w/w) of NaOH to the water in PHWE decreased the recovery of naringenin sharply, however, perhaps as a result of aglycon degradation (Table 2). For the future, smaller R.S.D. values for the PHWE method might be achieved by extending the extraction time, increasing the homogenization level or accelerating the hydrolysis of glycosides by acidification of the extracting water. Although all approaches require further study, they will make the extraction process more complicated without a marked increase in the efficiency of the method.

As shown in Table 2, the best result for naringenin was still obtained by Soxhlet extraction (48 h). However, the amount of naringenin recovered by the 24 h Soxhlet extraction was smaller than obtained with 35 min PHWE. Also, a greater amount of naringenin glycoside was found in the Soxhlet extracts, due to the lower extraction temperature. With short extraction times the most efficient method was PHWE.

Table 2 also summarizes the results obtained by ultrasonic extraction and reflux. As expected, recoveries obtained with these techniques were much lower. In general, better R.S.D. values were achieved with Soxhlet, sonication and reflux than with PHWE owing to the longer extraction time and higher sample amount.

The results of the analysis of Aspen knotwood extracts show a much larger amount of naringenin than in citrus fruits, which until now have been considered the primary source (Table 4).

In the chromatographic separation by HPLC–UV, the flavonoids were found to elute with distinct methanol/acidified water ratios. This finding was exploited in the purification of single compounds from the extracts by SPE. With use of the appropriate eluent, naringenin was separated from the PHWE extract by SPE with over 90% purity relative to the amount measured by HPLC–UV. Further optimization of the extraction conditions would improve the result.

4. Conclusions

Pressurized hot water extraction of aspen knotwood was carried out for the first time in this work and the results were compared with those obtained by other techniques. PHWE

proved to be a fast, environmentally friendly, cheap and effective technique for the extraction of biofunctional compounds from solid samples such as wood materials and especially knotwood. Since the technique can be automated, it is also suitable for the industrial preparation of extracts for different purposes. If required, compounds in the extracts can be purified by chromatography.

Since it possible to separate knotwood from the over-sized chip fraction in a pulp mill, the extractives in knotwood could be made available in large scale. As a side benefit, with the removal of knotwood, pulp quality would be improved. The phenolic compounds in knotwood constitute a valuable resource with potential for its use as active ingredients.

Acknowledgements

Funding from the University of Helsinki (project no. 2105040) is gratefully acknowledged. We would like to thank Professor Bjarne Holmbom and Doctor Stefan Willförer for their advice.

References

- [1] M.F. Ruh, T. Zacharewsky, K. Connor, J. Howell, J. Chen, S. Safe, *Biochem. Pharmacol.* 50 (1995) 1485–1493.
- [2] H. Adlercreutz, Y. Mousavi, J. Clark, K. Hoherstedt, E. Hamalainen, K. Wahala, T. Makela, T. Hase, *J. Steroid Biochem. Mol. Biol.* 41 (1992) 331–337.
- [3] N.M. Borradaile, K.K. Carroll, E.M. Kurowska, *Lipids* 34 (1999) 591–598.
- [4] F. Cheng, K. Breen, *Biomaterials* 13 (2000) 77–83.
- [5] F.A.A. Van Acker, O. Schouten, G.R. Haenen, W.J.F. Van der Vijgh, A. Bast, *FEBS Lett.* 473 (2000) 145–148.
- [6] A.K. Ratty, N.P. Das, *Biochem. Med. Metab. Biol.* 39 (1988) 69–79.
- [7] A. Saija, M. Scalese, M. Lanza, D. Marzullo, F. Bonina, F. Castelli, *Free Radical Biol. Med.* 19 (1995) 481–486.
- [8] Y.F. Ueng, Y.L. Chang, Y. Oda, S.S. Park, J.F. Liao, M.F. Lin, C.F. Chen, *Life Sci.* 65 (24) (1999) 2591–2602.
- [9] R.J. Grayer, *Method Plant Biochem.* 1 (1989) 287–288.
- [10] Y. Kai, in: D.N.-S. Hon, N. Shiraishi (Eds.), *Wood and Cellulosic Chemistry*, first ed., Marcel Dekker, New York, 1990, pp. 215–255.
- [11] M.P. Kahkonen, A.I. Hopia, H.J. Vuorela, J.-P. Rauha, K. Pihlaja, T.S. Kujala, M. Heinonen, *J. Agric. Food Chem.* 47 (1999) 3954–3962.
- [12] S. Willförer, J. Hemming, M. Reunanen, C. Eckerman, B. Holmbom, *Holzforchung* 57 (2003) 27–36.
- [13] S. Willförer, J. Hemming, M. Reunanen, B. Holmbom, *Holzforchung* 57 (2003) 359–372.
- [14] S. Pietarinen, S.M. Willförer, M.O. Ahotupa, J.E. Hemming, B.R. Holmbom, *J. Wood Sci.* 52 (2006) 436–444.
- [15] S. Willförer, M. Ahotupa, J. Hemming, M. Reunanen, P. Eklund, R. Sjöholm, C. Eckerman, S. Pohjamo, B. Holmbom, *J. Agric. Food Chem.* 51 (26) (2003) 7600–7606.
- [16] S. Pietarinen, S.M. Willförer, F.A. Vikstrom, B.R. Holmbom, *J. Wood Chem. Technol.* 26 (3) (2006) 245–258.
- [17] J. Peng, G. Fan, Y. Chai, Y. Wu, *J. Chromatogr. A* 1102 (2006) 44–50.
- [18] B. Holmbom, C. Eckerman, J. Hemming, M. Runanen, K. Sundberg, S. Willförer, *PCT Int. Appl.* (2002) 31 pp., WO 2002098830 A1.
- [19] Y. Yang, S.B. Hawthorne, D.J. Miller, *Environ. Sci. Technol.* 31 (1997) 430.
- [20] X. Lou, D.J. Miller, S.B. Hawthorne, *Anal. Chem.* 72 (2000) 481.
- [21] S.B. Hawthorne, Y. Yang, D.J. Miller, *Anal. Chem.* 66 (1994) 2912.

- [22] S.B. Hawthorne, C.B. Grabanski, E. Martin, D.J. Miller, *J. Chromatogr. A* 892 (2000) 421.
- [23] B. Van Bavel, K. Hartonen, Ch. Rappe, M.-L. Riekkola, *Analyst* 124 (1999) 1351.
- [24] C. Li-Hsun, C. Ya-Chuan, C. Chieh-Ming, *Food Chem.* 84 (2004) 279.
- [25] M. García-Marino, J.C. Rivas-Gonzalo, E. Ibañez, C. García-Moreno, *Anal. Chim. Acta* 563 (2006) 44.
- [26] K. Hartonen, K. Inkala, M. Kangas, M.-L. Riekkola, *J. Chromatogr. A* 785 (1997) 219–226.
- [27] T. Andersson, K. Hartonen, T. Hyötyläinen, M.-L. Riekkola, *Anal. Chim. Acta* 466 (2002) 93–100.
- [28] F. Örså, B. Holmbom, *J. Pulp Pap. Sci.* 20 (12) (1994) 361–366.
- [29] I. Erlund, E. Meririnne, G. Alfthan, A. Aro, *J. Nutr.* 131 (2001) 235–241.
- [30] P.R. Arabbi, M.I. Genovese, F.M. Lajolo, *J. Agric. Food Chem.* 52 (5) (2004) 1124–1131.

Lanthanide complexation with CMPO and CMPO-calix[4]arenes in solution: Spectrophotometric and electrospray mass spectrometric approaches

Haiko Herschbach^a, Frédéric Brisach^b, Jaouad Haddaoui^b,
Mohamed Saadioui^c, Emmanuelle Leize^a, Alain Van Dorsselaer^a,
Françoise Arnaud-Neu^{b,*}, Volker Böhmer^c

^a Laboratoire de spectrométrie de masse Bioorganique, DSA, IPHC, ULP, CNRS, 25 rue Becquerel, 67087 Strasbourg, France

^b Laboratoire de Chimie-Physique, DSA, IPHC, ULP, CNRS, ECPM, 25 rue Becquerel, 67087 Strasbourg, France

^c Fachbereich Chemie und Pharmazie, Abteilung Lehramt Chemie, Johannes-Gutenberg Universität, Duesbergweg 10-14, D-55099 Mainz, Germany

Received 16 February 2007; received in revised form 11 May 2007; accepted 18 May 2007

Available online 26 May 2007

Abstract

The binding of lanthanide(III) cations with organophosphorous ligands like CMPO and related calix[4]arene-based derivatives have been investigated using two experimental methods. The stability constants of the lanthanum, europium and ytterbium complexes were first determined in methanol by UV absorption spectrophotometry in the presence of nitrate or chloride anions. The results showed that the stoichiometry and the stability of the complexes formed depend on the position of the CMPO moieties either on the wide or the narrow rim of the calixarene scaffold, the nature of the medium, the conformational mobility of the ligands. Complexation of lanthanum was also followed by ESI-mass spectrometry in the same solvent. This method confirmed the stoichiometry of the complexes, giving also structural information, like coordination of anion or solvent molecules to the complexes, and allowed the calculation of distribution curves in good agreement with those derived from the spectrophotometric results. This is an important result showing that ESI-MS can be used to provide with quantitative information when absorption spectrophotometry is not applicable, i.e. for systems where complexation leads to weak spectral changes.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Complexation; Stability constants; Electrospray mass spectrometry; Spectrophotometry; Calixarenes; CMPO; Lanthanides

1. Introduction

During the treatment of waste issuing from nuclear plants two consecutive steps are concerned with (i) the simultaneous extraction of lanthanides and actinides followed by (ii) the separation of actinides from lanthanides [1]. Investigations have shown the potentialities of new calixarenes substituted by CMPO (*N,N'*-diisobutyl-carbamoylmethyl)-phenyloctyl-phosphine oxide) functions to perform these separations [2]. A great amount of extraction data have shown the remarkable abilities of such derivatives with CMPO groups attached either to the wide or to the narrow rim to extract lanthanides and actinides,

some of them having even favourable Am(III)/Eu(III) selectivities [3–10]. The acquisition of basic data on the binding properties of these ligands is very important for the understanding of their behaviour and their further implementation in industrial processes. However, there are only few complexation data available. Some stability constants have been published by us [8,10]. They were derived from UV absorption spectrophotometric titrations. This method is based on spectral differences between the ligand and the complex(es) which in some cases revealed to be not significant enough to allow satisfactory interpretations. In addition the results obtained so far showed different kinds of stoichiometries for the extracted species and for the complexes formed in a single medium: for instance, it was shown that ML₂ and ML europium complexes with **2b** [4,10] (Fig. 1) were extracted from 1 M HNO₃ into dichloromethane, whereas ML and M₂L complexes were shown

* Corresponding author. Tel.: +33 390242749; fax: +33 390242747.
E-mail address: farnaud@chimie.u-strasbg.fr (F. Arnaud-Neu).

to form in methanol [8]. Relaxivity and light scattering measurements suggested the formation of aggregates with Gd^{3+} cations and ligand **2b** [8].

In order to better assess the binding properties of these very complex systems we decided to use electrospray-ionization mass spectrometry (ESI-MS). This technique allowed us not only to identify all the species formed but also to get quantitative information on the equilibria in solution. To determine stability constants by absorption spectrophotometry, different models assuming possible stoichiometries of the complexes have to be fitted to the experimental data. The stability constants are then calculated using the model that gives the best fit. When several models lead to similar fits, the determination of stability constants is ambiguous. ESI-MS is able to establish the stoichiometries of the complexes and thus contributes to the determination of stability constants. This technique has been used with success on several studies [11–20].

For a quantitative treatment of the ESI-MS data, several conditions must be fulfilled to get similar response factors [21]:

- (i) appropriate solvents and concentrations have to be used thus maintaining the integrity of the complexes in solution;
- (ii) every kind of fragmentation has to be avoided;
- (iii) the formation mode of the ions has to be identical;
- (iv) the solvation energies of the ions should be similar;
- (v) the charge state of the ions has to be identical. Several studies have already shown that ESI-MS can be used to determine relative abundances of complexes, when these conditions are respected [11–14,22–25].

We report here on the binding of lanthanide(III) cations with the organophosphorus ligand CMPO **1**, the four calix[4]arenes **2a–c** and **3** substituted by four CMPO groups at the wide rim and the two calix[4]arenes **4a** and **4b**, bearing four CMPO groups at the narrow rim (Fig. 1). The stability constants of lanthanum, europium and ytterbium complexes were determined on the basis of spectrophotometric titrations in methanol in presence

of either nitrate or chloride ions. Complexation of lanthanum with ligands **1**, **2b**, **2c**, **3** and **4b** was also followed by ESI-MS in the same solvent, which allowed the identification of the species formed. Thus, our objectives in this paper are double: (i) to study the complexation of trivalent lanthanides with phosphorylated calix[4]arenes in solution; (ii) to compare the results obtained by the two methods and to discuss the added value of using ESI-MS.

2. Experimental

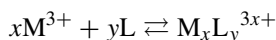
2.1. Materials

Methanol (Sigma Aldrich, 99.9% RS for HPLC) was used without further purification. The lanthanide salts were commercial nitrates and chlorides (Alfa Aesar): $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, $\text{Eu}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, $\text{Yb}(\text{NO}_3)_3 \cdot x\text{H}_2\text{O}$ ($x = 5–6$), $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$, $\text{EuCl}_3 \cdot x\text{H}_2\text{O}$ and $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$. The ligand **1** (*N,N*-diisobutylcarbamoylmethyl)-octylphenylphosphine oxide was provided by CEA, Cadarache and the CMPO calix[4]arene derivatives **2–4** were prepared as described previously [3,4,8,9].

According to the anions of the respective lanthanide salts either Et_4NCl (Fluka, purum) or Et_4NNO_3 (Acros Organics) were used as background salts in the spectrophotometric titrations to maintain the ionic strength constant at 5×10^{-2} M. In ESI-MS, no background salts could be used at this concentration due to the abundant formation of $[\text{Et}_4\text{N}(\text{Et}_4\text{NCl})]^+$ clusters which inhibited the observation of the expected complexes.

2.2. Spectrophotometric titrations

The apparent overall stability constants β corresponding to the complexation equilibria:



were determined in methanol by absorption spectrophotometry as previously described [26]. The spectra were recorded

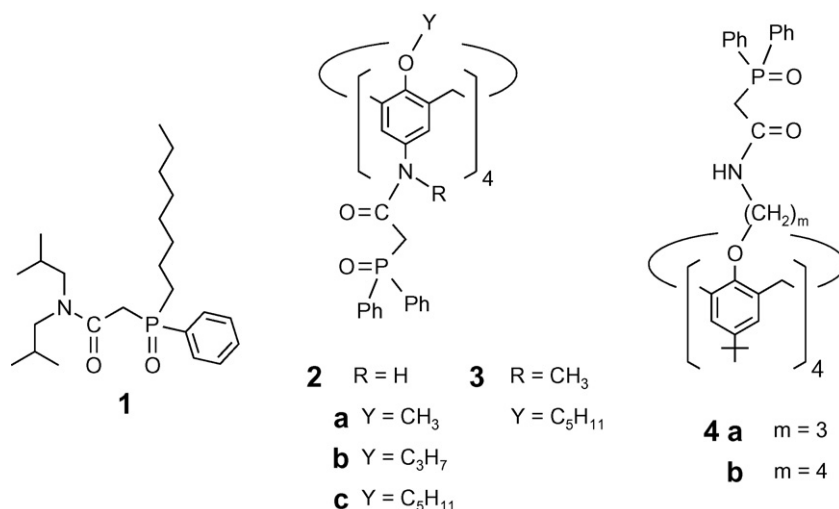


Fig. 1. Chemical structures of the CMPO and of the calixarenes studied: (**1**) CMPO; (**2a**) R = H, Y = CH₃; (**2b**) R = H, Y = C₃H₇; (**2c**) R = H, Y = C₅H₁₁; (**3**) R = CH₃, Y = C₅H₁₁; (**4a**) m = 3; (**4b**) m = 4.

Table 1

Stability constants ($\log \beta \pm \sigma_{N-1}$) of lanthanide complexes with CMPO and CMPO substituted calix[4]arenes in methanol, $I = 5 \times 10^{-2}$ M, $T = 25^\circ\text{C}$, determined by UV spectrophotometry

Ligands	Complexes	Cl^-			NO_3^-		
		La^{3+}	Eu^{3+}	Yb^{3+}	La^{3+}	Eu^{3+}	Yb^{3+}
1	ML	4.63 ± 0.05	4.99 ± 0.05	5.6 ± 0.3	^a	^a	^a
	ML ₂	8.5 ± 0.2	9.35 ± 0.05	10.5 ± 0.4	^a	^a	^a
	ML ₃	12.4 ± 0.2	13.5 ± 0.1	14.5 ± 0.5	^a	^a	^a
2a	ML	n.d.	7.00 ± 0.02	n.d.	n.d.	4.0 ± 0.1	n.d.
	M ₂ L	n.d.	11.5 ± 0.4	n.d.	n.d.	8.09 ± 0.02	n.d.
2b	ML	8.5 ± 0.1	7.2 ± 0.1	6.8 ± 0.5	6.0 ± 0.2	5.6 ± 0.3	3.5 ± 0.5
	M ₂ L	13.9 ± 0.3	11.9 ± 0.3	12.01 ± 0.03	10.6 ± 0.4	11.0 ± 0.3	8.6 ± 0.4
2c	ML	n.d.	n.d.	n.d.	6.9 ± 0.4	6.0 ± 0.3	5.7 ± 0.2
	M ₂ L	n.d.	n.d.	n.d.	12.2 ± 0.3	11.1 ± 0.4	10.3 ± 0.1
3	ML	7.1 ± 0.1	7.6 ± 0.2	^a	5.4 ± 0.2	5.3 ± 0.2	^a
4a	ML	5.77 ± 0.08	5.0 ± 0.5	5.0 ± 0.5	^a	^a	^a
4b	ML	6.4 ± 0.2	5.1 ± 0.1	5.1 ± 0.1	^a	^a	^a

n.d.: not determined.

^a Corresponds to small spectral variations (no constant could be determined).

between 250 and 300 nm with a Shimadzu UV-2101 spectrophotometer using quartz cells (Hellma) with optical path lengths of 1 or 2 cm. The ligand concentrations were 10^{-4} M for ligands **1** and **2**, 6×10^{-5} M for ligand **3** and ranging between 7×10^{-5} and 10^{-4} M for ligand **4**. The metal to ligand ratios which were reached at the end of the titrations were in the range of 2–10. The spectral changes were interpreted using the program SPECFIT [27]. The values of the stability constants given in Table 1 are arithmetic means of several experiments and the precision corresponds to the standard deviation of these mean values.

2.3. Electrospray mass spectrometry

Ligand solutions ($C_L = 10^{-4}$ M) with increasing metal/ligand ratios (up to 5) were prepared in methanol. The ESI mass spectra of these solutions were recorded with a Quattro II (Micromass) triple quadrupole mass spectrometer. Mass range from 200 to 3500 m/z was scanned in 33 s and five scans were summed to give the final spectrum. Desolvation temperature was 80°C and the sample injection flow rate $3 \mu\text{l}/\text{min}$. Several signal attributions were checked by simulations of the isotopic profiles.

The spectrometer was carefully tuned to ensure that the species detected correspond to those present in solution. Especially, the accelerating voltage applied in the interface of the ESI mass spectrometer (V_c) was carefully chosen and fixed at 10 V. To determine this value, the V_c was lowered until the relative abundances of the different complexes in the spectrum did not change any more, thus preventing any fragmentation of non-covalent species. On the other hand, 10 V is high enough to drive the ions through the interface with an acceptable yield (good sensitivity), to desolvate them and to destroy non-specific interactions. An increase of this voltage would lead to fragmentations of covalent or non-covalent bonds by collisions with nitrogen molecules.

For the quantitative exploitation of the ESI-MS spectra, three independent spectra acquisitions were averaged. The relative abundance of a complex was calculated dividing the sum of the signal intensities of the different forms of this complex (e.g. for ML: $[\text{ML}(\text{NO}_3)_m]^{n+} + [\text{ML } S_m]^{n+}$, with S = methanol) by the sum of the signal intensities of all complexes present in the spectrum. Tables S1 and S2 of the supplementary data section summarize the results obtained with all the ligands studied. These relative abundances were then plotted against the M/L ratios (Figs. 3, 6 and 7).

3. Results and discussion

3.1. Complexation of lanthanides with ligand **1**

3.1.1. UV absorption spectrophotometry

The ligand **1** is a well-known extractant of lanthanides and actinides used in the TRUEX process for the simultaneous separation of lanthanides and actinides [28]. The extracted complex of Am(III) involves three CMPO molecules, three co-extracted HNO_3 molecules and three nitrate counterions [29]. Although its extracting properties have been thoroughly investigated [3,30], no complexation data are available except preliminary results with europium in methanol containing nitrate ions derived from a competitive spectrophotometric method [10]. Complexation has now been followed in methanol in chloride medium by direct absorption spectrophotometry. With the three lanthanides the spectral changes could be interpreted by the formation of three species ML, ML₂ and ML₃. The corresponding stability constants are summarized in Table 1.

They show that the stability of the complexes increases in the series as expected from electrostatic considerations. In nitrate medium these changes revealed to be not significant enough to allow their satisfactory interpretation. The existence of these three complexes is in agreement with biphasic extraction

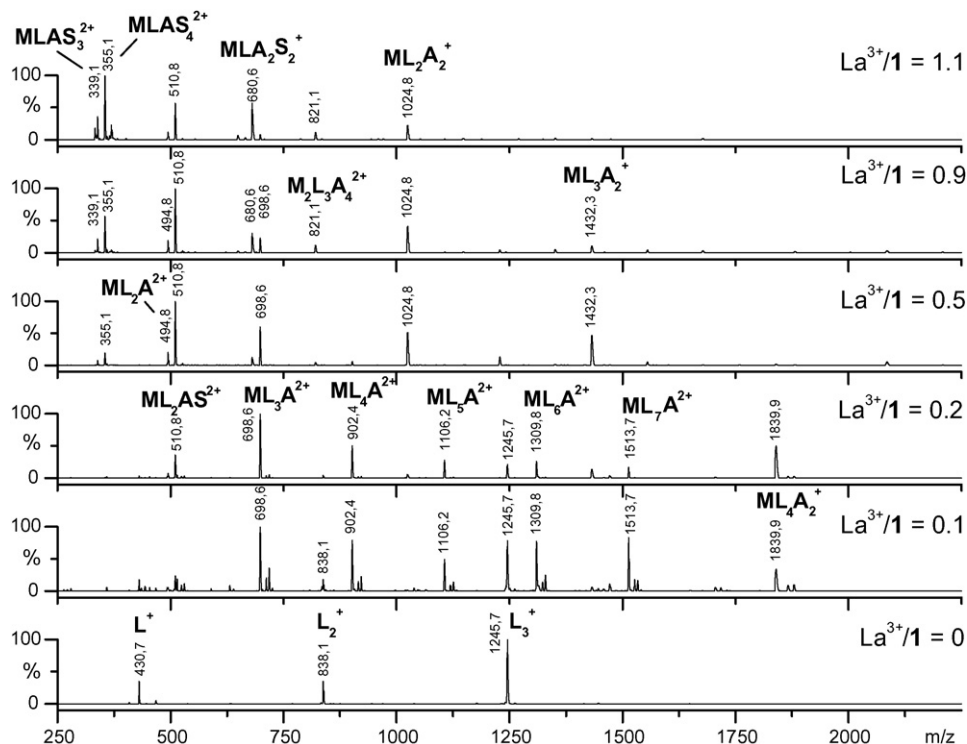


Fig. 2. ESI-MS titration of **1** with LaCl_3 in methanol ($C_L = 10^{-3}$ M, $V_c = 10$ V). M stands for lanthanum, L for ligand **1**, A for chloride ion and S for methanol.

experiments [31] and quantum mechanical and molecular dynamic simulations showing the formation of ML_3 species [32].

3.1.2. Electrospray mass spectrometry

The ligand **1** forms complexes with sodium ions present as impurities (Fig. 2). Signals for NaL , NaL_2 and NaL_3 are observed. It is interesting to note that no higher stoichiometries are observed thus indicating that the coordination sphere of sodium is saturated with three ligand molecules.

Complexation of **1** with both LaCl_3 and $\text{La}(\text{NO}_3)_3$ was monitored with ESI-MS by analysing solutions with increasing metal/ligand ratios (up to 1.1).

3.1.3. **1** + LaCl_3

Addition of 0.1 equiv. of LaCl_3 to **1** leads to the formation of ML_3 (base peak), ML_4 , ML_5 , ML_6 and ML_7 species, mainly doubly charged by coordination of one chloride anion to the La^{3+} cation.

At 0.5 equiv. of lanthanum, ML_4 and ML_5 have strongly decreased, and the higher stoichiometries have disappeared completely. At 0.9 equiv., the ML_2 species is dominant and a ML complex has appeared (about 50% of ML_2). At the end of the titration (1.1 equiv. of La^{3+}), ML is the base peak, and all species with higher stoichiometries than ML_2 have almost disappeared. Another point is that ML species were only detected with solvent molecules coordinated to the La^{3+} ion: the singly charged form (with two Cl^-) needs two methanol molecules, whereas the doubly charged complex (with one Cl^-) needs four solvent molecules. Almost no intermediate forms containing one

or three methanol molecules are observed under these conditions indicating that a chloride anion can replace two methanol molecules.

The UV spectrophotometric results could be interpreted by only ML , ML_2 and ML_3 complexes in the titration of **1** with La^{3+} , whereas ESI-MS evidenced complexes up to ML_7 . These differences might be explained by the lower solvation energies of ML_5 , ML_6 , etc., species in comparison to ML , thus facilitating their desolvation during the ESI process and leading to too high response factors [21]. Similar results were observed in the case of Mg^{2+} -crown ether complexes [13]. The different charge states of the complexes may also influence the solvation energy. Solvation energies of the different complexes cannot be established experimentally as they cannot be separated in solution. Sensitivity also plays a role: complexes like ML_5 or ML_6 may not be detected by UV spectrophotometry since they have very similar spectroscopic properties.

Distribution curves have been established by direct quantitative exploitation of the ESI-MS data (Fig. 3). As expected, the higher the $\text{La}^{3+}/\mathbf{1}$ ratio, the lower is the ligand content of the predominant complexes.

3.1.4. **1** + $\text{La}(\text{NO}_3)_3$

In order to get some information on the influence of the anion during complexation, the same titration experiment as with lanthanum chloride was carried out with lanthanum nitrate. The same tendencies are observed: at low metal/ligand ratios, complexes containing up to eight ligands are observed, whereas ML and ML_2 species are the dominant species at the end of titration. However, in the presence of nitrate, the proportion

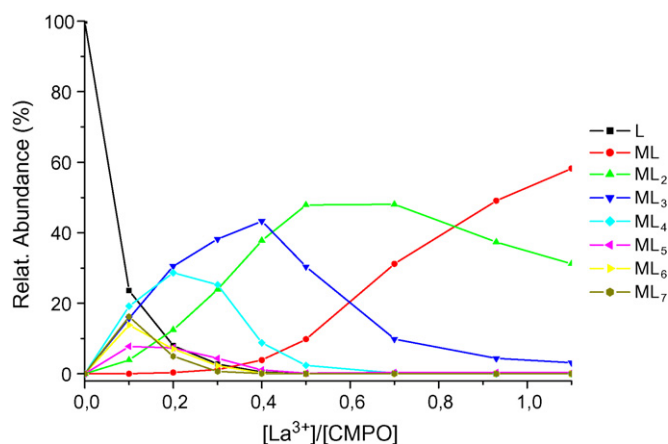


Fig. 3. Distribution curves of the complexes of the system **1**/ LaCl_3 in methanol ($C_L = 10^{-3}$ M).

of singly charged complexes (containing two nitrate anions) is much larger. From a metal/ligand ratio of 0.5 on, a complex $[\text{M}_2\text{L}_3]^{2+}$ is also observed at 874.5 m/z .

Distribution curves calculated from these data are similar to those established for the titration with lanthanum chloride.

3.2. Complexation of lanthanides with calix[4]arene CMPO derivatives

3.2.1. UV absorption spectrophotometry

The complexation of lanthanides with six different calixarene derivatives (Fig. 1) were investigated by spectrophotometry in both nitrate and chloride media: wide rim CMPO derivatives **2a–c** differ by the length of the ether residues, **3** is the *N*-methylated analogue of **2c** and **4a,b** carry the CMPO functions at the narrow rim. With the wide rim derivatives **2a** and **2b** a strong decrease in absorption intensity is observed until a metal to ligand ratio close to 1 followed by an increase for higher ratios in the presence of Et_4NCl . The spectral changes are similar though less intense in the presence of Et_4NNO_3 . These variations suggest the formation of two different absorbing species which are interpreted as ML and M_2L complexes by the program SPECFIT

[27]. Their stability constants are grouped in Table 1. With ligand **3**, the spectral changes are much weaker and no variation is noted above a ratio of 1. The spectrophotometric data in this case are best interpreted by the presence of a ML complex only. With the lower rim derivatives **4a,b**, significant spectral changes are observed only in chloride medium where a ML complex could be found. These results show:

- the high stability of the complexes formed with these ligands, the complexes with the wide rim derivatives being more stable than those with their narrow rim counterparts;
- the weak influence of the nature of the substituents at the narrow rim of the former ligands on the complex stability, except when these substituents are CH_3 groups. In this case, the conformational mobility of ligand **2a** leads to a decrease of the stability of the complexes, especially in nitrate medium (e.g. $\Delta \log \beta_{11} = 1.6\text{--}2$);
- the higher complex stability in chloride versus nitrate medium with the wide rim derivatives;
- a marked selectivity of the wide rim derivatives in the series, the complex stability decreasing from La^{3+} to Yb^{3+} . This is in contrast with the behaviour of the CMPO moieties and may be explained by the preorganization of the ligating groups.

3.2.2. Electrospray mass spectrometry

Complexing properties of ligands **2b**, **2c**, **3** and **4b** with $\text{La}(\text{NO}_3)_3$ have been investigated by this method. Fig. 4 presents the electrospray mass spectra recorded for ligand **2c** alone and in the presence of La^{3+} at five different $C_{\text{La}}/C_{\text{2c}}$ ratios.

In absence of La^{3+} , the ligand is observed in its form complexed with sodium and potassium ions present as impurities in the solution. In presence of La^{3+} , the spectra clearly show the formation of several lanthanum complexes in thermodynamic equilibrium. The ML complex is predominant until 1.5 equiv. of La^{3+} . At higher ratios, M_2L becomes the major species in the spectra. These species can be observed with different charge states, e.g. ML in the forms $[\text{La}2\text{c}(\text{NO}_3)_2]^+$ and $[\text{La}2\text{c}(\text{NO}_3)]^{2+}$, due to the successive loss of the nitrate anions introduced by

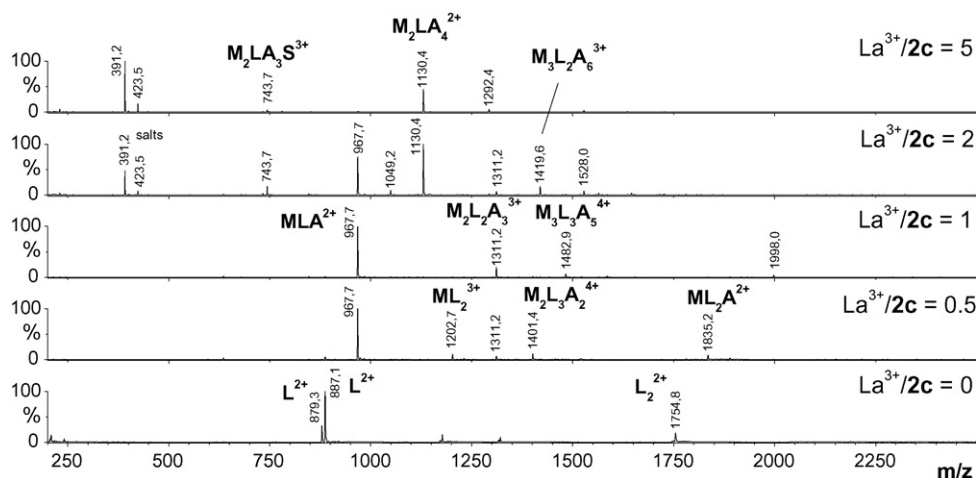


Fig. 4. ESI-MS titration of **2c** ($C_L = 10^{-4}$ M) with $\text{La}(\text{NO}_3)_3$ in methanol. M stands for lanthanum, L for ligand **2c**, A for nitrate ion and S for methanol.

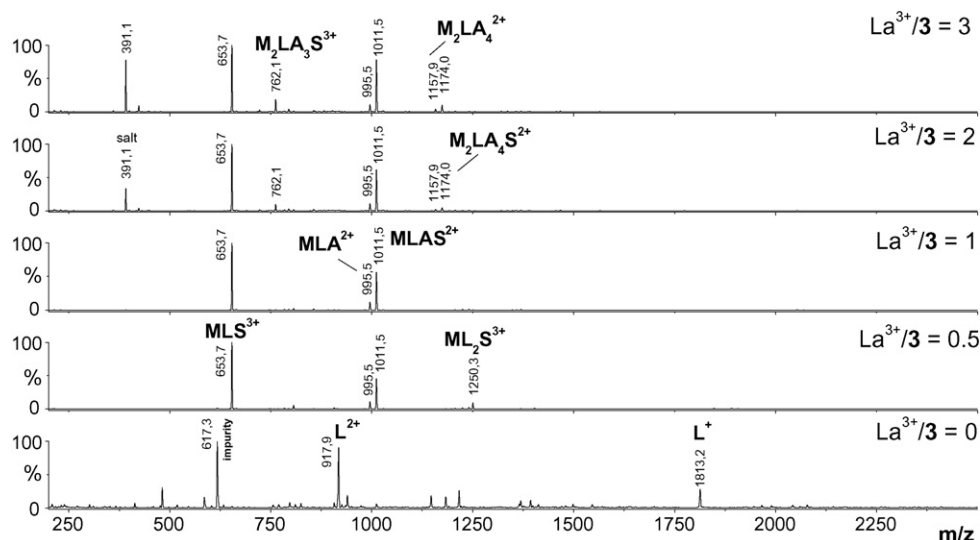


Fig. 5. ESI-MS titration of **3** ($C_L = 10^{-4}$ M) with $\text{La}(\text{NO}_3)_3$, in methanol. M stands for lanthanum, L for ligand **3**, A for nitrate ion and S for methanol.

the lanthanum salt. As mass spectrometry is a very sensitive technique, minor species like ML_2 , M_2L_3 or M_3L_2 can also be observed in very low quantities. They could not be found in UV spectrophotometric studies, most probably because this technique gives a global response and is not able to separate signals for each complex, especially when they are very similar. The replacement of pentyl groups of **2c** by propyl groups to give **2b** does not lead to significant changes in the mass spectra (see Table S2 and Fig. S1 of the supplementary data section). These chains do not play an important role in the complexation; both ligands behave similarly forming ML and M_2L species as shown by ESI-MS and UV spectrophotometric studies. Our findings confirm previous investigations for ligand **2b** [22]. Ligand **3** shows a very different behaviour (Fig. 5).

At all ratios, ML species are predominant in agreement with spectrophotometry. The *N*-methylation of the amides seems to inhibit the fixation of a second La^{3+} . Ligand **4b** shows formation of an ML complex in ESI-MS (Table S2) as in UV spectrophotometry. As the CMPO chains are attached to the lower rim, the distances between the chains can be smaller than in the case of calixarenes **2b**, **2c** and **3**, thus providing less space for the fixation of a second La^{3+} cation. Addition of LaCl_3 to ligand **4b** leads to similar mass spectra (Table S2).

SPECFIT software [27] allows to simulate distribution curves of the different complexes using stability constants and the total concentration of the ligand. We examined if direct quantitative exploitation of the electrospray mass spectra without using any spectrophotometric data gives similar results. Fig. 6 shows as an example the distribution curves of calixarene **2b** established separately by both techniques.

ESI-MS mainly shows the formation of two different complexes: an ML complex with a maximum at one equivalent of lanthanum and M_2L being predominant at higher $La^{3+}/2b$ ratios. The distribution curves of calixarene **3** (Fig. 7) are in strong contrast to those of **2b**. There is evidence for only the ML complex as major species. The slight difference between the ML curve from ESI-MS and from UV-vis can be explained by the fact

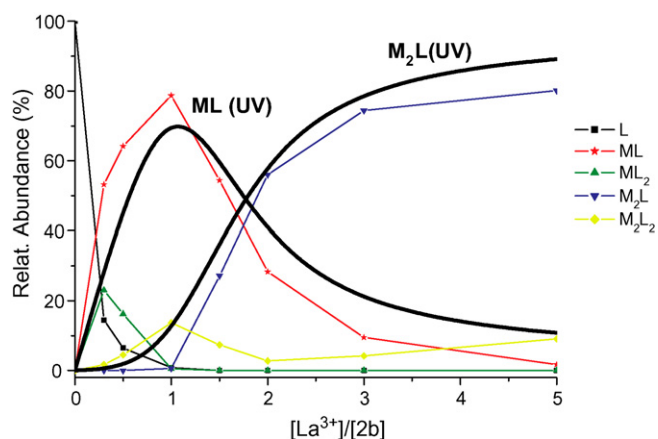


Fig. 6. Distribution curves of the system **2b**/La(NO₃)₃ in methanol ($C_L = 10^{-4}$ M) calculated from ESI-MS data and from UV spectrophotometric data (bold lines) corresponding to $\log \beta_1 = 6.0$, $\log \beta_2 = 10.6$.

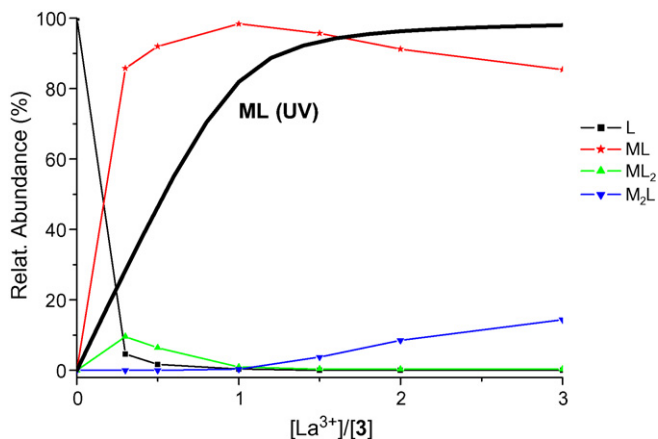


Fig. 7. Distribution curves of the system $3/\text{La}(\text{NO}_3)_3$ in methanol ($C_L = 10^{-4}$ M), calculated from ESI-MS data and from UV spectrophotometric data (bold lines) corresponding to $\log \beta_1 = 5.4$.

that the free ligand is neutral and thus cannot be seen directly in mass spectrometry. This leads to the phenomenon that from the moment on when a given complex is detected as the only species in the spectrum, its relative abundance will very soon get close to 100%. Thus, the quantitative exploitation of ESI-MS to establish distribution curves is especially interesting when there are several charged species present in equilibrium in the solution.

4. Conclusions

In the case of ligand **1**, UV spectrophotometric measurements in methanol show for the first time the formation of ML, ML₂ and ML₃ species with LaCl₃, whereas they could not be interpreted with La(NO₃)₃. The same stoichiometries are also observed in this solvent by ESI-MS studies for the major species. This technique could also provide additional structural information: for La(NO₃)₃ mainly singly charged species are observed (coordination of two NO₃[−]), while for LaCl₃, mainly doubly charged complexes are detected (coordination of one Cl[−]). The presence of two NO₃[−] ions and only one Cl[−] around the cation may explain why the complexes are less solvated in nitrate than in chloride media.

With the CMPO derivatives of calix[4]arene the same stoichiometries have been established by ESI-MS and UV spectrophotometry for the complexation of La(NO₃)₃, i.e. ML and M₂L with the wide rim derivatives **2b** and **2c** and ML for the wide rim **3** and the narrow rim **4b** derivatives.

The complex stability and the selectivity also depend on the position of the CMPO moieties on the calixarene scaffold, the nature of the medium and the mobility of the ligands. Among the 1:1 complexes the most stable are those formed with the wide rim derivatives in chloride medium. Their stability decreases remarkably in the series in contrast to the stability of the complexes with CMPO which “classically” increases with the electronic density on the cations.

Distribution curves, simulated from data obtained from both techniques, exhibit an excellent agreement. This is most interesting in cases where UV spectrophotometry cannot be applied due to limited differences in spectral properties of the species, as for instance in the case of large protein complexes. ESI-MS can also give precious information on the structure of complexes, like coordination of counterions or solvent molecules.

Acknowledgements

This work has been partially financed by the European Commission in the framework of the research programme on “Management and storage of radioactive waste” (Contracts No. F14W-CT96-0022). H.H. thanks the European Research Training Group 532 (GRK 532) for financing his PhD thesis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.talanta.2007.05.038](https://doi.org/10.1016/j.talanta.2007.05.038).

References

- [1] L. Cecille, M. Casarci, L. Pietrelli (Eds.), *New Separation Chemistry Techniques for Radioactive Waste and other Specific Applications*, Elsevier Applied Science, London, 1991.
- [2] J.F. Dozol, V. Böhmer, M.A. McKervey, F. Lopez-Calahorra, D. Reinhoudt, M.J. Schwing, R. Ungaro, G. Wipff, European Commission, Nuclear Science and Technology, Report EUR 17615 EN 1997; F. Arnaud-Neu, V. Böhmer, B. Casensky, A. Casnati, J.-F. Desreux, B. Gruner, C. Grüttner, J. De Mendoza, G. Pina, P. Selucky, W. Verboom, G. Wipff, *Selective Extraction of Minor Actinides from High Activity Liquid Waste by Organised Matrices (CALIXPART FIKW-CT-2000-00088)*, Final Technical Report no. 6466, March 31, 2004, available at <http://dbs.cordis.lu>.
- [3] F. Arnaud-Neu, V. Böhmer, J.F. Dozol, C. Grüttner, R.A. Jakobi, D. Kraft, O. Mauprivez, H. Rouquette, M.J. Schwing-Weill, N. Simon, W. Vogt, *J. Chem. Soc., Perkin Trans. 2* (1996) 1175–1182.
- [4] S.E. Matthews, M. Saadioui, V. Böhmer, S. Barbosa, F. Arnaud-Neu, M.J. Schwing-Weill, A. Garcia Carrera, J.F. Dozol, *J. Prakt. Chem.* 341 (1999) 264–273.
- [5] L.H. Delmau, N. Simon, M.J. Schwing-Weill, F. Arnaud-Neu, J.F. Dozol, S. Eymard, B. Tournois, V. Böhmer, C. Grüttner, C. Musigmann, A. Tunayar, *Chem. Commun.* (1998) 1627–1628.
- [6] L.H. Delmau, N. Simon, M.J. Schwing-Weill, F. Arnaud-Neu, J.F. Dozol, S. Eymard, B. Tournois, C. Grüttner, C. Musigmann, A. Tunayar, V. Böhmer, *Sep. Sci. Technol.* 34 (1999) 863–876.
- [7] F. Arnaud-Neu, S. Barbosa, V. Böhmer, F. Brisach, L. Delmeau, J.F. Dozol, O. Mogck, E.F. Paulus, M. Saadioui, *Aust. J. Chem.* 56 (2003) 1113–1119.
- [8] C. Schmidt, M. Saadioui, V. Böhmer, V. Host, M.R. Spirlet, J.F. Desreux, F. Brisach, F. Arnaud-Neu, J.F. Dozol, *Org. Biomol. Chem.* 1 (2003) 4089–4096.
- [9] S. Barbosa, A.G. Carrera, S.E. Matthews, F. Arnaud-Neu, V. Böhmer, J.F. Dozol, H. Rouquette, M.J. Schwing-Weill, *J. Chem. Soc., Perkin Trans. 2* (1999) 719–723.
- [10] F. Arnaud-Neu, S. Barbosa, D. Byrne, L.J. Charbonnière, M.J. Schwing-Weill, G. Ulrich, in: G.J. Lumetta, R.D. Rogers, A.S. Gopalan, (Eds.), *Calixarenes for Separations*, Symposium Series No. 757, American Chemical Society, 2000, pp. 150–164 (Chapter 12).
- [11] B. Hasenknopf, J.-M. Lehn, N. Boumediene, E. Leize, A. Van Dorsselaer, *Angew. Chem. Int. Ed.* 37 (1998) 3265–3268.
- [12] C. Garcia, J. Guyot, G. Jeminet, E. Leize-Wagner, H. Nierengarten, A. Van Dorsselaer, *Tetrahedron Lett.* 40 (1999) 4997–5000.
- [13] S.M. Blair, E.C. Kempen, J.S. Brodbelt, *J. Am. Soc. Mass Spectrom.* 9 (1998) 1049–1059.
- [14] S. Blanc, P. Yakirevitch, E. Leize, M. Meyer, J. Libman, A. Van Dorsselaer, A.M. Albrecht-Gary, A. Shanzer, *J. Am. Chem. Soc.* 119 (1997) 4934–4944.
- [15] A. Marquis, V. Smith, J. Harrowfield, J.-M. Lehn, H. Herschbach, R. Sanvito, E. Leize-Wagner, A. Van Dorsselaer, *Chem. Eur. J.* 12 (2006) 5632–5641.
- [16] J.-F. Nierengarten, U. Hahn, T. Figueira Duarte, F. Cardinali, N. Soladié, M.E. Walther, A. Van Dorsselaer, H. Herschbach, E. Leize, A.-M. Albrecht-Gary, A. Trabolsi, M. Elhabiri, *C.R. Chim.* 9 (2006) 1022–1030.
- [17] J.-F. Nierengarten, U. Hahn, A. Trabolsi, H. Herschbach, F. Cardinali, M. Elhabiri, E. Leize, A. Van Dorsselaer, A.-M. Albrecht-Gary, *Chem. Eur. J.* 12 (2006) 3365–3373.
- [18] N. Solladié, M.E. Walther, H. Herschbach, E. Leize, A. Van Dorsselaer, T.M. Figueira Duarte, J.-F. Nierengarten, *Tetrahedron* 62 (2006) 1979–1987.
- [19] U. Hahn, M. Elhabiri, A. Trabolsi, H. Herschbach, E. Leize, A. Van Dorsselaer, A.-M. Albrecht-Gary, J.-F. Nierengarten, *Angew. Chem. Int. Ed.* 44 (2005) 5338–5341.
- [20] D. Meshcheryakov, V. Böhmer, M. Bolte, V. Hubscher-Bruder, F. Arnaud-Neu, H. Herschbach, A. Van Dorsselaer, I. Thondorf, W. Mögelin, *Angew. Chem. Int. Ed.* 45 (2006) 1648–1652.
- [21] E. Leize, A. Jaffrezic, A. Van Dorsselaer, *J. Mass Spectrom.* 31 (1996) 537–544.

- [22] C. Lamouroux, S. Rateau, C. Moulin, *Rapid Commun. Mass Spectrom.* 20 (2006) 2041–2052.
- [23] S. Colette, B. Amekraz, C. Madic, L. Berthon, G. Cote, C. Moulin, *Inorg. Chem.* 41 (2002) 7031–7041.
- [24] S. Colette, B. Amekraz, C. Madic, L. Berthon, G. Cote, C. Moulin, *Inorg. Chem.* 42 (2003) 2215–2226.
- [25] C. Lamouroux, C. Moulin, J.-C. Tabet, C.-K. Jankowski, *Rapid Commun. Mass Spectrom.* 14 (2000) 1869–1877.
- [26] K. Ziat, F. Arnaud-Neu, M.J. Schwing-Weill, M.A. McKervey, *New J. Chem.* (1996) 31–33.
- [27] H. Gampp, M. Maeder, C.J. Meyer, A.D. Zuberbühler, *Talanta* 32 (1985) 257–264.
- [28] E.P. Horwitz, D.G. Kalina, H. Diamond, G.F. Vandegrift, W.W. Schulz, *Solvent Extr. Ion Exchange* 3 (1985) 75–109.
- [29] E.P. Horwitz, K.A. Martin, H. Diamond, L. Kaplan, *Solvent Extr. Ion Exchange* 4 (1986) 449–494.
- [30] W.W. Schulz, E.P. Horwitz, *Sep. Sci. Technol.* 23 (1998) 1191.
- [31] H. Naganawa, H. Suzuki, S. Tachimori, A. Nasu, T. Sekine, *Phys. Chem.* 3 (2001) 2509–2517.
- [32] P. Beudaert, V. Lamare, J.F. Dozol, L. Troxler, G. Wipff, *J. Chem. Soc., Perkin Trans. 2* (1999) 2515–2523.

A simple route to incorporate redox mediator into carbon nanotubes/ Nafion composite film and its application to determine NADH at low potential

Minghua Huang^{a,b}, Heqing Jiang^a, Junfeng Zhai^{a,b},
Baifeng Liu^a, Shaojun Dong^{a,*}

^a State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences,
Changchun, Jilin 130022, PR China

^b Graduate School of Chinese Academy of Sciences, Beijing 10039, PR China

Received 12 January 2007; received in revised form 21 May 2007; accepted 22 May 2007

Available online 31 May 2007

Abstract

Through a new and simple ion-exchange route, two-electron redox mediator thionine has been deliberately incorporated into the carbon nanotubes (CNTs)/Nafion composite film due to the fact that there is strong interaction between any of two among the three materials (ion-exchange process between thionine and Nafion, strong adsorption of thionine by CNTs, and wrapping and solubilizing of CNTs with Nafion). The good homogenization of electron conductor CNTs in the integrated films provides the possibility of three-dimensional electron conductive network. The resulting integrated films exhibited high and stable electrocatalytic activity toward NADH oxidation with the significant decrease of high overpotential, which responds more sensitively more than those modified by thionine or CNTs alone. Such high electrocatalytic activity facilitated the low potential determination of NADH (as low as -0.1 V), which eliminated the interferences from other easily oxidizable species. In a word, the immobilization approach is very simple, timesaving and effective, which could be extended to the immobilization of other cationic redox mediators into the CNTs/Nafion composite film. And these features may offer potential promise for the design of amperometric biosensors.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Redox mediator; Carbon nanotube; Nafion; NADH oxidation; Low potential determination

1. Introduction

The electrochemical oxidation of β -nicotinamide adenine dinucleotide (NADH) plays a vital role in developing amperometric biosensors or biofuel cells that use dehydrogenase-dependent enzymes, due to its ubiquitous use as cofactor in over 300 enzymatic reactions of NAD^+/NADH -dependent dehydrogenases [1]. However, the direct oxidation at bare electrodes in neutral media often requires high overpotential as large as 1.1 V versus SCE [2], which limits the selectivity of the determination in real samples. Moreover, the strong adsorption of not only reduced cofactor NADH itself but also of its oxidation

products NAD^+ leads to electrode surface fouling, ultimately producing a system which lacks sensitivity and stability [3]. A common approach to overcome the inherent difficulties is to modify the bare electrodes with suitable electrocatalysts that can facilitate the interfacial electron transfer kinetics. In this way, a number of electrocatalytic systems toward NADH oxidation have been identified [4–13]. For example, Wang's group [4,5] have demonstrated that carbon nanotubes (CNTs) modified electrodes exhibited good electrocatalytic activities toward NADH oxidation with the decrease of high overpotential and little electrode fouling. In addition, two-electron mediator phenothiazine derivatives have also been extensively used as the electrocatalysts for NADH oxidation by immobilizing them onto electrode surfaces using various methods, such as adsorption [14], covalent binding [15], and electropolymerization [16]. Despite the extensive work carried out, some difficulties remained. In many

* Corresponding author. Tel.: +86 431 5262101; fax: +86 431 5689711.
E-mail address: dongsj@ciac.jl.cn (S. Dong).

cases, the decrease of the overpotential is not sufficiently low to eliminate interferences from other easily oxidizable species in real samples [17].

Recently, the integration of CNTs and redox mediators has been explored to catalyze some molecules [18]. Some progresses have also been made in NADH oxidation reaction [19,20]. The integration of CNTs and redox mediators into polymeric matrix has been demonstrated to provide remarkable synergic electrocatalytic activities for NADH oxidation with long-term stability and a significant decrease in the overpotential [20]. The detection potential as low as -0.1 V was obtained in which no interference was observed, indicating high selectivity toward NADH. However, in this case, redox mediators, such as Toluidine Blue O and Azure A were covalently attached to a polymer chitosan with good film-forming ability by the formation of Schiff bases using glutaric dialdehyde as a tether and then interspersed with CNTs to form composite film for amperometric determination of NADH. Obviously, this covalent route was complicated and time-consuming, involving multistep synthetic procedures.

In the present work, a simple ion-exchange strategy was used to immobilize redox mediator phenothiazine derivative thionine into the CNTs/Nafion composite film. The choice of CNTs/Nafion composite is based on the following considerations. Nafion (a perfluorosulfonated cation-exchange polymer with good film-forming ability) displays unique ion-exchange property compared to another good membrane material chitosan, which can easily exchange positively charged ions, such as phenothiazine derivatives into Nafion films [21,22]. But the compact Nafion film is unfavorable for the analyte to diffuse in, and the rate of charge transfer was relatively low in pure Nafion film. Recently, it was reported that polymer Nafion has the ability to solubilize CNTs [23]. The resulting CNTs/Nafion composite film exhibited faster diffusion of the analyte and enhanced charge transport compared to the pure Nafion film [24]. In addition, it has been recently reported that CNTs showed a strong tendency to adsorb thionine molecules through donor–acceptor interaction and also π – π stacking force between these two kinds of conjugated frames [7,25]. Therefore, cationic dye thionine could be easily immobilized in the CNTs/Nafion composite film, due to the ion-exchange ability of Nafion and also the strong adsorption of CNTs. This immobilization approach is very simple, effective, and timesaving.

It is noted that the deliberate combination of thionine, CNTs, and Nafion is very interesting. There is strong interaction between any of two among the three materials (ion-exchange process between thionine and Nafion, strong adsorption of thionine by CNTs and wrapping and solubilizing of CNTs with Nafion), which will result in the homogenization of the three materials. And the good homogenization of CNTs within them may offer the possibility of three-dimensional electron conductive network. The resulting integrated films of thionine, and CNTs/Nafion exhibit high electrocatalytic response with the significant decrease of high overpotential, which responds more sensitively than those modified by thionine or CNTs alone. The kinetics investigation of NADH oxidation at such modified electrode indicates the formation of an intermediate charge

transfer complex. The analytical performance for amperometric detection of NADH at low-detection potential -0.1 V has been studied.

2. Experimental

2.1. Reagents

Phenothiazine derivative thionine and Nafion (perfluorinated ion-exchange resin, 5 wt.% in a mixture of lower aliphatic alcohols and water) were purchased from Aldrich. Reduced NADH was used as received. Multiwalled carbon nanotubes (95%) were purchased from Shenzhen Nanotech. Port. Co., Ltd. (Shenzhen, China), and were further purified before use. All other reagents were of analytical reagent grade and used as received. Water used for preparation of aqueous solution was purified using Millipore-Q water purification system. Buffer solutions were $\text{KH}_2\text{PO}_4 + \text{NaHPO}_4$ ($\text{pH} = 7.40$). The ion strengths of the solutions were adjusted through changing the electrolyte concentration with 0.01, 0.05, 0.10, and 0.50 M, respectively.

2.2. Purification of CNTs

The CNTs were purified by refluxing in 3 M HNO_3 for 48 h, then filtered with $2.5 \mu\text{m}$ pore size membrane with the aid of a pump and thoroughly washed with water to pH ca. 7, finally dried under vacuum at 60°C overnight.

2.3. Preparation of film electrodes

Three milligrams CNTs were dissolved in 1 ml 0.5 wt.% Nafion solutions with ultrasonication for 30–50 min to obtain a homogeneous, well-distributed solution of CNT/Nafion composite. The glassy carbon electrode (GCE) was cast a $12 \mu\text{l}$ aliquot of 0.5 wt.% pure Nafion solution or 3 mg/ml CNTs/Nafion suspension. The solvent in Nafion solution was allowed to evaporate under an infrared lamp. The resulting film was calculated to be ca. $3 \mu\text{m}$. After the solvent was evaporated, pure Nafion film or uniform film containing a network of CNTs was formed. Then, thus-prepared modified electrodes were immersed in 1 mM thionine dissolved in dimethylformamide (DMF) for 40 min, resulting in the incorporation of thionine. The modified electrodes were then soaked in DMF for 15 min, and thoroughly rinsed with water to remove the physically absorbed material, and dried with high purity nitrogen stream.

2.4. Instrumentation and measurements

Cyclic voltammetric experiments were performed with a CHI 660 electrochemical workstation (USA). An EG & PARC model 636 rotating ring-disk electrode system was used for rotating disk electrode (RDE) voltammetry experiments. All measurements were made at in a conventional three-electrode electrochemical cell using GCE (4 mm diameter) as the working electrode, twisted platinum wire as the auxiliary electrode, and Ag/AgCl as reference electrode. The GCE was polished

with 1.0 and 0.3 μm $\alpha\text{-Al}_2\text{O}_3$ powders, successively and sonicated in water for about 3 min after each polishing step. Finally, the electrode was sonicated in ethanol, washed with ethanol, and dried with high purity nitrogen stream immediately before use.

In steady-state amperometric measurements, the potential was set at -0.10 V in a magnetically stirred solution, and the current-time curves were recorded after a constant background current had been established. Scanning electron micrographs were obtained on an XL30 ESEM FEG field emission scanning electron microscopy.

3. Results and discussion

3.1. Scanning electron microscopy (SEM) measurement

SEM was employed to monitor morphological characteristics of the films. Fig. 1 presents the SEM images for the CNTs/Nafion composite film before (A) and after (B) immersing in thionine solution. From Fig. 1A, it can be seen that the CNTs are well distributed within the film and most of the CNTs are in the form of small bundles with 20–40 nm in diameter. And the profile of individual CNTs bundles is clearly seen. After incorporating thionine into the CNTs/Nafion composite film (Fig. 1B), the CNTs are still homogeneously distributed within the films. But the profile of individual CNTs bundles becomes indiscernible, maybe due to the incorporation of thionine. Moreover, there is no observation that thionine clusters in a distinct zone, which might be miscible throughout the matrix. It is noted that there is

strong interaction between any of two among the three materials (ion-exchange process between thionine and Nafion, strong adsorption of thionine by CNTs, and wrapping and solubilizing of CNTs with Nafion), which would lead to good homogenization of the three materials. And the good dispersion of electron conductor CNTs within them and highly intimate contact with thionine provides the possibility of three-dimensional electron conductive network, which would improve the charge transport.

3.2. Electrochemical characterization of the integrated film of thionine and CNTs/Nafion

Cyclic voltammetry was used to investigate the electrochemical behaviors of thionine incorporated in CNTs/Nafion composite film. After immersing in thionine solution for a certain period of time, the CNTs/Nafion composite film modified electrode exhibits a pair of distinct redox peak due to the redox of thionine at the formal potential (E_f) of ca. 0.26 V when it was cycled in 0.5 M H_2SO_4 solution (Fig. 2A) [7]. This result indicates that a large amount of thionine has been successfully incorporated into the CNTs/Nafion composite film. The modified electrode shows the characteristics of chemically reversible surface redox electrochemistry with a very small peak-to-peak

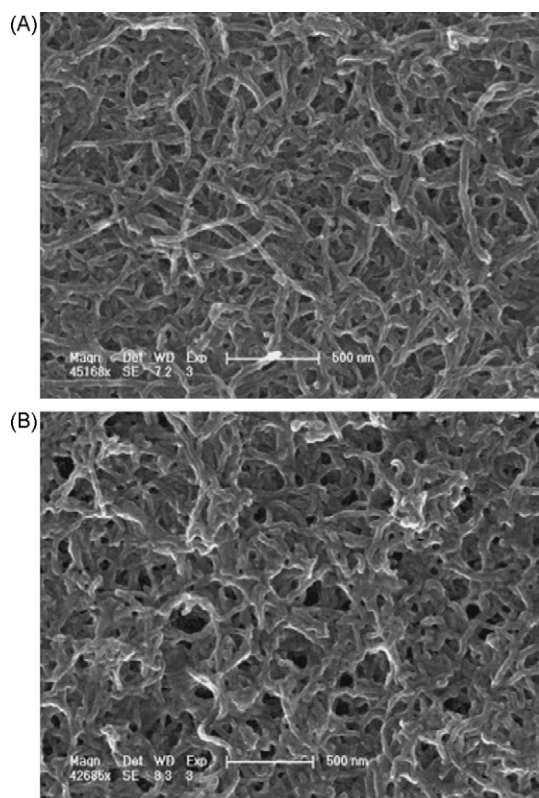


Fig. 1. Typical SEM images for the CNTs/Nafion composite film before (A) and after (B) immersing in thionine solution. The scale bar is 500 nm.

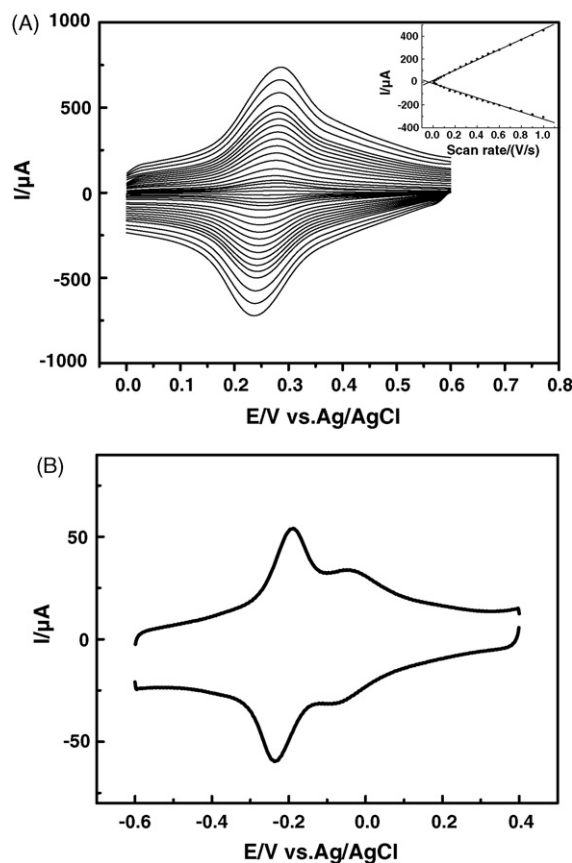
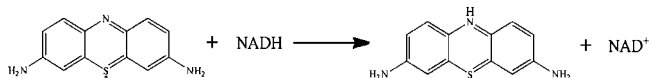


Fig. 2. (A) CVs of the integrated film of thionine and CNTs/Nafion with different scan rates: 10, 30, 50, 70, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, and 1000 mV s^{-1} in 0.5 M H_2SO_4 solution. The inset shows the relationship of oxidation peak and reduction current vs. scan rates, respectively. (B) CVs of the integrated film in 0.5 M pH 7.40 phosphate buffer solutions at scan rate of 50 mV s^{-1} .



Scheme 1. Scheme of the redox reaction between thionine and NADH.

separation. A plot of the cathodic and anodic peak currents as a function of scan rate is linear up to 1000 mV s^{-1} (the inset of Fig. 2A), suggesting facile surface-confined charge transfer kinetics. When the modified electrode was scanned in 0.5 M phosphate buffer (pH=7.40), two couples of redox peaks with the formal potential (E_f) of -0.06 and -0.21 V observed in Fig. 2B are attributed to pH effects. This has been demonstrated by previous reports [26]. With increasing pH of the supporting electrolyte, the formal potential of the redox couple shifts negatively. In pH=7.40 phosphate buffer, the redox peak currents are also proportional to the scan rate (figure not shown). In addition, the modified electrode was found to be stable during the whole electrochemical measurements even when continuous cycling between -0.4 and 0.6 V for 1 h. This facile electron accessibility and good stability of the integrated film offered the possibility to electrochemical catalysis with high and stable activities.

3.3. Electrocatalytic activities toward NADH oxidation on the modified electrodes

Here, the electrocatalytic activity of the integrated film of thionine and CNTs/Nafion for NADH oxidation was investigated. The redox reaction between thionine and NADH is presented in Scheme 1. In this case, NADH is oxidized by

the surface-confined thionine mediator. For comparison, the electrocatalytic oxidation of NADH at different modified electrodes is shown in Fig. 3. Cyclic voltammograms (CVs) in 0.5 M phosphate buffer solution (pH 7.40) with (solid line) and without 1.0 mM NADH (dotted line) are presented at a pure Nafion-modified electrode before (Fig. 3A) and after immersing in thionine solution (Fig. 3B), and a CNTs/Nafion composite film-modified electrode before (Fig. 3C) and after immersing in thionine solution (Fig. 3D). As expected, the pure Nafion-modified electrode did not show any characteristic voltammogram for the oxidation of NADH in the potential range used in the investigation (Fig. 3A, curve a), indicating that Nafion was electrochemically silent structural matrix. After exchanging thionine into the pure Nafion film, the Nafion/thionine film exhibited two couples of poorly defined peak with the formal potentials (E_f) of -0.04 and -0.18 V with the peak-to-peak separations ca. 85 mV (Fig. 3B, curve b1). Upon the addition of 1.0 mM NADH, there is a new broad anodic peak at 0.19 V on the CV (curve b), which indicates the thionine-mediated oxidation of NADH. In this mediation process, the NADH is oxidized by the surface-confined thionine, and the reduced thionine molecule is subsequently reoxidized at the electrode surface. The curve c in Fig. 3C shows that the oxidation of NADH at the CNTs/Nafion composite film-modified electrode yielded a current peak at 0.13 V . Compared to the pure Nafion film-modified electrode (Fig. 3A, curve a), such a large decrease in the overpotential for the oxidation of NADH may be ascribed to the high local density of electronic states in CNTs [6].

After incorporating thionine into the CNTs/Nafion composite film, it can be seen that there is two distinct redox peaks at -0.06

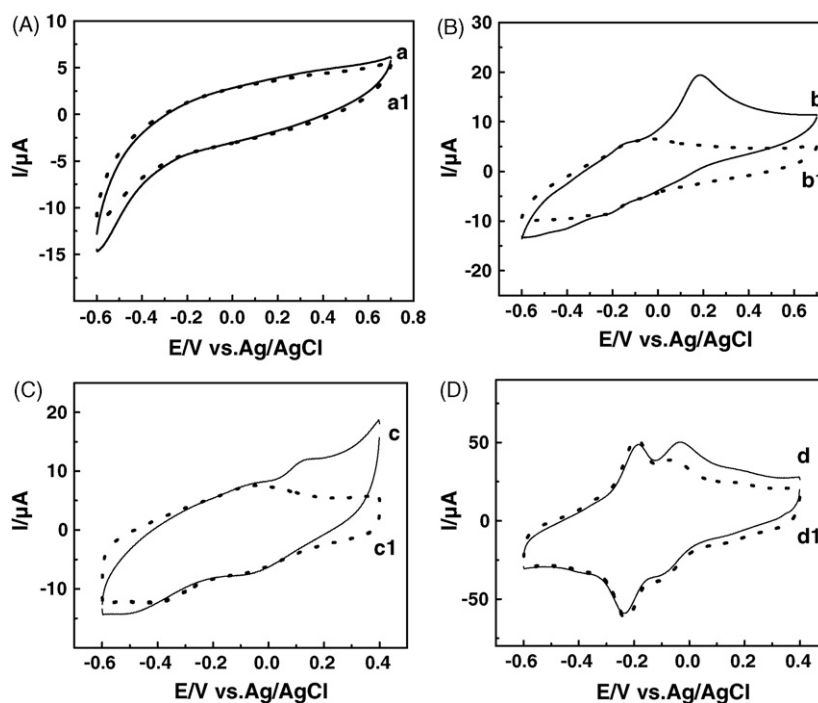


Fig. 3. CVs recorded in 0.5 M pH 7.40 phosphate buffer solution containing 1.0 mM NADH at the pure Nafion-modified electrode before (a) and after immersing in thionine solution (b), and the CNTs/Nafion composite film-modified electrode before (c) and after immersing in thionine solution (d). Background voltammograms for the corresponding film electrodes a1, b1, c1, and d1 were recorded in 0.5 M pH 7.40 phosphate buffer. Scan rate: 50 mV s^{-1} .

and -0.21 V due to the redox of thionine (Fig. 3D, curve d1). The peaks at -0.21 V were less separated with peak-to-peak separations being around 45 mV. Compared to the Nafion/thionine film (Fig. 3B, curve b1), the background current was enhanced after the interfusion of CNTs. This is most likely due to a larger electrode area undergoing double layer charging and background reactions [27]. And the peak current of thionine obtained on the CNTs/Nafion composite film-modified electrode at -0.21 V was almost 11 times larger than those yielded by pure Nafion films at -0.18 V (Fig. 3B, curve b1), while the active electrode area increased only ca. two times. In comparing these results, it becomes evident that the increase of the peak currents of thionine was not just due to the extra surface area provided by the CNTs for the redox of thionine [20]. In presence of NADH, a catalytic oxidation peak current starts at about -0.10 V with the peak potential at ca. -0.03 V (Fig. 3D, curve d), which shifts ca. 220 mV less positive than that of the Nafion/thionine film (Fig. 3B, curve b). Such a large decrease in the overpotential could not be ascribed to the direct oxidation of NADH at CNTs, and also not explained by the shift of original thionine peaks [20]. These indicate there is a positive synergy in the integrated films of thionine and CNTs/Nafion and the interfusion of CNTs facilitated the thionine-mediated oxidation of NADH. Such enhancement in the redox behavior of thionine and thionine-mediated oxidation of NADH caused by the introduction of electron conductor CNTs was likely due to the fact that the good dispersion of CNTs into the sensing film and highly intimate contact with thionine provided three-dimensional electron conductive network, and thus facilitated the charge transport.

3.4. Kinetic analysis of NADH oxidation on the integrated film of thionine and CNTs/Nafion

Rotating disk electrode (RDE) voltammetry technique was used for evaluating the kinetics of the reaction between NADH and the integrated film of thionine and CNTs/Nafion in order to obtain further information on the electrocatalytic system. Fig. 4A reveals current–potential curves of NADH oxidation at various rotation rates using the rotating glassy carbon disk electrode modified with the integrated films. It can be seen that potential-independent plateau currents are present at all rotation rate and that the catalytic currents increase gradually with the increase of the electrode rotation rate. Also, with increasing the concentration of NADH (from 0.10 to 0.50 mM) at a fixed rotation rate, the catalytic currents increase gradually (the inset of Fig. 4A). The corresponding Levich (figure not shown) and Koutecky–Levich plot (Fig. 4B) are obtained for the electrocatalytic oxidation of NADH in the presence of different amounts of NADH. The clear lack of linearity in the Levich plot suggests that the catalytic reaction is limited not by diffusion but by kinetics. Hence, it can be concluded that the rate-limiting step in this process is related to the oxidation of NADH at the composite film electrode. Under these conditions, the Koutecky–Levich plots can be used to determine the apparent rate constant (k_1) for the catalytic reaction between the films electrode and NADH (Fig. 4B) [28,29]. As expected, for several different NADH concentrations, the linearity was obtained between the inverse of the

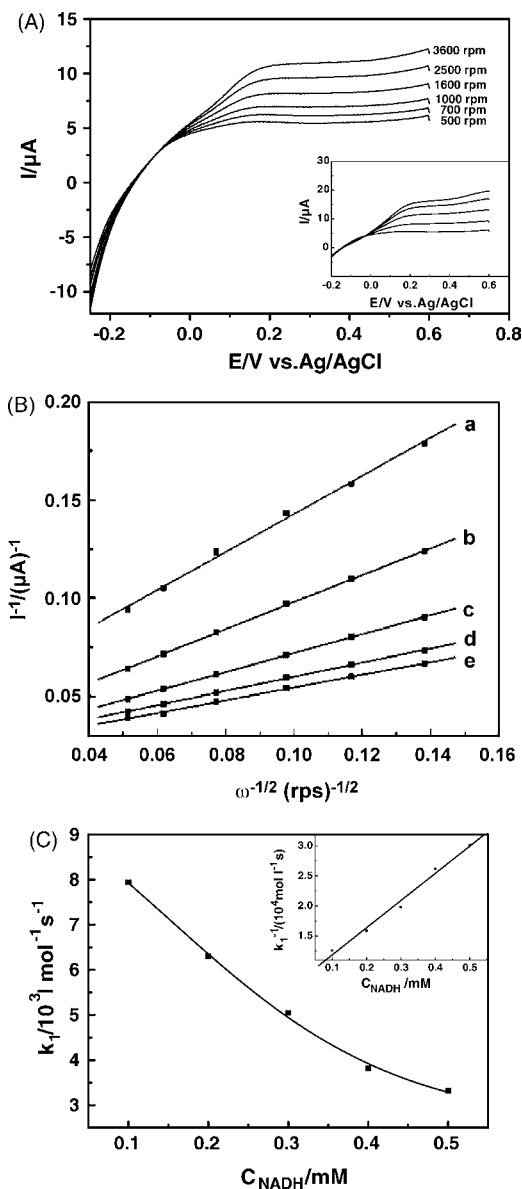


Fig. 4. (A) Current–potential curves of NADH oxidation at a rotating disk electrode modified with the integrated film with different rotating rate in 0.10 mM NADH. Inset shows current–potential curves with different NADH concentrations from bottom to top: 0.10, 0.20, 0.30, 0.40, and 0.50 mM at a fixed rotation rate 500 rpm. Koutecky–Levich plot (B) of the kinetic limiting currents of the voltammograms for the electrooxidation of (a) 0.10, (b) 0.20, (c) 0.30, (d) 0.40, and (e) 0.50 mM NADH. (C) Dependence of the rate constant k_1 and (inset) k_1^{-1} on the NADH concentration. Supporting electrolyte: 0.5 M phosphate buffer (pH 7.40).

limiting current and the inverse of the square root of the rotating rate (Koutecky–Levich plot).

From the intercepts of the Koutecky–Levich plots, the k_1 values at the different concentrations of NADH were determined as shown in Fig. 4C. It can be seen that the k_1 values decrease significantly with the increase of the NADH concentration. Such a concentration dependence of the rate constant indicates that the electrocatalytic oxidation reaction at the integrated film electrode supports the Michaelis–Menten (MM) model proposed by Gorton et al. [30]. The thionine–NADH complex is formed as an

intermediate that dissociates to produce the reduced thionine and NAD^+ , which has been observed in other modified electrodes with different redox mediators for the electrocatalytic oxidation of NADH [31]. The CNTs introduced into the Nafion/thionine film may make the electron transfer through the film easier and facilitate the thionine-mediated oxidation of NADH. This observation also provides additional evidence that the reaction between NADH and the catalyst is the sole rate-limiting step of the overall chemical reaction [12,30,31].

A plot of reciprocal apparent constant as a function of the concentration of NADH is linear as shown in the inset of Fig. 4C. The excellent correlation allows us to conclude that the electrooxidation of NADH at the modified electrode proceeds via the initial formation and concurrent rate-limiting decomposition of the intermediate complex between the catalyst and NADH. According to the slope and the intercept of this plot, the apparent Michaelis–Menten constant K_M and the rate constant for the charge transfer complex dissociation reaction k_{+2} can be evaluated. The values are found to be $1.62 \times 10^{-4} \text{ M}$ and 2.21 s^{-1} , respectively. The rate constant k_1 value obtained from the intercept of this plot is $1.36 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, which is comparable with those reported previously with such a catalytic functionality [12]. In addition, other plots could also be used as diagnostic criteria of such a MM model [30,31]. The plot of i_K against the concentration of NADH shows the characteristic MM shape. The double-reciprocal plot of these data results in a straight line, again indicating the electrooxidation of NADH proceeds via an intermediate complex.

3.5. Amperometric characterization of the modified electrodes

Such high electrocatalytic activity facilitates low potential amperometric determination of NADH at the integrated thionine and CNTs/Nafion composite film. For better comparison, Fig. 5A shows the typical steady-state current-time responses at the different modified electrodes on successive addition of 0.10 mM NADH at an applied potential -0.1 V in a magnetically stirred 0.5 M buffer solution (pH 7.40). As expected from the above voltammetric data, the pure Nafion film is not responsive to the oxidation of NADH using this low-detection potential (curve b). Almost no significant amperometric responses were also observed at the CNTs/Nafion film (curve a) and the Nafion/thionine film (curve c). From curve d, it can be seen that thionine exchanged within the CNTs/Nafion composite film responded significantly and rapidly to the changes in NADH concentrations. The current steps for each addition of NADH demonstrate a stable and efficient catalytic property of the electrocatalysts immobilized in the films. The time required to achieve 95% of the steady-state current is less than 10 s after the addition of NADH, which is much faster than that of the Nafion/thionine film (more than 30 s). Such a fast response may reflect the increased charge transport in the integrated films after the interfusion of CNTs. Based on the above results of CVs and steady-state plots, the current response at the integrated film of thionine and CNTs/Nafion is higher than those at individual Nafion/thionine or CNTs/Nafion films, which indicates that

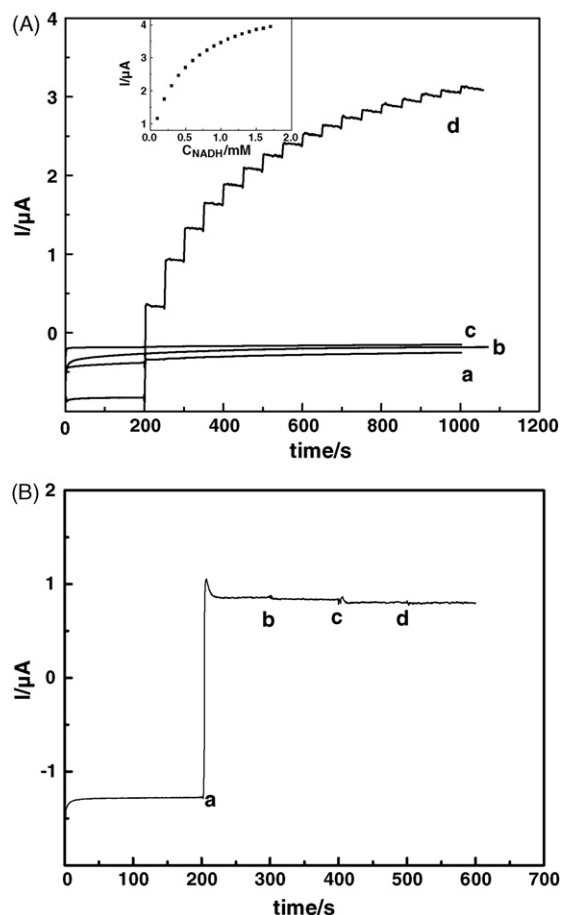


Fig. 5. (A) Typical steady-state response at (a) the CNTs/Nafion modified electrode, (b) the pure Nafion-modified electrode, (c) the Nafion/thionine modified electrode, and (d) the integrated film of thionine and CNTs/Nafion modified electrode upon successive addition of 0.10 mM NADH. Inset shows the calibration plot of the integrated film. (B) Amperometric responses of the integrated film upon successive addition of (a) 0.50 mM NADH, (b) 0.10 mM acetaminophen, (c) 0.10 mM dopamine, and (d) 0.10 mM ascorbic acid. Supporting electrolyte: 0.5 M phosphate buffer (pH 7.40); Applied potential: -0.1 V .

the synergistic amplification effects are present in the thionine immobilized into the CNTs/Nafion composite films-modified electrode. The composite film electrode displayed a sensitive (4.70 mA M^{-1}) and fast response (ca. 10 s) to NADH. The linear range of NADH is from 2 to $400 \mu\text{M}$ with a dynamic range up to 10 mM. The films provided a low-detection limit of $1 \mu\text{M}$ estimated at a signal-to-noise ratio of 3. Also, the reproducibility of the integrated film of thionine and CNTs/Nafion based NADH sensor was estimated by five measurements of 0.1 mM NADH under the applied potential of -0.1 V . The calculated relative standard deviation is found to be 4.0%. These results are better than those reported for other amperometric NADH sensors [12,32]. For example, although the reported highly boron-doped electrodes exhibited high sensitivity for NADH with the amperometric detection limit of 10 nM, the linear concentration range was rather limited (10–500 nM) [32]. Moreover, the detection potential was as high as 0.60 V (versus SCE). Recently, the products of electrochemical oxidation of 5-hydroxytryptophan can be used as catalyst for NADH oxidation, displaying the

low-detection limit of 27 nM [12]. However, the linear response was found between 0.05 and 6 μ M under the applied potential of 0.13 V (versus Ag/AgCl). The dynamic concentration range extended only to below 1 mM.

The selectivity advantage of the composite film electrode is demonstrated in Fig. 5B, which presents amperometric responses for relevant physiological levels of NADH, acetaminophen, dopamine, and ascorbic acid. Amperometric responses were obtained by a batch addition of interfering species from other redox-active molecules (0.10 mM acetaminophen, 0.10 mM dopamine, and 0.10 mM ascorbic acid) after the 0.50 mM NADH addition at the potential of -0.1 V. As seen from Fig. 5B, acetaminophen, dopamine and ascorbic acid exhibited no responses, whereas a large current response was observed for NADH. Therefore, the low-detection potential of the composite film electrode (-0.1 V) eliminated the interferences from these easily oxidizable species presented in the real samples, indicating high selectivity toward the NADH substrate. Further investigation into the application to real samples is underway. In addition, the CNTs-based modified electrodes exhibited relatively good stability, which is comparable to that of the reference [4]. As is well known, the bare GCE displays a rapid decay of the NADH response currents with a nearly complete electrode passivation from the strong adsorption of reaction intermediates, such as NAD^+ [4]. In contrast, the response of the composite film electrode to elevated levels of NADH remains stable during the entire experiment, with only 2, 8, and 17% current depressions at 10, 30, and 60 min, respectively, indicating a good resistance of the modified electrode to surface fouling. This may benefit from the coupling of the efficient resistance of CNTs to fouling and the antifouling/discriminative properties of Nafion films [4,23]. Also, the storage stability of the modified electrode was investigated by comparing the changes in amperometric response of before and after storing the modified electrode at 4°C in a refrigerator for 1 day. The sensor still retained about 88% of its original sensitivity. However, the long-term stability is not as good as in the reference [20]. In that case, Toluidine Blue O has been covalent with chitosan through glutaric dialdehyde, which must bear complicated synthetic process. It is well known that the covalent interaction is stronger than electrostatic attraction and π – π stacking. So it is reasonable that our stability is not better than theirs, maybe due to some confined thionine desorbed from the film during the long-term stability measurement.

We also note that the electrocatalytic activity of the integrated film of thionine and CNTs/Nafion can be tuned by changing ion strength of supporting electrolyte. Fig. 6 compares the amperometric responses at the integrated film on successive addition of 0.10 mM NADH in various electrolyte concentrations. We can see that the amperometric currents grow gradually with increasing the ion strength of supporting electrolyte. It is well known that a mobility of the counterions of the supporting electrolyte is necessary for the charge transport or to keep the electroneutrality at the electrode surface during the redox process. So, higher ion strength will produce positive effect on faster charge transport. The inset of Fig. 6 compares four calibration plots corresponding to the amperometric response of the integrated

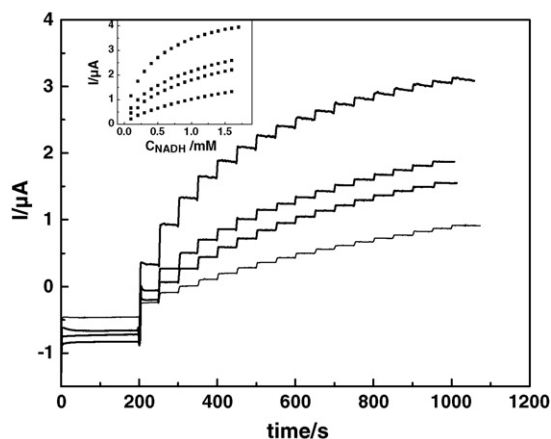


Fig. 6. Amperometric response of the integrated film upon successive addition of 0.10 mM NADH in pH 7.40 phosphate buffer with various electrolyte concentrations: 0.01, 0.05, 0.10, and 0.50 M (from bottom to top), respectively. Inset shows the corresponding calibration plots (from bottom to top). Applied potential: -0.1 V.

films in four different electrolyte concentrations. As seen, the sensitivity of the sensor increases gradually with increasing the ion strength of supporting electrolyte. But it would also reach a saturation state. And in the case we studied, the sensor exhibits the highest response in phosphate buffer solution with the ion strength of 0.5 M. In the above experiments, 0.5 M phosphate buffer solution (pH 7.40) was employed for cyclic voltammetric characterization and amperometric determination of NADH.

4. Conclusions

In this work, through a simple route redox mediator thionine was deliberately immobilized into the CNTs/Nafion composite film matrix to prepare a new electrochemical sensing platform, due to the ion-exchange ability of Nafion and the strong adsorption of CNTs. Benefiting from the coupling of the antifouling/discriminative properties of Nafion with three-dimensional electronic conductivity of CNTs and highly intimate contact with thionine, the integrated thionine and CNTs/Nafion composite film shows a stable and high electrocatalytic response toward the oxidation of NADH at a much lower potential with the amplification of redox mediator thionine-mediated oxidation of NADH. Compared to that of a pure Nafion film, the thionine incorporated into the CNTs/Nafion composite film matrix exhibited higher amperometric signal. Also, the kinetic studies of the electrocatalytic oxidation of NADH at such integrated films indicate that the oxidation reaction proceeds via the formation of an intermediate charge transfer complex. The interfusion of CNTs facilitated such thionine-mediated electrooxidation of NADH. And the high electron-transfer rate constant was obtained. The electrochemical sensor that was studied exhibited good analytical characteristics in terms of rapidity, sensitivity, selectivity, and stability. Additionally, the simple, timesaving and effective approach could be extended to the immobilization of other cationic redox mediators, which provides an excellent sensing platform and have potential applications in NADH transducer of a large group of NAD^+ -

dependent dehydrogenase-based electrochemical biosensors for the detection of species, such as ethanol, lactate and malate.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Nos. 20575064, 20427003).

References

- [1] A.P.F. Turner, I. Karube, G.S. Wilson (Eds.), *Biosensors—Fundamentals and Applications*, Oxford University Press, New York, 1987.
- [2] J. Moiroux, P.J. Elving, *Anal. Chem.* 50 (1978) 1056.
- [3] M.A. Hayes, W.G. Kuhr, *Anal. Chem.* 71 (1999) 1720.
- [4] M. Musameh, J. Wang, A. Merkoci, Y.H. Lin, *Electrochem. Commun.* 4 (2002) 743.
- [5] J. Wang, M. Musameh, *Anal. Chem.* 75 (2003) 2075.
- [6] M.G. Zhang, A. Smith, W. Gorski, *Anal. Chem.* 76 (2004) 5045.
- [7] M.H. Huang, H.Q. Jiang, X.H. Qu, Z.A. Xu, Y.L. Wang, S.J. Dong, *Chem. Commun.* (2005) 5560.
- [8] C.E. Banks, R.G. Compton, *Analyst* 130 (2005) 1232.
- [9] M. Yemini, M. Reches, E. Gazit, J. Rishpon, *Anal. Chem.* 77 (2005) 5155.
- [10] C.R. Raj, B.K. Jena, *Chem. Commun.* (2005) 2005.
- [11] A.A. Karyakin, Y.N. Ivanova, K.V. Revunova, E.E. Karyakina, *Anal. Chem.* 76 (2004) 2004.
- [12] N. de-los-Santos-Álvarez, M.J. Lobo-Castanón, A.J. Miranda-Ordieres, P. Tunón-Blanco, H.D. Abruña, *Anal. Chem.* 77 (2005) 2624.
- [13] (a) J. Liu, S. Tian, W. Knoll, *Langmuir* 21 (2005) 5596;
(b) S. Tian, A. Bara, J. Liu, Z. Wang, W. Knoll, M.K. Park, R. Advincula, *Adv. Funct. Mater.* 12 (2003) 473.
- [14] B. Persson, *J. Electroanal. Chem.* 287 (1990) 61.
- [15] M. Ohtani, S. Kuwabata, H. Yoneyama, *J. Electroanal. Chem.* 422 (1997) 45.
- [16] Q. Gao, X.Q. Cui, F. Yang, Y. Ma, X.R. Yang, *Biosens. Bioelectron.* 19 (2003) 277.
- [17] B. Prieto-Simón, E. Fàbregas, *Biosens. Bioelectron.* 19 (2004) 1131.
- [18] (a) Y. Xu, L. Yang, P.G. He, Y.Z. Fang, *Nanotechnology* 1 (2005) 202;
(b) D.R.S. Jeykumari, S.S. Kumar, S.S. Narayanan, *Pramana. J. Phys.* 65 (2005) 731;
(c) A. Salimi, A. Noorbakhsh, S. Soltanian, *Electroanalysis* 18 (2006) 703;
(d) K. Zhao, H.Y. Song, S.Q. Zhuang, L.M. Dai, P.G. He, Y.Z. Fang, *Electrochem. Commun.* 9 (2006) 65;
(e) A. Salimi, A. Noorbakhash, F.S. Karonian, *Int. J. Electrochem. Sci.* 1 (2006) 435.
- [19] N.S. Lawrence, J. Wang, *Electrochem. Commun.* 8 (2006) 71.
- [20] (a) M.G. Zhang, W. Gorski, *J. Am. Soc. Chem.* 127 (2005) 2058;
(b) M.G. Zhang, W. Gorski, *Anal. Chem.* 77 (2005) 3960.
- [21] V. Ganesan, S.A. John, R. Ramaraj, *J. Electroanal. Chem.* 502 (2001) 167.
- [22] A.R. Guadalupe, K.E. Liu, H.D. Abruña, *Electrochim. Acta.* 36 (1991) 881.
- [23] J. Wang, M. Musameh, Y.H. Lin, *J. Am. Chem. Soc.* 125 (2003) 2408.
- [24] Z.H. Guo, S.J. Dong, *Anal. Chem.* 76 (2004) 2683.
- [25] Q.W. Li, J. Zhang, H. Yan, M.S. He, Z.F. Liu, *Carbon* 42 (2004) 287.
- [26] H.Y. Chen, D.M. Zhou, J.J. Xu, H.Q. Fang, *J. Electroanal. Chem.* 422 (1997) 21.
- [27] P.P. Joshi, S.A. Merchant, Y.D. Wang, D.W. Schmidtke, *Anal. Chem.* 77 (2005) 3183.
- [28] A.J. Bard, L.R. Faulkner, *Electrochemical Methods—Fundamentals and Applications*, John Wiley and Sons, New York, 2000.
- [29] M.H. Huang, Y. Shao, X.P. Sun, H.J. Chen, B.F. Liu, S.J. Dong, *Langmuir* 21 (2005) 323.
- [30] L. Gorton, A. Torstensson, H. Jaegfeldt, G. Johansson, *J. Electroanal. Chem.* 161 (1984) 103.
- [31] F. Pariente, F. Tobalina, G. Moreno, L.E. Hernández, H.D. Abruña, *Anal. Chem.* 69 (1997) 4065 (and references therein).
- [32] T.N. Rao, I. Yagi, T. Miwa, D.A. Tryk, A. Fujishima, *Anal. Chem.* 71 (1999) 2506.

Supramolecular interaction of ethylenediamine linked β -cyclodextrin dimer and berberine hydrochloride by spectrofluorimetry and its analytical application

Fang Liu, Hui-ling Liang, Ke-hua Xu, Li-li Tong, Bo Tang*

College of Chemistry, Chemical Engineering and Materials Science, Engineering Research Center of Pesticide and Medicine Intermediate Clean Production, Ministry of Education, Shandong Normal University, Jinan 250014, PR China

Received 31 January 2007; received in revised form 18 May 2007; accepted 22 May 2007

Available online 3 June 2007

Abstract

The supramolecular interaction of β -cyclodextrin dimer with berberine hydrochloride was studied in aqueous $\text{KH}_2\text{PO}_4\text{--H}_3\text{PO}_4$ buffer solution of pH 2.00 at room temperature by spectrofluorimetry. The apparent association constant of the complex was $1.53 \times 10^4 \text{ L mol}^{-1}$. Based on the significant enhancement of fluorescence intensity of supramolecular sandwich complexes, a spectrofluorimetric method with high sensitivity and selectivity was developed for the determination of berberine hydrochloride in aqueous solution in presence of ethylenediamine linked β -CD dimer. The linear range of the method was $12.8\text{--}1.00 \times 10^4 \text{ ng mL}^{-1}$ with the detection limit 3.6 ng mL^{-1} . There was no interference from the normally used in tablets and serum constituents. The proposed method was successfully applied to the determination of berberine hydrochloride in tablets and serum. And then it has a promising potential in therapeutic drug monitoring, pharmacokinetics and clinical application.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Ethylenediamine linked β -CD dimer; Berberine hydrochloride; Supramolecular interaction; Spectrofluorimetry

1. Introduction

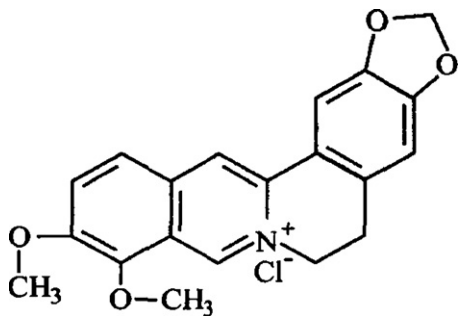
Cyclodextrins (CDs) are a class of cyclic oligosaccharides with six to eight D-glucose units linked by α -1,4-glucose bonds. They possess a hydrophobic cavity which is capable of including a variety of hydrophobic compounds via host–guest complexation [1–3]. But the apparent association constant (K_s) of the complex is not perfect ($10^1\text{--}10^3 \text{ L mol}^{-1}$) [4–6]. It is interesting to note that a cyclodextrin dimer (bis-CDs) connected with a linker can bind diverse molecules to form supramolecular sandwich complexes through the cooperative binding of two adjacent hydrophobic cavities. And these bis-CDs can give much higher binding abilities and molecular selectivity than those exhibited by parent native CDs or mono-modified CDs. The apparent association constant (K_s) of the complex can be up to $10^3\text{--}10^9 \text{ L mol}^{-1}$ [7–12]. Owing to these potential advantages, diverse functional groups, such as alkanedioates [11,12], disulfides [13,14], dipyrindines [15,16] and biquinolines

[17] have been used as the linker between two cyclodextrin units.

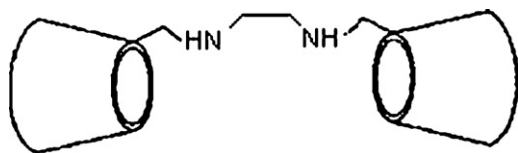
Berberine hydrochloride (Scheme 1), is a kind of isoquinoline alkaloid. It was widely employed as an antibacterial and antifungal drug in pharmaceutical products. At present, the analytical methods applied to the determination of berberine hydrochloride have HPLC [18], reversed-phase high performance liquid chromatography [19] and spectrofluorimetry [20]. Most of the methods need complicated extraction process [21], and also used organic solvent. Berberine hydrochloride could give off strong fluorescence in organic solvent. However, in the aqueous solution, it possesses a low fluorescence quantum yield. Therefore, we need to develop a simple, highly sensitive and selective method without any extraction process for the determination of berberine hydrochloride in the aqueous solution.

In this paper, we reported the supramolecular interaction of ethylenediamine linked β -CD dimer (Scheme 2) [22] with berberine hydrochloride. The molecular model of the host–guest complex was shown in Scheme 3 (it was described with Chem3D Ultra 8.03). When ethylenediamine linked β -CD dimer was added to the aqueous solution of berberine hydrochloride,

* Corresponding author. Tel.: +86 531 86180010; fax: +86 531 86180017.
E-mail address: tangb@sdu.edu.cn (B. Tang).

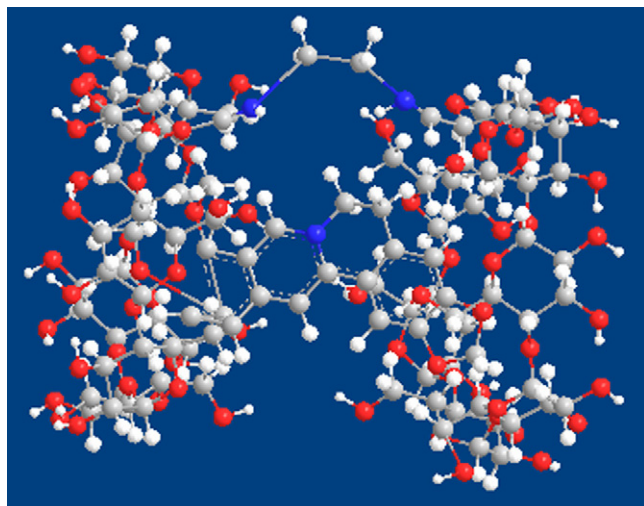


Scheme 1. The structure of berberine hydrochloride.

Scheme 2. The structure of ethylenediamine linked β -CD dimer.

ride, we could observe an obvious increase in fluorescence intensity of berberine hydrochloride. While the fluorescence intensity did not obvious enhance when β -CD was added. To our best knowledge, usage of β -CD dimer as the sensitizing agent for determination of berberine hydrochloride in the aqueous solution by spectrofluorimetry has not been reported.

Based on the supramolecular interaction, berberine hydrochloride in aqueous solution was determined with high sensitivity and selectivity by spectrofluorimetry. The linear range of the method was $12.8\text{--}1.00 \times 10^4 \text{ ng mL}^{-1}$ with the detection limit 3.6 ng mL^{-1} . The proposed method had been applied to the determination of berberine hydrochloride in tablets and serum with satisfactory results. And then it has a promising potential in therapeutic drug monitoring, pharmacokinetics and clinical application.



Scheme 3. The molecular model of the complex.

2. Experimental

2.1. Apparatus and reagents

All the spectrofluorimetric measurements were carried out on a Cary Eclipse (Varian, Australia) spectrofluorimeter equipped with a xenon lamp and 1.0 cm quartz cells. Absorption spectra were obtained from UV-1700 (Shimadzu) UV-vis spectrophotometer. pH measurements were made with a PHS-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode.

Berberine hydrochloride (99.8%) (purchased from Shandong Institute for the Control of Agrochemicals) was used as received without further purification. Its stock solution (2.00 mg mL^{-1}) was prepared with ethanol. Ethylenediamine linked β -CD dimer was prepared according to our recently reported procedures [23,24]. Other chemicals used were analytical reagent grade. Doubly distilled water was used throughout.

2.2. Experimental procedure

2.2.1. Calibration graph

Into a series of 10 mL colorimetric tube were added different aliquots of the berberine hydrochloride stock solution containing $0\text{--}1.00 \times 10^4 \text{ ng mL}^{-1}$ of berberine hydrochloride, 3.00 mL of $1.00 \times 10^{-3} \text{ mol L}^{-1}$ ethylenediamine linked β -CD dimer and 2.00 mL of $0.20 \text{ mol L}^{-1} \text{ KH}_2\text{PO}_4\text{--H}_3\text{PO}_4$ buffer solution (pH 2.00) sequentially. The mixture was diluted to mark with doubly distilled water, shaken thoroughly and equilibrated at room temperature for 15 min. Then the fluorescent intensity of the solution was measured at 445/535 nm against a reagent blank.

2.2.2. Determination of the apparent association constant

Into a series of 10 mL colorimetric tube were added 1.00 mL of $20.0 \mu\text{g mL}^{-1}$ berberine hydrochloride, varied amounts of $1.00 \times 10^{-3} \text{ mol L}^{-1}$ ethylenediamine linked β -CD dimer and 2.00 mL of $0.20 \text{ mol L}^{-1} \text{ KH}_2\text{PO}_4\text{--H}_3\text{PO}_4$ buffer solution (pH 2.00) sequentially. The mixed solution was diluted to 10 mL with doubly distilled water, shaken thoroughly and equilibrated at room temperature for 15 min. Then the fluorescent intensity of the solution was measured at 445/535 nm against a reagent blank.

3. Results and discussion

3.1. Excitation and emission spectra

According to the procedure above, the excitation and emission spectra were scanned (Fig. 1). The maximum excitation and emission wavelengths were 445 and 535 nm, respectively, and a significant increasing of the fluorescence intensity was observed when ethylenediamine linked β -CD dimer was added to the aqueous solution of berberine hydrochloride.

3.2. Influence of pH

Because of the instability of cyclodextrin at very low pH, the use of strongly acidic solution containing β -CD was avoided

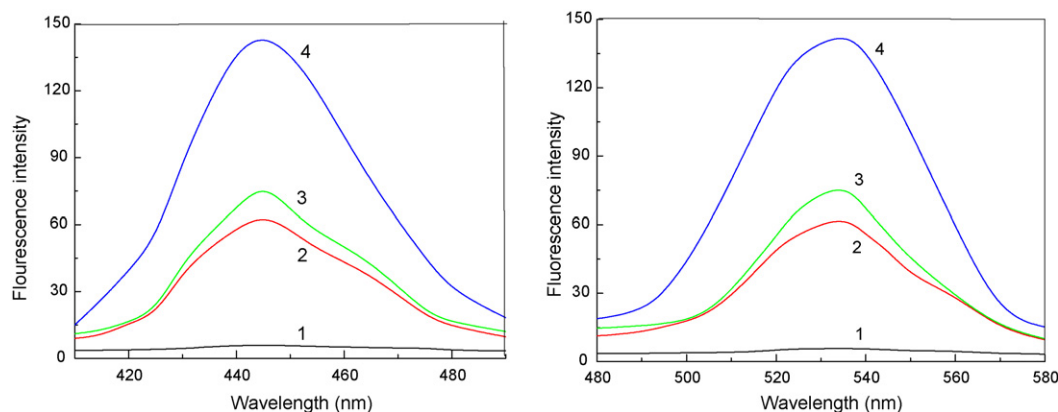


Fig. 1. Excitation and emission spectra: (1) reagent blank; (2) berberine hydrochloride solution; (3) berberine hydrochloride + β -CD; (4) berberine hydrochloride + β -CD dimer $C_{\text{berberine hydrochloride}} = 2.00 \times 10^{-4} \text{ mol L}^{-1}$; pH 2.00; $C_{\beta\text{-CD}} = 4.00 \times 10^{-4} \text{ mol L}^{-1}$; $C_{\beta\text{-CD dimer}} = 2.00 \times 10^{-4} \text{ mol L}^{-1}$.

[25]. Thus, the pH dependence of the system was studied over the range of 0.90–7.00. The results were shown in Fig. 2. As can be seen, the fluorescence intensity was relatively high and almost remained constant over the pH range of 1.60–3.00. A decrease in fluorescence intensity for values above pH 3.00 may due to the protonation of the host–guest complex. Therefore, a pH of 2.00 was fixed using $\text{KH}_2\text{PO}_4\text{--H}_3\text{PO}_4$ buffer solution.

The effect of the buffer will be lost as a result of a too small quantity of the buffer solution. While the buffer solution is excessive, the ionic strength will be too strong and then influence the fluorescence intensity. So the influence of the volume of the buffer solution was detected. As the volume of the buffer solution added (from 1.00 to 3.00 mL) had little effect on fluorescence intensity, 2.00 mL of buffer solution was used in subsequent experiments.

3.3. Influence of ethylenediamine linked β -CD dimer concentration

Influence of ethylenediamine linked β -CD dimer concentration on the fluorescence intensity was shown in Fig. 3. As can be seen, with increasing concentration of ethylenediamine

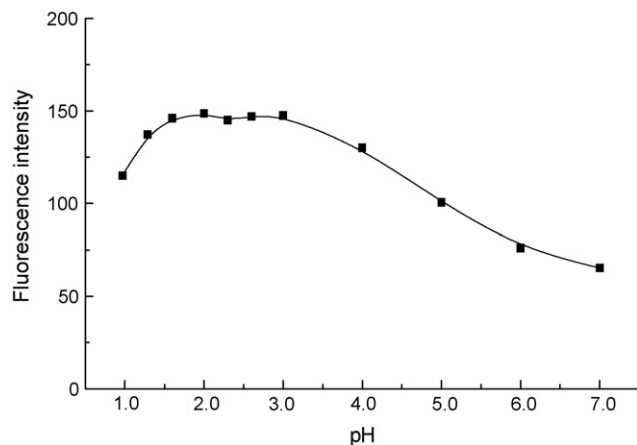


Fig. 2. Influence of pH on the fluorescence intensity. $C_{\text{berberine hydrochloride}} = 2.00 \mu\text{g mL}^{-1}$; $C_{\beta\text{-CD dimer}} = 2.00 \times 10^{-4} \text{ mol L}^{-1}$.

linked β -CD dimer the fluorescence intensity of the complex also increased. Thus, $3.00 \times 10^{-4} \text{ mol L}^{-1}$ ethylenediamine linked β -CD dimer was used.

3.4. Influence of temperature and reaction time

The influence of temperature was tested. The experimental results showed that higher temperature increased the collision of molecules, which made the collision probability of excited molecules and solvent molecules increased and resulted in the declination of fluorescent intensity. So room temperature was selected.

The effect of reaction time was studied. The results (Fig. 4) showed that the fluorescence intensity reached a maximum after the reagents had been added for about 15 min and remained constant for at least 45 min. Hence, after inclusive reaction was carried out for 15 min, the subsequent fluorescence measurement was made at room temperature.

3.5. Apparent association constant

The apparent association constant of the inclusion complex can be determined by the following method: assuming that the

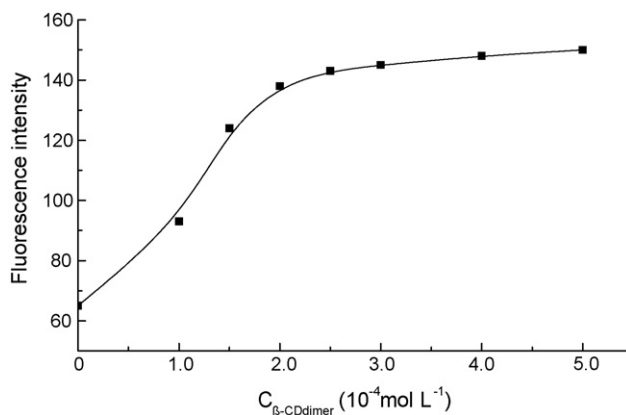


Fig. 3. Influence of ethylenediamine linked β -CD dimer on the fluorescence intensity. $C_{\text{berberine hydrochloride}} = 2.00 \mu\text{g mL}^{-1}$; pH 2.00.

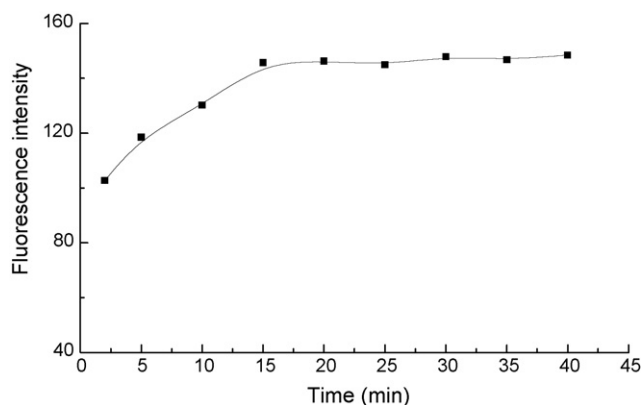
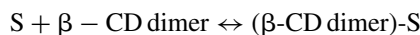


Fig. 4. Effect of reaction time on fluorescence intensity. $C_{\text{berberine hydrochloride}} = 2.00 \mu\text{g mL}^{-1}$; pH 2.00; $C_{\beta\text{-CD dimer}} = 3.00 \times 10^{-4} \text{ mol L}^{-1}$.

composition of the complex is 1:1, it can be written as:



where S denotes berberine hydrochloride. The formation constant of the complex (K) is given by:

$$K = \frac{[(\beta\text{-CD dimer})\text{-}S]}{[S][\beta\text{-CD dimer}]}$$

$[\beta\text{-CD dimer}]$, $[S]$ and $[(\beta\text{-CD dimer})\text{-}S]$ are equilibrium concentrations. An apparent association constant value of the inclusion complex can be determined through the typical double reciprocal plots [26]:

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)K C_{\beta\text{-CD dimer}}} + \frac{1}{F_\infty - F_0} \quad (1)$$

where F is the observed fluorescence intensity of the berberine hydrochloride solution at each ethylenediamine linked $\beta\text{-CD}$ dimer concentration tested, F_0 and F_∞ are the fluorescence intensity in the absence of ethylenediamine linked $\beta\text{-CD}$ dimer and when all the berberine hydrochloride molecules are included, respectively. It is taken into account that (1) ethylenediamine linked $\beta\text{-CD}$ dimer is in a large excess with respect to berberine hydrochloride, and therefore, its free and analytical concentration are same; (2) the variations in the fluorescence

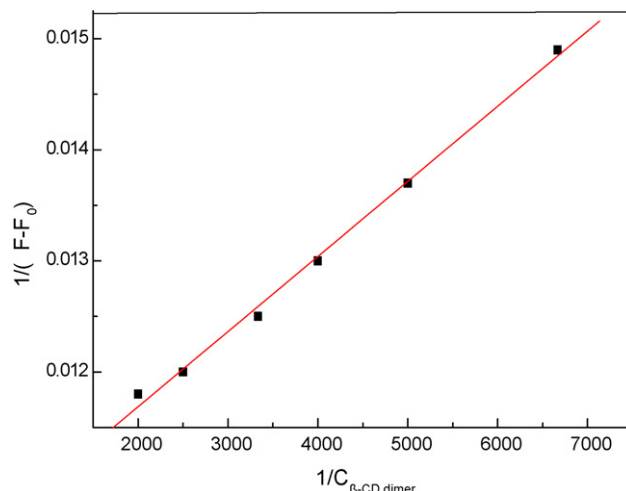


Fig. 5. Plot of $1/(F - F_0)$ vs. $1/C_{(\beta\text{-CD dimer})}$.

intensity are proportional to the complex concentrations and (3) at high ethylenediamine linked $\beta\text{-CD}$ dimer concentration essentially all of the berberine hydrochloride molecules are included.

The good linear relationship was obtained when $1/(F - F_0)$ was plotted against $1/C_{(\beta\text{-CD dimer})}$ supported the existence of a 1:1 complex ($R = 0.9986$, Fig. 5). Its apparent association constant was determined to be $1.53 \times 10^4 \text{ L mol}^{-1}$.

3.6. Influence of interference

The influence of the commonly used tablet excipients and the main constituents of serum on the determination of $2.00 \mu\text{g mL}^{-1}$ berberine hydrochloride were studied. A 3000-fold mass excess of them over $2.00 \mu\text{g mL}^{-1}$ berberine hydrochloride was tested firstly. If interference occurred, the ratio was reduced progressively until the interference ceased. The criterion for interference was fixed at a $\pm 5.0\%$ variation of the average fluorescence intensity calculated for the established level of berberine hydrochloride. The results were shown in Table 1 and it was obvious that the determination was free from interference of usual excipients and constituents of serum.

Table 1
Effect of interference (tolerance error $\pm 5.0\%$)

Interference	Tolerance ($\mu\text{g mL}^{-1}$)	Relative error (%)	Interference	Tolerance ($\mu\text{g mL}^{-1}$)	Relative error (%)
K^+	6000	2.7	Cl^-	1600	2.4
Na^+	6000	3.2	Methyl cellulose	1600	4.4
Ca^{2+}	5000	3.9	Sodium carboxymethyl cellulose	1600	4.7
Mg^{2+}	5000	4.2	Starch	1600	3.9
Zn^{2+}	5000	2.1	PO_4^{3-}	1200	-4.8
Glucose	5000	3.0	Mannitol	1200	2.6
Glycin	5000	-3.3	Sorbitol	1200	3.5
Polyethylene glycol	4000	2.5	Gum acacia power	1200	4.2
Sucrose	4000	3.0	Tryptophan	1200	-3.4
Boracic acid	2000	2.4	Fe^{3+}	800	4.9
Gelatin	2000	-2.2	Lactose	800	2.6
Casein	2000	-3.7	Sodium acetate	800	4.0

Table 2
Analytical characteristics compared with other spectrofluorimetry reported

Methods	Medium	Linear range	LOD
Using 1,4-bis(1,3-benzoxazol-2-yl)benzene (BBOB) as sensing material [28]	BBOB optode membrane	5.44–227.1 $\mu\text{g mL}^{-1}$	4435.2 ng mL^{-1}
Spectrofluorimetry with surfactant [29]	SDS solution	0.168–13.44 $\mu\text{g mL}^{-1}$	50.4 ng mL^{-1}
Resonance rayleigh scattering with fluorescent dyes [30]	Aqueous solution	0–1.68 $\mu\text{g mL}^{-1}$	14–215 ng mL^{-1}
Flow-injection analysis couples with liquid–liquid extraction [21]	Sodium perchlorate aqueous solution	0.001344–0.336 $\mu\text{g mL}^{-1}$	0.27 ng mL^{-1}
The proposed method	Aqueous solution	0.0128–10.0 $\mu\text{g mL}^{-1}$	3.6 ng mL^{-1}

Table 3
Determination of berberine hydrochloride in serum^a ($n = 5$, $p = 95\%$)

Sample no.	Berberine hydrochloride added (ng mL^{-1})	Berberine hydrochloride found (ng mL^{-1})	Recovery (%)	R.S.D. (%)
1	100	98.7 $\pm$ 0.07	98.7	1.5
	150	151 $\pm$ 0.14	101	2.4
	200	199 $\pm$ 0.05	99.5	0.7
	100	101.3 $\pm$ 0.21	101	3.4
2	150	148.8 $\pm$ 0.18	99.2	1.8
	200	198 $\pm$ 0.32	99.0	2.3

Sample 1 serum was not deproteinized with acetonitrile; Sample 2 serum was deproteinized with acetonitrile.

^a The serum matrix signal was eliminated.

3.7. Analytical characteristics

Under the optimum experimental conditions, there was a linear relationship between the fluorescence intensity and the concentration of berberine hydrochloride in the range of $12.8\text{--}1.00 \times 10^4 \text{ ng mL}^{-1}$ with a correlation coefficient of 0.9995. The regression equation was $\Delta F = 87.20 + 0.102 [\text{berberine hydrochloride}] (\text{ng mL}^{-1})$. The detection limit, as defined by IUPAC [27], was determined to be 3.6 ng mL^{-1} according to formula of $C = KS_0/S$, where the value of K was taken as 3, the standard deviation was 0.124 obtained from a series of 11 reagent blanks, and S was the slope of the standard curve. The relative standard deviation (R.S.D.) was 2.5% obtained from a series of 11 standards each containing $2.00 \mu\text{g mL}^{-1}$ of berberine hydrochloride. Compared with the spectrofluorimetric method without $\beta\text{-CD}$ [21,28–30], the proposed method has advantages, such as high sensitivity, wide linear range and also without any extraction process (Table 2).

3.8. Determination of berberine hydrochloride in serum

The procedure of serum sample analysis was used according to reference [31]. The standard solution containing 0.600 mg berberine hydrochloride was added into 8.00 mL serum of adult

man. Then the solution was divided into two parts. One was diverted into 100 mL volumetric flask and diluted to volume with doubly distilled water. The other was mixed with acetonitrile according to the volume ratio of 1:2 in order to remove protein. The mixture was centrifugated for 5 min. Then the centrifugate was transferred into a 100 mL volumetric flask and diluted to volume with doubly distilled water. The results were given in Table 3. As can be seen, there was no interference from the serum constituents.

4. Applications

4.1. Determination of berberine hydrochloride in tablets

The samples were prepared according to the reference [32]. Twenty tablets peeled off sugarcoat were finely powered and the power thoroughly mixed. An accurately weighed quantity of the power equivalent to 0.1 g of berberine hydrochloride was dissolved with about 150 mL of boiling water, and added 3 mL diluted hydrochloride acid. The cooled solution with the residue was transferred into a 250 mL volumetric flask, and then diluted to mark with doubly distilled water, shaken thoroughly and equilibrated at room temperature for 15 min. A 10 mL of the mixture was centrifugated for 10 min. A 2 mL of the centrifugate was diluted to volume in a 25 mL colorimetric tube, and this solution

Table 4
Determination of berberine hydrochloride in commercial tablets ($n = 5$, $p = 95\%$)

Pharmaceutical preparations	The proposed method		
	Label claim (mg tablet^{-1})	The proposed method (mg tablet^{-1})	R.S.D. (%)
Berberine hydrochloride tablets	100	98.8 $\pm$ 0.07	0.14
Fu fang huang lian su tablets	30	29.3 $\pm$ 0.31	0.60

was used for analytical determination. The results were shown in Table 4.

5. Conclusion

Based on the enhancement of the fluorescence intensity of berberine hydrochloride, a spectrofluorimetric method for the determination of berberine hydrochloride in aqueous solution in the presence of β -CD dimer was developed. The supramolecular interaction of berberine hydrochloride and ethylenediamine linked β -CD dimer has been studied by spectrofluorimetry. The results showed that β -CD dimer reacted with berberine hydrochloride to form a 1:1 (host:guest) complex with a apparent association constant of $1.53 \times 10^4 \text{ L mol}^{-1}$. The linear range of the method was $12.8\text{--}1.00 \times 10^4 \text{ ng mL}^{-1}$ with the detection limit 3.6 ng mL^{-1} .

Acknowledgements

This work was supported by Program for New Century Excellent Talents in University (NCET-04-0651), the National Natural Science Foundation of China (Nos. 20335030 and 20575036) and the Important Project of Natural Science Foundation of Shandong Province in China (No. Z2006B09).

References

- [1] W. Saenger, *Angew. Chem.* 92 (1980) 343.
- [2] G. Wenz, *Angew. Chem.* 106 (1994) 851.
- [3] J. Szejtli, *Chem. Rev.* 98 (1998) 1743.
- [4] Y. Liu, Y. Song, Y. Chen, Z.-X. Yang, F. Ding, *J. Phys. Chem. B* 109 (2005) 10717.
- [5] M. Shamsipour, A. Yari, H. Sharghi, *Spectrochim. Acta A* 62 (2005) 372.
- [6] I.N. Topchieva, P. Mischnick, G. Kuhn, V.A. Polyakov, S.V. Elezkava, G.I. Bystryzky, K.I. Karezin, *Bioconjugate. Chem.* 9 (1998) 676.
- [7] D. Wilson, L. Perlson, R. Breslow, *Bioorgan. Med. Chem.* 11 (2003) 2649.
- [8] Y. Liu, Y.-W. Yang, Y. Chen, F. Ding, *Bioorgan. Med. Chem.* 13 (2005) 963.
- [9] R. Breslow, Z.-W. Yang, R. Ching, G. Trojandt, F. Odobel, *J. Am. Chem. Soc.* 120 (1998) 3536.
- [10] Y. Liu, Y. Song, Y. Chen, X.-Q. Li, F. Ding, R.-Q. Zhong, *Chem. Eur. J.* 10 (2004) 3685.
- [11] A. Harada, M. Furue, S. Nozakura, *Polym. J.* 12 (1980) 29.
- [12] R. Breslow, N. Greenspoon, T. Guo, R. Zarzycki, *J. Am. Chem. Soc.* 111 (1989) 8296.
- [13] R. Breslow, S. Chung, *J. Am. Chem. Soc.* 112 (1990) 9659.
- [14] Y. Okabe, H. Yamamura, K. Obe, K. Ohta, M. Kawai, K. Fujita, *Chem. Commun.* (1995) 581.
- [15] R. Deschenaux, T. Ruch, P.-F. Deschenaux, A. Juris, R. Ziessel, *Helv. Chim. Acta* 78 (1995) 619.
- [16] Y. Liu, Y. Chen, S.-X. Liu, X.-D. Guan, T. Wada, Y. Inoue, *Organ. Lett.* 3 (2001) 1657.
- [17] Y. Liu, Y. Song, H. Wang, H.-Y. Zhang, T. Wada, Y. Inoue, *J. Org. Chem.* 68 (2003) 3687.
- [18] H.A. Weber, M.K. Zart, A.E. Hodges, H. Michael Molloy, B.M. O'Brien, L.A. Moody, A.P. Clark, R.K. Harris, J. Diane Overstreet, C.S. Smith, *J. Agric. Food Chem.* 51 (2003) 7352.
- [19] A.W. Smallwood, C.S. Tschee, R. Duane Satzger, *J. Agric. Food Chem.* 45 (1997) 3976.
- [20] F.P. Cossío, A. Arrieta, *Anal. Chem.* 72 (2000) 1759.
- [21] S. Tadao, O. Noriko, S.C. Yong, N. Harumitu, *Anal. Chim. Acta* 308 (1995) 329.
- [22] J.C. Beeson, A.W. Czarnik, *Bioorgan. Med. Chem.* 2 (1994) 297.
- [23] B. Tang, H.-L. Liang, K.-H. Xu, Z. Mao, X.-F. Shi, Z.-Z. Chen, *Anal. Chim. Acta* 554 (2005) 31.
- [24] B. Tang, H.-L. Liang, L.-L. Tong, P. Li, *Bioorgan. Med. Chem.* 14 (2006) 3947.
- [25] R.L. Vanetten, J.F. Sebastian, G.A. Clowes, M.A. Bender, *J. Am. Chem. Soc.* 89 (1967) 3242.
- [26] J.J. Berzas Nevado, J.A. Murillo Pulgarín, M.A. Gómez Laguna, *Talanta* 53 (2001) 951.
- [27] H.M.N.H. Irving, T.S. West (Eds.), *IUPAC Compendium of Analytical Nomenclature Definitive Rules*, Pergamon Press, Oxford, 1981.
- [28] Y. Wang, K. Wang, W.-H. Liu, G.-L. Shen, R.-Q. Yu, *Analyst* 122 (1997) 69.
- [29] M.O. Iwunze, *Monatsh. Chem.* 131 (2000) 429.
- [30] S.-P. Liu, P. Feng, *Microchim. Acta* 140 (2002) 189.
- [31] B. Tang, B.-X. Jia, X.-F. Shi, *Spectrochim. Acta A* 61 (2004) 2203.
- [32] X.-Y. Zheng, *Pharmacopoeia of the People's Republic of China. Part I., Chemical Engineering Press*, 2005, p. 534.

Performance of two monoclonal immunoassays in mixtures of cross-reacting dithiophosphorus pesticides

Josep V. Mercader¹, Angel Montoya^{*}

Instituto de Investigación e Innovación en Bioingeniería, Universitat Politècnica de València, Camí de Vera s/n, 46022 València, Spain

Received 22 February 2007; received in revised form 9 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

A statistical approach for the analysis of complex samples by immunoassay is proposed in this article. Two enzyme-linked immunosorbent assays (ELISAs), one of them in the conjugate-coated format and the other in the antibody-coated format, were evaluated for their suitability to the analysis of mixtures of three organodithiophosphorus pesticides: azinphos-methyl, azinphos-ethyl and phosmet. It was found that the apparent affinity of the antibody to each analyte changed in the presence of a cross-reacting compound in the antibody-coated ELISA format, but not when the conjugate-coated ELISA format was used. The assays were thereafter applied to the analysis of mixtures of the three recognized pesticides. With the conjugate-coated ELISA format, accurate and precise determinations of mixtures could be performed if an azinphos-methyl standard curve was employed, with recoveries between 71% and 130% and with coefficients of variation lower than 12.7%. Neither accurate nor precise measurements could be accomplished with the enzyme immunoassay using the antibody-coated ELISA format, independently of the standard curve used. It is thought that the study presented here will have applicability in a variety of cases where the analytical goal is semiquantitative screening based on the total quantity of an unknown mixture of related compounds.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Azinphos-methyl; Phosmet; Azinphos-ethyl; Cross-reactivity; Dithiophosphorus pesticides; ELISA

1. Introduction

Azinphos-methyl (AM) is an organophosphorus pesticide which has been applied in many crops throughout the world during decades. It poses potential risks of concern to farm workers, pesticide applicators and aquatic ecosystems. In fact, in a recent review of the European Council Directive 91/414/EEC, which regulates the placing of plant protection products on the market, it has been deemed that the use of this active substance can be accepted only if it is limited to a few specific crops for a limited time (7 years) and subject to strict risk reduction measures (http://ec.europa.eu/food/plant/index_en.htm). A similar decision has been taken in 2006 by the US EPA regarding the use of AM.

Biotechnology-based methods such as immunoassays have increasingly been reported for food and environmental analysis

since its promising application was first envisioned by Ercegovich [1]. Immunoassay format studies is nowadays a trend for the development of more sensitive, simpler, low-cost and faster assays [2]. The major disadvantage of immunochemical assays is their limited multianalyte capacity compared with separative techniques. Nevertheless, due to their high sample throughput, it is possible to design assays with multiple specificities just combining the appropriate selective reagents. During the past decade, the feasibility of immunoassays as analytical tools for environmental and food monitoring has been demonstrated [3,4]. Now, the time has come to use all the available reagents and the accumulated know-how in order to solve the major analytical limitation of immunoassays.

Recently, several enzyme-linked immunosorbent assays (ELISAs) for multiresidue analysis have been reported [5,6]. Also, biosensors and multiplexed immunoassays constitute promising tools for multianalyte detection. Some research groups have obtained antibodies with broad-specificities with the aim to develop class-specific tests [7,8]. Moreover, many immunoassays show certain cross-reactivity (CR) with other structurally related compounds or with decomposition products or metabolites [9,10]. This antibody unspecificity can be taken

^{*} Corresponding author. Tel.: +34 963877093; fax: +34 963877093.

E-mail address: amontoya@eln.upv.es (A. Montoya).

¹ Present address: Institut d'Agroquímica i Tecnologia d'Aliments (CSIC), Polígon Industrial La Coma s/n, 46980 Paterna, València, Spain.

into an advantage and become a convenient property so that the same immunoassay may be employed for the detection of different compounds [11]. Glass et al. [12] have reported the improvement of an immunoassay response to related polychlorinated biphenyl analytes by mixing antibodies with different CR patterns. Therefore, group-specific ELISAs can be developed as qualitative assays for large scale programs and multianalyte assays are possible if the suitable reagents are available and the appropriate statistical analysis is performed.

Some years ago, several papers were published describing the production of monoclonal antibodies (MAbs) to AM [13,14] and the optimization and characterization of two ELISAs for AM analysis in real samples [15–17] with limits of detection lower than the established maximum residue limits. These two immunoassays showed similar CRs with two structurally related dithiophosphate pesticides: azinphos-ethyl (AE) and phosmet (P). In many instances, AM, AE and P are alternatives for one another in controlling a similar spectrum of pests. In this paper, we have closely studied the performance of these tests with the aim to understand their behaviour when mixtures of these analytes occur. We provide experimental results which may contribute to the present challenge of immunoassays as multiresidue methods.

2. Experimental

2.1. Reagents and instrumentation

Pesticide standards (Pestanal grade) were purchased from Riedel-de-Haën AG (Seelze, Germany). All other chemicals were of reagent grade or better. Monoclonal antibodies, assay haptens (HBA: 2-[(4-oxo-1,2,3-benzotriazin-3-yl)oxy]acetic acid and MBH: 6-[(4-oxo-1,2,3-benzotriazin-3-yl)methylthio]hexanoic acid) and assay conjugates (ovalbumin coating conjugate: OVA-HBA and peroxidase enzyme tracer: HRP-MBH) were the same as those previously described [16,17]. Peroxidase-labelled rabbit anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). Flat-bottom polystyrene ELISA plates (High Binding Plates, catalogue no. 3590) were from Costar (Cambridge, MA, USA). Absorbances were read in a dual-wavelength mode with an Emax microplate reader controlled by a SOFTmax PRO software package, version 1.2.0, from Molecular Devices Corporation (Sunnyvale, CA, USA).

2.2. Enzyme-linked immunosorbent assays

Standard solutions were prepared in PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) by serial dilutions from a stock solution in *N,N*-dimethylformamide (DMF), using borosilicate glass tubes. A volume of 100 μ L per well was used throughout all assay steps. After each incubation, plates were washed four times with 0.15 M NaCl containing 0.05% Tween 20. Retained peroxidase activity was determined with freshly prepared substrate buffer (2 mg mL⁻¹ *o*-phenylenediamine, 0.012% H₂O₂ in 25 mM citrate, 62 mM sodium phosphate buffer, pH 5.4). The enzymatic reaction was stopped after 10 min at room temperature by adding 2.5 M sulphuric acid. Finally, the

absorbance was read at 490 nm with a reference wavelength at 650 nm. Standard curves were obtained by plotting absorbance values against the logarithm of analyte concentration and fitted to a four parameter logistic equation (1) using a Sigmaplot software package from Jandel Scientific (Erkrath, Germany):

$$y = \frac{A - D}{1 + (x/C)^B} + D \quad (1)$$

where A is the asymptotic maximum (absorbance in the absence of analyte, $A_{\max}$), B the curve slope at the inflexion point, C the x value at the inflection point and D is the asymptotic minimum (background signal). Assay sensitivity (I_{50}) was assumed as the analyte concentration that reduced the maximum absorbance signal by 50% in competitive assays, and corresponds to the C parameter in Eq. (1). The limit of quantitation was defined as the analyte concentration that produced a reduction of 20% of the maximum absorbance signal in competitive assays.

2.3. Assay I

This assay was conducted in the conjugate-coated ELISA format (Fig. 1) which, as determined in previous studies [17], showed an I_{50} value of 0.40 nM and a limit of quantitation of 0.14 nM (44 ng L⁻¹) for AM. Plates were coated with 100 μ L per well of the heterologous OVA-HBA hapten-conjugate solution at 200 ng mL⁻¹ in 50 mM carbonate-bicarbonate buffer, pH 9.6 (coating buffer) by overnight incubation at room temperature. Afterwards, competitive assays were executed by adding 50 μ L per well of standard or sample solution in triplicate wells, followed by 50 μ L per well of LIB-MFH14 MAb solution at 120 ng mL⁻¹ in PBS. The analytical performance of LIB-MFH14 MAb, including cross-reactivity, was previously reported [16,17]. The immunochemical reaction was allowed to occur for 1 h at room temperature. Thereafter, a 1/2000 dilution of HRP-labelled rabbit anti-mouse immunoglobulins in PBS containing 0.05% Tween 20 was added and plates were

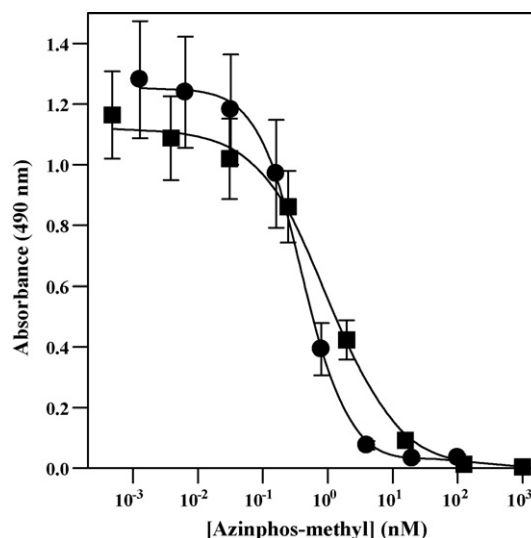


Fig. 1. Standard curves of Assay I (●) and Assay II (■).

incubated for 1 h at room temperature. Finally, the retained peroxidase activity was determined as described above.

2.4. Assay II

This assay employed the antibody-coated ELISA format (Fig. 1) which, as determined in previous studies [17], showed an I_{50} value of 1.01 nM and a limit of quantitation of 0.18 nM (57 ng L⁻¹) for AM. Plates were coated with 100 μ L per well of a LIB-MFH110 MAb solution at 1 μ g mL⁻¹ in coating buffer by overnight incubation at room temperature. The analytical performance of LIB-MFH110 MAb, including cross-reactivity, was previously reported [16,17]. Competitive assays were afterwards performed by adding 50 μ L per well of standard or sample solution in triplicate wells, followed by 50 μ L per well of heterologous HRP-MBH enzyme tracer solution at 40 ng mL⁻¹ in PBS with 40 μ g mL⁻¹ bovine serum albumin (BSA). The immunochemical reaction was allowed to occur for 1 h at room temperature. Thereafter, colour was developed as indicated previously.

2.5. Cross-reactivity data

Percent CR was determined at three different inhibition values of the standard curves, that is, at concentrations reducing the maximum signal by 30%, 50% and 70% in competitive assays (CR₃₀, CR₅₀ and CR₇₀, respectively). To this purpose, the I_{30} , I_{50} and I_{70} values of AM were compared with the I_{30} , I_{50} and I_{70} values of each analyte, respectively, according to the following equation:

$$CR_x = \frac{I_x \text{ for AM}}{I_x \text{ for analyte}} \times 100 \quad (2)$$

where I_x is defined as the concentration of analyte that reduces the maximum signal by $x\%$ in competitive assays.

2.6. Analysis of mixtures of pesticides

The I_{50} values of the standard curves for AM, AE and P were individually ascertained in the presence of several constant concentrations of one of the two other recognized pesticides in

Assay I and in Assay II. For instance, the I_{50} value of the standard curve for AM was calculated when the assay was performed in the presence of several constant concentrations of AE or P. For Assay I, PBS was spiked with AM or AE or P at 0.4, 0.3, 0.2 and 0.1 nM. For Assay II, PBS containing 40 μ g mL⁻¹ BSA was spiked with AM or AE or P at 1.0, 0.8, 0.6, 0.4 and 0.2 nM. Then, these spiked buffer samples were used to prepare the MAB (Assay I) or enzyme tracer (Assay II) solutions. For competitive assays, these spiked immunoreagent solutions were combined with a standard curve of each analyte.

In a second set of experiments, PBS samples spiked with mixtures of AM, AE and P were prepared and assayed with both ELISAs. Mixtures of the three pesticides were prepared in DMF and diluted to reach three final total concentrations equivalent to the three AM concentrations producing 30%, 50% and 70% inhibition of the maximum signal of the standard curve of AM, which were different for Assay I and Assay II. Moreover, those mixtures were equimolar or in a 4:2:1 molar proportion. Control samples containing only the pesticide used for each standard curve were prepared and analyzed as the mixtures. For each experiment three plates were used, each one containing the standard curve of a different analyte (AM, AE or P). Each sample was run in triplicate on each plate and the mean value was considered for calculation of the pesticide equivalents using the standard curve run also in triplicate in the same plate. Three independent determinations were conducted with the spiked samples.

3. Results and discussion

3.1. Cross-reactivity determination

A possible approach to the design of a class-specific immunoassay is the use of different antibodies showing a different CR pattern. Another approach is the use of an antibody that recognizes a group of analytes. The latter is the case of the ELISAs studied in the present work, which recognize similarly three organophosphorus pesticides [17]. For this multianalyte immunoassay to be feasible, the inhibition standard curves of the three compounds should be as parallel as possible along the working range. Furthermore, to be able to quantitate one of the analytes using as standard any of the cross-reacting pesticides

Table 1
CR values for AE and P calculated at 30%, 50% and 70% inhibition^a

	30% inhibition		50% inhibition		70% inhibition	
	CR ₃₀ ^b (%)	CV (%)	CR ₅₀ ^b (%)	CV (%)	CR ₇₀ ^b (%)	CV (%)
Assay I						
Phosmet	131.4 ^c	8.6	136.9	5.7	141.7 ^c	4.8
Azinphos-ethyl	92.2 ^d	16.1	82.7 ^e	9.2	75.2 ^{d,e}	6.3
Assay II						
Phosmet	171.9	25.3	169.4	14.3	153.3	8.3
Azinphos-ethyl	117.8	20.8	111.7	15.8	103.1	8.5

^a CR to AM is assumed as the reference value (100%).

^b CR values are the mean of ten independent determinations run in quadruplicate wells.

^c CR values statistically different to each other with $p < 0.05$.

^d CR values statistically different to each other with $p < 0.01$.

^e CR values statistically different to each other with $p < 0.02$.

the differences in sensitivity should be low (CR values close to 100%). Otherwise, the best standard curve among the curves of all cross-reacting analytes will be that one showing an average I_{50} value. Finally, the affinity of the MAb to any of the compounds should not change if any of the other cross-reactants is present.

To estimate the parallelism of the curves, CRs were calculated at three inhibition values of the standard curves. Table 1 shows the CR_x values for AE and P in Assay I and in Assay II, assuming a CR of 100% for AM. The statistical analysis of the Student's t distribution between CR_{30} , CR_{50} and CR_{70} values for AE and P provided different results for each assay. For Assay I, significant differences were found between the CR values, higher for those of AE. Statistical differences ($p < 0.02$) were calculated between CR_{50} and CR_{70} values for AE (82.7% and 75.2%, respectively). Significant deviations ($p < 0.01$ for AE and $p < 0.05$ for P) were also observed between the CR_{30} and CR_{70} values for AE (92.2% and 75.2%, respectively) and P (131.4% and 141.7%, respectively). Therefore, parallelism of the three standard curves in this assay cannot be assumed. Nevertheless, the CRs were not very far from 100%, which indicated that the sensitivity inside the working range was similar for the three analytes. The higher deviations (CR values diverting further from 100%) were observed at higher inhibition values (70%). For Assay II, no statistical differences were found between the calculated CRs at any inhibition value of the standard curves for any of the compounds. Nevertheless, higher deviations could be observed between the CR_{30} and CR_{70} values (Table 1). Although curves seemed to be parallel, statistical differences could be hidden because of the high coefficients of variation (CVs) of this assay.

3.2. Apparent affinity in mixtures of analytes

It has been reported that the apparent affinity of an antibody for an analyte may be lower in the presence of another recognized compound [18]. As described under Section 2, competitive assays were performed with both assays, Assay I and Assay II, in the presence of several constant concentrations of a cross-reacting pesticide (AM, AE and P) using either AM, AE or P as standards. As expected, it could be observed that the higher the concentration of the cross-reacting analyte in the assay buffer, the lower the maximum absorbance of the standard curve. As an example, when a concentration of AE or P equivalent to its I_{50} value was included in the buffer which was used to prepare the standard curve of AM, the maximum absorbance was half the absorbance of the standard curve for AM alone (results not shown). Standard curves were fitted to the four parameter logistic equation and their I_{50} values were plotted as a function of the concentration of the cross-reacting analyte present in the assay (Fig. 2). This study showed that the apparent affinity of the antibody in Assay I did not change as a consequence of the presence of a cross-reacting compound. On the contrary, in Assay II, higher concentrations of the cross-reacting analyte lowered the apparent affinity (increased I_{50} values) of the antibody for the standard compound, independently of the analyte used as cross-reacting or standard (AM, AE or P). This result suggested that

mixtures of cross-reacting pesticides could only be accurately measured with Assay I.

The slopes of the regression lines for the variation of the I_{50} values for AM and AE in Assay II (Fig. 2) were between 1.2 and 1.3 (AE had CR_x values close to 100%) while the corresponding slope for the experiment in which P was used as standard analyte was 0.6. This result is due to the fact that P was the analyte with higher CR_x values. Therefore, the variation of the apparent affinity seemed to depend on the analyte. Moreover, it seemed that the higher the affinity of the antibody for a pesticide, the lower the variation of the I_{50} value produced by the presence of a cross-reacting pesticide.

Either the assay format or the immunoreagent properties may explain the different performance of Assay I and Assay II when they were applied to the analysis of mixtures of pesticides. The antibodies used in both assays were revealed to be very similar

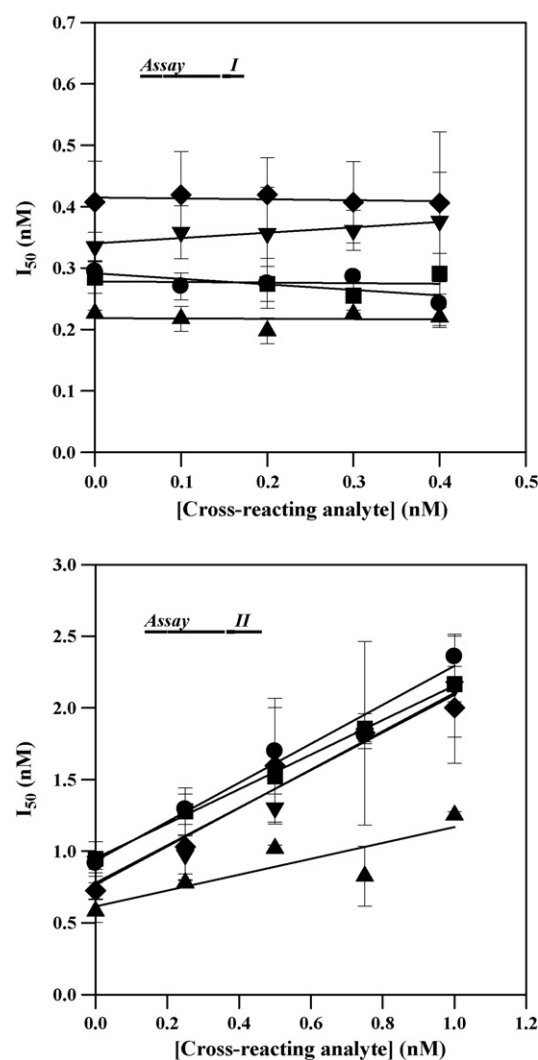


Fig. 2. Variation of the IC_{50} values in both Assay I and Assay II for a standard pesticide in the presence of a cross-reacting analyte. Symbols correspond to the competitive assays for AM in the presence of several constant concentrations of P (●) or AE (■), competitive assays for AE in the presence of several constant concentrations of P (◆) or AM (▼) and competitive assays for P in the presence of several constant concentrations of AM (▲). Each value is the mean $\pm$ S.D. of three independent determinations with points run in quadruplicate wells.

Table 2
Analysis of spiked buffer samples by Assay I with a standard curve of AM

Pesticide mixture	30% inhibition				50% inhibition				70% inhibition			
	Added ^a (nM)	Equiv. AM ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. AM ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. AM ^b (nM)	CV (%)	Recovery (%)
Control (AM)	0.20	0.20	6.6	98.0	0.40	0.44	11.2	109.8	0.80	0.95	8.9	118.1
Equimolar	0.21	0.15	12.7	71.0	0.42	0.38	6.6	91.5	0.84	0.88	3.5	105.2
AM + AE + P ^c	0.21	0.20	8.1	93.8	0.42	0.45	7.9	107.9	0.84	0.96	2.6	114.6
AM + P + AE	0.21	0.18	5.1	84.3	0.42	0.44	5.7	105.6	0.84	1.05	6.6	125.5
AE + AM + P	0.21	0.21	7.7	98.6	0.42	0.43	9.0	103.4	0.84	0.98	2.5	116.5
AE + P + AM	0.21	0.21	7.8	97.6	0.42	0.47	5.1	111.2	0.84	0.96	2.3	114.7
P + AM + AE	0.21	0.21	8.1	100.5	0.42	0.46	5.4	110.6	0.84	1.09	7.4	130.0
P + AE + AM	0.21	0.18	5.1	83.8	0.42	0.46	10.7	108.8	0.84	0.96	6.9	114.2

^a Concentration in the assay.

^b Each value is the mean of three independent determinations.

^c Mixtures of the three analytes in a 4:2:1 molar proportion (first analyte + second analyte + third analyte).

in previous optimization and characterization processes [17]. Therefore, the difference between Assay I and Assay II observed in this study might be preferentially related to the assay format employed in each case. Assay II is a direct ELISA, where the antibody is directly immobilized onto the solid support. This fact could, hypothetically, stabilize the antibody–analyte complexes or make the antibody less accessible to the enzyme conjugate which is in solution, making it difficult to achieve the chemical equilibrium between the immobilized antibody and any other recognized compounds.

3.3. Analysis of mixtures of pesticides

Real samples may contain complex mixtures of cross-reacting compounds. It is well known that the matrix can interfere in ELISA tests and modify some parameters such as the analytical range of quantitation. The influence of water and food matrices on the performance of Assay I and Assay II has been previously evaluated [15,17]. Nevertheless, in the present research we aim to understand the behavior of these two immunoassay formats in a model system, when different cross-reacting analytes are present in the sample. To this purpose, assay buffer was spiked with mixtures of the cross-reacting pesticides and analyzed using the optimized ELISAs. The analysis of these fortified samples was executed using standard curves of each of the three pesticides for Assay I and Assay II. Results were expressed as equivalents of the standard analyte used in each case. Tables 2–4 show the calculated equivalents for Assay I when a standard curve of AM, AE and P was used, respectively. Controls are the solutions containing a concentration of the analyte used as standard and they are equivalent to the I_{30} , I_{50} and I_{70} values of that analyte in the corresponding assay. Results shown in Table 2 indicate that these mixtures of pesticides can be accurately and precisely measured as AM equivalents (recoveries between 71.0% and 130.0% with CVs lower than 12.7%). These values are in the range usually found in routine methods for environmental and food matrices.

When a standard curve of AE was employed in Assay I (Table 3), measurements were accurate (recoveries between 83.8% and 117.6%) only if the total concentration of the mixture was 0.21 nM (around the I_{30} value of AE). Other mixtures provided too high recovery values (up to 184.7%). This is in accordance with the CR_{50} and CR_{70} values found for AE, which were lower than 100% (82.7% and 75.2%, respectively), while CR_{30} value was closer to 100% (92.2%). Finally, when a standard curve of P was used (Table 4), the best recoveries were obtained at high concentrations. Nevertheless, although they were acceptable for most samples, in this case the recoveries showed an underestimating tendency (down to 63.5%), which is again in accordance with the CR values found for this pesticide in this assay (higher than 100% in all cases, Table 1). It has been shown that CR values in Assay I are not far from 100% and that the AM standard curve is between those of AE and P. These results can probably explain that the most accurate measurements could be achieved when a standard curve of AM was used.

Table 3
Analysis of spiked buffer samples by Assay I with a standard curve of AE

Pesticide mixture	30% inhibition				50% inhibition				70% inhibition			
	Added ^a (nM)	Equiv. AE ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. AE ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. AE ^b (nM)	CV (%)	Recovery (%)
Control (AE)	0.23	0.21	2.4	92.2	0.50	0.52	12.8	103.4	1.10	1.39	12.1	126.4
Equimolar	0.21	0.18	2.3	83.8	0.42	0.50	0.4	119.8	0.84	1.29	4.6	153.0
AM + AE + P ^c	0.21	0.23	6.0	109.5	0.42	0.55	10.2	130.3	0.84	1.38	7.2	164.4
AM + P + AE	0.21	0.21	9.5	100.6	0.42	0.55	9.7	130.2	0.84	1.45	7.0	172.2
AE + AM + P	0.21	0.22	10.4	105.4	0.42	0.57	13.7	135.2	0.84	1.42	9.0	169.0
AE + P + AM	0.21	0.25	10.5	117.6	0.42	0.63	8.4	149.5	0.84	1.38	3.4	164.2
P + AM + AE	0.21	0.24	7.9	114.4	0.42	0.60	10.3	143.3	0.84	1.55	2.3	184.7
P + AE + AM	0.21	0.18	21.3	87.1	0.42	0.47	14.8	112.9	0.84	1.18	11.3	140.7

^a Concentration in the assay.

^b Each value is the mean of three independent determinations.

^c Mixtures of the three analytes in a 4:2:1 molar proportion (first analyte + second analyte + third analyte).

Table 4
Analysis of spiked buffer samples by Assay I with a standard curve of P

Pesticide mixture	30% inhibition				50% inhibition				70% inhibition			
	Added ^a (nM)	Equiv. P ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. P ^b (nM)	CV (%)	Recovery (%)	Added ^a (nM)	Equiv. P ^b (nM)	CV (%)	Recovery (%)
Control (P)	0.15	0.13	12.8	88.7	0.30	0.24	7.1	79.3	0.60	0.49	9.8	81.8
Equimolar	0.21	0.16	14.2	74.0	0.42	0.30	6.3	72.0	0.84	0.64	6.6	75.6
AM + AE + P ^c	0.21	0.18	9.1	84.0	0.42	0.34	4.2	80.2	0.84	0.69	3.3	82.0
AM + P + AE	0.21	0.17	10.8	79.7	0.42	0.34	3.0	79.8	0.84	0.79	1.8	94.1
AE + AM + P	0.21	0.17	5.9	80.6	0.42	0.33	3.1	77.7	0.84	0.69	7.4	82.3
AE + P + AM	0.21	0.17	16.0	80.5	0.42	0.37	7.7	86.8	0.84	0.74	2.3	88.7
P + AM + AE	0.21	0.17	12.6	79.5	0.42	0.34	7.3	82.0	0.84	0.81	4.2	96.8
P + AE + AM	0.21	0.13	14.3	63.5	0.42	0.32	3.7	77.0	0.84	0.63	15.0	75.1

^a Concentration in the assay.

^b Each value is the mean of three independent determinations.

^c Mixtures of the three analytes in a 4:2:1 molar proportion (first analyte + second analyte + third analyte).

The same experiment was carried out with Assay II (results not shown). Controls were prepared at concentrations corresponding to the I_{30} , I_{50} and I_{70} values of each standard analyte in this assay. CVs were overall very high (up to 98.9%) and recoveries showed important deviations. The most accurate measurements (recoveries from 62.3% to 159.0%) were obtained at concentrations near the I_{70} value, while recoveries between 11.8% and 135.3% were found at lower concentrations of analyte mixtures, even if a standard curve of AE (the compound that showed intermediate CR values) was used. Similar results had previously been obtained with a different MAb also used in the antibody-coated ELISA format [13]. These results agree with the hypothesis that the changes observed in the parameters of the inhibition curve of a certain analyte when mixtures of cross-reacting pesticides are analyzed can be attributed to the direct immobilization of the antibody onto the solid support.

4. Conclusions

A monoclonal immunoassay for the analysis of mixtures of azinphos-methyl, azinphos-ethyl and phosmet has been developed. Two immunoassays had previously been studied and evaluated in real samples. Assay I is a conjugate-coated ELISA while Assay II uses the antibody-coated format. For both assays, high cross-reactivity values for the three pesticides were found at three different points of their corresponding inhibition curves. Interestingly, it was observed that Assay I and Assay II behaved differently when the I_{50} values of the inhibition curve of each pesticide were determined in the presence of several constant concentrations of another cross-reacting analyte. This different behaviour could be attributed to the assay format employed in each case since important constraints to the receptor–ligand interaction are introduced upon immobilization of the antibody. Finally, accurate and precise analysis of buffer samples spiked with complex mixtures of the three recognized pesticides was accomplished using Assay I with a standard curve of azinphos-methyl. Hence, the conjugate-coated assay seems to be the most suitable format for the development of a rapid, class-

based immunochemical screening procedure for this group of organophosphorus pesticides.

Acknowledgement

J.V. Mercader was the recipient of a fellowship from Universitat Politècnica de València, Spain.

References

- [1] C.D. Ercegovich, Analysis of pesticide residues: Immunological techniques, in: F.J. Biros (Ed.), *Pesticide Identification at the Residue Level* (Advances in Chemistry, Series 104), American Chemical Society, Washington, DC, 1971, pp. 162–177.
- [2] D. Knopp, *Anal. Bioanal. Chem.* 385 (2006) 425.
- [3] J.A. Gabaldón, A. Maquieira, R. Puchades, *Crit. Rev. Food Sci. Nutr.* 39 (1999) 519.
- [4] F. Toldrá, M. Reig, *Trend Food Sci. Technol.* 17 (2006) 482.
- [5] A.J. Schuetz, M. Winklmair, M. Weller, M.G. Niessner, *Fresenius J. Anal. Chem.* 363 (1999) 625.
- [6] A. Montoya, A. Abad, M.J. Moreno, J.J. Manclús, J.V. Mercader, Validation of monoclonal single and multianalyte ELISAs for pesticide residue analysis in fruits and vegetables, in: L. Fukal, P. Jenč, P. Rauch (Eds.), *Proceedings of the VIth International Conference on Agri-Food Antibodies*, ITC Press, Prague, 2001, p. 37.
- [7] J.J. Manclús, A. Abad, M.Y. Lebedev, F. Mojarrad, B. Micková, J.V. Mercader, J. Primo, M.A. Miranda, A. Montoya, *J. Agric. Food Chem.* 52 (2004) 2776.
- [8] J. Ramón-Azcón, R. Galve, F. Sánchez-Baeza, M.P. Marco, *Anal. Chem.* 78 (2006) 71.
- [9] L. Anfossi, G. Giraudi, C. Tozzi, C. Giovannoli, C. Baggiani, A. Vanni, *Anal. Chim. Acta* 506 (2004) 87.
- [10] A. Abad, M.J. Moreno, A. Montoya, *J. Agric. Food Chem.* 47 (1999) 2475.
- [11] J.H. Skerritt, Analytical aspects of immunoassays of agrochemicals, in: D.A. Kurtz, J.H. Skerritt, L. Stanker (Eds.), *New Frontiers in Agrochemical Immunoassay*, AOAC International, Arlington, 1995, pp. 1–16.
- [12] T.R. Glass, N. Ohmura, K. Morita, K. Sasaki, H. Saiki, Y. Takagi, C. Kataoka, A. Ando, *Anal. Chem.* 78 (2006) 7240.
- [13] J.V. Mercader, J. Primo, A. Montoya, *J. Agric. Food Chem.* 43 (1995) 2789.
- [14] W.T. Jones, D. Harvey, S.D. Jones, G.B. Ryan, H. Wyberg, W. Ten Hoeve, P.H.S. Reynolds, *Food Agric. Immunol.* 7 (1995) 9.
- [15] J.V. Mercader, A. Montoya, *Anal. Chim. Acta* 347 (1997) 95.
- [16] J.V. Mercader, A. Montoya, *J. Agric. Food Chem.* 47 (1999) 1276.
- [17] J.V. Mercader, A. Montoya, *J. Agric. Food Chem.* 47 (1999) 1285.
- [18] A. Oubiña, J. Gascón, D. Barceló, *Environ. Sci. Technol.* 30 (1996) 513.

A new Cu(II)- 5-(4-sulphophenylazo)-8-aminoquinoline complex used for copper determination in presence of gold and silver in water and mineral samples

Libby Morales, M. Inés Toral*, M. José Álvarez

Department of Chemistry, Faculty of Sciences, University of Chile, PO Box 653, Santiago, Chile

Received 27 November 2006; received in revised form 17 May 2007; accepted 20 May 2007

Available online 26 May 2007

Abstract

In this work, a characterization of reagent chromophore 5-(4-sulphophenylazo)-8-aminoquinoline [SPA] by IR and ^1H RMN was carried out and a $\text{p}K_{\text{a}}$ value of 3.55 ± 0.03 was found as well. An 1:2 stoichiometry for the Cu(II)-SPA complex was determined at pH 9 by Job and molar ratio methods. A value of 1.4×10^{14} for the stability constant was also found. Based on the formation of this complex a new method for the copper determination in presence of gold and silver was developed by derivative spectrophotometry using a previous preconcentration on solid phase. In this method, the analytical measures were executed directly in the solid phase containing the complex. The Cu(II) reacts with the reagent chromophore SPA previously retained in the anionic exchange DEAE Sephadex A25. In this determination, the first derivative at 605 nm was used. The quantification range was between $(3.2 \pm 0.3 \times 10^{-1}) \times 10^{-8}$ and $(94.4 \pm 0.9) \times 10^{-8} \text{ mol L}^{-1}$ ($3.2 \pm 0.3 \times 10^{-1}) \times 10^{-8}$, and $(94.4 \pm 0.9) \times 10^{-8} \text{ mol L}^{-1}$. The repeatability expressed as RSD was between 1.1 and 2.0%. The method was applied successfully for the copper determination in mineral residuals and natural water samples. The results were consistent with those provided by ICP-mass spectrometry.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Characterization; 5-(4-sulphophenylazo)-8-aminoquinoline; Copper; Solid phase extraction; Derivative spectrophotometry

1. Introduction

The copper is extensively examined in environmental studies, industrial, biological applications, etc. However, there are many difficulties to determine traces in this type of samples due to insufficient sensitivity or matrix interferences. For this reason, a different preconcentration process has been employed together with diverse techniques. In this context, it was found that the use of solid phase with the incorporation of different chemical compounds or biological species that contain groups which permit the retention and preconcentration of different analytes, among them, the alga *Pilayella littoralis*, immobilized on silica gel employed as a biosorbent using flow injection system on line, and the amberlite XAD-2 functionalized with 2-aminothiophenol with a short column packed. This permits

the preconcentration of metallic ions being subsequent eluted and detected by inductively coupled plasma–atomic emission spectrophotometry (ICP–AES) [1,2].

On the other hand, chromatographic techniques have also incorporated preconcentration on solid phase or liquid–liquid in its methodologies for the determination of copper and other metallic ions, achieving the increase of the detection limits [3,4]. With the same purpose, in flame atomic absorption spectrometry (FAAS), a preconcentration has been used in batch and continuous [5–7]. All these methodologies incorporate preconcentration for the determination of Cu(II) at trace level. However, in spite of the use of a preconcentration and sophisticated instrumentation, the values of detection limits were found between 0.05 and $0.6 \mu\text{g L}^{-1}$. A disadvantage of the procedures that use a preconcentration is in the analyte elution because it diminishes the sensitivity for dilution effect and increases the previous stages of the analytic process, favoring the errors in the determinations.

The UV–vis spectrophotometry is an alternative technique that also allows carrying out the copper determination. Many

* Corresponding author. Tel.: +56 2 9787386; fax: +56 2 2713888.

E-mail address: analitic@uchile.cl (M.I. Toral).

analytic methods are based on this technique, due to the rapidness, simplicity and multiplicity of applications [8–10].

When kinetic reactions are used, it is possible to obtain a method by this technique with similar detection limit. Prasad [11] describes a highly-sensitive and selective catalytic kinetic spectrophotometric method for the determination of Cu(II) as low as $6 \mu\text{g L}^{-1}$ which is based on the catalytic effect of copper on the oxidation of citric acid by alkaline hexacyanoferrate(III) at 420 nm. A method has also been proposed for the determination of this metallic ion at 560 nm based on the catalytic effect of copper on the reduction of Ponceau S by sodium sulfide in an alkaline media. The method allows determination of copper at $2 \mu\text{g L}^{-1}$ levels [12]. The simultaneous determination of copper and iron, proposed by Ohno et al. [13], is based on their catalytic effects on the oxidative coupling of *p*-anisidine with *N,N*-dimethylaniline to form a colored compound in the presence of hydrogen peroxide in acid media. The quantification limit for copper was $0.16 \mu\text{g L}^{-1}$ and the method was successfully applied to the analyses of these ions in tap, well, river, and pond waters.

Nevertheless, when are not considered kinetic reactions, unfortunately, this technique presents a smaller sensitivity and selectivity, which limits its applicability. Some works have shown that it is possible to increase the sensitivity incorporating a preconcentration on solid phase (SPE), compensating the disadvantages of the liquid–liquid extraction [14,15]. Several methods have been described that include a preconcentration process, where the analytes are eluting being evaluated by their analytic signals later on, producing an increase of the previous stages [16]. Other methods require extended preconcentration times in order to obtain the quantitative extraction [17].

In the extraction and preconcentration process on solid phase, several supports have been used: chitosan microspheres [18], *Aspergillus niger* [19], polystyrene divinylbenzene resins, etc. The resins can be functionalised, as well as loaded with a variety of organic ligands having various functional groups [20–23]. Some resin beads are commercially available as Dowex and Amberlite, which differ in their cross-linkage degree, bead size, pore diameter, etc. The use of these resin types are in concordance when the analytes are eluted and then evaluated for the techniques previously indicated. However, the dilution effect, when the analytes are eluted, is a disadvantage for spectrophotometric determination.

This work proposes to use spectrophotometry in derivative modality to reach detection limits at trace level carrying out a previous preconcentration to the instrumental measure, obtaining the analytical signal directly in the solid phase, in order to increase the sensitivity and to diminish the previous stages, since the preconcentration and the formation of the complex is carried out in a single stage directly in the solid phase that contain to 5-(4-sulphophenylazo)-8-aminoquinoline ligand, previously retained on DEAE Sephadex A25 anionic exchange, since it was found that it is the most promising polymeric support, because it has a greater surface area for immobilizing the ligand. Further, the grains are small and uniform, whereby it is possible to obtain a good repeatability. The interaction is produced by electrostatic attraction between the group $[\dots\text{OCH}_2\text{CH}_2\text{NH}(\text{CH}_2\text{CH}_3)_2^+]$ of

the resin and the sulfonic groups of the ligand. The groups of the ligand $-\text{C}_5\text{H}_3\text{N}$ and $-\text{NH}_2$ reach a position in the solid phase that permits to form complex with Cu(II) and to obtain the preconcentration. This process makes it possible to reduce solvents, costs and time of extraction in the preparation of the samples and permits copper determination without highly sophisticated instruments [24,25].

The 5-(4-sulphophenylazo)-8-aminoquinoline (SPA) is a ligand that was synthesized and characterized only using elemental analysis by Zuotao and McCreedy [26]. This ligand only has been used in alkaline aqueous solution in presence of cetyltrimethylammonium bromide for the Au(III) determination by flow injection spectrophotometry.

In this work is presented the characterization and the structure of the Cu(II)-SPA complex in aqueous solution including a deep characterization of the SPA ligand by mean of IR, ^1H NMR and elemental analysis. In our work, the result of the last analysis differs with those obtained by Zuotao and McCreedy [26].

Based in this new Cu(II)-SPA complex a method for the copper determination in presence of silver and gold is proposed. The application of the method was realized in mineral residuals and natural water impacted by the mining activity.

2. Experimental

2.1. Apparatus

A Shimadzu UV–vis 1603 absorption spectrophotometer with a 1 mm quartz cell was used for absorbance measurements and derivative absorption spectra in solid phase. A 10 mm quartz cell was also used when the measurements were realized in aqueous solution. The derivative spectra were obtained using a software, Shimadzu kit version 3.7 (P/N 206-60570-04). An Orion Digital Research Ion-Analyzer 701 with glass and saturated calomel electrodes was used for pH determinations. A magnetic stirrer, HI 190 M Hanna Instrument, for the preconcentration step was also used.

An inductively coupled plasma–optical emission spectrometer (ICP–OES) Perkin–Elmer Optima 3300 XL was used to compare the proposed method.

2.2. Chemicals and reagents

All reagents were of analytical grade and the solutions were prepared with high-purity water from a NANO pure Barnstead ultrapure water system device.

Commercial standard solutions of 1000 mg L^{-1} Cu(II), Ag(I) and Au(III) were purchased from Merck®. The pH 9 was adjusted with H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.6 mol L^{-1} buffer solution. DEAE Sephadex A25 ionic exchanger was purchased from Aldrich®.

The chromogenic reagent SPA was synthesized, according to the procedure proposed by Zuotao and McCreedy [26]. Thus, SPA was prepared by dissolving sulphanilic acid (2.1 g) in 2 mL of ice-cold concentrated hydrochloric acid and 10 mL of ice-cold distilled water and was slowly diazotized with a solution of 0.8 g of sodium nitrite in 8 mL of water. The diazotized solution

was added drop-wise with stirring to an ice-cold solution of 8-aminoquinoline (1.6 g) in 50 mL of 2 mol L⁻¹ acetic acid, then mixed during 1.5 h with stirring in the ice-bath, neutralized with NaOH and filtered. The red precipitate was recrystallized and salted out with NaCl several times from the water.

The chromophore synthesized was used to prepare a 1.5 × 10⁻³ mol L⁻¹ SPA solution in water.

In the validation of the method a quality control solution High-Purity Standards® QCS-19 (Charleston, USA) was used. This sample had the following content in 100 g mL⁻¹ Sb, As, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Mo, Se, Tl, Ti, V, Zn, and Ni.

2.3. Characterization of SPA

The chromophore synthesized was characterized by elemental analysis, FT-IR and ¹H NMR spectroscopy. Elemental analysis (EA) of 5-(4-sulphophenylazo)-8-aminoquinoline dried in vacuo was performed using a CE Instrument EA 1108. The samples were analyzed in potassium bromide disks using an infrared Fourier transform spectrophotometer, Bruker vector 22 FT-IR, in the region from 4000 to 450 cm⁻¹. Nuclear Magnetic Resonance spectra of ¹H using d₆-DMSO were measured in a Bruker AMX-300 spectrophotometer.

The experimental data of elemental analysis of 5-(4-sulphophenylazo)-8-aminoquinoline (C₁₅H₁₁N₄NaO₃S) were 45.88% C, 14.34% N, 3.34% H, and 8.67% S. The IR spectrum shows bands corresponding to ν (S(=O)₂) 1193 cm⁻¹, ν (N–H) 3500–3250 cm⁻¹, ν (N=N) 1385 cm⁻¹, ν (C=C) 1621 cm⁻¹, and ν (C=N) 1511 cm⁻¹. The ¹H NMR shows signals corresponding to δ (–Ar–H) 6.95 ppm (1H), 9.25 ppm (1H), 7.7–7.9 ppm (4H); δ (C₅H₃N) 7.65 ppm (1H), 8 ppm (1H), 8.85 ppm (1H) and δ (NH₂) 7.1 ppm (2H).

2.4. Extraction procedure

2.4.1. Retention of SPA on DEAE Sephadex A25

The retention of SPA in the solid phase from 200 mL of aqueous solution containing 50 mL of ligand SPA

1.5 × 10⁻³ mol L⁻¹ was carried out using 10 g of DEAE Sephadex A25, stirring during 30 min. The solution was filtered and the solid was obtained with the retained ligand [SPA–DEAE Sephadex A25], finally was dried at 50 °C and used in the determinations.

2.4.2. Procedure for preconcentration and determination of metal ions

The formation of the complexes was generated in the ionic exchange with the previously retained ligand. The preparation of the samples was carried out in a final volume of solution of 200 mL and then were added between 20 to 120 μL of metal of 100 mg L⁻¹ and 10 mL of H₃BO₃ 1 mol L⁻¹/H₂BO₃Na 0.6 mol L⁻¹ buffer solution. To each solution was added 60 mg of SPA–DEAE Sephadex A25 and was stirred for 20 min. The solid phase was packed in 1 mm optical path quartz cells and the absorption spectra were carried out in wavelengths ranges from 250 to 800 nm. By means of graphic method at λ = 605 nm, the copper quantification was carried out by first derivative spectra. The first order derivative was obtained using a smoothing factor of 40,000 and a scale factor of 10,000.

2.5. ICP–OEC method

The copper determination was carried out by ICP–OES, following the US-EPA 6010 B method [27] in order to be used as comparative method.

3. Results and discussion

3.1. Characterization of SPA

The results of the characterization of chromophore synthesized are summarized in Section 2.3.

The results of elemental analysis are not concordant with theoretical data if the molecule is pure. However, taking account that the ¹H NMR spectrum presents a high peak at 3.3 ppm, it is possible to postulate that the SPA ligand contains crys-

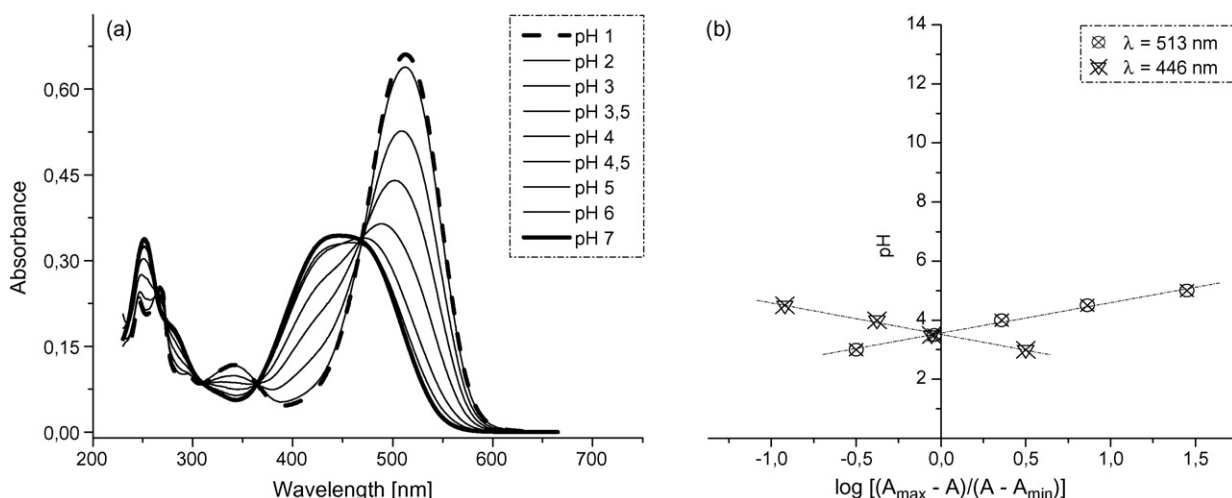


Fig. 1. (a) Absorption spectra of 5-(4-sulphophenylazo)-8-aminoquinoline 2 × 10⁻⁵ mol L⁻¹ in aqueous solution at different pH values and (b) pK_a determination of 5-(4-sulphophenylazo)-8-aminoquinoline by the Henderson–Hasselbalch equation.

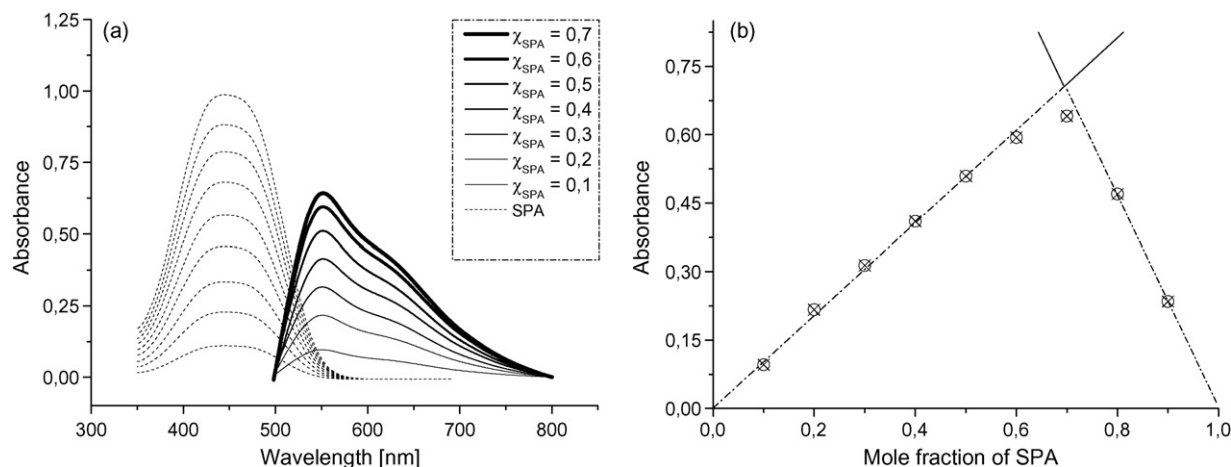


Fig. 2. Job's method of continuous variation of Cu(II)-SPA complex. Cu(II) or SPA $3.3 \times 10^{-4} \text{ mol L}^{-1}$, pH 9 (buffer H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.6 mol L^{-1}), $\lambda = 550 \text{ nm}$. (a) Spectra of the Cu(II)-SPA complex and reagent blank in aqueous solution. (b) Job's graphic.

tallization water. The theoretical data 47.2% C, 14.69% N, 2.9% H, 8.4% S has a good correspondence with the experimental data if the molecule has two crystallization water. The 5-(4-sulphophenylazo)-8-aminoquinoline pK_a was determined by spectrophotometric method. The Fig. 1a shows that spectra at 513 nm and 446 nm correspond to the acid and basic form of SPA, respectively. At 470 nm an isosbestic point was found. The quantification of pK_a value was carried out by using the Henderson–Hasselbach linear equation [28]. As can be seen, SPA presents a pK_a 3.55 ± 0.03 value (Fig. 1b).

3.2. Characterization of Cu(II)-SPA complex

Cu(II) reacts with SPA at room temperature in basic pH and it forms a brown-red complex with molar absorptivity (ϵ) of $3.4 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 550 nm. The absorption of this complex is not affected by the buffer addition. The stoichiometry of metal/ligand ratio 1:2 was determined by Job (Fig. 2)

and molar ratio methods (Fig. 3) [29]. These determinations were carried out in presence of H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.6 mol L^{-1} buffer solution in order to reach pH 9 and to maintain constant the ionic strength. In both cases the same stoichiometry was obtained. Mass spectrum of the complex shows the peaks at m/z value of 874.44, 856.11, 818.98, 761.34, and 719.79 corresponding to $(\text{C}_{30}\text{H}_{22}\text{CuN}_8\text{Na}_2\text{O}_6\text{S}_2 \cdot 6\text{H}_2\text{O})^+$, $(\text{C}_{30}\text{H}_{22}\text{CuN}_8\text{Na}_2\text{O}_6\text{S}_2 \cdot 5\text{H}_2\text{O})^+$, $(\text{C}_{30}\text{H}_{22}\text{CuN}_8\text{Na}_2\text{O}_6\text{S}_2 \cdot 3\text{H}_2\text{O})^+$, $(\text{C}_{30}\text{H}_{22}\text{CuN}_8\text{Na}_2\text{O}_6\text{S}_2)^+$, and $(\text{C}_{30}\text{H}_{22}\text{CuN}_8\text{O}_6\text{S}_2)^+$, respectively.

Taking into account the relation of the complex and the configuration d^9 of Cu^{2+} , it is possible to propose that $\text{Cu}(\text{SPA}x2\text{H}_2\text{O})_2$ bond with 2 molecules of water as axial ligands (long bonds) by mean of the O donor and to form a complex of the octahedral distorted structure, due to the Jahn–Teller effect. When the $\text{Cu}(\text{SPA}x2\text{H}_2\text{O})_2(\text{H}_2\text{O})_2$ complex is warmed loses water of coordination to give $\text{Cu}(\text{SPA}x2\text{H}_2\text{O})_2$ anhydrous com-

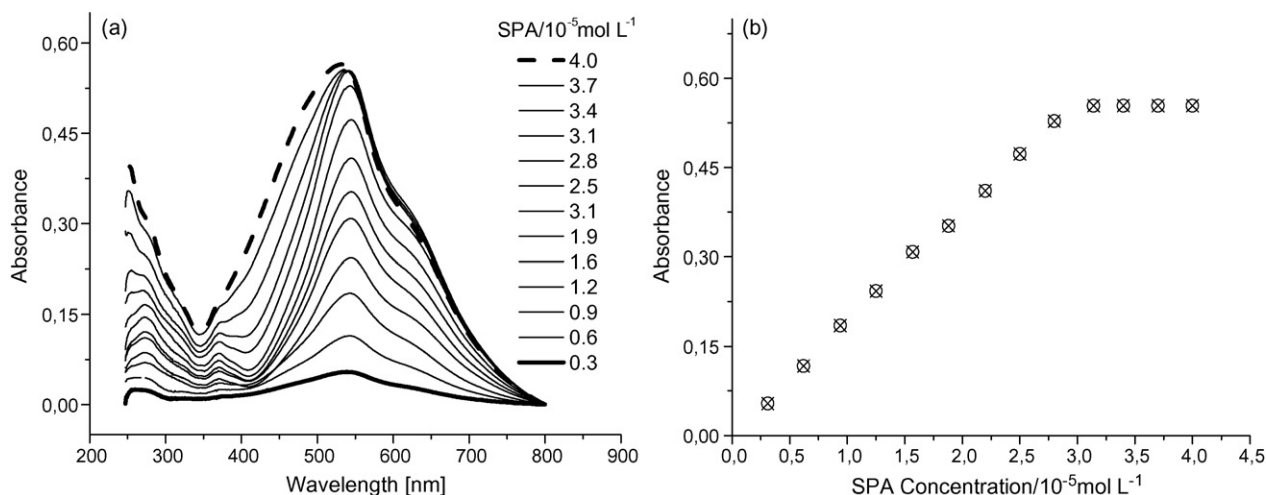


Fig. 3. Method of the molar relationships for the complex Cu(II)-SPA. (a). Absorption spectra of the complex formed by Cu(II) with different SPA ligand concentrations and (b) Effect of the addition of SPA on the formation of the complex. Cu(II) $1.5 \times 10^{-5} \text{ mol L}^{-1}$, buffer H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.63 mol L^{-1} , pH 9, $\lambda = 550 \text{ nm}$.

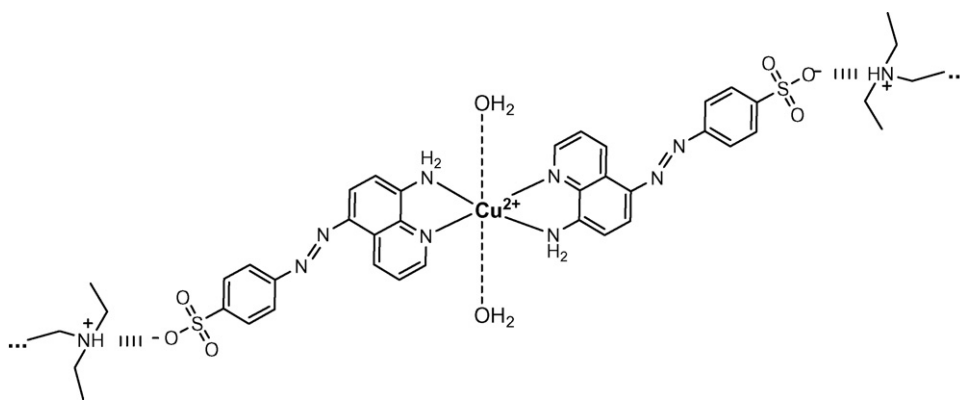


Fig. 4. Interaction Cu^{2+} with SPA-DEAE Sephadex A25. Formation of complex Cu(II)-SPA on solid phase.

plex, where the four molecules of water correspond to the water contained in the SPA ligand.

The stability constant of the complex obtained by Job and Likussar methods was 1.4×10^{14} at 18°C [30].

3.3. Concentration effect of SPA on DEAE Sephadex A25

The study of the concentration effect of SPA retained in the anionic exchange DEAE Sephadex A25 was carried out. The concentration of SPA in the solution was varied from $1.5 \times 10^{-6} \text{ mol L}^{-1}$ to $6.1 \times 10^{-6} \text{ mol L}^{-1}$, being retained successfully in the solid phase. Nevertheless, the spectral signals of SPA retained on Sephadex in the range $3.8 \times 10^{-6} \text{ mol L}^{-1}$ to $6.1 \times 10^{-6} \text{ mol L}^{-1}$, present a wide and intense band of absorption, diminishing the zone of absorption of the complex for instrumental limitations. For these reasons $2.3 \times 10^{-6} \text{ mol L}^{-1}$ was selected as work concentration.

3.4. Preconcentration of Cu(II) on the SPA-DEAE Sephadex A25

The extraction of the metallic ion from the solution generates the immediate formation of the complex in the solid phase (Fig. 4).

The complex Cu(II)-SPA on the anionic exchange DEAE Sephadex A25 shows a maximum of absorption to 590 nm. Carrying out the absorbance measure of the complex directly in the solid phase appears an increment of the sensitivity, which allows the determination of copper at trace levels. On the other hand, although Ag(I) and Au(III) form complex with SPA-DEAE Sephadex A25, their signals are of low intensity, which does not allow its quantification at low concentrations (Fig. 5).

Nevertheless, the presence of these cations at concentrations of $2.3 \times 10^{-7} \text{ mol L}^{-1}$ for Ag(I) and $6.3 \times 10^{-8} \text{ mol L}^{-1}$ for Au(III), produces an increment in the absorbance values near to 10%.

For these reasons a method is developed for the Cu(II) determination in those matrix in that these two ions or at least one of them is present, because the interference caused by Au(III) and Ag(I) can be eliminated with the use of derivative spectropho-

tometry [31], since this technique allows the resolution of the overlapped bands that absorb in common areas of the classic spectrum.

3.5. Optimization of chemical and preconcentration variables

3.5.1. pH Effect

The pH effect on the complex formation in the range 2–11 was studied. The acid medium does not interfere in the SPA retention on the anionic exchange, however, the complex Cu(II)-SPA is formed quantitatively at pH 9.0 reached with H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.6 mol L^{-1} buffer solution. Further, the effect of buffer concentration was studied on the complex formation selecting 10 mL of this buffer as optimum.

3.5.2. Mass of SPA-DEAE Sephadex A25 Effect

The effect of the mass on the analytical signal was optimized using different mass of solid phase. The signals exponentially decrease with the mass increment of the SPA-DEAE

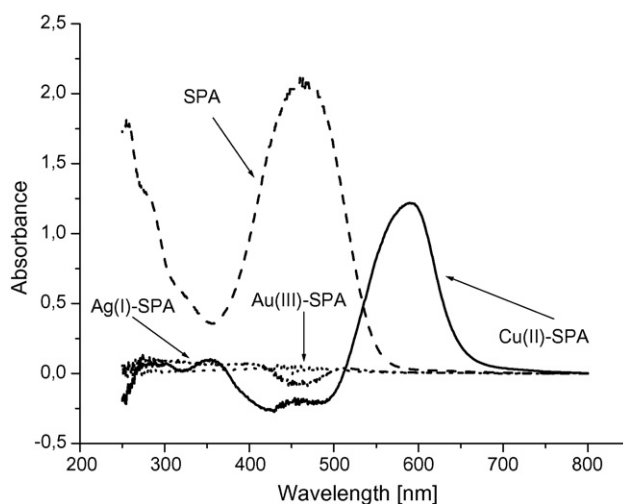


Fig. 5. Zero order spectra of the complexes retained on SPA-DEAE Sephadex A25, against reagent blank. Concentration Cu(II) $7.8 \times 10^{-7} \text{ mol L}^{-1}$, Ag(I) $2.3 \times 10^{-7} \text{ mol L}^{-1}$, Au(III) $6.3 \times 10^{-8} \text{ mol L}^{-1}$. Ten milliliter of buffer H_3BO_3 1 mol L^{-1} / $\text{H}_2\text{BO}_3\text{Na}$ 0.63 mol L^{-1} , 60 mg SPA-DEAE Sephadex A25.

Sephadex A25. In order to increase the sensibility a mass of $60 \text{ mg} \pm 0.01 \text{ mg}$ was selected.

3.5.3. Stirring time effect

The stirring time effect on the retention of the metal ion in the range 5–30 min was also studied. The stirring time for maximum copper retention was 15 min. However, 20 min was select to assure a quantitative retention.

3.6. Selection of spectral variables

3.6.1. Selection of the derivative order and λ analytical

Two modes of digital derivative spectrophotometry can be used: in the first mode, the derivatives correspond to $\Delta A/\Delta \lambda$ where the derivation process is obtained by a program using a two-column table. In this derivative type the $\Delta \lambda$ values can be varied, this mode is very useful when the spectra are strongly overlapped. In the second mode proposed by Savitzky and Golay [31], the derivative corresponds to $A/\Delta \lambda$, where A is absorbance, which must be measured in fixed points and $\Delta \lambda$ is the wavelength range used in the scanned spectral. In this type of digital derivative spectrophotometry, the minimum squares procedure to smooth and differentiate the spectra numerical data is carried out also by computer processing. The last alternative was adopted in this work, because with this mode it is possible to resolve the spectral bands and to obtain a control of the noise of the baseline and the analytical signal. Under this condition the higher signal/noise ratio was found.

Derivative spectra of different orders were obtained from stored zero order spectra using digital differentiation. The first derivative spectral allows the determination of Cu(II) in a sample that also contains Au(III) and Ag(I), since the first derivative spectra of these analytes are zero between 590 and 680 nm, allowing the Cu(II) determination by means of graphic method at analytical wavelength of 605 nm (Fig. 6). This value does

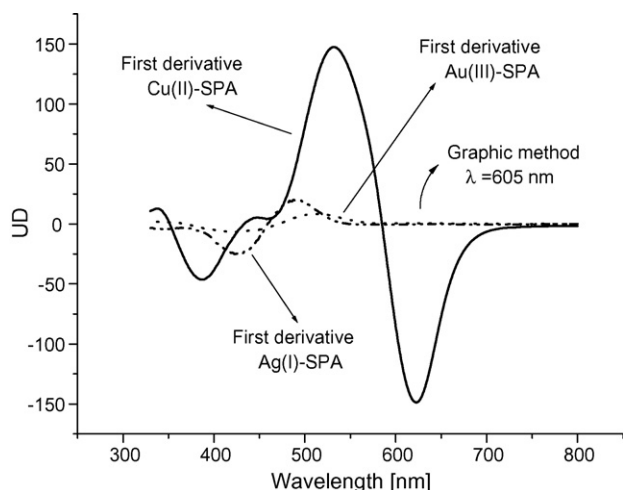


Fig. 6. Derivative spectra of the Cu(II), Au(III) y Ag(I) complexes on SPA-DEAE Sephadex A25. Concentration Cu(II) $7.8 \times 10^{-7} \text{ mol L}^{-1}$, Ag(I) $2.3 \times 10^{-7} \text{ mol L}^{-1}$, Au(III) $6.3 \times 10^{-8} \text{ mol L}^{-1}$. 10 mL of buffer H_3BO_3 $1 \text{ mol L}^{-1}/\text{H}_2\text{BO}_3\text{Na}$ 0.6 mol L^{-1} , 60 mg de SPA-DEAE Sephadex A25. First derivative, smoothing factor 40,000, scale factor 10,000.

not correspond to the maximum analytical signal of copper, however it was selected in order to increase the selectivity, because in this conditions, the interference of other ions is decreased.

3.6.2. Selection of the smoothing factor

The smoothing factor correspond to the points number used for the derivation zero order spectra together with to smooth the analytical signal. For the first derivative, the following values were used: 5000; 10,000; 20,000 and 40,000, these values are defined by default for the software, which are in relation with the wavelength range where the spectra were scanned. The $\Delta \lambda$ value is constant and correspond to 550 nm. Under these conditions a value of 40,000 points was selected. When the smoothing factor is increased the heights of the derivative signal decrease, but the noise decrease faster, increasing the signal/noise ratio.

3.6.3. Selection of the scale factor

The scale factor permits the amplification and improvement of the analytical signal in order to obtain a better lecture. This factor must be studied in order to observe if the system presents a distortion effect. Considering this fact, a scale factor of 10^4 was selected. It is important to state that this factor does not improve the analytical parameter because the signal of the analytes and the reagents blank increase in the same proportion.

3.7. Analytical features

The determination of the analytical features, for the determination of copper in presence of gold and silver was carried out by first order derivative spectrophotometry to 605 nm using a smoothing factor of 40,000 and an amplification factor of 10,000 in a total solution volume of 200 mL. The equation for the copper determination obtained by the method of least squares is $\text{DU} = (15.6 \pm 0.1) \times 10^6 C$ (mol L^{-1}) + $(1.2 \times 10^{-1} \pm 2.0 \times 10^{-3})$ with $r = 0.999$.

For a total volume of 200 mL the quantification range was between $(3.2 \pm 0.3 \times 10^{-1}) \times 10^{-8}$ to $(94.4 \pm 0.9) \times 10^{-8} \text{ mol L}^{-1}$. The detection limit (calculated by using the 3σ criterion) was found to be $(0.96 \pm 0.01) \times 10^{-8} \text{ mol L}^{-1}$.

The repeatability of the method expressed as relative standard deviation (RSD) for 10 samples containing $7.8 \times 10^{-7} \text{ mol L}^{-1}$, $2.3 \times 10^{-7} \text{ mol L}^{-1}$ and $6.3 \times 10^{-8} \text{ mol L}^{-1}$ of copper, silver, and gold, respectively, was between 1.1 and 2.0%.

The features of the proposed method are similar to those obtained using methods that require sophisticated instrumental.

3.8. Adsorption isotherm

For the determination of the adsorption behavior of the solid phase, the amount of adsorbed copper was studied as a function of copper concentration (Fig. 7a). The models proposed by Langmuir and Freundlich were analyzed. The linearization of Langmuir isotherm can be obtained by plotting C_e/q as a

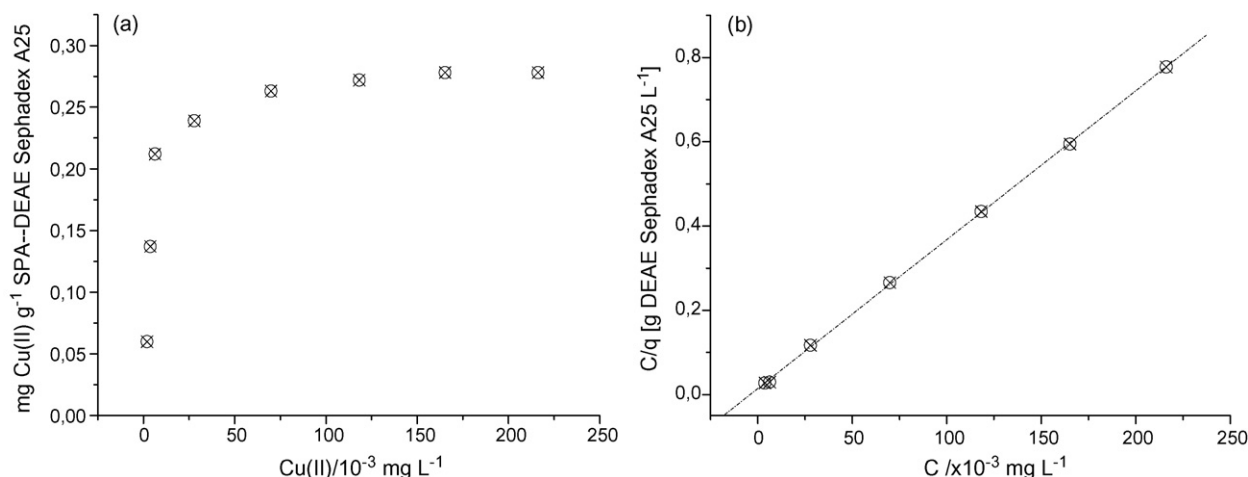


Fig. 7. (a) Adsorption isotherm of SPA-DEAE Sephadex A25 for copper. 60 mg of SPA-DEAE Sephadex A25, 10 mL of buffer H_3BO_3 $1 \text{ mol L}^{-1}/\text{H}_2\text{BO}_3\text{Na}$ 0.63 mol L^{-1} , $\lambda = 590 \text{ nm}$. and (b) Dependence of surface coverage SPA-DEAE Sephadex A25 on Cu(II) ion concentration determined from Fig. 7(a) data. The solid line represents the fit according to the Langmuir adsorption model.

function of C_e . The slope of the plot yields the maximum capacity (q_m) whereas its linear coefficient represents the Langmuir constant (K). The Freundlich equation can be linearized using logarithms to determine K_F and n from the slope and linear coefficient of the plot of $\log q$ versus $\log C_e$, respectively [32]. The correlation coefficients of the linear equations obtained in this study were 0.999 (Langmuir) and 0.847 (Freundlich). An analysis of the adsorption behavior comparing both models revealed that Langmuir equation yielded the best fit. Thus, the adsorption isotherm of copper is according to the Langmuir equation (Fig. 7b) [33] and the graphical treatment of the data yielded $1.71 \times 10^{-2} \text{ mg}$ of Cu(II) per gram of SPA-DEAE Sephadex A25 as maximum saturation capacity and equilibrium binding constant K $1.3 \times 10^7 \text{ mol}^{-1} \text{ L}$. The Langmuir treatment demonstrated that the surface is nearly fully covered with monomolecular layer of copper at high concentrations. Therefore, at high concentrations adsorption does not increase linearly. The main adsorption sites of the SPA-EAE Sephadex A25 were the $-\text{H}_2\text{N}$: and $-\text{N}$: groups forming a solid phase chelate.

3.9. Study of interferences

Potential interferences caused by several cations in the form of chlorides, nitrates or sulphates and anions as potassium, sodium or ammonium salts were studied. The interferences due to several cations and anions were studied thoroughly. In this study, different amounts of the ionic species were added together with Cu(II), Ag(I) and Au(III) containing $7.8 \times 10^{-7} \text{ mol L}^{-1}$, $2.3 \times 10^{-7} \text{ mol L}^{-1}$ and $6.3 \times 10^{-8} \text{ mol L}^{-1}$, respectively. The concentration of each interferent was progressively reduced until the ceasing of the interference.

Several cations interfered (variation of signal higher than 8%) at higher (K^+ , Mg^{2+} , Ca^{2+}) similar (Ni^{2+} , Co^{2+} , Hg^{2+}) or even lower (Fe^{3+}) molar concentration than copper concentration.

Although in many real samples, most of the interfering species appear at lower concentration than copper. However, in mineral residuals and natural water polluted, the concentration of Fe^{3+} is similar to that of Cu(II), whereby the interference of this cation was eliminated by means of the addition of NaOH 1 mol L^{-1} until reaching pH 5, since to this pH value, it is pos-

Table 1
Tolerance limits for cations and anions in copper determination

Cation	Interferent mol L^{-1}	Molar ratio I/Cu(II)	Anion	Interferent mol L^{-1}	Molar ratio I/Cu(II)
K^+	5.1×10^{-3}	6.5×10^3	SCN^-	3.3×10^{-3}	4.2×10^3
Ca^{2+} , Mg^{2+}	4.2×10^{-3}	5.4×10^3	Br^-	2.0×10^{-3}	2.5×10^3
Na^+	2.1×10^{-3}	2.7×10^3	I^-	1.0×10^{-3}	1.3×10^3
Al^{3+}	8.6×10^{-6}	10.9	Cl^-	3.0×10^{-3}	3.8×10^3
Cd^{2+}	6.2×10^{-6}	7.9	F^-	2.6×10^{-3}	3.3×10^3
Zn^{2+}	3.5×10^{-6}	4.5	Oxalate	3.5×10^{-6}	4.5
Bi^{3+}	3.4×10^{-6}	4.3	Tartrate	7.4×10^{-7}	0.95
Cr^{3+}	1.9×10^{-6}	2.5	EDTA	1.4×10^{-7}	0.18
Mn^{2+}	1.8×10^{-6}	2.3	Citrate	1.1×10^{-7}	0.14
Co^{2+} , Ni^{2+} , Pb^{2+}	1.7×10^{-6}	2.2			
Hg^{2+}	1.2×10^{-6}	1.5			
Fe^{3+}	3.6×10^{-7}	0.5			

Table 2
Determination of copper in real samples (comparison of the analytical methods)

Samples	ICP–MS spectrometry ^a Cu (mg L ⁻¹)*	Proposed method	
		Cu (mg L ⁻¹)**	Recovery (%)
Natural water ^b	0.424 ± 0.003	0.421 ± 0.008	99.3 ± 1.9
Industrial residuals	78.0 ± 0.6	79.2 ± 1.5	101.5 ± 1.9

ICP–MS spectrometry (USA-EPA 6020); * Three replicates. ** Seven replicates.

^a Provided by CEPEDEQ, Faculty of Sciences Chemical and Pharmaceutical University of Chile.

^b Coya river, VI Region of Chile.

sible to eliminate the interference of this cation by precipitation as Fe(OH)₃.

The tolerance limits for the different cations and studied anions are shown in the Table 1.

3.10. Validation and application of the method

The validation of the method was carried out using a control solution High-Purity Standards[®] enriched with gold 1.3×10^{-4} mol L⁻¹ and silver 4.6×10^{-4} mol L⁻¹. An aliquot of 0.1 mL of this sample was measured and diluted to 200 mL solution. The copper recovery was of 98.2%. The relative standard deviation was minor to 2%.

The method was applied on mineral residuals obtained from a confidential source and river water samples. The water samples were collected in the Coya river, VI Region of Chile, this aquatic system has been polluted by copper smelter processes for many years [34].

The results are shown in Table 2 and they are in agreement with ICP-mass spectrometry.

To compare both methods the *t* Student with a 95% threshold level was used. For ICP–MS method three replicates were used, obtaining an average of 0.424 mg L⁻¹ with a standard deviation of 3.1×10^{-3} . For the proposed method seven replicates were carried out obtaining an average of 0.421 mg L⁻¹ with a standard deviation of 7.5×10^{-3} . The calculated *t* (*t*_c) was 0.9 taking into account ten degrees of freedom and 95% confidence, the *t*_c is smaller than the tabulated *t* (2.228), which would be indicating that the methods are not significantly different and the results agree inside the experimental error.

4. Conclusions

In this work, it was found that the SPA ligand presents a p*K*_a value of 3.6. According to IR, elemental analysis and ¹H RMN data it is possible to establish that this ligand crystallized with 2 H₂O molecules. On the other hand, it was also found that Cu(II) reacts with two molecules of SPA, whereby it was proposed that the Cu(II)–SPA complex have an octahedral distorted structure.

Using the synthesized ligand, a method for the determination of copper in the range 3.2×10^{-8} to 94.4×10^{-8} mol L⁻¹ was developed. In this determination both the extraction of Cu(II) from aqueous solution and the formation of complex can be executed in only one step, under these conditions it is possible the copper determination in presence of silver and gold. Another feature of this method is that the consumption of organic

solvent is lower than in liquid–liquid extraction methods. This method offers diverse advantages such as simplicity, rapidity, low cost and sensitivity, reaching similar detection limits to those obtained by sophisticated techniques. This method requires good experimental strategies to achieve an appropriate sampling frequency, which could be a disadvantage. The method was applied successfully for the copper determination in industrial residuals and natural water samples.

Acknowledgements

The authors are grateful to the National Fund for Development of Sciences and Technology (FONDECYT), project 1020692 and 1070905 for the financial support. Libby Morales thanks to National Commission Investigation for Science and Technology (CONICYT) for her doctoral and AT-4040211 fellowships.

References

- [1] E. Carrillo, J. Nóbrega, T. Gilbert, Talanta 60 (2003) 1131.
- [2] Y. Guo, B. Din, Y. Liu, X. Chang, S. Meng, J. Liu, Talanta 62 (2004) 207.
- [3] H. Lu, Y. Yan, S. Tong, J. Riviello, J. Chromatogr. A 800 (1998) 247.
- [4] S. Igarashi, N. Ide, Y. Takagai, Analytica Chimica Acta 424 (2000) 263.
- [5] D. Afzali, A. Mostafavi, M. Taher, A. Moradian, Talanta 71 (2007) 971.
- [6] V. Azebedo, P. Xavier, Talanta 67 (2005) 564.
- [7] A. Anthemidis, G. Zachariadis, J. Stratis, Talanta 54 (2001) 935.
- [8] K. Hussain, N. Prasad, T. Sreenivasulu, Talanta 59 (2003) 425.
- [9] J. Ghasemi, Sh. Ahmadi, K. Torkestani, Analytica Chimica Acta 487 (2003) 181.
- [10] J. Pinto, C. Moreno, M. García, Talanta 64 (2004) 562.
- [11] S. Prasad, Analytica Chimica Acta 540 (2005) 173.
- [12] A. Safavi, N. Maleki, F. Farjami, Talanta 54 (2001) 397.
- [13] S. Ohno, N. Teshima, H. Zhang, T. Sakai, Talanta 60 (2003) 1177.
- [14] M.I. Toral, N. Lara, J. Gómez, P. Richter, Anal. Lett. 35 (2002) 153.
- [15] M.I. Toral, C. Paipa, J. Narváez, P. Richter, Microchem. J. 73 (2002) 317.
- [16] Y. Yamini, A. Tamaddon, Talanta 49 (1999) 119.
- [17] K. Turhan, C. Ekinci – Dogan, G. Akcin, A. Aslan, Fresenius Environmental 14 (2005) 1129.
- [18] T.C. Coelho, et al., React. Funct. Polym. 67 (2007) 468.
- [19] A. Dursun, Biochem. Eng. J. 28 (2006) 187.
- [20] O. Zaporozhets, N. Petruniok, V. Sukhan, Talanta 50 (1999) 865.
- [21] S. Zhang, Q. Pu, P. Liu, Q. Sun, Z. Su, Analytica Chimica Acta 452 (2002) 223.
- [22] I. Narin, M. Soylak, Talanta 60 (2003) 215.
- [23] P. Kumar, A. Kumar, The Analyst 124 (1999) 1847.
- [24] V. Camel, Spectrochim. Acta, Part B 58 (2003) 1177.
- [25] L. Hejazi, D. Mohammadi, Y. Yamini, R. Brereton, Talanta 62 (2004) 183.
- [26] Z. Zuotao, T. McCreedy, A. Analytica Chimica Acta 401 (1999) 237.

- [27] EPA 6010B method, U.S. Environmental Protection Agency. 1996.
- [28] K. Connors, *Curso de Análisis Farmacéutico* Reverté, 2nd edn., 1980, p. 211.
- [29] W. Vosburgh, G. Cooper, *J. Am. Chem. Soc.* 63 (1941) 437.
- [30] W. Likussar, D.F. Boltz, *J. Anal. Chem.* 43 (1971) 10.
- [31] G. Savitzky, M. Golay, *J. Anal. Chem.* 36 (1964) 1627.
- [32] E. Guibal, C. Milot, J. Tobin, *Ind. & Eng. Chemistry Research* 37 (1998) 1454.
- [33] P. Hiemenz, R. Rajagolapan, *Principles of Colloid and Surface Chemistry* Marcel-Dekker, 3rd edn., 1997, p. 336.
- [34] J. Narvaez, P. Richter, M.I. Toral, *Analytical and Bioanalytical Chemistry* 381 (2005) 1483.

Study of β -cyclodextrin/fluorinated trimethyl ammonium bromide surfactant inclusion complex by fluorinated surfactant ion selective electrode

Sandeep R. Patil^{a,b,c,1}, Mireille Turmine^{a,b,c}, Véronique Peyre^{a,b,c,d,*},
Grégory Durand^d, Bernard Pucci^d

^a Université Pierre et Marie Curie-Paris 6, UMR7575 (LECA), Case Courrier 39, 4 Place Jussieu,
75252 Cédex 05, Paris F-75005, France

^b ENSCP, UMR7575, Paris F-75005, France

^c CNRS, UMR7575, Paris F-75005, France

^d Laboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, Université d'Avignon
et des Pays du Vaucluse, Faculté des sciences, 33, rue Louis Pasteur, Avignon F-84000, France

Received 28 February 2007; received in revised form 7 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

The construction and performance of a liquid membrane electrode responsive to *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium bromide (FTABr) and its use for the study of β -cyclodextrin/fluorinated surfactant inclusion complex is described. The electrode is based on the use of tetrahydroperfluorooctyltrimethylammonium-tetraphenylborate ion pair as electro active material in polyvinyl chloride (PVC) matrix plasticized using 2-Nitrophenyl octyl ether (NPOE). The electrode exhibits a fast, stable, reproducible and “Nernstian” response (59 ± 2 mV) for FTABr over the concentration range of 10^{-5} to 2×10^{-3} mol L⁻¹ at 298 K. The lowest detection limit is 2×10^{-6} mol L⁻¹ and the response time is around 20–30 s. The validity of the electrode, for detection of fluorinated surfactant ions and hence to carry out electrochemical measurements to study micellization of fluorinated surfactant, is verified by comparing the critical micelle concentration (cmc) value of FTABr obtained by using the electrode, with that obtained by surface tension measurements. Association constant *K* for β -cyclodextrin/FTABr complex is evaluated from the potentiometric measurements carried out using this electrode and is observed to be $\sim 1.26 \times 10^5$. The results suggest that β -cyclodextrin forms an equimolar association complex with the FTA⁺ surfactant ion.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Ion selective electrode; Cationic fluorinated surfactant; β -cyclodextrin; Complexation constant

1. Introduction

Surfactants are important in various phenomena of interfacial science and continue to be critical in many applications in agrochemicals, water treatment, oil recovery, fire fighting, paper and plastic manufacturing [1]. Fluorinated surfactants are surface-active agents in which the hydrogen atoms of the hydrophobic moiety are partially or completely replaced by fluorine atoms [2]. The unique characteristic of fluorocarbon chains in fluorinated

surfactants leads to differences in their micellar solution properties as compared to those of their hydrocarbon analogues and hence is responsible for their specific performance properties. Fluorocarbon surfactants exhibit lower critical micelle concentration (cmc) as compared to hydrocarbon surfactants having the same tail length [3–4], display tendency to form aggregates having low curvatures, including cylindrical micelles and bilayer structures [5–8]. Fluorocarbon surfactants are, thus, very important from fundamental as well as industrial point of view and have been found useful in various high performance applications like electroplating, wetting, biological oxygen carriers in artificial blood formulations, emulsion polymerization, templates for synthesis of mesoporous structures and so on [9–12].

Handling of surfactants for use, formulation or production needs simple, reliable analytical technique to determine their

* Corresponding author. Tel.: +33 1 44 27 36 76; fax: +33 1 44 27 30 35.

E-mail address: peyre@ccr.jussieu.fr (V. Peyre).

¹ Current address: School of Chemical & Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland.

quantity precisely in reaction media. Surfactant ion selective electrodes (ISE) have been developed in the last three decades and have proven to be advantageous as an in-house analytical tool for direct determination of presence of surfactant even at low concentration as well as to study surfactant/dendrimer or surfactant/biomolecule interaction due to their low cost, high selectivity, rapid response and simplicity of operation. However, few articles reported the use of such ISE for fluorinated surfactants [13–14], and to our knowledge, no information is available regarding an ISE specific to cationic fluorinated surfactants. One of the limitation in the use of ISEs for cationic fluorinated surfactants is probably due to their low availability, since they are usually known as difficult to synthesize and are very unstable. In the present article, we report a convenient synthesis of a cationic fluorinated surfactant, *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium bromide (FTABr) and the construction and performance of stable, selective, quick response ion electrode of this fluorinated trimethylammonium compound. The stability and sensitivity offered by the set electrode are high enough to allow accurate determination of low concentrations of fluorinated cationic surfactant by direct potentiometric titration, facilitating precise investigation of micellization of cationic fluorinated surfactant. In order to illustrate the capacities of this electrode, we have determined the complexation constant of FTABr with β -cyclodextrin (β -CD).

β -cyclodextrin is a macrocyclic oligosaccharide consisting of seven D-(+)-glucopyranose units linked by $\alpha(1,4)$ interglucose bonds and shaped like a truncated cone with a relatively hydrophobic hollow cylindrical cavity [15–16]. Cyclodextrins are known to form inclusion compounds with many hydrophobic or especially amphiphilic species by capturing different guest molecules in their cavities. This peculiar behavior is responsible for their use in diverse applications like drug delivery systems, catalysis as well as taste and odor preservatives in food industry. Numerous studies are reported in literature which deals with the association of surfactants, including anionic fluorinated surfactants [17–20], with cyclodextrin using different methods [21–25]. However, the scattering of complexation constants determined for hydrogenated compounds is very large and also dependant on the number of complexation equilibrium considered. In the present article, we show that our newly designed ISE enables a fast and accurate determination of the complexation stoichiometry and related constant for the inclusion of a cationic fluorinated surfactant in the cavity of β -cyclodextrin.

2. Experimental

2.1. Reagents and apparatus

Most of the starting materials were purchased from Acros Chemicals except for 1H,1H,2H,2H-perfluorooctylidide which was from Fluorochem, β -cyclodextrin from Janssen-Chimica and used as received, low molecular weight ($M_w \sim 48,000$) polyvinyl chloride (PVC) and 2-Nitrophenyl octyl ether (NPOE) obtained from Fluka. Amberlite resin IRN78 was from Prolabo.

TLC analyses were performed on sheets precoated with silica gel 60F254 (Merck). All solvents were distilled and dried according to standard procedures. Compound detection was achieved by exposure to UV light (254 nm) and by I_2 vapor, then heating at 150 °C. Mass spectra were recorded on a Triple quadrupole spectrometer API III Plus Sciex for ESI+ and a triple quadrupole spectrometer Quattro I Micromass for ESI–. Melting points were measured on an Electrothermal IA9100 apparatus and have not been corrected. The ^1H , ^{13}C and ^{19}F NMR spectra were recorded on a Bruker AC-250 spectrometer. Chemical shifts are given in ppm relative to tetramethylsilane using the deuterium signal of the solvent (CDCl_3) as a heteronuclear reference for ^1H and ^{13}C . For the ^{19}F NMR spectra, the internal reference is CFCl_3 . Abbreviations used for signal patterns are: s, singlet; bs, broad singlet; t, triplet; m, multiplet. All aqueous solutions were prepared from distilled and deionized water with electrical resistivity 18 M Ω (Elga water purification system). Electromotive forces were measured with a Meterlab PHM250 Ion Analyzer System (Radiometer Analytical, Copenhagen). The surface tension measurements were carried out using a tensiometer (Krüss K10T) by the du Noüy ring method and the Harkins and Jordan correction; the probe was a 20 mm diameter standard platinum ring.

2.2. Synthesis

2.2.1. Synthesis of *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N*-dimethylammonium chloride (**1**)

Sodium azide (2.15 g, 31.6 mmol) was added to a solution of 1H,1H,2H,2H-perfluorooctylidide (5 g, 10.5 mmol) in DMF (20 mL). The mixture was stirred at room temperature for 48 h then poured into cold water (200 mL) and extracted with Et_2O (3 mL $\times$ 50 mL). The organic layer was washed with brine (2 mL $\times$ 50 mL), dried over Na_2SO_4 , filtered, and concentrated in vacuo to give 1H,1H,2H,2H-perfluorooctylazide (3.69 g, 9.5 mmol) as a yellow oil. The resulting azido compound was dissolved in MeOH (25 mL) with 0.6 g of 10% Pd/C and submitted to a hydrogen atmosphere (7 bars) overnight. Filtration of the catalyst through a pad of Celite and evaporation of the solvent gave 1H,1H,2H,2H-perfluorooctylamine (3.88 g, 10 mmol) as a yellow oil. The resulting amino compound was dissolved in a mixture formic acid/formaldehyde 37 wt.% in water stabilized by 10–15% methanol (60 mL, 1/1 (v/v)) and stirred at reflux for 48 h. Then the solvent was removed and the crude mixture was poured into 1N NaOH (100 mL) and extracted with Et_2O (3 mL $\times$ 30 mL). The organic layer was washed with 1N NaOH (2 mL $\times$ 50 mL), washed with brine (1 mL $\times$ 50 mL), dried over Na_2SO_4 and filtered. After bubbling of gaseous hydrochloric acid into the organic layer, the solution was cool down at –20 °C for 2 h and filtered to give *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N*-dimethylammonium chloride, **1** (3.59 g, 8.4 mmol) as a white powder. $R_f = 0.75$ in $\text{EtOAc}/\text{MeOH}/\text{H}_2\text{O}$ 7/2/1 (v/v/v). $\text{Mp} = 209.4\text{--}215.2$ °C (dec). ^1H NMR ($\text{DMSO}-d_6$, 250 MHz) δ 3.38 (6H, s), 3.0–2.6 (4H, m). ^{19}F NMR ($\text{DMSO}-d_6$, 235 MHz) δ –80.2 (3F, t, $J = 9.1$ Hz), –112.8 (2F, m), –121.7 (2F, m), –122.8, –123.5 (4F, m), –125.8 (2F, m). ^{13}C NMR ($\text{DMSO}-d_6$, 62.86 MHz) 48.5 (CH_2), 42.6 (CH_3), 26.1 (t, $J = 21$ Hz,

CH₂). HRMS (*m/z*) calcd for C₁₀H₁₁NF₁₃⁺: 392.0678. Found: 392.0678.

2.2.2. Synthesis of *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium bromide (2)

The ammonium salt was dissolved in 0.5N NaOH (100 mL) and extracted with Et₂O (2 mL × 50 mL). The organic layer was washed with brine (1 mL × 30 mL) dried over Na₂SO₄, filtered, and concentrated in vacuo. The resulting oil was dissolved in propanone (15 mL) in a sealed pressure tube. Methyl iodide (2 mL, 32.0 mmol) was added dropwise and the mixture was stirred at reflux overnight. After cooling at room temperature, the crude mixture was poured into cold Et₂O and filtered. The resulting solid was purified by three successive recrystallizations from MeOH/Et₂O to give *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium Iodide (2.79 g, 5.25 mmol) as a white powder. *R*_f = 0.74 in BuOH/AcOH/H₂O 4/1/5 (v/v/v). Mp = 243.9–244.3 °C. ¹H NMR (DMSO-*d*₆, 250 MHz) δ 3.73 (2H, bs), 3.16 (9H, s), 2.97 (2H, bs). ¹⁹F NMR (DMSO-*d*₆, 235 MHz) δ −80.2 (m, 3F), −112.3 (m, 2F), −121.7 (m, 2F), −122.4 (m, 2F), −122.6 (m, 2F), −125.8 (m, 2F). ¹³C NMR (DMSO-*d*₆, 62.86 MHz) 57.2 (CH₂), 53.0 (CH₃), 25.0 (t, *J* = 21 Hz, CH₂). HRMS (*m/z*) calcd for C₁₁H₁₃NF₁₃⁺: 406.0834. Found: 406.0834. FTAI (0.146 g, 0.274 mmol) was dissolved in water (20 mL) in the presence of resin Amberlite IRN78 (1 mL) under stirring. After 6 h the resin was filtered and the operation was repeated three times with new resin (1 mL). Then, the solution was lyophilized to give the *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium bromide **2** (0.102 g, 0.210 mmol) as a white powder. *R*_f = 0.55 in BuOH/AcOH/H₂O 4/1/5 (v/v/v). The exchange was checked by TLC and confirmed by mass spectrometry (*m/z* = 78.85 and 80.85).

2.2.3. Synthesis of *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium tetraphenylborate (FTAΦ₄)

FTABΦ₄ was prepared by mixing equimolar (0.01 mol L^{−1}) aqueous solutions of FTABr and sodium tetraphenylborate (NaBΦ₄). The insoluble salt obtained was extracted three times by liquid–liquid extractions in dichloromethane. After evaporation of the solvent, the residual salt was recrystallized twice from Et₂O/EtOH 9/1 (v/v).

2.3. Electrochemical measurements

2.3.1. Fluorinated cationic surfactant ion selective electrode

For electrochemical measurements, a membrane selective towards cationic fluorinated surfactant was prepared. This membrane was made from low molecular weight PVC mixed with

an equivalent quantity of plasticizer (NPOE). PVC (0.8 g) and NPOE (0.8 g) were mixed in 10 mL of tetrahydrofuran (THF) until PVC was fully soluble (solution A). The selectivity of the membrane is due to a “carrier” of the cationic fluorinated amphiphile, FTABΦ₄. A 0.02 mol L^{−1} solution of FTABΦ₄ was prepared in THF (solution B). Three milliliters of solution A were mixed with 0.2 mL of solution B and the clear solution was spread on clean and transparent glass surface of a flat dish. Then, THF was evaporated at room temperature forming the membrane in the form of thin film (~0.1 mm thick). The membrane was removed, cut into small pieces and fixed on the open end of a narrow glass tube of 1 mm diameter using PVC–THF paste as glue.

2.3.2. Electromotive force (emf) measurements

The experimental setup used for electrochemical measurements is as shown in Scheme 1.

As is shown in a previous work [26], when a single charged species is moving within the membrane, the emf of the cell can be written as

$$E = \text{const.} + \frac{RT}{F} \ln a_{\text{M}}^{+} \quad (1)$$

$$= \text{const.} - 2.3 \frac{RT}{F} \text{pM} \quad \text{with pM} = -\lg(a_{\text{M}}^{+}) \quad (2)$$

where a_{M}^{+} is the amphiphilic cation (i.e. FTA⁺) activity. In our experiments, due to the low value of the activities, they are assimilated to concentrations.

The reference electrodes (classical KCl-saturated calomel) were protected from amphiphile diffusion by a saline agar–agar gel made from 2 mol L^{−1} KCl solution contained in a Teflon capillary tube.

2.3.3. Calibration and cmc determination

Aliquot additions of known concentration of surfactant solution to a fixed quantity of water maintained at a constant temperature (298.0 ± 0.1) K were done and the corresponding emf values were measured. The reproducibility of the emf measurements was crosschecked by carrying out duplicate runs.

2.3.4. Complexation constant determination

A known volume of 10^{−3} mol L^{−1} aqueous FTABr solution was taken in a glass cell (maintained at constant temperature (298 ± 0.1) K) and the emf values were recorded when aliquots of β-CD 10^{−2} mol L^{−1} were added to this solution, until final concentration of β-CD reached 5 × 10^{−3} mol L^{−1}.

Cu (Φ ₁)	Reference electrode	KCl 2M Agar Agar Salt Bridge	Reference solution (10 ^{−3} M FTABr)	Selective Membrane	Aqueous unknown solution	KCl 2M Agar Agar Salt Bridge	Reference electrode	Cu (Φ ₂)
-------------------------	------------------------	---------------------------------------	--	-----------------------	--------------------------------	---------------------------------------	------------------------	-------------------------

Scheme 1. Electrochemical chain used for emf measurements.

2.4. Surface tension measurements

Aliquots of FTABr 0.1 mol L^{-1} were added to 12 mL water at a room temperature (300 K). The reproducibility of the surface tension measurements was crosschecked by carrying out triplicate runs.

3. Results and discussion

3.1. Synthesis of FTABr

As mentioned in the introduction, cationic fluorinated surfactants are usually recognized as difficult to synthesize and purify especially when increasing the alkyl chain length [27]. Moreover, some of them have shown instability in aqueous solution [28]. In this study, we present a convenient and short synthesis of a fluorinated trimethyl ammonium bromide surfactant (called FTABr) from commercially available 1H,1H,2H,2H-perfluorooctyl iodide [29] (Scheme 2). Firstly, according to our previously published procedure [30], the iodide group was quantitatively converted into an azido derivative which was reduced, without purification, in amino group by catalytic hydrogenation. The resulting 1H,1H,2H,2H-perfluorooctylamine was selectively converted in *N,N*-dimethyl tertiary amine by treatment with a refluxing mixture of formic acid/formaldehyde. Then, after precipitation in acidic conditions the *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N*-dimethylammonium chloride **1** was obtained as a white powder with 80% yield from perfluorooctyl iodide as starting material. Moreover, it has to be underlined that the ammonium chloride form of the tertiary perfluorooctylamine is stable and can be kept for months. Secondly, after treatment in basic condition, the compound **1** was quantitatively converted in quaternary ammonium iodide by refluxing in propanone with an excess of methyl iodide. Then, the fluorinated trimethyl ammonium iodide (FTAI) was purified by three successive recrystallizations. FTAI was fully characterized by ^1H , ^{13}C , ^{19}F NMR and mass spectrometry. At last, FTAI was converted to its bromide homologue FTABr **2** by using an anion exchange resin Amberlite IRN78 (Prolabo) and the counterion exchange was verified by TLC and mass spectrometry.

3.2. Response of the electrode and micellization of FTABr

Since the classical composition PVC/dinonylphtalate (DNP) 20/80 ([25,31,32]) led to irreproducible emf values, we realized

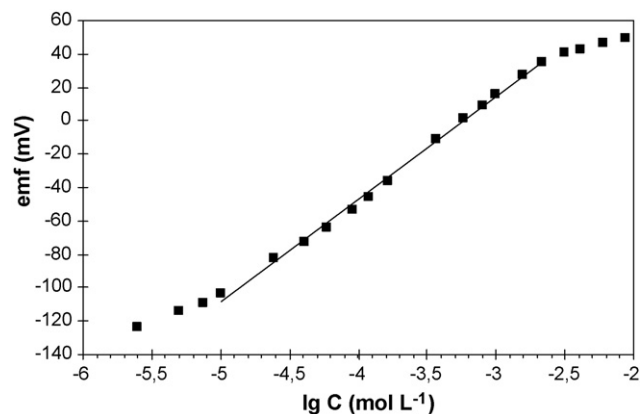
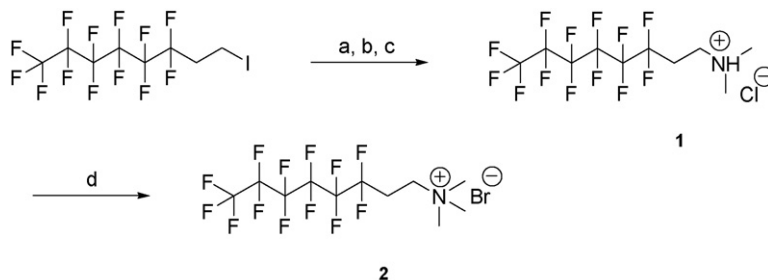


Fig. 1. Calibration curve of the FTA^+ selective electrode in water at 298 K.

several assays for the composition of the membrane based on the work of Gloton et al. [31]. We present in this work only the best system. Moreover, dipping a DTABr electrode in a FTABr solution in order to exchange the carriers, as is commonly done [32], is not sufficient. The carrier $\text{FTAB}\Phi 4$ must be carefully synthesized and purified in order to obtain reproducible results.

The emf values were plotted as a function of logarithm of concentration of fluorinated surfactant, $\Delta E = f(\lg[\text{FTA}^+])$ in aqueous medium (Fig. 1). The electrode exhibits a linear, reproducible ($\pm 2 \text{ mV}$) response in the concentration range 10^{-5} to $2.0 \times 10^{-3} \text{ mol L}^{-1}$ with a positive slope of $59 \pm 2 \text{ mV/decade}$ and a detection limit of $2 \times 10^{-6} \text{ mol L}^{-1}$. The response time is 20–30 s.

After the cmc, the activity and the concentration of the surfactant are no longer proportional, the activity remaining almost constant. Since the emf ΔE is indeed proportional to the logarithm of the activity, the break point in the plot $\Delta E = f(\lg[\text{FTA}^+])$ corresponds to the aggregation of the surfactant. It leads to a cmc value of $(2.0 \pm 0.2) \times 10^{-3} \text{ mol L}^{-1}$ for FTABr. The cmc value of FTABr was also determined by surface tension measurements. The addition of a surfactant in a solution lowers the surface tension, until micelles form. After the cmc, the chemical potential of the surfactant remaining constant, the surface tension reaches a plateau. The cmc values thus obtained from the break on the plot $\gamma = f(\lg[\text{FTA}^+])$ ($\text{cmc} = (2.8 \pm 0.4) \times 10^{-3} \text{ mol L}^{-1}$, Fig. 2) is coherent with the potentiometry data, indicating that the cmc determination carried out using the electrode is accurate.



Scheme 2. Synthesis of FTABr. (a) NaN_3 , DMF, RT, 48 h; (b) H_2 , Pd/C 10%, MeOH, RT, 14 h; (c) HCOOH/HCHO 1:1 (v/v), Δ , 48 h then HCl ; (d) MeI, acetone, Δ , 14 h, then anion exchange column Amberlite IRN78.

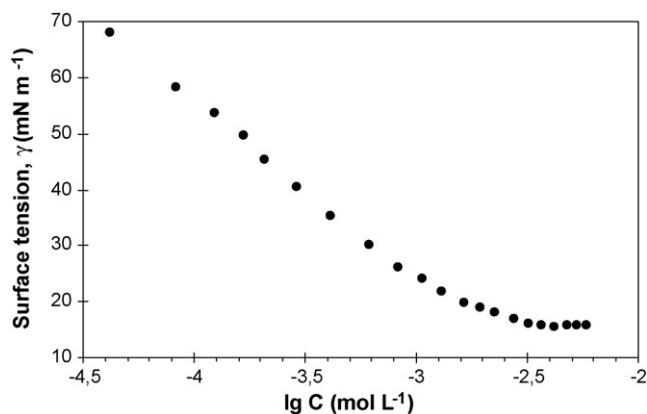
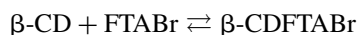


Fig. 2. Change in surface tension with concentration of FTABr at 300 K.

3.3. Association constant for β -cyclodextrin/FTABr complex

Development of the fluorinated surfactant ion selective electrode enabled the detection of FTA^+ activity in aqueous solution and hence its use for the investigation of β -cyclodextrin/FTABr complex. The emf values decrease when β -CD is added to a solution of FTABr. This is due to the consumption of free FTA^+ ions in solution resulting from their participation in complex formation with β -CD.

The emf values, thus, obtained were used to evaluate the association constant of β -CD/fluorinated surfactant inclusion complex with the help of a previously described procedure [25]. The interpretation of the experimental data used a calculation method which supposes the formation of a complex of 1:1 stoichiometry (β -CDFTABr) between β -CD and surfactant (FTABr). Hence, the complex can be represented as



The association constant is expressed as

$$K = \frac{[\beta\text{-CDFTABr}]}{[\beta\text{-CD}][\text{FTABr}]} \quad (3)$$

Conservation of mass results in the following equations:

$$[\text{FTABr}]_0 = [\text{FTABr}] + [\beta\text{-CDFTABr}] \quad (4)$$

and

$$[\beta\text{-CD}]_0 = [\beta\text{-CD}] + [\beta\text{-CDFTABr}] \quad (5)$$

where $[i]_0$ corresponds to the total concentration of species i in solution. The association constant can then be expressed as

$$K = \frac{[\text{FTABr}]_0 - [\text{FTABr}]}{([\beta\text{-CD}]_0 - [\text{FTABr}]_0 + [\text{FTABr}])[\text{FTABr}]} \quad (6)$$

This last relation can be written in a different way as

$$\lg(K) = \lg \left(\frac{[\text{FTABr}]_0 - [\text{FTABr}]}{([\beta\text{-CD}]_0 - [\text{FTABr}]_0 + [\text{FTABr}])} \right) \quad (7)$$

$$-\lg([\text{FTABr}]) = Y - \lg([\text{FTABr}])$$

[FTABr] was determined using the experimental emf values and the following expression derived from Eq. (2).

$$[\text{FTABr}] = 10^{E - \text{const./slope}} \quad (8)$$

Fig. 3 displays the plot of Y (defined in Eq. (7)) versus $\lg[\text{FTABr}]$, obtained by three different runs. Since the model requires a slope equal to one, the fit was performed imposing this value. $\lg K$ is then the only variable parameter. Leaving the slope parameter free to vary for the fit would lead to meaningless value for $\lg K$. The slope value 1 perfectly agrees with the data, which indicates that a 1:1 complex is sufficient to understand the data (no higher complex is needed). Fitting independently each run leads to $\lg K$ values of 5.16/5.03/5.16. Gathering all three runs for the fit leads to $\lg K = 5.11$. We will, thus, use the value $\lg K = 5.1 \pm 0.1$.

The interaction of this fluorinated surfactant with β -CD can be compared with the complexation of hydrogenated homologues with β -CD. The values of complexation constants published are quite scattered but as Jezequel et al. [25] carried out their assays with the same potentiometric technique that we used, we chose to take preferentially into account their results. We focused on tetradecyltrimethylammonium bromide (TTA^+ , Br^-) that has a cmc in water close to the cmc of FTABr ($\text{cmc}_{\text{TTA}} = 3.5 \text{ mM}$). The complexation of TTABr with β -CD is characterized by a complexation constant $\lg K = 4.60$ and a complex of higher order (2 β -CD:1 TTABr) was necessary in this case to explain such a result. This complex can be explained by the insertion of two β -CD on the same hydrophobic chain exhibiting a sufficient length, as in a (short) necklace [19]. This cannot happen with the short fluorinated chain we studied here.

Though they have similar cmc, indicating a similar hydrophobic effect, FTABr seems to have a higher affinity for β -CD than TTABr when used at concentration lower than its cmc. This trend was also observed by Palepu et al. [20–21]. They reported complexation constants of sodium dodecanoate and sodium perfluorooctanoate ($\lg K = 3.20$ and 3.65 , $\text{cmc} = 24 \text{ mM}$ [33] and 32 mM [2], respectively). Their hypothesis about the stability of such inclusion complexes is the closeness of the fit of the surfactant in the cavity of the β -CD. The inside diameter of the

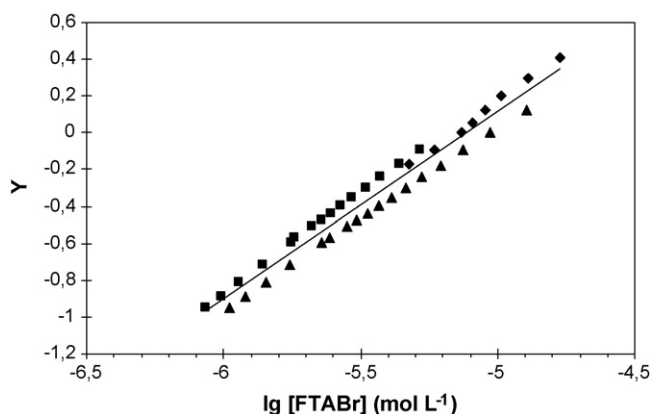


Fig. 3. Plot of Y vs. $\lg[\text{FTABr}]$. The y-intercept corresponds to $\lg K$ as envisaged by Eq. (7). The results of three runs are gathered on the same graph and used for the fitting procedure, in which a slope 1 was imposed.

cavity of β -cyclodextrin is ~ 0.7 nm whereas the diameter of CF_2 and CH_2 groups are 0.6 and 0.45 nm, respectively. It is expected that the fluorinated chain could fit more tightly within the cavity of β -cyclodextrin than a hydrogenated chain. Moreover, at a concentration lower than the fluorinated surfactant cmc, the higher hydrophobic environment within the interior of the cavity as compared to that in the aqueous region outside can also be a driving force for inclusion of fluorocarbon chain into such a cavity. Such an inclusion could facilitate reduction of contacts between fluorocarbon chain and aqueous surroundings resulting in an energetically viable phenomenon.

4. Conclusion

The construction and performance of a 1H,1H,2H,2H-perfluorooctyltrimethylammonium tetraphenylborate-PVC membrane electrode responsive to *N*-(1,1,2,2-tetrahydroperfluorooctyl)-*N,N,N*-trimethylammonium bromide (FTABr) and its application for the study of β -cyclodextrin/fluorinated surfactant inclusion complex was described. The electrode exhibits a fast, stable, reproducible and Nernstian response (59 ± 2 mV) for FTABr over the concentration range of 10^{-5} to 2×10^{-3} M at 298 K. The critical micelle concentration value for FTABr obtained using the electrode is almost equal to that obtained by surface tension measurements. The inherent advantages of the proposed electrode are its high selectivity, rapid response, simplicity of operation, accuracy in detection of fluorinated surfactant ions present even at a very low concentration, low cost and direct application for the study of interaction of fluorinated surfactant with β -cyclodextrin. The association constant value evaluated by carrying out potentiometric measurements using this electrode suggests that β -cyclodextrin forms a strong, stable and equimolar association complex with the 1H,1H,2H,2H-perfluorooctyltrimethyl ammonium surfactant.

Thus, the electrode can serve as a reliable tool for selective detection of cationic fluorinated surfactant ions in solution facilitating studies on interactions of such surfactants with different moieties like cyclodextrins, lactoglobulins and neutral fluorinated as well as hydrocarbon surfactants.

Acknowledgement

S.R.P. thanks the Ville de Paris for a Postdoctoral Fellowship.

References

- [1] J. Texter, T.A. Hatton, *Curr. Opin. Coll. Int. Sci.* 7 (2003) 253.
- [2] E. Kissa, *Fluorinated Surfactants and Repellents; Surfactant Science Series* 97, Marcel-Dekker, New York, 2001.
- [3] K. Shinoda, M. Hato, T. Hayashi, *J. Phys. Chem.* 76 (1972) 909.
- [4] H. Kunieda, K. Shinoda, *J. Phys. Chem.* 80 (1976) 2468.
- [5] H. Iijima, T. Kato, N. Yoshida, M. Mai, *J. Phys. Chem. B* 102 (1998) 990.
- [6] K. Wang, G. Karlsson, M. Almgren, T. Asakawa, *J. Phys. Chem. B* 103 (1999) 9237.
- [7] C. Oelschlaeger, G. Waton, E. Buhler, S.J. Candau, M.E. Cates, *Langmuir* 18 (2002) 3076.
- [8] A. Polidori, M. Presset, F. Lebaupain, B. Ameduri, J.-L. Popot, C. Breyton, B. Pucci, *Bioorg. Med. Chem. Lett.* 16 (2006) 5827.
- [9] P.D.I. Fletcher, *Fluorinated and semi-fluorinated surfactants*, in: I.D. Robb (Ed.), *Specialist Surfactants*, Blackie Academic and Professional, London, 1997, p. 104.
- [10] Yu. Han, J.Y. Ying, *Angew. Chem. Int. Ed.* 44 (2005) 288.
- [11] F. Lebaupain, A.G. Salvay, B. Olivier, G. Durand, A.-S. Fabiano, N. Michel, J.-L. Popot, C. Ebel, C. Breyton, B. Pucci, *Langmuir* 22 (2006) 8881.
- [12] L. Zarif, J.G. Riess, B. Pucci, C. Guedj, A.A. Pavia, *Artif. Blood Sub.* 22 (1994) 1485.
- [13] J. López-Fontán, F. Sarmiento, P. Schulz, *Colloid Polym. Sci.* 283 (2005) 862.
- [14] M. Kadi, P. Hansson, M. Almgren, *J. Phys. Chem. B.* 108 (2004) 7344.
- [15] W. Saenger, *Angew. Chem. Int. Ed. Engl.* 19 (1980) 344.
- [16] S. Hamai, *J. Phys. Chem.* 94 (1990) 2595.
- [17] W. Guo, B.M. Fung, S.D. Christian, *Langmuir* 8 (1992) 446.
- [18] E. Saint Aman, D. Serve, *J. Colloid Interface Sci.* 138 (1990) 365.
- [19] L.D. Wilson, R.E. Verrall, *Langmuir* 14 (1999) 4710.
- [20] R. Palepu, V.C. Reinsborough, *Can. J. Chem.* 67 (1989) 1550.
- [21] R. Palepu, J.M. Richardson, V.C. Reinsborough, *Langmuir* 5 (1989) 218.
- [22] W. Saenger, A. Muller-Fahmow, *Angew. Chem. Int. Ed. Engl.* 27 (1988) 393.
- [23] C. Cabaleiro-Lago, M. Nilsson, O. Söderman, *Langmuir* 21 (2005) 11637.
- [24] J.V. Martin, M. Turmine, P. Letellier, P. Hemery, *Electrochim. Acta* 40 (1995) 2749.
- [25] D. Jezequel, A. Mayaffre, P. Letellier, *Can. J. Chem.* 69 (1991) 1865.
- [26] J. Sirieix-Plenet, M. Turmine, P. Letellier, *Talanta* 60 (2003) 1071.
- [27] K. Matsuoka, Y. Moroi, *Curr. Opin. Coll. Int. Sci.* 8 (2003) 227.
- [28] T. Yamabe, Y. Moroi, Y. Abe, T. Takahasi, *Langmuir* 16 (2000) 9754.
- [29] Very similar compounds have already been described in Japanese patents: (a) K. Oshino, H. Mori, R. Nakai, H. Tajima, Japan Kokai Tokkyo Koho, JP04164027, 1992.; (b) Japan Kokai Tokkyo Koho, JP2003238500, 2003.
- [30] S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M.A. Pappolla, J. Böker, R. Hardeland, B. Pucci, *J. Med. Chem.* 49 (2006) 2812.
- [31] M.P. Gloton, M. Turmine, A. Mayaffre, P. Letellier, *Can. J. Chem.* 71 (1993) 371.
- [32] V. Peyre, S. Baillet, P. Letellier, *Anal. Chem.* 72 (2000) 2377.
- [33] P. Mukerjee, K.J. Mysels, *Critical Micelle Concentrations of Aqueous Surfactant Systems*, NSRDC-NBS 36, US Department of Commerce, Washington, DC, 1971.

Headspace single drop microextraction of methylcyclopentadienyl-manganese tricarbonyl from water samples followed by gas chromatography–mass spectrometry

Francisco Javier Pena Pereira^a, Carlos Bendicho^a,
Nicolas Kalogerakis^b, Eleftheria Psillakis^{b,*}

^a *Departamento de Química Analítica y Alimentaria, Facultad de Ciencias de Vigo, Universidad de Vigo As Lagoas-Marcosende, E-36200 Vigo, Spain*

^b *Department of Environmental Engineering, Technical University of Crete, Polytechniopolis, GR-73100 Chania, Greece*

Received 22 February 2007; received in revised form 10 May 2007; accepted 18 May 2007

Available online 24 May 2007

Abstract

Headspace single drop microextraction coupled to gas chromatography–mass spectrometry yielded a simple, fast and virtually solventless analytical protocol used for the headspace analysis of aqueous samples contaminated with methylcyclopentadienyl-manganese tricarbonyl (MMT). Initially, several experimental parameters were controlled and optimized and the optimum conditions found were 2.5 μ l octane microdrop exposed for 20 min to the headspace of a 10 ml aqueous sample (15 ml vial) containing 20% (w/v) NaCl and stirred at 1250 rpm. The calculated calibration curves gave a high level of linearity for MMT with correlation coefficients >0.9995 after conducting a 3-day study. The limit of detection was calculated to be $0.21 \mu\text{g l}^{-1}$. The proposed method achieved an enrichment factor of the order of 2100 and a 53% recovery after extracting the spiked aqueous solution for 20 min under the optimized experimental conditions. The repeatability and intra-day reproducibility of the proposed method, expressed as relative standard deviation were 8.4 and 6.4%, respectively. Finally, analysis of spiked tap and wastewater samples revealed that matrix had little effect upon extraction.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Headspace SDME; MMT; GC–MS; Liquid-phase microextraction

1. Introduction

There is increased concern within the scientific community concerning the neurotoxicity of manganese, owing part to the use of methylcyclopentadienyl-manganese tricarbonyl (MMT) as a gasoline fuel additive [1]. Indeed, MMT is highly toxic to some animals through inhalation, ingestion and skin absorption [1,2]. Although MMT is known to photolyze rapidly under sunlight, it is fairly stable in the absence of light with half-lives ranging from 0.2 to 1.5 years in aquifer materials at 25 °C [3].

Today, different analytical protocols are available for the determination of MMT in air, water and gasoline samples. These methods mainly use gas chromatography (GC) coupled to a variety of detectors, such as atmospheric pressure helium

microwave plasma emission system [4], direct current argon plasma emission [5], atomic absorption in a slotted quartz tube [6] and electron-capture detection [7]. The use of a high performance liquid chromatography system coupled with a laser-excited atomic fluorescence spectrometric detector [8] has also been reported. Recently, the use of solid phase microextraction (SPME) followed by gas chromatography and plasma atomic emission detection [9] or direct thermal desorption and detection by quartz furnace-atomic absorption spectrometry [10] has been proposed for the determination of MMT in water and gasoline samples following another publication dealing with the SPME analysis of MMT in beverages using SPME and gas chromatography coupled to atomic absorption spectrometry [11].

The inorganic applications of single drop microextraction (SDME) for sample preparation appeared in 2003 [12], following the inception of this technology in 1996 [13]. The limited numbers of reports dealing with these applications use a micro-liter drop of the extractant acceptor phase directly exposed to

* Corresponding author. Tel.: +30 28210 37810; fax: +30 28210 378527.
E-mail address: epsilaki@mred.tuc.gr (E. Psillakis).

the aqueous donor solution or to the headspace above it and represent an emerging field of study due to the inherent advantages of being fast, inexpensive, precise and virtually solventless. To date published methods were capable of determining trace levels of arsenic [12], arsine [14], organotin [15], methylmercury [16], selenium [17,18], organotin and organomercury [19], cadmium and lead [20], aluminium [21] or a mixture of metals [22] in environmental and biological matrices.

The objective of this work was to investigate the applicability of headspace SDME followed by GC with mass spectrometry used for the determination of MMT in water samples. Experimental parameters affecting the extraction process of MMT (namely organic solvent, organic drop volume, sample stirring, salt addition and sampling time) were controlled and optimized and the performance of the proposed method was evaluated. The developed method was applied to tap and wastewater samples in order to assess the effect of matrix upon extraction.

2. Experimental

2.1. Chemicals and aqueous samples

Methylcyclopentadienyl-manganese tricarbonyl (MMT) was purchased from Aldrich (Milwaukee, WI, USA), 1-octanol and methanol were purchased from Riedel-de Haën (Seelze, Germany) and *n*-octane ($\geq 99\%$ pure) was purchased from Flucka (Steinheim, Switzerland). Deionized water was prepared on a water purification system (EASYPure® RF) supplied by Barnstead/Thermolyne Corporation (Dubuque, IO, USA). Sodium chloride (Merck, Darmstadt, Germany) was used to adjust the ionic strength of the aqueous samples.

A 1000 mg l^{-1} of MMT in methanol was prepared and was used as a standard stock solution. From this a 10 mg l^{-1} of MMT in methanol was prepared each week and was used for the preparation of aqueous spiked solutions at the concentration levels of interest. All MMT methanolic solutions were stored at 4°C in the dark. Recovery studies were carried out using tap and wastewater samples taken from a car wash unit situated in a local petrol station (Chania, Greece). Samples were collected in 250 ml pyrex borosilicate amber glass containers with caps, lined with aluminium foil. They were stored in the dark at 4°C and were analyzed within 24 h of collection. Before extraction, samples were centrifuged in order to remove any solid particles and the ionic strength of the water samples was then adjusted to the one required by the extraction method used. Preliminary analyses of both samples under the full-scan and selective ion monitoring (SIM) mass spectrometry modes ensured that they were free of the target analyte.

2.2. Headspace SDME

For headspace SDME, 10 ml of a salted (20%, w/v, NaCl) aqueous solution spiked at a known concentration with MMT, was placed in a 15 ml crimp top glass vial covered with aluminium foil containing a PTFE-coated stirring bar and fitted with a Mininert valve (Supelco, Bellefonte, PA, USA). Aqueous spiked samples were freshly prepared, in order to eliminate

volatilization losses. In addition, before extraction each sample destined for analysis was stirred at the stirrer's maximum steering speed, allowing thus equilibrium to be attained between the aqueous and gaseous phases.

A $10 \mu\text{l}$ Hamilton Gastight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland), Model 1701, with a bevel needle tip (length: 5.1 cm, i.d.: 0.013 cm, bevel 22°), typically containing $2.5 \mu\text{l}$ of the appropriate organic solvent was clamped above the vial containing the water sample. For all quantification experiments, $2.5 \mu\text{l}$ of *n*-octane solution was used. The microsyringe was then lowered and its needle passed through the Mininert valve until the tip of the needle was 1 cm below the lower surface of valve. The plunger was depressed and the $2.5\text{-}\mu\text{l}$ drop of the organic phase was exposed to the headspace above the sample. The analytes were then allowed to partition between the headspace and the organic phase at room temperature for 20 min (unless otherwise stated within the text). After extraction, the microdrop was retracted into the microsyringe and transferred to the heated injection port of the gas chromatograph–mass spectrometer (GC–MS) for analysis.

2.3. GC–MS analysis

All analyses were carried-out on a Shimadzu GC-17A, Version 3, QP-5050A Gas Chromatograph/Mass Spectrometer system (Shimadzu Corporation, Kyoto, Japan) equipped with a $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ HP-5MS capillary column (Agilent Technologies). The injector was maintained at 250°C and operated in the splitless mode with the split closed for 5 min. Helium ($>99.999\%$ pure) was used as the carrier gas at a flow-rate of 1.2 ml min^{-1} . The column oven was initially set at 50°C for 2 min, programmed to 250°C at a $20^\circ\text{C min}^{-1}$ rate, where it was held for 1 min. The interface temperature was set at 290°C and the detector voltage at 1.50 kV. A 7 min solvent cut time was allowed for all analyses. The ionization mode was electron impact (70 eV). A SIM program was constructed for GC–MS acquisition and quantification. The latter was based on the target ion (m/z) 218. Prior to quantification in the SIM mode, the full scan mode (m/z 40–350) was used for identification of target compound based on their mass spectra and GC retention time. The enrichment factor and extraction recovery were determined by comparing the peak area obtained after extracting with the proposed method a $1 \mu\text{g l}^{-1}$ aqueous MMT solution, with the one obtained by direct injection of the same solvent volume of a 1 mg l^{-1} hexane solution of MMT.

3. Results and discussion

3.1. Optimization of headspace SDME

Initially, a step-by-step optimization scheme has been adopted and parameters, such as organic solvent, sample volume, organic drop volume, stirring rate, salt effect and sampling time were controlled and optimized.

Two solvents (namely octane and 1-octanol) were initially tested and used for extracting the target analyte. Solvent selectivity was evaluated after exposing for 5 min a $3 \mu\text{l}$ organic solvent

drop to the headspace of a 15 ml glass vial containing 5 ml deionized water samples, stirred at 1000 rpm and spiked at $100 \mu\text{g l}^{-1}$ with MMT. Both examined solvents had a low volatility restricting thus solvent evaporation during extraction. However, elution of 1-octanol was found to interfere with the target eluting analyte. Octane on the other hand provided a high solubility for MMT and exhibited an excellent chromatographic behaviour. Accordingly, it was decided to use octane as the extraction solvent for all subsequent experiments.

In general, during headspace SDME, increasing the sample volume and consequently decreasing the headspace volume when the same type of vial is used, enhances the extraction of target analytes and improves the sensitivity of the method [23]. Accordingly, in the present studies, the headspace of 15-ml vials containing sample volumes of 5, 7 and 10 ml, stirred at 1000 rpm and spiked at $100 \mu\text{g l}^{-1}$ with MMT, was extracted with $3 \mu\text{l}$ of octane for 5 min. As expected the results revealed that extraction is optimized for 10 ml sample volumes.

Although, the use of larger drops is expected to result in an increase of the analytical response of the instrument, their use has been reported as less reliable and more difficult to manipulate [24]. In a separate set of experiments the effect of drop volume upon extraction was investigated. The headspace of 10 ml sample volumes contained in 15 ml vials spiked at $100 \mu\text{g l}^{-1}$ MMT stirred at 1000 rpm was extracted for 5 min using octane drop volumes ranging from 1.5 to $3 \mu\text{l}$. As can be seen in Fig. 1, increasing the organic drop volume from 1.5 to $2.5 \mu\text{l}$, resulted in a small increase of the extraction efficiency. However, a further increase of the octane drop from 2.5 to $3 \mu\text{l}$ resulted in a decrease of the extraction efficiency. This observation is in agreement with previous published reports dealing with the headspace SDME extraction of analytes present in water samples [23,25]. Accordingly, it was decided to use a $2.5 \mu\text{l}$ octane drop for all subsequent experiments.

Next, the effect of sample agitation upon extraction was investigated. It has been repeatedly reported that sample agitation enhances extraction and reduces the time to thermodynamic equilibrium [24]. For the purpose of the present investigations, $2.5 \mu\text{l}$ octane drops were exposed each time for 5 min to the

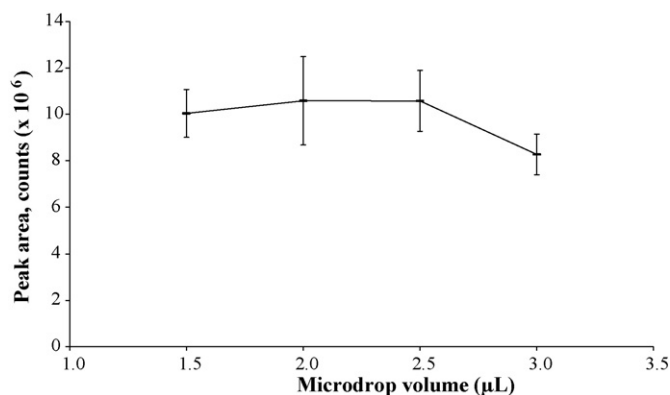


Fig. 1. Effect of drop volume on the extraction efficiency of headspace SDME used for the analysis of MMT in water samples. Other experimental conditions: $100 \mu\text{g l}^{-1}$ concentration level; 10 ml sample volume contained in 15 ml glass vial; 5 min sampling time; 1000 rpm stirring rate.

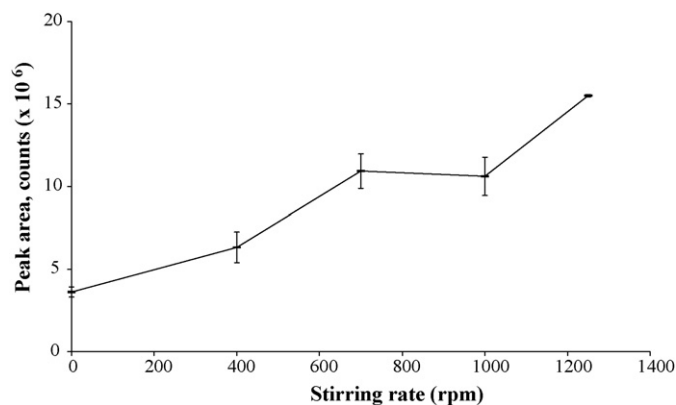


Fig. 2. Effect of sample agitation on the extraction efficiency of headspace SDME used for the analysis of MMT in water samples. Other experimental conditions: $100 \mu\text{g l}^{-1}$ concentration level; 10 ml sample volume in 15 ml glass vial; $2.5 \mu\text{l}$ octane drop; 5 min sampling time.

headspace of 10 ml water samples spiked at $100 \mu\text{g l}^{-1}$ with MMT and stirred at different agitation rates (namely: 0, 400, 700, 1000 and 1250 rpm). As expected, the results revealed that agitation dramatically enhanced extraction reaching a maximum at 1250 rpm (Fig. 2). Based on the above observations 1250 rpm was selected as the optimum agitation rate.

Addition of salt to the sample and accordingly increasing the ionic strength of the aqueous solution may have several effects on extraction. Depending on the solubility of the target analytes, extraction is usually enhanced with increased salt concentration and increased polarity of the target compounds (salting-out effect). In a separate set of experiments the effect of ionic strength on extraction was investigated. Experiments were carried out using 10 ml aqueous solutions spiked at $100 \mu\text{g l}^{-1}$ with MMT and having a salt content ranging from 0 to 30% (w/v) NaCl. Samples were extracted using each time a $2.5 \mu\text{l}$ octane drop exposed to the headspace for 5 min whilst applying a 1250 rpm agitation rate. Fig. 3 shows the results of this study. As can be seen, the response of the instrument increased when increasing the salt content of the aqueous samples. It appears that addition of NaCl in the aqueous solution enhanced extrac-

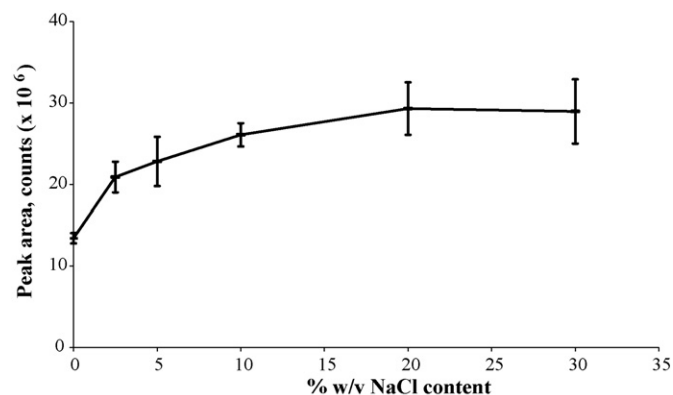


Fig. 3. Effect of ionic strength on the extraction efficiency of headspace SDME used for the analysis of MMT in water samples. Other experimental conditions: $100 \mu\text{g l}^{-1}$ concentration level; 10 ml sample volume in 15 ml glass vial; $2.5 \mu\text{l}$ octane drop; 5 min sampling time; 1250 rpm stirring rate.

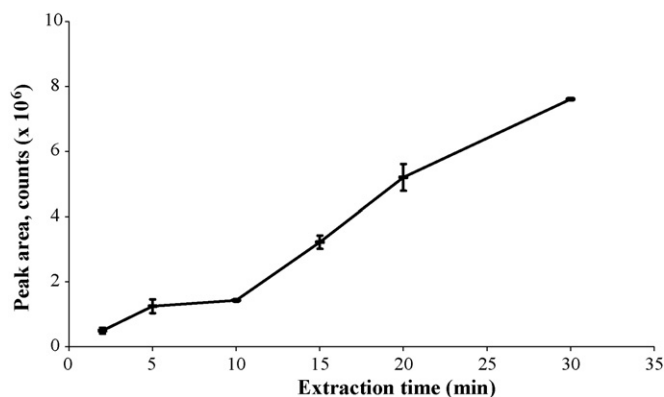


Fig. 4. Effect of sampling time on the extraction efficiency of headspace SDME used for the analysis of MMT in water samples. Other experimental conditions: $10 \mu\text{g l}^{-1}$ concentration level; 10 ml sample volume in 15 ml glass vial; $2.5 \mu\text{l}$ octane drop; 1250 rpm stirring rate; 20% (w/v) NaCl.

tion due to the salting-out effect where fewer water molecules are available for dissolving the analyte molecules, increasing thus the total amount of analytes transferred in the headspace and as such enhance extraction [24]. Accordingly a 20% (w/v) NaCl content was selected for all subsequent experiments.

Finally, the effect of sampling time upon extraction was investigated. During this set of experiments samples were spiked at $10 \mu\text{g l}^{-1}$ instead of $100 \mu\text{g l}^{-1}$ due to the high response of the instrument obtained with increased sampling times. Accordingly, each time a $2.5 \mu\text{l}$ octane drop was exposed to the headspace for a pre-set period of time (ranging between 2 and 30 min) of 10 ml aqueous sample containing $10 \mu\text{g l}^{-1}$ of MMT, having a 20% (w/v) NaCl content and stirred at 1250 rpm. Fig. 4 shows the response of the analytical instrument as a function of time. As can be seen, even after sampling the headspace of the spiked salted aqueous solutions for 30 min equilibrium is not attained. However, for the microdrop-based extraction methods equilibrium needs not to be attained [26]. It was decided to use a 20 min sampling time matching thus the chromatographic run and maximizing the sample throughput.

3.2. Performance of the headspace SDME method

The performance of the proposed method was evaluated using five concentration levels ranging from 0.1 to $25 \mu\text{g l}^{-1}$. Samples were extracted under the optimized experimental conditions ($2.5 \mu\text{l}$ octane drop exposed for 20 min to the headspace of a 10 ml aqueous solutions containing 20% (w/v) NaCl stirred at 1250 rpm). The calculated calibration curves gave a high level of linearity and the correlation coefficients (r) for MMT after conducting a 3-day study were found to be >0.9995 . The limit of detection (LOD) for MMT was calculated by using the calibration curves and the Long and Winefordner criterion [27] and was calculated to be $0.21 \mu\text{g l}^{-1}$ (corresponding to approximately 53 ng l^{-1} when expressed as Mn). A previous report by Yang and Chau yielded a 0.3 pg l^{-1} (as Mn) detection limit for MMT in water samples whilst using SPME as a sample treatment method followed by gas chromatography-plasma atomic emission detection (a highly selective and sensitive detection

tool for most elements) [9]. In addition, Fragueiro et al. recently published an analytical protocol based on SPME and direct thermal desorption–quartz furnace atomic absorption spectrometry and the authors reported a $0.71 \mu\text{g l}^{-1}$ (as Mn) detection limit of MMT in aqueous samples [10].

The repeatability of the proposed method expressed as relative standard deviation (R.S.D.), was evaluated by extracting five consecutive aqueous samples spiked at $1 \mu\text{g l}^{-1}$ under the optimized experimental conditions and was found to be 8.4%. Intra-day reproducibility was calculated by analyzing each day three aqueous solutions spiked at $1 \mu\text{g l}^{-1}$ with MMT for 3 consecutive days and the R.S.D. value found was 6.4%.

The enrichment factor defined as the ratio between the final analyte concentration in the organic acceptor phase and the initial aqueous sample concentration was found to be approximately 2100 after extracting for 20 min the headspace of a sample spiked at $1 \mu\text{g l}^{-1}$ with MMT. Furthermore, recovery, defined as the percentage of the amount of analyte extracted into the organic acceptor phase was found to be approximately 53%.

Finally, the effect of matrix on the proposed headspace SDME method was evaluated by extracting under the optimized experimental conditions five replicate samples of tap water and wastewater each one spiked at $1 \mu\text{g l}^{-1}$ with MMT. Relative recoveries (defined as the ratio of the concentrations found in environmental and deionized water samples spiked with the same amount of analyte) were 102% (R.S.D. = 17%) and 112% (R.S.D. = 12%) for tap water and wastewater, respectively. As expected using the headspace sampling mode resulted in a method that appeared not affected by the matrix of the aqueous samples.

4. Conclusions

A new analytical method comprising headspace SDME coupled with GC–MS has been developed, quantifying trace levels of MMT in water samples. Sample preparation time as well as consumption of toxic organic solvents was minimized without affecting the sensitivity of the method. This easy to use and cost-effective method represents an attractive alternative to traditional and recently introduced methods.

References

- [1] D.C. Dorman, M.F. Struve, H.J. Clewell III, M.E. Andersen, *NeuroToxicology* 27 (2006) 752.
- [2] E. Browning, *Toxicology of Metals*, Butterworth, London, 1982, pp. 185–196.
- [3] A.W. Garrison, M.G. Cipollone, N.L. Wolfe, R.R. Swank Jr., *Environ. Toxicol. Chem.* 14 (1995) 1859.
- [4] B.D. Quimby, P.C. Uden, R.M. Barnes, *Anal. Chem.* 50 (1978) 2112.
- [5] P.C. Uden, R.M. Barnes, F.P. Di Sanzo, *Anal. Chem.* 50 (1978) 852.
- [6] M. Coe, R. Cruz, J.C. Van Loon, *Anal. Chim. Acta* 120 (1980) 171.
- [7] V.S. Gaiind, K. Vohra, F. Chai, *Analyst* 117 (1992) 161.
- [8] A.P. Walton, G.T. Wei, Z. Liang, R.G. Michel, J.B. Morris, *Anal. Chem.* 63 (1991) 232.
- [9] F. Yang, Y.K. Chau, *Analyst* 124 (1998) 71.
- [10] M.S. Fragueiro, F. Alava-Moreno, I. Lavilla, C. Bendicho, *Spectrochim. Acta B* 56 (2001) 215.

- [11] D.S. Forsyth, L. Dusseault, *Food Addit. Contam.* 14 (1997) 301.
- [12] M. Chamsaz, M.H. Arbab-Zavar, S. Nazari, *J. Anal. At. Spectrom.* 18 (2003) 1279.
- [13] M.A. Jeannot, F.F. Cantwell, *Anal. Chem.* 68 (1996) 2236.
- [14] M.S. Fragueiro, I. Lavilla, C. Bendicho, *Spectrochim. Acta B* 59 (2004) 851.
- [15] V.C.V. Colombini, C. Bancon-Montigny, L. Yang, P. Maxwell, R.E. Sturgeon, Z. Mester, *Talanta* 63 (2004) 555.
- [16] S. Gil, S. Fragueiro, I. Lavilla, C. Bendicho, *Spectrochim. Acta B* 60 (2005) 145.
- [17] R. Figueroa, M. Garcia, I. Lavilla, C. Bendicho, *Spectrochim. Acta B* 60 (2005) 1556.
- [18] S. Fragueiro, I. Lavilla, C. Bendicho, *Talanta* 68 (2006) 1096.
- [19] J.F. Liu, Y.G. Chi, G.B. Jiang, *J. Separ. Sci.* 28 (2005) 87.
- [20] L. Li, B. Hu, L. Xia, Z. Jiang, *Talanta* 70 (2006) 468.
- [21] L. Xia, B. Hu, Z. Jiang, Y. Wu, L. Li, R. Chen, *J. Anal. At. Spectrom.* 20 (2005) 441.
- [22] L. Xia, B. Hu, Z. Jiang, Y. Wu, Y. Liang, *Anal. Chem.* 76 (2004) 2910.
- [23] L. Vidal, A. Canals, N. Kalogerakis, E. Psillakis, *J. Chromatogr. A* 1089 (2005) 25.
- [24] E. Psillakis, N. Kalogerakis, *TrAC Trends Anal. Chem.* 21 (2002) 53.
- [25] A. Przyjazny, J.M. Kokosa, *J. Chromatogr. A* 977 (2002) 143.
- [26] W. Wardencki, J. Curyło, T. Namieśnik, *J. Biochem. Biophys. Meth.* 70 (2007) 275.
- [27] G.L. Long, J.D. Winefordner, *Anal. Chem.* 55 (1983) A712.

Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase microextraction–gas chromatography–quadrupole mass spectrometry

M. Pontes, J.C. Marques, J.S. Câmara *

*Centro de Química da Madeira, Departamento de Química da Universidade da Madeira,
Campus Universitário da Penteada, 9000-390 Funchal, Portugal*

Received 15 March 2007; received in revised form 14 May 2007; accepted 18 May 2007
Available online 26 May 2007

Abstract

The volatile composition from four types of multifloral Portuguese (produced in Madeira Island) honeys was investigated by a suitable analytical procedure based on dynamic headspace solid-phase microextraction (HS-SPME) followed by thermal desorption gas chromatography–quadrupole mass spectrometry detection (GC–qMS). The performance of five commercially available SPME fibres: 100 μm polydimethylsiloxane, PDMS; 85 μm polyacrylate, PA; 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane, DVB/CAR/PDMS (StableFlex); 75 μm carboxen/polydimethylsiloxane, CAR/PDMS, and 65 μm carbowax/divinylbenzene, CW/DVB; were evaluated and compared. The highest amounts of extract, in terms of the maximum signal obtained for the total volatile composition, were obtained with a DVB/CAR/PDMS coating fibre at 60 °C during an extraction time of 40 min with a constant stirring at 750 rpm, after saturating the sample with NaCl (30%). Using this methodology more than one hundred volatile compounds, belonging to different biosynthetic pathways were identified, including monoterpenols, C₁₃-norisoprenoids, sesquiterpenes, higher alcohols, ethyl esters and fatty acids. The main components of the HS-SPME samples of honey were in average ethanol, hotrienol, benzeneacetaldehyde, furfural, *trans*-linalool oxide and 1,3-dihydroxy-2-propanone.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Honey; Volatile compounds; Solid-phase microextraction; GC–qMS

1. Introduction

Honey is a natural product produced by *Apis mellifera* bees from the nectar of plants and has for long been an excellent nutritional option for many generations due to its health benefits (one of the traditional sources for treatment of flu and common cold in the region) [1], has been reported to be effective in gastrointestinal disorders, in healing of wounds and burns, as an anti-microbial agent [2]. The healing effect of honey is due to the enzyme glucose oxidase, this enzyme is virtually inactive in full-density honey but becomes active in diluted honey producing hydrogen peroxide and gluconic acid from glucose. In addition, many natural antibacterial compounds have been identified from different types of honey [2]. Honey, as a source of antioxidants has been proven to be effective against deteriorative

oxidation reaction in food [3]. The antibacterial activity of honey is attributed both to physical factors, acidity and osmolarity and chemical factors, hydrogen peroxide, volatiles, beeswax, nectar, pollen and propolis [2,3].

Honey includes over 400 different chemical compounds, more than 95% mainly formed by sugars and water, whereas proteins, vitamins (mainly vitamin B6, thiamin, niacin, riboflavin, and pantothenic acid), essential minerals (including calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), pigments, flavours, free amino acids and volatile compounds constitute minor components [4]. The sugars present in honey are mainly fructose (about 38.5%) and glucose (about 31.0%). The remaining carbohydrates include maltose, sucrose, and other complex carbohydrates.

The chemical composition of honey is highly dependent to the nectar source and the botanical origin of the nectar foraged by bees [1]. Aroma compounds are present in honey at very low concentrations as complex mixtures of volatile components of different chemical families belonging, in general, to

* Corresponding author. Tel.: +351 291705112; fax: +351 291705149.
E-mail address: jsc@uma.pt (J.S. Câmara).

monoterpenes, C₁₃-norisoprenoids, sesquiterpenoids, benzene derivatives [5] and in lowest content, higher alcohols, esters, fatty acids, ketones, terpenes and aldehydes. Some of these substances are present in honey collected by bees, and have been described as characteristics of the floral source (could be related to plant characteristics), and other compounds, like some alcohols, branched aldehydes, and furfural derivatives, may be related to the microbial purity of processing and storage conditions of honey [6]. The quantitative analysis of volatile compounds present in such samples is extremely demanding due: (1) to complex chemical composition of the volatile fraction and (2) the fact of individual volatile compounds can be present in a wide range of concentration. Honey volatile fractions have been used as quality markers for the authenticity of the floral origin [7,8]. It also prevents overpayment and helps to identify frauds [7].

Traditional analytical methods employing organic solvents such as liquid–liquid extraction [9], simultaneous distillation–extraction (SDE) [10], supercritical fluid extraction (SFE) [11], solid-phase extraction (SPE) [12] and ultrasound extraction [13] were commonly used. These are hazardous since requires large amounts of toxic and expensive solvents, are labour-intensive and time-consuming and requires the pre-concentration of the extract. Each procedure of the sample preparation is subject to inconveniences, but offers specific advantages under determined circumstances. Nowadays, alternative to these classical methods that may overcome their disadvantages, more easier and selective, are used such as solid-phase microextraction (SPME), developed by Pawliszyn and coworker [14,15] in the early 1990s and more recently stir bar sorptive extraction (SBSE) developed in the late 1990s by Baltussen et al. [16]. This technique uses a TwisterTM, a glass stir bar onto which is bonded a sorptive phase, often polydimethylsiloxane (PDMS), in quantities far in excess of those found on SPME fibres [17]. SPME is an equilibrium technique that requires a previous optimisation of the extraction parameters that can affect extraction efficiencies, in order to obtain high recoveries of volatiles. SPME sampling can be performed in three basic modes: direct extraction (the analytes were transported directly from matrix to the extracting phase), headspace extraction (the analytes are extracted from the gas phase equilibrated with the sample) and extraction with membrane protection (the fibre is separated from the sample with a selective membrane). The selection of the sampling mode is dependent of the nature of the compounds to be analysed and the sample type. Bearing in mind that one of the goals of this study was to screen volatile compounds from honeys, the headspace sampling mode was the most appropriate. The headspace SPME process protects the fibre from adverse effects caused by non-volatile compounds present in the sample matrix namely sugars, and allows modifications, as for example: pH, with no effect in the fibre. Moreover, the equilibration times for volatile compounds are shorter for headspace SPME extraction than for direct extraction under the same conditions.

Since the first SPME fibres becomes commercially available, it has been more and more used and the fields of

application have been continuously growing, including a wide range of food analysis, namely the volatile composition of wines [18–22], beers [23], whiskeys [24–26], several kinds of fruits [27–31] and honeys [32–36], with nowadays about 3000 research papers published. The technique gained growing acceptance and increasing use in routine laboratories and industrial applications. This method shows clear advantages compared with traditional techniques, eliminates the use of (toxic) organic solvents, allows the quantification of a large number of molecules, no or little manipulation/preparation of samples, substantially shortness the time of analysis and moreover are simple and faster techniques, and covers a wide range of sampling techniques, including field, *in situ* and air sampling. Generally accepted disadvantages are relatively lot-to-lot variations, sensitivity against organic solvents and the limited range of stationary phases which are commercially available.

In this study, headspace SPME combined with GC–qMS, was developed and applied to evaluating the volatile composition profile of different multifloral honey samples (H1–H4). A preliminary screening of fibre of various polarities was carried out in order to select the best coating for the matrix. Comparison between the performance of the five sorbent materials is given. To confirm the applicability of the SPME, comparative study on the characteristic GC–qMS volatile honey profiles were performed. The possibility of differentiation from the investigated honeys was evaluated.

2. Experimental

2.1. Chemicals and materials

All reagents used were analytical quality and all solvents were HPLC grade. Sodium chloride (99.5%) used to obtain the adequate ionic strength (decrease the solubility of the aroma molecules which then partition more readily into the headspace improving the adsorption of analytes in SPME analysis), was supplied by Merck (Darmstadt, Germany). Water was purified through a Milli-Q purification system (Millipore). The C₈–C₂₀ *n*-alkanes series, and the chemical standard used as internal standard, 3-octanol, were supplied by Sigma–Aldrich (Spain).

The SPME fibres tested and compared in this work, polydimethylsiloxane layer (PDMS, 100 µm), recommended for nonpolar volatiles; polyacrylate (PA, 85 µm) with high selectivity for polar semivolatile compounds; divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) on a 1 cm StableFlex fibre, recommended for flavours (volatiles and semivolatiles); carboxen–polydimethylsiloxane (CAR/PDMS, 75 µm); carbowax–divinylbenzene (CW/DVB, 65 µm) and the SPME holder for manual sampling, were obtained from Supelco (Bellefonte, PA, USA). The coating of all fibres was 1 cm long. The fibres were conditioned prior to use according to the manufacturer's instructions by inserting them into the GC injector port. Before the first daily analysis the fibres were conditioned for 20 min. A blank test was performed to check possible carry-over.

2.2. Honey samples

This study was carried out on 16 multifloral honeys, H1–H4 (four samples from each honey) from four different regions of Madeira Island (Portugal) where predominates the wild flora, eucalyptus, hissed and rosemary. All samples were obtained from local stores and were processed using the traditional procedures. None of these samples underwent any treatment that could alter their composition.

All samples were contained in glass bottles and stored at 4 °C until analysis. All analysis, were carried out four times.

2.3. SPME methodology

In order to select the best fibre for honey volatile composition, five SPME coatings of different polarity and extraction mechanisms were tested. The methodology developed and optimised by Câmara et al. [37] for the volatile compounds extraction using manual SPME was used in this study with minor modifications. The H1 honey sample was selected as the matrix for comparison of the performance of the fibres. The fibre that presented the most complete profile of H1 honey volatile compounds was chosen for the study. The extraction was carried out at 60 °C (controlled temperature) and each measurement was repeated four times.

For each extraction 15 g of sample was diluted 1:1 with deionised water (Milli-Q). The dilution decrease the density of the matrix making easy the evaporation of analytes of the interest otherwise retained for sugars. For headspace sampling, 30 mL of the sample was immediately placed in a 60 mL ambar glass vial (volume ratio headspace/solution was 1:1) hermetically sealed and spiked with 3-octanol (250 µL at 422 mg L⁻¹) and 4-methyl-2-pentanol (125 µL at 422 mg L⁻¹) (Sigma–Aldrich) used as internal standards, and equilibrated during 40 min in a thermostatic bath on a stirrer. The extractions were carried out without adjust of pH. The ionic strength was increased using NaCl (30%, w/v) to improve the extraction efficiency by decreasing the solubility of hydrophilic compounds in the aqueous phase. The SPME fibre was then exposed to the headspace of the sample and kept for 40 min at 60 °C. As stirring usually improves the extraction, because the static layer resistant to mass transfer is destroyed (facilitate mass transport between the bulk of the aqueous sample and the fibre), all the experiments were performed under constant stirring velocity (750 rpm).

After sampling, the SPME fibre was withdrawn into the needle, removed from the vial and inserted into the hot injector port (260 °C) of the GC–qMS system for 6 min where the extracted analytes were thermally desorbed and transferred directly to the analytical column.

2.4. Gas chromatography–quadrupole mass spectrometry detection (GC–qMS)

The volatile compounds extracted by dynamic headspace SPME procedure from honey, were tentatively identified using an Agilent 6890N (Palo Alto, CA, USA) gas chromatograph system coupled to an Agilent 5975 quadrupole inert mass selective

detector equipped with a 30 m × 0.25 mm i.d., with a 0.25 µm film thickness, BP-20 fused silica capillary column, according to the method described by Câmara et al. [37]. Helium (Helium N60, Air Liquid, Portugal) was used as the carrier gas at a flow rate ≈ 1 mL min⁻¹ (column-head pressure: 13 psi). An insert of 0.75 mm i.d. was used and the injector temperature was fixed at 260 °C. Splitless injections were used. The temperature used included the following settings: initial temperature of 50 °C was held for 1 min and then increased in three steps: 50–100 °C, at 2.5 °C min⁻¹; 100–180 °C, at 2 °C min⁻¹ and 180–220 °C, at 15 °C min⁻¹. Each step was preceded by a small period at constant temperature for 2, 1 and 10 min, respectively. The manifold, GC–qMS interface and quadrupole temperatures were held at 180, 220 and 180 °C, respectively. The detection was performed by a 5975 mass spectrometer in the electronic impact (EI) mode (ionization energy, 70 eV; source temperature, 180 °C). The electron multiplier was set to the auto tune procedure. The mass acquisition range, made in full scan mode, was 30–300 *m/z*; 1.9 spectra/s.

Compounds were identified by comparing the MS fragmentation pattern with those of the pure standards and mass spectrum of the unknown peaks with those stored in the NIST GC/MS library, retention time of the pure standards obtained under the same conditions, and Kováts retention indices (RI). For the determination of the RI a C₈–C₂₀ *n*-alkanes series was used. The relative amounts of individual components are expressed as percent peak areas (RPA, %) relative to the total peak area.

2.5. Statistical analysis

Significant differences among the honey samples were determined by one-way analysis of variance (ANOVA) using a SPSS Program, Version 14.0 (SPSS Inc., 2006). Principal component analysis (PCA) and stepwise linear discriminant analysis (SLDA) were performed using the same SPSS program. These techniques were applied to the normalized total peak areas from different chemical classes.

3. Results and discussion

Sixteen honey samples from different multifloral origins were analysed with the objective to identify and compare their volatile compounds profiles. Differences in the total ion current (TIC) chromatographic profiles were observed when comparing the studied honeys. TIC chromatograms from H1–H4 samples were compared in terms of total areas of the volatile compounds and number of compounds. The identified compounds were organized in different groups according to their chemical structure. This was done for C₁₃-norisoprenoids/monoterpenes, sesquiterpenoids, higher alcohols, fatty acids, ethyl esters, furanic compounds, carbonyl compounds, aliphatic and aromatic hydrocarbons, while compounds of different structures were considered together as the class “miscellaneous”.

From these identification we found that carbonyl compounds, furanic compounds, higher alcohols and C₁₃-

norisoprenoids/monoterpenes, constitute a main part of flavour studied honeys, and they probably play a significant role in their flowery notes. These findings are in good agreement with previous reports [34,35]. As predominant compounds *trans*-linalool oxide, furfural, hotrienol and in minor extent, 1,3-dihydroxy-2-propanone, 5-hydroxymethylfurfural, benzeneacetaldehyde, ethyl decanoate, ethyl dodecanoate, *o*-methoxyacetophenone and 2-ethyl hexanoic acid were found. The two fatty acid ethyl esters may contribute with sweet and fruity notes while benzeneacetaldehyde may contribute with flowery, rose and honey notes.

Furanic compounds such as furfural, 1-(2-furanyl)-ethanone and 5-hydroxymethylfurfural were presented in all samples. Several aliphatic and aromatic hydrocarbons arise from bee wax which has not been completely separated during harvest and processing, were identified.

3.1. Selection of SPME fibre coating

The fibre coating used influences the chemical nature of the extracted analyte that is established by their characteristic polarity and volatility. To evaluate the extraction yields of the honey volatile components by HS-SPME, and taking account of the physico/chemical characteristics of the targets under consideration, we tested five types of fibre (CAR/DVB, CW/PDMS, PDMS, PA, and DVB/CAR/PDMS) among those used most routinely for assaying volatiles. A few key experimental factors which influence the HS-SPME extraction yield, namely time required for the target analytes to reach equilibrium and extraction temperature, were previously evaluated by Câmara et al. [37], and applied in this work. Bearing in mind the obtained results, each fibre was exposed to the headspace at the same temperature (60 °C) during the same extraction time (40 min). For reasons of comparability all tests were carried out with the same honey sample (H1). The comparison of the SPME fibre performance was made in terms of extraction efficiency, number of identifiable compounds in the extract and reproducibility. The chromatographic profiles presented in Fig. 1, obtained for a H1 honey sample by using different coatings in same experimental conditions, shows the different extraction efficiency of the fibres. The results obtained using the five fibres on the same H1 sample, in rigorously reproduced temperature and exposure time conditions, are reported in Table 1. The qualitative composition of honey volatiles and the number of identified compounds using the five fibres under study is very different. As can be seen in Table 1 with DVB/CAR/PDMS fibre a total of 46 compounds were identified whilst with the PA, CW/DVB, CAR/PDMS and PDMS coatings, were detected only, 32, 30, 35 and 17 compounds, respectively. The semi-polar DVB/CAR/PDMS fibre allowed the best efficiency of extraction for volatile compounds while under the same conditions PDMS fibre had the lowest sorption capacity. The former fibre provided the best sensitivity in terms of total compound peak areas, highest number of detected compounds and high reproducibility, hence this fibre was chosen for evaluation of volatile compounds in honeys (Table 2). PA fibre coating extracted

71.8% of DVB/CAR/PDMS while PDMS coating extracted the lowest amount (about 32.0% of DVB/CAR/PDMS). Similar amounts of volatiles (57.4–60.2% of DVB/CAR/PDMS) were extracted when using either, CW/DVB or CAR/PDMS coatings (Table 2). From Fig. 2, it can be observed that the fibres show different selectivity to different target compounds.

Higher alcohols (HA) have a larger affinity for PA fibre. This coating also presents better sensitivity for furanic compounds (FC), carbonyl compounds (CaC) and miscellaneous. DVB/CAR/PDMS coating showed a strong extraction capacity for aliphatic and aromatic hydrocarbons (HSHAr), C₁₃-norisoprenoids/monoterpenes (NT), sesquiterpenoids (S) and ethyl esters (EEs).

Some characteristic honey compounds were isolated by the five fibres, such as toluene, *trans*-linalool oxide, furfural, linalool, hotrienol, benzeneacetaldehyde, 1,3-dihydroxy-2-propanone, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) and 5-hydroxymethylfurfural.

3.2. Volatile compounds in honey samples

The proposed HS-SPME method, previously optimised and validated, was applied to determine the content of volatile patterns in four commercial available Portuguese honeys. Each honey was analysed four times using the best sampling conditions described above. A characteristic GC-qMS profile of each honey obtained with DVB/CAR/PDMS using the experimental conditions discussed above is shown in Fig. 3. More than 100 volatile compounds belonging to several chemical classes were positively identified, including monoterpenes, C₁₃-norisoprenoids, sesquiterpenoids, higher alcohols, fatty acids ethyl esters, fatty acids, furanic compounds, carbonyl compounds and aliphatic/aromatic hydrocarbons. Most of the volatile compounds were identified by a library search NIST. In some cases a comparison with authentic compounds was performed. The Kováts retention indices were calculated for each peak and compared with the literature in order to ensure the correct identification of the compounds. Table 3 summarises the average ($n = 4$) relative composition determined in the multifloral studied honeys. The relative composition of every flavour compound was calculated as the percent ratio of the respective peak area relative to the total peak area (RPA, %) and relative to the compound with highest peak area in each honey (RPC, %).

A total of 110 volatile compounds were identified in investigated honey samples. Fifteen of these volatiles: ethanol, toluene, 1-methyl-2-(1-methylethyl)-benzene, *trans*-linalool oxide, acetic acid, furfural, 1-(2-furanyl)-ethanone, linalool, hotrienol, benzeneacetaldehyde, 1-nonanol, 1,3-dihydroxy-2-propanone, nonanoic acid, DDMP and 5-hydroxymethylfurfural, were detected in all samples (Table 3) but the ratio between the single components were different for each honey. Some of these 15 compounds ethanol, toluene, *trans*-linalool oxide, furfural, hotrienol and benzeneacetaldehyde, have been reported as common components of various honeys [32–36].

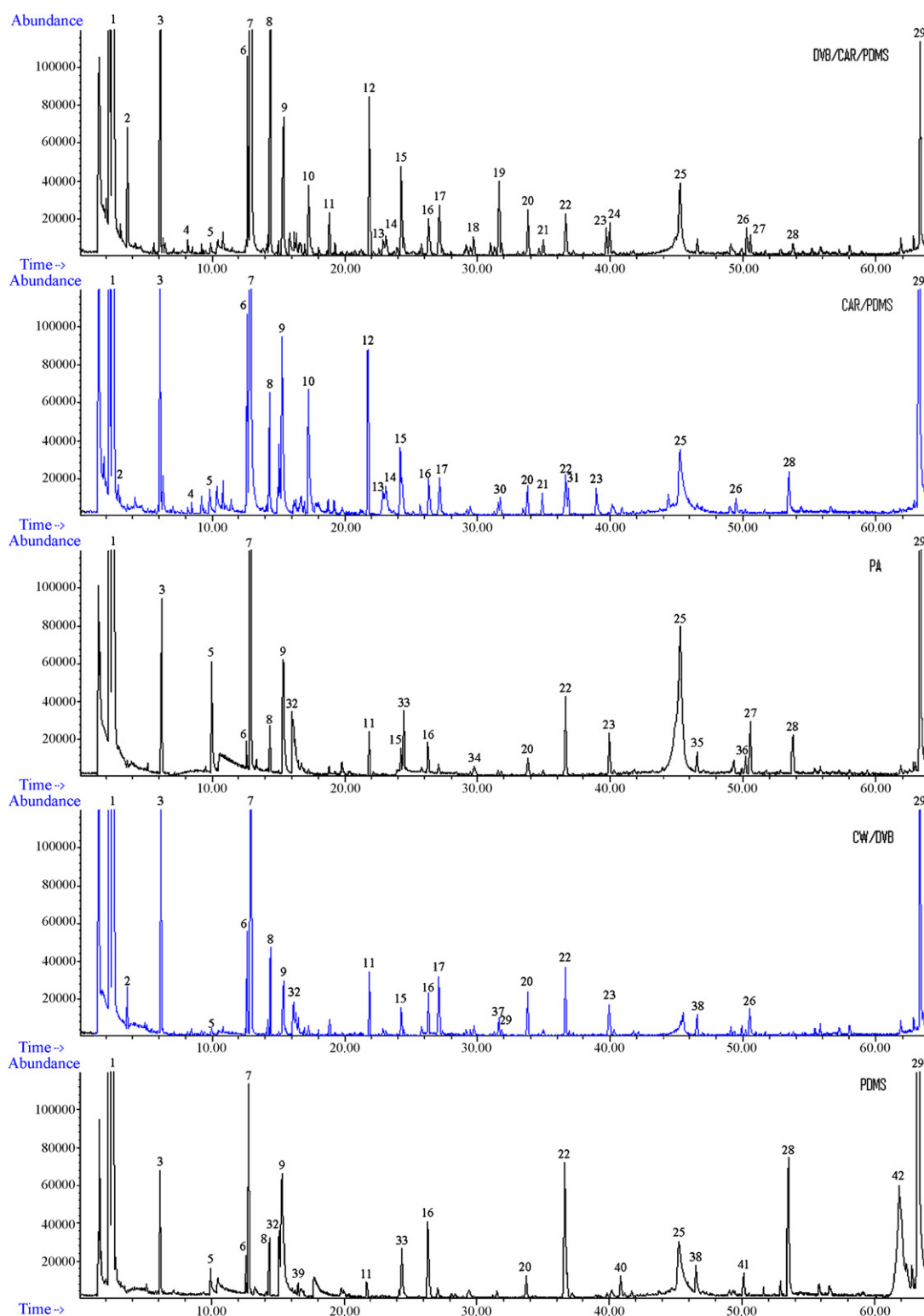


Fig. 1. Chromatograms obtained by SPME analysis of H1 honey with different fibres using in the headspace sampling mode with 30% NaCl and at 60 °C during 40 min. Peak identification: (1) ethanol; (2) toluene; (3) 4-methyl-2-pentanol (IS); (4) 1,3-butanediol; (5) 1-hydroxy-2-propanone; (6) nonanal; (7) 3-octanol (IS); (8) *trans*-linalool oxide; (9) furfural; (10) benzaldehyde; (11) linalool; (12) hotrienol; (13) benzeneacetaldehyde; (14) acetophenone; (15) 1-nonanol; (16) eicosane; (17) naphthalene; (18) 3-ethyl-2-pentene; (19) 2-methyl-2-phenylethyl ester propanoic acid; (20) *E*-6,10-dimethyl-5,9-undecadien-2-one; (21) α -2-propenyl-benzenemethanol; (22) heptadecane; (23) 2-ethyl-hexanoic acid; (24) 1-ethyl-2-heptyl-cyclopropane; (25) 1,3-dihydroxy-2-propanone; (26) nonanoic acid; (27) *N*-(4-hydroxyphenyl)acetamide; (28) 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one; (29) 5-hydroxymethylfurfural; (30) α -methyl benzenemethanol; (31) α -ionol; (32) acid acetic; (33) furanmethanol; (34) 2-hydroxy-2-cyclopenten-1-one; (35) tridecane; (36) acetoxymethyl-2-furaldehyde; (37) ethyl ester benzenepropanoic acid; (38) nonadecane; (39) hexadecane; (40) furyl hydroxymethyl ketone; (41) 5-acetoxymethylfurfural; (42) 4-(2-hydroxyethyl)phenol.

Table 1
Volatile compounds identified in H1 honey after dynamic headspace solid-phase microextraction using different coatings (extraction temperature: 60 °C; extraction time: 40 min)

RT (min)	KI ^a	Compound	Identity ^b	SPME coating				
				PA	CW/DVB	CAR/PDMS	PDMS	DVB/CAR/PDMS
2.575	929	Ethanol	A, B	x	x	x	x	x
3.614	1023	Toluene	A, B		x			x
4.186	1064	Hexanal	A, B			x		
6.295	1178	Heptanal	A, B			x		x
8.259	1254	1,3-Butanediol	A					x
8.481	1261	1-Methyl-3-(1-methylethyl)-benzene	A, B	x		x		x
9.233	1285	2,4,6,8-Tetramethyl-1-undecane	B	x		x		
9.942	1307	1-Hydroxy-2-propane	B	x		x	x	x
10.864	1337	Linalool	B	x		x		
12.668	1389	Nonanal	A, B	x	x	x		x
12.796	1392	(E)-2-Nonen-1-ol	A, B				x	x
14.130	1432	Heptanoic acid ethyl ester	B	x		x		x
14.274	1436	1,4-Diclorobenzene	A, B	x	x	x		x
14.401	1439	Trans-linalool oxide	B	x	x	x	x	x
15.025	1457	Acetic acid	B		x		x	x
15.368	1466	Cis-linalool oxide	B		x			
15.368	1466	Furfural	A, B	x		x	x	x
15.767	1477	α-Cubebeno	A, B					x
16.149	1486	Methyl ester nonanoic acid	A, B	x		x		
16.578	1497	Hexadecane	B		x			x
16.764	1502	1-(2-Furanyl)-ethanone	A, B	x		x		x
17.009	1508	TBH ^c	B		x			x
17.009	1508	(E,E,E)-2,4,6-Octatriene	A, B	x		x		
17.300	1515	Benzaldehyde	A, B		x	x		x
18.849	1549	(S)-Linalool	B		x			x
18.849	1549	5-Methyl-1,3,6-heptatriene	A, B	x		x		
19.248	1558	3-Methyl-1,5-pentenediol	B					x
19.248	1558	1-Hexene	A, B	x		x		
19.801	1569	5-Methylfurfural	B					x
20.052	1574	β-Elementene	B		x			
21.343	1600	α-4-Dimethyl-3-cyclohexene-1-acetaldehyde	B					x
21.840	1611	Hotrienol	B	x	x	x	x	x
22.144	1617	Dihydro-cis-α-copaene-8-ol	B					x
22.589	1626	Pulegone	B					x
22.893	1633	Phenylacetaldehyde	B	x	x	x		x
23.122	1637	Acetophenone	B	x	x	x		x
23.475	1644	3-Carene	A, B					x
23.981	1654	1-Nonanol	A, B	x	x	x		x
24.252	1660	2-Furanmethanol	A, B	x	x	x	x	x
25.472	1683	n-Germacrene	B					x
26.312	1698	Heptadecane	B	x	x	x	x	x
27.090	1714	Naphthalene	A, B	x	x	x		x
28.038	1733	β-Cubebene	A, B					x
29.198	1756	Methyl ester 2-hidroxy-benzoic acid	A,B					x
29.368	1759	Cis-α-bisabolene	A, B					x
29.368	1759	α-Ocimene	B		x			
29.734	1766	(Z)-Heptene	A, B					x
31.591	1801	DPEPA ^d	B					x
31.591	1801	β-Damascenone	A, B		x			
31.591	1801	TMCHB ^e	B	x		x		
31.819	1805	α-Methyl-benzenemethanol	A, B	x	x	x		x
33.474	1839	Ethyl ester dodecanoic acid	B	x		x		
33.774	1845	(E)-6,10-Dimethyl-5,9-undecadien-2-one	B	x	x	x	x	x
35.010	1869	α-2-Propenyl-benzenemethanol	B	x		x		x
36.628	1898	Nonadecane	B	x	x	x	x	x
36.918	1904	α-Ionol	A, B				x	
36.918	1904	DDMMP ^f	A, B					x
40.004	1967	2-Ethyl-hexanoic acid	A, B		x			
42.142	2008	2-Methyl-pentanal	B		x			
45.000	2068	1,3-Dihydroxy-2-propanone	A, B	x		x	x	x
46.548	2098	10-Methyl eicosane	B		x			x

Table 1 (Continued)

RT (min)	KI ^a	Compound	Identity ^b	SPME coating				
				PA	CW/DVB	CAR/PDMS	PDMS	DVB/CAR/PDMS
49.500	2162	Nonanoic acid	B	x	x	x		x
53.494	2225	DDMP ^g	A, B	x		x	x	x
55.814	2251	2,3,4-Trimethyl-hexane	A, B				x	x
58.057	2275	2-Acetylbenzoic acid	B		x			
61.825	2345	4-Hydroxy-benzenmethanol	B				x	
63.381	2395	5-Hydroxymethylfurfural	B	x		x	x	x
64.113	2418	2,6-Dimethoxy-4-(2-propenyl)-phenol	B		x			
65.352	2457	Dihydro-4-hydroxy-2(3H)-furanone	A, B					x
Total compounds identified by fibre				32	30	35	17	50

^a Experimentally determined Kovats indices on the BP-20 column, relative to C₈–C₂₀ hydrocarbons.

^b (A) Components identified on the basis of the retention time and EI mass spectra of pure standard; (B) components identified on the basis of their EI mass spectra only.

^c 1,7,7-Trimethyl-bicyclo[2.2.1]hepten-2-ene.

^d 2,2-Dimethyl-2-phenylethyl ester propanoic acid.

^e 1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one.

^f 4,6-Di(1,1-dimethylethyl)-2-methylphenol.

^g 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one.

Each type of honey showed a typical composition. H1 honey (Fig. 3) is characterized by a high amount of ethanol, furfural, nonanal, 1,3-dihydroxy-2-propanone, *trans*-linalool oxide, benzaldehyde, hotrienol, and nonanol. Some identified compounds are specific of this sample. Therefore, heptanal, ethyl heptanoate, α - and β -cububene, (Z)-heptene, α -propenyl-2-benzenemethanol, pulegone, acetophenone, 3-carene, D-germacrene and 2,3,4-trimethylhexane, were identified only in this honey sample. This sample is also characterized by the lowest level of 5-hydroxymethylfurfural.

H2 (Fig. 3) is characterized by a high amount of hotrienol. This compound was identified in each honey analysed but at lower levels. Also 5-hydroxymethylfurfural, benzeneacetaldehyde, furfural and *trans*-linalool oxide, are present in significantly high amounts, when compared with the H1,

H3 and H4 honey samples. 1,3,8-*p*-menthatriene, 1-ethyl-2-methylcyclopropane, cycloheptane, and β -phenylethanol, are present only in H2 honey type.

In H3 honey were identified 60 volatile compounds. As shows Fig. 3, the most intense peaks correspond to *o*-methoxyacetophenone, benzeneacetaldehyde, hotrienol, furfural, ethyl dodecanoate and 2-ethyl hexanoic acid. *o*-methoxyacetophenone and 2-ethyl hexanoic acid can be used to differentiate these honey type from H1, H2 and H4 samples, since were detected, at high levels, only in this sample. 5-Hydroxymethylfurfural, 1,3-dihydroxy-2-propanone, *trans*-linalool oxide, furfural, hotrienol and benzeneacetaldehyde, are the dominating components found in the H4 sample. Forty-eight compounds were positively identified in this honey with the experimental conditions used. 3-Methyl-1-butanol, (*E*)-2-hexen-1-ol, 3-methyl-1-pentanol, 5-acetoxymethylfurfural and

Table 2

Sorption capacity of different fibres for extraction of H1 honey volatile compounds during dynamic HS-SPME extraction, expressed as peak area (40 min at 60 °C with salt saturation)

Class of compounds	SPME fibre				
	PA	CW/DVB	DVB/CAR/PMS	PDMS	CAR/PDMS
Higher alcohols	1.10×10^8	1.12×10^8	8.07×10^7	5.34×10^7	1.00×10^8
Fatty acids	7.78×10^6	3.53×10^6	1.23×10^6	8.04×10^5	5.71×10^5
Ethyl esters	—	—	2.85×10^6	—	6.70×10^5
NT ^a	2.16×10^6	6.08×10^6	8.95×10^6	1.99×10^6	8.34×10^6
Sesquiterpenoids	—	5.21×10^5	1.63×10^6	—	—
Furanic compounds	1.04×10^8	1.43×10^7	7.16×10^6	1.33×10^7	3.60×10^7
Carbonyl compounds	4.71×10^7	3.05×10^6	1.19×10^7	5.75×10^5	1.91×10^7
HSHAr ^b	2.98×10^6	7.57×10^6	1.38×10^7	5.06×10^6	5.67×10^6
Miscellaneous	1.95×10^6	—	5.64×10^5	—	3.10×10^5
Sum	2.04×10^8	1.63×10^8	2.84×10^8	9.09×10^7	1.71×10^8
% R.S.D. ($n = 4$) on sum	4.66	4.89	2.81	1.67	11.63

^a C₁₃-norisoprenoids and monoterpenes.

^b Aliphatic and aromatic hydrocarbons.

Table 3
Volatile compounds identified in different types of Portuguese honeys after dynamic headspace solid-phase microextraction using DVB/CAR/PDMS coating (extraction temperature: 60 °C; extraction time: 40 min)

RT (min)	KI ^a	Compound	RPA (%)				RPC (%)			
			H1	H2	H3	H4	H1	H2	H3	H4
2.575	929	Ethanol	56.16	41.86	44.27	29.30	51	69	100	98
3.228	992	Tricloromethane	–	–	1.25	–	–	–	100	–
3.614	1023	Toluene	3.99	2.95	2.32	1.61	48	72	80	100
5.941	1162	2,6-Dimethyl-4-heptanone	–	–	–	0.09	–	–	–	100
6.295	1178	Heptanal	0.60	–	–	–	100	–	–	–
7.105	1212	3-Methyl-1-butanol	–	–	–	0.16	–	–	–	100
8.187	1252	3-Octanone	–	0.35	–	0.10	–	100	–	80
8.252	1254	Styrene	–	–	0.16	–	–	–	100	–
8.259	1254	1,3-Butanediol	0.25	–	–	–	100	–	–	–
8.481	1261	1-Methyl-2-(1-methylethyl)-benzene	0.19	0.14	0.13	0.12	35	41	58	100
9.377	1289	1-Methoxy-2-methyl-propane	–	–	–	0.26	–	–	–	100
9.942	1307	1-Hydroxy-2-propane	0.95	0.54	–	0.22	100	55	–	96
10.387	1322	2-Heptanol	–	–	–	0.14	–	–	–	100
12.524	1385	Methyl ester octanoic acid	–	–	0.49	0.15	–	–	100	57
12.668	1389	Nonanal	6.94	–	–	–	100	–	–	–
12.629	1388	(E)-2-Hexen-1-ol	–	–	–	0.36	–	–	–	100
12.796	1392	(E)-2-Nonen-1-ol	0.93	1.25	1.55	–	17	53	100	–
14.100	1431	1-Methyl-4-(1-methylethenyl)-benzene	–	0.67	1.05	0.36	–	43	100	64
14.13	1432	Heptanoic acid ethyl ester	0.20	–	–	–	100	–	–	–
14.267	1436	1,4-Dichloro-benzene	1.05	–	–	–	100	–	–	–
14.401	1439	Trans-linalool oxide	4.99	7.83	4.92	3.90	30	73	69	100
14.469	1441	1,3,8- <i>p</i> -Menthatriene	–	0.39	–	–	–	100	–	–
15.025	1457	Acetic acid	1.12	2.11	0.90	0.78	35	99	66	100
15.368	1466	Furfural	8.09	11.31	6.10	3.62	32	100	82	88
15.767	1477	α -Cubebene	1.67	–	–	–	100	–	–	–
16.13	1486	Methyl ester nonanoic acid	–	–	0.55	0.13	–	–	100	43
16.241	1489	2-Ethyl-1-hexanol	–	–	0.29	–	–	–	100	–
16.358	1492	Decanal	–	0.86	0.31	0.14	–	100	53	45
16.535	1496	Benzofuran	–	–	0.85	–	–	–	100	–
16.578	1497	Hexadecane	0.34	–	–	0.19	29	–	–	100
16.764	1502	1-(2-Furanyl)-ethanone	0.20	0.92	0.60	0.27	14	100	98	80
16.917	1505	1-(1,4-Dimethyl-3-cyclohexen-1-yl)-ethanone	–	–	0.41	0.18	–	–	100	79
17.009	1508	1,7,7-Trimethyl-bicyclo[2.2.1]hepten-2-ene	0.35	–	–	–	100	–	–	–
17.3	1515	Benzaldehyde	4.60	2.80	0.80	–	73	100	44	–
17.47	1518	2-Nonanol	–	–	0.76	–	–	–	100	–
18.009	1531	Ethyl ester octanoic acid	–	–	0.34	–	–	–	100	–
18.849	1549	(S)-Linalool	1.46	0.78	1.16	0.56	51	44	100	88
19.189	1557	Cis-1-ethyl-2-methyl-cyclopropane	–	0.37	–	–	–	100	–	–
19.192	1557	(S)-(+)-3-Methyl-1-pentanol	–	–	–	0.17	–	–	–	100
19.801	1569	5-Methylfurfural	0.32	0.5	0.32	–	28	99	100	–
20.774	1589	2-Methyl benzofuran	–	–	0.86	–	–	–	100	–
21.343	1600	α -4-Dimethyl-3-cyclohexene-1-acetaldehyde	0.10	0.84	–	0.17	21	100	–	55
21.840	1611	Hotrienol	3.79	30.33	7.20	4.98	8	100	36	45
22.144	1617	Dihydro- <i>cis</i> - α -copaene-8-ol	0.02	–	–	–	100	–	–	–
22.562	1626	1-Ethenyl-4-methoxybenzene	–	–	0.72	–	–	–	100	–
22.589	1626	Pulegone	0.14	–	–	–	100	–	–	–
22.893	1633	Phenylacetaldehyde	0.50	12.44	7.92	3.69	2	100	89	82
23.122	1637	Acetophenone	1.24	–	–	–	100	–	–	–
23.475	1644	3-Carene	0.29	–	–	–	100	–	–	–
23.661	1648	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	–	0.87	–	0.50	–	68	–	100
24.252	1660	1-Nonanol	4.04	1.18	1.00	0.66	100	60	83	99
24.436	1663	2-Furanmethanol	1.52	1.51	–	1.32	27	36	–	100
25.105	1676	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	–	0.38	0.55	0.27	–	60	100	89
25.472	1683	<i>n</i> -Germacrene	0.60	–	–	–	100	–	–	–
25.681	1687	1-Methyl-4-(1-methylethylidene)-cyclohexene	–	–	0.20	–	–	–	100	–
25.703	1687	2,6-Dimethyl-5,7-octadien-2-ol	–	0.14	–	0.11	–	10	100	16
26.272	1697	Eicosane	–	1.22	1.26	–	–	100	95	–
26.312	1698	Heptadecane	2.40	–	–	0.64	84	–	–	100
27.011	1712	8-Heptadecane	–	–	0.29	–	–	–	100	–
27.090	1714	Naphthalene	4.45	–	0.14	–	100	–	11	–
27.318	1719	2-Heptadecanol	–	–	0.78	–	–	–	100	–

Table 3 (Continued)

RT (min)	KI ^a	Compound	RPA (%)				RPC (%)			
			H1	H2	H3	H4	H1	H2	H3	H4
27.328	1719	2-Tetradecanol	–	0.26	–	–	–	100	–	–
27.357	1720	1,2-Dihydro-3,6,8-trimethyl-naphthalene	–	0.46	–	–	–	100	–	–
28.038	1733	β-Cubebene	0.35	–	–	–	100	–	–	–
29.172	1756	2-Hydroxy methyl ester benzoic acid	–	2.02	–	0.36	–	100	–	48
29.198	1756	Methyl ester 2-hidroxy-benzoic acid	0.52	–	–	–	100	–	–	–
29.368	1759	Cis-α-bisabolene	0.25	–	–	–	100	–	–	–
29.446	1761	Cycloheptane	–	0.56	–	–	–	100	–	–
29.453	1761	2-Methyl-cyclopentanone	–	–	–	0.33	–	–	–	100
29.734	1766	(Z)-Heptene	0.96	–	–	–	100	–	–	–
30.400	1779	4-Hydroxy-acetophenone	–	–	0.25	–	–	–	100	–
31.182	1793	Methyl ester dodecanoic acid	–	–	0.81	–	–	–	100	–
31.587	1800	2-Phenylethyl ester propanoic acid	–	–	0.33	0.30	–	–	61	100
31.591	1801	2,2-Dimethyl-2-phenylethyl ester propanoic acid	4.99	–	–	–	100	–	–	–
31.819	1805	(S)-α-Methyl-benzenemethanol	0.57	–	0.42	–	55	–	100	–
33.395	1837	Ethyl ester dodecanoic acid	–	1.38	6.80	–	–	15	100	–
33.424	1838	Ethyl ester decanoic acid	–	–	–	0.22	–	–	–	100
33.594	1841	α-4-Trimethyl-benzenemethanol	–	–	–	0.11	–	–	–	100
33.774	1845	(E)-6,10-Dimethyl-5,9-undecadien-2-one	2.15	–	0.96	–	91	–	100	–
34.336	1856	3-Methylbutyl ester pentadecanoic acid	–	–	0.24	–	–	–	100	–
34.901	1866	Phenol	–	–	0.64	–	–	–	100	–
35.010	1869	α-2-Propenyl-benzenmethanol	0.78	–	–	–	100	–	–	–
36.565	1897	β-Phenylethanol	–	1.84	–	–	–	100	–	–
36.628	1898	Nonadecane	2.53	–	1.90	0.89	54	–	100	85
36.830	1902	α-Ionol	–	–	0.56	–	–	–	100	–
36.918	1904	4,6-Di-(1,1-dimethylethyl)-2-methyl phenol	1.80	–	–	–	100	–	–	–
37.052	1907	Benzyl nitrile	–	–	0.26	0.16	–	–	91	100
38.602	1939	2,3,4,5-Tetramethyl-1,4-hexadiene	–	–	–	0.97	–	–	–	100
38.951	1946	2-Ethyl hexanoic acid	–	–	6.08	–	–	–	100	–
40.203	1971	o-Methoxyacetophenone	–	–	19.25	–	–	–	100	–
40.664	1979	MFPOE ^b	–	–	–	0.29	–	–	–	100
40.906	1984	Furyl hydroxymethyl ketone	–	–	–	0.41	–	–	–	100
41.71	1999	Methyl tetradecanoate	–	–	0.48	–	–	–	100	–
43.727	2042	Ethyl ester tetradecanoic acid	–	–	0.55	–	–	–	100	–
44.358	2055	Octanoic acid	–	0.81	1.61	0.68	–	46	100	78
45.355	2075	1,3-Dihydroxy-2-propanone	6.20	6.97	5.61	6.90	18	53	45	100
46.548	2098	10-Methyleicosane	0.75	–	–	–	100	–	–	–
49.862	2170	Cyclododecane	–	–	0.42	–	–	–	100	–
50.189	2176	5-Acetoxyethylfurfural	–	–	–	0.29	–	–	–	100
50.679	2186	Nonanoic acid	0.70	0.75	1.01	0.34	26	57	100	63
51.673	2204	15-Methyl ester hexadecanoic acid	–	–	0.40	–	–	–	100	–
53.461	2225	Ethyl ester hexadecanoic acid	–	–	0.72	–	–	–	100	–
53.824	2229	DDMP ^c	1.61	2.23	0.70	1.61	24	57	25	100
54.464	2236	Ethyl ester E-11 hexadecanoic acid	–	–	1.02	–	–	–	100	–
54.732	2239	2,3-Dimethoxy-naphthalene	–	–	1.67	–	–	–	100	–
55.814	2251	2,3,4-Trimethyl-hexane	6.06	–	–	–	100	–	–	–
59.217	2287	3,5-Dimethoxy methyl ester benzoic acid	–	–	0.82	–	–	–	100	–
63.074	2345	Dodecanoic acid	–	–	1.12	–	–	–	100	–
63.381	2395	5-Hydroxymethylfurfural	0.31	14.71	4.86	10.93	1	59	29	100
65.352	2457	Dihydro-4-hydroxy-2-(3H)-furanone	0.33	–	–	0.37	21	–	–	100
Total compounds identified by honey							50	37	60	48
Sum of total area		2.8 × 10 ⁸ 4.3 × 10 ⁸	5.2 × 10 ⁸		5.12 × 10 ⁸					
% R.S.D. (n = 4)		2.81 1.95	7.35		7.30					

^a Experimentally determined Kovats indices on the BP-20 column, relative to C₈–C₂₀ hydrocarbons.^b 1-[3-[2-Methyl-2-[5-methyl-2-furanyl]propyl]oxiranyl]-ethanone.^c 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one.

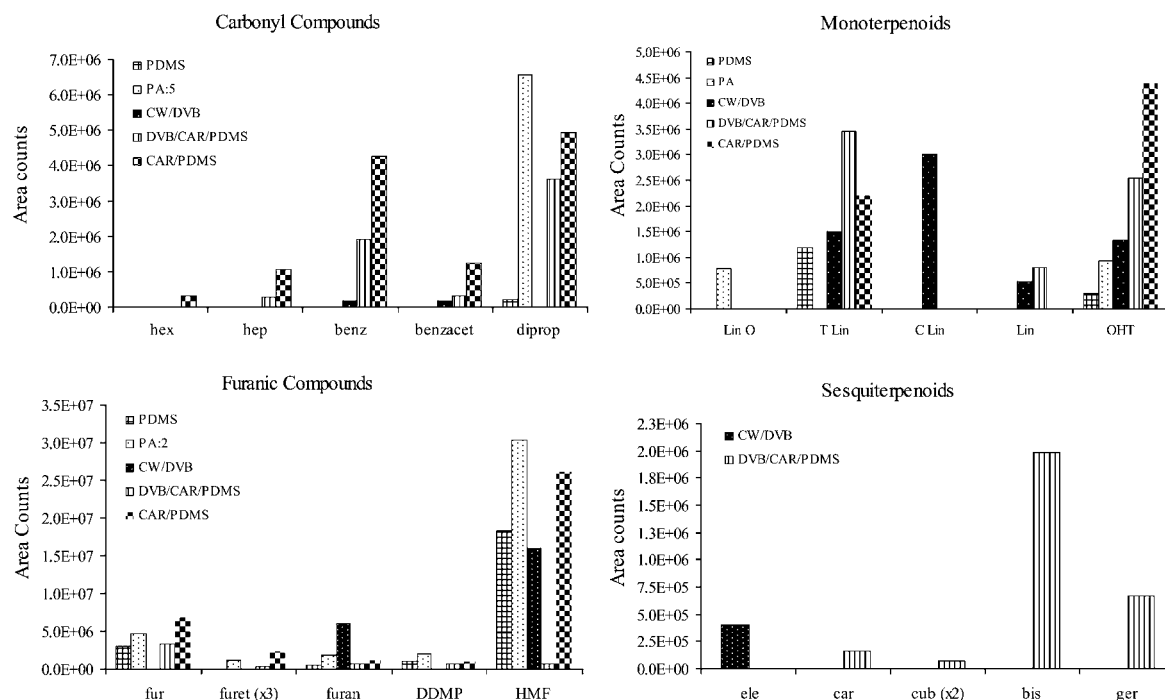


Fig. 2. Comparison of the performance of different SPME coatings on the carbonyl compounds, monoterpene, furanic compounds and sesquiterpenoids extraction (hex: hexane; hep: heptane; benz: benzaldehyde; benzacet: benzeneacetaldehyde; diprop: 1,3-hydroxy-2-propanone; Lin O: linalool oxide; T Lin: *trans*-linalool oxide; C Lin: *cis*-linalool oxide; Lin: linalool; OHT: hotrienol; fur: furfural; furet (x3): 1-(2-furanyl)-ethanone; furan: furanmethanol; DDMP: 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one; HMF: 5-hydroxymethylfurfural; ele: β -elemene; car: 3-carene; cub (x2): β -cubebene; bis: α -bisabolene; ger: δ -germacrene).

2-methylcyclopentanone, has been detected only in this honey sample. Fig. 4 presents the distribution of compounds classes by honey sample.

The presence of several honey constituents found in this study have been previously reported. The presence of hotrienol in the *Eucryphia lucida* honey [38]. We have also found, for instance aromatic aldehydes such as benzaldehyde and benzeneacetaldehyde, which have been reported as common components of various unifloral honey [39].

3.3. Multivariate analysis

The proposed headspace SPME_{DVB/CAR/PDMS} method was applied to four different commercial honeys. Evidently, the different concentrations of the volatile constituents determined in these samples allow their differentiation. Although the volatile compounds studied showed important data for the characterization of honey samples, the differentiation is quite difficult to establish from the direct observation of Table 3. Multivariate

techniques of data analysis represent a powerful statistical tool to explain this differentiation. The total peak area of each chemical group, higher alcohols (HA), fatty acids (FAc), ethyl esters (EEs), C_{13} -norisoprenoids/monoterpenols/sesquiterpenoids (NTS1), furanic compounds (FC), carbonyl compounds (CaC) and aliphatic/aromatic hydrocarbons (HSHAr), were used as variable vectors for multivariate analysis in order to obtain more detailed information. When PCA was applied to the total peak area different chemical classes, three factors were extracted and 98.3% of the total variance was explained (Table 4). As can be seen in Fig. 5a, a clear separation can be observed. Considering the factor loadings of the variables (Table 5), the most influential variables (chemical groups) to the first component (PC1, 50.1%), are carbonyl compounds, ethyl esters and fatty acids, while NTS1, HSHAr (25.9%) and furan derivatives (22.3%), are the variables that most contribute to the PC2 and PC3, respectively. Fig. 5a shows the scores scatter plot of the first two principal components (50.1% of the total variability) that represents the

Table 4
Percentage of variance and percentage of cumulative variance explained by the three first principal components

Component	Total variance explained			Rotation sums of squared loadings		
	Extraction sums of squared loadings					
	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	3.771	53.877	53.877	3.509	50.124	50.124
2	2.449	34.988	88.864	1.814	25.917	76.041
3	0.664	9.491	98.355	1.562	22.314	98.355

Extraction method: principal component analysis.

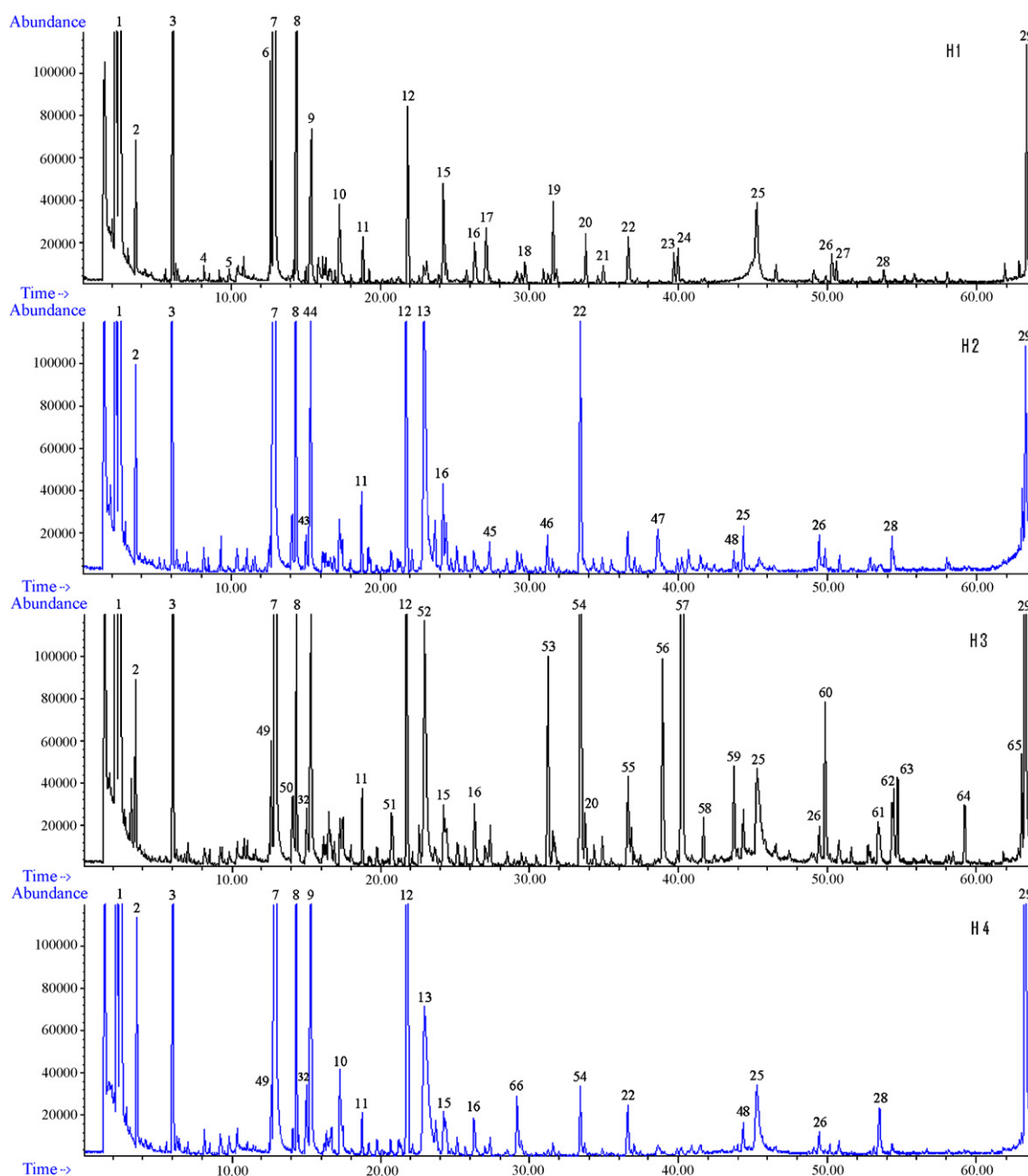


Fig. 3. Comparison of GC-qMS profiles of HS-SPME_{DVB/CAR/PDMS} H1–H4 honey extracts obtained at 60 °C during 40 min. Peak identification: (1) ethanol; (2) toluene; (3) 4-methyl-2-pentanol (IS); (4) 1,3-butanediol; (5) 1-hydroxy-2-propanone; (6) nonanal; (7) 3-octanol (IS); (8) *trans*-linalool oxide; (9) furfural; (10) benzaldehyde; (11) linalool; (12) hotrienol; (13) benzeneacetaldehyde; (14) acetophenone; (15) 1-nonanol; (16) eicosane; (17) naphthalene; (18) 3-ethyl-2-pentene; (19) 2-methyl-2-phenylethyl ester propanoic acid; (20) *E*-6,10-dimethyl-5,9-undecadien-2-one; (21) α -2-propenyl-benzenemethanol; (22) heptadecane; (23) 2-ethyl-hexanoic acid; (24) 1-ethyl-2-heptyl-cyclopropane; (25) 1,3-dihydroxy-2-propanone; (26) nonanoic acid; (27) *N*-(4-hydroxyphenyl)acetamide; (28) 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; (29) 5-hydroxymethylfurfural; (31) α -ionol (32) acetic acid; (33)–(42) (compounds from 33 to 42 were not detected with this fibre); (43) 1-methyl-4-(1-methylethyl)-benzene; (44) *cis* linalool oxide; (45) 2-undecanol; (46) 2-methyl-cyclopentanone; (47) 2,3,4,5-tetramethyl-1,4-hexadiene; (48) octanoic acid; (49) 2-nonen-1-ol; (50) ethyl ester octanoic acid; (51) methyl ester decanoic acid; (52) ethyl ester decanoic acid; (53) methyl ester dodecanoic acid; (54) ethyl ester dodecanoic acid; (55) phenol; (56) 2-ethyl hexanoic acid; (57) *o*-methoxyacetophenone; (58) methyl tetradecanoate; (59) ethyl ester tetradecanoic acid; (60) cyclododecane; (61) ethyl ester hexadecanoic acid; (62) ethyl ester hexadecanoic acid E11; (63) 5,7,8-trimethyl coumarin; (64) 3,5-dimethoxy-methyl ester benzoic acid; (65) dodecanoic acid; (66) 2-hydroxy methyl ester benzoic acid.

distinction among the honey samples. Fig. 5b represents the corresponding loadings plot that established the relative importance of each chemical group. H1 and H2 honeys (third quadrant) are characterized by the carbonyl compounds (CaC), ethyl esters (EEs) and in lower extent, by fatty acids

(FAc). The H3 honey is related to the positive PC1 side. Higher alcohols, is the variable which characterize them. H4 honey represented in the second quadrant being characterized by carbonyl compounds (CaC) and fatty acids (FAc) (Fig. 5b).

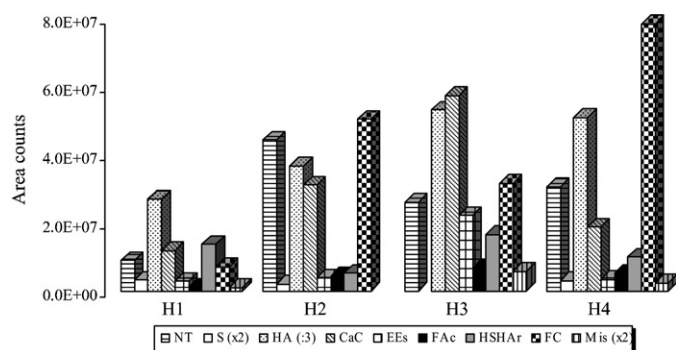


Fig. 4. Distribution of compounds classes by honey samples (NT: C₁₃-norisoprenoids and monoterpenoids; S: sesquiterpenoids; HA: higher alcohols; CaC: carbonyl compounds; EEs: ethyl esters; FAc: fatty acids; HSHAr: aliphatic and aromatic hydrocarbons; FC: furanic compounds; Mis: miscellaneous).

Table 5
Loadings of volatiles in the first three principal components

	Rotated component matrix ^a		
	Component 1	Component 2	Component 3
CC	0.994	0.081	−0.019
EE	0.960	−0.220	−0.060
FA	0.783	0.208	0.579
Alc	0.781	−0.169	0.556
NTS	0.186	0.938	0.284
HSHA	0.579	−0.802	−0.124
F	−0.057	0.403	0.904

(1) PC1; (2) PC2 and (3) PC3; rotation method: Varimax with Kaiser normalization; CC: carbonyl compounds; EE: ethyl esters; FA: fatty acids; Alc: higher alcohols; NTS: C₁₃-norisoprenoids, monoterpenoids and sesquiterpenoids; HSHA: aliphatic and aromatic hydrocarbons; F: furanic compounds. Extraction method: principal component analysis. Rotation method: Varimax with Kaiser normalization.

^a Rotation converged in five iterations.

After PCA, a linear discriminant analysis (LDA) was run, using the above-mentioned variables, in order to obtain suitable classification rules. Fig. 6 shows a projection of the honeys in two-dimensional space, generated by the two first discriminate

Table 6
Prediction abilities for the different honeys, using stepwise discriminant analysis

HM		Classification results ^{a,b}				Total
		Predicted group membership				
		H1	H2	H3	H4	
Original						
Count	H1	4	0	0	0	4
	H2	0	4	0	0	4
	H3	0	0	4	0	4
	H4	0	0	0	4	4
%	H1	100.0	0	0	0	100.0
	H2	0	100.0	0	0	100.0
	H3	0	0	100.0	0	100.0
	H4	0	0	0	100.0	100.0
Cross-validated ^c						
Count	H1	4	0	0	0	4
	H2	0	4	0	0	4
	H3	0	0	4	0	4
	H4	0	0	0	4	4
%	H1	100.0	0	0	0	100.0
	H2	0	100.0	0	0	100.0
	H3	0	0	100.0	0	100.0
	H4	0	0	0	100.0	100.0

^a 100% of original grouped cases correctly classified.

^b 100% of cross-validated grouped cases correctly classified.

^c Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.

functions that explain 97.9% of the total variance. Four groups representing each honey, H1–H4, were clearly observed. The good agreement achieved indicates that very acceptable classification functions can be deduced. The leave one out method was used as cross-validation procedure to evaluate the classification performance (Table 6).

From the results it can be concluded that headspace SPME coupled to GC–qMS and chemometrics is a very appropriate sampling technique to distinguish the different Portuguese honeys studied based on their volatile profile.

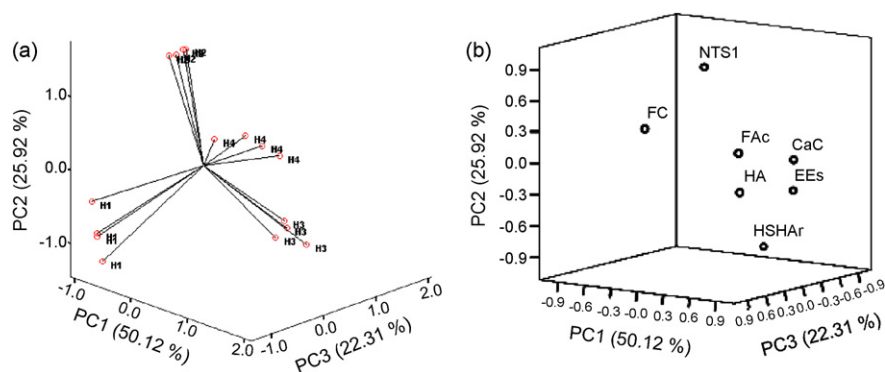


Fig. 5. PC1 and PC2 scatter plot of the main sources of variability between honey samples (H1–H4). (a) Distinction between the samples (scores); (b) relation between the chemical classes (loadings); Variables identification: CaC: carbonyl compounds; EEs: ethyl esters; FAc: fatty acids; HA: higher alcohols; NTS1: C₁₃-norisoprenoids/monoterpenes/sesquiterpenoids; HSHAr: aliphatic and aromatic hydrocarbons; FC: furanic compounds.

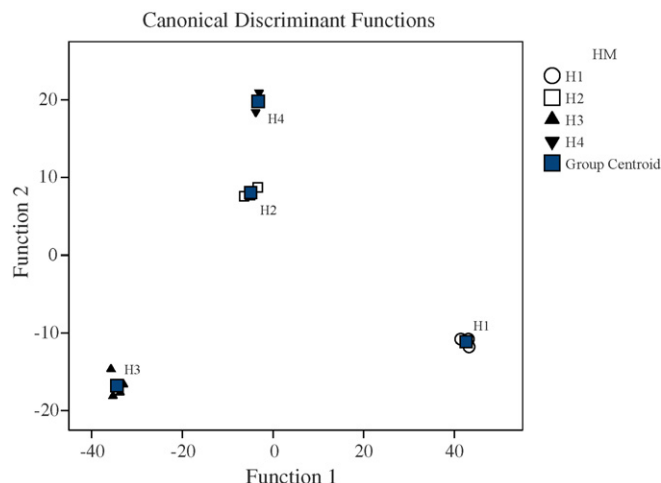


Fig. 6. Differentiation between H1–H4 honeys by applying LDA.

4. Conclusions

Headspace solid-phase microextraction sampling followed by GC–qMS analysis provides an appropriate and selective way to characterize the volatile compounds in honey. Is a simple procedure of extraction with a great capacity of concentration and combines extraction to a rapid, sensitive and solvent-free method suitable for determination of volatile and semivolatile compounds. The chromatographic profiles obtained after extraction with PDMS, PA, CAR/PDMS, CW/DVB and DVB/CAR/PDMS coatings suggest that the later was the most suitable for the SPME analysis of honey volatiles. The more polar fibre, PA, shows no more effective extraction for polar such as higher alcohols, fatty acids, furan derivatives and carbonyl compounds, while DVB/CAR/PDMS favours the extraction of less polar compounds like ethyl esters, C_{13} -norisoprenoids/monoterpenes, sesquiterpenoids and aliphatic and aromatic hydrocarbons. More than 100 volatile compounds, including higher alcohols, ethyl esters and fatty acids, were identified. The higher alcohols, fatty acids and C_{13} -norisoprenoids/monoterpenes constitute important aroma groups compounds which contributes with “fruity”, “cheese/fatty” and “flowery” notes to honey sensory properties. Using a chemometric approach (PCA and LDA), the volatile composition in terms of average peak areas, provides a suitable tool to differentiate between the analysed honeys.

References

- [1] L. Cuevas-Glory, J. Pino, L. Santiago, E. Sauri-Duch, Food Chem. 103 (2007) 1032.
- [2] M. Al-Mamary, A. Al-Meer, M. Al-Habori, Nutr. Res. 22 (2002) 1041.

- [3] S. Buratti, S. Benedetti, M. Cosio, Talanta 71 (2007) 1387.
- [4] E. Alissandrakis, P. Tarantilis, P. Harizanis, M. Moschos, J. Sci. Food Agric. 85 (2005) 91.
- [5] B. D’Arcy, G. Rintoul, C. Rowland, A. Blackman, J. Agric. Food Chem. 45 (1997) 1834.
- [6] R. Pérez, C. Sánchez-Brunete, R. Calvo, J. Tadeo, J. Agric. Food Chem. 50 (2002) 2633.
- [7] F. Bianchi, M. Carreri, M. Musci, Food Chem. 89 (2005) 527.
- [8] C. Guyot, V. Scheirman, S. Collin, Food Chem. 64 (1999) 3.
- [9] V. Ferreira, N. Ortin, A. Escudero, R. Lopez, J. Cacho, J. Agric. Food Chem. 50 (2002) 4048.
- [10] J. Nuñez, H. Bemelmans, Chromatogr. A 294 (1984) 361.
- [11] G. Blanch, G. Reglero, M. Herraiz, J. Agric. Food Chem. 43 (1995) 1251.
- [12] R. López, M. Aznar, J. Cacho, V. Ferreira, J. Chromatogr. A 966 (2002) 166.
- [13] C. Cocito, G. Gaetano, C. Delfini, Food Chem. 52 (1995) 311.
- [14] H. Lord, J. Pawliszyn, J. Chromatogr. A 885 (2000) 153.
- [15] Z. Zhang, J. Pawliszyn, Anal. Chem. 65 (1993) 1843.
- [16] E. Baltussen, P. Sandra, F. David, C. Cramers, J. Microcolumn Sep. 11 (1999) 737.
- [17] R. Alves, A. Nascimento, J. Nogueira, Anal. Chim. Acta 546 (2005) 11.
- [18] E. Coelho, S.M. Rocha, I. Delgadillo, M.A. Coimbra, Anal. Chim. Acta 563 (2006) 204.
- [19] S.M. Rocha, P. Coutinho, A. Barros, I. Delgadillo, M.A. Coimbra, J. Chromatogr. A 1114 (2006) 188.
- [20] J. Demyttenaere, C. Dagherb, P. Sandra, S. Kallithraka, R. Verhé, N. Kimpe, J. Chromatogr. A 985 (2003) 233.
- [21] M. Begala, L. Corda, G. Podda, M. Fedrido, P. Traldi, Rapid Commun. Mass Spectrom. 16 (2002) 1086.
- [22] M. Burmeister, C. Drumond, E. Pfisterer, D. Hysert, J. Am. Soc. Brew. Chem. 50 (1992) 53.
- [23] O. Pinho, I. Ferreira, L. Santos, J. Chromatogr. A 1121 (2006) 145.
- [24] J. Câmara, J. Marques, R. Perestrelo, F. Rodrigues, L. Oliveira, P. Andrade, M. Caldeira, J. Chromatogr. A 1150 (2007) 198.
- [25] J. Demyttenaere, C. Dagherb, P. Sandra, S. Kallithraka, R. Verhé, N. Kimpe, J. Chromatogr. A 985 (2003) 221.
- [26] J. Pino, M. Martí, M. Mestres, J. Pérez, O. Busto, J. Guasch, J. Chromatogr. A 954 (2002) 51.
- [27] J. Pino, R. Marbot, C. Vázquez, J. Agric. Food Chem. 49 (2001) 5883.
- [28] J. Pino, R. Marbot, C. Vázquez, J. Agric. Food Chem. 50 (2002) 6023.
- [29] M. Lopez, G. Guzmán, A. Dorantes, J. Chromatogr. A 1036 (2004) 87.
- [30] A. Gioacchini, M. Menotta, L. Bertini, I. Rossi, S. Zeppa, A. Zambonelli, G. Piccoli, V. Stocchi, Rapid Commun. Mass Spectrom. 19 (2005) 2365.
- [31] F. Augusto, A. Valente, E. Tada, S. Rivellino, J. Chromatogr. A 873 (2000) 117.
- [32] E. Alissandrakis, P. Tarantilis, P. Harizanis, M. Polissiou, Food Chem. 100 (2007) 396.
- [33] M. Baroni, M. Nore, M. Díaz, G. Chiabrando, J. Fassano, C. Costa, D. Wunderlin, J. Agric. Food Chem. 54 (2006) 7235.
- [34] A. Soria, I. Martínez-Castro, J. Sanz, J. Sep. Sci. 26 (2003) 793.
- [35] I. Jerković, J. Mastelić, Z. Marijanović, Chem. Biodivers. 3 (2006) 1307.
- [36] R. Peña, J. Barciela, C. Herrero, S. Garcia-Martín, J. Sep. Sci. 27 (2004) 1540.
- [37] J.S. Câmara, M.A. Alves, J.C. Marques, Anal. Chim. Acta 555 (2006) 191.
- [38] C.Y. Rowland, A.J. Blackman, B.R. D’Arcy, G.B. Rintoul, J. Agric. Food Chem. 43 (1995) 753.
- [39] L. Piasenzotto, L. Gracco, L. Conte, J. Sci. Food Agric. 83 (2003) 1037.

Chemometric analysis of the water purification process data

I. Stanimirova^a, M. Połowniak^b, R. Skorek^b, A. Kita^b,
E. John^b, F. Buhl^b, B. Walczak^{a,*}

^a Department of Chemometrics, Institute of Chemistry, The University of Silesia, 9 Szkolna Street, 40-006 Katowice, Poland

^b Department of Analytical Chemistry, Institute of Chemistry, The University of Silesia, 9 Szkolna Street, 40-006 Katowice, Poland

Received 2 February 2007; received in revised form 20 May 2007; accepted 24 May 2007

Available online 2 June 2007

Abstract

The aim of this work was to show usefulness of chemometric analysis in processing of the data describing production of drinking water in the Silesian region of Poland. Water samples have been collected within the period of 1 year and the quality of water was characterized by a number of physical, chemical and microbiological parameters. Principal component analysis (PCA) and STATIS (Structuration des Tableaux A Trois Indices de la Statistique) were employed to obtain the knowledge about the complete water treatment process. PCA makes it possible to uncover seasonal changes influencing the water treatment process. In particular, it was found out that the salt content, hardness and conductivity of water tend to obtain higher levels in winter rather than in summer, and the relatively lower acidity is also to be expected in winter. The sensory quality of water is considerably improved over the consecutive purification steps. Complementary information about the individual technological units of the process is gained with the STATIS approach. The obtained results show that the water produced by the two independent filtering branches of the water plant is of similar quality and the prescribed quality characteristics of drinking water are fulfilled.

© 2007 Elsevier B.V. All rights reserved.

Keywords: STATIS; PCA; Drinking water; Quality control

1. Introduction

Nowadays, the quality control of drinking water is of major concern. The Silesian region situated in the urban area of South Poland is supplied with drinking water by the water treatment plants of the Upper Silesian Waterworks. This company is one of the biggest in Europe and embraces several surface and deep water treatment plants. It has a capacity of an average combined process flow of 1.5 million m³ day⁻¹. Up to date, over 0.5 million m³ day⁻¹ of drinking water are redistributed among the three and a half million inhabitants of the Upper Silesian agglomeration. In order to meet the required quality specifications, raw water passes through several filtering systems before reaching the municipal pipeline system of 1030 km length. The natural water sources for the Goczałkowice surface water plant are the Vistula and the Sola rivers collected by the Goczałkowice and Czaniec reservoirs, respectively. Water from each

source flows through an individual technological system of general treatment. Then the parallel purification systems merge into one that utilizes secondary ozonation, granular activated carbon filtration and chlorination as a disinfection process. The two parallel systems of water filtering allow periodical conservation and backflushing of the filters of one of the two systems, if necessary, without breaking the purification process and water supply [1].

The aim of this work is to obtain more detailed information about the global changes of the monitored physico-chemical parameters and the levels of inorganic elements at different water treatment units. Moreover, it is important to trace a possible seasonal impact upon the filtering process and consequently, upon the quality of the final product. It is also essential to confirm that the water passing through the two independent filtering branches is of comparable quality.

In order to meet the aims of this study, water samples were collected at different steps of the technological process twice per month within the 1-year period (24.10.2005–11.09.2006) and a comprehensive analysis of the various different water

* Corresponding author. Tel.: +48 32 359 2115; fax: +48 32 259 9978.
E-mail address: beata@us.edu.pl (B. Walczak).

quality parameters was performed, like assessing the levels of the main inorganic components (F^- , Cl^- , NO_3^- , SO_4^{2-} , Na^+ , K^+), trace elements (Al, Cu, Ba, Fe, Mn, Zn, Ca, Mg, Li), turbidity, color, hardness, biochemical oxygen demand, conductivity, pH, quantity of dissolved oxygen, alkalinity, acidity and the quantity of total solids. Although each step of the process is controlled at the water plant separately, this study aims to produce a global picture of the process and the factors that influence it. Two chemometric techniques are going to be employed to analyze the collected analytical data. These are the classical principal component analysis, PCA [2], and a less popular technique known as STATIS (the acronym holds for Structuration des Tableaux A Trois Indices de la Statistique) [3,4]. PCA is one of the most often applied dimensionality reduction techniques used for data compression and visualization. It can reveal similarity among the water samples collected at each filtering step and the relationship among the measured parameters. However, classical PCA can be applied to the two-way data tables only, for instance to the data organized as (time of sampling $\times$ filtering steps) $\times$ parameters. Complementary information can be obtained exploring the three-way data structure with the STATIS approach, i.e., the data that are arranged as, e.g., filtering steps $\times$ time of sampling $\times$ parameters. Application of STATIS to such type of the data is interesting, because the method allows uncovering and relatively easy interpretation of complex relationships among the parameters measured at the consecutive filtering steps, what often cannot be achieved with the conventional two-way methods.

2. Experimental

2.1. Stages in water purification

The first technological system of the Goczałkowice water plant, called Go-cza I, processes water of the Goczałkowice reservoir located on the Vistula river. It utilizes pre-ozonation (Fig. 1, step 1), coagulation with alum ($Al_2(SO_4)_3$) (step 3), and the flocculation and sedimentation units. After a rapid sand filtration (step 4), water flows through the pumps (step 7) and is mixed with the water passing through the second filtering system. The second filtering system, Go-cza II, uses raw water from both the Goczałkowice and the Czaniec reservoir. Technologically, it consists of the analogous water treatment units, i.e., of pre-ozonation (step 2), coagulation with $Al_2(SO_4)_3$ at step 5 and the rapid sand filtration of water (step 6). However, a more effective, advanced and compact clarifier/flocculator unit (Degremont) is employed for the coagulation process. The consecutive step is a common secondary ozonation (step 8). Then the water passes through the granular activated carbon filtration unit (step 9) and finally, the process of chlorination (step 10) takes place. Eventually, the water enters the municipal pipeline system [5]. The consecutive steps of water filtering are schematically given in Fig. 1.

2.2. Sampling and analysis

All water samples were collected directly at each of the 10 filtering units schematically presented in Fig. 1. They were stored

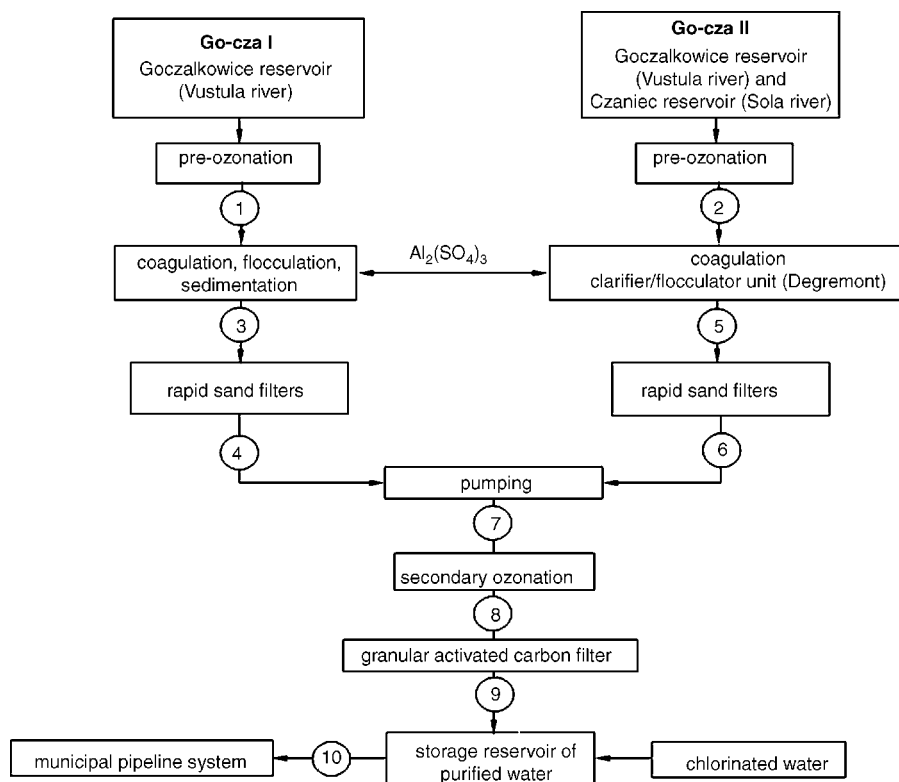


Fig. 1. Flowchart of the stages in water treatment at the Goczałkowice water plant.

in plastic bottles for an easy and secure transportation to the laboratory [6]. The sampling campaign took place twice per month within the 1-year period (24.10.2005, 7.11.2005, 21.11.2005, 5.12.2005, 19.12.2005, 4.01.2006, 16.01.2006, 30.01.2006, 13.02.2006, 27.02.2006, 13.03.2006, 27.03.2006, 10.04.2006, 24.04.2006, 8.05.2006, 22.05.2006, 5.05.2006, 19.06.2006, 3.07.2006, 17.07.2006, 7.08.2006, 28.08.2006, 11.09.2006). Due to the renovation process, the coagulation and rapid sand filtration units at Go-cza I (see Fig. 1) were closed within the period from 27.03.2006 till 5.06.2006 and for this reason the respective measurements were not performed.

The quality of drinking water was characterized by 29 chemical, physical and microbiological parameters such as dissolved oxygen (DOXY), biochemical oxygen demand (BOD₅), the percentage of saturated oxygen (SOXY), total hardness (HARD), chemical oxygen demand (COD), alkalinity (ALK), acidity (AC), the content of total solids (TS), the content of non-volatile (NVS) and volatile (VS) substances, temperature (TEMP), pH, conductivity (COND), turbidity (TUR), color (COL), anions (F⁻, Cl⁻, NO₃⁻, SO₄²⁻), cations (Na⁺, K⁺) and metals (Al, Cu, Ba, Fe, Mn, Zn, Ca, Mg, Li). The employed analytical procedures were performed according to the current Polish standards issued by the Polish Committee for Standardization [7–21]. In general, the ion chromatographic method (IC) was used for determination of the cations and anions. The relative standard deviation of the IC method is less than 5% for all the ions. All metals were analysed by the inductively coupled plasma optical emission spectrometry (ICP-OES). The standard calibration procedure using five standards was adopted and all the measurements were done in triplicate. The relative standard deviation of the technique was 3–5% for all the elements. Several parameters like saturated oxygen, pH, turbidity, color, conductivity and temperature were measured directly at the sampling place. The uncertainty of the measurements is reported in Refs. [6,14,15,17,20]. Titration methods were adopted for determination of the dissolved oxygen, hardness, biochemical oxygen demand, chemical oxygen demand, acidity and alkalinity with the accuracy ± 0.05 ml determined in replicates. The contents of the total solids and of the non-volatile and volatile substances were determined by use of the routine gravimetric analytical method. The measurements were performed in replicates with the accuracy $\pm 1.10^{-5}$ g.

3. Theory

3.1. Principal component analysis

Principal component analysis (PCA) is usually chosen to uncover information hidden in the complex data sets [2]. For the studied data set, it allows to reveal the relationship between the water samples collected over the investigated time span and the parameters measured for the consecutive filtering units, and also it can elucidate the relationships among these parameters. PCA allows projection of the data from a high dimensional space onto a space spanned by a few uncorrelated factors, called principal components (PCs). These principal components are obtained as a weighted sum of the original variables. The weights are

called loadings and show the importance of the parameters in construction of the PCs. Several algorithms can be used to perform PCA like, e.g., the singular value decomposition (SVD) approach [2] and the non-linear iterative partial least squares (NIPALS) algorithm [22]. In recent years, the so-called kernel PCA approach has gained a lot of attention in chemometrics, due to its computational efficiency [23,24].

PCA is a very widely applied method and therefore it will not be further explained in this place.

3.2. STATIS

The acronym STATIS stands for Structuration des Tableaux A Trois Indices de la Statistique [3,4,25], which can be translated as “structuring the three-way data sets in statistics”. As the name suggests, STATIS explores the three-way structure of the data. It highlights similarities among the individual data tables constituting the three-way array. This is done with use of a special weighting scheme. STATIS can be viewed as PCA of the unfolded data, where the variance of each data table constituting the three-way array is weighted. In fact, the weighting scheme is responsible for the three-way character of the method. The weights are used to define the so-called STATIS compromise, as a weighted sum of the individual cross-product matrices [3]. They reflect an “agreement” between each individual data table and the compromise. Including the weights that reflect an agreement between the individual tables and the compromise makes the STATIS compromise robust. The table that agrees with the compromise the least has the weight close to zero. In that way, the influence of atypical observations is diminished.

The weights are determined as the normalized elements (their sum is equal to one) of the first eigenvector of the so-called RV matrix, which holds the RV coefficients. The RV coefficient [26] is a measure of closeness between any two cross-product matrices. It is positive and its value varies in the range of (0, 1). The two cross-product matrices are more similar, if their respective RV coefficient is closer to one.

STATIS has very good visualization properties. Distribution of objects can be visualized on the plane spanned by the principal components selected after PCA of the compromise matrix and the representation is called the compromise plot. Because of the weighting scheme, another way of data representation can additionally be used. A plot displaying each individual cross-product matrix projected on the compromise plot can be drawn, i.e., the plot that shows the position of each point on the compromise plot as a weighted center of the individual positions of this point. Such plot enables, e.g., an easy interpretation of the possible changes in chemical composition at each step of purification in the water treatment plant.

Several STATIS compromises can be obtained by reorganizing the three-way array in various different ways. If the data have the same dimension for the columns or rows, three different arrangements of the data array are possible, resulting in the three different ways of data analysis with STATIS. Since the STATIS method uses the cross-product matrices, it also allows working with the data that do not have the same dimensions for the columns or rows. In this latter case, the compromise is

obtained for the objects, for which all the data tables have the same dimensions.

4. Results and discussion

For a preliminary inspection of the data structure, principal component analysis (PCA) was applied. For this purpose, the analytical data were organized in a two-way table (sampling time $\times$ filtering steps) $\times$ parameters of the dimension 218×29 . Each object in this table corresponds with the sample collected at a given technological unit at a definite sampling time and is characterized by 29 measured parameters. As it has been mentioned before, the measurements of the coagulation and the rapid sand filtration units were not recorded at Go-cza I within the period from 27.03.2006 till 5.06.2006, due to the ongoing renovation activities. Then the data were preprocessed, using the so-called autoscaling procedure [2]. This data transformation was required, since the variables have been measured in different units and appeared at the various different ranges. Autoscaling is performed by subtracting the corresponding column mean from each data element and dividing it by the corresponding column standard deviation. As a result, the variables have the unitary variances (scale) and consequently, they have the same importance in the PCA analysis. The results obtained from PCA on the autoscaled data are shown in Fig. 2.

The first three principal components explain about 60% of the total data variance (see Fig. 2a). The total data variance is distributed over all PCs, which indicates that the data compression is not very efficient, i.e., there is no high correlation structure in the data. However, the PCA analysis allows drawing some general conclusions about the studied water purification process. The first principal component, PC 1, accounts for 30.48% of the total data variance. It is associated mainly with conductivity (COND), the quantity of the total solids (TS), Ca, Cl^- , hardness (HARD), the quantity of the non-volatile solids (NVS) and to a lesser degree with Na^+ , F^- and Ba (see Fig. 2b). Therefore, this factor reflects the salt content in the water samples and can be conditionally called the ‘salt’ factor. The second principal component, PC 2 (explaining 20.18% of the total data variance), contrasts the two groups of the parameters: color (COL), turbidity (TUR), Fe, chemical oxygen demand (COD), pH and BOD_5 with the negative loading values on the one hand, and the saturated (SOXY), and dissolved oxygen (DOXY) with the positive loading values on the other (see Fig. 2b). These parameters are associated with the smell, taste and appearance of drinking water. The third principal component, PC 3 (see Fig. 2c), is associated with the Mg content (the negative loading value) and the water acidity, AC (the positive loading value).

A diffuse distribution of samples is observed in the PC 1–PC 2 score plot (see Fig. 2d and e). To enable better visualization and easier interpretation of the general effects, the objects are colored (in gray scale). The intensity of the color corresponds with the sampling time (Fig. 2d) and the filtering units (Fig. 2e). Fig. 2d shows a clear seasonal tendency along PC 1. The samples found on the left-hand side of the PC 1–PC 2 plot (light gray) were collected in the winter period (21.11.2005–10.04.2006), whereas those placed on the right-hand side of the plot were col-

lected in summer. Combining information from the PCA score and the loading plots, it comes out that the salt content, hardness and conductivity of the water samples appear higher in winter than in the summer at all the technological units. This is not a striking observation though, since coagulation, flocculation, sedimentation, and the activated carbon and sand filtration are the strongly temperature-dependent processes [24]. As with the temperature decrease the viscosity of water increases, the rate of sedimentation decreases. Therefore, the coagulation and sedimentation processes may be less efficient in winter than in summer. Filtration of water is also slowed down at lower temperatures. Adsorptivity of the activated carbon increases as the temperature decreases. It has been reported that in general, hardness and conductivity of water decrease with an increase of temperature at all water purification steps. The same tendency was observed within the framework of this study.

The change of parameters that reflect the sensory quality of water at the consecutive technological units is the main effect observed along PC 2 (see Fig. 2e). The levels of Fe, COD, pH and BOD_5 decrease, and the color and turbidity diminish in the consecutive filtering steps, while the quantities of both the saturated and dissolved oxygen increase. The quantities of the saturated and dissolved oxygen are essential to interpreting biological and chemical processes going on in water. The higher levels thereof in water are indicative of lower contamination. From Fig. 2e it comes out that the quality of water is considerably better in the checkpoints located after the parallel pre-ozonation units 1 and 2 at Go-cza I and Go-cza II, respectively, than before these units.

An additional seasonal effect related to the acidity of water takes place along PC 3. The samples situated in the lower part of the PC 1–PC 3 plot (see Fig. 2f) have been collected mainly in the autumn and winter (24.10.2005–16.01.2006). They are characterized by a low acidity and high levels of Mg. In general, pH decreases with an increase of the temperature and consequently, the lower acidity of water is expected in winter rather than in summer. However, the presence of carbonates and of the other acidic species in water can modify this effect. In an agreement with this observation, the highest acidity was found for the samples collected in the summer (24.04.06–11.09.06), for which the conductivity, the quantity of the total solids, Ca, Cl^- , hardness, and the quantity of the non-volatile solids are at the low levels (see Fig. 2f). The effect of an increased temperature contributes to the increased concentrations of the organic acids and CO_2 that ultimately might result in an increase of microbiological action, consequently leading to a higher acidity.

The results of PCA demonstrate the main effects influencing production of drinking water. However, PCA does not allow for a detailed description of the processes at the individual technological steps. Such possibility arises, when exploring the three-way structure of the collected analytical data with aid of the STATIS approach. For this purpose, the autoscaled data were organized in a three-way array as parameters $\times$ sampling time $\times$ filtering steps. As it has already been mentioned before, certain data for the coagulation and rapid sand filtration units at Go-cza I are missing, which results in smaller dimensions of the columns in the third and

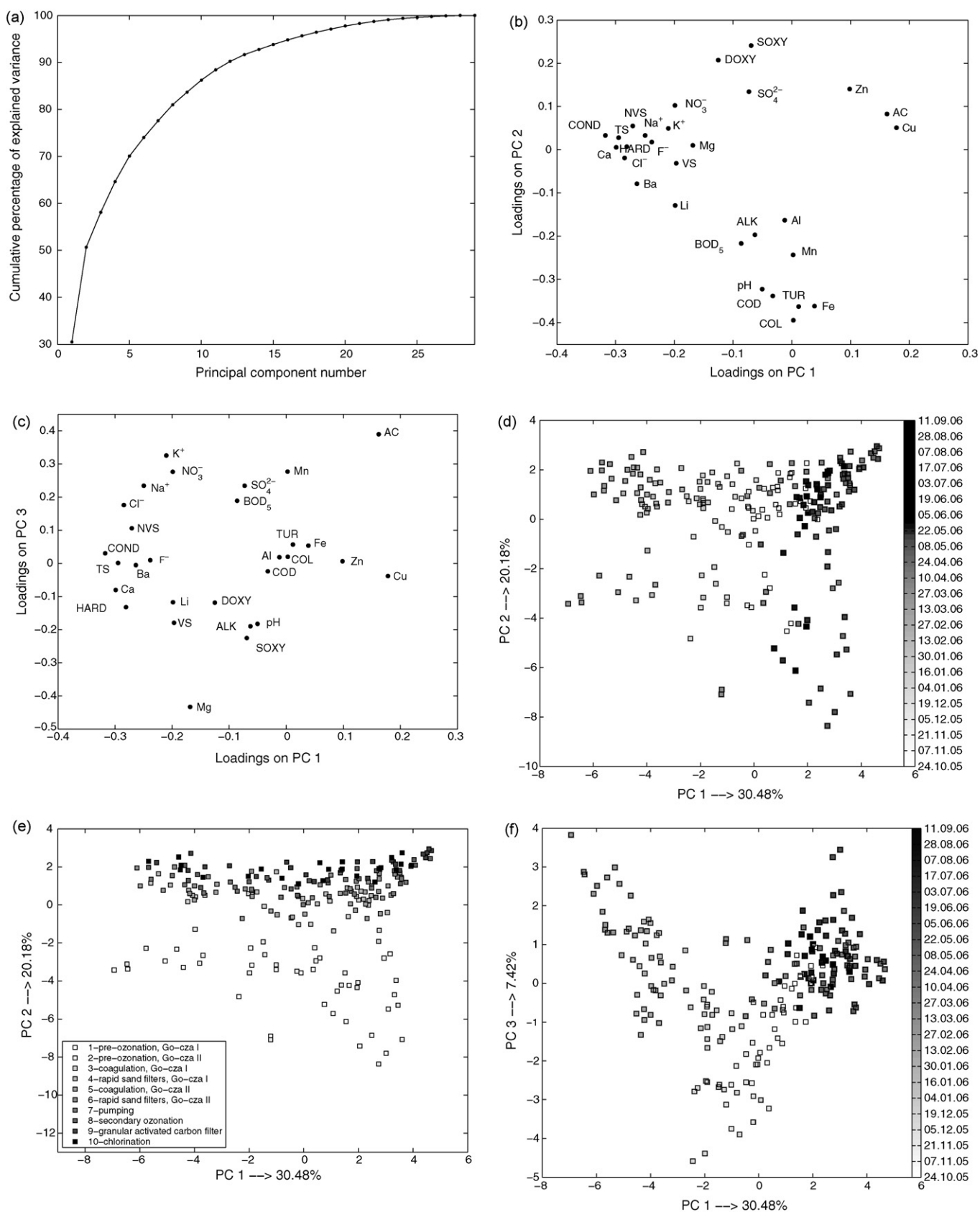


Fig. 2. Results of PCA for the autoscaled data: (a) cumulative percentage of the explained data variance as a function of the principal components number. (b) Projection of parameters on the plane spanned by PC 1 and PC 2. (c) Projection of parameters on the plane spanned by PC 1 and PC 3. (d) Projection of samples on the plane spanned by PC 1 and PC 2. The samples are presented in gray scale according to the sampling time. (e) Projection of samples on the plane spanned by PC 1 and PC 2. The samples are presented in gray scale according to the purification steps. (f) Projection of samples on the plane spanned by PC 1 and PC 3. The samples are presented in gray scale according to the sampling time.

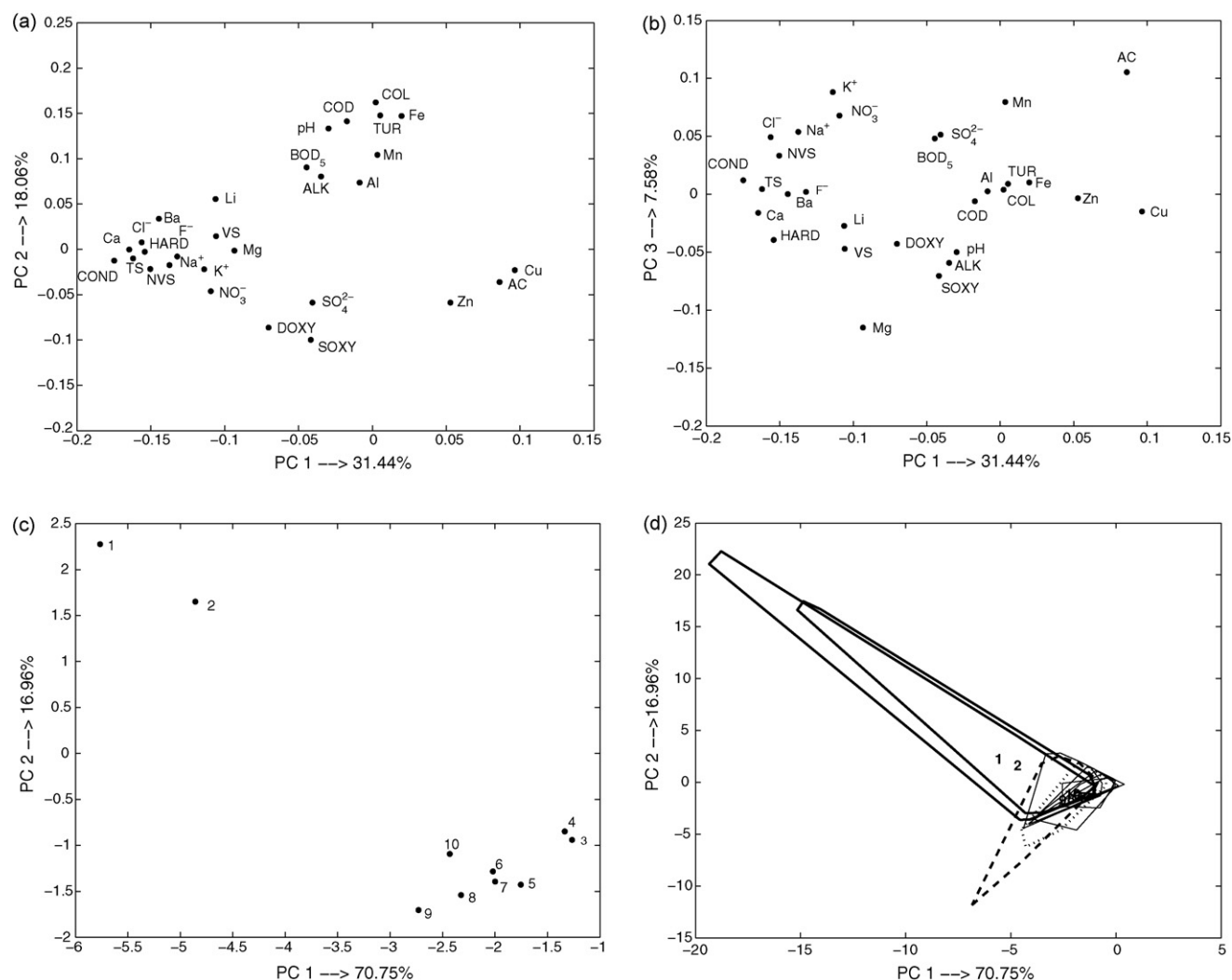


Fig. 3. Results of STATIS for the autoscaled data: (a) the PC 1–PC 2 compromise plot for the parameters, (b) the PC 1–PC 3 compromise plot for the parameters, (c) the PC 1–PC 2 compromise plot for the water purification units and (d) convex hulls of parameters in the PC 1–PC 2 compromise plot for the water purification units.

the fourth table. The PC 1–PC 2 and PC 1–PC 3 compromise plots for the parameters are presented in Fig. 3a and b.

Distributions of the parameters in the plots resemble distributions of the parameters observed on the loading plots of PCA, except for the fact that now the PC 2 axis has a reversed sign. However, the sign change does not influence an overall interpretation. The principal components explain the same relationships between the explanatory parameters.

The compromise plot presented in Fig. 3c shows the changes in the quality of the water passing through the various filtering steps. It is obtained from STATIS using the data array, each table of which is organized as filtering steps $\times$ parameters. Three principal components explain about 87% of the data variance. Assessing the two figures (Fig. 3a and c), it comes out that after the pre-ozonation steps at Go-cza I (step 1) and Go-cza II (step 2), the water still has the high levels of Fe, COD, pH, BOD₅ and turbidity, as well as an intensive color, whereas the quantities of the saturated and dissolved oxygen are at the low

levels. At these two steps, the water is additionally characterized by the high conductivity (COND), the quantity of total solids (TS), Ca, Cl⁻, hardness (HARD) and the quantity of the non-volatile solids (NVS). However, the quality of water considerably improves with the successive filtering units. A decrease of these parameters combined with a quantitative increase of the saturated and dissolved oxygen is observed. This conclusion has already been drawn, when describing the results of PCA, but it is even more apparent, when applying the STATIS approach.

As mentioned in the theory part, each point on the compromise plot is a weighted center of the individual locations of this point. Therefore each filtering step in Fig. 3c can be presented as a weighted center of all the parameters at this step. Thus a convex hull passing through individual parameters can be drawn to better visualize the relationship between the parameters. Fig. 3d shows that the convex hulls for step 1 (solid line), step 2 (solid line), step 8 (dashed line) and step 9 (dotted line) do not considerably overlap, which points out to certain global changes of

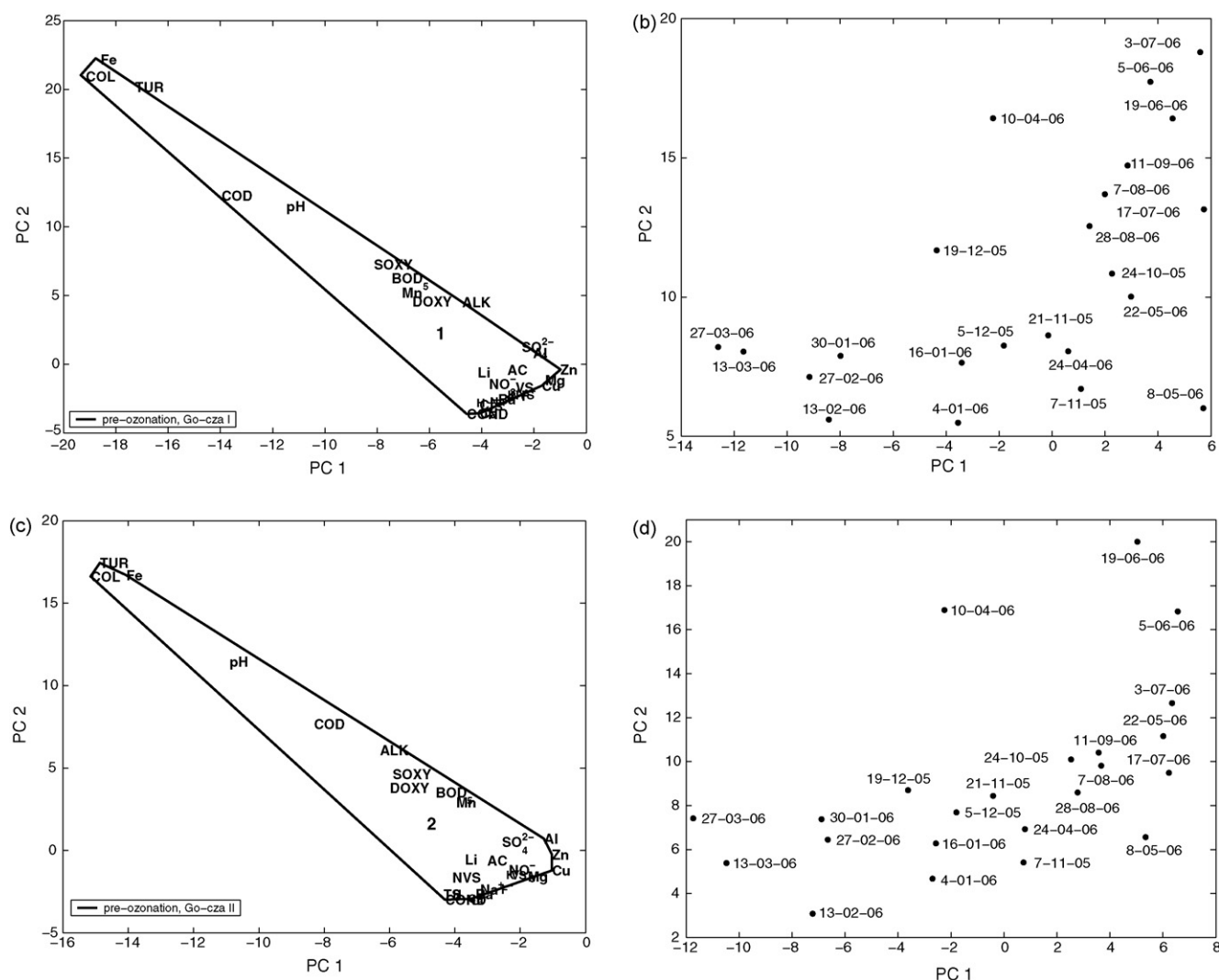


Fig. 4. A comparison of pre-ozonation steps at Go-cza I and Go-cza II: (a) a convex hull of parameters on the PC 1–PC 2 compromise plot for the pre-ozonation step at Go-cza I, (b) the PC 1–PC 2 plot of sampling times for the pre-ozonation step at Go-cza I, (c) a convex hull of parameters in the PC 1–PC 2 compromise plot for pre-ozonation step at Go-cza II and (d) the PC 1–PC 2 plot of sampling times for the pre-ozonation step at Go-cza II.

the parameters at these purification steps. However, such a complex plot is not easily legible and therefore interpretation will be continued, using separate plots. Because of the difference in sampling times, the plot describing the time trends at each filtering unit can also be constructed.

Let us consider filtering steps 1 and 2. Fig. 4 shows distribution of the parameters (Fig. 4a and c) and time (Fig. 4b and d).

In Fig. 4a, projections of the parameters Fe, color intensity, turbidity, COD and pH are placed far away from the compromise point along PC 1 and PC 2. They have the high absolute score values. Combining information about the distribution of the parameters and the time in Fig. 4a and b, it comes out that Fe, color intensity, turbidity, COD and pH are at the high levels within the whole sampling period. However, the highest levels of these parameters were found on 3.07.06, 5.06.06, 19.06.06 and 10.04.06. A similar conclusion can also be drawn from Fig. 4c and d.

A comparison of water quality after the coagulation process at Go-cza I (step 3) and Go-cza II (step 5) is made, based on Fig. 5a and c.

Fig. 5a shows that conductivity (COND), Cl^- , Ca, hardness and NVS have the high absolute score values on PC 1 and PC 2, while Mn has the high absolute score value on PC 1. The high absolute score value on PC 2 was also found for Al. Fig. 5c describes similar tendency for these parameters, except for Mn, which has a low absolute score value. Taking into the account the distribution of time (see Fig. 5b and d), it can be pointed out that these parameters are at high levels within the whole sampling period. However, the higher levels are characteristic of the winter rather than the summer time. As it was already mentioned before, this is so due to the strong temperature effect on coagulation. The presence of Al in the water is in a sense not surprising, since alum is used as a coagulant. At the same time, such parameters as Fe, color intensity, turbidity, COD and pH are found at the low levels indicating a considerable improvement of the water quality. The

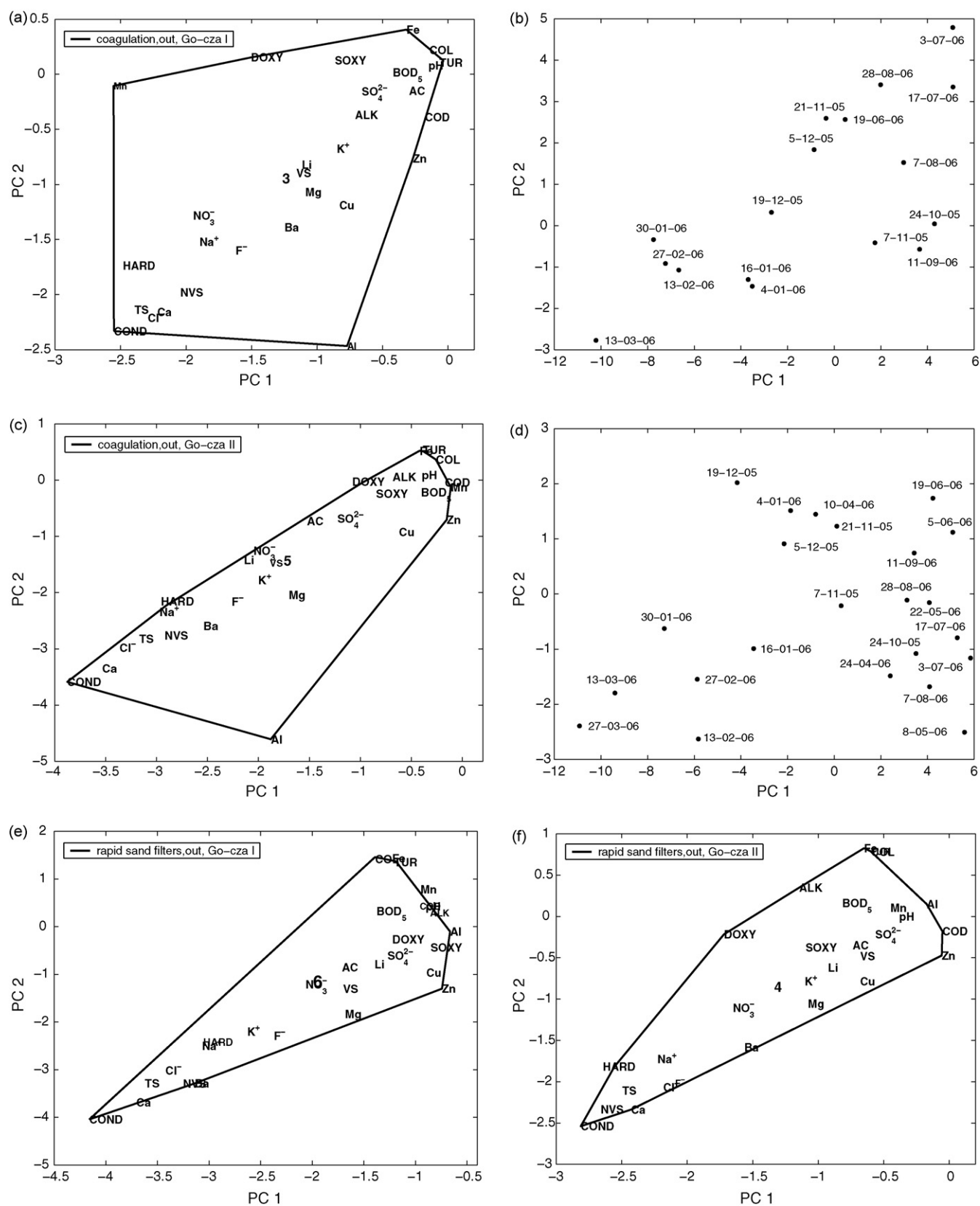


Fig. 5. (a) A convex hull of parameters in the PC 1–PC 2 compromise plot for the coagulation at Go-cza I, (b) the PC 1–PC 2 plot of sampling times for the coagulation at Go-cza I, (c) a convex hull of parameters in the PC 1–PC 2 compromise plot for the coagulation at Go-cza II, (d) the PC 1–PC 2 plot of sampling times for the coagulation at Go-cza II, (e) a convex hull of parameters in the PC 1–PC 2 compromise plot for the rapid sand filtration at Go-cza I and (f) a convex hull of parameters in the PC 1–PC 2 compromise plot for the rapid sand filtration at Go-cza II.

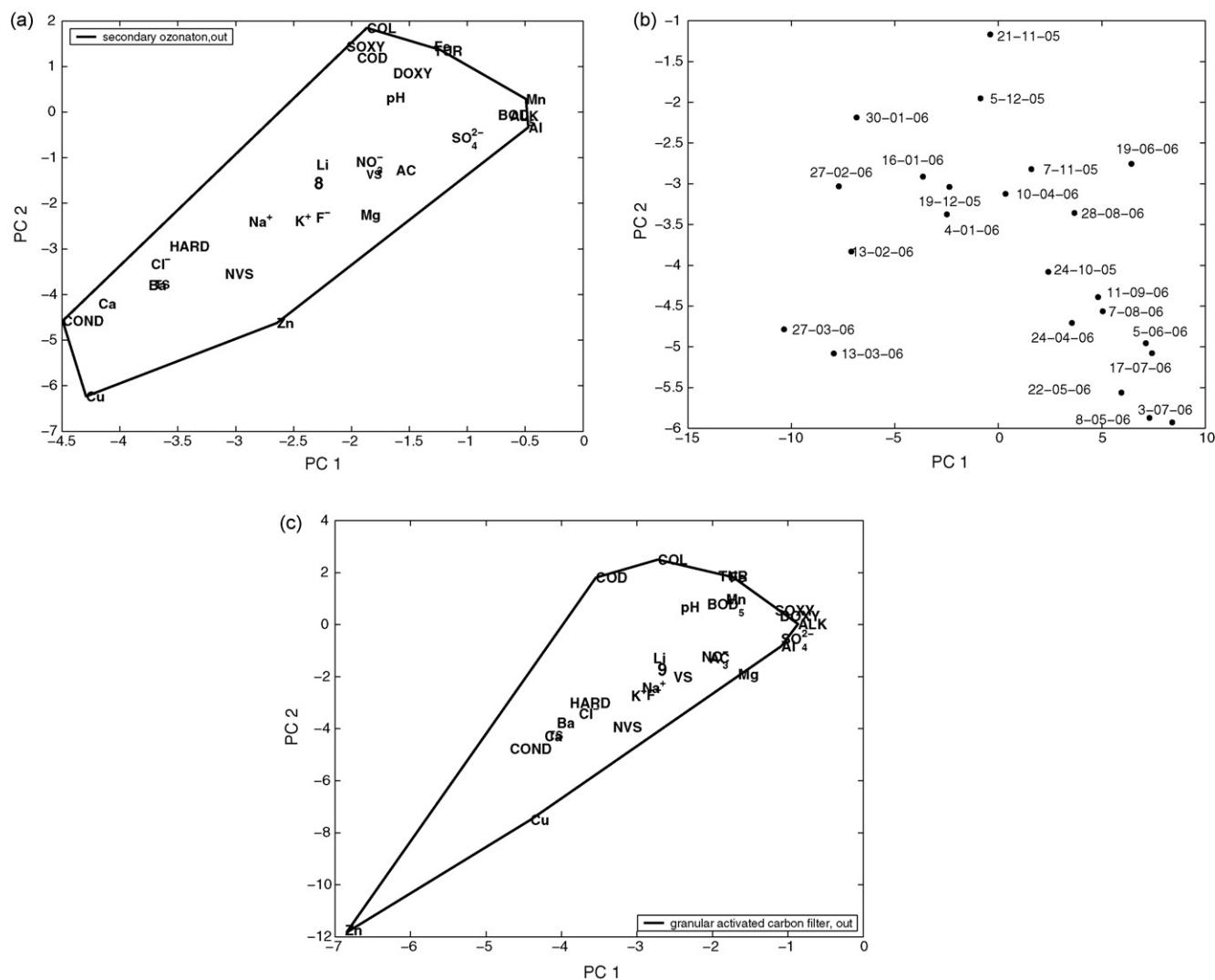


Fig. 6. (a) A convex hull of parameters in the PC 1–PC 2 compromise plot for the secondary ozonation, (b) the PC 1–PC 2 plot of sampling times for the secondary ozonation and (c) a convex hull of parameters in the PC 1–PC 2 compromise plot for the granular activated carbon filtration.

consecutive rapid sand filtration allows a decrease of the Al and Mn concentrations, which is revealed from the projections in Fig. 5e and f. Both figures show the low absolute score values of Al and Mn. For the sake of brevity, figures that describe the tendency that involves the sampling time were omitted. It is important to emphasize that the water from the parallel purifying units is of a similar quality. The quality of the water does not change after the pumping unit (step 7). Surprisingly enough, after the secondary ozonation there appear the increased levels of Cu and Zn. An increase of these concentrations is indicated by their higher score values on PC 1 and PC 2 (see Fig. 6a), when compared with the score values at the preceding water filtering steps (see Fig. 5e and f). This tendency proves true for the entire sampling period. The higher levels are characteristic of the winter rather than the summer period (see Fig. 6b).

After the granular activated carbon filtration, the even higher levels of Zn are observed (see Fig. 6c). The reason might be related to the strong desorption of zinc from the carbon filter surface. A substantial decrease of the Zn concentration is noticed after chlorination of the water. Presently, neither the

national nor the European regulations require the control of the Zn concentration in drinking water. However, the presence of Zn lends the water an undesired taste and appearance. It should be emphasized that the drinking water quality in the Upper Silesian region is fulfilled according to the national and European standards. This means that Cu, Mn, Na, Mg, Fe, Cl^- , chemical oxygen demand and color are found in the aliquots 30 times lower than those indicated by the norms, whereas the levels of Al, F^- , NO_3^- , SO_4^{2-} , hardness, turbidity and conductivity are 10 times lower than those required by the national regulations.

5. Conclusions

The analysis of the collected data describing production of drinking water shows that the major improvement of the water quality is achieved after the coagulation steps 3 and 5 at Go-cza I and Go-cza II, respectively. The two parallel technological units produce water of a comparable quality. However, the Go-cza II unit is more efficient, compared with Go-cza I, due to the faster coagulation process.

The PCA analysis helps to get a general insight in the studied water purification treatment. The STATIS method provides complementary and detailed information by considering the three-way data structure. The effect of the temperature changes, influencing the water treatment process within the 1-year period is also revealed with the PCA and the STATIS approach.

The STATIS approach can further be employed as a tool for the water quality control. However, such a control requires regular and frequent water sampling at a given purification unit. Similar application of STATIS to the process control data was described in Ref. [27].

Acknowledgments

The Authors would like to thank the Upper Silesian Waterworks in Katowice and the Colleagues of the Goczalkowice water treatment plant for the opportunity to collect water samples and for the fruitful discussions.

Dr. R. Michalski from the Institute of Environmental Engineering, the Polish Academy of Science in Zabrze, Poland, is kindly acknowledged for providing the ion chromatographic data.

References

- [1] I. Zimoch, B. Koba, K. Trybulec, in: M. Elektorowicz, M.M. Sozański (Eds.), Proceedings of the XVIIIth National, the VIth International Scientific and Technical Conference on Water Supply and Water Quality, PZiTS, Poznań, Poland, 2004, pp. 743–749.
- [2] B.M.G. Vandeginste, D.L. Massart, L.M.C. Buydens, S. de Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics, Part B, Elsevier, Amsterdam, The Netherlands, 1998.
- [3] Ch. Lavit, Y. Escoufier, R. Sabatier, P. Traissac, Comput. Stat. Data Anal. 18 (1994) 97.
- [4] I. Stanimirova, B. Walczak, D.L. Massart, V. Simeonov, C.A. Saby, E. Di Crescenzo, Chemomet. Intell. Lab. Syst. 73 (2004) 219.
- [5] I. Zimoch, A. Szostak, A. Gawlik, M. Czechowski, in: M. Elektorowicz, M.M. Sozański (Eds.), Water Supply and Water Quality, Proceedings of the XVIIIth National, the VIth International Scientific and Technical Conference, PZiTS, Poznań, Poland, 2004, pp. 751–762.
- [6] Water Quality, Sampling, PN-EN 25667-1, 2003.; Water Quality, Sampling, PN-EN 25667-2, 1999.; Water Quality, Sampling, PN-EN ISO 5667-3, 2002.
- [7] Water Quality, Determination of dissolved fluoride, chloride, nitrite, orthophosphate, bromide, nitrate and sulfate ions using liquid chromatography of ions. Part 1 Method for water with low contamination, PN-ISO 10304-1, 1992.
- [8] Water Quality, Determination of dissolved Li^+ , Na^+ , NH_4^+ , K^+ , Mn^{2+} , Ca^{2+} , Mg^{2+} , Sr^{2+} and Ba^{2+} using ion chromatography method (ISO 14911-1:1998), PN-ISO 14911, 2001.
- [9] Water Quality, Determination of dissolved oxygen—iodometric method, (ISO 5813:1983), PN-EN 25813, PN-ISO 5813, 1997.
- [10] Water and Waste Water, Tests for oxygen demand and of organic carbon content—DETERMINATION of biochemical oxygen demand (BOD_n) without sample dilution, PN-84/C-04578/04, 1984.
- [11] Water and Waste Water, Tests for chemical oxygen demand and of organic carbon content—determination of chemical oxygen demand (COD) by permanganate method, PN-85/C-04578/02, 1985.
- [12] Water Quality, Determination of the sum of calcium and magnesium—EDTA titrimetric method, PN-ISO 6059, 1999.
- [13] Water Quality, Determination of alkalinity. Part 1: Determination of total and composite alkalinity (ISO 9963-1:1994), PN-EN ISO 9963-1, 2001.
- [14] Water and waste water, Test for pH, acidity and alkalinity—determination of mineral and total acidity and alkalinity with titration indicator method, PN-90/C-04540/03, 1990.
- [15] Water and waste water, Determination of solid residue, residue after ignition, ignition loss and dissolved, dissolved mineral and dissolved volatile matter, PN-78/C-04541, 1978.
- [16] Water quality, Determination of turbidity (ISO 7027:1999), PN-EN ISO 7027, 2003.
- [17] Water and waste water, Measurement of color, PN-74/C-04558, 1974.
- [18] Water and waste water, Measurement of temperature, PN-77/C-04584, 1977.
- [19] Water and waste water, Test for pH, acidity and alkalinity—electrometric pH determination of water and waste water with specific conductivity $10 \mu\text{S}/\text{cm}$ and higher, PN-90/C-04540/01, 1990.
- [20] Water quality, Determination of electrical conductivity (ISO 7888:1985), PN-EN 27888, 1999.
- [21] Water quality, Determination of 33 elements by inductively coupled plasma atomic emission spectroscopy, PN-EN ISO 11885, 1996.
- [22] W. Wu, D.L. Massart, S. de Jong, Chemomet. Intell. Lab. Syst. 36 (1997) 165.
- [23] W. Wu, D.L. Massart, S. de Jong, Chemomet. Intell. Lab. Syst. 37 (1997) 271.
- [24] T.R. Camp, D.A. Root, B.V. Bhoota, J. Am. Water Assoc. 60 (1968) 460.
- [25] P. Schlich, in: T. Naes, E. Risvik (Eds.), Multivariate Analysis of Data in Sensory Science, Elsevier, Amsterdam, The Netherlands, 1996, pp. 259–305.
- [26] P. Robert, Y. Escoufier, Appl. Stat. 25 (1976) 257.
- [27] S. Gourvenec, I. Stanimirova, C.-A. Saby, C.Y. Airiau, D.L. Massart, J. Chemomet. 19 (2005) 288.

Determination of polyphenol components in herbal medicines by micellar electrokinetic capillary chromatography with Tween 20

Xiao-Kui Wang, You-Zhao He*, Li-Li Qian

Department of Chemistry, University of Science and Technology of China, Hefei, Anhui 230026, China

Received 15 February 2007; received in revised form 15 May 2007; accepted 16 May 2007

Available online 24 May 2007

Abstract

A simple and convenient method of micellar electrokinetic capillary chromatography (MEKC) using polyoxyethylene sorbitan monolaurate (Tween 20) to form single micelle and methanol as a buffer additive was introduced for the simultaneous determination of five polyphenols, including scopoletin, rutin, esculetin, chlorogenic acid and caffeic acid. A running buffer solution of pH 9.3, 20 mmol/L sodium tetraborate containing 64 mmol/L Tween 20 and 9% (v/v) methanol was adopted in the separation. Because rutin and esculetin were difficult to be separated by capillary zone electrophoresis (CZE) and SDS-based MEKC, Tween 20-based MEKC was adopted and the polyphenols were separated satisfactorily. The proposed method was used to determine the polyphenol components in the herbal medicine of *Cortex fraxini*. The separation mechanism of Tween 20-based MEKC for the polyphenols was discussed preliminarily.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Micellar electrokinetic capillary chromatography; Non-ionic surfactant; Tween 20; Polyphenols; Herbal medicine

1. Introduction

Traditional Chinese medicine has been used to prevent and cure many diseases over millennium. *Cortex fraxini*, called “Qin-pi” in Chinese, is an important natural herb in Chinese medicine, which is adopted primarily to cure diarrhea and dysentery of intense heat type, and lung heat syndrome with cough and dyspnea [1,2]. It was demonstrated that esculetin was an important component in *C. fraxini* and was considered as a quality index for *C. fraxini* [3]. Some analytical methods, such as high performance liquid chromatography (HPLC) [4], thin-layer chromatography (TLC) [5] and capillary electrophoresis (CE) [2,6], were employed in the determination of esculetin.

Micellar electrokinetic capillary chromatography (MEKC), based on the analyte partition between aqueous and micellar phase, viz. pseudo-stationary one, was developed by Terabe et al. [7]. Being suitable for fused-silica capillary, an anionic micelle of sodium dodecyl sulfate (SDS) was used in MEKC frequently [7–14]. Tween 20, a non-ionic surfactant of polyether, was adopted as a buffer additive in CZE [15–18] and micellar

affinity capillary electrophoresis (MACE) [19], and was used to form its micelle in mixed MEKC [16,20,21]. Since esculetin and rutin were difficult to be separated by CE and were only reported by non-aqueous CZE [6], the separation of five polyphenols, including scopoletin, rutin, esculetin, chlorogenic acid and caffeic acid, was investigated by CZE and MEKC with SDS, Tween 20, Tween 80 and their mixtures. In this paper, a Tween 20-based MEKC method was proposed and used in the simultaneous determination of the polyphenols in *C. fraxini* samples. The separation mechanism of the polyphenols was discussed preliminarily.

2. Experimental

2.1. Apparatus and procedure

A capillary electrophoresis analyzer (Model 1229, Institute New Tech. Appl., Beijing, China) detecting at 214 nm and a double-channel chromatography processor (N-2000, Institute Info. Engineering, Zhejiang Univ., Zhejiang, China) were employed throughout the separation. A spectrophotometer (U-3010, Hitachi, Japan) was used for differential spectrophotometry. A fused-silica capillary of 50 μm i.d., 50 cm effective length and 65 cm total length was purchased from Yongnian

* Corresponding author. Tel.: +86 551 3607072; fax: +86 551 3603388.
E-mail address: yzhe@ustc.edu.cn (Y.-Z. He).

Chromatogr. Components Ltd. (Hebei, China). Before the separation, the capillary was washed in the order of 0.1 mol/L HCl (10 min), distilled water (1 min), 0.1 mol/L NaOH (15 min), distilled water (1 min) and a running buffer (15 min) by pressure. Between the runs, the capillary was washed with the running buffer for 2 min by pressure. Standard and sample solutions were introduced with 10 cm height difference for 15 s and the separation voltage was 22 kV.

2.2. Reagents and solutions

All the reagents were of analytical reagent grade. Scopoletin, rutin, esculetin, chlorogenic acid and caffeic acid were obtained from Fluka (USA). One milligram per millilitre stock solutions of the polyphenols were prepared with 80% (v/v) methanol and their mixed calibration solutions were obtained by diluting the stock solutions with the running buffer. Tween 20, Tween 80, SDS, sodium tetraborate, hydrochloric acid, sodium hydroxide, etc. were purchased from Chem. Reagent Co. (Shanghai, China). The herbal samples of *C. fraxini* were obtained from local drugstores.

Required solutions were prepared with tri-distilled water (SZ-3 tri-distilled water system, Huxi Anal. Instr. Factory, Shanghai, China). The running buffer solution was pH 9.3, 20 mmol/L sodium tetraborate containing 64 mmol/L Tween 20 and 9% (v/v) methanol, and its pH value was adjusted with 0.5 mol/L NaOH.

2.3. Sample preparation

The herbal samples were dried at 60 °C for 6 h, powdered and passed through a sieve (mesh 40). 1.250 g treated sample was weighed accurately and extracted with 10 mL 80% (v/v) methanol for 30 min by an ultrasonic bath (35 kHz, 120 W, J & L Ultrasonics, Shanghai, China). After precipitation, the supernatant was filtered with a filter paper and 0.45 µm membrane. The precipitated sample was extracted with 8.0 mL 80% (v/v) methanol and the slurry solution was filtered with the filter paper and 0.45 µm membrane again. Then the filtration residue, filter paper and membrane, etc. were washed with 5.0 mL 80% (v/v) methanol. The collected solution was diluted to 25 mL with 80% (v/v) methanol and preserved in a refrigerator at 4 °C. The analytical solutions were prepared by diluting the sample solutions with the running buffer.

3. Results and discussion

3.1. Influence of Tween 20 and methanol concentration on MEKC separation

Scopoletin, rutin, esculetin, chlorogenic acid and caffeic acid are polyphenol compounds, and their structures are presented in Fig. 1. The separation of five polyphenols was investigated by CZE firstly. A buffer solution of pH 9.3, 20 mmol/L sodium tetraborate was selected by optimizing the buffer conditions of CZE. However, it was found that rutin and esculetin could not be separated by CZE.

To improve the resolution of rutin and esculetin, the polyphenols was separated by MEKC with different surfactant added into the buffer solution mentioned above, including SDS, Tween 20, Tween 80 and their mixtures. According to the experimental results with the optimized conditions in Fig. 2, rutin and esculetin could not be separated by SDS-based MEKC, as shown in Fig. 2A; but be separated partially with mixed micelles of SDS and Tween 20, as shown in Fig. 2B. Finally, it was found that MEKC with Tween 20 could separate rutin and esculetin satisfactorily. Based on the resolution between rutin and esculetin, 64 mmol/L Tween 20 was used in the buffer solution after examining the concentration range of Tween 20 from 21 to 96 mmol/L, as shown in Fig. 2C. Tween 80-based MEKC caused higher noises than others.

Methanol was used as an additive in the separation and could influence the migration time and resolution of the polyphenols. Its concentration was examined from 0 to 11.0% (v/v). The migration time of the polyphenols was prolonged by increasing the methanol concentration because of the decrease of electroosmotic flow (EOF). Especially, the migration time of caffeic acid increased to 28 min with 11.0% (v/v) methanol. Moreover, the resolution of rutin and esculetin increased slightly from 1.0 to 1.2 by increasing the methanol concentration up to 9% (v/v), which may result from the change of the polyphenol solubility. Consequently, 9.0% (v/v) methanol was selected in the subsequent separation.

The influence of applied voltage was examined from 18 to 26 kV, 22 kV was chosen as the running voltage by compromising the migration time and thermal effect.

3.2. Determination of critical micelle concentration of Tween 20 in running buffer

The critical micelle concentration (CMC) of Tween 20 in water is 0.056 mmol/L (25 °C) [23]. However, it can be changed with temperature, solvent and salt concentration, etc. In this work, the CMC value in pH 9.3, 20 mmol/L sodium tetraborate buffer containing 9.0% methanol (v/v) was measured by surface tension – maximal bubble method at 25 °C [24]. The experimental data are illustrated in Fig. 3, in which the CMC was 0.013 mmol/L Tween 20 (25 °C). Since 64 mmol/L Tween 20 was much higher than its CMC, there existed Tween 20 micelles predominantly in the running buffer. The separation mode belonged to MEKC, in which the micelles of the non-ionic surfactant were migrated by EOF. It implied that the polyphenols were separated by the polyphenol partition between Tween 20 micelle and aqueous phase, and the electrophoretic mobility of the polyphenols.

3.3. Interaction between polyphenol and Tween 20

To investigate the interaction and binding ability between the polyphenols and Tween 20 (or tetraborate), ultraviolet-visible differential spectrometry was adopted to measure their equilibrium constants and binding numbers [22].

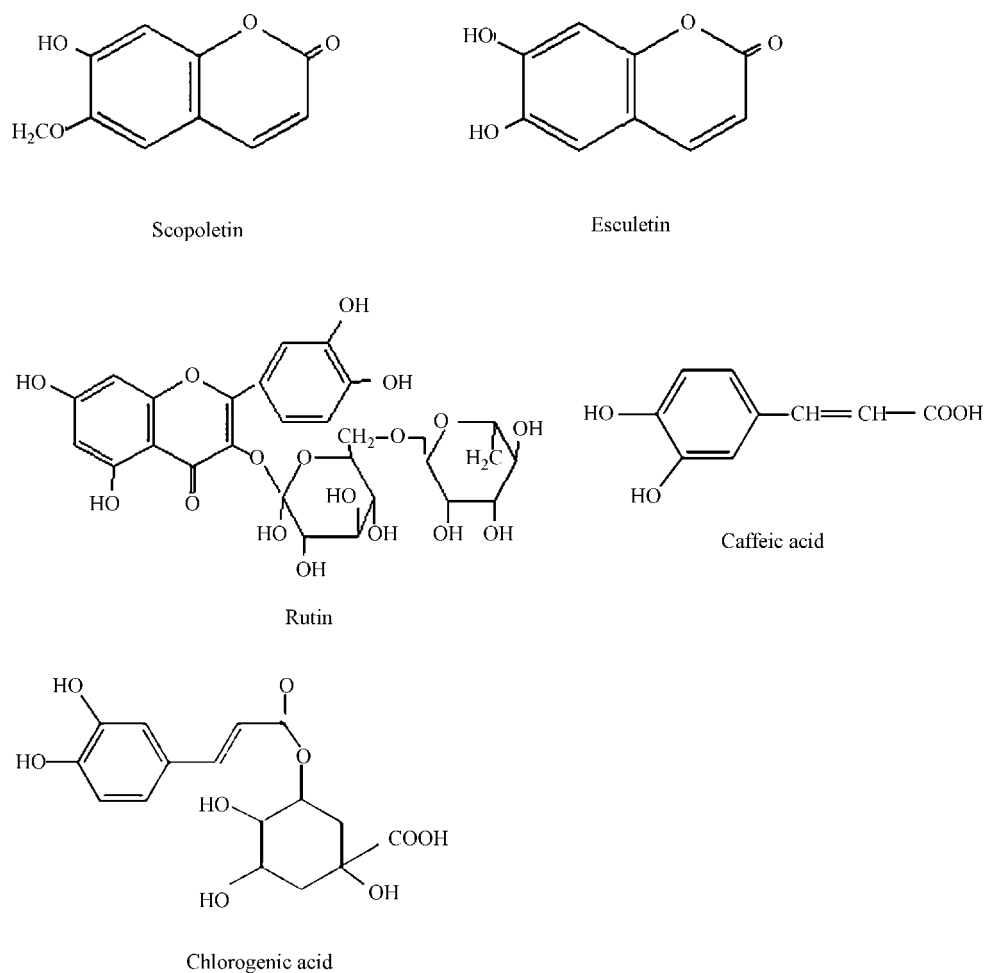


Fig. 1. Molecular structure of five polyphenols.

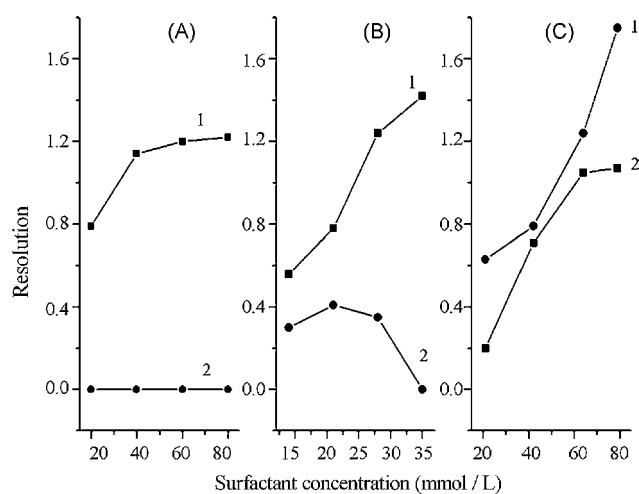


Fig. 2. Influence of surfactant concentration in running buffer on polyphenol resolution: (1) the resolution between scopoletin and rutin and (2) the resolution between rutin and esculetin. Separating conditions: running voltage, 22 kV; detection wavelength, 214 nm; running buffer, pH 9.3; 20 mmol/L sodium tetraborate containing different surfactant. (A) SDS concentration, (B) Tween 20 concentration with 40 mmol/L SDS and (C) Tween 20 concentration.

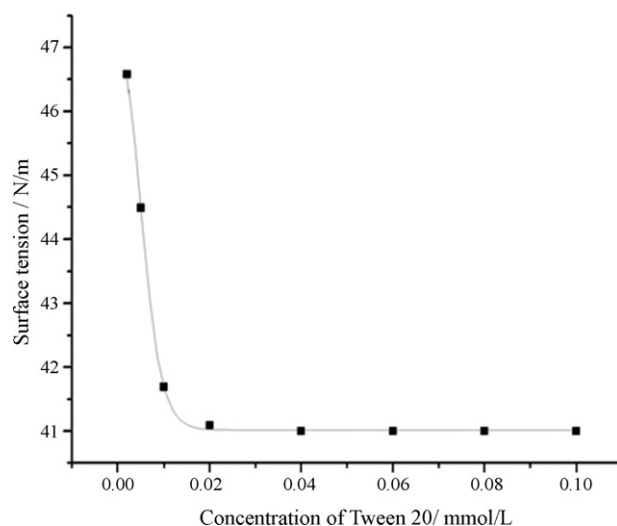


Fig. 3. Effect of Tween 20 concentration in running buffer containing 9.0% methanol (v/v) on surface tension at 25 °C.

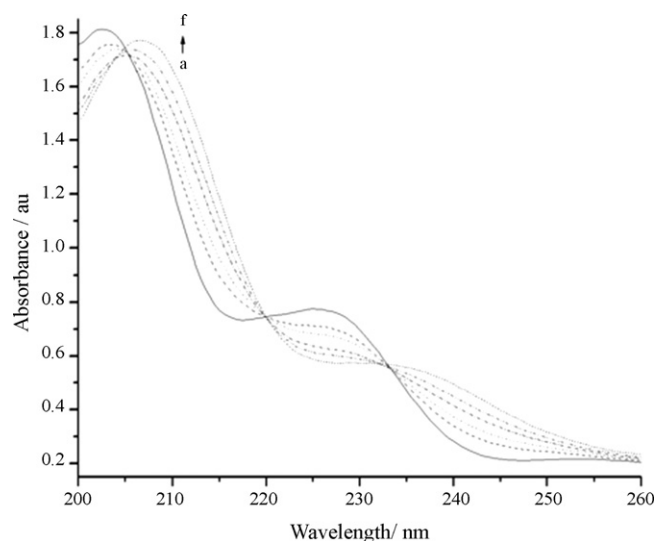


Fig. 4. Absorption spectra of esculetin with different concentration of sodium tetraborate.

The binding reaction can be expressed with the following equation:



where X is polyphenol, Y represents Tween 20 (or tetraborate) and XY_n the product. n is the binding number of Y . The equilibrium constant can be calculated from the following equation:

$$\frac{\Delta A}{b_0^n} = -K \Delta A + Ka_0 \Delta \varepsilon l \quad (2)$$

where ΔA is the differential absorbance between the reacted and original polyphenol; a_0 and b_0 represent total concentration of the polyphenol and Tween 20 (or tetraborate); $\Delta \varepsilon = \varepsilon_2 - \varepsilon_1$, ε_1 and ε_2 are the molar absorption coefficient of X and XY_n , respectively; l represents the length of absorption path.

By estimating a binding number ($n = 1, 2, 3, \dots$) to accord with the linear relation of Eq. (2) and illustrating the data of $\Delta A/b_0^n$ and ΔA measured in the binding reaction of a polyphenol, a linear regressive equation of Eq. (2) and its equilibrium constant K can be obtained. Due to the low resolutions of scopoletin, rutin and esculetin in CZE and MEKC, the separation mechanism was studied on these polyphenols only.

The absorption spectra of scanning wavelength were measured by the spectrophotometer as shown in Figs. 4 and 5. In Fig. 4, the concentration of esculetin was 0.05 mmol/L and those of tetraborate were 0, 0.05, 0.10, 0.15, 0.20 and 0.25 mmol/L (Fig. 4a–f), respectively. The maximal absorption wavelength was shifted to red from 203 ($\varepsilon = 3.00 \times 10^4$ to 3.19×10^4) to 207 nm by increasing the concentration of sodium tetraborate. It indicated that the transition energy ($\pi-\pi^*$) of esculetin was reduced, which may result from the binding effect between esculetin and tetraborate anions. In accordance with the absorption spectra in Fig. 4, the binding number n of tetraborate was not a constant and the equilibrium constant K could not be obtained. The absorption spectra of scopoletin and rutin (not shown) were similar to that of esculetin.

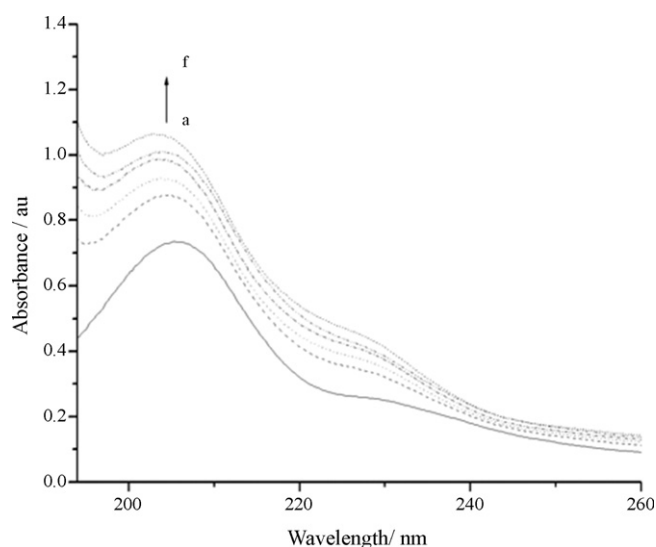


Fig. 5. Absorption spectra of esculetin with different concentration of Tween 20.

In Fig. 5, 0.05 mmol/L esculetin and 20 mmol/L tetraborate were adopted and different Tween 20 concentrations of 0, 0.58, 0.90, 1.12, 1.28 and 1.60 mmol/L were examined (Fig. 5a–f). Although their maximal absorption wavelength at 203 nm did not change a lot by increasing the concentration of Tween 20, the absorbance was enhanced obviously. The absorbance at 203 nm ($\varepsilon = 1.26 \times 10^4$ to 1.84×10^4) represented the transition energy of $\pi-\pi^*$. According to the absorbance of the reacted esculetin subtracted with those of Tween 20, we can estimate a binding number n , establish a linear regressive equation of $\Delta A/b_0^n$ versus ΔA and calculate the equilibrium constant K of the interaction between esculetin and Tween 20. When the binding number n was 2, a linear Eq. (2) could be obtained. In the same way, the binding numbers of Tween 20 with rutin and scopoletin were 2 and 1, respectively. Three linear curves are illustrated in Fig. 6.

The linear regression equations, correlation coefficients, binding numbers and equilibrium constants of the interac-

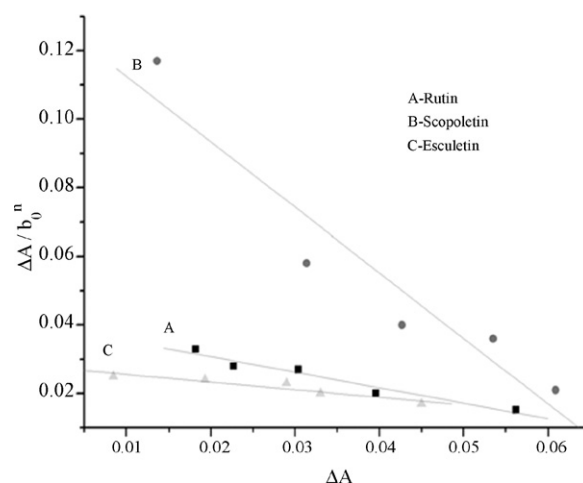


Fig. 6. Linear curves of $\Delta A/b_0^n$ vs. ΔA for rutin (A), scopoletin (B) and esculetin (C). The detection wavelength is 204 nm.

Table 1
Interaction characteristics between polyphenols and Tween 20

Polyphenol	Regressive equation	<i>r</i>	<i>n</i>	<i>K</i> (L/mol) ^{<i>n</i>}
Rutin	$y = 0.040 - 0.453x$	0.9764	2	4.53×10^5
Esculetin	$y = 0.028 - 0.223x$	0.9444	2	2.23×10^5
Scopoletin	$y = 0.122 - 1.911x$	0.9550	1	1.91×10^3

In the regressive equations, *x* is ΔA , *y* is $\Delta A/b_0^n$ and *n* is the binding number in (L/mol)^{*n*}.

tions between three polyphenols and Tween 20 are listed in Table 1.

The experimental results indicate that the polyphenols can interact with Tween 20 and tetraborate in aqueous solution. During the separation, the polyphenols were combined with tetraborate to form anions and move toward anode by electrophoresis. Tween 20 micelles were migrated to cathode by EOF. The peak of rutin moved to the detection window faster than esculetin. It was evident that the separation of rutin and esculetin resulted from the stronger interaction between rutin and Tween 20 than that between esculetin and Tween 20, while the electrophoretic mobilities of rutin and esculetin were similar. Although the interaction between escopoletin and Tween 20 with different binding number (*n* = 1) cannot be directly compared with those between rutin (or esculetin) and Tween 20 (*n* = 2), the separation peak of escopoletin appeared in

front, which resulted from its lowest electrophoretic mobility observed in CZE. So different interaction ability between polyphenols and Tween 20 causes the improvement of the resolution between rutin and esculetin in Tween 20-based MEKC.

3.4. Method evaluation and sample analysis

The optimal separation conditions involved pH 9.3, 20 mmol/L sodium tetraborate buffer solution containing 64 mmol/L Tween 20 and 9.0% (v/v) methanol, hydrodynamic injection of 10 cm height difference for 15 s, and separation voltage of 22 kV. A typical electropherogram of a mixed standard solution is shown in Fig. 7A. The analytical characteristics, including linear regression equations, correlative coefficients, relative standard deviations (R.S.D) and linear concentration ranges are listed in Table 2.

The proposed method of Tween 20-based MEKC was applied to determine five polyphenols in three *C. fraxini* samples under the selected conditions mentioned above. The electropherogram of a sample is shown in Fig. 7B and the analytical results are listed in Table 3. The polyphenol peaks were identified by standard addition method in the sample analysis. The average recoveries of the polyphenols ranged from 95 to 112% given in Table 3, which were determined by adding the

Table 2
Analytical characteristics of polyphenols with Tween 20-based MEKC

Compound	Regressive equation	<i>r</i>	R.S.D. (%)		Concentration range (mg/L)	LOD (mg/L)
			Time (<i>n</i> = 5)	Area (<i>n</i> = 5)		
Scopoletin	$y = 618.0x - 39.1$	0.9972	0.45	1.32	2.7–500	0.9
Rutin	$y = 1308x - 43$	0.9797	0.32	1.25	5.7–400	1.9
Esculetin	$y = 540.6x - 22.2$	0.9936	0.44	1.56	2.3–80	0.8
Chlorogenic acid	$y = 1556x - 75$	0.9943	0.55	1.56	3.2–200	1.0
Caffeic acid	$y = 353.3x - 35.6$	0.9968	0.56	1.54	1.7–200	0.6

In the regressive equations, *x* is the concentration of analytes (mg/L) and *y* is the peak area of analyte response (V s).

Table 3
Analytical results of polyphenols in *Cortex fraxini* samples (*n* = 5)

Sample	Compound	Original (mg/L)	Added (mg/L)	Recovery (%)	R.S.D. (%)	Polyphenol conc. in dry sample (g/100 g)
Sample 1	Scopoletin	425	100	102	2.3	0.85
	Rutin	260	100	96	3.8	0.52
	Esculetin	35	40	98	2.1	0.07
	Chlorogenic acid	15	40	102	3.6	0.03
	Caffeic acid	58	100	105	5.6	0.12
Sample 2	Scopoletin	405	100	106	2.4	0.81
	Rutin	170	100	96	2.7	0.34
	Esculetin	45	40	95	1.4	0.09
	Chlorogenic acid	ND	40	98	3.9	ND
	Caffeic acid	36	100	100	3.4	0.07
Sample 3	Scopoletin	375	100	99	2.2	0.75
	Rutin	280	100	101	3.8	0.56
	Esculetin	65	40	97	2.1	0.12
	Chlorogenic acid	ND	40	112	3.7	ND
	Caffeic acid	51	100	104	4.9	0.10

ND: not detected.

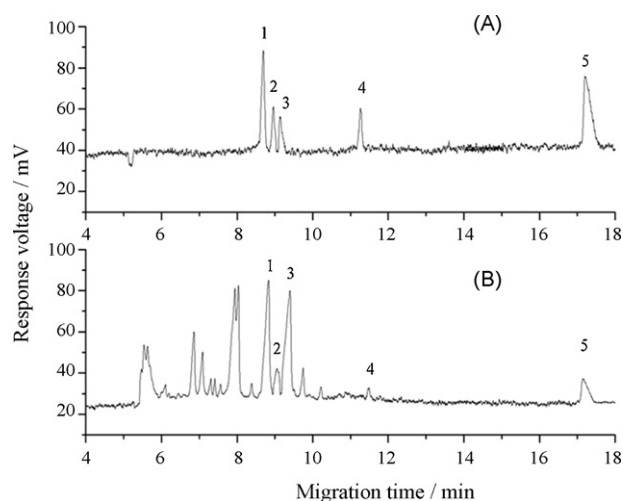


Fig. 7. Electropherograms of mixed standard and real sample. (A) Mixed standard solution and (B) *C. fraxini* sample. Analyte identification: (1) scopoletin (100 mg/L), (2) rutin (100 mg/L), (3) esculetin (40 mg/L), (4) chlorogenic acid (100 mg/L) and (5) caffeic acid (100 mg/L). Separating conditions: running buffer, pH 9.3, 20 mmol/L sodium tetraborate containing 64 mmol/L Tween 20 and 9% (v/v) methanol; running voltage, 22 kV.

standard solutions into powder samples before their extraction. It means that the proposed method is suitable for the simultaneous determination of the polyphenols in the herbal samples.

4. Conclusion

In this paper, a separation method of MEKC with Tween 20 single micelle was proposed. Five polyphenols were separated satisfactorily and determined simultaneously in the herbal medicine of *C. fraxini*. Its separation mechanism was also studied by ultraviolet-visible differential spectrophotometry, which was based on the interaction between polyphenols and Tween 20, and the electrophoretic mobility of the polyphenols.

Acknowledgment

The authors gratefully acknowledge the support of this work by the Natural Science Foundation of China (nos. 20275035 and 20675075).

References

- [1] J.S. Li, Study on the Identification of Chinese Traditional Medicine, Shanghai Science and Technology Press, 1995, p. 316.
- [2] H.Y. Zhang, Q.F. Li, Z.H. Shi, Z.D. Hu, R. Wang, Talanta 52 (2000) 607.
- [3] Pharmacopoeia Committee of People's Republic of China, Pharmacopoeia of People's Republic of China, vol. 1, Chemistry Industry Publisher, 1995, p. 241.
- [4] Z.T. Zhang, Q.G. Liu, Q. He, Z.W. Gao, Chin. J. Anal. Chem. 30 (2002) 327.
- [5] B.S. Yu, X.P. Yan, G.B. Zhen, Y.P. Rao, J. Pharm. Biomed. Anal. 30 (2002) 843.
- [6] C.H. Li, A.J. Chen, X.F. Chen, X.G. Chen, Z.D. Hu, J. Pharm. Biomed. Anal. 39 (2005) 125.
- [7] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
- [8] S. Baskan, A. Daut-özdemiş, K. Günaydın, F.B. Erım, Talanta 71 (2007) 747.
- [9] S.L. Zhao, Y. Song, Y.M. Liu, Talanta 67 (2005) 212.
- [10] M.G. Khaledi, C. Quang, R.S. Sahota, J.K. Strasters, S.C. Smith, in: N. Guzman (Ed.), Capillary Electrophoresis Technology, Part 1, Marcel Dekker, New York, NY, 1993, p. 187.
- [11] L.G. Liu, X.G. Chen, Z. Hu, Talanta 71 (2007) 155.
- [12] X. Zhou, L.Y. Fan, W. Zhang, C.X. Cao, Talanta 71 (2007) 1541.
- [13] A. Balchunas, M. Sepaniak, Anal. Chem. 59 (1987) 1466.
- [14] A. Mohanty, J. Dey, Talanta 71 (2007) 1211.
- [15] A.W. Adamson, Physical Chemistry of Surfaces, 3rd ed., John Wiley & Sons, New York, 1976, pp. 6–80.
- [16] L.H. Liu, X.M. Liu, X.G. Chen, Z.D. Hu, J. Chromatogr. A 1098 (2005) 177.
- [17] S.P. Wang, W.T. Lee, J. Chromatogr. A 987 (2003) 269.
- [18] A. Saccomani, C. Gelfi, H. Wajcman, P.G. Righetti, J. Chromatogr. A 832 (1999) 225.
- [19] Y. Mrestani, R.H.H. Neubert, J. Pharm. Biomed. Anal. 24 (2001) 637.
- [20] Y. Esaka, K. Tanaka, B. Uno, M. Goto, Anal. Chem. 69 (1997) 1332.
- [21] H.H. Steen, B. Inga, T. Jette, J. Pharm. Biomed. Anal. 13 (1995) 687.
- [22] J. Zhou, X.W. He, Anal. Chim. Acta 381 (1999) 85.
- [23] L. Schweitz, L.I. Andersson, S. Nilsson, Analyst 127 (2002) 22.
- [24] L. Suntornsuk, S. Kasemsook, S. Wongyai, Electrophoresis 24 (2003) 1236.

Surface plasmon resonance and electrochemistry characterization of layer-by-layer self-assembled DNA and Zr^{4+} thin films, and their interaction with cytochrome c

Jianlong Wang, Fuan Wang, Zhiai Xu, Yizhe Wang, Shaojun Dong*

*State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry,
Graduate School of the Chinese Academy of Sciences, Changchun Jilin 130022, China*

Received 25 February 2007; received in revised form 17 May 2007; accepted 19 May 2007

Available online 26 May 2007

Abstract

Through electrostatic layer-by-layer (LbL) assembly, negatively charged calf thymus double stranded DNA (CTds-DNA), and positively charged Zr^{4+} ions were alternately deposited on gold substrate modified with chemisorbed cysteamine. Thus-prepared three-dimensional DNA networks were characterized by surface plasmon resonance (SPR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and infrared reflection–absorption spectroscopy (IR-RAS). SPR spectroscopy indicates that the effective thickness of DNA monolayer in the $(\text{DNA}/\text{Zr}^{4+})_1$ bilayer was 1.5 ± 0.1 nm, which corresponds to the surface coverage of 79% of its full packed monolayer. At the same time, a linear increase of film thickness with increasing number of layers was also confirmed by SPR characterizations. The data of XPS and IR-RAS show that Zr^{4+} ions interact with both the phosphate groups and nitrogenous bases of DNA and load into the framework of DNA. Furthermore, the interactions between this composite film and heme protein cytochrome c (Cyt c) were investigated by SPR spectroscopy and electrochemistry. Compared with the adsorption of Cyt c on DNA monolayer, this composite multilayer film can obviously enhance the amount of immobilized Cyt c confirmed by SPR reflectivity–incident angle ($R-\theta$) curves. Cyclic voltammetry (CV) indicates the Cyt c adsorbed on the composite film is electroactive, and the enhancement of peak current in CV indirectly verifies the increase of the amount of immobilized Cyt c.

© 2007 Published by Elsevier B.V.

Keywords: Layer-by-layer; Surface plasmon resonance; Electrochemistry; DNA; Cytochrome c

1. Introduction

The construction of functional and organized ultrathin multilayer films in predetermined ways is an important area of research in biosensor and material science [1]. One promising method for fabricating structured thin films on the surface of solid substrates is the layer-by-layer (LbL) self-assembly technique. This method has been receiving increased attention because of its potential as a simple, yet powerful, approach for constructing building blocks of different compositions into ultrathin multilayer films with controlled thicknesses and controlled molecular substrates on essentially arbitrary solid substrates [2]. In the past decades, this method has been extensively applied to prepare multilayer composite materials, including polymer, both

conductive polymer and usual polymer [3–7] inorganic nanoparticles such as Au [8–10], Ag [11,12], other metal or metal oxide [13–15], and biomolecules including special proteins [16], enzymes [8–10,17,18], viruses [19], and RNA [20,21]. These films have shown a great potential application in composites, electronics, nonlinear optics, catalysis, active biomaterial coatings, and biosensing [2,22–26].

Recently, this LbL self-assembly method has also been applied to DNA assembly. DNA is a natural polymer consisting of three parts: a phosphate acid group, a basic group (adenine (A), guanine (G), cytosine (C), thymine (T)), and a sugar unit [27]. These units form a well-defined linear helix and repetitive structure by hydrogen bonds between basic groups. This special structure causes DNA to be an interesting anionic polyelectrolyte that can be used to make ultrathin films with cationic materials by the LbL technique. Because of the importance of DNA in controlling gene transfection, a lot of work has been done to construct higher ordered structures of DNA films with differ-

* Corresponding author. Tel.: +86 431 5262101; fax: +86 431 5689711.
E-mail address: dongsj@ns.ciac.jl.cn (S. Dong).

ent kinds of molecules through the LbL method, such as Shi et al. have studied the influence of ionic strength of DNA solutions on the interaction between DNA and Polylysine by LbL method [28]. Shang et al. constructed a multilayer DNA composite film by alternately assembled heme protein hemoglobin and DNA [29]. Lang and co-workers observed the interactions of the DNA assemblies with small organic dyes in film states [30]. Zhou et al. reported the alternate assembly of DNA and metal Eu^{3+} ions and studied the interaction between this multilayer film and small molecules [31]. Here, we analyzed the layer-by-layer assembly of DNA and metal Zr^{4+} ions, and evaluated the interaction of this multilayer film with cytochrome c (Cyt c) by electrochemistry and SPR spectroscopy.

As a surface-sensitive spectroscopic technique, SPR has shown the strong ability in the study of the formation process of multilayer film [32–37]. Different from usual characterization methods, SPR spectroscopy not only provides single information on the film growth, but also monitors the formation of multilayer in solution in real-time and detects the effective thickness of each monolayer in multilayer film. Since DNA is a polyanion, whereas Zr^{4+} is a tetravalent metal cation, the electrostatic attraction enabled the alternate assembly of DNA and Zr^{4+} on the surface of SPR substrate modified with cysteamine. Numerous studies have confirmed the strong ionic and coordinative interaction between Zr^{4+} ions and phosphate groups [38,39]. However, these works mainly focus on the assembly of Zr^{4+} ions and low molecular organic phosphonic acids or phosphoric acid itself, the assembly of negatively charged DNA and positively charged Zr^{4+} ions was seldom reported. In this paper, the multilayer films containing DNA and Zr^{4+} were built and the interaction of this composite film with Cyt c were investigated. SPR shows that the adsorption of Cyt c on the composite film is much larger than that of Cyt c on DNA monolayer. The redox peak of Cyt c indicates the Cyt c adsorbed on the composite film are stable and electroactive. Thus, this $(\text{DNA}/\text{Zr}^{4+})_n$ composite film may preserve the activity of biomolecules, and can act as a substrate to immobilize biomolecules for developing biological sensors.

2. Experimental section

2.1. Reagents

All solutions were made with deionized water, which was further purified with a Milli-Q system (Millipore). Calf thymus DNA (CTds-DNA), 2-aminoethanethiol hydrochloride and Cyt c were obtained from Sigma. zirconyl chloride octahydrate ($\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$) and glass microscope slides ($1.8\text{ cm} \times 1.8\text{ cm}$) were obtained from China. The phosphate-buffered saline solution (pH 7.0, 0.01 M) was used as electrolyte for electrochemical experiment. All of the chemicals, unless mentioned otherwise, were of analytical grade and were used as received.

2.2. Assembly of $\text{DNA}/\text{Zr}^{4+}$ film

Multilayer films were assembled on SPR gold film electrodes. A positively charged gold surface was first formed by

immersing the gold substrates in 20 mM aqueous solution of 2-aminoethanethiol hydrochloride for >10 h. Films were grown on cysteamine-modified gold surface by repeated alternate adsorption from aqueous solutions of CT ds-DNA and Zr^{4+} ions. Gold electrodes were first immersed in aqueous solutions of polyanionic CT ds-DNA (0.2 mg/mL, containing 0.01 M NaCl) for 20 min. After being washed with water, the electrodes were then soaked in 2 mM/L Zr^{4+} solutions for 10 min. This cycle was repeated to make $(\text{DNA}/\text{Zr}^{4+})_n$ multilayer films, with immediate washing with pure water after each adsorption step. Unless mentioned, $(\text{DNA}/\text{Zr}^{4+})_7$ multilayer film with DNA as the outer layer was used to adsorb Cyt c.

2.3. In situ SPR measurements

SPR measurements were performed with a home-built electrochemical SPR system [40]. It was based on the Kretschmann configuration to achieve the resonant condition by attenuated total internal reflection spectroscopy (ATR). The SPR-active substrates were prepared by sputtering of gold. In details, the gold films with the thickness of $\sim 50\text{ nm}$ were sputtered on one side of glass slide for the excitation of surface plasmons. An underlayer of chromium (1.5 nm) was used to ensure mechanical stability of the gold film. For further experiments, SPR-active substrate cleaned through piranha solution was pressed onto the base of a half-cylindrical lens (ZK_7 , $n = 1.61$) via an index matching oil ($n = 1.61$). Linearly p -polarized light having a wavelength of 670 nm from a diode laser was directed through the prism onto the gold film. The intensity of the reflected light was measured as a function of the angle of incidence, θ , using a photodiode with a chopper/lock-in amplifier technique. The gold surface of the glass slide was mounted against the Teflon cell with use of a Kalrez O-ring (the apparent electrode area was 0.38 cm^2). The small, single-compartment, three-electrode Teflon cell provided a liquid-tight seal and an electrolyte contact. The Teflon cell allowed for the simultaneous recording of SPR and electrochemical data along with applied voltage to the sample.

2.4. Electrochemical experiments

Electrochemical experiments were carried out with a CHI 800 electrochemical system in a Teflon cell. The Teflon cell was provided with a saturated Ag/AgCl reference electrode and a platinum counter electrode. Electrochemical measurements were all recorded and reported versus the KCl-saturated Ag/AgCl reference electrode. Buffers were purged with highly purified nitrogen for at least 20 min, prior to a series of experiments. All experiments were done at room temperature.

2.5. Measurements of infrared reflection–absorption spectroscopy (IR-RAS)

IR-RAS spectra for DNA and $(\text{DNA}/\text{Zr}^{4+})_n$ film on gold substrate were obtained using a 50° incidence angle in a Bruker IFS 66v/s spectrometer. This system uses an MCT-A detector cooled with liquid nitrogen to collect the reflected light. IR-RAS experiments were run in a vacuum at less than 5 mbar pressure

and referenced to background spectra determined on a similar bare gold substrate. All spectra were collected at a resolution of 4 cm^{-1} over 128 scans in the $2000\text{--}1000\text{ cm}^{-1}$ range.

3. Results and discussion

3.1. Assembly of $(\text{DNA}/\text{Zr}^{4+})_n$ multilayer film

The electrostatic attraction between negatively charged DNA and positively charged Zr^{4+} ions was used as the driving force to build up the 3D DNA multilayer films. The *in situ* R-*t* mode of SPR measurements was used for observing adsorption kinetics of DNA and Zr^{4+} to establish the time for adsorption saturation. When the SPR instrument works at a fixed angle near the θ_{SPR} , the reflected light intensities are varied by any changes in film thickness or indexes of refraction resulted from the adsorption of molecules onto the modified gold surface. Fig. 1 shows *in situ* SPR kinetic curves of DNA and Zr^{4+} adsorption. Upon addition of DNA to Zr^{4+} monolayer on gold surface, the SPR reflectance signals sharply increase during the first 2 min. Two reasons are responsible for this phenomenon. One is the refractive index change caused by solutions, and the other is the high surface density of DNA that was adsorbed on the Zr^{4+} monolayer. And then, a slower change of reflectance is observed as adsorption saturation gradually sets in. After about 20 min, a constant reflectance levels off. On the other hand, the SPR reflectance signals quickly increase during the first 30 s when the Zr^{4+} ions were added to the surface of DNA, and a constant reflectance is achieved after 10 min. The different adsorption behaviors are reasonable, allowing for the fact that the molecular weights of DNA and Zr^{4+} are high and low, respectively. The insert of Fig. 1 shows the SPR R- θ curves *in situ* recorded after various surface modification steps in Fig. 1, the cysteamine-modified, positively charged gold electrode (Curve 0), after 20 min adsorption of CT ds-DNA (Curve 1), and after another 10 min adsorption

of Zr^{4+} ions (Curve 2). All the spectra were recorded in pure water after thoroughly rinsing to move weakly bound polyions or metal ions. The reflectance minimum is right shifted about 0.15° in comparison to the basal surface when the DNA was absorbed on the cysteamine-modified SPR substrate. Upon saturation adsorption of the Zr^{4+} ions to negatively charged DNA surface, another right-shift of $\sim 0.04^\circ$ in the reflectance minimum occurred. An *N*-phase complex Fresnel calculation was used to determine the film thickness, and a refractive index of 1.45 was assumed for DNA layers measured here. This index of refraction is a typical value used previously by others to interpret SPR and ellipsometry data from biomacromolecules [41]. The other refractive index values used for the SPR modeling calculations are 1.61 and 1.33, for ZK_7 lens and water, respectively. At a wavelength of 670 nm, the real part of dielectric constant used for bulk gold film is -0.1372 , and the imaginary part of dielectric constant is 3.7852. The thickness of bulk gold film is 55 nm. Thickness calculated from SPR data are reported as effective thickness at a particular refraction index since they measure an average thickness over the size of the incident beam, and small errors in estimated refraction index can result in relatively large errors in absolute calculated thickness. The value of effective thickness measured by *in situ* SPR R- θ curves for DNA monolayers is $1.5 \pm 0.1\text{ nm}$. This value is slight smaller than those reported by Rusling et al. from QCM frequency, but reasonable [42]. Indeed, the hydrogel-like thin viscoelastic DNA films with a fraction of internally trapped water cause a somewhat larger mass uptake, (and then calculated thickness) measured by QCM as compared to that measured by SPR, because QCM measures hydration water and trapped water [43]. Taking into account double-stranded DNA diameter of 1.9 nm [44], we conclude that the surface coverage of each DNA corresponds to 79% of its full packed monolayer. Fig. 2 shows the changes of SPR R- θ curves after seven successive bilayers of DNA/ Zr^{4+} are adsorbed on SPR substrate. A right shift of 1.71° in the reflectance minimum was observed after the formation of $(\text{DNA}/\text{Zr}^{4+})_7$. By detecting

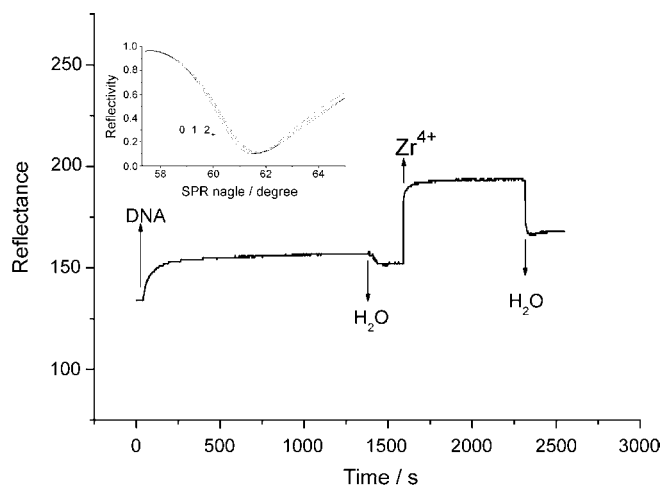


Fig. 1. *In situ* kinetics curve of alternating injections of DNA and Zr^{4+} solution monitored by SPR R-*t* spectroscopy (SPR angle was fixed at 61.5° for collecting SPR R-*t* curve). Inset: The SPR R- θ curves corresponding to aforementioned kinetics change process. (0) The SPR gold film modified with cysteamine. (1) Adsorbed DNA on the SPR gold film modified with cysteamine. (2) Adsorbed Zr^{4+} on the gold film modified with DNA.

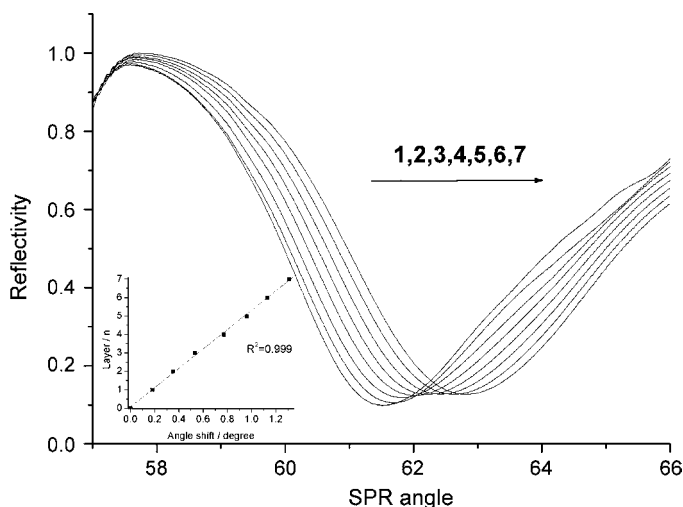


Fig. 2. The changes of SPR R- θ curves after seven successive DNA/ Zr^{4+} bilayer were deposited. Inset: Correlation of changes of SPR reflectance minimum with the number of DNA/ Zr^{4+} bilayers.

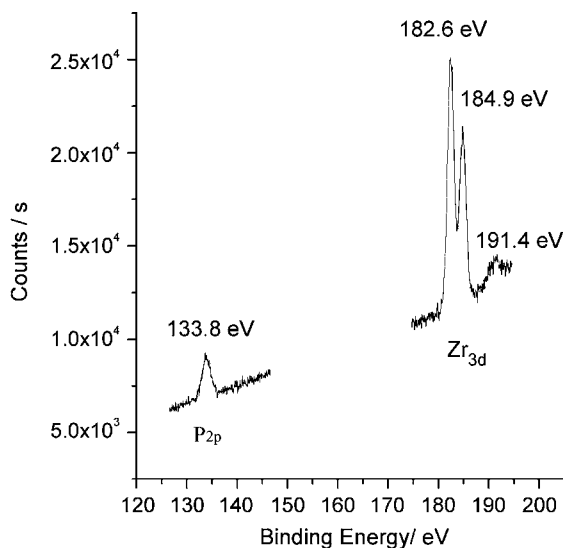


Fig. 3. The data of XPS spectra for DNA and multilayers of $(\text{DNA}/\text{Zr}^{4+})_n$. The peaks at 182.6 eV and 184.9 eV are attributed to $\text{Zr}_{3d_{3/2}}$ and $\text{Zr}_{3d_{5/2}}$; The peak of P_{2p} and P_{2s} are occurred at 133.8 eV and 191.4 eV, respectively.

the change of SPR resonant angle against the number of bilayers, a dependent relationship can be obtained. A good linear relationship was observed from the inset in Fig. 2. This linear relationship indicates that the $(\text{DNA}/\text{Zr}^{4+})_n$ films are uniformly assembled on SPR substrate. Thus, it is possible to precisely control the amount of materials deposited simply by controlling the bilayer numbers.

3.2. Characterization of $(\text{DNA}/\text{Zr}^{4+})_n$ multilayer film

X-ray photoelectron spectroscopy (XPS) and IR-RAS were used to characterize the component of $(\text{DNA}/\text{Zr}^{4+})_n$ films. The P elements present in DNA and Zr element were detected by XPS spectra to confirm the components of multilayer composite films. Fig. 3 shows XPS spectra of the Zr and P elements for a $(\text{DNA}/\text{Zr}^{4+})_n$ composite film. The peaks are attributed to Zr ($3d_{3/2}$ at 182.6 eV; $3d_{5/2}$ at 184.9 eV) and P (2s at 191.4 eV; 2p at 133.8 eV). These data show that this film was made up of DNA and Zr^{4+} ions. Furthermore, the interaction between DNA and Zr^{4+} ions was analyzed by IR-RAS spectra. The data of IR-RAS spectra for DNA and multilayers of $(\text{DNA}/\text{Zr}^{4+})_n$ are shown in Fig. 4. As we can see, the LbL assembly of $(\text{DNA}/\text{Zr}^{4+})_n$ induces the change of absorption bands in the DNA spectrum. Changes are major in the shift of the characteristic absorption bands of the phosphate groups and bases (the absorption bands at 1088, 1248, and 1712 cm^{-1} shift to 1095, 1252, and 1709 cm^{-1} , respectively), indicating that Zr^{4+} interacts with both the phosphate groups and nitrogenous bases of DNA.

3.3. Interactions of $(\text{DNA}/\text{Zr}^{4+})_n$ multilayer film with heme protein Cyt c

Proteins and nucleic acid have relations with each other of intermutation, interdependence and interrestriction. For example, DNA and redox proteins are coexistent in the mito-

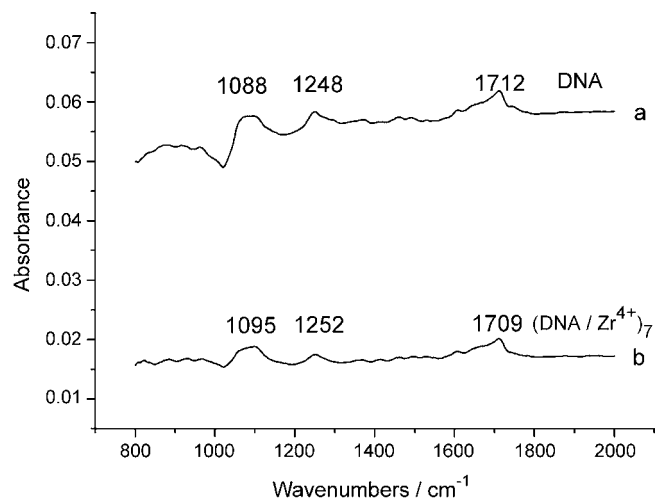


Fig. 4. The data of IR-RAS spectra for DNA and multilayers of $(\text{DNA}/\text{Zr}^{4+})_n$. (a) The absorption bands of the phosphate groups and bases for DNA. (b) The absorption bands of the phosphate groups and bases for multilayers of $(\text{DNA}/\text{Zr}^{4+})_n$.

chondrion. Such proteins are generally located on or in the biological membranes, and play a basic function of electron transport in the electron transport chain by virtue of the redox reaction of iron ion in its iron–porphyrin prosthetic group. Therefore, it is wondrously significant to investigate the electron transport between redox proteins and DNA for apprehending the biological oxidation, energy production, and conversion in respiratory chain, etc. In the present study, we investigated the interactions of both DNA monolayer and $(\text{DNA}/\text{Zr}^{4+})_7$ composite film with redox protein Cyt c by SPR spectroscopy and electrochemistry. Due to the strong electrostatic interaction, Cyt c was adsorbed onto the surface of DNA monolayer. This interaction was confirmed by SPR spectroscopy. Fig. 5a shows SPR R- θ curves for the adsorption of Cyt c to DNA monolayer. The change in SPR angle shift observed upon the adsorption of Cyt c to DNA monolayer is 0.28° , which is close to the surface coverage calculated for a fully packed monolayer of protein by Fresnel equation (taking the molecule of Cyt c as a sphere of 2.5 nm diameter [45]). The refractive index of Cyt c used for Fresnel calculation is 1.45, which is a typical value used to interpret SPR and ellipsometry data from biomacromolecules [41]. The value of angle shift is slightly larger than the value in our previous work [46], but reasonable. According to the results reported by Chen et al. [47], the amount and the redox behavior of Cyt c adsorbed to gold electrodes modified with self-assembled monolayers mainly depends on the self-assembled monolayers. Compared with the monolayer formed by small molecule, DNA adsorbed on gold substrate possesses well-defined space structure and more negative charges, these character resulted in the increase of the adsorption of Cyt c. Interestingly, the adsorption amount of Cyt c will be further enhanced when $(\text{DNA}/\text{Zr}^{4+})_7$ composite film was used as an adsorbed layers. As shown in Fig. 5b, the change in SPR angle shift before and after the adsorption of Cyt c to $\text{DNA}/\text{Zr}^{4+}$ composite film is 1.12° , which is much larger than the adsorption of Cyt c to DNA monolayer. This strong adsorption is attributed to the synergetic effect of DNA

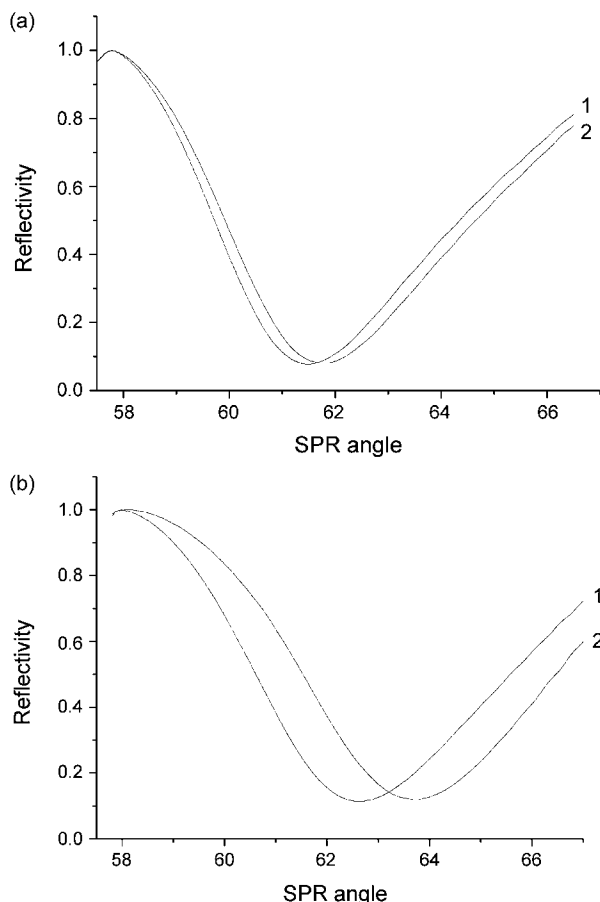


Fig. 5. (a) The SPR R- θ curves for the adsorption of Cyt c on DNA monolayer. (b) The SPR R- θ curves for the adsorption of Cyt c on (DNA/Zr⁴⁺)_n multilayers.

and Zr⁴⁺ ions. Ichinose et al. reported that the ZrO₂-gel layers could adsorb an excess amount of Cyt c [45]. Else, Cyt c can also be encapsulated in the galleries of α -zirconium phosphate under mild conditions, and its thermal stability, peroxidase activity, and the spectral features of the bound proteins were largely preserved in these galleries [48]. These studies show that the material containing Zr element is ideal for immobilizing Cyt c because of the strong affinity between Zr and oxygen group in biomolecule. However, the direct electrochemistry of Cyt c on the surface of these materials is seldom reported. The DNA/Zr⁴⁺ composite film here reported not only enhanced the immobilizing amount of Cyt c, but also come through the direct electrochemistry of Cyt c with DNA as the electron transfer promoter. Fig. 6 compared the cyclic voltammetry (CV)s of Cyt c on a DNA monolayer and a DNA/Zr⁴⁺ composite film. Fig. 6a shows the CVs of Cyt c on a DNA monolayer at different scan rates. The reversible redox couples of Cyt c were observed in the blank buffer solution at different scan rates, the shape of the peaks is nearly symmetric, with equal reduction and oxidation peak height. The inset of Fig. 6a shows that the slope of a plot of I_{pc} (the anodic peak current) versus the scan rate has a linear dependence. This behavior is consistent with a diffusionless electron transfer process at a low scan rate. The peak current and scan rate are related as: $I_p = n^2 F^2 v A \Gamma_o / 4RT$ [49], where Γ_o , v , A , and I_p represent the surface coverage concentration,

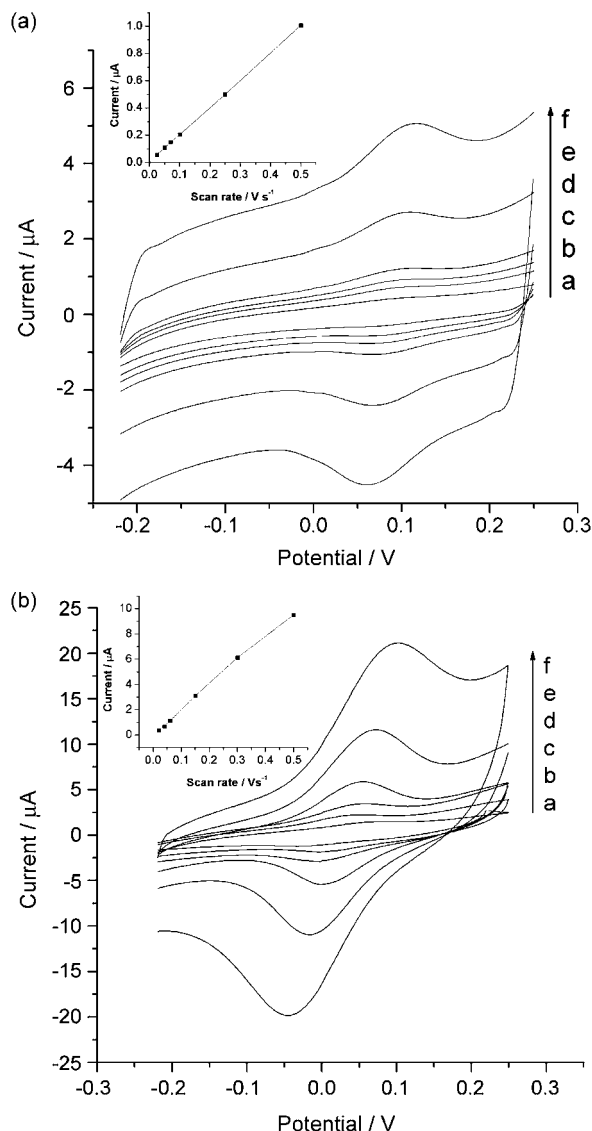


Fig. 6. (a) The CVs of Cyt c on the DNA monolayer. (b) The CVs of Cyt c on the (DNA/Zr⁴⁺)_n composite film.

scan rate, electrode area, and peak current, respectively. Fig. 6b shows the CV curves of Cyt c on a (DNA/Zr⁴⁺)_n composite film at different scan rates. Compared with Fig. 6a, the peak currents both anodic and cathodic peaks are obvious enhanced at the same scan rate. This enhancement mainly comes from the increase of the amount of Cyt c immobilized on the gold electrode. Another change in Fig. 6b is the increase of the reduction–oxidation peak separation. The reduction–oxidation peak separation of 0.145 V is larger than 0.055 V in Fig. 6a (the CV curves with the scan rate of 0.5 V/s are used to calculate the reduction–oxidation peak separation). This may be due to a high transfer resistance of electrons across (DNA/Zr⁴⁺)_n composite film. The inset of Fig. 6b shows the relation between peak current and scan rate. The peak current increases linearly with scan rate. This result suggests thin-layer voltammetry, in which all of the electroactive Fe²⁺ in the film are converted to Fe³⁺ on the reduction scan, with full conversion of Fe³⁺ back to Fe²⁺ on the oxidation scan. It must be pointed out that this thin-layer electrochemistry is differ-

ent from the results reported by McKenzie and Marken [50], but similar to the report by Jin et al. [36] This difference may be due to the electron transport ability of DNA. These results also indicate that the redox process of Cyt c was confined to the surface of the (DNA/Zr⁴⁺)_n/gold film, and the (DNA/Zr⁴⁺)_n composite film is a suitable substrate for immobilizing biomolecules.

4. Conclusions

The assembly of alternating DNA and Zr⁴⁺ multilayer films by electrostatic layer-by-layer adsorption has been studied in this article. Real-time SPR technique monitored layer-by-layer assembly of multilayer films in real time in solution. The results show that the uniform DNA/Zr⁴⁺ multilayer can be fabricated on SPR gold substrate, and the precise control of the thickness of each layer in multilayer film can be obtained. Moreover, the interaction of this (DNA/Zr⁴⁺)_n composite film with Cyt c was investigated by SPR and CV method. SPR results confirm that the amount of Cyt c adsorbed on this composite film is much larger than that on DNA monolayer. The redox peak of Cyt c proved that this composite film could preserve the activity of biomolecules, and this composite film is a good material for immobilizing biomolecules and can be used as a substrate for constructing biological sensors to study biocatalyzed process.

Acknowledgement

This work was supported by special funds for major state basic research of China. (Ns. 2002CB713803, 20427003, 20575064)

References

- [1] R.J. Pei, X.Q. Cui, X.R. Yang, E.K. Wang, *Biomacromolecules* 2 (2001) 463.
- [2] G. Decher, *Science* 277 (1997) 1232.
- [3] E. Kim, Y. Kim, *Mol. Cryst. Liq. Cryst.* 447 (2006) 491.
- [4] B. Bushm, G.H. Xu, C. Carraro, R. Maboudian, *Sensors and Actuators: A-Physical* 126 (2006) 194.
- [5] G.B. Sukhorukov, H. Mohwald, G. Decher, Y.M. Lvov, *Thin Solid Films* 285 (1996) 220.
- [6] D. Laurent, J.B. Schlenoff, *Langmuir* 13 (1997) 1552.
- [7] F. Caruso, K. Niikura, D.N. Furlong, Y. Okahata, *Langmuir* 13 (1997) 3422.
- [8] Y. Bai, S. Zhao, K. Zhang, C.Q. Sun, *Colloids Surf. A* 281 (2006) 105.
- [9] W.W. Yang, J.X. Wang, S. Zhan, Y.Y. Sun, C.Q. Sun, *Electrochem. Commun.* 8 (2006) 665.
- [10] Y. Zhuo, R. Yuan, Y.Q. Chai, Y. Zhang, X.L. Li, N. Wang, et al., *Sens. Actuators: B* 114 (2006) 631.
- [11] P.J.G. Goulet, D.S. Santos, R.A. Alvarez-Puebla, O.N. Oliveira, R.F. Aroca, *Langmuir* 21 (2005) 5576.
- [12] C.H. Lu, S. Bai, D.B. Zhang, L. Huang, J.M. Ma, C.Q. Luo, et al., *Nanotechnology* 14 (2003) 680.
- [13] T. Trung, T.H. Trung, C.S. Ha, *Electrochim. Acta* 51 (2005) 984.
- [14] F. Huguenin, V. Zucolotto, A.J.F. Carvalho, E.R. Gonzalez, O.N. Oliveira, *Chem. Mater.* 17 (2005) 6739.
- [15] Y. Zhou, R.Z. Ma, Y. Ebina, K. Takada, T. Sasaki, *Chem. Mater.* 18 (2006) 1235.
- [16] J.A. He, L. Samuelson, L. Li, J. Kumar, S.K. Tripathy, *Langmuir* 14 (1998) 1674.
- [17] M. Onda, Y. Lvov, K. Ariga, T. Kunitake, *Biotechnol. Bioeng.* 51 (1996) 163.
- [18] F. Caruso, D. Trau, H. Mohwald, R. Renneberg, *Langmuir* 16 (2000) 1485.
- [19] Y. Lvov, H. Flaas, G. Decher, *Langmuir* 10 (1994) 4232.
- [20] C.L. Recksiedler, B.A. Deore, M.S. Freund, *Langmuir* 22 (2006) 2811.
- [21] A. Ishibashi, Y. Yamaguchi, H. Murakami, N. Nakashima, *Chem. Phys. Lett.* 419 (2006) 574.
- [22] H.M. Li, Y.S. Zhou, Y.L. Li, Y.L. Song, H.J. Fang, S.Q. Xiao, et al., *Chem. Phys. Lett.* 383 (2004) 230.
- [23] A. Facchetti, A. Abboto, L. Beverina, M.E. van der Boom, P. Dutta, G. Evmenenko, et al., *Chem. Mater.* 15 (2003) 1064.
- [24] B.Q. Wang, J.F. Rusling, *Anal. Chem.* 75 (2003) 4229.
- [25] F. Caruso, C. Schuler, *Langmuir* 16 (2000) 9595.
- [26] S.Q. Liu, Z. Tang, Z.X. Wang, Z.Q. Peng, E.K. Wang, S.J. Dong, *J. Mater. Chem.* 10 (2000) 2727.
- [27] S.M. Chen, S.V. Chen, *Electrochim. Acta* 48 (2003) 513.
- [28] X.Y. Shi, R.J. Sanedrin, F.M. Zhou, *J. Phys. Chem. B* 106 (2002) 1173.
- [29] L.B. Shang, X.J. Liu, J. Zhong, C.H. Fan, I. Suzuki, G.X. Li, *Chem. Lett.* 32 (2003) 296.
- [30] J. Lang, M.H. Liu, *J. Phys. Chem. B* 103 (1999) 11393.
- [31] Y.L. Zhou, Y.Z. Li, *Langmuir* 20 (2004) 7208.
- [32] V. Zucolotto, A.P.A. Pinto, T. Tumolo, M.L. Moraes, M.S. Baptista, A. Riul, et al., *Biosens. Bioelectron.* 21 (2006) 1320.
- [33] M. Szekeres, A. Szechenyi, K. Stepan, T. Haraszti, I. Dekany, *Colloid Polym. Sci.* 283 (2005) 937.
- [34] M.H. Huang, Y. Shao, X.P. Sun, H.J. Chen, B.F. Liu, S.J. Dong, *Langmuir* 21 (2005) 323.
- [35] J.Y. Liu, Y.D. Jin, A.G. Wu, Z. Li, S.J. Dong, *Electroanalysis* 16 (2004) 1931.
- [36] Y.D. Jin, Y. Shao, S.J. Dong, *Langmuir* 19 (2003) 4771.
- [37] A. Palumbo, C. Pearson, J. Nagel, M.C. Petty, *Sens. Actuators: B* 91 (2003) 291.
- [38] P. OliveraPastor, P. MaireslesTorres, E. RodriguezCastellon, A. Jimenezlopez, T. Cassagneau, D.J. Joner, et al., *Chem. Mater.* 8 (1996) 1758.
- [39] M.M. Fang, D.M. Kaschak, A.C. Sutorik, T.E. Mallouk, *J. Am. Chem. Soc.* 119 (1997) 12184.
- [40] X.F. Kang, Y.D. Jin, G.J. Cheng, S.J. Dong, *Langmuir* 18 (2002) 1713.
- [41] J.M. Brockman, A.G. Frutos, R.M. Corn, *J. Am. Chem. Soc.* 121 (1999) 8044.
- [42] Y.M. Lvov, Z.Q. Lu, J.B. Schenkman, X.L. Zu, J.F. Rusling, *J. Am. Chem. Soc.* 120 (1998) 4073.
- [43] F. Hook, B. Kasemo, T. Nylander, C. Fant, K. Sott, H. Elwing, *Anal. Chem.* 73 (2001) 5796.
- [44] Y. Lvov, G. Decher, G. Sukhorukov, *Macromol.* 26 (1993) 5396.
- [45] I. Ichinose, R. Takaki, K. Kuroiwa, T. Kunitake, *Langmuir* 19 (2003) 3883.
- [46] J.L. Wang, Y. Shao, Y.D. Jin, F.A. Wang, S. Dong, *J. Anal. Chem.* 77 (2005) 5760.
- [47] X.X. Chen, R. Ferrigno, J. Yang, G.M. Whitesides, *Langmuir* 18 (2002) 7009.
- [48] C.V. Kumar, G.L. McLendon, *Chem. Mater.* 9 (1997) 863.
- [49] A.P. Brown, F.C. Anson, *Anal. Chem.* 49 (1977) 1589.
- [50] K.J. McKenzie, F. Marken, *Langmuir* 19 (2003) 4327.

Novel potentiometric membrane sensor based on 6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo[3,1,0]hex-2-ene for detection of strontium (II) ions at trace levels

M.A. Zanjanchi*, M. Arvand, A. Islamnezhad, N.O. Mahmoodi

Department of Chemistry, Faculty of Science, University of Guilan, P.O. Box 1914, Rasht, Iran

Received 28 February 2007; received in revised form 17 May 2007; accepted 20 May 2007

Available online 26 May 2007

Abstract

A new PVC membrane strontium ion-selective electrode has been constructed using 6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo[3,1,0] hex-2-ene (NPDBH) as a neutral ionophore. The electrode was prepared with 7% NPDBH (as ionophore), 57% acetophenone (as plasticizer), 30% PVC and 6% oleic acid (as lipophilic additive). The electrode responds to Sr^{2+} ion with a sensitivity of 28.2 ± 0.5 mV/decade over the range 1.0×10^{-6} – 1.0×10^{-1} mol L $^{-1}$ and in a pH range of 3.0–10.0. The limit of detection was 2.4×10^{-7} mol L $^{-1}$. It has a response time of <20 s and can be used for at least three months without any divergence in potentials. The proposed electrode shows good discrimination of Sr^{2+} ion from several cations. The effect of organic solvents on electrode response was examined. The results show that this electrode can be used in ethanol media up to 15% (v/v) concentration without interference. The isothermal temperature coefficient of this electrode amounted to 0.00019 V/°C. The electrode was found to work well under laboratory conditions. It was successfully applied to the determination of strontium ions in human urine and bone digests.

© 2007 Published by Elsevier B.V.

Keywords: Strontium; Sr^{2+} -selective electrode; PVC membrane; Neutral carrier; ISEs; Isothermal temperature coefficient

1. Introduction

Ion-selective electrodes (ISEs) have been known for the past three decades. They are well established in the most frequent routine application in the fields of environmental, agricultural, industrial and clinical analysis. This is due to their several advantages, such as speed, ease of preparation, simple instrumentation, relatively fast response, wide dynamic range, selectivity and low cost. ISEs are also appropriate for on-site monitoring. Therefore, the analysis by ISEs is a preferred procedure for those ions for which selective electrodes are available. That is the reason why researchers in the field of ISEs attempt to prepare highly selective electrodes for trace determination of the species. The capabilities of ion-selective electrodes have been changed dramatically in recent years so that the lower limit of detection of ISEs has improved by a factor of up to one million and the discrimination factor of interferences from ions by up to one

billion [1]. Unlike most other advances in trace determination, this has not been achieved by significant changes in instrumentation, but instead by the development of new materials [2]. Therefore, it is expected that the range of application of ISEs will increase in the near future coinciding with devising new materials.

Strontium belongs to the alkaline earth elements of group 2A of the periodic table and ranks fifteenth in abundance among the elements. It is widely distributed in the environment, soils, plants and humans tissues. It is used in a variety of optical materials, paints, plastics, bricks, tiles and ferrite magnets. It is used in pyrotechnics and signaling devices due to its brilliant red color formation. The determination of strontium, therefore, is important and common instrumental methods such as inductively coupled plasma(ICP)-AES [3,4] and atomic absorption spectroscopy [5,6] can be used for its measurement. However, these methods are time consuming and are not suitable for large scale monitoring because of high cost and tedious requirement of sample preparation. In these circumstances, a convenient potentiometric determination by ion-selective electrode is beneficial.

* Corresponding author. Tel.: +98 131 3226643; fax: +98 131 3220066.
E-mail address: zanjanchi@guilan.ac.ir (M.A. Zanjanchi).

Over the past few years, several Sr^{2+} -selective electrode based on the use of neutral carrier have been introduced [7–15]. All the reported electrodes generally have relatively acceptable performance for monitoring strontium but they suffer from some limitations. Gupta et al. who have reported several Sr^{2+} -selective electrodes, have improved the performance of the sensor by structural modification of the ionophore used for construction of the electrodes [8,14]. Their electrode based on 4-*tert*-butylcalix [8] arene(III) with the limitation on low pH range (2.4–4.8) and considerable interference by Na^+ and Pb^{2+} [8] was improved using the modified calix [6]arene to develop a better sensor with a wider pH range (3–10) and less interference of the mentioned ions [14]. The working concentration range of the most electrodes is satisfactory in the vicinity of $\approx 2 \times 10^{-5}$ – $1 \times 10^{-1} \text{ mol L}^{-1}$ [7–14]. This range is 4×10^{-6} – $1 \times 10^{-1} \text{ mol L}^{-1}$ in the most recent report using a new PVC membrane electrode based on a macrocyclic tetramide [15]. This electrode has a relatively low response time (<10 s) but suffers from Ca^{2+} interference. Therefore, it is obvious that there is still a need for developing better sensor for Sr^{2+} ion.

In the present work, a new synthetic heteroaromatic compound has been employed as ionophore for construction of a strontium ion-selective electrode. This electrode exhibits higher performance in terms of working concentration range and selectivity compared to those reported so far.

2. Experimental

2.1. Reagents and materials

Reagent grade acetophenone (AP), oleic acid (OA), tetrahydrofuran (THF), dibutyl phthalate (DBP), high-molecular weight PVC (all from Merck), 4-heptanone, 4-nitrobenzaldehyde, ammonium acetate, absolute EtOH, anhydrous gaseous ammonia, ammonium bromide (from Fluka or Aldrich) were used as received. The chloride and nitrate salts of all the cations used (all from Merck) were of the highest purity available and utilized without any further purification. Double distilled deionized water was used throughout.

The ionophore 6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo [3,1,0] hex-2-ene (Fig. 1), used in this work, was synthesized according to our previously reported method with some modification in the described procedure [16]. To prepare the compound, 2-benzoyl-3-(4-nitrophenyl)-aziridine (0.268 g, 1 mmol), NH_4Br (0.1 g, 1 mmol) and 4-heptanone (1 mmol) were dissolved in 6 ml of absolute ethanol and stirred at room temperature. Anhydrous gaseous ammonia is gently blown into reaction mixture for several hours. A color change in the reaction mixture from orange into blue or greenish blue is characteristic for product formation. The reaction mixture was filtered, washed with ethanol, dried in the air and the resulting solid recovered 0.36 g, purified by silica gel column chromatography and recrystallized from ethanol (65.5% yield). The product has mp = 138–140 °C, IR (KBr): 3100, 3080, 2950, 2920, 2880, 1600, 1570, 1510, 1440, 1340, 1240, 1140, 960, 860, 830, 760, 740 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3): 0.93–0.99

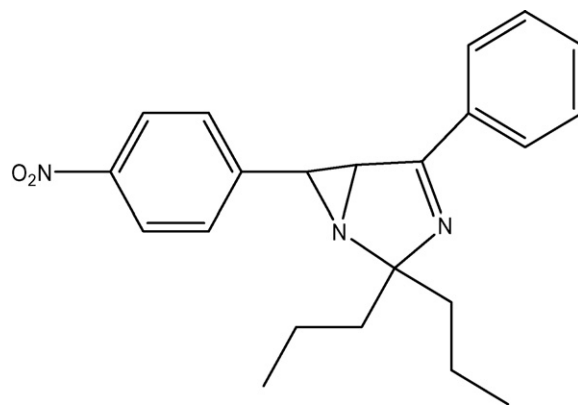


Fig. 1. Structure of 6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo [3,1,0] hex-2-ene (NPDBH) used as ionophore.

(tt, $J = 7$, 7.3 Hz, 6H), 1.28–1.36 (m, 1H), 1.51–1.57 (m, 2H), 1.65–1.7 (m, 2H), 1.88–1.94 (m, 3H), 2.65 (s, 1H), 3.54 (s, 1H), 7.45–7.54 (m, 5H), 7.89 (d, $J = 7.5$ Hz, 2H), 8.23 (d, $J = 8.5$ Hz, 2H).

2.2. Instrumentation

Potentials were measured with a Corning ion analyzer pH/mV meter relative to a double junction saturated calomel electrode (SCE) with the chamber filled with an ammonium nitrate solution at constant temperature (25 ± 0.1 °C). A silver/silver chloride electrode containing a 3 mol L^{-1} solution of KCl was used as the internal reference electrode. The electrode cell assembly of the following type was used:

$\text{Ag}-\text{AgCl} \mid \text{KCl} (3 \text{ mol L}^{-1}) \mid \text{internal solution}, 1.0 \times 10^{-3} \text{ mol L}^{-1} \text{ Sr}^{2+} \mid \text{PVC membrane} \mid \text{test solution} \mid \text{Hg}-\text{Hg}_2\text{Cl}_2, \text{KCl (saturated)}.$

A simultaneous inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Vista-PRO, Australia) was also used for measurement of strontium in real samples.

2.3. Procedures

Membranes containing different PVC/plasticizer ratios were prepared and were studied. The optimum composition found was 30.0 wt.% of powdered PVC, 57 wt.% of plasticizer (AP), 6 wt.% of additive (OA) and 7 wt.% of the corresponding ionophore (NPDBH). These were mixed in 1.5 mL of THF and the solution was mixed well. The solvent was evaporated slowly until an oily concentrated mixture was obtained. A Pyrex tube (4 mm o.d.) was dipped into the mixture for about 15 s so that a transparent membrane of about 0.4 mm thickness was formed. The tube was then pulled out from the solution and kept at room temperature for about 2 h. The tube was then filled with internal solution $1.0 \times 10^{-3} \text{ mol L}^{-1} \text{ Sr}^{2+}$. The electrode was finally conditioned for 24 h by soaking in a $1.0 \times 10^{-2} \text{ mol L}^{-1} \text{ Sr}^{2+}$ solution. The ratios of various ingredients, concentration of equilibrating solution and time of contact were optimized to provide a membrane that was reproducible, noiseless and acquire stable potential.

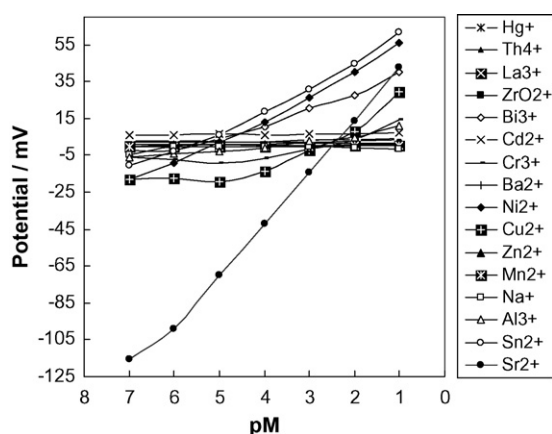


Fig. 2. Potential response of various ion-selective membranes based on NPDBH.

3. Results and discussion

3.1. Effect of membrane composition on the electrode response

The potential responses of various ion-selective electrodes based on NPDBH as an ionophore in the PVC-membranes were obtained for the ions separately and are shown in Fig. 2. Except for the Sr^{2+} ion-selective electrode, in all other cases the slope of the corresponding potential–pM plots is much lower than the expected Nernstian slopes. This means that Sr^{2+} ions can be suitably determined with this ISE with a high sensitivity. It is well known that the sensitivity as well as selectivity and linearity of ISEs are affected not only by the nature of ionophore but also significantly by membrane composition and the nature of solvent mediator and lipophilic additive used [17–20]. The composition of membranes with different proportional ingredients and the performance and characteristics of the electrodes is given in Table 1. Our experience on using several plasticizers including AP and DBP for preparation of the membrane showed that AP plasticizer performs best as it exhibits the widest working concentration range and near–Nernstian slope. The nature of plasticizer influences both the dielectric constant of the membrane and the mobility of ionophore and its interaction with Sr^{2+} ions [21]. The effect of ionophore (NPDBH) amount on the functioning of membrane was also investigated. It was found that

the membrane having a composition as NPDBH:AP:PVC:OA as 7:57:30:6 wt.% exhibits the best results (no. 8 in Table 1). The sensitivity of electrode response increases with increasing ionophore content from 2 to 7%. Further addition of ionophore to 10% will, however, results in diminished response of the electrode, most probably due to some inhomogeneities and possible saturation of the membrane.

3.2. Influence of internal reference solution

The working of membrane electrode in relation to variation of reference solutions was investigated. It was found that, the variation of the concentration of the internal solution from 10^{-1} to 10^{-4} mol L $^{-1}$ of Sr^{2+} solution did not cause any significant difference in potential response except for an expected change in the intercept of the resulting plots. Therefore, a solution of 10^{-3} mol L $^{-1}$ Sr^{2+} would be quite satisfactory as a suitable internal solution for proper functioning of the electrode system. The electrode works with a good slope of 28.2 ± 0.5 mV/decade.

3.3. Effect of pH

The effect of pH on the response of the electrode was studied over the pH range from 1 to 13 at different concentrations (10^{-2} – 10^{-3} mol L $^{-1}$) of Sr^{2+} solution. The pH of solutions was adjusted with either HCl or NaOH solutions. Potentials remain constant at pH range from 3 to 10 (Fig. 3). Below pH 3, the change in the potential is due to cofluxing of hydrogen ions and above pH 10, the variation of potential may be due to formation of some hydroxyl complex of the Sr^{2+} ions in solution.

3.4. Response time, lifetime and reversibility

The response time of the electrodes, tested by measuring the time required to achieve a steady potential (within ± 1 mV), was less than 20 s and was sustained for about 10 min over the linear range of the concentration (Fig. 4). The detection system was very stable, and after a period of two months, calibration sensitivity decreased about 1.1 mV without any considerable change in its linear range. The reproducibility of the slope of calibration graphs was within ± 0.5 mV/decade over a period of two months ($n = 6$).

Table 1
Composition and optimization of membrane ingredients

No.	composition (% w/w)				Slope (mV/decade)	DLR (mol L $^{-1}$)	R^2
	NPDBH	Plasticizer	PVC	Additive (OA)			
1	–	60(AP)	35	5	1.7	1×10^{-1} – 1×10^{-5}	0.9001
2	2	63(AP)	30	5	18.1	1×10^{-1} – 1×10^{-6}	0.9215
3	5	60(AP)	30	5	24.9	1×10^{-1} – 1×10^{-6}	0.9741
4	7	58(AP)	30	5	27.8	1×10^{-1} – 1×10^{-5}	0.9914
5	10	55(AP)	30	5	22.3	1×10^{-1} – 1×10^{-5}	0.9796
6	7	60(AP)	30	3	18.4	1×10^{-1} – 1×10^{-5}	0.9309
7	7	55(AP)	30	8	21.9	1×10^{-1} – 1×10^{-5}	0.9854
8	7	57(AP)	30	6	28.2	1×10^{-1} – 1×10^{-6}	0.9998
9	7	57(DBP)	30	6	24.6	1×10^{-1} – 1×10^{-6}	0.9800

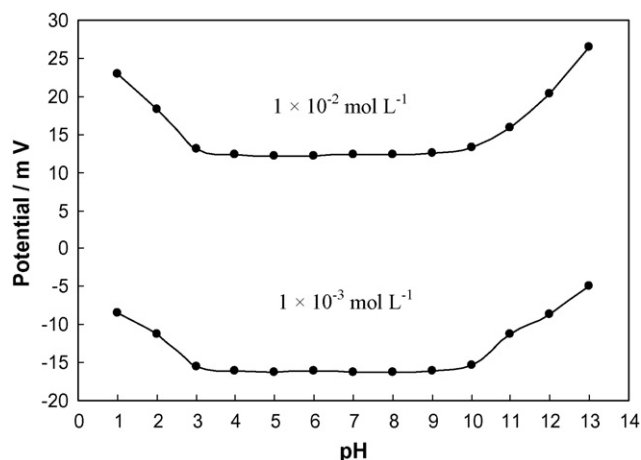


Fig. 3. Effect of pH at 1.0×10^{-2} and 1.0×10^{-3} mol L $^{-1}$ Sr $^{2+}$ solutions on the potential response of membrane no. 8.

Reversibility was studied by performing potential measurement in the sequence of high-to-low sample concentration [22]. Fig. 5 shows that the potentiometric response of the electrode is reversible. The detection limit, taken at the point of intersection of the extrapolated linear segment of the calibration curve, was 2.4×10^{-7} mol L $^{-1}$.

3.5. Selectivity

The influence of interfering ions on the response behavior of ion-selective membrane electrode is usually described in terms of selectivity coefficient ($k_{Sr,j}^{pot}$). The potentiometric selectivity coefficients $k_{Sr,j}^{pot}$ of strontium electrode were evaluated by fixed interference method (FIM) [23–25]. This coefficient is calculated from the following equation

$$k_{Sr,j}^{pot} = a_{Sr}/(a_j)^{Z_{Sr}/Z_j}$$

Where, a_{Sr} is the activity of strontium ion; a_j the activity of interfering ion; Z_{Sr} and Z_j are their respective charges. In this method a fixed concentration of interfering ions (1.0×10^{-2} mol L $^{-1}$) and a varying concentration of Sr $^{2+}$ ion was used. The resulting

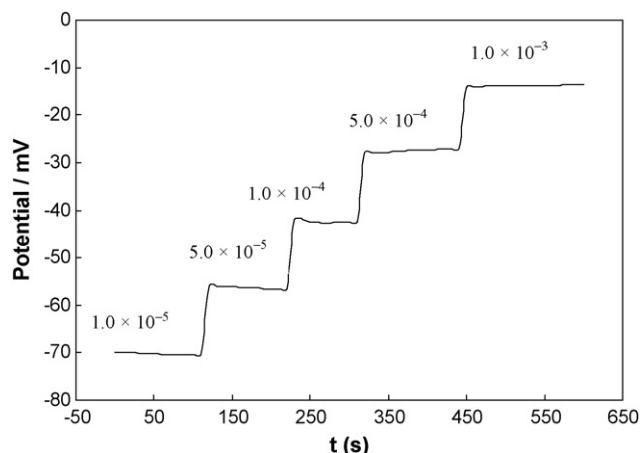


Fig. 4. Dynamic response of membrane electrode for step change in concentration of Sr $^{2+}$.

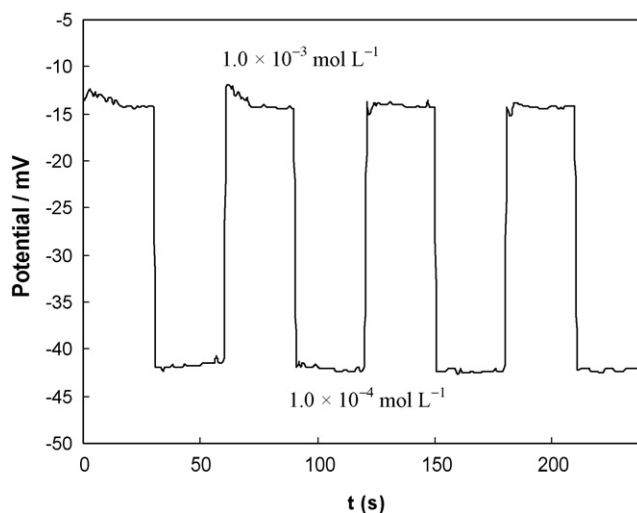


Fig. 5. Dynamic response characteristics of the Sr $^{2+}$ -electrode for several high-to-low sample cycles.

values of the selectivity coefficients are summarized in Table 2. It is evident from the selectivity coefficients data, that the sensor exhibits a high performance for Sr $^{2+}$ ion compared with alkali, alkaline earth, transition and heavy metal ions. Comparison of the selectivity of the most recently described strontium ion selective electrodes (Table 3) with our Sr $^{2+}$ -selective electrode reveals that, the present electrode exhibits a better selectivity, especially in the presence of alkaline and alkaline earth cations.

3.6. Effect of nonaqueous media

The functioning of the electrode was investigated in partially nonaqueous media using acetone–water, methanol–water and ethanol–water mixtures and the results obtained are presented in Table 4. It is observed that in the presence of methanol, the slope decreases remarkably. The slope is acceptable in the presence of ethanol until about 15% (v/v) in the water and for the higher percentage of ethanol, the slope decreases. This effect is drastic in acetone–water mixture where the slope is considerably decreased even in 5% (v/v) acetone. Therefore, the electrode is not suitable for using in acetone–water and methanol–water mixtures. However, in ethanol–water mixture (up to 15%), there is only a small decrease in slope and working concentration range

Table 2
Selectivity coefficients ($k_{Sr,j}^{pot}$) of various interfering ions for strontium using fixed interference method (FIM)

Interfering ion	$k_{Sr,j}^{pot}$	Interfering ion	$k_{Sr,j}^{pot}$
Na $^{+}$	1.8×10^{-3}	Cd $^{2+}$	2.1×10^{-3}
K $^{+}$	5.1×10^{-2}	Sn $^{2+}$	2.1×10^{-2}
NH $_4^{+}$	8.5×10^{-3}	Zn $^{2+}$	1.4×10^{-3}
Hg $^{+}$	5.0×10^{-3}	Cu $^{2+}$	7.6×10^{-3}
Mg $^{2+}$	3.0×10^{-2}	ZrO $^{2+}$	3.0×10^{-2}
Ca $^{2+}$	9.3×10^{-3}	Al $^{3+}$	4.0×10^{-2}
Ba $^{2+}$	1.9×10^{-2}	Cr $^{3+}$	5.0×10^{-2}
Pb $^{2+}$	4.9×10^{-3}	La $^{3+}$	4.6×10^{-2}
Ni $^{2+}$	2.5×10^{-2}	Bi $^{3+}$	2.9×10^{-2}
Mn $^{2+}$	3.3×10^{-2}	Th $^{4+}$	2.7×10^{-2}

Table 3

Comparison of the selectivity coefficient ($k_{\text{Sr},j}^{\text{pot}}$) of the most recently described strontium ion selective electrodes with our Sr^{2+} -selective electrode based on FIM data

Interfering ion	Reference [11]	Reference [13]	Reference [14]	Reference [15]	This work
Na^+	3.0×10^{-1}	2.0×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	1.8×10^{-3}
K^+	3.0×10^{-1}	2.5×10^{-2}	2.0×10^{-2}	7.8×10^{-2}	5.1×10^{-2}
NH_4^+	2.0×10^{-1}	3.2×10^{-2}	1.2×10^{-1}	–	8.5×10^{-3}
Mg^{2+}	2.6×10^{-2}	5.0×10^{-2}	3.1×10^{-2}	5.2×10^{-2}	3.0×10^{-2}
Ca^{2+}	2.6×10^{-2}	3.2×10^{-2}	3.5×10^{-2}	3.8×10^{-1}	9.3×10^{-3}
Ba^{2+}	2.8×10^{-2}	3.9×10^{-2}	3.4×10^{-2}	3.4×10^{-2}	1.9×10^{-2}
Pb^{2+}	3.1×10^{-2}	6.3×10^{-2}	4.4×10^{-2}	4.1×10^{-2}	4.9×10^{-2}
Cd^{2+}	2.5×10^{-2}	6.3×10^{-2}	7.5×10^{-2}	5.8×10^{-2}	2.1×10^{-2}

Table 4

Performance of the Sr^{2+} -selective electrode in partially nonaqueous media

Nonaqueous content (% v/v)	Slope (mV/decade)	DLR (mol L^{-1})
Methanol		
5	16.61	$1 \times 10^{-1} - 1 \times 10^{-5}$
10	16.01	$1 \times 10^{-1} - 1 \times 10^{-4}$
15	15.83	$1 \times 10^{-1} - 1 \times 10^{-4}$
20	15.32	$1 \times 10^{-1} - 5 \times 10^{-4}$
Ethanol		
5	27.12	$1 \times 10^{-1} - 1 \times 10^{-5}$
10	27.10	$1 \times 10^{-1} - 1 \times 10^{-5}$
15	27.00	$1 \times 10^{-1} - 5 \times 10^{-5}$
20	24.95	$1 \times 10^{-1} - 5 \times 10^{-5}$
Acetone		
5	18.60	$1 \times 10^{-1} - 1 \times 10^{-5}$
10	18.08	$1 \times 10^{-1} - 1 \times 10^{-5}$
15	18.23	$1 \times 10^{-1} - 1 \times 10^{-5}$
20	18.71	$1 \times 10^{-1} - 5 \times 10^{-5}$

and hence the electrode can be satisfactorily used in this media with above-mentioned percentages.

3.7. Effect of temperature

Drift of changes of electrode performance with temperature, at test solution temperatures 10, 20, 30, 40 and 50 °C for the Sr^{2+} -selective electrode was studied. The electrode exhibits good Nernstian behavior in the temperature range (10–50 °C). At higher temperatures, the slope of electrode deviates from a good Nernstian behavior. This behavior may be due to the disturbances occurring in phase boundary equilibrium at the membrane layer-test solution interface produced by the thermal agitation of the

solution [26]. The standard cell potentials (E_{cell}^0), were determined at different temperatures from the respective calibration plots as the intercepts of these plots at $p \text{ Sr} = 0$, and were used to determine the isothermal temperature coefficient (dE^0/dt) of the cell with the aid of the following equation [27]:

$$E_{\text{cell}}^0 = E_{\text{cell}(25^\circ\text{C})}^0 \pm (dE^0/dt)_{\text{cell}}(t - 25)$$

Plot of E_{cell}^0 versus $(t-25)$ produces a straight line, which its slope was taken as the isothermal temperature coefficient of the cell. It amounts to 0.00063 V/°C. The standard potentials of the reference electrode ($\text{Hg}/\text{Hg}_2\text{Cl}_2$; KCl (saturated)) were calculated using the following equation:

$$E_{\text{Hg}/\text{Hg}_2\text{Cl}_2}^0 = 0.241 - 0.00066(t - 25)$$

The values of the standard potentials of Sr^{2+} -selective electrode were calculated at the different temperatures from the following relation:

$$E_{\text{cell}}^0 + E_{\text{reference}}^0 = E_{\text{electrode}}^0$$

Plot of E^0 electrode versus $(t-25)$ produces a straight line. The slope of the line was taken as the isothermal temperature coefficient of our strontium selective electrode. It equals to 0.00019 V/°C. The small values of $(dE^0/dt)_{\text{cell}}$ and $(dE^0/dt)_{\text{electrode}}$ reveal the high thermal stability of the electrode within the investigated temperature range.

4. Analytical application

The proposed Sr^{2+} -selective electrode has been successfully used for the determination of Sr^{2+} in aqueous solution by using the standard addition method [28,29], and the results are summa-

Table 5

Potentiometric determination of Sr^{2+} in aqueous solutions in presence of interfering ion(s)^a by the standard addition method, at 25 °C

Sample	Sr^{2+} concentration (mol L^{-1})/added	Sr^{2+} concentration (mol L^{-1})/ found	Recovery (% of nominal value)	R.S.D. (%)
Sr^{2+} , Ni^{2+}	5.0×10^{-3}	4.81×10^{-3}	96.2	1.2
Sr^{2+} , Sn^{2+}	5.0×10^{-3}	4.90×10^{-3}	98.0	1.0
Sr^{2+} , Ba^{2+}	5.0×10^{-3}	4.92×10^{-3}	98.4	1.1
Sr^{2+} , Ni^{2+} , Sn^{2+}	5.0×10^{-3}	4.76×10^{-3}	95.2	1.3
Sr^{2+} , Ni^{2+} , Ba^{2+}	5.0×10^{-3}	4.79×10^{-3}	95.8	0.8
Sr^{2+} , Sn^{2+} , Ba^{2+}	5.0×10^{-3}	4.80×10^{-3}	96.0	0.9
Sr^{2+} , Ni^{2+} , Sn^{2+} , Ba^{2+}	5.0×10^{-3}	4.72×10^{-3}	94.4	1.4

^a The concentration of interfering ion(s) in each sample: $1.0 \times 10^{-3} \text{ mol L}^{-1}$.

Table 6

Determination of strontium ion in human biological samples using Sr^{2+} -selective electrode and ICP method

Sample ^a	Sr^{2+} -selective electrode	ICP
Urine ^a	1.39 ± 0.06	1.42 ± 0.03
Bone ^b	48.1 ± 0.5	45.3 ± 0.2

^a Results are based on triplicate measurements.

^a $\mu\text{g mL}^{-1}$.

^b $\mu\text{g g}^{-1}$.

rized in Table 5. The recovery and relative standard deviation values given in the table were calculated from eight determinations. Collective results, given in Table 5, indicate the high accuracy and precision of the present work.

The optimized strontium selective electrode was found to work well for determination of Sr^{2+} ions in real samples. To assess the applicability of the electrode, an attempt was made to determine Sr^{2+} in biological samples. The urine and bone samples from apparently healthy human individuals were collected from a hospital in Rasht and were pre-treated by a suitable method [30]. They were analyzed by both our Sr^{2+} -selective electrode and ICP method. The results are summarized in Table 6. As is seen from the table, the results determined with the proposed electrode are in good agreement with those obtained by ICP method. Therefore, it can be concluded that, the electrode may have applications in the biological analysis of Sr^{2+} .

The main analytical features of the recently reported Sr^{2+} -selective electrodes in comparison with our electrode is shown in Table 7. The table compares the performance of the electrodes with regards to the slope, linear range, detection limit and interfering ions for the strontium selective electrodes. As can be seen from the table, the figure of merits obtained for the

proposed electrode are comparable or superior to those reported so far.

5. Conclusion

The PVC-based membrane incorporating 6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo [3,1,0] hex-2-ene (NPDBH) as ionophore, acetophenone as plasticizer and oleic acid as lipophilic additive can be used for construction of a selective electrode for determination of strontium(II) ion in solution. The results show that oleic acid is a good lipophilic additive for electrode construction. The proposed electrode responds to Sr^{2+} in a Nernstian fashion and presents high selectivity and sensitivity to Sr^{2+} ion, relatively wide working concentration (1.0×10^{-6} – $1.0 \times 10^{-1} \text{ mol L}^{-1}$), wide pH range (3–10), low detection limit ($2.4 \times 10^{-7} \text{ mol L}^{-1}$), long lifetime and moderately fast response time (<20 s). The proposed electrode reveals excellent selectivity for Sr^{2+} over a wide variety of alkali, alkaline earth, some transitions and heavy metal ions. Analytical features of the electrode are either comparable or better than those previously reported. The thermal stability and performance of the electrode is adequate within the tested temperature of 10–50 °C. The electrode works successfully in partially nonaqueous medium of ethanol–water (up to 15%). The proposed Sr^{2+} ion-selective electrode was found to work well under laboratory conditions and it was successfully applied to the determination of Sr^{2+} ions in real biological samples.

Acknowledgment

The authors are thankful to the Research Council of Guilan University for providing financial assistance.

Table 7

Comparison of our Sr^{2+} -selective electrode with the recently reported electrodes

Ionophore	Slope (mV/decade)	Linear range (mol L^{-1})	Limit of detection (mol L^{-1})	Interfering ions	Reference
<i>N,N,N',N'</i> -tetracyclohexyl-2,6-pyridine-bis (methylenoxy acetamide)	29.0	2.0×10^{-5} – 1.0×10^{-2}	8.0×10^{-6}	Ag^+ , Pb^{2+}	7
4- <i>tert</i> -butylcalix[8]arene	31.3 ± 1.0	2.0×10^{-5} – 1.0×10^{-1}	^a	Ba^{2+} , Pb^{2+}	8
1,13-diaza-2,3;11,12-dibenzo-4,7,10-trioxo-Cyclopentadecane-14,15-dione	30.0	3.2×10^{-5} – 1.0×10^{-1}	8.0×10^{-6}	K^+ , Ca^{2+}	9
2,9-dimethyl-4,11-diphenyl-1,5,8,12-tetraazacyclotetradeca-1,4,8,11-tetraene	28.6 ± 0.3	1.4×10^{-6} – 1.0×10^{-1}	^a	Li^+ , Na^+ , K^+	11
Dibenzo-30-crown-10	29.2 ± 0.3	9.0×10^{-6} – 1.0×10^{-1}	5.0×10^{-6}	Na^+	12
4- <i>tert</i> -butylcalix(8)arene-octaacetic acid Octaethyl ester (I)	30.0	3.2×10^{-5} – 1.0×10^{-1}	^a	Cd^{2+} , Pb^{2+}	13
5,11,17,23,29,35-Hexakis (1,1,3,3-tetramethyl-butyl)-37,38,39,40,41,42-hexakis(carboxy methoxy)calyx[6]arene (I)	30.0	1.9×10^{-5} – 1.0×10^{-1}	^a	NH_4^+	14
5,7,12,14-dibenzo-2,3,9,10-tetraoxa-1,4,8,11-tetraazacyclotetradecane	29.0 ± 0.1	3.98×10^{-6} – 1.0×10^{-1}	2.82×10^{-6}	Ca^{2+}	15
6-(4-nitrophenyl)-2-phenyl-4,4-dipropyl-3,5-diaza-bicyclo[3,1,0]hex-2-ene	28.2 ± 0.5	1.0×10^{-6} – 1.0×10^{-1}	2.4×10^{-7}	La^{3+}	This work

^a Not reported.

References

- [1] E. Pretsch, *Trends Anal. Chem.* 26 (2007) 46.
- [2] R.J.C. Brown, M.J.T. Milton, *Trends Anal. Chem.* 24 (2005) 266.
- [3] K. Usuda, K. Kono, S. Hayashi, T. Kawasaki, G. Mitsu, T. Shibutani, E. Dote, K. Adachi, M. Fujihara, Y. Shimbo, W. Sun, B. Lu, K. Nakasuji, *Environ. Health Prev. Med.* 11 (2006) 11.
- [4] D.K. Tarun, M. Sarin, *Geostd. Newslett.* 26 (2002) 301.
- [5] Z. Arslan, J.F. Tyson, *Talanta* 50 (1999) 929.
- [6] M. Shamsipur, F. Raoufi, *Mikrochim. Acta* 137 (2001) 163.
- [7] G. Qian, M.B. Wu, G. Wu, S. Huang, Y. Yan, B. Tain, *Talanta* 47 (1998) 1149.
- [8] V.K. Gupta, A.K. Jain, U. Khurana, L.P. Singh, *Sens. Actuators, B* 55 (1999) 201.
- [9] M. Shamsipur, S. Rouhani, H. Sharghi, M.R. Ganjali, H. Eshghi, *Anal. Chem.* 71 (1999) 4938.
- [10] A.K. Singh, G. Bhattacharjee, S. Baniwal, M. Singh, *J. Indian Chem. Soc.* 76 (1999) 53.
- [11] A. Panwar, S. Baniwal, R. Singh, C.L. Sharma, A.K. Singh, *Anal. Lett.* 34 (2001) 2415.
- [12] M.R. Ganjali, R. Kiani, M. Yousefi, M. Faal-Rastegar, *Anal. Lett.* 36 (2003) 2134.
- [13] A.K. Jain, V.K. Gupta, J.R. Raison, *Sensors* 4 (2004) 115.
- [14] V.K. Gupta, R. Ludwig, S. Agarwal, *Anal. Sci.* 21 (2005) 293.
- [15] A.K. Singh, P. Saxena, S. Mehtab, B. Gupta, *Talanta* 69 (2006) 521.
- [16] N.O. Mahmoodi, M.A. Zanjanchi, H. Kiyani, *J. Chem. Res.* (2004) 438.
- [17] E. Bakker, P. Buhlmann, E. Pretsch, *Chem. Rev.* 97 (1997) 3083.
- [18] X. Yang, N. Kumar, H. Chi, D.B. Hibbert, P.N.W. Alexander, *Electroanal.* 9 (1997) 549.
- [19] U. Schaller, E. Bakker, U.E. Spischiger, E. Pretsch, *Anal. Chem.* 41 (1994) 379.
- [20] W. Kim, D.D. Sung, G.S. Cha, S.B. Park, *Analyst* 123 (1998) 379.
- [21] D. Ammann, E. Pretsch, W. Simon, E. Lindner, A. Bezegh, E. Pungor, *Anal. Chim. Acta* 171 (1991) 1380.
- [22] S. Matysik, F.M. Matysik, J. Mattusch, W.D. Einicke, *Electroanal.* 10 (1998) 98.
- [23] R.P. Buck, E. Lindner, *Pure Appl. Chem.* 66 (1995) 2527.
- [24] Y. Umezawa, K. Umezawa, H. Sato, *Pure Appl. Chem.* 67 (1995) 507.
- [25] G.G. Guilbault, R.A. Durst, M.S. Frant, H. Freiser, E.H. Hansen, T.S. Light, E. Pungor, G. Rechnitz, N.M. Rice, J.J. Rohm, W. Simon, J.D.R. Thomas, *Pure Appl. Chem.* 48 (1976) 127.
- [26] S. Khalil, S. Abd El-Aliem, *J. Pharm. Biomed. Anal.* 27 (2002) 25.
- [27] L.I. Antropov, *Theoretical Electrochemistry*, Mir Publishing, Moscow, 1972.
- [28] M. Arvand, M.F. Mousavi, M.A. Zanjanchi, M. Shamsipur, *J. Pharm. Biomed. Anal.* 33 (2003) 975.
- [29] M.B. Saleh, A.A. Abdel Gaber, M.M.R. Khalaf, A.M. Tawfeek, *Sens. Actuators, B* 119 (2006) 275.
- [30] M. Burguera, J.L. Burguera, C. Rondón, M.L. di Bernardo, M. Gallignani, E. Nieto, J. Salinas, *Spectrochim. Acta, Part B* 54 (1999) 805.